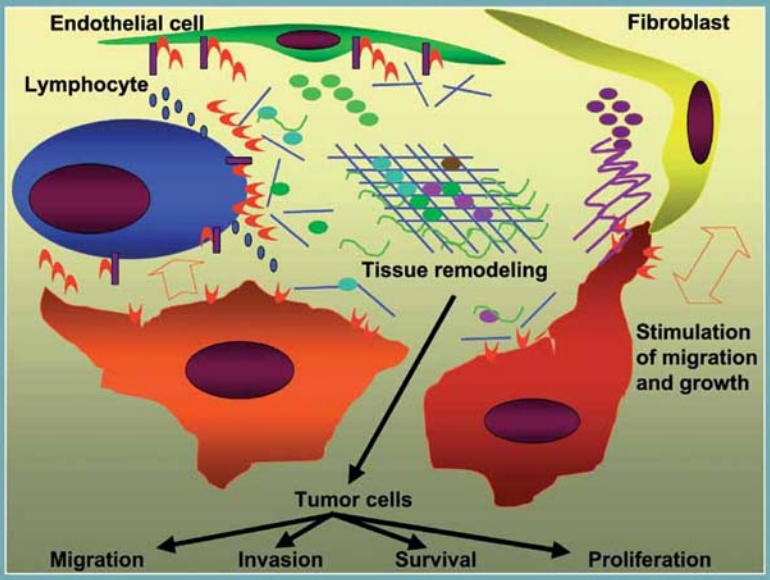
CANCER GROWTH AND PROGRESSION

Series Editor: Hans E. Kaiser

Integration/Interaction of Oncologic Growth

Edited by

Gary G. Meadows



Springer

INTEGRATION/INTERACTION OF ONCOLOGIC GROWTH

Cancer Growth and Progression

Volume 15

Integration/Interaction of Oncologic Growth

Edited by

Gary G. Meadows
Washington State University, Pullman, WA, U.S.A.

Springer

A C.I.P. Catalogue record for this book is available from the Library of Congress.

ISBN-10 1-4020-3413-X (HB) Springer Dordrecht, Berlin, Heidelberg, New York
ISBN-10 1-4020-3414-8 (e-book) Springer Dordrecht, Berlin, Heidelberg, New York
ISBN-13 978-1-4020-3413-8 (HB) Springer Dordrecht, Berlin, Heidelberg, New York
ISBN-13 978-1-4020-3414-5 (e-book) Springer Dordrecht, Berlin, Heidelberg, New York

Published by Springer,
P.O. Box 17, 3300 AA Dordrecht, The Netherlands.

Printed on acid-free paper

Printed in the Netherlands.

Table of Contents

Series Preface

The present multi-volume Book Series, CANCER GROWTH AND PROGRESSION, encompasses the widest possible framework of cutting edge research in the field of neoplastic pathology and other integrated fields. Normal and pathologic growth is one of the most intensively studied yet challenging areas in pathology. Thus the individual volumes in this series focus on the topics of highest scientific interest for basic and clinical researchers, pathologists, medical and surgical oncologists and allied multidisciplinary teams interested in the study of these aspects of neoplastic growth, progression and inhibition. The range of topics covered is extensive, including but not limited to autonomous growth characteristics of malignancy, phenomena of progression of malignant growth involving the various body systems, and recent advances being made in successful neoplastic inhibition and control.

Cell function may be described as producing progression or regression, often found as alternating features in tumors or as variations between normal tissues and tumors. The source of regression in normal melanin producing cells may not be the same as in melanomas. These functions of living matter persist in all phyla of eumetazoans vascular plants as well as in particular species of fungi. However, *homo sapiens* are the eumetazoan species, which interest us the most.

Normal growth processes cannot be entirely understood in all its diversity until we have a thorough knowledge of what constitutes normal growth in various organisms. Complex cellular metabolic pathways are the fundamental elements of growth processes with wide variation in different tissues and organs subjected to a host of carcinogenic influences. The etiology of neoplasms that includes inherent/acquired gene defects, chemical and physical carcinogens, radioactive emissions, viruses, bacteria and parasites are too numerous to catalogue. Therefore, it would be a challenging task to address every aspect of the diverse processes of neoplastic growth and progression. In order to accomplish this goal in the most practical manner, I have invited a highly select team of distinguished authors who are among the most knowledgeable authorities in their fields, to share their expertise in various areas of normal and neoplastic growth, progression, inhibition and control.

A specific purpose of this Book Series is to provide a broad yet comprehensive review of the topics covered that will enrich the reader with the latest and most authentic information at the cutting edge of the field from the world authorities that will be of practical utility to a wide range of professionals in the field of cancer. I have dedicated all my life to the study, teaching and research in cancer. It is my utmost desire to wish you all a great success in your fight against cancer. I hope the second edition of this series will serve as a landmark in our continued efforts to unravel the complexities of the neoplastic phenomena at the one end and to improve the quality of life and minimize the suffering of the patients with cancer on the other!

Hans E. Kaiser. D.Sc.
Series Editor

Preface

Cell proliferation, apoptosis, invasion, and cellular remodeling are elements in the development of normal cells. It is remarkable how well these intricate processes are controlled. During the development of cancer, normal cells develop a number of genetic mutations and alterations that alter normal mechanisms such that they no longer are subject to the environmental cues that control proliferation, survival, angiogenesis, and invasion. Thus, a genetically instable cell is born "out of control", and it must struggle vigorously for existence. Tumor cells are not autonomously growing cells. Rather they are extremely dependent on the host microenvironment for their survival. An early response that ensues after limited *in situ* proliferation is angiogenesis, a reaction that will help to insure survival of a growing nexus of hypoxic tumor cells. The important interactions and interrelationships between the host and the tumor become even more vital as the battle between the tumor and host continues. Ultimately, without intervention, the invasive characteristics of the tumor and the ability of the tumor to overcome host defenses will prevail, and metastasis will occur. Once this transpires additional host-tumor interactions take place and if the tumor finds the appropriate "soil", the process starts all over again. Without intervention a metastatic tumor can and does almost always leads to death of the host.

This book addresses the interactions and interrelationships between tumor and host that modulate tumor progression and metastasis. A continuous theme in the book is highlighting the host and tumor processes/signaling mechanisms that control metastasis in order to find new therapeutic approaches to control cancer progression. One promising approach is to target the host rather than the tumor itself, and several authors examine the potential for this novel tactic to oppress the manipulative cancer cell.

In Chapter 1 the authors discuss differences in the microenvironment between tumors and normal cells and the genomic changes that modulate cell survival, angiogenesis, migration and invasion, and metastasis. They also discuss the possibility of using genes regulated by the microenvironment as intrinsic prognostic tumor markers. Chapter 2 discusses the interactions between stromal cells and the tumor microenvironment in angiogenesis and the potential for development of novel targeted therapies. Chapter 3 examines various imaging techniques for studying angiogenesis *in vivo*. The role of the microenvironment on the molecular mechanisms that underlie vasculogenic mimicry and tumor cell transdifferentiation is reviewed in Chapter 4. In Chapter 5, the author's examine the regulatory role of cell adhesion interactions that occur

during melanoma development, since they control proliferation, differentiation, migration, apoptosis, and vasculogenic mimicry.

Important mediators of invasion include the matrix metalloproteinases and their inhibitors, and their role in remodeling the extracellular matrix and as targets for therapy are evaluated in Chapter 6. It is well known that every organ of the body has its own unique microenvironment, and comprehension of this environment is important to finding new approaches to control tumor metastasis. The role of the brain microenvironment in brain metastasis is examined in Chapter 7. Influence of the bone microenvironment on breast cancer metastasis is examined in Chapter 8, and the interaction between the tumor and liver stroma are reviewed in Chapter 9.

It is well known that activation of the cellular immune system, at least in animals, can mediate tumor rejection; however complete success in humans has been illusive. The microenvironment within the tumor is generally prohibitory to T cell activation and this is reviewed in Chapter 10. Inflammation and chemokines play an important role in control of tumor progression. The role of inflammatory mediators and chemokines in breast cancer progression is discussed in Chapter 11. The interactions between tumor and host cells mediated by chemokines in the microenvironment is examined in Chapter 12, and the specific role of CXCR4, a chemokine receptor, in promoting the outgrowth of micrometastasis is reviewed in Chapter 13.

Energy homeostasis is important to the survival of both host and tumor cells and many of the metabolic disturbances that occur during tumor progression could involve the brain. The role of the brain in host defense and survival is understudied, and potential strategies that protect the brain from the consequences of tumor growth are discussed in Chapter 14. Anti-angiogenic and pro-apoptotic mechanisms can be triggered by moderate dietary restriction. In Chapter 15, the authors discuss the mechanisms associated with these effects. The tumor and host interactions associated with dietary restriction of specific amino acids that limit invasion and metastasis are presented in Chapter 16.

Drug resistance is a major problem encountered by cancer patients during therapy. The contribution of tumor microenvironmental factors to drug resistance is reviewed in Chapter 17. In Chapter 18, the integrin-mediated mechanisms of drug resistance are reviewed. Chapter 19 contains an analysis of the mechanisms involving the microenvironment of the bone and the refractoriness of stage D3 prostate cancer to therapy. In these chapters the possibilities for developing new therapeutic approaches to augment chemosensitivity and to develop novel therapeutic approaches through targeting the metastatic microenvironment are discussed.

The local microenvironment and the interface between the tumor and host in the therapeutic intervention of cancer are not well understood. Chapter 20 addresses current and future directions to target the tumor and host microenvironment and discusses an important new approach by targeting the stroma. Chapter 21 considers bone stromal cells as targets to eliminate bone metastasis. Lung cancer in particular, metastasizes to several organs. The therapy of multiple organ metastases and the mechanisms underlying organotropism are not understood. In Chapter 22, the authors examine through c-DNA-microarray analyses the factors regulating organotropism of metastasis in four different organs, and discuss the potential for developing multi-organ, molecular targeted, antimetastatic therapy. Maspin is a novel serine protease inhibitor that functions in many steps of tumor progression. The research progress toward the potential use of this inhibitor clinically in anti-cancer therapies is reviewed in Chapter 23. Metastasis suppressor

genes could have a significant impact in controlling metastasis and are unique therapeutic targets. In Chapter 24, updated information regarding thirteen defined metastasis suppressor genes is reviewed and in Chapter 25, the important host-tumor interactions involving MKK4, a metastasis suppressor gene important to prostate and ovarian cancer are reviewed. Understanding the mechanisms underlying the suppression and progression of metastasis by these metastasis suppressor genes could ultimately lead to new treatment paradigms.

The editors would like to thank all the contributing authors for their time and effort in preparing the chapters for this book. We would especially like to acknowledge Dr. Cristina dos Santos, the publishing editor from Kluwer Academic Publishers (now Springer Life Sciences – Biomedical Unit) for her assistance and especially Ms. Melania Ruiz, her assistant for help with formatting the chapters. Finally the editors are indebted to Mr. Daniel Campbell, Ms. Yvonne Rivers, and Ms. Melissa McGraw for their support in editing, proof reading, and general assistance in preparing the final versions of the chapters.

Gary G. Meadows

List of Contributors

Alejandro, E. Molecular Signalling Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, Bethesda, MD, 20892, USA

Anderson, R.L. Trescowthick Research Laboratories, Peter MacCalhum Cancer Centre, East Melbourne, Victoria, 3002, Australia

Au, J.L.-S. College of Pharmacy, Ohio State University, Columbus, OH, 43210, USA

Balian, G. Departments of Orthopedics & Biochemistry and Molecular Genetics, School of Medicine, University of Virginia Health System, USA

Beck, G.L. Departments of Orthopedics & Biochemistry and Molecular Genetics, School of Medicine, University of Virginia Health System, USA

Ben-Baruch, A. Department of Cell Research and Immunology, Tel-Aviv University, Tel-Aviv, Israel

Berger, J.C. The Pritzker School of Medicine, The University of Chicago, USA

Bogdanos, J. Department of Experimental Physiology, Medical School, University of Athens, Goudi-Athens, 115 27, Greece

Brown, M.L. Department of Chemistry, University of Virginia, USA

Cooper, C.R. Cancer Biology Laboratory, Department of Biological Sciences, University of Delaware, USA

Coussens, L.M. Cancer Research Institute, Department of Pathology, and Comprehensive Cancer Center, University of California, San Francisco, CA, USA

Diaz, R.J. Cancer Research Institute, University of California, San Francisco, CA, USA

Eckhardt, B.L. Trescowthick Research Laboratories, Peter MacCalhum Cancer Centre, East Melbourne, Victoria, 3002, Australia

Eichten, A. Cancer Research Institute, University of California, San Francisco, CA, USA

da Silva, C.C. Centre de Biophysique Moléculaire, UPR 4301 CNRS, Glycogiology of Cell Recognition, 45071, Orléans, France

de Visser, K.E. Cancer Research Institute, University of California, San Francisco, CA, USA

Fu, Y.-M. Cancer Prevention & Research Center, Department of Pharmaceutical Sciences, The Pharmacology & Toxicology Graduate Program, and the College of Pharmacy, Box 646510, Washington State University, Pullman, WA 99164-6510, USA

Fukumura, D. Edwin L. Steele Laboratory, Department of Radiation Oncology, Massachusetts General Hospital and Harvard Medical School, Boston, MA, 02114, USA

Gotley, D.C. University of Queensland, Brisbane, Australia

Grunfield, J. Department of Neuro-Oncology, University of Texas M.D. Anderson Cancer Center, Houston, TX, USA

Hendrix, M.J.C. Children's Memorial Institute for Education and Research, Children's Memorial Hospital, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA

Herlyn, M. The Wistar Institute, 3601 Spruce Street, Philadelphia, PA, 19104, USA

Hickson, J. A. Section of Urology, Department of Surgery, Department of Biochemistry and Molecular Biology, Markey Molecular Medicine Program, The University of Chicago, USA

Hill, R.P. Experimental Therapeutics Division, Ontario Cancer Institute/Princess Margaret Hospital, Departments of Medical Biophysics and Radiation Oncology, University of Toronto, 610 University Avenue, Toronto, Ontario, M5G 2M9, Canada

Hoffman, R.M. AntiCancer, Inc., 7917 Ostrow St., San Diego, CA, 92111, USA

Kakiuchi, S. Department of Internal Medicine and Molecular Therapeutics, University of Tokushima Graduate School, Tokushima, Japan

Kalliomäki, T. Experimental Therapeutics Division, Ontario Cancer Institute/Princess Margaret Hospital, Department of Medical Biophysics, 610 University Avenue, Toronto, Ontario, M5G 2M9, Canada

Kassis, J. Molecular Signalling Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, Bethesda, MD, 20892, USA

Kieda, C. Centre de Biophysique Moléculaire, UPR 4301 CNRS, Glycogiology of Cell Recognition, 45071, Orléans, France

Kirschmann, D.A. Children's Memorial Institute for Education and Research, Children's Memorial Hospital, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA

Kohn, E.C. Molecular Signalling Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, Bethesda, MD, 20892, USA

Koutsilieris, M. Department of Experimental Physiology, Medical School, University of Athens, Goudi-Athens, 115 27, Greece

Lamerant-Fayel, N. Centre de Biophysique Moléculaire, UPR 4301 CNRS, Glycogiology of Cell Recognition, 45071, Orléans, France

Lembessis, P. Department of Experimental Physiology, Medical School, University of Athens, Goudi-Athens, 115 27, Greece
Li, G. The Vistar Institute, 3601 Spruce Street, Philadelphia, PA, 19104, USA

Lockwood, D.S.R. University of Queensland, Brisbane, Australia

Marincola, R.M. Immunogenetics Section, Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, MD, USA

Måseide, K. Experimental Therapeutics Division, Ontario Cancer Institute/Princess Margaret Hospital, Department of Medical Biophysics, University of Toronto, 610 University Avenue, Toronto, Ontario, M5G 2M9, Canada

Meadows, G.G. Cancer Prevention & Research Center, Department of Pharmaceutical Sciences, The Pharmacology & Toxicology Graduate Program, and the College of Pharmacy, Box 646510, Washington State University, Pullman, WA, 99164-6510, USA

Meltzer, P.S. Cancer Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA

Milathianakis, C. Department of Experimental Physiology, Medical School, University of Athens, Goudi-Athens, 115 27, Greece

Mukherjee, P. Biology Department, Boston College, Chestnut Hill, MA, 02467, USA

Nagorsen, D. Department of Haematology, Oncology and Transfusion Medicine, Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany

Pitulis, N. Department of Experimental Physiology, Medical School, University of Athens, Goudi-Athens, 115 27, Greece

Pouliot, N. Trescowthick Research Laboratories, Peter MacCalhum Cancer Centre, East Melbourne, Victoria, 3002, Australia

Pruitt, F. Cancer Biology Laboratory, Department of Biological Sciences, University of Delaware, USA

Puduvali, V.K. Department of Neuro-Oncology, University of Texas M.D. Anderson Cancer Center, Houston, TX, USA

Richert, M.M. Department of Pathology, Comprehensive Cancer Center, and the National Foundation for Cancer Research- Center for Metastasis Research, University of Alabama at Birmingham, Birminghan, AL, USA

Rinker-Schaeffer, C.W. Section of Urology, Department of Surgery, The Committee on Cancer Biology, Department of Biochemistry and Molecular Biology, Markey Molecular Medicine Program, The University of Chicago, USA

Rintoul, R.C. Department of Respiratory Medicine, Royal Infirmary of Edinburgh, Edinburgh, UK

Robinson, V.L. Section of Urology, Department of Surgery, The Committee on Cancer Biology, The University of Chicago, USA

Roos, E. Division of Cell Biology, The Netherlands Cancer Institute, 121 Plesmanlann, 1066CX, Amsterdam

Seftor E.A. Children's Memorial Institute for Education and Research, Children's Memorial Hospital, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA

Sethi T. Department of Respiratory Medicine and Cancer Cell Biology, Royal Infirmary of Edinburgh, Edinburgh, UK

Seyfried, T.N. Biology Department, Boston College, Chestnut Hill, MA 02467, USA

Sheng, S. Department of Pathology, Wayne State University School of Medicine, Protease Program of the Karmanos Cancer Insitute, Detroit, MI, USA

Sikes, R.A. Laboratory for Cancer Ontogeny and Therapeutics, Department of Biological Sciences, University of Delaware, USA

Sone, S. Department of Internal Medicine and Molecular Therapeutics, University of Tokushima Graduate School, Tokushima, Japan

Sotiriou, E. Department of Experimental Physiology, Medical School, University of Athens, Goudi-Athens, 115 27, Greece

Sourla, A. Department of Experimental Physiology, Medical School, University of Athens, Goudi-Athens, 115 27, Greece

Tenta, R. Department of Experimental Physiology, Medical School, University of Athens, Goudi-Athens, 115 27, Greece

Tiblalexi, D. Department of Experimental Physiology, Medical School, University of Athens, Goudi-Athens, 115 27, Greece

Vander Griend, D. Section of Urology, Department of Surgery, The Committee on Cancer Biology, University of Chicago, USA

Virador, V. Molecular Signalling Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, Bethesda, MD, 20892, USA

Wei, Y. College of Pharmacy, Ohio State University, Columbus, OH, 43210, USA

Welch, D.R. Department of Pathology, Comprehensive Cancer Center, and the National Foundation for Cancer Research- Center for Metastasis Research, University of Alabama at Birmingham, Birminghan, AL, USA

Wiater, M.F. Program in Neuroscience, College of Veterinary Medicine, Washington State University, Pullman, WA, 99164-6510, USA

Yano, S. Department of Internal Medicine and Molecular Therapeutics, University of Tokushima Graduate School, Tokushima, Japan

Zeelenberg, I.S. Division of Cell Biology, The Netherlands Cancer Institute, 121 Plesmanlann, 1066CX, Amsterdam

Zhang, H. Department of Internal Medicine and Molecular Therapeutics, University of Tokushima Graduate School, Tokushima, Japan

Chapter 1

Microenvironmental Effects on Tumour Progression and Metastasis

Kårstein Måseide[1,2], Tuula Kalliomäki[1,2] and Richard P. Hill[1,2,3]
[1]Experimental Therapeutics Division, Ontario Cancer Institute/Princess Margaret Hospital, [2]Departments of Medical Biophysics and [3]Radiation Oncology, University of Toronto, 610 University Avenue, Toronto, Ontario, M5G 2M9, Canada

Abstract: The microenvironment of a tumour differs significantly from that of a normal tissue due to abnormal vasculature and altered cellular metabolism in tumours. Microenvironmental conditions in tumours, which include hypoxia, acidity, nutrient deprivation and high interstitial fluid pressure, have been associated with metastatic tumour progression both experimentally and clinically. The mechanisms by which this occurs are believed to include genomic instability, changes in gene expression levels and microenvironment-mediated selective pressure. These genomic changes modify cell survival and growth during the angiogenic, migratory and invasive stages of tumour progression and metastasis. This chapter summarises the data examining links between tumour microenvironment and metastasis and discusses the possibility of using microenvironmentally regulated genes as intrinsic prognostic markers.

Key words: Tumour microenvironment, hypoxia, acidity, IFP, nutrient deprivation, genomic instability, metastasis and prognostic markers, Apoptosis, angiogenesis, tumour progression, gene expression, intravasion, extravasion, ECM, epigenetic, VEGF, melanoma, fibrosarcoma, cervical carcinoma, lung carcinoma, lymphatic metastasis, head and neck carcinoma, soft tissue carcinoma, soft tissue sarcoma, rectal adenocarcinoma, oral squamous cell carcinoma, breast carcinoma, apoptosis, genomic stability, UPAR, UPA, TIMP, C-MET, AMF, chemokine, ovarian cancer, bladder cancer carcinoma, pancreatic carcinoma, colorectal carcinoma, gastric, non-small cell lung, plasminogen activator inhibitor, osteopontin

1. INTRODUCTION

As tumours grow they tend to progress to a more aggressive phenotype and to spread to distant organs, a process known as metastasis. This is a major cause of treatment failure and death in cancer patients. Tumour progression involves a series of genetic and epigenetic changes in the tumour cells that occur at increased frequency because of genomic instability. This results in heterogeneity in the genomic and phenotypic properties of the cells from different tumours and even between the cells within a single tumour. Although there is uncertainty about the mechanisms by which tumour cells can acquire genomic instability, there is wide agreement that most tumour cells possess a significant number of genomic changes. The microenvironment of cells in tumours involves both the immediate interactions with surrounding cells and extracellular matrix, and exposure to pathophysiological conditions, such as low oxygen tension (hypoxia), low glucose concentrations, high lactate concentrations, low extracellular pH (acidity) and high interstitial fluid pressure that can vary between different tumour regions. There is increasing clinical and experimental evidence that the pathophysiological tumour microenvironment plays an important role in tumour progression. This chapter provides an overview of this evidence and discusses experimental studies that are shedding light on the

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 1-22.

underlying mechanisms, with an emphasis on epigenetic factors.

2. THE TUMOUR MICROENVIRONMENT AND METASTASIS – CLINICAL AND EXPERIMENTAL STUDIES

The pathophysiological microenvironment of cells in solid tumours is complex, primarily because of the structural and functional abnormalities of the vasculature in tumours. The blood vessels that develop during tumour neoangiogenesis often have highly irregular architecture that includes such features as blind ends, arterio-venous shunts and high angle branching patterns. They can also lack smooth muscle and enervation, and may have an incomplete endothelial lining and basement membrane, which makes them more permeable than vessels in normal tissues. The result is a heterogeneous tumour microenvironment characterized by regions of hypoxia, low glucose concentrations, high lactate concentrations, acidity and high interstitial fluid pressure (Figure 1).

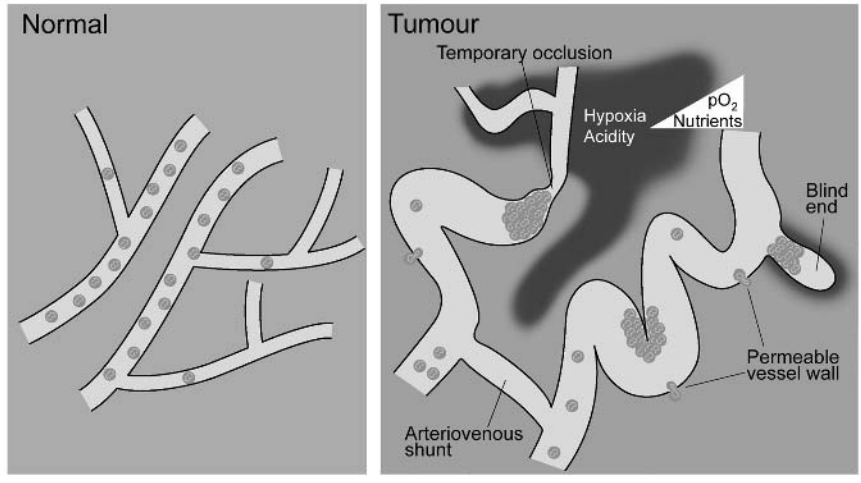


Figure 1. Illustration of the tumour microvasculature. Whereas normal tissues have relatively uniform blood vessels that are sufficiently close together to supply all of the tissue with oxygen and other nutrients, tumour blood vessels are tortuous and have sluggish and irregular blood flow. Consequently, tumours have regions of hypoxia, nutrient deprivation, build-up of catabolic products and acidity. Hypoxia might occur as a result of diffusion limitations at a distance from blood vessels (chronic hypoxia) or adjacent to temporarily closed vessels (acute hypoxia). Modified from Brown (1).

2.1 Hypoxia

Regions of low oxygen tension (pO2), or hypoxia, are found in most solid tumours. The extent of hypoxic regions is heterogeneous even amongst tumours of identical histopathological type, and does not correlate with standard prognostic factors such as tumour size, stage and grade (2, 3). Although the definition of hypoxia depends on the effect being studied and varies between different studies, pO2 < 10 mmHg is generally considered to be associated with changes in the expression of a number of genes and has also been associated with poor prognosis in a number of clinical studies. A significant proportion of tumour cells are in hypoxic regions beyond the maximum diffusion distance of oxygen from a capillary. This distance is usually in the range 50-150 μm, depending on the pO2 in the capillary and on the oxygen consumption rate of the surrounding cells in the tumour (Figure 1). These cells may be exposed chronically to low oxygen tensions (chronic hypoxia) for hours to days. Tumour hypoxia can also occur transiently due to the substantial instability in microregional blood flow and tissue oxygenation that can occur in animal and human tumours. These fluctuations are thought to be due to transient

occlusion and narrowing of vessels and to arteriolar vasomotion. Also, the abnormal architecture of the vascular system itself may produce variations in red cell flow. High interstitial fluid pressure may further exacerbate the situation. This blood flow instability, in the context of an already poorly organized and regulated vascular system, can produce short-term (5-60 minutes) fluctuations in oxygenation (acute hypoxia) in substantial volumes of solid tumours (4).

Since the early 1980s the Eppendorf pO2 histograph has been available for clinical measurement of tumour oxygenation in patients. As a result, there have been an increasing number of clinical studies of tumour oxygenation. These studies have all demonstrated the presence of regions with low levels of oxygen in human tumours (Figure 2a). Many of these studies have also provided evidence for a negative impact of tumour hypoxia on treatment outcome in several tumour types, including soft tissue sarcoma, cervical carcinoma, and head and neck carcinoma (Table 1). Höckel et al. (20) demonstrated that tumour hypoxia was a poor prognostic factor in cervical carcinoma regardless of whether patients were treated with radiotherapy or with surgery alone, suggesting that hypoxia might be associated with poorer treatment outcome independently of its effect on reducing radiation sensitivity (21). Similarly, an association was found between hypoxia and metastatic disease in soft tissue sarcoma (22). Most of these studies involved radiotherapy with or without chemotherapy and surgery, and they demonstrated the predictive value of pre-treatment pO2 measurements for both local and distant (metastatic) relapse.

A link between hypoxia and metastasis has also been demonstrated in experimental tumour models as discussed in a number of recent reviews (23-25). For example, a relation between pO2 levels of small primary tumours and micrometastases was found in the highly metastatic KHT murine fibrosarcoma when grown intramuscularly in mice. Furthermore, exposure of murine KHT cells, SCC-VII cells and B16F10 melanoma cells to hypoxia *in vitro* for 18-24 hrs followed by 18 hr reoxygenation resulted in an enhanced number of lung metastases when the cells were injected intravenously into mice. Notably this effect was transient, suggesting a potential role for regulation of gene expression. Recent results have identified that in KHT cells, the mouse double minute 2 (*Mdm2)* gene is transiently up-regulated by hypoxic exposure independently of p53, and that transient over-expression of Mdm2 protein in oxygenated KHT cells can increase their metastatic potential26. An increase in the number of metastases was also observed in nude mice when D-12 human melanoma cells were injected intravenously after a 24 hr hypoxia treatment *in vitro*. In this study the increase in metastases was observed without any period of reoxygenation and was found to be associated with a simultaneous induction of vascular endothelial growth factor (VEGF) secretion and enhanced angiogenic potential. No effect of hypoxic exposure was observed in A-07 melanoma cells, which have a constitutively high VEGF level. Taken together these data suggest a role of hypoxia in enhancing the metastatic potential of tumour cells, but they also suggest that this effect may occur through different mechanisms in different cell types.

Currently there is no method to routinely measure and discriminate between chronic and acute hypoxia in tumours. Consequently, the extent of chronic versus acute hypoxia in human tumours and their respective importance for clinical outcome is unknown. Clinical studies have suggested a correlation between proteins that are regarded as markers of chronic hypoxia (see section 5) and metastases in cervical carcinomas (9, 27). Experimental studies using the D-12 human melanoma xenografts have shown that vascular hot spots were induced in foci of chronic hypoxia and associated with subsequent spontaneous lung metastasis formation (28). Other studies exposing mice bearing KHT murine fibrosarcomas or ME180 human cervix carcinomas to cycles of low oxygen breathing (12 cycles of 10 min @ 7% O2 followed by 10 min air) every day during tumour growth resulted in an enhanced number of microscopic lung metastases or lymph node metastasis respectively (29, 30), while no such effect was seen after daily chronic treatment (2 hr @ 7% O2) of KHT-bearing mice. Since the tumours became severely hypoxic during the low oxygen part of these breathing cycles, these data suggest a role for acute hypoxia in the development of both blood-borne and lymphatic metastasis.

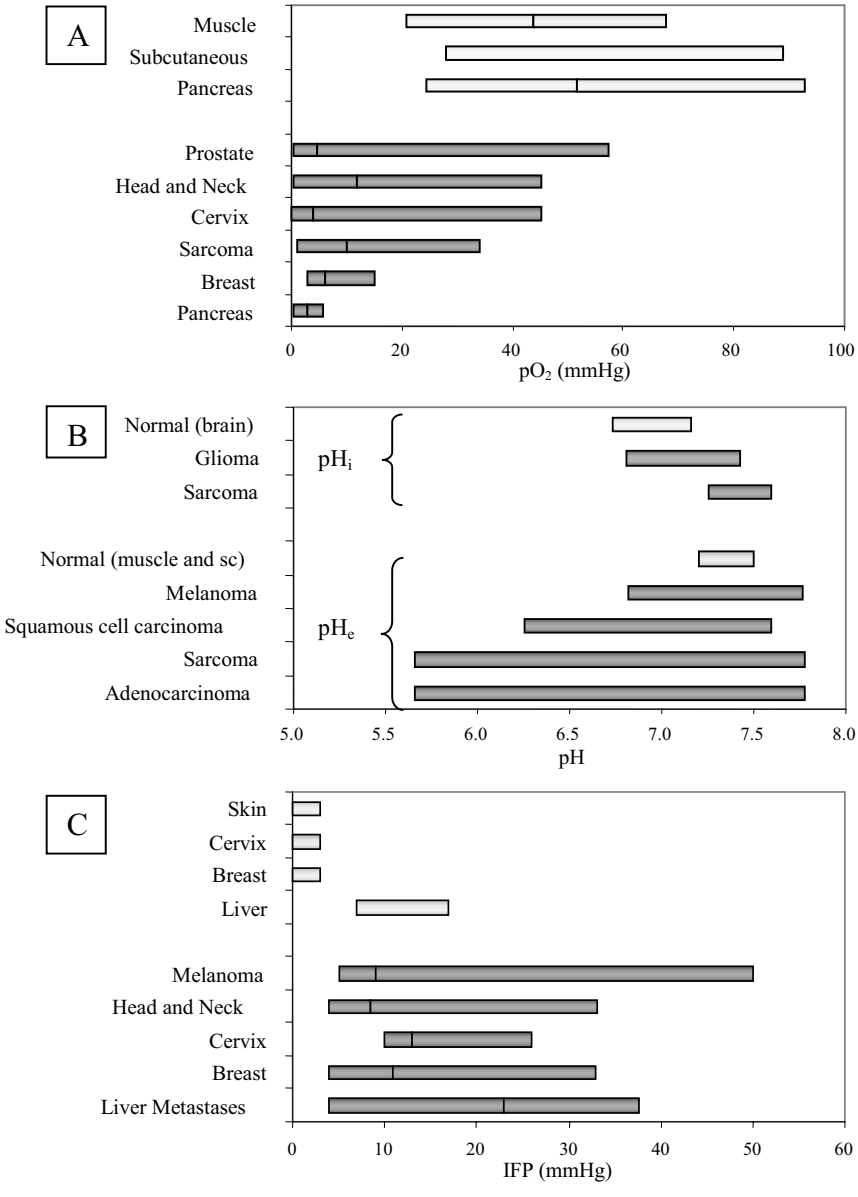


Figure 2. Levels of oxygenation (pO2), pH and interstitial fluid pressure (IFP) in human tumours (dark bars) and normal tissues (light bars). (a) Ranges of median pO2 values for individual patients5-11. (b) Ranges of pH values found in extracellular (pHe) and intracellular (pHi) spaces12-15. (c) Ranges of IFP values16-19. Overall medians are indicated with a vertical line in each panel.

2.2 Acidity

Initial measurements of pH in patients used needle-sheathed probes inserted into the tissue of interest and primarily recorded the pH of the extracellular space (pH_e). Such measurements have demonstrated severe acidity in many tumours and mildly alkaline levels in normal tissues (Figure 2b). Tumour acidity is likely due to the accumulation of lactic acid and/or carbonic acid, resulting from

enhanced production of these acids by glycolysis and hydration of carbon dioxide, respectively, and/or insufficient removal by the abnormal vasculature and lymphatic drainage (31). Intracellular pH (pH_i), measured by magnetic resonance spectroscopy (MRS), is usually found to be mildly alkaline in tumours and neutral in normal tissues (32). Maintenance of pHi within a physiologically normal range is critical to ensure proper function of intracellular proteins and preservation of genomic integrity of a cell. It requires the activity of several energy-dependent cellular transmembrane ion pumps and ports, such as Na+/H+ antiporter/exchanger and HClO3-/Cl- exchanger, the concentration and activity of which vary with pHe and between cell lines.

There is relatively sparse clinical information about the relation between tumour acidity and metastasis. Studies using an enzymatic bioluminescent technique demonstrated that elevated tumour lactate levels were associated with increased metastatic disease and reduced survival in patients with head and neck, cervical and rectal carcinoma (Table 1). However, lactate concentrations in the metastatic and non-metastatic patient groups overlapped, suggesting that elevated tumour lactate concentration alone is not sufficient for induction of metastasis. Tumour acidity might potentially be associated with regions of hypoxia since both parameters are linked to metabolism and the abnormal vascular network. Indeed, experimental studies have indicated a correlation of hypoxia with pHe and lactate concentrations, although local pO2 and pHe gradients around individual microvessels showed no close correlation (33, 34).

Experimental studies have demonstrated that murine KHT and B16F1 tumour cells grown at acidic conditions (pH = 6.5 for 1-2 days) *in vitro* and injected intravenously into mice form more lung metastases than cells cultured at physiologic pH (pH = 7.4) (35,36). However, a recent study showed that exogenously induced acidification of KHT and B16F1 solid tumours (pH = 6.9) had no effect on the number of spontaneous lung metastases (37). It remains unknown whether tumour hypoxia and acidity promote metastasis by different mechanisms.

2.3 Glucose and nutrient deprivation

Many tumours have a higher demand for glucose than normal tissues, as demonstrated by increased uptake of (18)-F-2-deoxy-2-fluoro-D-glucose (FDG) imaged by positron emission tomography (PET). The high glucose demand by tumour cells is likely due to a preferential use of glycolysis in the generation of cellular energy. Tumour cells have been demonstrated to use glycolysis even under well-oxygenated conditions (Warburg effect), in contrast to normal cells, which use glycolysis only under anaerobic conditions, thus amplifying the imbalance of nutrient supply and demand (55).

Low glucose levels in tumours have been linked to metastasis both clinically and experimentally. Experimental studies with KHT, B16F1 and SCC VII murine tumour cells cultured in glucose-deficient medium for 1-2 days followed by recovery in glucose-supplemented medium for 0-2 days demonstrated a significantly increased lung colonization ability when the cells were injected intravenously into mice (35, 36). Increased glucose uptake and reduced glucose concentration in tumours have been shown to be poor prognostic factors in patients with carcinomas of the head and neck, oral cavity, breast and rectum, but not of the cervix. Interestingly, no correlation was found between tumour ATP concentration and survival (Table 1), even though clinical data suggest a correlation between ATP, glucose and lactate concentrations in cervical carcinoma (41). Hypoxia-inducible glucose transporter-1 (Glut-1) correlates with hypoxia in cervical carcinoma, but other clinical and experimental studies have not found consistent correlations between levels of hypoxia and glucose uptake or Glut-1 expression in tumours (27, 56, 57).

Table 1. Association between microenvironmental parameters measured at the time of diagnosis and clinical outcome. Abbreviations used are: ATP: adenosine triphosphate, IFP: interstitial fluid pressure, DFS: disease-free survival, DSS: disease-specific survival, DM: distant metastasis, DMFS: distant metastasis-free survival, LRC: loco-regional control, LRLNM: loco-regional lymph node metastasis. The clinical end-points DFS, DMFS, DSS and LRC refer to analysis of time-dependent follow-up data (Kaplan-Meier), while DM and LRLNM refer to correlation analysis between studies reporting overall survival only are excluded from this table. Studies marked with an asterisk were performed on the same patient population.

Parameter	Treatment	Association	Reference
Cervical carcinoma			
Hypoxia	Radiation or surgery	↓ DFS	20
Hypoxia	Radiation	↓ DFS	38
Hypoxia	Radiation	↓ DFS, ↓ LRC	39
Hypoxia	Radiation	↓ DFS, ↓ DMFS	40*
Lactate	Radiation	↓ DFS, ↑ DM	41
Glucose	Radiation	– DFS, – DM	41
ATP	Radiation	– DFS, – DM	41
IFP	Radiation	↓ DFS, ↓ DMFS	42*
Head and neck carcinoma			
Hypoxia	Radiation +/- radiation sensitizer	↓ LRC	43
Hypoxia	Radiation +/- chemo	↓ DFS, ↓ LRC	44
Hypoxia	Radiation	– LRC	45
Hypoxia	Radiation +/- radiation sensitizer	↓ LRC	46
Hypoxia	Radiation +/- chemo	↓ DSS	47
Lactate	Radiation	↑ LRLNM	48
Lactate	Radiation or surgery + radiation	↓ DMFS	49
Head and neck carcinoma			
Glucose	Radiation	– LRLNM	48
Glucose uptake	Surgery +/- radiation or radiation +/- chemo	↓ DFS, ↓ LRC	50
ATP	Radiation	– LRLNM	48
Soft tissue sarcoma			
Hypoxia	Radiation + surgery + hyperthermia	↓ DMFS	22
Hypoxia	Surgery +/- pre- or postoperative radiation	↓ DSS	51
Rectal adenocarcinoma			
Lactate	Surgery	↑ DM	52
Glucose	Surgery	↓ DM	52
ATP	Surgery	– DM	52
Oral squamous cell carcinoma			
Glucose uptake	Radiation + chemo + surgery	↓ DSS, ↓ LRC	53
Breast carcinoma			
Glucose uptake	Surgery +/- endocrine +/- chemo	↓ DFS	54

2.4 Interstitial fluid pressure

Interstitial fluid pressure (IFP) is elevated in many human tumours including head and neck, breast, colorectal and cervical carcinoma, and metastatic melanoma (Figure 2c). Elevated IFP in tumours is a consequence of the abnormal tumour vasculature (reviewed by Milosevic (58). Tumour vessels are hyperpermeable with low resistance to transcapillary fluid flow and are of highly variable size and organization, resulting in high resistance to capillary blood flow. Hence, the microvascular hydrostatic pressure can drive fluid from the blood vessels into the tumour interstitium. Tumours generally have impaired lymphatic drainage due to incompletely formed or compressed lymphatic vessels, and as a result, fluid will accumulate and distend the extracellular matrix. The resulting elevated IFP may have severe consequences for the delivery and distribution of therapeutic macromolecules (59).

High pretreatment IFP was reported to be a poor prognostic factor for disease-free survival and distant relapse (metastases) in cervical carcinoma patients treated with radical radiotherapy (Table 1). However, there was no correlation between IFP and lymph node metastasis at diagnosis similar to the findings of a smaller study in breast cancer patients (60). There was no correlation between tumour hypoxia (Eppendorf pO2 measurements) and IFP in the cervix study, and similarly no correlation has been observed between such measurements in experimental tumours (58). Such a correlation could have been expected since both IFP and pO2 are closely related to the structural and functional abnormalities of the vascular network, and high IFP is believed to cause vessel narrowing that can reduce blood red cell flow.

A recent experimental study showed that high tumour IFP was associated with the development of pulmonary and lymph node metastases in A-07 human melanoma xenografts (61). These tumours were not hypoxic, suggesting that high IFP might promote metastasis by mechanisms independent of tumour hypoxia, possibly by facilitating tumour cell intravasation (see Section 4). The IFP drops dramatically at the periphery of solid tumours towards the normal tissue, creating a fluid flow out of the tumour tissue that could assist peripheral tumour cells to migrate towards functional lymphatics (62).

3. TUMOUR PROGRESSION MEDIATED BY THE MICROENVIRONMENT

As described above, both clinical and experimental studies have suggested a causal link between tumour hypoxia, acidity, or IFP, and metastatic potential. There are several mechanisms by which tumour microenvironmental factors may contribute to tumour progression as illustrated in Figure 3. Tumour cells from adverse microenvironments may acquire a number of genomic changes and exposure to these conditions in tumours may induce gene expression changes by activating transcription factors or specific signal transduction pathways. The tumour microenvironment may also enable preferential survival of tumour cells expressing specific phenotypic changes under the adverse conditions encountered in that environment, possibly leading to a more aggressive tumour. These mechanisms of tumour progression are discussed in more detail below.

3.1 Genomic instability

In contrast to normal cells where a damaged genome is usually deleted through apoptosis or restored through repair pathways, tumour cells commonly harbour multiple mutations. The types of mutations include a variety of chromosomal aberrations, such as aneuploidy, translocations, amplifications and loss of heterozygosity, as well as smaller alterations such as insertions, deletions and base changes. Hypoxia and nutrient deprivation of cells *in vitro* have been shown to increase the number of mutations. Repeated rounds of hypoxia-treatment *in vitro* generated mutation spectra resembling those found in solid tumours (63). One mechanism by which this may occur is via reactive oxygen or nitrogen species (ROS/RNOS), i.e. superoxide radical (O2.-), hydrogen peroxide

(H2O2), hydroxyl radical (.OH), hydroperoxyl radical (HO2.) and peroxynitrite (ONOO-), and their interaction with cellular macromolecules, such as DNA and lipids.

In the absence of adequate anti-oxidant responses to ROS-mediated oxidative stress, more than 40 different DNA adducts can be formed by ROS interaction with DNA. One of the best-studied lesions, 8-hydroxy-2'-deoxyguanosine (8-OHdG), is mutagenic and causes G:C to T:A transversions by preventing accurate DNA template reading by polymerases (64). ROS may also attack polyunsaturated membrane lipids causing an oxidising chain reaction and the generation of secondary mutagenic end products such as malondialdehyde (MDA), which in turn can form adducts with DNA (65). ROS can also contribute to tumour progression by inactivating tumour suppressor genes such as p53 or by activating oncogenes, such as c-FOS, c-JUN and c-MYC, or transcription factors such as nuclear factor κB (NF-κB), activator protein 1 (AP-1) and hypoxia-inducible factor-1 (HIF-1) (66, 67). ROS can function as signaling molecules and induce expression of growth factors, such as VEGF and interleukin-8 (IL-8), or activate proteinases, such as matrix metalloproteinase-2 (MMP-2) (68). Alternatively, ROS and acidic pH may contribute to tumour progression by reacting with and inactivating proteins, including DNA repair proteins. Diminished repair of UV-induced DNA damage has been demonstrated in cells exposed to hypoxic and acidic culture conditions, and hypoxia has been shown to decrease the expression of two mismatch repair (MMR) proteins, MutL protein homolog-1 (MLH1) and postmeiotic segregation increased 2 (PMS2)(69,70).

The tumour microenvironment may also enhance genomic instability and promote tumour progression by selecting for genetic cell variants that have a survival advantage under the adverse microenvironmental conditions. Hypoxia has been identified as a selective pressure for MMR-deficient colorectal cell populations that had increased mutation frequencies (71). It has also been demonstrated that exposure of some transformed cells to hypoxia *in vitro* induces apoptosis, but that repeated cycles of hypoxia and reoxygenation can select for cells with mutated p53 that are resistant to apoptosis. A spatial correlation was observed between hypoxic and highly apoptotic regions in p53 wild-type tumours grown in mice, whereas few apoptotic cells were seen in hypoxic regions of p53-deficient tumours (72, 73). Apoptotic cell death has also been reported to depend on p53 under acidic conditions *in vitro* (74), which may suggest a growth advantage for p53 deficient cells under acidic conditions *in vivo*.

The role of oxidative stress in tumour progression is supported by clinical studies demonstrating decreased antioxidant responses in certain human tumours relative to normal tissues, increased plasma MDA concentration in patients with metastatic versus non-metastatic disease and increased hydroxyl radical-induced DNA damage in metastatic versus non-metastatic breast tumours (75,76). Clinical data have also linked low apoptotic index and pronounced hypoxia to a high probability of lymphatic spread and recurrence in a group of cervical carcinoma patients (77). However, no correlation was found between tumour oxygenation and p53 status in soft tissue sarcoma patients even though hypoxia was a poor prognostic factor and potentially linked to metastatic spread (51). Interpretation of these observations is complicated by studies demonstrating an absence of DNA damage despite increased oxidative stress and by studies showing a lack of correlation between DNA adduct levels and mutagenicity (78, 79). Such data highlight the importance of interactive mechanisms controlling DNA repair, antioxidant levels and cellular proliferation rates.

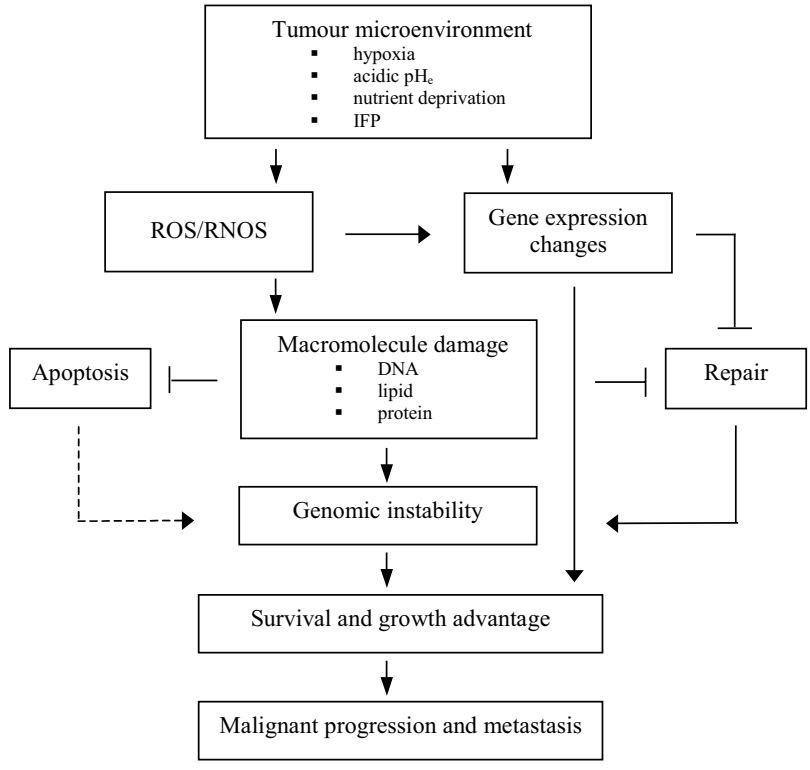


Figure 3. Schematic illustration of possible effects of the tumour microenvironment on malignant progression and metastasis. Microenvironmental conditions in tumours can lead to gene expression changes that will improve tumour cell survival. Alternatively, the tumour microenvironment may generate reactive oxygen or nitrogen species (ROS/RNOS) in excess of neutralizing anti-oxidants and damage repair proteins leading to macromolecule damage, which in the absence of functional repair and damage removal mechanisms induces genomic instability. Apoptosis preserves genomic stability therefore, its inhibition may promote genomic instability.

3.2 Gene expression

Although global protein synthesis is reduced in cells exposed to hypoxia, there is evidence that a number of genes are specifically up-regulated under hypoxic conditions both in normal and malignant cells, either through transcriptional activation or through post-transcriptional modifications. These responses promote cell adaptation to the adverse microenvironment, and thus promote essential functions for cell survival and growth under such conditions. Some of these genes may be involved in metastasis as discussed in Section 4. One of the key hypoxia regulatory transcription factors is HIF-1, which regulates more than 60 target genes involved in angiogenesis, metabolism, proliferation and survival (80). HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits. While the HIF-1β protein subunit is constitutively expressed, the HIF-1α protein subunit is negatively regulated under normoxic conditions through several post-translational modifications. First, under oxygenated conditions proline residues 402 and 564 are hydroxylated by oxygen-dependent prolyl hydroxylases. This modification facilitates the binding of the von Hippel-Lindau (VHL) tumour suppressor protein (an E3 ubiquitin ligase) that targets the HIF-1α protein for ubiquitylation and proteosomal degradation. Second, acetylation of lysine 532 by acetyltransferase stimulates HIF-1α degradation by inducing its interaction with VHL. Third, oxygen-dependent hydroxylation of asparagine 803 by aspargyl hydroxylase/factor inhibiting HIF-1 (FIH-1) sterically inhibits the interaction of HIF-1α with its co-activator CBP/p300 and prevents the initiation of

transcription. Finally, HIF-1 stability is regulated by several non-hydroxylase proteins, such as Hsp90, Cdc42, Rac1 and RhoA, of which the latter is also ROS-inducible (81).

The response of HIF-1α protein levels in cells to changes in oxygen levels is rapid with stabilization by hypoxic exposure and a half-life upon reoxygenation of a few minutes (81). The maximal protein levels are achieved at oxygen concentrations of 0.5% O2 (~ 3.8 mmHg). HIF-1α can also be activated by ROS, lactate, cytokines and growth factors. Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and insulin-like growth factors (IGF) act through a mitogen-activated protein kinase (MAPK) cascade resulting in increased HIF-1α transcriptional activity, or through a phosphatidyl inositol 3-kinase (PI3K) cascade leading to increased HIF-1α protein synthesis. In tumours, HIF-1 may also be activated as a result of the inactivation of the *VHL* gene (renal tumours) or by activation of oncogenes such as *Ras* or *Src*, which can up-regulate the MAPK or PI3K pathways.

Transcriptional regulation occurs by HIF-1 binding in a sequence-specific manner to hypoxia responsive elements (HRE) in target genes. Although stabilization of HIF-1α protein can up-regulate downstream genes such as *VEGF*, the constitutive *HIF-1α* mRNA levels can vary between different cell lines and might be of importance for the early induction of hypoxia-regulated genes and for the development of metastatic disease (82). However, the exact role of HIF-1 in tumour development is unclear. Some studies have reported reduced growth in allografted tumours from *HIF-1α* and *HIF-1β* deficient cell lines, but others have reported opposite findings (80). Immunohistological analysis has shown an over-expression of HIF-1α in many human cancers and their metastases. HIF-1α has also been associated with poor outcome in many, but not all studies of human cancers (Table 3, Section 5). Although HIF-1 is a key regulator of gene expression under hypoxic conditions, other stress-responsive transcription factors have also been shown to be up-regulated by hypoxia, such as AP-1, NF-κB, early growth response-1 (Egr-1), specificity protein-1 (SP-1), cyclic AMP-response-element-binding protein (CREB), p53, CAAT enhancer binding protein-β (C/EPB)-β and activating transcription factor-4 (ATF-4)(83,84).

4. THE METASTATIC PROCESS – ROLE OF THE TUMOUR MICROENVIRONMENT

The process of metastasis is often described as a series of stages that include invasion of tumour cells from the primary tumour into blood or lymph vessels, survival of the cells in the circulation, arrest in the capillary bed of a secondary organ, extravasation from the vessel into the surrounding tissue of the secondary organ and proliferation of the cells in the secondary organ (Figure 4). Each of these stages involves interactions between the tumour cells and the host. The molecular mechanisms involved in these stages are diverse, but the factors that are generally accepted to play important roles include cell-cell and cell-matrix adhesion, degradation of extracellular matrix by proteinases, survival in the circulation, and the initiation and maintenance of early growth at new sites.

4.1 Intravasation and extravasation

To metastasize, a tumour cell needs to invade a blood or lymphatic vessel, a process known as intravasation. The tumour cell has to migrate through the extracellular matrix and transverse the basement membrane. This involves the processes of detachment, mediated by adhesion proteins responsible for cell-cell and cell-matrix interactions such as cadherins and integrins, migration mediated by chemokines, and degradation of the extracellular matrix mediated by a number of proteinases and their inhibitors. Many of these molecules can be regulated by the hypoxic or acidic microenvironment, suggesting a direct role of the microenvironment in invasion (86, 87).

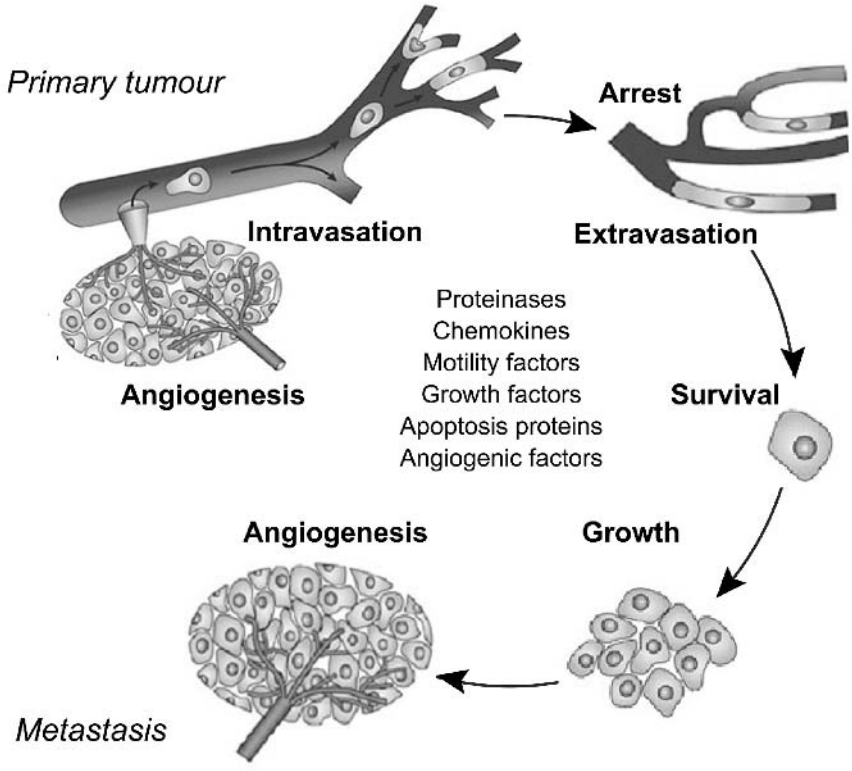


Figure 4. The metastatic process. Indicated are groups of genes that are known to be regulated by the tumour microenvironment. Modified by R.A. Cairns from Chambers et al. (85).

A number of proteinases that have been implicated in invasion and metastasis, have been found to be regulated by the tumour microenvironment (Table 2). Plasmin, a serine protease that is formed by activation of plasminogen by the plasminogen activators, tPA and uPA, is often found in tumours as a result of vascular hyperpermeability. It facilitates tumour cell migration and invasion by degrading fibrin and other matrix proteins and by activating MMPs. The plasminogen activator uPA binds to a receptor, uPAR, on the tumour cell surface and can localize the activity of plasmin to the local tumour cell microenvironment. Recently uPAR has been shown to be up-regulated by hypoxia resulting in enhanced invasion and the promotion of lymph node metastasis (88, 89). Hypoxia-induced up-regulation of MMP-9 and down-regulation of tissue inhibitor of metalloproteinase-1 (TIMP-1) was demonstrated to be associated with enhanced invasion by MDA MB231 breast carcinoma cells *in vitro*, suggesting that hypoxia can modify tumour cell invasiveness by regulating the balance between proteinases and their inhibitors.

There is also evidence that hypoxia might have a role in enhancing cell motility. Hepatocyte growth factor (HGF) is a cytokine involved in normal and neoplastic cell growth. Its biological effect is mediated by a tyrosine kinase receptor encoded by the *c-MET* proto-oncogene, which is over-expressed in several types of cancer. Recently it was shown that hypoxia sensitizes cells to HGF stimulation by up-regulating the c-MET receptor, resulting in cell migration and extracellular matrix invasion *in vitro* (90). Also, autocrine motility factor (AMF) has been reported to be up-regulated by hypoxia, resulting in enhanced random motility of pancreatic cancer cells (91). Tumour cells may home to specific target organs by the expression of certain chemokines or their receptors. The chemokine C-X-C receptor type

4 (CXCR4) has been reported to be up-regulated by hypoxia via HIF-1, and was associated with poor disease-specific survival in renal cell carcinoma (92,93). This receptor has also been associated with metastasis in other tumour types such as colon and breast carcinoma. Similarly, nutrient deprivation and acidic pHe have been reported to increase motility and invasiveness of human tumour cells through PI3K-dependent inhibition of the pHi-regulating Na+/H+ exchanger and activated MMPs, respectively (94,95).

Table 2. Metastasis- or invasion-associated genes or gene products regulated by the tumour microenvironment. Referenced are experimental studies showing an association between the gene or gene product and the microenvironment in vivo or in vitro.

Gene ID	Gene name	Regulation	Reference
Intravasation/ extravasation			
MMP-9	Matrix metalloproteinase-9	Hypoxia, acidity	96,97
TIMP-1	Tissue inhibitor of metalloproteinase-1	Hypoxia	97
uPAR	Urokinase type plasminogen activator receptor	Hypoxia	88,89
PAI-1	Plasminogen activator inhibitor-1	Hypoxia	98
c-MET		Hypoxia	90
AMF	Autocrine motility factor	Hypoxia	91
CXCR4	C-X-C receptor type 4	Hypoxia	92,93
CatB	Cathepsin B	Hypoxia, acidity	99,100
Survival and growth			
Mdm2	Mouse double minute 2	Hypoxia	26
ILK	Integrin-linked kinase	Hypoxia	101
OPN	Osteopontin	Hypoxia	102
Angiogenesis			
VEGF	Vascular endothelial growth factor	Hypoxia, acidity, nutrient deprivation	103,104
IL-8	Interleukin-8	Hypoxia, acidity, nutrient deprivation	28,104-106
ANG	Angiogenin	Hypoxia	107
bFGF	Basic fibroblast growth factor	Hypoxia	108
PD-ECGF	Platelet derived endothelial cell growth factor	Hypoxia, acidity	109

4.2 Survival and growth

Only a small proportion of tumour cells released into the circulation form macroscopic metastases, thus the efficiency of metastasis is low. Cells may be destroyed in the circulation either by the immune system, by hemodynamic forces, or stress-induced apoptosis. Cells can arrest due to size constraints or by attachment to the vascular wall at the secondary site, which is usually the first capillary bed they encounter. It has been generally thought that the ability of tumour cells to extravasate is an important limiting step in metastasis formation (85), although some studies have indicated that micrometastases can form in blood vessels without initial extravasation (110, 111). Furthermore, intravital microscopy studies have shown that, for some tumour cells, extravasation can be efficient, allowing for most of the cells (> 80%) that enter the circulation to move from the vasculature into the interstitial space. On the other hand, only a small proportion of these cells were found to initiate cell

division to form micrometastases, and only a proportion of these became vascularized and grew into macroscopic metastases (85). These data indicate that growth after extravasation can be a critical limiting factor in metastatic spread. Recently CXCR4, which can be induced by hypoxia, was identified as one factor required for the outgrowth of colon carcinoma micrometastasis in the lung (112).

Apoptosis can be an important determinant for metastatic inefficiency, as a significantly higher number of apoptotic cells was demonstrated among cells arrested in the lung following intraveneous injection of a low-metastatic versus a high-metastatic cell line (113). Although hypoxia can induce apoptosis through both HIF-mediated and -independent pathways (114), hypoxia and acidic pHe may also select for cells with diminished apoptotic potential that have a survival advantage as discussed in Section 3. Apoptotic resistance of hypoxic cells can be mediated by several mechanisms, such as up-regulation of inhibitor of apoptosis protein-2 (IAP-2), or up-regulation of telomerase activity (115,116). Recent data suggest that hypoxia-induced resistance to apoptosis might be an important mechanism for the enhanced number of lung metastases observed after intravenous injection of hypoxia-pretreated KHT tumour cells. This effect was mediated through increased expression of the *Mdm2* gene, independently of p53 (26).

Another reason for metastatic inefficiency is lack of growth (dormancy) of solitary tumour cells or, in small microscopic lesions, a balance between cell proliferation and cell death (117). Growth of tumour cells at a metastatic site requires the presence of growth factors either produced by the tumour cells (autocrine) or present in the surrounding tissue. Inhibitory factors may also be present in the tissue. Thus, tumour growth is a result of a positive interaction between the tumour cell phenotype and the tissue in which the tumour is growing (85). A number of metastasis suppressor genes have been identified that can suppress growth at a secondary site without suppressing primary tumour growth (118). The role of the tumour microenvironment, if any, in regulating these metastasis suppressor genes remains unknown. Recent work has suggested that proteases, such as those involved in intra- and extravasation, may play an additional role in metastasis by facilitating the release of growth factors from the extracellular matrix to promote metastatic growth at a secondary site (119). Up-regulation of such molecules by exposure to the tumour microenvironment may play a role at more than one stage of the metastatic process (Figure 4).

4.3 Angiogenesis

Angiogenesis is important for metastasis both in terms of new vessel formation in the primary tumour, allowing tumour cells to more easily enter the circulation, and for the ability of tumour cells to survive and grow into macroscopic nodules at the metastatic site. A number of angiogenic factors have been shown to be induced by the tumour microenvironment, including VEGF, IL-8, angiogenin (ANG), bFGF and platelet derived endothelial cell growth factor (PD-ECGF) (Table 2), although inhibition of vascular development by acidic pH has also been reported (120). A number of clinical studies have reported that regions of high vascular density (hot spots) are associated with more aggressive disease (121). Experimentally, vascular hot spots, induced in hypoxic foci, were found to be associated with subsequent spontaneous lung metastasis formation. This effect was mediated by the angiogenic factors VEGF and IL-8, the latter of which co-localized with the hypoxic foci (28). A direct role of microenvironmentally-induced angiogenesis at the secondary site in enhancing initial metastasis formation has not been demonstrated. However, it was shown that human melanoma cells exposed to hypoxia *in vitro* could form an increased number of lung metastases when injected into mice, and this effect was associated with an up-regulation of VEGF and could be blocked by anti-VEGF antibody (122). The ability of tumour cells to produce angiogenic factors and recruit new blood vessels is crucial for microscopic metastases to grow into macroscopic lesions.

5. MICROENVIRONMENTALLY-REGULATED GENES AS INTRINSIC PROGNOSTIC MARKERS

A large number of genes or gene products have been found to be associated with metastasis or poor prognosis in human tumours. The expression of some of these genes has also been found to be regulated by the pathophysiological tumour microenvironment as discussed above. Hence, they are potentially useful as intrinsic markers of these conditions and might be used as joint prognostic markers (Table 3). The microregional distribution of the markers can be assessed on histological sections in relation to other morphological or molecular features, even retrospectively, and thus might provide detailed information about the local tumour microenvironment and the potential for metastatic spread.

Table 3. Microenvironmentally-regulated genes or gene products as intrinsic prognostic markers for solid tumours. ↑ good prognosis, ↓ poor prognosis, ~ no effect. Listed are recent studies or recent review articles. The list is not comprehensive, and the exact definition of the end-points might vary from study to study.

Gene ID	Gene name	Tumour type	Reference
ANG	Angiogenin	Bladder↓, breast↓ and pancreatic↓	123
AMF	Autocrine motility factor	Lung↓	124
bFGF	Basic fibroblast growth factor	Bladder↓, breast↑–, colorectal↓–, gastric↓, head and neck↓–, hepatocellular↓, lung↓–, ovarian↓↑ and pancreatic↓	123
CA IX	Carbonic anhydrase IX	Breast↓, cervical↓↑–, head and neck↓–, nasopharyngeal–, non-small-cell lung↓ and renal clear cell↑	125-128
CA XII	Carbonic anhydrase XII	Breast↓↑	129
CatB	Cathepsin B	Breast–, non-small-cell lung↓ and ovarian↓	130-132
CXCR4	C-X-C receptor type 4	Clear renal cell↓ and non-small-cell lung↓	92,133
Glut-1	Glucose transporter-1	Bladder↓, cervical↓, colorectal↓, gastric↓, non-small-cell lung↓, ovarian↓ and rectal↓	125,126
HIF-1α	Hypoxia-inducible factor-1α	Breast↓, cervical↓–, endometrial↓, head and neck↓↑, nasopharyngeal–, non-small-cell lung–↑, oropharyngeal↓, ovarian– and oligodendroglioma↓	80,125
IL-8	Interleukin-8	hepatocellular↓, melanoma↓ , non-small-cell lung↓ and ovarian↓	134-137
ILK	Integrin-linked kinase	Melanoma↓	138
c-MET		Breast↓ and prostate↓	139
MMP-9	Matrix metalloproteinase-9	Colorectal↓, gastric↓, head and neck↓, lung↓, non-small-cell lung↓ and ovarian↓	140
OPN	Osteopontin	Breast↓, colorectal↓, head and neck↓, hepatocellular↓, lung↓ and prostate↓	141
PAI-1	Plasminogen activator inhibitor-1	Breast↓, cervical↓, gastric↓ and lung↓–, ovarian↓, renal cell↓,	142,143
PD-ECGF	Platelet derived endothelial cell growth factor	Bladder↓, breast↓, colorectal↓↑–, gastric↓, head and neck↓, hepatocellular↓–, lung↓, ovarian↓ and pancreatic↓	123
TIMP-1	Tissue inhibitor of metalloproteinase-1	Bladder↓, breast↓, colorectal↓, gastric↓, prostate↓↑ and lung↓	144,145
uPAR	Urokinase type plasminogen activator receptor	Bladder↓, breast↓–, colorectal↓, endometrial–, hepatocellular↓, non-small-cell lung–, ovarian–, renal cell↓, squamous cell lung↓ and chondrosarcoma↓	146
VEGF	Vascular endothelial growth factor	Bladder↓–, breast↓, colorectal↓, gastric↓–, head and neck↓–, hepatocellular↓–, lung↓, pancreatic↓, prostate↓, ovarian↓, melanoma↓ and osteosarcoma↓	123

Indeed, the expression of a number of genes or gene products regulated by hypoxic or acidic conditions *in vitro* such as HIF-1, VEGF, carbonic anhydrase IX (CA IX) and Glut-1 have recently been shown to correlate with hypoxia or acidosis *in vivo*. Although correlations between these markers and poor outcome have been demonstrated in a number of tumour types, this is not seen in all studies (Table 3). For instance, an association between CA IX and hypoxia was demonstrated in two independent studies of cervical carcinoma, although a correlation between CA IX levels and poor outcome was found in only one of these studies. Co-localization between different markers is not always observed, possibly reflecting that different levels and durations of microenvironmental stress are required to induce different proteins, and/or that these proteins can be regulated by alternate pathways (126). A recent study showed that CA IX up-regulation by hypoxia was further enhanced by glucose or bicarbonate deprivation, showing that CA IX up-regulation can reflect other microenvironmental stresses in addition to hypoxia (147). There are also inconsistencies in the prognostic value of the same marker between different tumour types (Table 3). While most studies report CA IX to be a marker of poor prognosis, it was associated with good prognosis in renal carcinoma. These tumours have a high frequency of *VHL* mutations, and hence, a constitutive up-regulation of HIF-1α.

In addition to being indicative of prognosis, microenvironmentally-regulated gene products can also have a direct role in promoting tumour progression. The prognostic markers identified in Table 3 include genes that are known to have a role in angiogenesis, cell-cell and cell-matrix interactions, and/or cell survival and growth. Indeed, some of these genes have been shown to promote metastasis when studied in experimental models, as was the case for the angiogenic factors, VEGF, IL-8, PD-ECGF and bFGF (122). Some gene products might have several functions. Plasminogen activator inhibitor-1 (PAI-1), an inhibitor of tPA and uPA activity, is implicated in malignant progression and has also been shown to be up-regulated by hypoxia (98). It has been shown to be involved in cell adhesion, signalling and tissue remodelling, and may therefore play a role at more than one stage of the metastatic process.

The complexity of the metastatic process suggests that enhanced metastasis most likely occurs as a result of coordinated regulation of many genes. The recent development of methods for large-scale analysis of gene expression such as representational difference analysis (RDA), serial analysis of gene expression (SAGE) and high density cDNA microarrays have allowed for studies of differences in expression in large groups of genes between different cell populations. This type of analysis allows for faster identification of novel genes that are regulated by the tumour microenvironment or identification of a role of the tumour microenvironment in regulating already known genes (recently reviewed by Subarsky (24)).

From studies of normal and transformed cell lines it has been estimated that approximately 1.5% of the genome is transcriptionally responsive to hypoxic treatment *in vitro (*83). Recent studies have identified the metastasis-related genes PAI-1 and osteopontin (OPN) as being up-regulated in hypoxic tumours and suggest that they may be useful as serum markers for tumour hypoxia (98,102). Another study identified integrin-linked kinase (ILK) as being hypoxia-responsive (Table 2). It localizes to focal adhesion plaques by interacting with the cytoplasmic domains of integrin β1 and β3, and over-expression of ILK has been shown to inhibit anoikis, suggesting that hypoxic induction of ILK might enhance anchorage-independent survival. An association between ILK and poor prognosis was reported for human melanoma (Table 3).

Coordinated regulation of many genes after exposure to microenvironmental stress conditions can also help in identifying specific functional responses. A recent study classified hypoxia-regulated genes into functional categories, and showed that the largest functional groups represented genes involved in metabolism/transport, angiogenesis, tissue remodeling, apoptosis, proliferation/differentiation and gene expression (83). To address microenvironment-specific versus cell-type specific changes in gene expression, another study used cDNA microarray technology to identify functional groups of genes that were differentially regulated when a lung adenocarcinoma

and a glioblastoma cell line were grown subcutaneously *in vivo* versus *in vitro* (148). *In vitro* growth resulted predominantly in up-regulation of genes associated with cell division and metabolism, while *in vivo* growth resulted in up-regulation of genes associated with the extracellular matrix, cell adhesion, cytokine and metalloendopeptidase activity, and neovascularization. However, the two cell lines expressed different sets of these genes, suggesting that different genes are involved in similar mechanisms in different cancers. These results emphasize the importance of the microenvironment as well as the cell type in gene regulation. Similar strategies could be used to identify functional groups of genes of importance for tumour progression and metastasis after exposures to different microenvironments *in vitro* and *in vivo*.

6. SUMMARY

The tumour microenvironment can contribute to tumour progression and metastasis. Clinical investigations in cervical carcinoma and soft tissue sarcoma have suggested that the development of metastatic disease is associated with low oxygen levels, high lactate levels, or elevated IFP in the primary tumour. Experimental studies have shown that exposure of murine and human tumour cell lines to hypoxic or acidic conditions lead to increased metastases. Hypoxia may contribute to metastatic progression through alternate mechanisms, such as the induction of genomic instability or epigenetic regulation of gene expression. Furthermore, the tumour microenvironment may provide a selective pressure enriching for cells that are more aggressive or resistant to apoptosis. Evidence suggests that altered gene expression in response to the hypoxic microenvironment is a contributing factor to increased metastatic efficiency. The genes implicated are involved in processes such as adhesion to and degradation of the extracellular matrix, stimulation of growth and angiogenesis. The molecular aspects of metastasis are diverse, due to the complex interactions that occur during all stages of the process between the tumour cell and its immediate and more extended microenvironment.

REFERENCES

1. Brown, J. M., 2000, Exploiting the hypoxic cancer cell: mechanisms and therapeutic strategies. Mol Med Today, 7:157-162.
2. Hockel, M., and Vaupel, P., 2001, Biological consequences of tumor hypoxia. Semin Oncol, 28:36-41.
3. Hockel, M., and Vaupel, P., 2001, Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst, 93:266-276
4. Dewhirst, M. W., 1998, Concepts of oxygen transport at the microcirculatory level. Semin Radiat Oncol, 8:143-150.
5. Becker, A., Hansgen, G., Bloching, M., Weigel, C., Lautenschlager, C., and Dunst, J., 1998, Oxygenation of squamous cell carcinoma of the head and neck: comparison of primary tumors, neck node metastases, and normal tissue. Int J Radiat Oncol Biol Phys, 42:35-41.
6. Le, Q. T., Kovacs, M. S., Dorie, M. J., Koong, A., Terris, D. J., Pinto, H. A., Goffinet, D. R., Nowels, K., Bloch, D., and Brown, J. M., 2003, Comparison of the comet assay and the oxygen microelectrode for measuring tumor oxygenation in head-and-neck cancer patients. Int J Radiat Oncol Biol Phys, 56:375-383.
7. Koong, A. C., Mehta, V. K., Le, Q. T., Fisher, G. A., Terris, D. J., Brown, J. M., Bastidas, A. J., and Vierra, M., 2000, Pancreatic tumors show high levels of hypoxia. Int J Radiat Oncol Biol Phys, 48:919-922.
8. Parker, C., Milosevic, M., Toi, A., Sweet, J., Panzarella, T., Bristow, R., Catton, C., Catton, P., Crook, J., Gospodarowicz, M., et al., 2004, Polarographic electrode study of tumor oxygenation in clinically localized prostate cancer. Int J Radiat Oncol Biol Phys, 58:750-757.
9. Loncaster, J. A., Harris, A. L., Davidson, S. E., Logue, J. P., Hunter, R. D., Wycoff, C. C., Pastorek, J., Ratcliffe, P. J., Stratford, I. J., and West, C. M., 2001, Carbonic anhydrase (CA IX) expression, a potential new intrinsic marker of hypoxia: correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. Cancer Res, 61:6394-6399.
10. Bentzen, L., Keiding, S., Nordsmark, M., Falborg, L., Hansen, S. B., Keller, J., Nielsen, O. S., and Overgaard, J., 2003, Tumour oxygenation assessed by 18F-fluoromisonidazole PET and polarographic needle electrodes in human soft tissue tumours. Radiother Oncol, 67:339-344.
11. Vaupel, P., Mayer, A., Briest, S., and Hockel, M., 2003, Oxygenation gain factor: a novel parameter characterizing the association between hemoglobin

level and the oxygenation status of breast cancers. Cancer Res, 63:7634-7637.

12. Tyler, J. L., Diksic, M., Villemure, J. G., Evans, A. C., Meyer, E., Yamamoto, Y. L., and Feindel, W., 1987, Metabolic and hemodynamic evaluation of gliomas using positron emission tomography. J Nucl Med, 28:1123-1133.
13. Shinkwin, M. A., Lenkinski, R. E., Daly, J. M., Zlatkin, M. B., Frank, T. S., Holland, G. A., and Kressel, H. Y., 1991, Integrated magnetic resonance imaging and phosphorus spectroscopy of soft tissue tumors. Cancer, 67:1849-1858.
14. van de Merwe, S. A., van den Berg, A. P., Kroon, B. B., van den Berge, A. W., Klaase, J. M., and van der Zee, J., 1993, Modification of human tumour and normal tissue pH during hyperthermic and normothermic antiblastic regional isolation perfusion for malignant melanoma: a pilot study. Int J Hyperthermia, 9:205-217.
15. Engin, K., Leeper, D. B., Cater, J. R., Thistlethwaite, A. J., Tupchong, L., and McFarlane, J. D., 1995, Extracellular pH distribution in human tumours. Int J Hyperthermia, 11:211-216.
16. Boucher, Y., Kirkwood, J. M., Opacic, D., Desantis, M., and Jain, R. K., 1991, Interstitial hypertension in superficial metastatic melanomas in humans. Cancer Res, 51:6691-6694.
17. Roh, H. D., Boucher, Y., Kalnicki, S., Buchsbaum, R., Bloomer, W. D., and Jain, R. K., 1991, Interstitial hypertension in carcinoma of uterine cervix in patients: possible correlation with tumor oxygenation and radiation response. Cancer Res, 51:6695-6698.
18. Less, J. R., Posner, M. C., Boucher, Y., Borochovitz, D., Wolmark, N., and Jain, R. K., 1992, Interstitial hypertension in human breast and colorectal tumors. Cancer Res, 52:6371-6374.
19. Gutmann, R., Leunig, M., Feyh, J., Goetz, A. E., Messmer, K., Kastenbauer, E., and Jain, R. K., 1992, Interstitial hypertension in head and neck tumors in patients: correlation with tumor size. Cancer Res, 52:1993-1995.
20. Höckel, M., Schlenger, K., Aral, B., Mitze, M., Schaffer, U., and Vaupel, P., 1996, Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res, 56:4509-4515.
21. Hall, E. J., 2000, Radiobiology for the Radiologist, 5th edition. Lippincott Williams and Wilkins, Philadelphia PA.
22. Brizel, D. M., Scully, S. P., Harrelson, J. M., Layfield, L. J., Bean, J. M., Prosnitz, L. R., and Dewhirst, M. W., 1996, Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. Cancer Res, 56:941-943.
23. Rofstad, E. K., 2000, Microenvironment-induced cancer metastasis. Int J Radiat Biol, 76:589-605.
24. Subarsky, P. and Hill, R. P., 2003, The hypoxic tumour microenvironment and metastatic progression. Clin Exp Metastasis, 20:237-250.
25. Coomber, B. L., Yu, J. L., Fathers, K. E., Plumb, C., and Rak, J. W., 2003, Angiogenesis and the role of epigenetics in metastasis. Clin Exp Metastasis, 20:215-227.
26. Zhang, L. and Hill, R. P., 2004, Hypoxia enhances metastatic efficiency by up regulating mdm2 in KHT cells and increasing resistance to apoptosis.
27. Airley, R., Loncaster, J., Davidson, S., Bromley, M., Roberts, S., Patterson, A., Hunter, R., Stratford, I., and West, C., 2001, Glucose transporter glut-1 expression correlates with tumor hypoxia and predicts metastasis-free survival in advanced carcinoma of the cervix. Clin Cancer Res, 7:928-934.
28. Rofstad, E. K. and Halsør, E. F., 2002, Hypoxia-associated spontaneous pulmonary metastasis in human melanoma xenografts: involvement of microvascular hot spots induced in hypoxic foci by interleukin 8. Br J Cancer, 86:301-308.
29. Cairns, R. A., Kalliomaki, T., and Hill, R. P., 2001, Acute (cyclic) hypoxia enhances spontaneous metastasis of KHT murine tumors. Cancer Res, 61:8903-8908.
30. Cairns, R. A., and Hill, R. P., 2004, Acute hypoxia enhances spontaneous lymph node metastasis in an orthotopic murine model of human cervical carcinoma. Cancer Res, 64:2054-2061.
31. Stubbs, M., McSheehy, P. M., Griffiths, J. R., and Bashford, C. L., 2000, Causes and consequences of tumour acidity and implications for treatment. Mol Med Today, 6:15-19.
32. Griffiths, J. R., 1991, Are cancer cells acidic? Br J Cancer, 64:425-427.
33. Walenta, S., Snyder, S., Haroon, Z. A., Braun, R. D., Amin, K., Brizel, D., Mueller-Klieser, W., Chance, B., and Dewhirst, M. W., 2001, Tissue gradients of energy metabolites mirror oxygen tension gradients in a rat mammary carcinoma model. Int J Radiat Oncol Biol Phys, 51:840-848.
34. Helmlinger, G., Yuan, F., Dellian, M., and Jain, R. K., 1997, Interstitial pH and pO2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. Nat Med, 3:177-182.
35. Schlappack, O. K., Zimmermann, A., and Hill, R. P., 1991, Glucose starvation and acidosis: effect on experimental metastatic potential, DNA content and MTX resistance of murine tumour cells. Br J Cancer, 64:663-670.
36. Jang, A., and Hill, R. P., 1997, An examination of the effects of hypoxia, acidosis, and glucose starvation on the expression of metastasis-associated genes in murine tumor cells. Clin Exp Metastasis, 15:469-483.

37. Kalliomaki, T. and Hill, R. P., 2004, Effects of tumour acidification with glucose+MIBG on the spontaneous metastatic potential of two murine cell lines. Br J Cancer, 13:13.
38. Knocke, T. H., Weitmann, H. D., Feldmann, H. J., Selzer, E., and Potter, R., 1999, Intratumoral pO2-measurements as predictive assay in the treatment of carcinoma of the uterine cervix. Radiother Oncol, 53:99-104.
39. Sundfør, K., Lyng, H., Trope, C. G., and Rofstad, E. K., 2000, Treatment outcome in advanced squamous cell carcinoma of the uterine cervix: relationships to pretreatment tumor oxygenation and vascularization. Radiother Oncol, 54:101-107.
40. Fyles, A., Milosevic, M., Hedley, D., Pintilie, M., Levin, W., Manchul, L., and Hill, R. P., 2002, Tumor hypoxia has independent predictor impact only in patients with node-negative cervix cancer. J Clin Oncol, 20:680-687.
41. Walenta, S., Wetterling, M., Lehrke, M., Schwickert, G., Sundfor, K., Rofstad, E. K., and Mueller-Klieser, W., 2000, High lactate levels predict likelihood of metastases, tumor recurrence, and restricted patient survival in human cervical cancers. Cancer Res, 60:916-921.
42. Milosevic, M., Fyles, A., Hedley, D., Pintilie, M., Levin, W., Manchul, L., and Hill, R., 2001, Interstitial fluid pressure predicts survival in patients with cervix cancer independent of clinical prognostic factors and tumor oxygen measurements. Cancer Res, 61:6400-6405.
43. Nordsmark, M., Overgaard, M., and Overgaard, J., 1996, Pretreatment oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. Radiother Oncol, 41:31-39.
44. Brizel, D. M., Dodge, R. K., Clough, R. W., and Dewhirst, M. W., 1999, Oxygenation of head and neck cancer: changes during radiotherapy and impact on treatment outcome. Radiother Oncol, 53:113-117.
45. Adam, M. F., Gabalski, E. C., Bloch, D. A., Oehlert, J. W., Brown, J. M., Elsaid, A. A., Pinto, H. A., and Terris, D. J., 1999, Tissue oxygen distribution in head and neck cancer patients. Head Neck, 21:146-153.
46. Nordsmark, M. and Overgaard, J., 2000, A confirmatory prognostic study on oxygenation status and loco-regional control in advanced head and neck squamous cell carcinoma treated by radiation therapy. Radiother Oncol, 57:39-43.
47. Rudat, V., Stadler, P., Becker, A., Vanselow, B., Dietz, A., Wannenmacher, M., Molls, M., Dunst, J., and Feldmann, H. J., 2001, Predictive value of the tumor oxygenation by means of pO2 histography in patients with advanced head and neck cancer. Strahlenther Onkol, 177:462-468.
48. Walenta, S., Salameh, A., Lyng, H., Evensen, J. F., Mitze, M., Rofstad, E. K., and Mueller-Klieser, W., 1997, Correlation of high lactate levels in head and neck tumors with incidence of metastasis. Am J Pathol, 150:409-415.
49. Brizel, D. M., Schroeder, T., Scher, R. L., Walenta, S., Clough, R. W., Dewhirst, M. W., and Mueller-Klieser, W., 2001, Elevated tumor lactate concentrations predict for an increased risk of metastases in head-and-neck cancer. Int J Radiat Oncol Biol Phys, 51:349-353.
50. Allal, A. S., Dulguerov, P., Allaoua, M., Haenggeli, C. A., El-Ghazi el, A., Lehmann, W., and Slosman, D. O., 2002, Standardized uptake value of 2-[(18)F] fluoro-2-deoxy-D-glucose in predicting outcome in head and neck carcinomas treated by radiotherapy with or without chemotherapy. J Clin Oncol, 20:1398-1404.
51. Nordsmark, M., Alsner, J., Keller, J., Nielsen, O. S., Jensen, O. M., Horsman, M. R., and Overgaard, J., 2001, Hypoxia in human soft tissue sarcomas: adverse impact on survival and no association with p53 mutations. Br J Cancer, 84:1070-1075.
52. Walenta, S., Chau, T. V., Schroeder, T., Lehr, H. A., Kunz-Schughart, L. A., Fuerst, A., and Mueller-Klieser, W., 2003, Metabolic classification of human rectal adenocarcinomas: a novel guideline for clinical oncologists? J Cancer Res Clin Oncol, 129:321-326. (Epub 2003, Jun 2025.)
53. Kunkel, M., Forster, G. J., Reichert, T. E., Kutzner, J., Benz, P., Bartenstein, P., and Wagner, W., 2003, Radiation response non-invasively imaged by [18F]FDG-PET predicts local tumor control and survival in advanced oral squamous cell carcinoma. Oral Oncol, 39:170-177.
54. Inoue, T., Yutani, K., Taguchi, T., Tamaki, Y., Shiba, E., and Noguchi, S., 2004, Preoperative evaluation of prognosis in breast cancer patients by [(18)F]2-Deoxy-2-fluoro-D-glucose-positron emission tomography. J Cancer Res Clin Oncol, 24:24.
55. Vaupel, P., Kallinowski, F., and Okunieff, P., 1989, Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Res, 49:6449-6465.
56. Thews, O., Kelleher, D. K., Esser, N., Kraus, S., and Vaupel, P., 2003, Lack of association between tumor hypoxia, GLUT-1 expression and glucose uptake in experimental sarcomas. Adv Exp Med Biol, 510:57-61.
57. Rajendran, J. G., Mankoff, D. A., O'Sullivan, F., Peterson, L. M., Schwartz, D. L., Conrad, E. U., Spence, A. M., Muzi, M., Farwell, D. G., and Krohn, K. A., 2004, Hypoxia and glucose metabolism in malignant tumors: evaluation by [(18)f]fluoromisonidazole and [(18)f]fluorodeoxyglucose positron emission

tomography imaging. Clin Cancer Res, 10:2245-2252.

58. Milosevic, M., Fyles, A., and Hill, R., 2001, Interstitial fluid pressure in cervical cancer: guide to targeted therapy. Am J Clin Oncol, 24:516-521.
59. Jain, R. K., 1994, Barriers to drug delivery in solid tumors. Sci Am, 271:58-65.
60. Nathanson, S. D., and Nelson, L., 1994, Interstitial fluid pressure in breast cancer, benign breast conditions, and breast parenchyma. Ann Surg Oncol, 1:333-338.
61. Rofstad, E. K., Tunheim, S. H., Mathiesen, B., Graff, B. A., Halsør, E. F., Nilsen, K., and Galappathi, K., 2002, Pulmonary and lymph node metastasis is associated with primary tumor interstitial fluid pressure in human melanoma xenografts. Cancer Res, 62:661-664.
62. Padera, T. P., Kadambi, A., di Tomaso, E., Carreira, C. M., Brown, E. B., Boucher, Y., Choi, N. C., Mathisen, D., Wain, J., Mark, E. J., et al., 2002, Lymphatic metastasis in the absence of functional intratumor lymphatics. Science, 296:1883-1886. (Epub 2002 Apr 1825.)
63. Yuan, J., and Glazer, P. M., 1998, Mutagenesis induced by the tumor microenvironment. Mutat Res, 400:439-446.
64. Jackson, A. L., and Loeb, L. A., 2001, The contribution of endogenous sources of DNA damage to the multiple mutations in cancer. Mutat Res, 477:7-21.
65. Marnett, L. J., 2002, Oxy radicals, lipid peroxidation and DNA damage. Toxicology, 181-182:219-222.
66. Cobbs, C. S., Whisenhunt, T. R., Wesemann, D. R., Harkins, L. E., Van Meir, E. G., and Samanta, M., 2003, Inactivation of wild-type p53 protein function by reactive oxygen and nitrogen species in malignant glioma cells. Cancer Res, 63:8670-8673.
67. Turpaev, K. T., 2002, Reactive oxygen species and regulation of gene expression. Biochemistry (Mosc), 67:281-292.
68. Brown, N. S., and Bicknell, R., 2001, Hypoxia and oxidative stress in breast cancer. Oxidative stress: its effects on the growth, metastatic potential and response to therapy of breast cancer. Breast Cancer Res, 3:323-327. (Epub 2001 Jul 2023.)
69. Yuan, J., Narayanan, L., Rockwell, S., and Glazer, P. M., 2000, Diminished DNA repair and elevated mutagenesis in mammalian cells exposed to hypoxia and low pH. Cancer Res, 60:4372-4376.
70. Mihaylova, V. T., Bindra, R. S., Yuan, J., Campisi, D., Narayanan, L., Jensen, R., Giordano, F., Johnson, R. S., Rockwell, S., and Glazer, P. M., 2003, Decreased expression of the DNA mismatch repair gene Mlh1 under hypoxic stress in mammalian cells. Mol Cell Biol, 23:3265-3273.
71. Kondo, A., Safaei, R., Mishima, M., Niedner, H., Lin, X., and Howell, S. B., 2001, Hypoxia-induced enrichment and mutagenesis of cells that have lost DNA mismatch repair. Cancer Res, 61:7603-7607.
72. Graeber, T. G., Osmanian, C., Jacks, T., Housman, D. E., Koch, C. J., Lowe, S. W., and Giaccia, A. J., 1996, Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature, 379:88-91.
73. Kim, C. Y., Tsai, M. H., Osmanian, C., Graeber, T. G., Lee, J. E., Giffard, R. G., DiPaolo, J. A., Peehl, D. M., and Giaccia, A. J., 1997, Selection of human cervical epithelial cells that possess reduced apoptotic potential to low-oxygen conditions. Cancer Res, 57:4200-4204.
74. Williams, A. C., Collard, T. J., and Paraskeva, C., 1999, An acidic environment leads to p53 dependent induction of apoptosis in human adenoma and carcinoma cell lines: implications for clonal selection during colorectal carcinogenesis. Oncogene, 18:3199-3204.
75. Malins, D. C., Polissar, N. L., and Gunselman, S. J. 1996, Progression of human breast cancers to the metastatic state is linked to hydroxyl radical-induced DNA damage. Proc Natl Acad Sci U S A 93: 2557-2563.
76. Guven, M., Ozturk, B., Sayal, A., Ozeturk, A., and Ulutin, T., 1999, Lipid peroxidation and antioxidant system in the blood of cancerous patients with metastasis. Cancer Biochem Biophys, 17:155-162.
77. Hockel, M., Schlenger, K., Hockel, S., and Vaupel, P., 1999, Hypoxic cervical cancers with low apoptotic index are highly aggressive. Cancer Res, 59:4525-4528.
78. Nagao, M., Ochiai, M., Okochi, E., Ushijima, T., and Sugimura, T., 2001, LacI transgenic animal study: relationships among DNA-adduct levels, mutant frequencies and cancer incidences. Mutat Res, 477:119-124.
79. Limoli, C. L., Giedzinski, E., Morgan, W. F., Swarts, S. G., Jones, G. D., and Hyun, W., 2003, Persistent oxidative stress in chromosomally unstable cells. Cancer Res, 63:3107-3111.
80. Semenza, G. L., 2003, Targeting HIF-1 for cancer therapy. Nat Rev Cancer, 3:721-732.
81. Hopfl, G., Ogunshola, O., and Gassmann, M., 2004, HIFs and tumors--causes and consequences. Am J Physiol Regul Integr Comp Physiol, 286:R608-623.
82. Koshikawa, N., Iyozumi, A., Gassmann, M., and Takenaga, K., 2003, Constitutive upregulation of hypoxia-inducible factor-1alpha mRNA occurring in highly metastatic lung carcinoma cells leads to vascular endothelial growth factor overexpression upon hypoxic exposure. Oncogene, 22:6717-6724.
83. Denko, N. C., Fontana, L. A., Hudson, K. M., Sutphin, P. D., Raychaudhuri, S., Altman, R., and Giaccia, A. J., 2003, Investigating hypoxic tumor physiology through gene expression patterns. Oncogene, 22:5907-5914.

84. Abramovitch, R., Tavor, E., Jacob-Hirsch, J., Zeira, E., Amariglio, N., Pappo, O., Rechavi, G., Galun, E., and Honigman, A., 2004, A pivotal role of cyclic AMP-responsive element binding protein in tumor progression. Cancer Res, 64:1338-1346.
85. Chambers, A. F., Groom, A. C., and MacDonald, I. C., 2002, Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer, 2:563-572.
86. Cairns, R. A., Khokha, R., and Hill, R. P., 2003, Molecular mechanisms of tumor invasion and metastasis: an integrated view. Curr Mol Med, 3:659-671.
87. Liotta, L. A. and Kohn, E. C., 2001, The microenvironment of the tumour-host interface. Nature, 411:375-379.
88. Postovit, L. M., Adams, M. A., Lash, G. E., Heaton, J. P., and Graham, C. H., 2002, Oxygen-mediated regulation of tumor cell invasiveness. Involvement of a nitric oxide signaling pathway. J Biol Chem, 277:35730-35737. (Epub 32002 Jul 35739.)
89. Rofstad, E. K., Rasmussen, H., Galappathi, K., Mathiesen, B., Nilsen, K., and Graff, B. A., 2002, Hypoxia promotes lymph node metastasis in human melanoma xenografts by up-regulating the urokinase-type plasminogen activator receptor. Cancer Res, 62:1847-1853.
90. Pennacchietti, S., Michieli, P., Galluzzo, M., Mazzone, M., Giordano, S., and Comoglio, P. M., 2003, Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell, 3:347-361.
91. Niizeki, H., Kobayashi, M., Horiuchi, I., Akakura, N., Chen, J., Wang, J., Hamada, J. I., Seth, P., Katoh, H., Watanabe, H., et al., 2002, Hypoxia enhances the expression of autocrine motility factor and the motility of human pancreatic cancer cells. Br J Cancer, 86:1914-1919.
92. Staller, P., Sulitkova, J., Lisztwan, J., Moch, H., Oakeley, E. J., and Krek, W., 2003, Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. Nature, 425:307-311.
93. Schioppa, T., Uranchimeg, B., Saccani, A., Biswas, S. K., Doni, A., Rapisarda, A., Bernasconi, S., Saccani, S., Nebuloni, M., Vago, L., et al., 2003, Regulation of the chemokine receptor CXCR4 by hypoxia. J Exp Med, 198:1391-1402.
94. Martinez-Zaguilan, R., Seftor, E. A., Seftor, R. E., Chu, Y. W., Gillies, R. J., and Hendrix, M. J., 1996, Acidic pH enhances the invasive behavior of human melanoma cells. Clin Exp Metastasis, 14:176-186.
95. Reshkin, S. J., Bellizzi, A., Albarani, V., Guerra, L., Tommasino, M., Paradiso, A., and Casavola, V., 2000, Phosphoinositide 3-kinase is involved in the tumor-specific activation of human breast cancer cell Na(+)/H(+) exchange, motility, and invasion induced by serum deprivation. J Biol Chem, 275:5361-5369.
96. Kato, Y., Nakayama, Y., Umeda, M., and Miyazaki, K., 1992, Induction of 103-kDa gelatinase/type IV collagenase by acidic culture conditions in mouse metastatic melanoma cell lines. J Biol Chem, 267:11424-11430.
97. Canning, M. T., Postovit, L. M., Clarke, S. H., and Graham, C. H., 2001, Oxygen-mediated regulation of gelatinase and tissue inhibitor of metalloproteinases-1 expression by invasive cells. Exp Cell Res, 267:88-94.
98. Koong, A. C., Denko, N. C., Hudson, K. M., Schindler, C., Swiersz, L., Koch, C., Evans, S., Ibrahim, H., Le, Q. T., Terris, D. J., et al., 2000, Candidate genes for the hypoxic tumor phenotype. Cancer Res, 60:883-887.
99. Cuvier, C., Jang, A., and Hill, R. P., 1997, Exposure to hypoxia, glucose starvation and acidosis: effect on invasive capacity of murine tumor cells and correlation with cathepsin (L + B) secretion. Clin Exp Metastasis, 15:19-25.
100. Sloane, B. F., Rozhin, J., Krepela, E., Ziegler, G., and Sameni, M., 1991, The malignant phenotype and cysteine proteinases. Biomed Biochim Acta, 50:549-554.
101. Scandurro, A. B., Weldon, C. W., Figueroa, Y. G., Alam, J., and Beckman, B. S., 2001, Gene microarray analysis reveals a novel hypoxia signal transduction pathway in human hepatocellular carcinoma cells. Int J Oncol, 19:129-135.
102. Le, Q. T., Sutphin, P. D., Raychaudhuri, S., Yu, S. C., Terris, D. J., Lin, H. S., Lum, B., Pinto, H. A., Koong, A. C., and Giaccia, A. J., 2003, Identification of osteopontin as a prognostic plasma marker for head and neck squamous cell carcinomas. Clin Cancer Res, 9:59-67.
103. Fukumura, D., Xu, L., Chen, Y., Gohongi, T., Seed, B., and Jain, R. K., 2001, Hypoxia and acidosis independently up-regulate vascular endothelial growth factor transcription in brain tumors in vivo. Cancer Res, 61:6020-6024.
104. Marjon, P. L., Bobrovnikova-Marjon, E. V., and Abcouwer, S. F., 2004, Expression of the pro-angiogenic factors vascular endothelial growth factor and interleukin-8/CXCL8 by human breast carcinomas is responsive to nutrient deprivation and endoplasmic reticulum stress. Mol Cancer, 3:4.
105. Shi, Q., Abbruzzese, J. L., Huang, S., Fidler, I. J., Xiong, Q., and Xie, K., 1999, Constitutive and inducible interleukin 8 expression by hypoxia and acidosis renders human pancreatic cancer cells more tumorigenic and metastatic. Clin Cancer Res, 5:3711-3721.
106. Karashima, T., Sweeney, P., Kamat, A., Huang, S., Kim, S. J., Bar-Eli, M., McConkey, D. J., and Dinney, C. P., 2003, Nuclear factor-kappaB

mediates angiogenesis and metastasis of human bladder cancer through the regulation of interleukin-8. Clin Cancer Res, 9:2786-2797.

107. Hartmann, A., Kunz, M., Kostlin, S., Gillitzer, R., Toksoy, A., Brocker, E. B., and Klein, C. E., 1999, Hypoxia-induced up-regulation of angiogenin in human malignant melanoma. Cancer Res, 59:1578-1583.
108. Le, Y. J. and Corry, P. M., 1999, Hypoxia-induced bFGF gene expression is mediated through the JNK signal transduction pathway. Mol Cell Biochem, 202:1-8.
109. Griffiths, L., Dachs, G. U., Bicknell, R., Harris, A. L., and Stratford, I. J., 1997, The influence of oxygen tension and pH on the expression of platelet-derived endothelial cell growth factor/thymidine phosphorylase in human breast tumor cells grown in vitro and in vivo. Cancer Res, 57:570-572.
110. Al-Mehdi, A. B., Tozawa, K., Fisher, A. B., Shientag, L., Lee, A., and Muschel, R. J., 2000, Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: a new model for metastasis. Nat Med, 6:100-102.
111. Wong, C. W., Song, C., Grimes, M. M., Fu, W., Dewhirst, M. W., Muschel, R. J., and Al-Mehdi, A. B., 2002, Intravascular location of breast cancer cells after spontaneous metastasis to the lung. Am J Pathol, 161:749-753.
112. Zeelenberg, I. S., Ruuls-Van Stalle, L., and Roos, E., 2003, The chemokine receptor CXCR4 is required for outgrowth of colon carcinoma micrometastases. Cancer Res, 63:3833-3839.
113. Wong, C. W., Lee, A., Shientag, L., Yu, J., Dong, Y., Kao, G., Al-Mehdi, A. B., Bernhard, E. J., and Muschel, R. J., 2001, Apoptosis: an early event in metastatic inefficiency. Cancer Res, 61:333-338.
114. Harris, A. L., 2002, Hypoxia--a key regulatory factor in tumour growth. Nat Rev Cancer, 2:38-47.
115. Dong, Z., Wang, J. Z., Yu, F., and Venkatachalam, M. A., 2003, Apoptosis-resistance of hypoxic cells: multiple factors involved and a role for IAP-2. Am J Pathol, 163:663-671.
116. Seimiya, H., Tanji, M., Oh-hara, T., Tomida, A., Naasani, I., and Tsuruo, T., 1999, Hypoxia up-regulates telomerase activity via mitogen-activated protein kinase signaling in human solid tumor cells. Biochem Biophys Res Commun, 260:365-370.
117. Luzzi, K. J., MacDonald, I. C., Schmidt, E. E., Kerkvliet, N., Morris, V. L., Chambers, A. F., and Groom, A. C., 1998, Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. Am J Pathol, 153:865-873.
118. Yoshida, B. A., Sokoloff, M. M., Welch, D. R., and Rinker-Schaeffer, C. W., 2000, Metastasis-suppressor genes: a review and perspective on an emerging field. J Natl Cancer Inst, 92:1717-1730.
119. Egeblad, M. and Werb, Z., 2002, New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer, 2:161-174.
120. Burbridge, M. F., West, D. C., Atassi, G., and Tucker, G. C., 1999, The effect of extracellular pH on angiogenesis in vitro. Angiogenesis, 3:281-288.
121. Weidner, N., 1998, Tumoural vascularity as a prognostic factor in cancer patients: the evidence continues to grow. J Pathol, 184:119-122.
122. Rofstad, E. K. and Halsor, E. F., 2000, Vascular endothelial growth factor, interleukin 8, platelet-derived endothelial cell growth factor, and basic fibroblast growth factor promote angiogenesis and metastasis in human melanoma xenografts. Cancer Res, 60:4932-4938.
123. Poon, R. T., Fan, S. T., and Wong, J., 2001, Clinical implications of circulating angiogenic factors in cancer patients. J Clin Oncol, 19:1207-1225.
124. Takanami, I., Takeuchi, K., Naruke, M., Kodaira, S., Tanaka, F., Watanabe, H., and Raz, A., 1998, Autocrine motility factor in pulmonary adenocarcinomas: results of an immunohistochemical study. Tumour Biol, 19:384-389.
125. Vordermark, D. and Brown, J. M., 2003, Endogenous Markers of Tumor Hypoxia. Predictors of Clinical Radiation Resistance? Strahlenther Onkol, 179:801-811.
126. Bussink, J., Kaanders, J. H., and van der Kogel, A. J., 2003, Tumor hypoxia at the micro-regional level: clinical relevance and predictive value of exogenous and endogenous hypoxic cell markers. Radiother Oncol, 67:3-15.
127. Hedley, D., Pintilie, M., Woo, J., Morrison, A., Birle, D., Fyles, A., Milosevic, M., and Hill, R., 2003, Carbonic anhydrase IX expression, hypoxia, and prognosis in patients with uterine cervical carcinomas. Clin Cancer Res, 9:5666-5674.
128. Bui, M. H., Seligson, D., Han, K. R., Pantuck, A. J., Dorey, F. J., Huang, Y., Horvath, S., Leibovich, B. C., Chopra, S., Liao, S. Y., et al., 2003, Carbonic anhydrase IX is an independent predictor of survival in advanced renal clear cell carcinoma: implications for prognosis and therapy. Clin Cancer Res, 9:802-811.
129. Watson, P. H., Chia, S. K., Wykoff, C. C., Han, C., Leek, R. D., Sly, W. S., Gatter, K. C., Ratcliffe, P., and Harris, A. L., 2003, Carbonic anhydrase XII is a marker of good prognosis in invasive breast carcinoma. Br J Cancer, 88:1065-1070.
130. Levicar, N., Kos, J., Blejec, A., Golouh, R., Vrhovec, I., Frkovic-Grazio, S., and Lah, T. T., 2002, Comparison of potential biological markers cathepsin B, cathepsin L, stefin A and stefin B with urokinase and plasminogen activator inhibitor-1 and clinicopathological data of breast carcinoma patients. Cancer Detect Prev, 26:42-49.

131. Kayser, K., Richter, N., Hufnagl, P., Kayser, G., Kos, J., and Werle, B., 2003, Expression, proliferation activity and clinical significance of cathepsin B and cathepsin L in operated lung cancer. Anticancer Res, 23:2767-2772.
132. Scorilas, A., Fotiou, S., Tsiambas, E., Yotis, J., Kotsiandri, F., Sameni, M., Sloane, B. F., and Talieri, M., 2002, Determination of cathepsin B expression may offer additional prognostic information for ovarian cancer patients. Biol Chem, 383:1297-1303.
133. Spano, J. P., Andre, F., Morat, L., Sabatier, L., Besse, B., Combadiere, C., Deterre, P., Martin, A., Azorin, J., Valeyre, D., et al., 2004, Chemokine receptor CXCR4 and early-stage non-small cell lung cancer: pattern of expression and correlation with outcome. Ann Oncol, 15:613-617.
134. Yuan, A., Yang, P. C., Yu, C. J., Chen, W. J., Lin, F. Y., Kuo, S. H., and Luh, K. T., 2000, Interleukin-8 messenger ribonucleic acid expression correlates with tumor progression, tumor angiogenesis, patient survival, and timing of relapse in non-small-cell lung cancer. Am J Respir Crit Care Med, 162:1957-1963.
135. Kassim, S. K., El-Salahy, E. M., Fayed, S. T., Helal, S. A., Helal, T., Azzam Eel, D., and Khalifa, A., 2004, Vascular endothelial growth factor and interleukin-8 are associated with poor prognosis in epithelial ovarian cancer patients. Clin Biochem, 37:363-369.
136. Ren, Y., Poon, R. T., Tsui, H. T., Chen, W. H., Li, Z., Lau, C., Yu, W. C., and Fan, S. T., 2003, Interleukin-8 serum levels in patients with hepatocellular carcinoma: correlations with clinicopathological features and prognosis. Clin Cancer Res, 9:5996-6001.
137. Nurnberg, W., Tobias, D., Otto, F., Henz, B. M., and Schadendorf, D., 1999, Expression of interleukin-8 detected by in situ hybridization correlates with worse prognosis in primary cutaneous melanoma. J Pathol, 189:546-551.
138. Dai, D. L., Makretsov, N., Campos, E. I., Huang, C., Zhou, Y., Huntsman, D., Martinka, M., and Li, G., 2003, Increased expression of integrin-linked kinase is correlated with melanoma progression and poor patient survival. Clin Cancer Res, 9:4409-4414.
139. Hay, R., Cao, B., Tsarfaty, I., Tsarfaty, G., Resau, J., and Woude, G. V., 2002, Grappling with metastatic risk: bringing molecular imaging of Met expression toward clinical use. J Cell Biochem Suppl, 39:184-193.
140. Vihinen, P. and Kahari, V. M., 2002, Matrix metalloproteinases in cancer: prognostic markers and therapeutic targets. Int J Cancer, 99:157-166.
141. Rittling, S. R., and Chambers, A. F., 2004, Role of osteopontin in tumour progression. Br J Cancer, 90:1877-1881.
142. Andreasen, P. A., Kjoller, L., Christensen, L., and Duffy, M. J., 1997, The urokinase-type plasminogen activator system in cancer metastasis: a review. Int J Cancer, 72:1-22.
143. Schmitt, M., Harbeck, N., Thomssen, C., Wilhelm, O., Magdolen, V., Reuning, U., Ulm, K., Hofler, H., Janicke, F., and Graeff, H., 1997, Clinical impact of the plasminogen activation system in tumor invasion and metastasis: prognostic relevance and target for therapy. Thromb Haemost, 78:285-296.
144. Jiang, Y., Goldberg, I. D., and Shi, Y. E., 2002, Complex roles of tissue inhibitors of metalloproteinases in cancer. Oncogene, 21:2245-2252.
145. Zucker, S., Hymowitz, M., Conner, C., Zarrabi, H. M., Hurewitz, A. N., Matrisian, L., Boyd, D., Nicolson, G., and Montana, S., 1999, Measurement of matrix metalloproteinases and tissue inhibitors of metalloproteinases in blood and tissues. Clinical and experimental applications. Ann N Y Acad Sci, 878:212-227.
146. de Bock, C. E., and Wang, Y., 2004, Clinical significance of urokinase-type plasminogen activator receptor (uPAR) expression in cancer. Med Res Rev, 24:13-39.
147. Rafajova, M., Zatovicova, M., Kettmann, R., Pastorek, J., and Pastorekova, S., 2004, Induction by hypoxia combined with low glucose or low bicarbonate and high posttranslational stability upon reoxygenation contribute to carbonic anhydrase IX expression in cancer cells. Int J Oncol, 24:995-1004.
148. Creighton, C., Kuick, R., Misek, D. E., Rickman, D. S., Brichory, F. M., Rouillard, J. M., Omenn, G. S., and Hanash, S., 2003, Profiling of pathway-specific changes in gene expression following growth of human cancer cell lines transplanted into mice. Genome Biol, 4:R46.

Chapter 2

Role of Microenvironment on Gene Expression, Angiogenesis and Microvascular Function in Tumors

Dai Fukumura
Edwin L. Steele Laboratory, Department of Radiation Oncology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, USA

Abstract: Solid tumors are organ-like entities. In addition to neoplastic cells, they consist of non-transformed host stromal cells such as endothelial cells, fibroblasts and inflammatory cells. All of these cells are embedded in a characteristic extracellular matrix and are surrounded by specific molecular and metabolic microenvironments. Blood and lymphatic vessels, which are important for maintaining the homeostasis of living organisms, are compromised in solid tumors, causing various physiological barriers to the delivery of therapeutic agents to tumors in sufficient quantity and under optimal conditions. There is a growing body of evidence that stromal cells are not quiescent bystanders; instead, they significantly influence the pathophysiology of tumors. Both stromal cells and tumor cells participate in the formation of this milieu, and the microenvironment, which includes the expression of positive and negative regulators of angiogenesis, influences the biology of these cells. Any of these factors – tumor cells, stromal cells, and the local microenvironment of particular organs – may vary during treatment and may influence the efficiency of various treatment modalities. Therefore, stromal cells and the tumor microenvironment offer novel targets for tumor detection and treatment. A better understanding of host-tumor interaction and formation, as well as of the function of blood and lymphatic vessels in tumors in different microenvironments, is warranted in order to facilitate the development of such strategies.

Key words: Microenvironment, angiogenesis, stromal cells, extracellular matrix, fibroblasts, lymphocytes macrophages, hypoxia, intravital microscopy, VEGF, blood vessels, colon carcinoma, AKT, PI3K, acidosis, urokinase plasminogen activator, hypoxia inducing factor, gene expression

1. INTRODUCTION

Tumors consist not only of cancer cells but also of host stromal cells – non-malignant cells which include endothelial cells, peri-vascular cells, fibroblasts, macrophages, lymphocytes, dendritic cells and mast cells. These cells, embedded within a protein-rich extracellular matrix, face a hostile metabolic microenvironment characterized by hypoxia and acidosis (Figure 1). Each of these cells is capable of producing positive and negative regulators of angiogenesis in response to microenvironmental cues (1, 2). These local interactions vary with tumor type and site of growth (host organ), and may change during the course of tumor growth and treatment. In this chapter, we will discuss the characteristics of tumor vasculature and the resulting microenvironment, as well as the roles of the metabolic microenvironment, the host stromal cells, and the host organ microenvironment in the regulation of tumor angiogenesis and physiological functions.

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 23-36.

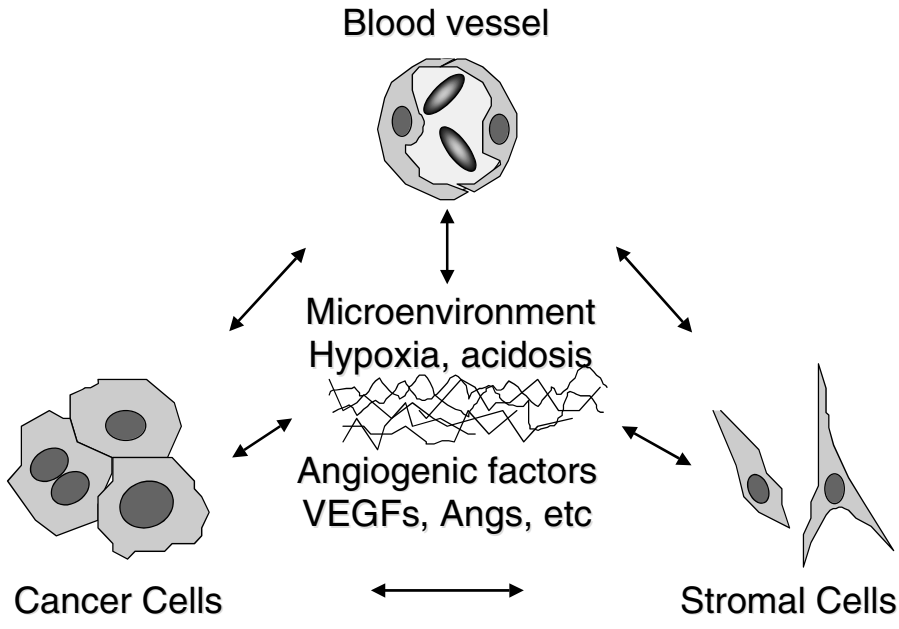


Figure 1. Composition of solid tumor.

2. INTRAVITAL MICROSCOPY FOR THE DISSECTION OF TUMOR PATHOPHYSIOLOGY

In vivo tumor models and their monitoring systems must be used in order to understand the inner workings of solid tumors (i.e. angiogenesis and the functions of newly formed blood and lymphatic vessels). Intravital microscopy provides molecular, cellular, structural, and functional insights *in vivo* with high spatial and temporal resolutions, and is thus ideal for the investigation of tumor pathophysiology (3). Intravital microscopy requires four essential components: a) appropriate animal models that allow optical access to tissues of interest, b) molecular probes (usually fluorescent) that can be imaged, c) microscopes and detection systems, and d) computer-assisted image processing and analysis systems (Figure 2). Our laboratory has established and modified many animal tumor models for intravital microscopy, including transparent window models such as the mouse dorsal skin chamber (4) and cranial window (5), and orthotopic tumor models such as liver (6), gall bladder (7), pancreas (8), mammary gland (9) and lung tumors (10). Molecular probes include the green fluorescent protein (GFP) driven by a promoter of interest (11), optical probes that are activated by specific enzymes (12), or microenvironments (for example, pO_2 and pH-activated probes) (13) and endogenous and exogenous tracers that label specific target cells (14, 15) or molecules (for example, second harmonic generation by fibrillar collagen) (16). Conventional fluorescent microscopes (15, 17), confocal laser scanning microscopes (11), and, more recently, multiphoton laser-scanning microscopes (10, 18) have been used for intravital studies. The development and application of multiphoton laser-scanning microscopy has provided significant advances because it allows deep tissue penetration, high signal-to-noise ratio, and minimal photo-damage.

Structural analyses reveal quantitative information regarding tumor growth and angiogenesis (vessel density, diameter, volume), as well as information about the size of pores in the vessel walls and in the extracellular matrix (3, 10). In addition, functional analyses can evaluate hemo- and lympho-dynamics (blood and lymph flow, vasomotor action), leukocyte-endothelial interaction,

vascular permeability, cell migration (of tumor cells, lymphocytes, macrophages, fibroblasts, etc), and interstitial transport (3, 10). The combination of these tumor models with intravital microscopy techniques has allowed us to discern the regulation of tumor angiogenesis and microcirculation as well as the effects of various treatments on this regulation.

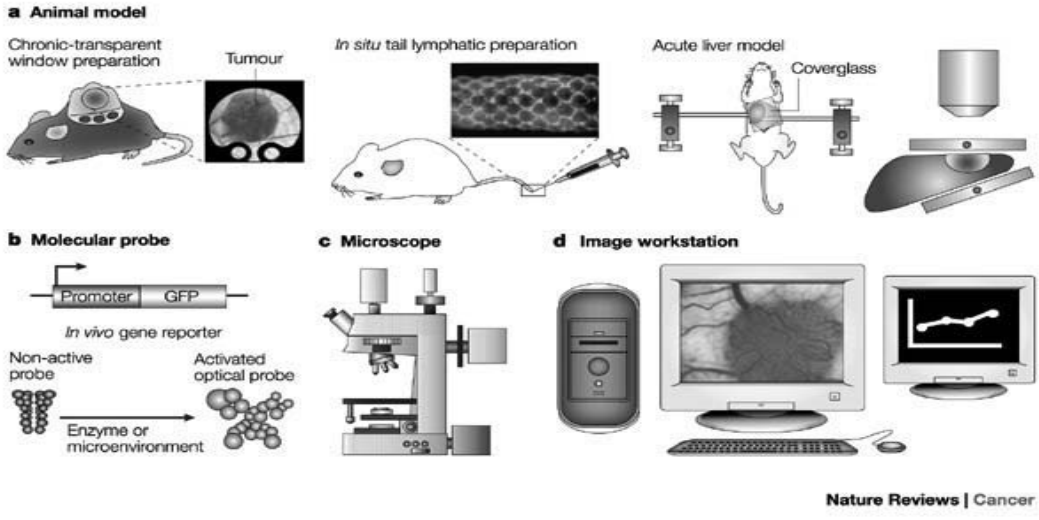


Figure 2. Four requirements for intravital microscopy. Reproduced from Reference (3).

3. CHARACTERISTICS OF TUMOR VASCULATURE AND THE RESULTING MICROENVIRONMENT

Vascular function is an important determinant of the local microenvironment. There are two vascular systems in the body: blood vessels and lymphatic vessels. In tumors, both types of vessels are morphologically and functionally abnormal (3). The normal microcirculation has a well-organized architecture consisting of arterioles, capillaries, and venules. Arterioles, the upstream element, have circumferential pericyte coverage and higher vessel tone. From arterioles, blood flows into capillaries, which represent the major portion of the microvasculature and have the smallest diameter and the thinnest vessel wall. Capillaries maintain tissue homeostasis by mediating the exchange of nutrients, gas, and waste. From the capillaries, the blood is collected into venules, the downstream portion of the microcirculation. Venules have intermediate wall thickness and non-circumferential pericyte coverage. In contrast to these highly organized, functional networks from normal tissue, the tumor vascular network is immature and mesh-like, similar to the primarily vascular plexus in early stage embryos. Tumor vessels are dilated, tortuous, and have an irregular surface and a heterogeneous spatial distribution. Recent studies show the presence of pericyte-like cells in tumor vessels. However, their morphology and association with vessels are abnormal (19), which results in poorly regulated vascular function. In normal vessels, arterioles have a higher flow velocity and nutrient/oxygen level, while venules have lower velocity and oxygen level. In each vessel category, flow velocity correlates well with vessel size. In tumor vessels, blood flow is very slow, is sometimes static, and may even change direction over time. Moreover, there is no correlation between tumor vessel diameter and flow rate or oxygen level (5, 13). Some tumor vessels contain almost no oxygen or other nutrients despite relatively good perfusion. This abnormal and non-

homogenous blood flow creates a physiological barrier to the delivery of therapeutic agents to tumors (20) and can also lead to the hypoxia and acidosis which are often seen in tumors (13). Such a severe metabolic microenvironment reduces the efficacy of anti-tumor therapies. In addition, the abnormality of tumor vessels also results in high vascular permeability, and leaked plasma proteins such as fibronectin form an optimal provisional matrix for angiogenesis. Finally, leukocyte adhesion in tumor vessels is generally low, hiding tumors from immune surveillance. All of these vascular, interstitial, and cellular barriers have to be overcome for efficient delivery of anti-tumor therapies.

By transporting both immune cells and interstitial fluid out of tissue, the normal lymphatic network plays an important role in immune function and in the maintenance of tissue interstitial fluid balance. Tumor cells grow in a confined space and, thus, create a mechanical stress (solid stress), which compresses the intratumor blood and lymph vessels (21). Consequently, there are no functional lymphatic vessels inside solid tumors (22). High permeability of intratumor blood vessels and impaired lymphatic drainage cause significant elevation of interstitial fluid pressure and oncotic pressure in solid tumors. As a result, the pressure gradient between blood vessel and tumor tissue is lost (23). High interstitial fluid pressure and the loss of pressure gradients constitute additional physiological barriers to the delivery of therapeutic agents to tumors. Nevertheless, in the peripheral region of the tumor, lymph-angiogenesis, lymphatic hypertrophy, and lymphatic dilatation are often found (22, 24, 25). Dysfunction of the lymphatic valves allows retrograde flow in these lymphatic vessels (25). Tumor cells can invade these peripheral lymphatic vessels and form metastases within the lymphatic system. Hence, a better understanding of the formation and function of blood and lymphatic vessels in tumors is necessary in order to develop new strategies to overcome these barriers to tumor treatment.

4. VEGF, THE MAIN REGULATOR OF NEW BLOOD VESSEL FORMATION

Because tumor cells depend on a nutrient supply from blood vessels, neovascularization (angiogenesis) is required for tumors to grow beyond 1-2 mm in diameter (26). Newly formed blood vessels are important not only for the growth of primary tumors, but also for the metastatic spread of cancers (27). A variety of positive and negative regulators govern vasculogenesis, angiogenesis, and subsequent vessel maturation (1, 2, 28). More than 20 angiogenic stimulators and inhibitors have been discovered in the past two decades, including vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), and angiopoietin (Ang) (1, 2, 28, 29). These factors not only mediate tumor vessel formation but also affect the function of these vessels. VEGF is one of the most potent angiogenic factors and is the target of the first FDA approved anti-angiogenic agent, Avastin (approved for colorectal cancer in 2004) (30). VEGF expression levels in tumors, and the concentrations of this protein in the bodily fluids (serum, urine, CSF) of cancer patients, show significant correlation with the extent of angiogenesis and/or metastasis and have therefore been suggested as diagnostic and prognostic markers (29, 31). VEGF is a mitogen that acts primarily on endothelial cells (29). VEGF signaling is critical for the development of a nascent vascular network via physiological vasculogenesis as well as pathological angiogenesis (2, 29). VEGF induces nitric oxide-mediated relaxation of the arterial vessels (32), signaled through PI3K and Akt, and has a role as a survival factor in maintaining the vasculature (29, 33). VEGF is also believed to contribute to an angiogenic phenotype by increasing the permeability of existing vessels (31). This leads to the extravasation of fibrin, plasmin, and clotting factors, resulting in a fibrin-rich stroma that supports the migration of endothelial and peri-endothelial cells and the formation of new vasculature (31). Local variation in the concentrations of VEGF in the tumor may explain the heterogeneous angiogenesis and vascular dysfunction in tumor vessels as well the non-uniform response of these vessels to various therapies. The tumor microenvironment, in turn,

regulates the expression of VEGF and thereby regulates the formation and function of blood vessels in tumors.

5. REGULATION OF VEGF AND ANGIOGENESIS BY THE METABOLIC MICROENVIRONMENT

Hypoxia and acidosis are hallmarks of the metabolic environment in solid tumors (34-37). Both oxygen tension (pO_2) and pH are important determinants of tumor growth, metabolism, and response to a variety of therapies such as radiation therapy, chemotherapy, hyperthermia, and photodynamic therapy (34-39). Hypoxia upregulates various angiogenic growth factors, including VEGF, Ang2, PDGF, Placenta growth factor (PlGF), transforming growth factor (TGF), interleukin (IL)-8, and hepatocyte growth factor (35, 40). Hypoxia inducible factor (HIF) is a transcriptional factor that binds to the hypoxia responsive element (HRE) in the promoter of hypoxia-responsive genes such as VEGF, PDGF and TGF (35, 40). HIF-1 also mediates hypoxia-induced apoptosis via p53 and other mechanisms (35, 41). Tumor cells have developed many mechanisms to evade HIF-1-mediated cell death under hypoxic conditions (35). A few other factors, such as IL-8 and PlGF, are activated by HIF-independent mechanisms (35, 42). Several lines of evidence have shown that microenvironmental hypoxia upregulates VEGF, both in tumors *in vivo* and in multicellular tumor spheroids *in vitro* (43, 44). Hypoxia may play an important role in the angiogenic switch (45) which is required for tumor growth and expansion. However, an immunohiostochemical study revealed a lack of spatial correlation between the staining of redox marker and VEGF in squamous cell carcinomas (46). There is a wide heterogeneity in intratumor and intertumor pO_2 distributions (13, 47). To understand the extent of hypoxia which is required to upregulate VEGF *in vivo*, pO_2 distributions should be measured simultaneously with VEGF profiles *in vivo*.

Low extracellular pH, another characteristic of solid tumors, causes stress-induced alteration of gene expression, including the upregulation of VEGF in macrophages and in tumor cells *in vitro* (48, 49). Furthermore, low pH synergistically enhances the hypoxia-induced upregulation of VEGF in cancer cells *in vitro* (49). Despite its importance, the effect of the low and heterogeneous interstitial pH on hypoxia-induced VEGF production *in vivo* remained unknown for many years due to the lack of appropriate techniques and animal models. However, two non-invasive optical techniques have now been developed: fluorescence ratio imaging microscopy for pH measurements (50) and phosphorescence quenching microscopy for pO_2 measurements (51). These two techniques provide high spatial resolution and are routinely used in combination to map temporal and spatial pH and pO_2 profiles at the same tumor locations (13). Recently, we also developed a GFP reporter system (*$VEGF^p$-GFP*) that monitors VEGF promoter activity *in vivo* (11). The combination of these techniques allows the coordinated study of pH, pO_2, and VEGF expression *in vivo* (52).

We first determined the effect of hypoxia on *$VEGF^p$-GFP* transfected cells *in vitro,* as a means of confirming the system. Both the endogenous VEGF gene and exogenous construct-derived GFP were comparably upregulated by hypoxia (52). Then, *$VEGF^p$-GFP* U87 tumors were implanted in SCID mouse cranial windows (5) and grown into well-vascularized tumors over a period of 7-8 days. GFP fluorescence in U87 tumor cells was visualized by fluorescence microscopy (10). To translate GFP fluorescence intensity into instantaneous GFP concentration, we generated calibration curves using known quantities of recombinant EGFP and the same intravital microscopy set-up. Tissue pO_2 and pH were determined by phosphorescence quenching microscopy and ratio imaging microscopy, respectively (13). We analyzed the relationship between pO_2, pH, and VEGF expression in *$VEGF^p$-GFP* U87 tumors by dividing the measurements into either hypoxic ($pO_2 < 30$ mmHg) or oxygenated ($pO_2 > 30$ mmHg) groups and either low pH ($pH < 6.8$) or neutral ($6.8 < pH < 7.4$) groups. This allowed for the comparison of GFP expression under each of these conditions using linear regression. Figures 3A and 3D show that, under hypoxic conditions or neutral pH conditions, pO_2, but not pH, is correlated

to GFP expression. Conversely, Figures 3B and 3C show that, under low pH or oxygenated conditions, pH and not pO_2 is related to GFP expression. These results indicated, for the first time, that VEGF transcription in brain tumors is independently regulated by the tissue pO_2 and pH (52).

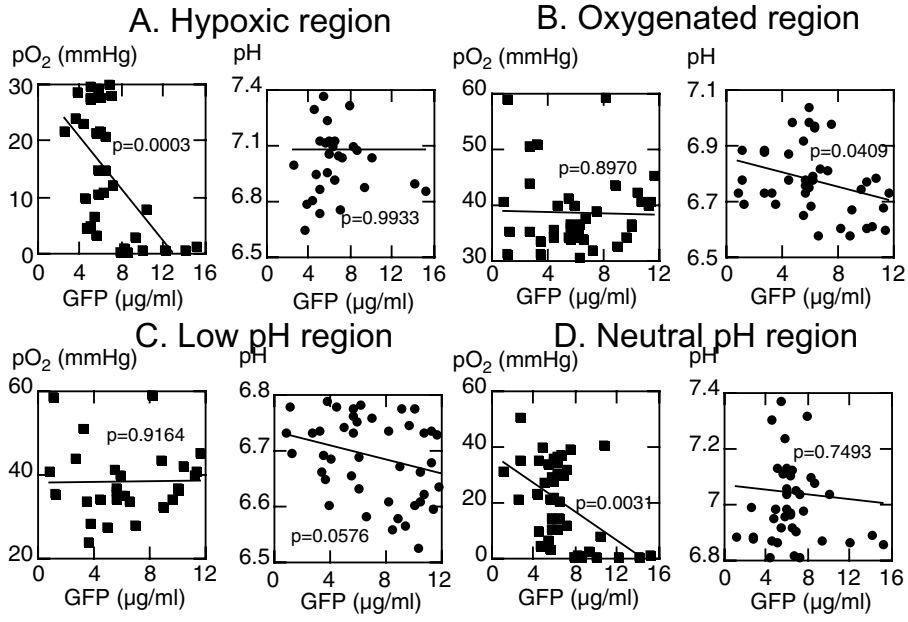


Figure 3. Relationships between VEGF promoter activity, tissue pO2, and extracellular pH. Reproduced from (52).

Our findings prompted us to study the signaling pathway in order to mediate acidic-pH-induced VEGF upregulation. Serial deletion of the VEGF promoter showed that the promoter region contains the activating protein (AP)-1 binding site but not HRE or AP-2, and also indicated that stimulatory protein-1 binding sites were crucial for acidic-pH-induced VEGF upregulation. This is clearly a different signaling pathway from that which regulates hypoxia-induced VEGF expression, which is mediated by HIF-1 complex binding to HRE. Acidic pH also stabilized VEGF mRNA. Furthermore, the acidic-pH-activated *Ras* oncogene and downstream signaling requires extracellular signal-related kinase1/2 but not p38 or Jun amino-terminal kinase (53). These data confirmed that two major metabolic environments in solid tumors regulate VEGF expression in a complimentary manner via distinct signaling pathways.

6. INVOLVEMENT OF HOST STROMAL CELLS IN TUMOR ANGIOGENESIS

Traditionally, cancer researchers have focused their studies on genetically transformed neoplastic cells. However, it is becoming increasingly apparent that the development and pathophysiology of a tumor cannot be explained simply by the genes carried by the tumor cells (54). The cell population in a tumor includes numerous non-neoplastic bystanders (stromal cells) such as endothelial cells, peri-vascular cells, fibroblasts, and inflammatory cells (Figure 1). We are beginning to understand that stromal cells profoundly influence many steps of tumor progression, such as tumor cell proliferation, invasion, angiogenesis, metastasis, and even malignant transformation (11, 55-60). Crosstalk between the diverse cell types within a tumor, via both soluble factors and direct cell-to-cell contact,

plays an important role in the induction, selection, and expansion of the neoplastic cells. Successful tumor cells are those that have acquired the ability to co-opt their normal neighbors by inducing them to release abundant fluxes of growth-stimulating signals (54, 58, 60).

Although the importance of angiogenesis in tumor development has been appreciated for some time, the involvement of host stromal cells in tumor angiogenesis was overlooked due to a lack of methodology for determining the specific contributions of stromal cells. To this end, we have engineered transgenic mice bearing the VEGF promoter-GFP reporter construct (*VEGF^p-GFP* mice). *VEGF^p-GFP* mice showed green cellular fluorescence around the healing margins and throughout the granulation tissue of superficial ulcerative wounds (11). Tumor implantation in the *VEGF^p-GFP* mice led to an accumulation of green fluorescence resulting from the tumor induction of stromal VEGF promoter activity (15). Initially, surface-weighed confocal laser scanning microscopy of both wounds and tumors revealed that GFP-positive cells are mainly spindle shaped fibroblast-like cells (11). Subsequently, we used multiphoton laser-scanning microscopy to determine gene expression and function at depths of over 400 microns in the tumor tissue (Figure 4). We then found that VEGF-expressing stromal cells co-localize with the vasculature and even surround tumor blood vessels deep inside the tumor (18). These findings suggest that activated fibroblasts are involved in angiogenesis, the fortification of blood vessels, and the function of these vessels. In fact, co-implantation of fibroblasts enhanced the tumorigenicity of breast cancer cells *in vivo* (61) and fibroblastic expression of three components of the urokinase-type plasminogen activator system (uPA, uPA receptor, PA inhibitor-1), which are also known to be angiogenic factors, showed a positive correlation with clinical parameters of breast cancers such as tumor size and grade (62). In addition to fibroblasts, some inflammatory cells recruited to tumors may also promote (rather than eliminate) angiogenesis and tumor cell growth (59).

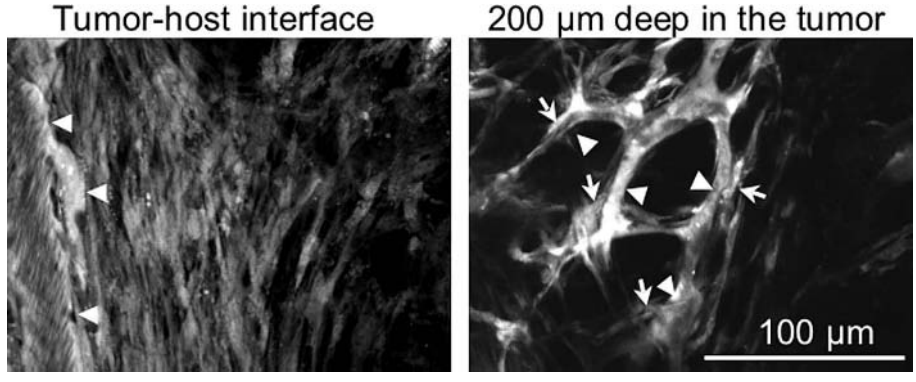


Figure 4. Imaging VEGF promoter activity in vivo. MCaIV murine breast tumor was grown in the dorsal skin chamber in VEGFp-GFP mice. The tumor vasculature was highlighted by injection of tetramethylrhodamine-labeled BSA (arrow head). Fluorescence of GFP and rhodamine was visualized by multiphoton laser-scanning microscopy (18). Deep inside the tumor (~200 μm) GFP positive cells (arrow) were often associated with angiogenic vessels (arrow). Adapted from Reference (18).

The next question would be the extent of stromal cell contribution in tumor angiogenesis. To answer this question, we determined angiogenesis and tissue VEGF protein level in various tumors derived from genetically engineered embryonic stem cells (ES cells). We compared ES cells with mutations that can influence VEGF expression, including mutations in HIF-1, in HRE in the VEGF promoter, and in VEGF itself (63). We found that angiogenesis in these tumors correlated well with their VEGF levels,

i.e., $VEGF^{-/-} \approx HRE^{-/-} < HIF\text{-}1^{-/-} <$ wild type (WT). VEGF protein levels in $VEGF^{-/-}$ ES cell-derived tumors, which have VEGF only from host stromal cells, were approximately half of those in WT ES cell-derived tumors, which contain both tumor cell-derived and stromal cell-derived VEGF. This suggests that host stromal cells make a significant contribution (~50%) to the production of VEGF in these tumors (63). The ratio of tumor/host-derived VEGF may vary depending on tumor type, stage, and organ site. For an example, late stage orthotopic pancreatic tumors expressed significantly higher tumor cell-derived VEGF compared to early stage or ectopically-grown tumors (8).

The contribution of host stromal cells to tumor angiogenesis was also altered by tumor treatments. The blockade of human epidermal growth factor receptor (HER)-2 signaling by a neutralizing antibody (Herceptin) downregulates VEGF, TGFα, Ang1, and PAI-1, and also induces thrombospondin-1, producing significant inhibition of angiogenesis and tumor growth (Figure 5) (64). Based on these findings, we proposed that Herceptin mimics an anti-angiogenic cocktail (64). It is noteworthy that although Herceptin significantly inhibited VEGF expression in tumor cells, the overall VEGF expression in tumor tissue did not change, due to compensation by the host stromal cells. Increased host stromal contribution to VEGF-promoter activity was observed following various anti-angiogenesis/anti-vascular treatments such as hormone withdrawal (65) and photodynamic therapy (66). These findings suggest that host stromal cells may compensate for the loss of critical growth factors during anti-tumor treatment, and will thus provide a survival window for repopulation with treatment-resistant tumor cells.

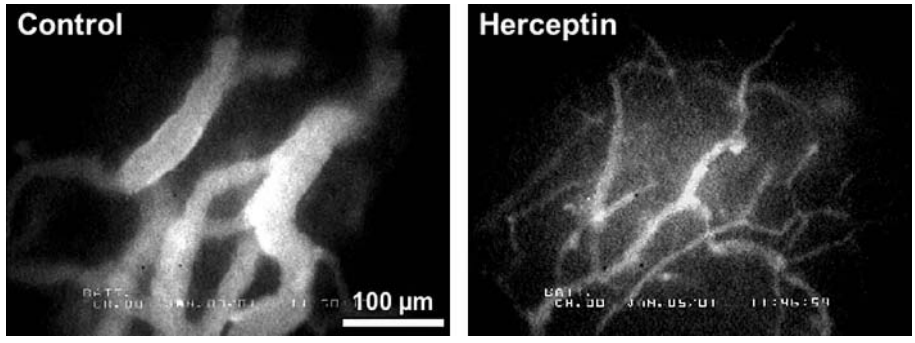


Figure 5. Effect of Herceptin on tumor vessels. Vasculature of control (left) and Herceptin (right) treated MDA-MB-361HK tumor grown in the cranial windows of SCID mice on day-15. The blood vessels are contrast enhanced by i.v. injection of FITC-dextran. Reproduced from Reference (64).

In addition to primary tumor angiogenesis and growth, host stromal cells may also contribute to the spread of disease. Stromal cells and cancer cells exchange enzymes and cytokines that modify the local extracellular matrix and stimulate migration (56, 58). Macrophages have been shown to induce tumor cell intravasation (59). Fibroblasts promote tumor cell invasion by upregulation of matrix metalloproteinase-9 production (67), and proliferative activity of fibroblasts closely correlates with metastasis of breast carcinoma (68). The fact that stromal cells can survive and proliferate in distant organs (69) is particularly important. Heterotypic multicellular tumor fragments have been found in blood and lymphatic vessels (57, 70). Tumor cell aggregates form more metastatic tumors than single tumor cells (70-72). Co-injection of non-neoplastic cells such as fibroblasts or embryo cells significantly increase metastasis in experimental tumor models (71, 73). Taken together these findings support the provocative idea that primary tumor-derived stromal cells may form the provisional stroma for the initial survival and growth of metastasic tumors. Stromal cells should subsequently be considered as an additional target

for both anti-tumor treatments and prevention of metastasis.

7. REGULATION OF ANGIOGENESIS AND VESSEL FUNCTIONS BY ORGAN MICROENVIRONMENT

Gene expression, angiogenesis, and microcirculatory functions differ significantly between sites of implantation when the same tumor cells are grown in different host organs (3, 74). These observations imply that growth factor expression and function are determined by a complicated interaction between tumor cells, host stromal cells, and the organ microenvironment where the tumor is growing. This hypothesis is supported by the facts that various human tumors have particular organ preferences for their metastases (75) and that tumor growth, angiogenesis, metastatic potential, and response to treatment differs among various host organs (76-78). These preferences/differences seem to be due to the organ-specific microenvironment. Organ-specific upregulation of bFGF (79), IL-8 (80), VEGF (6, 8), and various metastasis-related genes (81) in tumors has been reported (Table 1). Human renal cell carcinoma (HRCC) xenografts grown in the kidneys of immunodeficient mice were highly vascularized and metastatic, and they expressed levels of bFGF mRNA which were 10- to 20-fold higher than those from the same tumor grown subcutaneously (79). We found that LS174T tumors (human colon adenocarcinoma) grown in the liver expressed lower levels of VEGF mRNA and had a lower vessel density than those in subcutaneous tissue (6). Similarly, the levels of IL-8 mRNA were always high in A375 tumors (human melanoma) re-established in the skin and were low in the tumors re-established in the liver (80).

Table 1. Role of host organ microenvironment on angiogenesis and vascular function.

Comparison	Key conclusions	Reference
Cranium *vs.* skin	Faster angiogenesis in a collagen gel in the cranial window.	(82)
	Smaller pore cut off size in the cranial tumors.	(83)
	Lower vascular permeability in baseline and after VEGF superfusion in pial vessels.	(84)
	Higher interstitial diffusion in the cranial tumors due to less collagen (fibroblast) involvement.	(85)
	Tumor blood barrier formation in HGL21 human glioma only when grown in the cranial window.	(86)
Liver *vs.* skin	Higher IL-8 expression in skin tumors and induction of IL-8 by co-culture with keratinocytes.	(80)
	Lower VEGF expression and angiogenesis but higher vascular permeability in the liver tumor.	(6)
Colon *vs.* skin	Higher EGFR, FGF, collagenase, mdr-1 gene expression, and metastatic potential in colon tumors.	(81)
Kidney *vs.* skin	Higher bFGF expression, angiogenesis, and metastatic potential in kidney tumors.	(79)
Gall bladder *vs.* skin	Higher production of anti-angiogenesis factor (TGFβ1) in the gall bladder tumor.	(7)
Pancreas *vs.* abdominal wall	Higher VEGF protein level, angiogenesis, and tumor growth in tumors grown in the pancreas.	(8)
Cranium vs. mammary fat pad	Higher VEGF/receptor expression and permeability but lower angiogenesis in the mammary tumor.	(9)

The expression of endogenous anti-angiogenic factors is also regulated by organ specific host-tumor interaction. Human gall bladder primary tumors inhibit angiogenesis and growth of secondary tumors at a distant site. However, this result was only observed when the primary tumor was grown in the gall bladder (orthotopic), rather than the subcutaneous space (ectopic) (7). TGFβ1 mediates this process. Murine fibrosarcoma FsaII can also inhibit angiogenesis in secondary tumors *in vivo*. Surgical removal of the primary tumor cancelled this effect. On the other hand, irradiation of the primary tumor induced a more pronounced inhibitory effect on angiogenesis in the distant site (87). Plasma endostatin levels correlate well with these endogenous antiangiogenic effects caused by tumor burden and/or treatments. Knowledge of organ-dependent profiles of gene expression and protein level, in stromal cells and tumor cells from different organ microenvironments, will provide new insight into tumor biology and should allow us to understand why a given tumor behaves differently in different organs.

Knowledge of gene expression alone, without corresponding functional analysis, provides an incomplete understanding at best of the putative role of the gene and/or its product *in vivo*. For example, it is widely accepted that VEGF is responsible for high permeability in tumors, but the organ microenvironment also plays a role: HGL21, a human glioblastoma, has high vascular permeability (similar to that in the majority of other tumors) when it is grown in subcutaneous tissue, but these tumors are no longer as permeable when grown in the cranium (88), despite similar expression levels of VEGF and its receptors in HGL21 tumors grown in both sites. Furthermore, the vascular pore cut-off size (the maximum functional pore size for trans-vascular transport of macromolecules through the vessel wall) in various tumors decreased when the tumors were grown in the cranial window as compared to the dorsal skin chamber (89). Similarly, with the presence of a blood-brain-barrier, a significantly higher amount of VEGF was required to induce vascular hyperpermeability in normal vessels in the cranial window than in the dorsal skin chamber (84). On the other hand, the cranial environment is more angiogenic and forms new vessels faster than the subcutaneous tissue does in response to a given angiogenic factor (82). These differences are presumably due to differences in the phenotype of vascular endothelial cells, which is defined by their origin, by cell-cell and cell-matrix interactions, and by the surrounding microenvironment. These findings indicate that VEGF level alone may not be a sufficient predictor of angiogenesis or vascular permeability in the tumors of different organs. Indeed, the vascular permeability of LS174T human colon cancer grown in the liver versus subcutaneous space was inversely correlated with the expression levels of VEGF at these sites, while angiogenesis was parallel to VEGF levels (6). Conversely, higher VEGF expression and permeability but lower angiogenesis were observed in ZR75 human breast cancers grown in the mammary fat pad (primary site) compared to those grown in the cranial window (metastatic site) (9). These findings underscore the need for functional studies in conjunction with gene expression studies.

In conclusion, host-tumor interaction influences the biology of both tumor cells and host stromal cells, including their expression of positive and negative regulators of angiogenesis. This interaction depends on the cross-talk between tumor cells and stromal cells. In addition, the local microenvironment of different organs may vary during treatment, which in turn will influence the efficiency of various treatment modalities. Unlike neighboring malignant cells, stromal cells are genetically stable, yet they play important roles in multiple steps of tumor progression. Thus, targeting stromal cells may be a superior strategy for tumor detection and treatment. The cellular, molecular, and metabolic environment of solid tumors activates stromal cells and tumor cells, inducing angiogenesis, tumor cell proliferation, invasion, and metastasis and reducing the therapeutic efficacy of conventional anti-tumor treatments. However, these characteristic tumor environments may offer novel targets for tumor detection and treatment. A better understanding of host-tumor interaction during tumor growth, response to treatments, regression, and regrowth would facilitate the development of innovative tumor treatment strategies.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health.

This chapter is based on four previous, related reviews:

Jain, Munn, Fukumura, and Melder. *In vitro* and *in vivo* quantification of adhesion between leukocytes and vascular endothelium. In: Morgan and Yarmush, eds. Methods in Molecular Medicine - Tissue Engineering Methods and Protocols. Vol. 18. Totowa, NJ: Humana Press; (1998) 553-575.

Jain, Munn, and Fukumura. Transparent window models and intravital microscopy. In: Teicher, ed. Tumor Models in Cancer Research. Totowa, NJ: Humana Press; (2001) 647-671.

Jain, Munn, and Fukumura. Dissecting tumor pathophysiology using intravital microscopy. Nature Reviews Cancer (2002) 2: 266-276.

Jain, Brown, Munn, and Fukumura. Intravital microscopy of normal and diseased tissues in the mouse. In *Live cell imaging: A laboratory manual*: Cold Spring Harbor Press; in press.

We thank the publishers for allowing us to reproduce the relevant material. We also thank Drs. M.F. Booth, D.G. Duda, R.K. Jain and T. Padera for their helpful comments, and Ms. T. Belezos for the preparation of manuscript.

REFERENCES

1. Carmeliet, P., and Jain, R.K., 2000, Angiogenesis in cancer and other diseases: from genes to function to therapy. Nature, 407:249-257.
2. Jain, R.K., 2003, Molecular regulation of vessel maturation. Nature Medicine, 9:685-693.
3. Jain, R.K., Munn, L.L., and Fukumura, D., 2002, Dissecting tumor pathophysiology using intravital microscopy. Nature Reviews Cancer, 2:266-276.
4. Leunig, M., Yuan, F., Menger, M.D., Boucher, Y., Goetz, A.E., Messmer, K., and Jain, R.K., 1992, Angiogenesis, microvascular architecture, microhemodynamics, and interstitial fluid pressure during early growth of human adenocarcinoma LS174T in SCID mice. Cancer Research, 52:6553-6560.
5. Yuan, F., Salehi, H.A., Boucher, Y., Vasthare, U.S., Tuma, R.F., and Jain, R.K., 1994, Vascular permeability and microcirculation of gliomas and mammary carcinomas transplanted in rat and mouse cranial window. Cancer Research, 54:4564-4568.
6. Fukumura, D., Yuan, F., Monsky, W.L., Chen, Y., and Jain, R.K., 1997, Effect of host microenvironment on the microcirculation of human colon adenocarcinoma. American Journal of Pathology, 151:679-688.
7. Gohongi, T., Fukumura, D., Boucher, Y., Yun, C.-O., Soff, G.A., Compton, C., Todoroki, T., and Jain, R.K., 1999, Tumor-host interactions in the gallbladder suppress distal angiogenesis and tumor growth: Involvement of transforming growth factor β1. Nature Medicine, 5:1203-1208.
8. Tsuzuki, Y., Carreira, C.M., Bockhorn, M., Xu, L., Jain, R.K., and Fukumura, D., 2001, Pancreas microenvironment promotes VEGF expression and tumor growth: Novel window models for pancreatic tumor angiogenesis and microcirculation. Laboratory Investigation, 81:1439-1452.
9. Monsky, W.L., Carreira, C.M., Tsuzuki, Y., Gohongi, T., Fukumura, D., and Jain, R.K., 2002, Role of host microenvironment in angiogenesis and microvascular functions in human breast cancer xenografts: mammary fat pad vs. cranial tumors. Clinical Cancer Research, 8:1008-1013.
10. Jain, R.K., Brown, E.B., Munn, L.L., and Fukumura, D., in press, Intravital microscopy of normal and diseased tissues in the mouse. In Live cell imaging: A laboratory manual. Cold Spring Harbor Press.
11. Fukumura, D., Xavier, R., Sugiura, T., Chen, Y., Park, E., Lu, N., Selig, M., Nielsen, G., Taksir, T., Jain, R.K., et al., 1998, Tumor induction of VEGF promoter in stromal cells. Cell, 94:715-725.
12. Weissleder, R., Tung, C.H., Mahmood, U., and Bogdanov, A.J., 1999, In vivo imaging of tumors with protease-activated near-infrared fluorescent probes. Nat Biotechnol, 17:375-378.
13. Helmlinger, G., Yuan, F., Dellian, M., and Jain, R.K., 1997, Interstitial pH and pO_2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. Nature Medicine, 3:177-182.
14. Fukumura, D., Salehi, H.A., Witwer, B., Tuma, R.F., Melder, R.J., and Jain, R.K., 1995, Tumor necrosis factor α-induced leukocyte adhesion in normal and tumor vessels: Effect of tumor type, transplantation site, and host strain. Cancer Research, 55:4824-4829.
15. Jain, R.K., Munn, L.L., Fukumura, D., and Melder, R.J., 1998, In vitro and in vivo quaqntification of adhesion between leukocytes and vascular endothelium. In Methods in molecular medicine, Tissue Engineering methods and protocols. 18:553-575. J.R. Morgan, and M.L. Yarmush, ed, Totowa: Humana Press Inc..
16. Brown, E., McKee, T., di Tomaso, E., Seed, B., Boucher, Y., and Jain, R.K., 2003, Dynamic imaging of collagen and its modulation in tumors in vivo

using second harmonic generation. Nature Medicine, 9:796-800.
17. Jain, R.K., Munn, L.L., and Fukumura, D., 2001, Transparent window models and intravital microscopy. In Tumor models in cancer research. B.A. Teicher, ed, 647-671. Totowa: Humana Press Inc.
18. Brown, E.B., Campbell, R.B., Tsuzuki, Y., Xu, L., Carmeliet, P., Fukumura, D., and Jain, R.K., 2001, In vivo measurement of gene expression, angiogenesis, and physiological function in tumors using multiphoton laser scanning microscopy. Nature Medicine, 7:864-868.
19. Morikawa, S., Baluk, P., Kaidoh, T., Haskell, A., Jain, R.K., and McDonald, D.M., 2002, Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. American Journal of Pathology, 160:985-1000.
20. Jain, R.K., 1998, The next frontier of molecular medicine: delivery of therapeutics. Nature Medicine, 4:655-657.
21. Padera, T.P., Stoll, B.R., Tooredman, J.B., Capen, D., di Tomaso, E., and Jain, R.K., 2004, Cancer cells compress intratumor vessels. Nature, 427:695.
22. Padera, T.P., Kadambi, A., diTomaso, E., Carreira, C.M., Brown, E.B., Munn, L.L., and Jain, R.K., 2002, Lymphatic metastasis in the absence of functional intratumor lymphatics. Science, 296:1883-1886.
23. Tong, R., Boucher, Y., Kozin, S.V., Winkler, F., Hincklin, D.J., and Jain, R.K., 2004, Vessel normalization by VEGFR-2 blockade lowers interstitial hypertension and improves drug penetration in tumors. Cancer Research, 64:3731-3736.
24. Alitalo, K., and Carmeliet, P., 2002, Molecular mechanisms of lymphangiogenesis in health and disease. Cancer Cell, 1:219-227.
25. Isaka, N., Padera, T.P., Hagendoorn, J., Fukumura, D., and Jain, R.K., 2004, Peritumor lymphatics induced by vascular endothelial growth factor-C exhibit abnormal function. Cancer Research, 64:4400–4404.
26. Folkman, J., 2000, Tumor angiogenesis. In Cancer Medicine, 5th Edition, J.F. Holand, E.I. Frei, R.C.J. Bast, D.W. Kufe, P.E. Pollock, and R.R. Weichselbauum, eds, 132-152. Decker Inc., Ontario, B.C.
27. Kerbel, R., and Folkman, J., 2002, Clinical translation of angiogenesis inhibitors. Nature Reviews Cancer, 2:727-739.
28. Yancopoulos, G.D., Davis, S., Gale, N.W., Rudge, J.S., Wiegand, S.J., and Holash, J., 2000, Vascular-specific growth factors and blood vessel formation. Nature, 407:242-248.
29. Ferrara, N., Gerber, H.P., and LeCouter, J., 2003, The biology of VEGF and its receptors. Nature Medicine, 9:669-676.
30. Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Baron, A., Griffing, S., Holmgren, E., et al., 2004, Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med, 350:2335-2342.
31. Dvorak, H.F., 2002, Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. Journal of Clinical Oncology, 20:4368-4380.
32. Fulton, D., Gratton, J.-P., McCabe, T.J., Fontana, J., Fujio, Y., Walsh, K., Franke, T.F., Papapetropoulos, A., and Sessa, W.C., 1999, Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature, 399:597-601.
33. Alon, T., Hemo, I., Itin, A., and Pe'er, J., 1995, Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. Nature Medicine, 1:1024-1028.
34. 2001, Causes and consequences of acidic pH in tumors. Gillies, R.J., ed, John Eiley & Sons Ltd., West Sussex.
35. Harris, A.L., 2002, Hypoxia – A key regulatory factor in tumor growth. Nat Rev Cancer, 2:38-47.
36. Brown, J.M., and Giaccia, A.J., 1998, The unique physiology of solid tumors: Opportunities (and problems) for cancer therapy. Cancer Res, 58:1408-1416.
37. Tannock, I.F., and Rotin, D., 1989, Acid pH in tumors and its potential for therapeutic exploitation. Cancer Res, 49:4373-4384.
38. Skarsgard, L.D., Skwarchuk, M.W., Vinczan, A., Kristl, J., and Chaplin, D.J., 1995, The cytotoxicity of melphalan and its relationship to pH, hypoxia and drug uptake. Anticancer Res, 15:219-224.
39. Wike-Hooley, J.L., Haveman, J., and Rheinhold, H.S., 1984, The relevance of tumor pH to the treatment of malignant disease. Radiother Oncol, 2:343-366.
40. Semenza, G.L., 2003, Targeting HIF-1 for cancer therapy. Nat Rev Cancer, 3:721-732.
41. Carmeliet, P., Dor, Y., Herbert, J.M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., et al., 1998, Role of HIF-1α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature, 394:485-490.
42. Xu, L., Pathak, P.S., Jain, R.K., and Fukumura, D., 2004, Hypoxia-induced activation of p38 mitogen-activated protein kinase and phosphatidylinositol 3'-kinase signaling pathways contributes to expression

of interleukin-8 in human ovarian carcinoma cells. Clinical Cancer Research, 10:701-707.

43. Waleh, N.S., Brody, M.D., Knapp, M.A., Mendonca, H.L., Lord, E.M., Koch, C.J., Laderoute, K.R., and Sutherland, R.M., 1995, Mapping of the vascular endothelial growth factor-producing hypoxic cells in multicellular tumor spheroids using a hypoxia-specific marker. Cancer Res, 55:6222-6226.
44. Shweiki, D., Itin, A., Soffer, D., and Keshet, E., 1992, Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature, 359:843-845.
45. Hanahan, D., and Folkman, J., 1996, Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell, 86:353-364.
46. Raleigh, J.A., Calkins-Adams, D.P., Rinker, L.H., Ballenger, C.A., Weissler, M.C., Fowler, W.C.J., Novotony, D.B., and Varia, M.A., 1998, Hypoxia and vascular endothelial growth factor expression in human squamous cell carcinoma using pimonidazole as a hypoxia marker. Cancer Res, 58:3765-3768.
47. Vaupel, P.W., 1993, Oxygenation of solid tumors. In Drug resistance in oncology. B. Teicher, ed, 53-85 Dekker, M., Inc., New York, NY.
48. Jensen, J.A., Hunt, T.K., Scheuenstuhl, H., and Banda, M.J., 1986, Effect of lactate, pyruvate and pH on the secretion of angiogenesis and mitogenesis factors by macrophages. Lab Invest, 54:574-578.
49. Xie, K., Huang, S., Xu, L., and Fidler, I.J., 1998, Molecular mechanisms for the regulation of vascular endothelial growth factor expression by extracellular and intracellular pH. Proceedings of American Association of Cancer Research, 39:378.
50. Martin, G.R., and Jain, R.K., 1993, Fluorescence ratio imaging measurement of pH gradients: calibration and application in normal and tumor tissues. Microvasc Res, 46:216-230.
51. Torres-Filho, I.P., Leunig, M., Yuan, F., Intaglietta, M., and Jain, R.K., 1994, Noninvasive measurement of microvascular and interstitial oxygen profiles in a human tumor in SCID mice. Proc Natl Acad Sci USA, 91:2081-2085.
52. Fukumura, D., Xu, L., Chen, Y., Gohongi, T., Seed, B., and Jain, R.K., 2001, Hypoxia and acidosis independently up-regulate vascular endothelial growth factor transcription in brain tumors in vivo. Cancer Research, 61:6020-6024.
53. Xu, L., Fukumura, D., and Jain, R.K., 2002, Acidic extracellular pH induces vascular endothelial growth factor (VEGF) in human glioblastoma cells via ERK1/2 MAPK signaling pathway. Mechanism of low pH induced VEGF. Journal of Biological Chemistry, 277:11368-11374.
54. Hanahan, D., and Weinberg, R.A., 2000, The hallmarks of cancer. Cell, 100:57-70.
55. Elenbaas, B., and Weinberg, R.A., 2001, Heterotypic signaling beteen epithelial tumor cells and fibroblasts in carcinoma formation. Experimental Cell Research, 264:169-184.
56. Liotta, L.A., and Kohn, E.C., 2001, The microenvironment of the tumour–host interface. Nature, 411:375-379.
57. Ruiter, D.J., van Krieken, J.H., van Muijen, G.N., and de Waal, R.M., 2001, Tumour metastasis: is tissue an issue? Lancet Oncology, 2:109-112.
58. Li, G., Satyamoorthy, K., Meier, F., Berking, C., Bogenrieder, T., and Herlyn, M., 2003, Function and regulation of melanoma–stromal fibroblast interactions: when seeds meet soil. Oncogene, 22:3162-3171.
59. Pollard, J.W., 2004, Tumour-educated macrophages promote tumour progression and metastasis. Nat rev Cancer, 4:71-78.
60. Tlsty, T.D., 2001, Stromal cells can contribute oncogenic signals. Cancer Biology, 11:97-104.
61. Noel, A., De Pauw-Gillet, M.C., Purnell, G., Nusgens, B., Lapiere, C.M., and Foidart, J.M., 1993, Enhancement of tumorigenicity of human breast adenocarcinoma cells in nude mice by matrigel and fibroblasts. British Journal of Cancer, 68:909-915.
62. Dublin, E., Hanby, A., Patel, N.K., Liebman, R., and Barnes, D., 2000, Immunohistochemical expression of uPA, uPAR, and PAI-1 in breast carcinoma: Fibroblastic expression has strong association with tumor pathology. Am J Pathol, 157:1219-1227.
63. Tsuzuki, Y., Fukumura, D., Oosthuyse, B., Koike, C., Carmeliet, P., and Jain, R.K., 2000, Vascular endothelial growth factor (VEGF) modulation by targeting hypoxia inducible factor-1α —> Hypoxia response element —>VEGF cascade differentially regulates vascular response and growth rate in tumors. Cancer Research, 60:6248-6252.
64. Izumi, Y., Xu, L., diTomaso, E., Fukumura, D., and Jain, R.K., 2002, Herceptin acts as an anti-angiogenic cocktail. Nature, 416:279-280.
65. Hansen-Algenstaedt, N., Stoll, B.R., Padera, T.P., Dolmans, D.E.G.J., Hicklin, D.J., Fukumura, D., and Jain, R.K., 2000, Tumor oxygenation in hormone-dependent tumors during vascular endothelial growth factor receptor-2 blockage, hormone ablation, and chemotherapy. Cancer Research, 60:4556-4560.
66. Dolmans, D.E.J.G.J., Xu, L., Fukumura, D., and Jain, R.K., 2003, Host versus tumor derived vascular endothelial growth factor after photodynamic therapy. Proceedings American Association for Cancer Research, 44:6.
67. Wang, T.N., Albo, D., and Tuszynski, G.P., 2002, Fibroblasts promote breast cancer cell invasin by upregulating tumor matrix metalloproteinase-9 production. Surgery, 132:220-225.
68. Hasebe, T., Sasaki, S., Imoto, S., and Ochiai, A., 2000, Proliferative activity of intratumoral fibroblasts is closely correlated with lymph node and

distant organ metastases of invasive ductal carcinoma of the breast. American Journal of Pathology, 156:1701-1710.

69. Duda, D.G., Fukumura, D., Munn, L.L., Booth, M.F., Huang, P., Seed, B., and Jain, R.K., 2004, Differential transplantability of tumor-associated stromal cells: endothelial vs. non-endothelial cells. Cancer Res, in press.
70. Glaves, D., 1983, Correlation between circulating cancer cells and incidence of metastasis. Br J Cancer, 48:665-673.
71. Fidler, I.J., 1973, The relationship of embolic homgeneity, number, size and viability to the incidence of experimental metastasis. European Journal of Cancer, 9:223-227.
72. Liotta, L.A., Saidel, M.G., and Kleinerman, J., 1976, The significance of hematogeneous tumor cell clumps in the metastatic process. Cancer Res, 36:889-894.
73. Picard, O., Rolland, Y., and Poupon, M.F., 1986, Fibroblast-dependent tumorigenicity of cells in nude mice: Implication for implantation of metastasis. Cancer Res, 46:3290-3294.
74. Fidler, I.J., 2001, Angiogenic heterogenity: regulation of neoplastic angiogenesis by the organ microenvironment. Journal of the National Cancer Institute, 93:1040-1041.
75. Paget, S., 1889, The distribution of secondary growths in cancer of the breast. Lancet, 1:571-573.
76. Fidler, I.J., 1995, Modulation of the organ microenvironment for treatment of cancer metastasis. Journal of the National Cancer Institute, 87:1588-1592.
77. Helmlinger, G., Netti, P.A., Lichtenbeld, H.C., Melder, R.J., and Jain, R.K., 1997 Solid stress inhibits the growth of multicellular tumor spheroids. Nat Biotech, 15:778-783.
78. Kerbel, R.S., 1995, Significance of tumor-host interactions in cancer growth and metastasis. Cancer and Metastasis Reviews, 259-262.
79. Singh, R.K., Bucana, C.D., Gutman, M., Fan, D., Wilsaon, M.R., and Fidler, I.J., 1994, Organ site-dependent expression of basic fibroblast growth factor in human renal cell carcinoma cells. American Journal of Pathology, 145:365-374.
80. Gutman, M., Singh, R.K., Xie, K., Bucana, C.D., and Fidler, I.J., 1995, Regulation of interleukin-8 expression in human melanoma cells by the organ environment. Cancer Research, 55:2470-2475.
81. Kitadai, Y., Bucana, C.D., Ellis, L.M., Anzai, H., Tahara, E., and Fidler, I.J., 1995, In situ mRNA hybridization technique for analysis of metastasis-related genes in human colon carcinoma cells. American Journal of Pathology, 147:1238-1247.
82. Dellian, M., Witwer, B.P., Salehi, H.A., Yuan, F., and Jain, R.K., 1996, Quantitation and physiological characterization of angiogenic vessels in mice: Effect of basic fibroblast growth factor, vascular endothelial growth factor/vascular permeability factor, and host microenvironment. Am J Pathol, 149:59-72.
83. Hobbs, S.K., Monsky, W.L., Yuan, F., Roberts, G., Griffith, L., Torchillin, V., and Jain, R.K., 1998, Regulation of transport pathways in tumor vessels: role of tumor type and host microenvironment. Proceedings of the National Academy of Sciences of the United States of America, 95:4607-4612.
84. Monsky, W.L., Fukumura, D., Gohongi, T., Ancukiewcz, M., Weich, H.A., Torchilin, V.P., Yuan, F., and Jain, R.K., 1999, Augmentation of transvascular transport of macromolecules and nanoparticles in tumors using vascular endothelial growth factor. Cancer Research, 59:4129-4135.
85. Pluen, A., Boucher, Y., Ramanujan, S., McKee, T.D., Gohongi, T., diTomasso, E., Brown, E.B., Izumi, Y., Campbell, R.B., Berk, D.A., et al., 2001, Role of tumor-host interactions in interstitial diffusion of macromolecules: Cranial vs. subcutaneous tumors. Proceedings of the National Academy of Sciences of the United States of America, 98:4628-4633.
86. Jain, R.K., Yuan, F., Brown, L.F., Detmar, M., and Dvorak, H.F. 2001. Relationship between VPF/VEGF and vascular permeability in tumors is host-organ dependent. Microvas Res submitted.
87. Hartford, A.C., Gohongi, T., Fukumura, D., and Jain, R.K., 2000, Irradiation of a primary tumor, unlike surgical removal, enhances angiogenesis suppression at a distal site: Potential role of host-tumor interaction. Cancer Res, 60:2128-2131.
88. Jain, R.K., 1997, The Eugene M. Landis Award Lecture. Delivery of molecular and cellular medicine to solid tumors. Microcirculation, 4:1-23.
89. Hobbs, S.K., Yuan, F., Griffith, L., and Jain, R.K., 1997, Pore cutoff size of tumor microvessels: Effect of tumor type, treatment, and host microenvironment. Proceedings of American Association of Cancer Research, 38:263-264.

Chapter 3

Imaging of Angiogenesis In Vivo with Fluorescent Proteins

Robert M. Hoffman
AntiCancer, Inc., 7917 Ostrow St., San Diego, CA 92111, USA

Abstract: We have adapted the surgical orthotopic implantation (SOI) model to image angiogenesis of human tumors labeled with green fluorescent protein (GFP) in nude mice. The nonluminous induced capillaries are clearly visible against the very bright tumor fluorescence examined either intravitally or by whole-body imaging in real time. The fluorescence shadowing replaces the laborious histological techniques for determining blood vessel density. Intravital images of an SOI model of human pancreatic tumors expressing GFP visualized angiogenic capillaries at both primary and metastatic sites. Whole-body optical imaging showed that blood vessel density increased linearly over a 20-week period in an SOI model of human breast cancer expressing GFP. Opening a reversible skin-flap in the light path markedly reduces signal attenuation, increasing detection sensitivity many-fold. The observable depth of tissue is thereby greatly increased. With dual-color fluorescence imaging, effected by using red fluorescent protein (RFP)-expressing tumors growing in GFP-expressing transgenic mice that express GFP in all cells, great clarity the details of the tumor-stroma interaction, especially tumor-induced angiogenesis are visualized. The GFP-expressing tumor vasculature, both nascent and mature, are readily distinguished interacting with the RFP-expressing tumor cells. Using a spectral imaging system based on liquid crystal tunable filters, we were able to separate individual spectral species on a pixel-by-pixel basis. Such techniques non-invasively visualized the presence of host GFP-expressing vessels within the RFP-labeled tumor by whole-body imaging. This new differential dual-colored fluorescence imaging tumor-host model, along with spectral unmixing, can non-invasively visualize in real-time the onset and progression of angiogenesis in a tumor. Thus, fluorescent proteins expressed *in vivo* offer the highest resolution and sensitivity for real-time whole-body imaging of angiogenesis.

Key words: Green fluorescent protein, red fluorescent protein, imaging, mouse models

1. INTRODUCTION

1.1 Formation and Nature of Blood Vessels

Vasculogenesis is the formation of new blood vessels by endothelial progenitors. Angiogenesis is the sprouting and growth of existing vessels. Capillaries distribute the blood flow while proximal arterioles provide the bulk flow to tissue. Capillaries consist only of endothelial cells (ECs), whereas larger vessels are surrounded by mural cells such as pericytes and smooth muscle cells (1). ECs differentiate from angioblasts in the embryo as well as from endothelial progenitor cells (EPCs) in the adult bone marrow. Vascular endothelial growth factor (VEGF), angiopoietin (Ang)-1, cytokines, and other signals stimulate vasculogenesis and angiogenesis (1).

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 37-45.

1.2 Tumor Blood Vessels

Vessel walls are abnormal in tumors and have uneven diameters. ECs form an imperfect and uneven vessel lining, with wide junctions at some locations and stacked layers of ECs at others. Some ECs do not express endothelial markers such as CD31. ECs in tumors can undergo apoptosis, allowing cancer cells into the lumen resulting in mosaic vessels. Heterogeneity of tumor vessels is very common (2). Tumor vessels do not have the hierarchical branching pattern of normal vascular networks, resulting in avascular, hypoxic areas in the tumor (2). The abnormal organization and ultrastructure of tumor vessels makes the blood flow in tumor vessels chaotic and the vessels leaky (2). Due to remodeling of the vasculature, blood flow varies between a tumor and its metastases as well as within a given tumor from one location to another (2). A relative deficiency of pericytes, or pericyte function, could be responsible for the morphological features of tumor vascuature including their tortuous pattern (3).

Cancer cells may be part of vessel-like structures found within some tumors. This concept is known as 'vasculogenic mimicry' (4). Melanomas often have a histological pattern that is characterized by a network of periodic acid-schiff (PAS)-positive structures that appear to be channels formed without endothelial cells, which is different from cancer cells in the walls of blood vessels. Cancer cells have been reported to make up as much as 25% of the luminal surface of some tumor vessels, the remaining surface which is covered by an endothelium. The tumor cells in the vessel lining might be in transit, entering or exiting the vessel (3).

1.3 Methods of Imaging Blood Vessels (5)

Magnetic resonance imaging and computed tomography have resolutions of 100 to 500 μm. PET and ultrasonography have resolutions of a few millimeters. X-ray images, has a resolution of ~100 μm. Computed tomographic angiography and magnetic resonance imaging angiography require intravascular contrast agents, and resolution is insufficient to resolve microvasculature. Macromolecular magnetic resonance imaging contrast agents enable resolution approaching 200 μm in animals and thus most angiogenic blood vessels are not resolved. Micromagnetic resonance imaging has a resolution of approximately 10 μm (5). Fluorescence has a resolution of approximately 100 nm (5) and therefore seems best suited for imaging blood vessels.

1.4 Models to Visualize Angiogenesis

Tumor angiogenesis is a critical step in tumor growth, progression, and metastasis. As such, angiogenesis promises a uniquely effective yet remarkably benign target for cancer chemotherapy. A major requirement for the effective discovery of angiogenesis-related drugs is an assay system that is accurate, rapid, and economical. We have developed model systems that meet these requirements (6).

The discovery and evaluation of antiangiogenic substances initially relied on *in vivo* methods such as the chorioallantoic membrane assay (7, 8), the monkey iris neovascularization model (9), the disk angiogenesis assay (10), and various models that use the cornea to assess blood vessel growth (11-16). Although they are important for understanding the mechanisms of blood vessel induction, these models did not deal with tumor angiogenesis and are poorly suited to drug discovery.

Subcutaneous tumor xenograft angiogenesis models have been developed to study tumor angiogenesis, but these require cumbersome pathological examination procedures such as histology and immunohistochemistry. Measurements require animal sacrifice and therefore preclude ongoing angiogenesis studies in individual, live, tumor-bearing animals. Moreover, xenografts are not representative models of human disease.

Tumors transplanted in the cornea of the rodents (17-19) and rodent skin-fold window chambers have also been used for angiogenesis studies (20-26). The cornea and skin-fold chamber models provide a means for studying angiogenesis in living animals. However, quantification requires specialized procedures, and the sites do not represent natural environments for tumor growth. The cornea and skin-fold window chamber tumor models do not allow orthotopic and metastatic angiogenesis, which

may involve mechanisms of angiogenesis (27) that are qualitatively different from these ectopic models.

1.5 Orthotopic Tumor Models Expressing GFP to Visualize Tumor Angiogenesis

A suitable model for drug discovery will accurately represent clinical cancer as well as enable real-time visualization of the angiogenesis process and its inhibition by effective agents. To develop realistic and real-time tumor angiogenesis models, the we have used surgical orthotopic implantation (SOI) metastatic models of human cancer (28). These models place tumors in natural microenvironments and replicate clinical tumor behavior more closely than do ectopic implantation models (28). For these studies the tumors implanted in the orthotopic model have been transduced and selected to strongly express green fluorescent protein (GFP) *in vivo* (28).

GFP expression in primary tumors and in their metastases in the mouse models can be detected by an intense fluorescence seen by intravital or by whole-body imaging. The nonluminous angiogenic blood vessels appear as sharply defined dark networks against this bright background. The high image resolution permits quantitative measurements of total vessel length. These genetically fluorescent tumor models thereby allow quantitative optical imaging of angiogenesis *in vivo*. Tumor growth, vascularization, and metastasis can now be followed in real time (28).

1.6 Intravital Images of Angiogenesis of Arthotopic Pancreas Cancer

The clarity of angiogenic blood vessel imaging was illustrated by intravital examination of the orthotopic growth of a Bx-PC-3-GFP pancreatic tumor. The nonluminous blood vessels were clearly visible against the GFP fluorescence of the primary tumor. Angiogenesis associated with metastatic growths was also easily imaged by intravital examination (28).

1.7 Intravital Imaging of Angiogenesis of Orthotopic Prostate Cancer

Because angiogenesis could be measured without animal sacrifice, it was possible determine a time course for individual animals. Sequential intravital images of angiogenesis for the human prostate tumor PC-3-GFP growing orthotopically in a single nude mouse were acquired. The tumor-associated blood vessels were clearly visible by day 7 and continued to increase at least until day 20 (28).

1.8 Whole-Body Imaging of Angiogenesis in Orthotopic Breast Cancer

We have demonstrated whole-body images and quantitation of the time course of angiogenesis of the MDA-MB-435-GFP human breast cancer growing orthotopically in the breast fat pad in a nude mouse. The development of the tumor and its angiogenesis could be imaged in a completely noninvasive manner (28). The mouse breast fat pad is the orthotopic environment for the implanted MDA-MB-435-GFP breast cancer and allows noninvasive, whole-body imaging of tumor angiogenesis. The quantitative angiogenesis data show that microvessel density increased over 20 weeks. Thus, tumors, even in their natural microenvironment, growing orthotopically in sites such as the fatpad and presumably others, can be whole-body imaged for quantitative angiogenesis studies (28).

1.9 Comparative Advantages of Fluorescent Tumor Imaging

Subcutaneous implantation sites (29-32) are not normal sites for tumor growth, and their microenvironments are very different from orthotopic sites with regard to regulation not only of tumor growth but of the angiogenesis process itself (27). Orthotopically implanted GFP-labeled tumors also allow the study of angiogenesis for metastasis. The orthotopically growing tumors, in contrast to most other models, give rise to spontaneous metastases that resemble, both in target tissues and in frequency of occurrence, the clinical behavior of

the original human tumor (33). Moreover, the extreme detection sensitivity afforded by the strong GFP fluorescence allows imaging of very early events in blood vessel induction. As Li *et al.* (24) point out, angiogenesis initiation in metastatic tumors may be very different from that of primary tumors and require different interventions.

1.10 Skin Flaps Enable Ultra-High Resolution External Imaging of Angiogenesis

Opening a reversible skin-flap in the light path markedly reduced signal attenuation, increasing detection sensitivity many-fold. The observable depth of tissue is thereby greatly increased (34). The brilliance of the tumor GFP fluorescence, facilitated by the reduced absorption through the skin-flap window, allowed imaging of the induced microvessels as dark against a bright background. The orthotopically growing BxPC 3-GFP human pancreatic tumor was visualized surrounded by its microvessels visible by their dark shadows (34).

1.11 Dual Color Tumor-Host Models

Okabe et al. (35) produced transgenic mice with GFP under the control of a chicken beta-actin promoter and cytomegalovirus enhancer. All of the tissues from these transgenic mice, with the exception of erythrocytes and hair, fluoresce green.

Tumor cells to be transplanted in the GFP mouse were made visible by transforming them with the red fluorescent protein (RFP) (28). In order to gain further insight into tumor-host interaction in the living state, including tumor angiogenesis, we have visualized RFP-expressing tumors transplanted in the GFP-expressing transgenic mice under dual-color fluorescence microscopy. The dual-color fluorescence made it possible to visualize the tumor growth in the host by whole-body imaging as well as to visibly distinguish interacting tumor and host cells in fresh tissue. The dual-color approach affords a powerful means of both visualizing and distinguishing the components of the host-tumor interaction (36).

1.12 Visualizing Angiogenesis Onset and Development by Dual-Color Imaging

Dual-color images of early events in tumor angiogenesis induced by a B16F10 mouse melanoma in the transgenic GFP expressing mouse were acquired in fresh tissue preparations. Host-derived GFP-expressing fibroblast cells and endothelial cells form nascent blood vessels were visualized clearly against the red fluorescent background of the RFP-expressing mouse melanoma. Host-derived GFP-expressing blood vessels within the RFP-expressing mouse melanoma became visible. The images were acquired three weeks after subcutaneous injection of B16F10-RFP melanoma cells in the GFP mouse.

1.13 Dual-Color Imaging with Spectral Resolution with Ultra-High Resolution Whole-Body Imaging of Angiogenesis

The MDA-MB-435 human breast tumor expressing RFP was orthotopically transplanted to the transgenic GFP nude mice. The RFP tumors growing in the GFP-mice were visualized using excitation centered at 470 nm and appropriate >500-nm emission filters. Using a spectral imaging system based on liquid crystal tunable filters, we were able to perform whole-body imaging yielding high-resolution spectral information at each pixel of the resulting image. Powerful analysis algorithms allow the separation (unmixing) of individual spectral species on a pixel-by-pixel basis. Such techniques non-invasively visualized the presence of host GFP-expressing stroma within the RFP-labeled tumor. Moreover, fluorescence spectra emitted in the far-red, allow the whole-body imaging of tumor angiogenesis. This new differential dual-colored fluorescence imaging tumor-host model, along with spectral unmixing, can non-invasively visualize in real-time the onset and progression of angiogenesis in a tumor. Other host cells and structures in the tumor may also be visualized by whole-body spectral imaging (37).

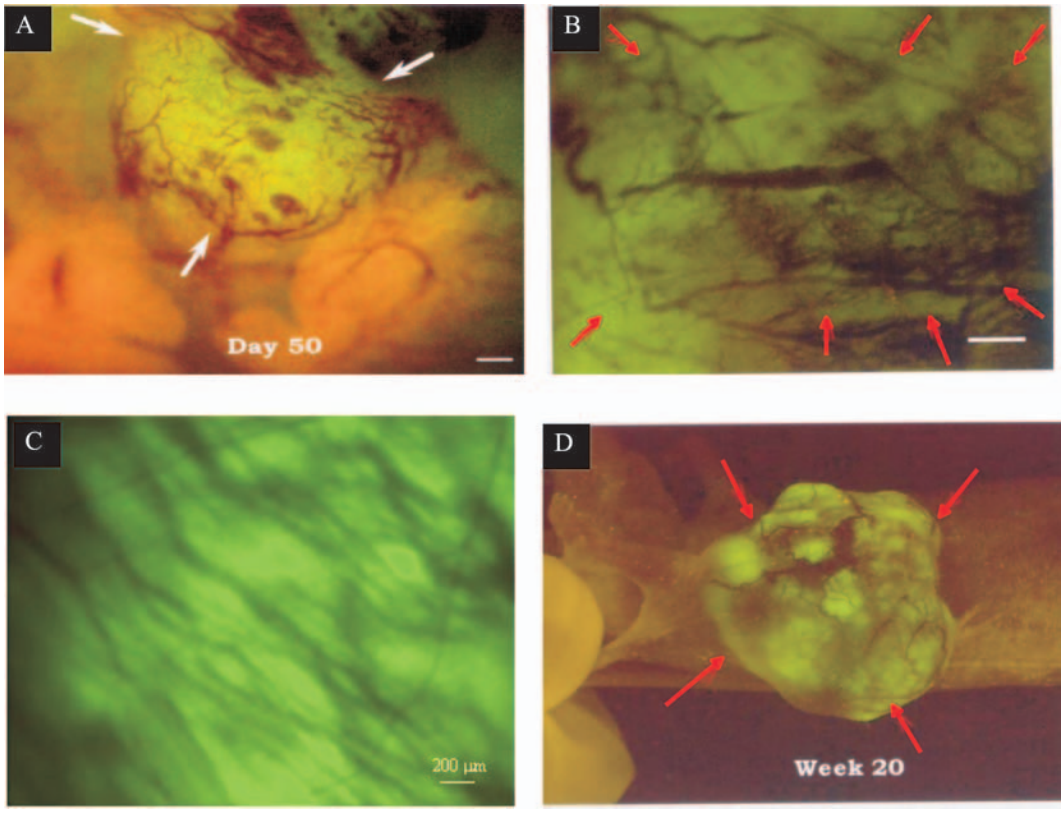


Figure 1. **A.** Intra vital fluorescence imaging of Bx PC 3 GFP human pancreas cancer angiogenesis in an orthotopic tumor. The GFP expressing human tumor was transplanted to nude mice by surgical orthotopic implantation (SOI) and intravitally imaged 50 days later. Bar = 200 µm. **B.** Intra vital fluorescence imaging of PC 3 human prostate cancer angiogenesis in orthotopic primary tumor. The GFP expressing human tumor was transplanted to nude mice by SOI and imaged at day 19. Bar = 470 µm. **C.** Direct view of microvessels of orthotopically-growing pancreatic cancer. The human Bx PC-3-GFP pancreatic tumor, microvessels were directly viewed on day-58 after SOI and highly resolved through the skin flap window at higher magnification. Bar = 200 µm. **D.** Whole body fluorescence imaging of MDA MB 435 human breast cancer angiogenesis in orthotopic primary tumor. The GFP expressing human tumor was transplanted by SOI in the fat pad of nude mice and whole body imaged at week 20.

2. MATERIALS AND METHODS

2.1 Fluorescence Optical Imaging (6)

A Leica fluorescence stereo microscope model LZ12 equipped with a mercury lamp and a 50-W power supply was used. Selective excitation of GFP was produced through a D425y60 band-pass filter and a 470 DCXR dichroic mirror. Emitted fluorescence was collected through a long-pass filter (GG475; Chroma Technology, Brattleboro, VT) on a Hamamatsu C5810 3-chip cooled color charge-coupled device camera (Hamamatsu Photonics, Bridgewater, NJ). Images were processed for contrast and brightness and analyzed with the use of IMAGE PRO PLUS 3.1 software (Media Cybernetics, Silver Spring, MD). High-resolution images of 1024 3 724 pixels were captured directly on an IBM PC or continuously through video output

on a high-resolution Sony VCR (model SLVR1000; Sony, Tokyo).

2.2 Expression Cectors (38)

The pLNCX$_2$ vectors were purchased from Clontech Laboratories (Palo Alto, CA). the pLNCX$_2$ vector contains the neomycin resistance gene for antibiotic selection in eukaryotic cells. The RFP gene (DsRed2; Clontech Laboratories) was inserted in the pLNCX$_2$ vector at the Egl II and Not I sites.

2.3 GFP and RFP Vector Production (6, 36)

GFP expression vector. The pLEIN retroviral vector (CLONTECH) expressing enhanced GFP and the neomycin resistance gene on the same bicistronic message, which contains an internal ribosome entry site (6), was used to transduce tumor cells.

RFP expression vector. PT67, a NIH 3T3-derived packaging cell line expressing the 10 Al viral envelope, was purchased from CLONTECH. PT67 cells were cultured in DMEM (Irvine Scientific) supplemented with 10% heat-inactivated FBS (Gemini Biological Products, Calabasas, CA). For vector production, packaging cells (PT67), at 70% confluence, were incubated with a precipitated mixture of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*trimethylammonium methylsulfate reagent (Roche Molecular Biochemicals) and saturating amounts of pLEIN plasmid for 18 h. Fresh medium was replenished at this time. The cells were examined by fluorescence microscopy 48 h after transfection. For selection, the cells were cultured in the presence of 500-2000 mgyml of G418 (Life Technologies, Grand Island, NY) for 7 days (6).

2.4 Retroviral GFP and RFP Transduction of Tumor Cells (6)

For GFP and RFP gene transduction, 25% confluent cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells and RPMI 1640 (GIBCO) containing 10% FBS (Gemini Biological Products) for 72 h. Fresh medium was replenished at this time. Cells were harvested by trypsin EDTA 72 h after transduction and subcultured at a ratio of 1:15 into selective medium, which contained 200 mg/ml of G418. The level of G418 was increased stepwise up to 1000 mg/ml. Clones stably expressing GFP or RFP were isolated with cloning cylinders (Bel-Art Products) with the use of trypsin/EDTA and were then amplified and transferred by conventional culture methods.

2.5 Animals (6)

Six-week-old BALB/c *nu*/*nu* male and female *nude* mice were used. Transgenic C57/B6-GFP mice (35) were obtained from Research Institute for Microbial Diseases, Osaka University, Osaka, Japan. The C57/B6-GFP mice express GFP under the control of the chicken beta-actin promoter and cytomegalovirus enhancer. All of the tissues from this transgenic line, with the exception of erythrocytes and hair, fluoresce green under excitation light. The GFP gene, regulated as described above, was crossed in to nude mice on the C57/B6 background. Both immunocompetent and nude GFP transgenic mice were used.

All animal studies were conducted in accordance with the principles and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals under assurance number A3873-1. Animals were kept in a barrier facility under HEPA filtration. Mice were fed with autoclaved laboratory rodent diet (Tecklad LM-485, Western Research Products, Orange, CA).

2.6 SOI Tumor Models (33)

Tumor fragments (1 mm^3), stably expressing GFP or RFP, previously grown s.c. in nude mice, were implanted by SOI on the appropriate organ in nude mice. After proper exposure of the organ to be implanted, 8–0 surgical sutures were used to penetrate the tumor pieces and attach them to the appropriate orthotopic organ. The incision in the skin was closed with a 7–0 surgical suture in one layer. The animals are kept under isoflurane anesthesia during surgery. All procedures of the operation described above were performed with a 37 magnification microscope (MZ6; Leica, Nussloch, Germany).

2.7 Cutaneous Melanoma Model (36)

Six-week-old male C57/B6-GFP mice were injected subcutaneously with 10^6 RFP-expressing mouse B16F0 melanoma cells. Cells were first harvested by trypsinization and washed 3 times with cold serum-containing medium, then kept on ice. Cells were inoculated by intradermal injection of the dorsal skin of the animal in a total volume of 50 µl within 40 minutes of harvesting.

2.8 Quantitative Analysis of Angiogenesis (6)

Periodically, the tumor-bearing mice were examined by intravital or whole-body fluorescence imaging. The extent of blood vessel development in a tumor was evaluated based on the total length of blood vessels (*L*) in chosen areas: areas containing the highest number of vessels were identified by scanning the tumors by intravital or whole-body imaging. To compare the level of vascularization during tumor growth, the ‘‘hot’’ areas with the maximum development of vessels per unit area were then quantitated for *L* expressed in pixels. Captured images were corrected for unevenness in illumination. Then the total number of pixels derived from the blood vessels was quantified with IMAGE PRO PLUS software.

2.9 Skin-Flap Windows (34)

Tumor cells on the various internal organs were visualized through the skull or body wall through different skin-flap windows over the scalp, chest wall, upper abdomen, and lower abdomen. The animals were anesthetized with the ketaminemixture. An arc-shaped incision was made in the skin, and s.c. connective tissue was separated to free the skin flap. The skin flap could be opened repeatedly to image tumor cells on the internal organs through the nearly transparent mouse body walls or skull and simply closed with an 6–0 suture. This procedure greatly reduced the scatter of fluorescent photons.

2.10 Tumor Tissue Sampling (36)

Tumor tissue biopsies were processed from three days to four weeks after inoculation of tumor cells. Fresh tissue were cut into ~1mm^3 pieces and pressed on slides for fluorescence microscopy. For analyzing tumor angiogenesis, the tissues were digested with trypsin/EDTA at 37°C for 5 minutes before examination. After trypsinization, tissues were put on pre-cleaned microscope slides (Fisher Scientific, Pittsburgh, PA 15219) and covered with another microscope slide.

ACKNOWLEDGEMENT

These studies were funded in part by National Cancer Institute grant number 1 R43 CA099258-01 and 1 R43 CA103563-01.

REFERENCES

1. Carmeliet, P., 2003, Angiogenesis in health and disease. Nature Med 9:653-660.
2. Jain, R.K., 2003, Molecular regulation of vessel maturation. Nature Med 9:685-693.
3. Ruoslahti, E., 2002, Specialization of tumour vasculature. Na Rev Cancer, 2:83-90.
4. Hendrix, M. J., Seftor, E.A., Hess, A.R., and Seftor, R. E., 2003, Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. Nat Rev Cancer 3:411-21.
5. McDonald, D.M., and Choyke, P.L., 2003, Imaging of angiogenesis: from microscope to clinic. Nat Med 9:713-725.
6. Yang, M., Baranov, E., Li, X-M., Wang, J-W., Jiang, P., Li, L., Moossa, A.R., Penman, S., and Hoffman, R.M., 2001, Whole-body and intravital optical imaging of angiogenesis in orthotopically implanted tumors. Proc Natl Acad Sci USA, 98:2616-2621.
7. Auerbach, R., Kubai, L., Knighton, D., and Folkman, J., 1974, A simple procedure for the long-term cultivation of chicken embryos. Dev Biol 41:391–394.
8. Crum, R., Szabo, S., and Folkman, J., 1985, A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. Science, 230:1375-1378.
9. Miller, J. W., Stinson, W. G., and Folkman, J., 1993, Regression of experimental iris neovascularization

with systemic alpha-interferon. Ophthalmology, 100:9–14.
10. Passaniti, A., Taylor, R. M., Pili, R., Guo, Y., Long, P. V., Haney, J. A., Pauly, R. R., Grant, D. S., and Martin, G. R., 1992, A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. Lab Invest, 67:519–528.
11. Alessandri, G., Raju, F., and Gullino, P. M., 1983, Mobilization of capillary endothelium in vitro induced by effectors of angiogenesis in vivo. Cancer Res, 43:1790–1797.
12. Deutsch, T. A., and Hughes, W. F., 1979, Suppressive effects of indomethacin on thermally induced neovascularization of rabbit corneas. Am J Ophthalmol, 87:536–540.
13. Korey, M., Peyman, G. A., and Berkowitz, R., 1977, The effect of hypertonic ointments on corneal alkali burns. Ann Ophthalmol, 9:1383–1387.
14. Mahoney, J. M., and Waterbury, L. D., 1985, Drug effects on the neovascularization response to silver nitrate cauterization of the rat cornea. Curr Eye Res, 4:531–535.
15. Li, W. W., Grayson, G., Folkman, J., and D'Amore, P. A., 1991, Sustained-release endotoxin. A model for inducing corneal neovascularization. Invest. Ophthalmol Vis Sci, 32:2906–2911.
16. Epstein, R. J., Hendricks, R. L., and Stulting, R. D., 1990, Interleukin-2 induces corneal neovascularization in A/J mice. Cornea, 9:318–323.
17. Gimbrone, M. A., Cotran, I. S., Leapman, S. B., and Folkman, J., 1974, Tumor growth and neovascularization: an experimental model using the rabbit cornea. J Natl Cancer Inst, 52:413–427.
18. Fournier, G. A., Lutty, G. A., Watt, S., Fenselau, A., and Patz, A., 1981, A corneal micropocket assay for angiogenesis in the rat eye. Invest Ophthalmol Vis Sci, 21:351–354.
19. Muthukkaruppan, V., and Auerbach, R., 1979, Angiogenesis in the mouse cornea. Science, 205:1416–1418.
20. Papenfuss, H. D., Gross, J. F., Intaglietta, M., and Treese, F. A., 1979, A transparent access chamber for the rat dorsal skin fold. Microvasc Res 18:311–318.
21. Gross, J., Roemer, R., Dewhirst, M., and Meyer, T., 1982, Int J Heat Mass Transfer, 25:1313–1320.
22. Dewhirst, M., Gross, J., Sim, D., Arnold, P., and Boyer, D., 1984, The effect of rate of heating or cooling prior to heating on tumor and normal tissue microcirculatory blood flow. Biorheology, 21:539–558.
23. Fukumura, D., Xavier, R., Sugiura, T., Chen, Y., Park, E. C., Lu, N., Selig, M., Nielsen, G., Taksir, T., Jain, R. K., et al., 1998, Tumor induction of VEGF promoter activity in stromal cells. Cell, 94:715–725.
24. Li, C. Y., Shan, S., Huang, Q., Braun, R. D., Lanzen, J., Hu, K., Lin, P., and Dewhirst, M. W., 2000, Initial stages of tumor cell-induced angiogenesis: evaluation via skin window chambers in rodent models. J Natl Cancer Inst, 92:143–147.
25. Al-Mehdi, A. B., Tozawa, K., Fisher, A. B., Shientag, L., Lee, A., and Muschel, R. J., 2000, Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: a new model for metastasis. Nat Med, 6:100–102.
26. Huang, Q., Shan, S., Braun, R. D., Lanzen, J., Anyrhambatla, G., Kong, G., Borelli, M., Corry, P., Dewhirst, M. W., and Li, C. Y., 1999, Noninvasive visualization of tumors in rodent dorsal skin window chambers. Nat Biotechnol, 17:1033–1035.
27. Cowen, S. E., Bibby, M. C., and Double, J. A., 1995, Characterisation of the vasculature within a murine adenocarcinoma growing in different sites to evaluate the potential of vascular therapies. Acta Oncol, 34:357–360.
28. Hoffman, R.M., 2002, Green fluorescent protein imaging of tumour growth, metastasis, and angiogenesis in mouse models. Lancet Oncology, 3:546-556.
29. O' Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J., 1997, Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell, 88, 277–285.
30. Drevs, J., Hofmann, I., Hugenschmidt, H., Wittig, C., Madjar, H., Muller, W., Wood, J., Martiny-Baron, G., Unger, C., and Marme, D., 2000, Effects of PTK787/ZK 222584, a specific inhibitor of vascular endothelial growth factor receptor tyrosine kinases, on primary tumor, metastasis, vessel density, and blood flow in a murine renal cell carcinoma model. Cancer Res, 60:4819–4824.
31. Prewett, M., Huber, J., Li, Y., Santiago, A., O'Connor, W., King, K., Overholser, J., Hooper, A., Pytowski, B., Witte, L., et al., 1999, Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. Cancer Res, 59:5209–5218.
32. Kurebayashi, J., Kunisue, H., Yamamoto, S., Kurosumi, M., Otsuki, T., and Sonoo, H., 2000, Paradoxical hormone responses of KPL-1 breast cancer cells in vivo: a significant role of angiogenesis in tumor growth. Oncology, 59:158–165.
33. Hoffman, R.M., 1999, Orthotopic metastatic mouse models for anticancer drug discovery and evaluation: a bridge to the clinic. Investigational New Drugs 17:343-359.
34. Yang, M., Baranov, E., Wang, J-W., Jiang, P., Wang, X., Sun, F-X., Bouvet, M., Moossa, A.R., Penman, S., and Hoffman, R.M., 2002, Direct external imaging of nascent cancer, tumor progression, angiogenesis, and metastasis on internal organs in the

fluorescent orthotopic model. Proc Natl Acad Sci USA 99:3824-3829.

35. Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T., and Nishimune, T., 1997, 'Green mice' as a source of ubiquitous green cells. FEBS Letters, 407: 313-319.
36. Yang, M., Li, L., Jiang, P., Moossa, A.R., Penman, S., and Hoffman, R.M., 2003, Dual-color fluorescence imaging distinguishes tumor cells from induced host angiogenic vessels and stromal cells. Proc Natl Acad Sci USA, 100:14259-14262.
37. Levenson, R., Yang, M., and Hoffman, R.M., 2004, Whole-body dual-color differential fluorescence imaging of tumor angiogenesis enhanced by spectral unmixing. Proc Am Assoc Cancer Res, accepted abstract.
38. Yamamoto, N., Yang, M., Jiang, P., Xu, M., Tsuchiya, H., Tomita, K., Moossa, A.R., and Hoffman, R.M., 2003, Determination of clonality of metastasis by cell-specific color-coded fluorescent-protein imaging. Cancer Res, 63:7785-7790.

Chapter 4

The Epigenetic Influence of the Tumor Microenvironment on Melanoma Plasticity

E.A. Seftor[1], P.S. Meltzer[2], D.A. Kirschmann[1], R.E.B. Seftor[1] and M.J.C.Hendrix[1]
[1]Children's Memorial Institute for Education and Research, Children's Memorial Hospital, Feinberg School of Medicine, Northwestern University, Chicago, Illinois USA and [2]Cancer Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA

Abstract: Melanoma represents a growing public health burden worldwide, and like most other cancers, is a disease of the tumor-host microenvironment. An innovative cellular and molecular analysis has been used to study the epigenetic induction of a transdifferentiated phenotype in poorly aggressive melanoma cells exposed to the microenvironment of aggressive melanoma cells, including the acquisition of a plastic and invasive phenotype. These findings offer a unique perspective of the inductive properties associated with an aggressive melanoma microenvironment that might provide new insights into the regulation of tumor cell plasticity and differentiation, as well as mechanisms that could be targeted for novel therapeutic strategies. A dynamic, complex relationship exists between tumor cells and their microenvironment, which plays a pivotal role in cancer progression, yet remains poorly understood. Particularly perplexing is the revelation that aggressive melanoma cells express genes associated with multiple cellular phenotypes, in addition to their ability to form vasculogenic-like networks in three-dimensional (3-D) matrix -- vasculogenic mimicry. Key to identifying the molecular mechanisms underlying vasculogenic mimicry and tumor cell transdifferentiation is understanding the unique role of the tumor microenvironment in this process. This chapter will review the epigenetic effect of the microenvironment of aggressive melanoma cells. The data reveal profound changes in the global gene expression in poorly aggressive melanoma cells exposed to 3-D matrices preconditioned by aggressive melanoma cells, including the acquisition of a vasculogenic cell phenotype, upregulation of ECM remodeling genes, and increased migratory/invasive potential -- indicative of microenvironment-induced transdifferentiation.

Key words: Melanoma, epigenetic, plasticity, tumor microenvironment, vasculogenic mimicry, microarray, transdifferentiation, angiogenesis, cell signalling, 3-D matrix

1. PLASTICITY OF HUMAN MELANOMA CELLS

Cancer is a disease of the tumor-host microenvironment consisting of a complex, dynamic relationship that remains enigmatic. Cutaneous melanoma is considered one of the few remaining cancers escalating in incidence (1, 2), and thus represents a growing public health burden worldwide (3-5). Uveal melanoma is considered the most common primary intraocular cancer in adults (6), and metastasis occurs in an unpredictable manner in approximately 50% of patients with a primary tumor originating in the choroid or ciliary body of the eye (6). Indeed, the clinical management of cutaneous and uveal melanoma, and many other types of cancer, would benefit significantly from the identification of valid predictors of disease progression and metastatic potential. Recent reports directed toward unveiling the molecular signature of

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 47-63.

melanoma tumor cells have resulted in important classification schemes for cutaneous (7) and uveal (8, 9) melanoma. Indeed, translational studies are beginning to emerge that support the promise of microarray technology in melanoma care (10; for review, see 11); however, the precise biological function of aberrantly expressed genes and their role(s) in tumor cell interactions with the microenvironment are yet to be fully comprehended.

To date, comparative global gene analyses of aggressive and poorly aggressive human cutaneous and uveal melanoma cell lines have revealed the unexpected finding that aggressive tumor cells express genes that are associated with multiple cellular phenotypes (8, 12, 13). These include genes that are usually expressed by epithelial, endothelial, pericyte, fibroblast, hematopoietic, kidney, neuronal, muscle, and several other cell types, and their respective precursor stem cells. These intriguing findings strongly suggest that aggressive melanoma cells revert to an undifferentiated, embryonic-like phenotype, a concept that challenges our current thinking of how to identify and target tumor cells with a "plastic" phenotype that can possibly masquerade as other cell types. A remarkable example of melanoma cell plasticity is vasculogenic mimicry, which describes the unique ability of aggressive melanoma cells (but not poorly aggressive melanoma cells) to express endothelia-associated genes and form extracellular matrix (ECM)-rich vasculogenic-like networks in three-dimensional (3-D) culture (14; for review, see 12). The formation of these networks recapitulates the embryonic development of vasculogenic networks, and they are associated with the distinctly patterned, ECM-rich networks observed in aggressive tumors of patients with melanoma (8, 14-17). Additional studies have reported vasculogenic mimicry in various tumor types (for review, see 12), including the demonstration of blood flow and fluid exchange between tumor cell-lined vascular spaces and endothelium-lined vasculature (18-20). Indeed, there is growing evidence for the existence of an intratumoral, tumor-cell-lined, ECM-rich, patterned network that can provide an extravascular fluid pathway, referred to as the "fluid-conducting meshwork" (19, 21).

1.1 Vasculogenic Mimicry as an Example of Plasticity

The etiology of vasculogenic mimicry remains unclear; however, it appears to involve dysregulation of the tumor-specific phenotype and the concomitant transdifferentiation of aggressive tumor cells into other cell types -- such as endothelial cells. Furthermore, select angiogenesis inhibitors are ineffective in destroying tumor cell vasculogenic mimicry (12, 22), which is an important consideration in the design of anti-vascular therapies. Key to our identifying the molecular mechanisms underlying vasculogenic mimicry and tumor cell plasticity is to first understand the unique role the tumor microenvironment plays in this process. Thus, the purpose of this chapter is to review the evidence demonstrating the potential epigenetic effect of the microenvironment of aggressive melanoma cells on the transdifferentiation of poorly aggressive melanoma cells. The data reveal profound changes in the global expression of genes in poorly aggressive melanoma cells exposed to 3-D collagen I matrices preconditioned by aggressive melanoma cells -- indicative of microenvironment-induced transdifferentiation. In addition, the poorly aggressive melanoma cells acquired characteristics associated with an aggressive phenotype, including the expression of various cell phenotype associated genes, ECM remodeling genes, and migratory/invasive potential. It is anticipated that these findings will offer an unique perspective of the inductive properties associated with the microenvironment of aggressive tumor cells that might provide new targets and paradigms for drug discovery.

Using an unique experimental strategy, presented in Figure 1, we sought to determine whether the microenvironment of aggressive melanoma tumor cells could influence the phenotype, migratory and invasive potential of poorly aggressive melanoma cells exposed to a "preconditioned" ECM microenvironment. The biological properties of the human uveal melanoma cells used in this study are listed in Table 1. The cell phenotype was determined by immunohistochemistry and Northern blot analyses

for the expression of cell-type-specific intermediate filament markers: vimentin for the mesenchymal phenotype, and keratins 8, 18 for an epithelial phenotype. Additional assessments were conducted for in vitro invasive potential and for the ability of the tumor cells to form vasculogenic-like networks (vasculogenic mimicry) in 3-D collagen I matrices. The data presented in Table 1 revealed that the human uveal melanoma cells could be classified into two distinctive categories (based on their biological behavior) -- poorly aggressive and aggressive. Specifically, the poorly aggressive MUM-2C cells (derived from the MUM-2 heterogeneous metastatic cell line) expressed vimentin only, a classical melanoma mesenchymal marker, were poorly invasive in vitro, and did not engage in vasculogenic mimicry. In contrast, the aggressive MUM-2B cells (derived from the MUM-2 metastatic cell line) coexpressed vimentin and keratin(s), indicative of a dedifferentiated, interconverted phenotype, previously described by our laboratory (8, 23). In addition, these aggressive melanoma cells were highly invasive in vitro and formed vasculogenic-like networks in 3-D collagen I matrix.

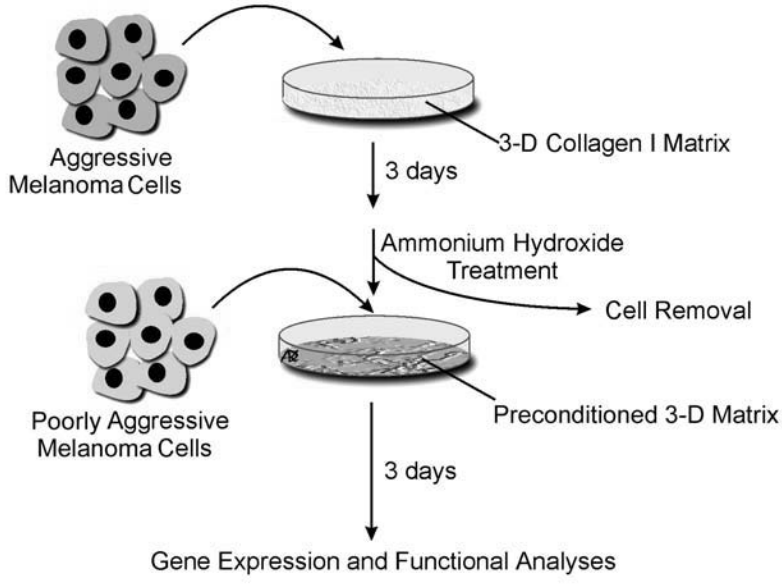


Figure 1. Experimental plan. As shown in this model, aggressive melanoma cells are cultured on a 3-D, collagen I matrix for three days, after which the cells are removed using ammonium hydroxide followed by extensive washing of the matrix. This was followed by seeding the poorly aggressive melanoma cells onto the preconditioned matrix, then analyzing the cells for changes in gene expression and functional changes compared to the poorly aggressive cells cultured on a non-inductive matrix.

Table 1. Biological Properties of Human Uveal Melanoma Cells. [a]Scoring of tumor cell phenotype using classical pathology markers of vimentin (mesenchymal) and cytokeratins 8 and 18 (epithelial) intermediate filaments (IFs) was based on a positive (+) and negative (-) ranking system, determined by immunohistochemistry and Northern blot analysis. [b]Invasiveness was calculated as the percentage of cells capable of invading a collagenous matrix-coated polycarbonate membrane over 24 hours within a membrane invasion culture system (MICS) chamber compared with the total number of cells seeded (± SE; n=6 wells per parameter and run in duplicate experiments). [c]Vasculogenic mimicry was assessed based on the ability of cells seeded onto a three-dimensional collagen I matrix to form tubular vasculogenic-like networks over 7 days. [d]Derived from the MUM-2 heterogeneous cell line obtained from a liver metastasis.

Culture designation	**Cell phenotype[a]**	**Invasive potential[b]**	**Vasculogenic mimicry[c]**
MUM-2B[d]	vimentin+keratin(s)	High (13.3-15±0.6)	+
MUM-2C[d]	vimentin only	Poor (2.0-2.3±0.06)	–

To address the potential epigenetic effect(s) of the tumor cell microenvironment (associated with aggressive melanoma cells) on the possible transdifferentiation of poorly aggressive melanoma cells, we allowed the aggressive MUM-2B cells to "precondition" a defined 3-D microenvironment consisting of collagen I for up to 3 days, then removed the tumor cells and seeded the poorly aggressive MUM-2C cells on the "preconditioned matrix". An additional experimental combination tested included MUM-2B cells incubated on a 3-D matrix preconditioned by poorly aggressive MUM-2C cells. The results from these experiments are shown in Figure 2.

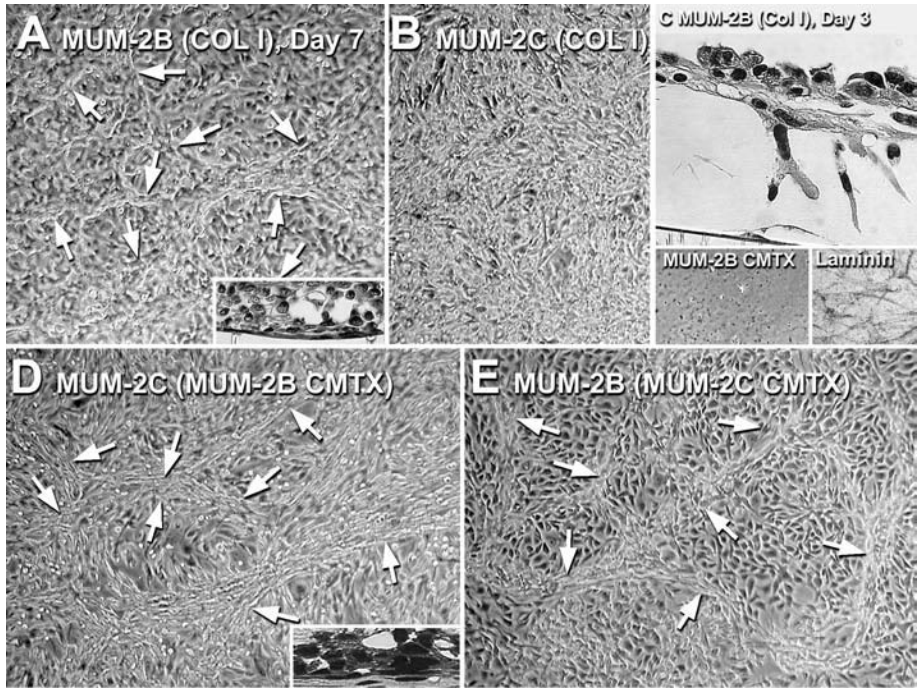


Figure 2. Phase contrast microscopy of human melanoma cells cultured on collagen I 3-D matrices under varying conditions. (A) Highly aggressive, metastatic MUM-2B cells cultured on a collagen I (COL-1) 3-D matrix for seven days form patterned, vasculogenic-like networks that appear tubular when examined by cross-section and stained with H&E (inset). (B) Poorly aggressive, metastatic MUM-2C cells do not form these networks when cultured under the same conditions for seven days. (C) Cross-section of an H&E stained culture of MUM-2B cells on a 3-D matrix after three days and phase contrast image of the matrix after removal of the cells (left inset) and subsequent staining of the preconditioned matrix for laminin (right inset). (D) Poorly aggressive MUM-2C cells form patterned, vasculogenic-like networks when cultured on a matrix preconditioned by the MUM-2B cells (MUM-2B CMTX) and the structures appear tubular in cross-section when stained with H&E (inset). (E) MUM-2B cells cultured on a MUM-2C preconditioned matrix are not inhibited in the formation of the patterned networks by the poorly aggressive cell preconditioned matrix.

The aggressive MUM-2B cells formed mature, patterned, vasculogenic-like networks by 7 days on 3-D collagen I matrix, and histological cross-sections of these cultures revealed the tubular nature of the network structures (Figure 2A, inset). By comparison, the poorly aggressive MUM-2C cells were unable to engage in vasculogenic mimicry under similar experimental conditions (Figure 2B). In preparation for the next set of experiments involving the incubation of poorly aggressive melanoma cells on 3-D matrices preconditioned by aggressive tumor cells, it was important to first determine the morphological status of the aggressive melanoma cell cultures after 3 days of interacting with the 3-D collagen I matrix. As shown in Figure 2C, a representative example of aggressive

melanoma cells (MUM-2B) interacting for 3 days with a 3-D collagen I matrix demonstrates that the tumor cells are invading into the 3-D matrix, but have not yet formed mature, tubular, vasculogenic-like networks. The two insets in Figure 2C show that ammonium hydroxide treatment efficiently removed tumor cells from their respective 3-D matrices, and proteins, such as laminin, can be detected deposited in tracks within the preconditioned matrix. When the poorly aggressive MUM-2C cells were incubated on the 3-D matrix preconditioned by the aggressive MUM-2B cells, they formed patterned vasculogenic-like networks -- for the first time -- some of which were tubular in nature as shown by cross-sectional histological analysis (Figure 2D, inset). We then tested whether the preconditioning of a 3-D matrix by poorly aggressive MUM-2C cells could affect the vasculogenic mimicry potential of MUM-2B cells, and it did not (Figure 2E). Collectively, these data support the hypothesis that the microenvironment preconditioned by aggressive melanoma tumor cells can induce a vasculogenic phenotype in poorly aggressive melanoma cells.

2. INDUCTION OF DIFFERENTIAL GENE EXPRESSION BY THE MICROENVIRONMENT OF AGGRESSIVE MELANOMA CELLS

To achieve a global gene analysis of the epigenetic changes associated with poorly aggressive melanoma cells exposed to 3-D collagen I matrices preconditioned by aggressive melanoma cells, we employed microarray analysis followed with hierarchical clustering using the average linkage method. Highlights of these data are shown in Tables 2 and 3 based on the hierarchical clustering of melanoma gene expression patterns under varying culture conditions. The culture condition parameters consisted of: 1) Aggressive MUM-2B or poorly aggressive MUM-2C cells grown on tissue culture plastic *versus* their respective incubation on unconditioned 3-D collagen I matrix; 2) MUM-2B *versus* MUM-2C cells cultured on either tissue culture plastic or on unconditioned 3-D collagen I matrix; and 3) MUM-2C cells grown on 3-D collagen I matrix preconditioned by MUM-2B cells *versus* MUM-2C cells grown on an unconditioned (control) collagen I matrix. The complete global gene analysis is available at http://www.hgri.nih.gov.

Comparative analysis of genes from the various cell lines grown on plastic relative to unconditioned collagen I matrix showed minimal to no change in their molecular profile or phenotype (data not shown). However, the microarray data demonstrate that the matrix microenvironment preconditioned by aggressive MUM-2B melanoma cells, exerted (in most instances) a profound, epigenetic effect on the poorly aggressive MUM-2C cells exposed to these preconditioned 3-D collagen I matrices. There are several categories of genes that are shown to be upregulated in the poorly aggressive melanoma cells indicative of a transdifferentiated phenotype, including genes associated with the ECM and remodeling, endothelial, epithelial, neuronal, and bone marrow mesenchymal stem cell phenotypes, growth factors, signal transduction and transcription factors, and a highly invasive/aggressive cell phenotype.

The change in gene expression resulting from the epigenetic influence of the aggressive melanoma microenvironment on poorly aggressive cells coincides with many of the constitutively upregulated genes in the MUM-2B cells (relative to MUM-2C). Interestingly, many of the down-regulated genes observed in the poorly aggressive melanoma cells exposed to the matrix microenvironment preconditioned by aggressive melanoma cells are associated with melanoma-specific antigens -- similar to their constitutive down-regulation observed in the aggressive MUM-2B cells.

Table 2. Microarray analysis of aggressive versus poorly aggressive uveal melanoma cells on preconditioned versus control collagen I matrices: Upregulated genes. Altered gene expression in human uveal melanoma cells was identified by cDNA microarray analysis. [a]Selected genes with a differential expression of 1.4-fold or greater are reported as a ratio of aggressive to poorly aggressive uveal melanoma cells. [b]Differential expression of selected genes reported as a ratio of poorly aggressive uveal melanoma cells grown on a Col I matrix (cmtx) preconditioned by aggressive uveal melanoma cells compared to cells grown on an unconditioned control Col I matrix.

Gene Name	MUM-2B/ MUM-2C[a]	MUM-2C on MUM-2B cmtx/ MUM-2C Col I[b]
Cell phenotype associated genes		
Aminopeptidase N, CD13	18.8	11
Vascular endothelial (VE)-cadherin	3.6	1.5
Melanoma cell adhesion molecule	>20	6.2
EphA2 protein tyrosine kinase	6.5	1.3
Keratin 7	9.8	2.6
Epithelial membrane protein 1	9.3	1.9
Putative lymphocyte G0/G1 switch gene	>20	7.8
ECM-related genes		
Fibronectin 1	>20	11.6
Laminin 5 β3	18.6	7.5
Laminin 5 γ2	8.9	2.5
Integrin, α_3 -subunit	>20	2.8
Lysyl oxidase-like 2	5.5	2.4
Matrix metalloproteinase-2 (MMP-2)	4.2	1.5
Matrix metalloproteinase-14 (MT1-MP)	3.3	1.7
Urokinase	9.5	1.5
Transcription/signal transduction/growth factor-related genes		
c-met protooncogene	3.2	2.8
Interleukin-8	9.7	>20
Paired box gene 8	8.7	4.5
Colony stimulating factor 3	> 20	5.2
Cysteine-rich, angiogenic inducer, 61	16.9	2.0
GRO I oncogene	11.0	1.8
Interleukin-1β	>20	5.8
Rho GDP dissociation inhibitor (GDI) beta	7.9	2.4
Urokinase Receptor	4.5	2.9
Inhibin, beta A	15.7	6.1
Zinc finger protein 41	>20	8.4
Transmembrane 4 superfamily member 1	11.7	4.4
Thymosin, beta 4, X chromosome	>20	4.8
Thymosin, beta 4, Y chromosome	>20	3.4
Cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 4	>20	4.6

Table 3. Microarray analysis of aggressive versus poorly aggressive uveal melanoma cells on preconditioned versus control collagen I matrices: Down-regulated genes. Altered gene expression in human uveal melanoma cells was identified by cDNA microarray analysis. [a]Selected genes with a differential expression of 1.4-fold or greater are reported as a ratio of aggressive to poorly aggressive uveal melanoma cells. [b]Differential expression of selected genes reported as a ratio of poorly aggressive uveal melanoma cells grown on a Col I matrix (cmtx) preconditioned by aggressive uveal melanoma cells compared to cells grown on an unconditioned control Col I matrix.

Gene Name	MUM-2B/ MUM-2C[a]	MUM-2C onMUM-2B cmtx/ MUM-2C Col I[b]
Melanoma antigen, family A, 8	0.53	0.49
Melanoma antigen, family D, 2	0.65	0.66
Preferred Expression in Melanoma	0.09	0.79
Tyrosinase-related protein I	0.05	0.62
Melan-A	0.25	0.41

Confirmation of select differentially expressed genes was accomplished by semi-quantitative RT-PCR analysis of human melanoma cells under various experimental conditions that underwent microarray analysis (Figure 3).

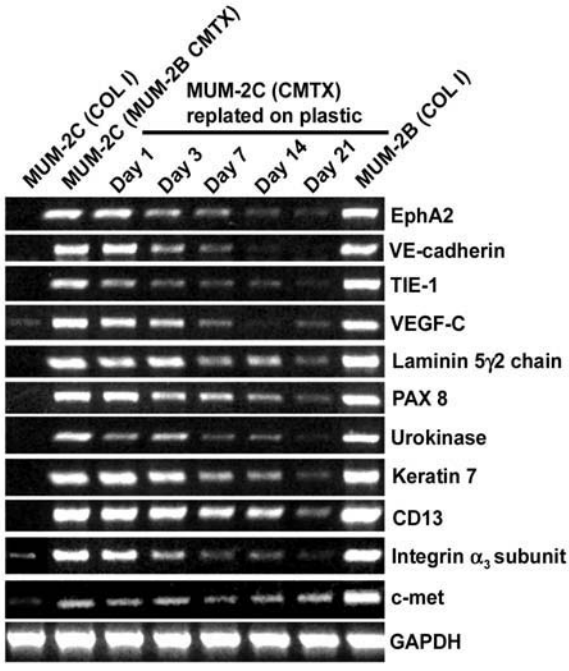


Figure 3. Select genes were studied further by RT-PCR to determine the longevity of EphA2, VE-cadherin, TIE-1, VEGF-C, laminin 5γ2 chain, PAX8, urokinase, keratin 7, CD13, integrin 3-subunit and c-met gene expression in MUM-2C cells grown on a 3-D collagen I matrix preconditioned by MUM-2B cells (MUM-2B CMTX), and subsequently removed from this "inductive" matrix and replated onto tissue culture plastic for 1, 3, 7, 14 and 21 days, respectively, and then compared with the gene expression profiles of MUM-2C or MUM-2B cells grown on collagen I only (COL-I).

The categories of genes tested consisted of: endothelial/vascular-associated genes [EphA2 (erythropoietin-producing hepatocellular carcinoma-A2), VE-cadherin (vascular endothelial), TIE-1 (tyrosine kinase with Ig and epidermal growth factor homology domains-1), VEGF-C (vascular endothelial growth factor-C)]; ECM proteins associated with an aggressive cancer cell phenotype (laminin 5γ2 chain and an associated receptor, α_3-containing integrin); a homeobox gene (PAX 8); an epithelial phenotype-specific gene (keratin 7); a matrix remodeling gene (urokinase); a mesenchymal stem cell-associated gene (CD13); and a proto-oncogene associated with a migratory cell phenotype (c-met). Most noteworthy is the upregulation of genes (mentioned above) by the poorly aggressive MUM-2C cells exposed to matrices preconditioned by the aggressive, metastasis-derived MUM-2B cells, thus validating select data presented in Table 2. In most cases, these genes are not expressed by the poorly aggressive melanoma cells grown on the control 3-D collagen I matrices, but their induced expression is similar (in many instances) to the genes expressed constitutively by the aggressive melanoma cells.

The next question we addressed pertained to the stability of the epigenetic induction of a transdifferentiated phenotype. We focused our observations on the poorly aggressive MUM-2C cells exposed to the matrix microenvironment preconditioned by the aggressive MUM-2B cells, as shown in Figure 3. Following exposure to the preconditioned matrices, the MUM-2C cells were replated onto tissue culture plastic from 1 day to 21 days. By 21 days, expression of VE-cadherin, TIE-1, urokinase, and the integrin α_3-subunit appears to be gone; expression of EphA2, VEGF-C, laminin 5 γ2 chain, PAX 8, keratin 7, and CD13 is reduced, while c-met remains elevated. These data indicate that the epigenetic effect of the aggressive tumor cell microenvironment on poorly aggressive cells is long-lasting with respect to the induction of certain genes and transient in other cases.

3. EPIGENETIC EFFECT OF THE TUMOR MICROENVIRONMENT

The tumor microenvironment plays a critical role in cancer progression (24-26). However, most molecular analyses employed in cancer biology focus on differential gene expression of either cells in an artificial environment (such as tissue culture plastic) or whole tumors, where the contributions of individual cells and their respective microenvironment(s) are undeterminable. Thus, our study was designed to introduce an innovative approach to investigate the potential epigenetic

effect of the microenvironment of aggressive melanoma cells on the possible transdifferentiation of poorly aggressive melanoma cells exposed to it. The findings generated from this strategic approach showed profound changes in the phenotype, genetic profile, and biological activity of poorly aggressive melanoma cells exposed to a metastatic melanoma microenvironment, and indicated their transdifferentiation into an aggressive melanoma cell phenotype. Particularly noteworthy was the observation that some of the genes induced in the poorly aggressive melanoma cells through this epigenetic event remained upregulated 21 days after the cells were removed from an inductive microenvironment and maintained on tissue culture plastic, suggesting more than a transient response to the microenvironment.

The selection of cell lines for the study allowed the comparative analyses of the vasculogenic phenotype, molecular profile, and migratory and invasive potential associated with aggressive *versus* poorly aggressive uveal melanoma cells. Although previous studies from our laboratory had confirmed that both aggressive cutaneous and uveal melanoma cells formed vasculogenic-like networks in 3-D collagen I matrices, whereas poorly aggressive melanoma cells were incapable of vasculogenic mimicry (13, 14, 27), a global comparative molecular analysis of the possible epigenetic effect(s) of an aggressive melanoma cell microenvironment had not been attempted. Prior microarray analyses of human melanoma cell lines (grown on tissue culture plastic) derived from cutaneous and uveal melanomas showed comparable gene expression profiles (7, 8). However, our current work described in this chapter utilized only uveal melanoma as the experimental model because the aggressive and poorly aggressive clones were isolated from the same patient.

The morphological analyses presented in Figure 2 clearly demonstrate that by day 3, aggressive melanoma cells had formed cord-like structures with evidence of highly migratory cells invading the 3-D collagen I matrices. Furthermore, these cells deposited ECM proteins, such as laminin, in tracks throughout the 3-D matrix. Removal of cells from the preconditioned matrices was efficient and did not compromise the integrity of the collagen I matrix. Most noteworthy was the observation that poorly aggressive melanoma cells (MUM-2C) acquired a vasculogenic phenotype and formed tubular vasculogenic-like networks in response to a metastatic microenvironment (preconditioned by MUM-2B). Also intriguing was the finding that the matrix microenvironment preconditioned by the poorly aggressive MUM-2C cells offered no apparent informative cues with respect to inhibiting or enhancing the vasculogenic phenotype of the aggressive MUM-2B cells which demonstrated the predominance of the aggressive cell phenotype under these experimental conditions.

To determine the molecular epigenetic effects induced by the matrix microenvironment preconditioned by aggressive melanoma cells, we utilized a nonbiased global gene analysis approach. Highlighted genes from the 14,000 cDNA element microarray are presented in Tables 2 and 3 subdivided into the following categories to better appreciate their respective biological significance: Cell phenotype associated genes; ECM-related genes; and transcription/signal transduction/growth factor-related genes.

In the category of cell phenotype associated genes, the poorly aggressive MUM-2C melanoma cells exposed to the metastatic-derived MUM-2B cell microenvironment showed a significant down-regulation of several melanoma-specific markers -- similar to those constitutively down-regulated in the aggressive MUM-2B tumor cells. For example, melanoma antigen (family A,8 and family D,2), preferred expression in melanoma, tyrosinase-related protein 1, and melan-A were all down-regulated in MUM-2B aggressive melanoma cells, but not in MUM-2C poorly aggressive cells -- until they were exposed to the MUM-2B preconditioned matrix microenvironment. The expression of melanoma associated antigen genes has been linked to a favorable disease outcome in advanced stage melanoma (28), and several of these antigens are used as important markers in the diagnosis of melanoma (29, 30). Collectively, these data suggest that melanoma cells appear to dedifferentiate as they acquire an aggressive phenotype, which might make them more difficult to identify using routine histopathological markers for diagnosis. However, it is interesting to note that melanoma cell adhesion

molecule, upregulated constitutively in the aggressive melanoma cells, was epigenetically induced in the poorly aggressive MUM-2C cells exposed to the MUM-2B-preconditioned matrix microenvironment. This adhesion molecule, also referred to as MCAM, MUC18, and CD146, is a member of the immunoglobulin supergene family and has been shown to mediate melanoma-endothelial cell heterophilic ligand adhesion integral in the metastatic cascade and can facilitate cell-host interactions (31).

Other cell phenotype associated genes that were upregulated in the aggressive melanoma cells and epigenetically induced in the poorly aggressive melanoma cells exposed to the MUM-2B microenvironment are involved in angiogenesis, lymphangiogenesis and vasculogenesis, including EphA2, VE-cadherin, TIE-1, and VEGF-C. These molecules, with their binding partners, are a few of the factors that are required for the formation and maintenance of the vasculature (32-35). At the protein level, VE-cadherin and EphA2 are expressed only by aggressive melanoma cells, and not by poorly aggressive melanoma cells (35, 36). Furthermore, down-regulation of VE-cadherin or EphA2 expression results in the complete inability of aggressive melanoma cells to form vasculogenic-like networks in 3-D culture. These previous observations from our laboratory, together with the current findings, suggest that the metastasis-derived MUM-2B cells preconditioned their matrix microenvironment in a manner that induced the poorly aggressive cells to acquire a vascular phenotype as demonstrated by the expression of vascular cell-associated genes and ability to form vasculogenic-like networks for the first time in 3-D culture. Additional intriguing vascular cell phenotype associated genes that were induced epigenetically in the poorly aggressive melanoma cells are CD13 and putative lymphocyte G0/G1 switch gene. CD13, a surface-bound metallopeptidase, also referred to as alanyl aminopeptidase (APN), has been identified as the leukocyte surface differentiation antigen (predominantly expressed on cells of myelo-monocytic lineage), and most recently implicated as an angiogenic regulator and transcriptional target of Ras signaling pathways in endothelial morphogenesis and a prime target for anti-angiogenic tumor-homing peptides (37-40). Of particular note in the current study was the observation that the epigenetically induced expression of CD13 endured 21 days after the poorly aggressive melanoma cells were removed from their inductive matrix preconditioned by MUM-2B cells. Thus, it is tempting to speculate that the upregulation of this gene might represent an early event in the differentiation pathway of vascular cells, possibly upstream of VE-cadherin, EphA2, and TIE-1. Coincident with the strong epigenetically induced expression of CD13 is a similar induction of the putative lymphocyte G0/G1 switch gene. Although this gene was originally thought to be the lectin-induced switch of lymphocytes from the G0 to the G1 phase of the cell cycle (41), more recent findings indicate its involvement in mesenchymal progenitor developmental events leading to the differentiation of osteogenic, chondrogenic and predominantly adipogenic lineages (42). The strong induction of the putative lymphocyte G0/G1 switch gene in the poorly aggressive melanoma cells supports the hypothesis that these cells acquire a dedifferentiated, embryonic-like phenotype, similar to that associated with the aggressive melanoma cells.

The epithelial-associated genes -- epithelial membrane protein 1 (EMP1) and keratin 7, were also upregulated in the poorly aggressive melanoma cells exposed to the MUM-2B preconditioned matrix microenvironment. EMP1, alternatively referred to as tumor-associated membrane protein, has been detected in embryonic kidney, brain, gut and is linked to cell-cell interactions and the regulation of cell proliferation, in addition to neuronal differentiation and neurite outgrowth (43-45). Similarly, the induced expression of keratin 7, indicative of a simple epithelial cell type, in the mesenchymally-derived poorly aggressive melanoma cells, strongly suggests their transition to a dedifferentiated, interconverted phenotype, previously shown by our laboratory and others to be closely associated with aggressive behavior and metastatic disease (23, 46-48).

Many of the biological properties germane to embryogenesis, particularly the plasticity associated with stem cells, are also important in tumor

progression. The unexpected microarray profile of aggressive *versus* poorly aggressive melanoma cells revealed the coexpression of multiple phenotype-specific genes by the aggressive tumor cells (7, 8, 27), and current studies are addressing the functional significance of the melanoma vasculogenic phenotype in various models. One such study introduced fluorescently-tagged aggressive melanoma cells into an ischemic limb (non-tumor environment) and then assessed whether the tumor cells could participate in neovascularization (49). This investigation revealed that the limb was reperfused within 5 days with newly formed vasculature containing melanoma and endothelial cells forming chimeric vessels, thus demonstrating the influence of the microenvironment on the transendothelial differentiation of aggressive melanoma cells that were biologically functional. Although the current study indicates that poorly aggressive melanoma cells exposed to the metastatic cell-derived microenvironment were induced to express genes associated with a vascular cell phenotype and form vasculogenic-like networks, it is unknown whether they are fully competent to provide a vascular function similar to that demonstrated previously by aggressive melanoma cells. We are just beginning to appreciate the complexity of cell fate restriction and transdifferentiation (50-52), and the data generated in the present study strongly suggest that epigenetic regulation of cell phenotype specific genes plays a critical role in transdifferentiation and dedifferentiation. Understanding the lineage of tumor cells and the significance of their altered circuitry is critical in elucidating cancer as a disease of altered cellular behavior (53). Achieving a better perspective of the common transcriptional pathways regulating phenotype control genes, particularly with respect to melanocyte development, may hold the key to formulating new strategies to harness the aggressive melanoma cell phenotype.

The epigenetic induction of ECM-related genes in poorly aggressive melanoma cells exposed to the matrix preconditioned by metastasis-derived aggressive MUM-2B cells (highlighted in Table 2) suggests a newly acquired potential to remodel the microenvironment. It is well accepted that the cellular microenvironment can directly modulate cell fate (54-55), but the molecular details governing this dynamic interplay remain poorly understood. However, we are gaining a greater appreciation of the cooperative roles of specific ECM components and matrix remodeling proteases involved in the interactions between tumor cells and their microenvironment (26, 56, 57). In the present study, the most robust epigenetic induction of an ECM-related gene observed in poorly aggressive melanoma cells was fibronectin. Emerging evidence points to fibronectin as a critical regulator of ECM organization and stability with broader implications in cellular migration, differentiation, and metastasis (58-60). Of additional significance is earlier work uncovering the metalloproteinase stimulating ability of cleaved fragments of fibronectin (61), which may provide a biological mechanism for the increased migratory and invasive potential acquired by the poorly aggressive melanoma cells exposed to a metastatic melanoma cell preconditioned microenvironment. Other genes that were upregulated in the poorly aggressive melanoma cells exposed to the microenvironment preconditioned by metastasis-derived MUM-2B cells are associated with matrix remodeling: urokinase, lysyl oxidase-like 2, and matrix metalloproteinases-2 and -14 (MT1-MMP) (26, 62-67). The epigenetic upregulation of laminin 5 and an associated α_3-containing integrin observed in the poorly aggressive melanoma cells was of particular interest based on previous studies from our laboratory demonstrating the requirement for cooperative interactions of laminin 5 γ2 chain, MMP-2 and MT1-MMP for vasculogenic mimicry by aggressive melanoma cells (68-69). Laminins are important components of basement membranes that are involved in regulating differentiation, tumor metastasis, cell attachment, migration and angiogenesis (70-72). Proteolytic cleavage of the laminin 5 γ2 chain by MT1-MMP and MMP-2 results in the formation of laminin 5 γ2' and γ2x promigratory fragments (68, 73-75). Laminin is also an integral component of vasculogenic-like networks or fluid-conducting meshwork formed by aggressive melanoma cells *in vitro* and *in vivo*, respectively (12, 19, 21, 69). Thus, it is tempting to speculate that the acquired ability of poorly aggressive melanoma cells to upregulate laminin 5 (and an associated α_3-

containing integrin) and the MMPs necessary to cleave it into promigratory signals in their microenvironment provides additional evidence that they have assumed a transdifferentiated phenotype that resembles a more aggressive melanoma cell, with possible implications in altered signaling capabilities as well (76). Quite interestingly, the inductive potential of the microenvironment preconditioned by aggressive metastatic melanoma cells can be neutralized by treatment with a chemically modified tetracycline (CMT-3 or COL-3), which is a potent inhibitor of MMP activity, inhibits the cleavage of laminin 5 chain to promigratory fragments, and down-regulates MMP-2, MMP-9, MT1-MMP, VE-cadherin, VEGF-C, and TIE-1 (27). Indeed, these are important biological findings that may be useful in targeting molecular cues in the microenvironment of aggressive tumors, ultimately inhibiting the triggering of the angiogenic/vasculogenic switch thought to initiate critical control pathways (77, 78).

The third category of microarray data consists of transcriptional/signal transduction/growth factor-related genes. Although these genes have been categorized separately from those related to the cell phenotype and the ECM, it is plausible to assume that they are interrelated. For example, as a tumor cell transdifferentiates and acquires different matrix remodeling capabilities, it may also gain the ability to activate poorly diffusible matrix-sequestered growth factors that regulate biological function and trigger various signal transduction pathways, as previously suggested in other models (56). There was quite a robust response by poorly aggressive melanoma cells, exposed to the matrix microenvironment preconditioned by metastatic MUM-2B cells, for the upregulation of genes involved in proliferation/survival, motility, and activation of important signaling pathways. Previous work from our laboratory correlated the expression of c-met proto-oncogene (receptor for hepatocyte growth factor/scatter factor; HGF/SF) in aggressive uveal melanoma cells expressing an interconverted/dedifferentiated phenotype (cells coexpressing vimentin and keratins), and suggested that HGF/SF may play an important role in the metastatic dissemination of this tumor (79). The significance of c-met as a regulator of mitogenesis, motility, and morphogenesis and its critical role in metastasis has been demonstrated in other studies as well (80, 81). Furthermore, the c-met/HGF/SF signaling pathway holds great promise as a therapeutic target for intervention strategies (82, 83). In the present study, it is interesting that the poorly aggressive melanoma cells retained a robust expression for c-met 21 days after their removal from the inductive metastatic preconditioned matrix microenvironment.

Additional genes in this category that have significant implications in cellular migration and invasion include Rho GDP dissociation inhibitor, the thymosin β4 family and the transmembrane 4 superfamily (84-87). The urokinase receptor (uPAR), also upregulated in the poorly aggressive melanoma cells exposed to inductive matrices, has been linked to cellular migration through its ability to promote pericellular proteolysis, mediate cell signaling, and regulate integrin function, and most recently has been identified as a preferential binding partner for $\alpha_3\beta_1$ (88, 89). The upregulation of uPAR coincides with the increased expression of the α_3-containing integrin -- also considered a binding partner for laminin. uPAR also activates $\alpha_5\beta_1$ (a fibronectin-specific integrin), which coincides with the upregulation of fibronectin by these cells (90). Another potentially related upregulated gene that could provide a transduction role is cysteine-rich, angiogenic inducer, 61 (CYR61), which acts as an ECM-associated signaling molecule and promotes endothelial cell adhesion and neovascularization through an integrin-dependent pathway (91). Thus, the potential for multiple signaling interactions and down-stream events associated with the upregulation of key molecules may help to elucidate the pathways underlying the transdifferentiation of poorly aggressive melanoma cells. In conjunction with the upregulated signaling molecules were genes associated with potent cytokines and growth factors, including interleukin 8 (IL8), interleukin 1-beta (IL1β), colony stimulating factor 3 (CSF3; granulocyte colony-stimulating factor, GCSF), inhibin, beta A, and the GRO1 oncogene (melanoma growth stimulatory activity). IL8 is a member of the CXC chemokine family and is a proinflammatory mediator of neutrophil activation and migration (92). IL8 expression has been shown to be stimulated by

IL1β -- primarily produced by blood monocytes and most recently implicated in the development of hepatic metastases of melanoma (93). CSF3 (also called GCSF), is known to stimulate the proliferation and differentiation of the progenitor cells for granulocytes (94-95), in a manner similar to the biological activity on inhibin, beta A (also called activin A), whose ligands act as growth and differentiation factors in many cells (96). The GRO1 oncogene, formerly called melanoma growth stimulatory activity, is a mitogenic polypeptide secreted by human melanoma cells and important to their growth and survival (97). Also intriguing was the upregulation (by poorly aggressive melanoma cells exposed to aggressive melanoma cell preconditioned matrices) of zinc finger protein 41 that encodes regulatory proteins (98), paired box gene 8 important in differentiation (99), and cytochrome P450, subfamily 3A4 which plays a central role in the metabolism of drugs (100).

Overall, the cellular and molecular analyses employed in this study to measure the effects of a microenvironment preconditioned by aggressive melanoma cells on poorly aggressive melanoma cells revealed an intriguing epigenetic induction of a transdifferentiated phenotype. The implications of these findings pose important clinical challenges involving: 1) the detection of tumor cells -- as they may phenotypically mimic other cell types; and 2) the targeting of aggressive tumor cells within a heterogeneous tumor that have the potential to modify their microenvironment is such a manner as to epigenetically induce transdifferentiation and a more aggressive phenotype in other tumor cells. It is interesting to note that a previous study comparing gene expression profiles between metastatic derivatives and their poorly metastatic parental cells implicated genes involved in matrix remodeling and signal transduction (101). Also germane to the interpretation of results from the current work are previous reports highlighting the molecular signature of metastasis-associated genes in primary tumors (102) and also raising questions about the concept of metastasis genes (103).

Transdifferentiation is emerging as an important phenomenon that adds a new level of complexity to developing rational therapeutic strategies (50, 104-106). It is interesting to note that during the development of Kaposi's sarcoma, endothelial cells transdifferentiate into tumor cells (107), whereas aggressive melanoma cells, and poorly aggressive melanoma cells exposed to metastatic inductive matrices, transdifferentiate to an endothelial cell type. These observations raise the intriguing possibility that these two tumor cell types could have a common origin or lineage. Paramount to our efforts to manage melanoma is addressing the major problem of drug resistance (108). Compounding these efforts are recent *in vitro* and *in vivo* data showing that aggressive melanoma cells engaged in vasculogenic mimicry are relatively unaffected by angiogenesis inhibitors (12, 22). Additional evidence indicates that tumor cells may remodel their microenvironment with extra ECM to increase their survival in the presence of therapeutic agents (109), which has been shown to adversely affect interstitial transport in solid tumors (110). MMP inhibitors have also experienced challenges in clinical trials, but these proteinases are still worth consideration in the development of strategies to target the tumor microenvironment (24, 26, 27, 111). As we elucidate the pro-migratory inductive potential of proteolytically cleaved fragments of the ECM, it is clear that these partially degraded molecules could be prime targets for therapeutic intervention -- potentially for the use in a combinatorial manner with other therapies (72). Successful management of malignant melanoma and other cancers will benefit from the identification of essential regulatory pathways and molecular switches underlying the dedifferentiated, plastic tumor cell phenotype and its unique interactions with the microenvironment.

ACKNOWLEDGMENT

The authors gratefully acknowledge access to human uveal melanoma cell lines and tissue explants kindly provided by Drs. Daniels, Folberg, Kan-Mitchell and Pe'er, in addition to the technical expertise of Lynn Gruman, and the support from the National Cancer Institute/NIH, CA59702 and CA80318 to MJCH, and Department of Defense grant DAMD17-99-1-9225 to DAK.

REFERENCES

1. Wingo, P.A., Ries, L.A., Giovino, G.A., Miller, D.S., Rosenberg, H.M., Shopland, D.R., Thun, M.J., and Edwards, B.K., 1999, Annual report to the nation on the status of cancer, 1973-1996, with a special section on lung cancer and tobacco smoking. J Natl Cancer Inst, 91:675-690.
2. Houghton, A.N., and Polsky, D., 2002, Focus on melanoma. Cancer Cell, 2:275-278.
3. Greenlee, R.T., Murray, T., Bolden, S., and Wingo, P.A., 2000, Cancer statistics, 2000. CA Cancer J Clin, 50:7-33.
4. Rigel, D.S., and Carucci, J.A., 2000, Malignant melanoma: Prevention, early detection, and treatment in the 21st century. CA Cancer J Clin, 50:215-236.
5. Chin, L., Merlino, G., and DePinho, R.A., 1998, Malignant melanoma: modern black plague and genetic black box. Genes Dev, 12(22):3467-3481.
6. Zimmerman, L., and McClean, I., 1984, Do growth and onset of symptoms of uveal melanoma indicate subclinical metastasis?. Ophthalmology, 92:685-691.
7. Bittner, M., Meltzer, P., Chen, Y., Jiang, Y., Seftor, E., Hendrix, M., Radmacher, M., Simon, R., Yakhini, Z., Ben-Dor, A., et al., 2000, Molecular classification of cutaneous malignant melanoma by gene expression profiling. Nature, 406:536-540.
8. Seftor, E.A., Meltzer, P.S., Kirschmann, D.A., Pe'er, J., Maniotis, A.J., Trent, J.M., Folberg, R., and Hendrix, M.J.C., 2002, Molecular determinants of human uveal melanoma invasion and metastasis. Clin Exp Metastas, 19:233-246.
9. Tschentscher, F., Husing, J., Holter, T., Kruse, E., Dresen, I.G., Jockel, K.-H., Anastassiour, G., Schilling, H., Bornfeld, N., Horsthemke. B., et al., 2003, Tumor classification based on gene expression profiling shows that uveal melanoma with and without monosomy 3 represent two distinct entities. Cancer Res, 63:2578-2584.
10. Kim, C.J., Reintgen, D.S., and Yeatman, T.J., 2002, The promise of microarray technology in melanoma care. Cancer Control, 9(1):49-53.
11. Carr, K.M., Bittner, M., and Trent, J.M., 2003, Gene-expression profiling in human cutaneous melanoma. Oncogene, 22:3076-3080.
12. Hendrix, M.J.C., Sefto,r E.A., Hess, A.R., and Seftor, R.E.B., 2003, Molecular plasticity of human melanoma cells. Oncogene, 22:3070-3075.
13. Seftor, E.A., Meltzer, P.S., Schatteman, G.C., Gruman, L.M., Hess, A.R., Kirschmann, D.A., Seftor, R.E.B., and Hendrix, M.J.C., 2002, Expression of multiple molecular phenotypes by aggressive melanoma tumor cells: role in vasculogenic mimicry. Crit Rev Oncology Hematol, 44:17-27.
14. Maniotis, A.J., Folberg, R., Hess, A., Seftor, E.A., Gardner, L.M.G., Pe-er, J., Trent, J.M., Meltzer, P.S., and Hendrix, M.J.C.. 1999, Vascular channel formation by human melanoma cells in vivo and in vitro. Am J Pathol, 155:739-752.
15. Makitie, T., Summanen, P., Tarkkanen, A., and Kivela, T., 1999, Microvascular loops and networks as prognostic indicators in choroidal and ciliary body melanomas. J Natl Cancer Inst, 91:359-367.
16. Thies, A., Mangold, U., Moll, I., and Schumache,r U., 2001, PAS-positive loops and networks as a prognostic indicator in cutaneous malignant melanoma. J Pathol, 195:537-542.
17. Warso, M.A., Maniotis, A.J., Chen, X., Majumdar, D., Patel, M.K., Shikaitis, A., Das Gupta, T.K., and Folberg, R., 2001, Prognostic significance of periodic acid-Schiff-positive patterns in primary cutaneous melanoma. Clin Cancer Res, 7:473-477.
18. Shirakawa, K., Kobayashi, H., Heike, Y., Kawamoto, S., Brechbiel, M.W., Kasumi, F., Iwanaga, T., Konishi, F., Terada, M., and Wakasugi, H., 2002, Hemodynamics in vasculogenic mimicry and angiogenesis of inflammatory breast cancer xenografts. Cancer Res, 62:560-566.
19. Maniotis, A.J., Chen, X., Garcia, C., DeChristopher, P.J., Wu, D., Pe'er, J., and Folberg, R., 2002, Control of melanoma morphogenesis endothelial survival, and perfusion by extracellular matrix. Lab Invest, 82:1031-1043.
20. Ruf, W., Seftor, E.A., Petrovan, R., Weiss, R.M., Gruman, L.M., Margaryan, N.V., Seftor, R.E.B., Miyagi, Y., and Hendrix, M.J.C., 2003, Differential role of tissue factor pathway inhibitor-1 and 2 (TFPI-1 and 2) in melanoma vasculogenic mimicry. Cancer Res, 63:5381-5389.
21. Clarijs, R., Otte-Holler, I., Ruiter, D.J., and de Waal, R.M.W., 2002, Presence of a fluid-conducting meshwork in xenografted cutaneous and primary human uveal melanoma. Inv Ophthalmol Vis Sci, 43:912-918.
22. Ryback, S.M., Sanovich, E., Hollingshead, M.G., Borgel, S.D., Newton, D.L., Melillo, G., Kong, D., Kaur, G., and Sausville, E.A., 2003, "Vasocrine" formation of tumor cell-lined vascular spaces: Implications for rationale design of antiangiogenic therapies. Cancer Res, 63:2812-2819.
23. Hendrix, M.J.C., Seftor, E.A., Seftor, R.E.B., Gardner, L.M., Boldt, H.C., Meyer, M., Pe'er, J., and Folberg, R., 1998, Biologic determinants of uveal melanoma metastatic phenotype: Role of intermediate filaments as predictive markers. Lab Investig, 78(2):153-163.
24. Liotta, L.A., and Kohn, E.C., 2001, The microenvironment of the tumour-host interface. Nature, 411:375-379.

25. Bissell, M.J., and Radisky, D., 2001, Putting tumours in context. Nature Rev Cancer, 1:46-54.
26. Egeblad, M., and Werb, Z., 2002, New functions for the matrix metalloproteinases in cancer progression. Nature Rev Cancer, 2:161-174.
27. Seftor, R.E.B., Seftor, E.A., Kirschmann, D.A., and Hendrix, M.J.C., 2002, Targeting the tumor microenvironment with chemically modified tetracyclines: inhibition of laminin 5 γ2 chain promigratory fragments and vasculogenic mimicry. Mol Cancer Therapeut 2002, 1:1173-1179.
28. Takeuchi, H., Kuo, C., Morton, D.L., Wang, H.-J., and Hoon, D.S.B., 2003, Expression of differentiation melanoma-associated antigen genes is associated with favorable disease outcome in advanced-stage melanomas. Cancer Res, 63:441-448.
29. Sheffield, M.V., Yee, H., Dorvault, C.C., Weilbaecher, K.N., Eltoum, I.A., Siegal, G.P., Fisher, D.E., and Chhieng, D.C., 2002, Comparison of five antibodies as markers in the diagnosis of melanoma in cytologic preparations. Am J Pathol, 118(6):930-936.
30. Du, J., Miller, A.J., Widlund, H.R., Horstmann, M.A., Ramaswamy, S., and Fisher, D.E., MLANA/MART1 and SILV/PMEL17/GP100 are transcriptionally regulated by MITF in melanocytes and melanoma. Am J Pathol, 163(1):333-343.
31. Shih, I.-M., Speicher, D., Hsu, M.-Y., Levine, E., and Herlyn, M., 1997, Melanoma cell-cell interactions are mediated through heterophilic Mel-CAM/ligand adhesion. Cancer Res, 57:3835-3840.
32. 2002, Assembly of the Vasculature and Its Regulation. Tomanek, R.J. ed, Birkhauser, Boston.
33. Risau, W., 1997, Mechanisms of angiogenesis. Nature, 386:671-674.
34. Carmeliet, P., 2000, Mechanisms of angiogenesis and arteriogenesis. Nature Med, 6:389-395.
35. Hynes, R.O., Bader, B.L., and Hodivala-Diike, K., 1999, Integrins in vascular development. Braz J Med Biol Res, 32:501-510.
36. Hess, A.R., Seftor, E.A., Gardner, L.M.G., Carles-Kinch, K., Schneider, G.B., Seftor, R.E.B., Kinch, M.S., and Hendrix, M.J.C., 2001, Molecular regulation of tumor cell vasculogenic mimicry by tyrosine phosphorylation: role of epithelial cell kinase (Eck/EphA2). Cancer Res, 61:3250-3255.
37. Look, A.T., Ashmun, R.A., Shapiro, L.H., and Peiper, S.C., 1990, Human myeloid plasma membrane glycoprotein CD13 (gp150) is identical to aminopeptidase. J Clin Invest, 83:1299-1307.
38. Ashmun, R.A., and Look, A.T., 1990, Metalloprotease activity of CD13/aminopeptidase N on the surface of human myeloid cells. Blood, 75:462-469.
39. Bhagwat, S.V., Petrovic, N., Okamoto, Y., and Shapiro, L.H., 2003, The angiogenic regulator CD13/APN is a transcriptional target of Ras signaling pathways in endothelial morphogenesis. Blood, 101(5):1818-1826.
40. Pasqualini, R., Koivunen, E., Kain, R., Lahdenranta, J., Sakamoto, M., Stryhn, A., Ashmun, R.A., Shapiro, L.H., Arap, W., and Ruoslahti, E., 2000, Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. Cancer Res, 60:722-727.
41. Russell, L., and Forsdyke, D.R., 1991, A human putative lymphocyte G0/G1 switch gene containing a CpG-rich island encodes a small basic protein with the potential to be phosphorylated DNA. Cell Biol, 10(8):581-591.
42. Bachner, D., Ahrens, M., Schroder, D., Hoffmann, A., Laube,r J., Betat, N., Steinert, P., Flohe, L., and Gross, G., 1998, Bmp-2 downstream targets in mesenchymal development identified by subtractive cloning from recombinant mesenchymal progenitors (C3H10T1/2). Dev Dyn, 213(4):398-411.
43. Chen, Y., Medvedev, A., Ruzanov, P., Marvin, K.W., and Jetten, A.M., 1997, cDNA cloning, genomic structure, and chromosome mapping of the human epithelial membrane protein CL-20 gene (EMP1), a member of the PMP22 family. Genomics, 41(1):40-48.
44. Ben-Porath, I., Kozak, C.A., and Benvenisty, N., 1998, Chromosomal mapping of Tmp (Emp1), Xmp (Emp2), and Ymp (Emp3), genes encoding membrane proteins related to Pmp22. Genomics, 49:443-447.
45. Wulf, P., and Suter, U., 1999, Embryonic expression of epithelial membrane protein 1 in early neurons. Brain Res Dev Brain Res, 116(2):169-180.
46. Hendrix, M.J.C., Seftor, E.A., Chu, Y.-W., Seftor, R.E.B., Nagle, R.B., McDaniel, K.M., Leong. S.P.L. Yohem, K.H., Leibovitz. A,M,, Meyskens. F.L., Jr, et al., 1992, Coexpression of vimentin and keratins by human melanoma tumor cells: correlations with invasive and metastatic potential. J Natl Cancer Inst, 84:165-174.
47. Miettinen, M., and Fransella, K., 1989, Immunohistochemical spectrum of malignant melanoma: the common presence of keratins. Lab Invest, 61:623-628.
48. Zarbo, R.J., Gown, A.M., Nagle, R.B., Visscher, D.W., and Crissman, J.D., 1990, Anomalous cytokeratin expression in malignant melanoma: one- and two-dimensional western blot analysis and immunohistochemical survey of 100 melanomas. Mod Pathol, 3:494-501.
49. Hendrix, M.J.C., Seftor, R.E.B., Seftor, E.A., Gruman, L.M., Lee, L.M.L., Nickoloff, B.J., Miele, L., Sheriff, D.D., and Schatteman, G.C., 2002, Transendothelial function of human metastatic melanoma cells: role of the microenvironment in cell-fate determination. Cancer Res, 62:665-668.

50. Stocum, D.L., 2002, A tail of transdifferentiation. Science, 298:1901-1903.
51. Blau, H.M., 2002 A twist of fate. Nature, 419:437.
52. Echeverri, K., and Tanaka, E.M., 2002, Ectoderm to mesoderm lineage switching during axolotl tail regeneration. Science, 298:1993-1996.
53. Klausner, R.D., 2002, The fabric of cancer cell biology - Weaving together the strands. Cancer Cell, 1:3-10.
54. 1991, Cell Biology of Extracellular Matrix, 2nd edition. Hay, E.D., ed, Plenum Press, New York.
55. Boudreau, N., and Bissell, M.J., 1998, Extracellular matrix signaling: integration of form and function in normal and malignant cells. Curr Opin Cell Biol, 10(5):640-646.
56. Van Kempen, L.C.L., Rhee, J.-S., Dehne, K., Lee, J., Edwards, D.R., and Coussens, L.M., 2002, Epithelial carcinogenesis: dynamic interplay between neoplastic cells and their microenvironment. Differentiation, 70:610-623.
57. Chambers, A.F., and Matrisian, L.M., 1997, Changing views of the role of matrix metalloproteinases in metastasis. J Natl Cancer Inst, 89(17):1260-1270.
58. Sottile, J., and Hocking, D.C., 2002, Fibronectin polymerization regulates the composition and stability of extracellular matrix fibrils and cell-matrix adhesions. Mol Biol Cell, 13:3546-3559.
59. Clark, E.A., Golub, T.R., Lander, E.S., and Hynes, R.O., 2000, Genomic analysis of metastasis reveals an essential role for RhoC. Nature, 406:532-535.
60. Ruoslahti, E., 1999, Fibronectin and its integrin receptors in cancer. Adv Cancer Res, 76:1-20.
61. Tremble, P.M., Damsky, and C.H., Werb, Z., 1992, Fibronectin fragments, but not intact fibronectin, signalling through the fibronectin receptor induce metalloproteinase gene expression in fibroblasts. Matrix Suppl, 1:212-214.
62. Montgomery, A.M., De Clerck, Y.A., Langley, K.E., Reisfeld, R.A., and Mueller, B.M., 1993, Melanoma-mediated dissolution of extracellular matrix: contribution of urokinase-dependent and metalloproteinase-dependent proteolytic pathways. Cancer Res, 53(3):693-700.
63. Kirschmann, D.A., Seftor, E.A., Fong S.F.T., Nieva, D.R.C., Sullivan, C.M., Edwards, E.M., Sommer, P., Csiszar, K., and Hendrix, M.J.C., 2002, A molecular role for lysyl oxidase in breast cancer invasion. Cancer Res, 62:4478-4483.
64. Akiri, G., Sabo, E., Dafni, H., Vadasc, A., Kartvelishvily, T., Gan, N., Kessle,r O., Cohen, T., Resnick, M., Meeman, M., and Neufeld, G., 2003, Lysyl oxidase-related protein-1 promotes tumor fibrosis and tumor progression in vivo. Cancer Res, 63(7):1657-1666.
65. Seiki, M., 2002, The cell surface: the stage for matrix metalloproteinase regulation of migration. Curr Opin Cell Biol, 14:624-632.
66. Hotary, K., Allen, E., Punturieri, A., Yana, I., and Weiss, S.J., 2000, Regulation of cell invasion and morphogenesis in a three-dimensional type collagen matrix by membrane-type matrix metalloproteinases 1, 2, and 3 J. Cell Biol, 149(6):1309-1323.
67. Aimes, R.T., and Quigley, J.P., 1995, Matrix metalloproteinase-2 is an interstitial collagenase. J Biol Chem, 270:5872-5876.
68. Seftor, R.E.B., Seftor, E.A., and Hendrix, M.J.C., 1999, Molecular role(s) for integrins in human melanoma invasion. Cancer Metast Rev, 18:359-375.
69. Seftor, R.E.B., Seftor, E.A., Koshikawa, N., Meltzer, P.S., Gardner, L.M.G., Bilban, M., Stetler-Stevenson, W.G., Quaranta, V., and Hendrix, M.J.C., 2001, Cooperative interactions of laminin 5γ2 chain, matrix metalloproteinase-2, and membrane type-1 matrix/metalloproteinase are required for mimicry of embryonic vasculogenesis by aggressive melanoma. Cancer Res, 61:6322-6327.
70. Malinda, K.M., and Kleinman, H.K., 1996, The laminins. Int J Biochem Cell Biol, 28:957-959.
71. Colagnato, H., and Yurchenco, P.D., 2000, Form and function: the laminin family of heterotrimers. Dev Dynamics, 218:213-234.
72. Kalluri, R., 2003, Basement membranes: structure, assembly and role in tumour angiogenesis. Nature Rev Cancer, 3:422-433.
73. Malinda, K.M., Nomizu, M., Chung, M., Delgado, M., Kuratomi, Y., Yamada, Y., Kleinman, H.K., and Ponce, M.L., 1999, Identification of laminin α1 and β1 chain peptides active for endothelial cell adhesion, tube formation, and aortic sprouting. FASEB J, 13:53-62.
74. Koshikawa, N., Giannelli, G., Cirulli, V., Miyazaki, K., and Quaranta, V., 2000, Role of cell surface metalloprotease MT1-MMP in epithelial cell migration over laminin-5. J Cell Biol, 148:615-624.
75. Giannelli, G., Falk-Marzillier, J., Schiraldi, O., Stetler-Stevenson, W.G., and Quaranta, V., 1997, Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. Science, 277:225-228.
76. Hynes, R.O., 2002, Integrins: bidirectional, allosteric signaling machines. Cell, 110:673-667.
77. Giordano, F.J., and Johnson, R.S., 2001, Angiogenesis: the role of the microenvironment in flipping the switch. Curr Opin Genetics Dev, 11:35-40.
78. Bergers, G., Brekken, R., McMahon, G., Vu, T.H., Itoh, T., Tamaki, K., Tanzawa, K., Thorpe, P., Itohara, S., Werb, Z., and Hanahan, D., 2000, Matrix metalloproteinase-9 triggers and angiogenic switch

during carcinogenesis. Nature Cell Biol, 2(10):737-744.
79. Hendrix, M.J.C., Seftor, E.A., Seftor, R.E.B., Kirschmann, D.A., Gardner, L.M., Boldt, H.C., Meyer, M., Pe'er, J., and Folberg, R., 1998, Regulation of uveal melanoma interconverted phenotype by hepatocyte growth factor/scatter factor. Am J Pathol, 152:855-863.
80. Yu, Y., and Merlino, G., 2002, Constitutive c-Met signaling through a nonautocrine mechanism promotes metastasis in a transgenic transplantation model. Cancer Res, 62:2951-2956.
81. Jeffers, M., Rong, S., and Vande Woude, G.F., 1996, Hepatocyte growth factor/scatter factor-Met signaling in tumorigenicity and invasion/metastasis. J Mol Med, 74:505-513.
82. Hammond, D.E., Urbe, S, Vande Woude, G.F., and Clague, M.J., 2001, Down-regulation of MET, the receptor for hepatocyte growth factor. Oncogene, 20(22):2761-2770.
83. Cao, B., Su, U.Y., Oskarsson, M., Zhao, P., Kort, E.J., Fisher, R.J., Wang, L.-M., and Vande Woude, G.F., 2001, Neutralizing monoclonal antibodies to hepatocyte growth factor/scatter factor (HGF/SF) display antitumor activity in animal models. Proc Natl Acad Sci, USA, 98(13):7443-7448.
84. Sahai, E., and Marshall, C.J., 2003, Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signaling and extracellular proteolysis. Nature Cell Biol, 5(8):711-719.
85. Chen, H., Bernstein, B.W., and Bamburg, J.R., 2000, Regulating actin-filament dynamics in vivo. Trends Biochem Sci, 25(1):19-23.
86. Shiomi, T., and Okada, Y., 2003, MT1-MMP and MMP-7 in invasion and metastasis of human cancers. Cancer Metast Rev, 22(2-3):145-152.
87. Marken, J.S., Schieven, G.L., Hellstrom, I., Hellstrom, N.K., and Aruffo, A., 1992, Cloning and expression of the tumor-associated antigen L6. Proc Natl Acad Sci, USA, 89:3503-3507.
88. Wei, Y., Eble, J.A., Wang, Z., Kreidberg, J.A., and Chapman, H.A., 2001, Urokinase receptors promote β1 integrin function through interactions with integrin $\alpha_3\beta_1$. Mol Biol Cell, 12:2975-2986.
89. Blasi, F., Carmeliet, P., 2002, uPAR: a versatile signaling orchestrator. Nature Rev Mol Cell Biol, 3:932-943.
90. Liu, D., Aguirre Ghiso, J.A., Estrada, Y., and Ossowski, L., 2002, EGFR is a transducer of the urokinase receptor initiated signal that is required for in vivo growth of a human carcinoma. Cancer Cell, 1:445-457.
91. Babic, A.M., Kireeva, M.L., Kolesnikova, T.V., and Lau, L.F., 1998, CYR61, a product of growth factor-inducible immediate early gene, promotes angiogenesis and tumor growth. Proc Natl Acad Sci, USA, 95:6355-6360.
92. Modi, W.S., and Chen, Z.-Q., 1998, Localization of human CXC chemokine subfamily on the long arm of chromosome 4 using radiation hybrids. Proc Natl Acad Sci, USA, 47:136-136.
93. Vidal-Vanaclocha, F., Fantuzzi, G., Mendoza, L., Fuentes, A.M., Anasagasti, M.J., Martin, J., Carrascal, T., Walsh, P., Reznikov, L.L., Kim, S.-H., et al., 2000, IL-18 regulates IL-1-beta-dependent hepatic melanoma metastasis via vascular cell adhesion molecule-1. Proc Natl Acad Sci, USA, 97:734-739.
94. Metcalf, D., 1985, The granulocyte-macrophage colony-stimulating factors. Science, 229:16-22.
95. Nagata, S., Tsuchiya, M., Asano, S., Kaziro, Y., Yamazaki, T., Yamamoto, O., Hirata, Y., Kubota, N., Oheda, M., Nomura, H., and Ono, M., 1986, Molecular cloning and expression of cDNA for the human granulocyte colony-stimulating factor. Nature, 319:415-418.
96. Mellor SL, Cranfield M, Ries R, Pedersen J, Cancilla B, de Kretser D, Groome NP, Mason AJ, Risbridger GP. Localization of activin beta(A)-, beta(B)-, and beta(C)-subunits in human prostate and evidence for formation of new activin heterodimers of beta(C)-subunit. J Clin Endocr Metab 2000, 85:4851-4858.
97. Richmond, A., Balentien, E., Thomas, H.G., Flaggs, G., Baron, D.E., Spiess, J., Bordoni, R., Francke, U., and Derynck, R., 1988, Molecular characterization and chromosomal mapping of melanoma growth stimulatory activity, a growth factor structurally related to beta-thromboglobulin. EMBO J, 7:2025-2033.
98. Franze, A., Archidiacono, N., Rocchi, M., Marino, M., and Grimaldi, G., 1991, Isolation and expression analysis of a human zinc finger gene (ZNF41) located on the short arm of the X chromosome. Genomics, 9:728-736.
99. Di Magliano, M., Di Lauro, R., and Zannini, M., 2000, Pax 8 has a key role in thyroid cell differentiation. Proc Natl Acad Sci, USA, 97(24):144-149.
100. Wrighton, S.A., and Stevens, J.C., 1992, The human hepatic cytochromes P450 involved in drug metabolism. Crit Rev Toxicol, 22:1-21.
101. Chen, Z., Zhang, K., Zhang, X., Yuan, X.H., Yuan, Z., Jin, L., and Xiong, M., 2003, Comparison of gene expression between metastatic derivatives and their poorly metastatic parental cells implicates crucial tumor-environment interaction in metastasis of head and neck squamous cell carcinoma. Clin Exp Metastas, 20:335-342.
102. Ramaswamy, S., Ross, K.N., Lander, E.S., and Golub, T.R., 2003, A molecular signature of metastasis in primary solid tumors. Nature Genet, 33:49-54.

103. Bernards, R., and Weinberg, R.A., 2002, A progression puzzle. Nature, 418:823.
104. LaBarge, M.A., and Blau, H.M., 2002, Biological progression from adult bone marrow to mononucleate muscle fiber in response to injury. Cell, 111:589-601.
105. Kon, K., and Fujirawa, T., 1994, Transformation of fibroblasts into endothelial cells during angiogenesis. Cell Tissue Res, 278:625-628.
106. Condorelli, G., Borillo, U., De Angelis, L., Latronico, M., Sirabella, D., Coletta, M., Galli, R., Balcón, G., Follenzi, A., Frati, G., et al., 2001, Cardiomyocytes induce endothelial cells to transdifferentiate into cardiac muscle. Proc Natl Acad Sci, USA, 98:10733-10738.
107. Nickoloff, B.J., and Foreman, K.E., 2002, Etiology and pathogenesis of Kaposi's sarcoma Recent Results. Cancer Res, 160:332-342.
108. Grossman, D., and Altieri, D.C., 2001, Drug resistance in melanoma: mechanisms, apoptosis, and new potential therapeutic targets. Cancer Metast Rev, 20:3-11.
109. Sherman-Baust, C.A., Weeraratna, A.T., Rangel, L.B.A., Pizer, E.S., Cho, K.R., Schwatz, D.R., Shock, T., and Morin, P.J., 2003, Remodeling of the extracellular matrix through overexpression of collagen VI contributes to cisplatin resistance in ovarian cancer cells. Cancer Cell, 3:377-386.
110. Netti, P.A., Berk, D.A., Swartz, M.A., Grodzinsky, A.J., and Jain, R.K., 2000, Role of extracellular matrix assembly in interstitial transport in solid tumors. Cancer Res, 60:2497-2503.
111. Coussens, L.M., Fingleton, B., and Matrisian, L.M., 2002, Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science, 295:2387-2392.

Chapter 5

Dynamics of Cell Adhesion Interactions during Melanoma Development

G. Li and M. Herlyn
The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA

Abstract: Cell adhesion interactions are important processes during normal development and malignant transformation. Recent studies have shown that in melanoma, cell adhesion interactions play a critical role in regulating melanocytic cell proliferation, differentiation, migration, apoptosis and even vasculature mimicry. In the review we will discuss recent progresses in the study of the dynamic expression and function of the adhesion molecules in melanoma development.

Key Words: Melanoma, cell adhesion, metastasis, cadherins, integrins, intercellular interactions, cadherin switch, gap junction, CAM, MELCAM, L1 CAM, ICAM-1

1. INTRODUCTION

Cutaneous melanoma is one of the fastest growing cancers in the last several decades in the United States and around the world (1). In contrast to many other cancers, melanoma affects a relatively younger population and is notorious for its propensity to metastasize and for its poor response to current therapeutical regiments. The morbidity in melanoma patients is mainly due to metastatic disease. Thus, understanding the progression of the tumor to the metastatic state and the changes taking place during the progression is critical for the development of effective methods to diagnose and treat melanoma.

Human skin is a complex organ composed of two layers, the epidermis and the dermis, separated by a basement membrane. The pigment-producing melanocytes reside in the epidermal-dermal junction and are interspersed among every 5-10 basal keratinocytes, forming the "epidermal-melanin unit". Within this unit, each melanocyte transports melanin-containing melanosomes to surrounding keratinocytes through its multiple dendrites. Despite the dynamic nature of epidermal shedding, involving constant proliferation, differentiation, and migration of keratinocytes, proliferation of melanocytes is strictly controlled and rarely observed under physiologic conditions.

The transition from normal melanocytes to benign lesions and to metastatic cancers usually occurs through a stepwise process involving increase in the expression and function of oncogenes or decrease in tumor suppressors, as well as changes that provide tumor cells the ability to overcome cell-cell adhesion and micro-environmental controls by the host, to invade surrounding tissues and to translocate to distant locations (2, 3). Based on clinical and histopathological features, five steps of melanoma progression have been proposed (4, 5): common acquired and congenital nevi with cytologically normal melanocytes; dysplastic nevus with structural atypia; early radial growth phase (RGP) primary melanoma, which is non-tumorigenic

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 65-79.

but may have the potential of uncontrolled growth; vertical growth phase (VGP) primary melanoma, which is tumorigenic and may have competence for metastasis; and metastatic melanoma. During the step-wise transition from normal melanocytes to RGP, VGP and metastatic melanoma, the transformed cells interact with their microenvironmental components such as keratinocytes, fibroblasts, endothelial cells and extracellular matrix (6, 7). These microenvironmental factors form a complex context that may either inhibit (6) or promote tumorigenesis (8), depending on the nature of the interactions (7, 9). In multicellular organisms, homeostasis is governed by three major forms of communications: extracellular communication via soluble factors including hormones, growth factors, and cytokines; intracellular communication via complex signal transduction networks; and intercellular communication via cell-cell adhesion (7). Together these interactions provide the information that is critical to control normal cellular behavior and to maintain tissue structure integrity (9). Therefore, defining intercellular dialogues in human skin may provide key information for the development of novel treatment strategies that target the structural and functional unit of tumor and its stroma.

2. INTERCELLULAR INTERACTIONS BY DIRECT CONTACTS

Generally, intercellular adhesion junctions utilize four structural types of adhesion receptors. The first group consists of cadherins which mediate homophilic cell-cell adhesion (10, 11). The second structural type includes proteins with four transmembrane regions and intracellular amino- and carboxyl- termini. This diverse group consists of the gap junction proteins, connexins (12) and the tight junction proteins, occludin (13) and claudins (14). The third group includes those adhesion molecules with immunoglobulin-like repeats, such as MelCAM (MUC18, CD146) (15), L1-CAM and ICAM-1 (16). The fourth group contains integrins. Although integrins are primarily involved in cell-matrix interactions (17), they also participate in cell-cell adhesion. For example, integrin $\alpha 4\beta 1$ expression is characteristic of advanced primary tumors and mediates interaction of the tumor cells with vascular cell adhesion molecule-1 (VCAM-1) on vascular endothelium (18).

There are extensive crosstalks between different forms of cell adhesion. Cadherin-mediated cell adhesion appears to be the critical one because it can, at least in some cases, form the foundation for other cell-cell junctional complexes, including tight junctions (19), desmosomes (20) and gap junctions (21, 22). On the other hand, the expression and function of the adhesion molecules is a dynamic process exist not only in normal development and morphogenesis but also in tumor initiation and progression (Figure 1).

3. CADHERINS IN NORMAL EPIDERMAL DEVELOPMENT AND MAINTENANCE

Cadherins form a family of cell surface glycoproteins that function in promoting calcium-dependent cell-cell adhesion and serve as the transmembrane components of cell-cell adherens junctions (3). The isotypes of classical cadherins, such as E-, N-, P-cadherin, and the non-classical cadherins, such as VE-cadherin, are expressed in a cell-, tissue-, and development-specific manner. For example, in normal human skin, E-cadherin is expressed on the surfaces of all epidermal cells, including keratinocytes, melanocytes and Langerhans cells, while P- cadherin is expressed only on the surface of basal layer keratinocytes (24). During human skin development, P-cadherin expression is spatiotemporally controlled and is closely related to the segregation of the basal layers as well as to the arrangement of epidermal cells into eccrine ducts (25). N-cadherin is expressed in the human skin by dermal fibroblasts and vascular endothelial cells, but not by keratinocytes or melanocytes (26). Cadherin molecules and cadherin-catenin complexes are involved during embryogenesis and morphogenesis in cell recognition, motility, tissue integrity, and homeostasis (27). The links between cadherin-mediated cell-cell adhesion, growth factor/receptor

tyrosine kinases, and WNT signaling pathway form a complex network (28). The organization of such a hierarchy of multiple components allows cadherins to function as cell adhesion machinery as well as signaling receptors for intercellular and intracellular communication regulating cell growth, differentiation, and apoptosis.

During embryonic development, expression of the cadherin subtypes correlates with the migration and segregation of different cell layers and cell populations (29). The development and migration of melanoblasts/melanocytes serves as a good example how microenvironmental factors affect the behavior of the cells. Melanocytes and their progenitor melanoblasts are derived from the neural crest and migrate along the dorsolateral pathway to their final destination. In the mouse, at 11.5 days postcoitum, melanoblasts are in the dermis and are E-cad-P-cad-. During the next 48 h, a 200-fold increase of E-cadherin expression is induced on the surface of melanoblasts prior to their entry into the epidermis, thereby forming a homogeneous E-cadhighP-cad-/low population. The cadherin expression pattern then diversifies, giving rise to three populations, an E-cad-P-cad- dermal population, E-cadhighP-cadlow epidermal population, and E-cad-P-cadmed-high follicular population. In all three populations, the patterns of expression are region-specific, being identical with those of surrounding cells such as keratinocytes and fibroblasts. These findings suggest a role for E- and P-cadherins in guiding melanocyte progenitors to their final destinations (30), particularly during and after melanocyte entry into the epithelial layer where the epidermal architecture of keratinocytes is maintained by E- and P-cadherins (31, 32). In the human skin, E-cadherin has been shown to be critical in the control of melanocytes by keratinocytes(6).

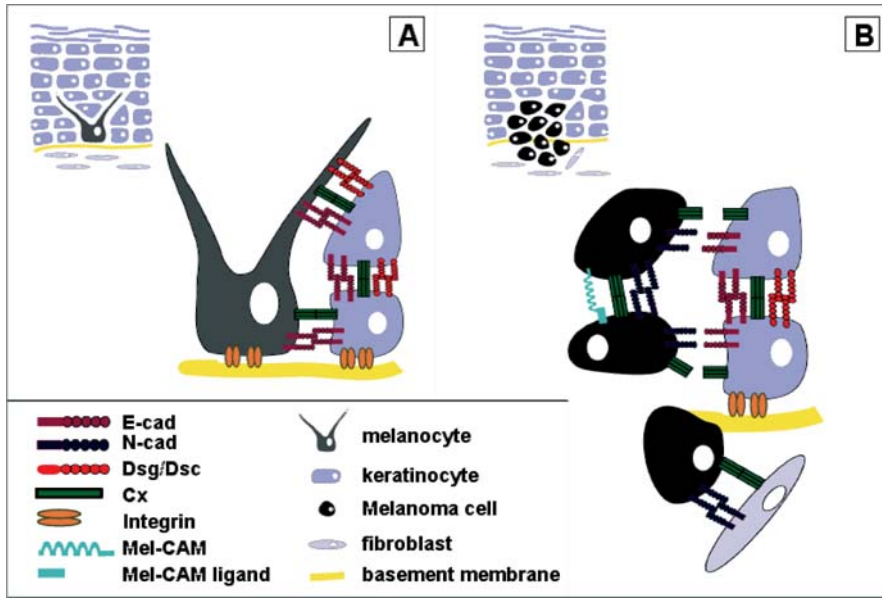


Figure 1. Dynamics of intercellular interactions under normal and pathological situations during melanoma development**. (A)** Normal melanocytes reside close to the basement membrane and form a "epidermal melanin unit" that contains one melanocyte and five to eight keratinocytes. Melanocytes interact with adjacent keratinocytes through E-cadherin, Desmoglein 1 and connexins. This contact-dependent interaction is required for the growth and phenotypic control of melanocytes by keratinocytes. **(B)** Malignant melanoma cells proliferate, penetrate basement membrane, and invade into dermis. A shift of cadherin profile from E- to N- during melanoma development not only frees the cells from epidermal keratinocytes, but also confers new adhesive properties. Melanoma cells form N-cadherin-mediated adhesion and connexin-mediated gap junction with N-cadherin-expressing fibroblasts, endothelial cells and adjacent melanoma cells. Mel-CAM and its unknown ligand are also involved in melanoma-melanoma cell interaction, which is implicated to play a role in the progression of melanoma (Reprinted from Li et al. (23), with permission by Nature Publishing Group).

Besides E-cadherin-mediated adherens junctions, desmosomes are also believed to play an important role in maintaining human skin integrity (33). Desmosomes contain specialized cadherin adhesion molecules, Desmogleins and Desmocollins, associating with various cytoplasmic proteins such as Desmoplakins and Plakoglobin (11, 33-35). Desmosomes provide the cells with binding domains for intermediate filaments network and are required for tissue organization (36-38). Plakoglobin (γ-catenin) is also part of the cadherin-catenin complex in adherens junctions (39-41) and may mediate crosstalk between adherens junctions and desmosomes (41). Reduction or loss of desmosomes may contribute to the invasive and metastatic behavior of various tumors, for example transitional cell carcinoma of the bladder (42) and squamous cell carcinomas (43-45). Reduction in desmosome formation was correlated with invasion and with reduction in E-cadherin staining (45). Therefore, desmosomes appear to have tumor-suppressor properties. In fact, overexpressing desmosomal cadherins in squamous cell carcinoma cells inhibited invasiveness (46, 47).

4. LOSS OF E-CADHERINS IN MELANOMA

During melanoma development, a progressive loss of E-cadherin expression has been observed: superficial compartments of nevi show heterogeneous membranous E-cadherin immunoreactivity (48), while junctional nevus cell nests display heterogeneous or diffuse cytoplasmic staining (48); melanoma cells, with few exceptions, do not express E-cadherin (26, 49, 50).

Loss of E-cadherin appears to be one of the critical steps in progression of melanoma, because loss of functional E-cadherin could trigger the release of cancer cells from the primary focus (51). This process is probably not only due to loss of physical adhesion, but also due to multiple events that lead to uncontrolled proliferation and migration/invasion (52). Forced expression of E-cadherin in E-cadherin-negative melanoma cells restored their adhesion to keratinocytes and rendered them susceptible for keratinocyte-mediated control (6). After co-culture with keratinocytes, E-cadherin-expressing melanoma cells no longer expressed αvβ3 or MelCAM. In a skin reconstruction model, ectopic E-cadherin expression inhibited the invasion of melanoma cells into dermis and induced tumor cell apoptosis (6).

5. REGULATION OF CADHERIN EXPRESSION

The regulation of the state of adherens junctions can occur at different levels and through different mechanisms (2, 10, 51). Studies have unveiled multiple potential causes for the loss of functional E-cadherin, including: 1. deletion or mutational inactivation of E-cadherin gene (2); 2. abnormal expression and abnormal subcellular localization of cadherin or the components of the cadherin-containing adhesion complex (10); 3. silenced expression by hypermethylation (53-55); 4. expression of transcriptional repressor, such as Snail family of transcription factors (56-59); 5. disruption of cadherin/catenin complex due to phosphorylation of catenins by activated receptor tyrosine kinases (2); 6. degradation of E-cadherin's extracellular portion by proteases such as stromelysin 1 (60). However, in contrast to other cancer types (51), studies (7, 61, 62) have shown that loss of E-cadherin in melanoma cell lines does not usually involve mutations in the E-cadherin gene or promoter methylation. The mechanisms of E-cadherin downregulation remained a mystery until recently when several studies suggested that multiple factors are involved.

One involves aberrant expression of growth factor hepatocyte growth factor (HGF) (63). HGF is a multifunctional cytokine acting as mitogen, motogen and morphogen for many cell types (64) through its tyrosine kinase receptor c-Met, which is present in epithelial cells and melanocytes (63, 65, 66). HGF is physiologically secreted by cells of mesenchymal origin and acts on neighboring epithelial cells through a paracrine loop (67). However, coexpression of HGF and c-Met has been identified in a variety of transformed cells and tumors both in vitro and in vivo and shown to be involved in tumor development and invasion (68-

72). Normal melanocytes do not produce HGF (63). However, a HGF/c-Met autocrine loop has been identified in melanoma cells, and shown to be involved in tumor development and invasion by downregulating E-cadherin and Desmoglein 1 expression (63). Interestingly, the cadherin switch from E- to N-cadherin in epiblast cells can be recapitulated in vitro by treating the cells with HGF (73).

Besides the downregulation of E-cadherin and Desmoglein, an HGF autocrine loop in melanoma has other important implications. For example, a recent study showed that activation of αvβ3 can be induced by HGF (74). Up-regulation of αvβ3 has been implicated in melanomas (8, 75, 76). The involvement of αvβ3 will be discussed in more detail later.

Lately, it was found that the Snail family of zinc finger transcription factors repress E-cadherin expression by interacting with the E-pal element of the E-cadherin promoter through its E2-box sequence (56, 58). Snail was originally implicated in the epithelial-mesenchymal transition required for the emigration of the neural crest from the neural tube and of the early mesoderm from the primitive streak during embryonic development (57, 77). Endogenous Snail protein is present in invasive mouse and human carcinoma cell lines and tumors in which E-cadherin expression has been lost (56, 58, 59). Recent study by Hajra et al. (78) showed that Slug (another member of Snail family of zinc finger transcription factor (79) represses E-cadherin in breast cancer cells. Results from screening melanoma cells of different progression stages (23, 62) indicated that the expression pattern of Snail negatively correlates with the expression of E-cadherin, while there is no clear correlation between the expression of Slug and E-cadherin.

6. CADHERIN SWITCH: EXPRESSION AND FUNCTION OF N-CADHERIN IN MELANOMA

Whereas E-cadherin expression is downregulated in melanoma, the expression of another cadherin, N-cadherin is increased during melanoma progression (26). The phenomena of cadherin class switching have been observed in various biological processes such as cell differentiation and migration (30, 80-82) during embryonic development and tumor progression (26, 83-85). It is possible that, by switching classes or subtypes of adhesion molecules, tumor cells could reconcile their changing requirements for variations in local microenvironment during metastasis, and adjust to different biological processes.

What is the biological significance of the N-cadherin expressed in melanoma cells? It has been demonstrated that N-cadherin expression in melanoma cells allows communication with N-cadherin-expressing fibroblasts through gap junctions (86). N-cadherin can also mediate homotypic aggregation among melanoma cells as well as heterotypic adhesion of melanoma cells to dermal fibroblasts and vascular endothelial cells (61).

N-cadherin-mediated adhesion between melanoma cells and vascular endothelial cells may facilitate transmigration of cancer cells through the vascular endothelium during metastasis. The two major cadherins of vascular endothelial cells are N-cadherin and VE-cadherin (87). VE-cadherin predominantly promotes the homotypic interaction between endothelial cells, whereas N-cadherin is responsible for the interaction between endothelial cells and other N-cadherin-expressing cells (87). Our data suggest that expression of N-cadherin in melanoma cells increase their adherence to the endothelium, thus promoting a critical step in the breaching of blood vessels by tumor cells (88). Anti-N-cadherin antibodies can delay the transendothelial migration of melanoma cells (89, 90).

N-cadherin-mediated adhesion between melanoma cells and the stromal fibroblasts may facilitate dynamic processes such as cell migration and outgrowth. Cell migration depends on a delicate balance of cell adhesion and detachment. Under different physiological conditions and microenvironments, cell adhesion molecules can either promote or inhibit migration (91). Our studies show that N-cadherin mediates melanoma cells migration on fibroblasts, and that forced expression of N-cadherin in otherwise E-cadherin-positive, N-cadherin-negative melanocytes promotes migration

relative to fibroblasts. N-cadherin has been postulated to promote both stable and labile cellular interactions (92-94). N-cadherin-expressing cells retained their adhesive properties in short-term aggregation assays and possibly in the apparently tightly clustered metastases in vivo (95-97), suggesting that reduced cell adhesion does not underlie N-cadherin–induced invasiveness. Rather, it is likely that N-cadherin promotes a state of dynamic adhesion that allows both attachment and detachment of cells from the primary tumor and selective association with tissues such as the stroma or the endothelium.

In vivo observations showed that tumor cells predominantly invade the surrounding tissue as coherent clusters or cell nests (97). Cohort migration is a major form of migration in melanoma. Our data provide evidence that melanoma cells may benefit from N-cadherin-mediated aggregation with increased viability and resistance to apoptosis. N-cadherin promoted anchorage-independent survival by activation of Akt/PKB and inactivation of the pro-apoptotic protein Bad (61). The mechanism(s) whereby cadherins stimulate those biochemical routes is not well understood; however, our findings indicate that N-cadherin can initiate outside-in signal transduction pathways that ultimately benefit melanoma cells.

Thus, cadherin subtype switching from E- to N-cadherin during melanoma development not only frees melanocytic cells from the control of keratinocytes but also provides growth and possibly metastatic advantages to melanoma cells (7, 96, 98, 99).

7. GAP JUNCTION

The observed cadherin subtype switch during melanoma development was also found to impact on partner-specific gap junctional communication among skin cells (86). Gap junctions are formed by clusters of connexin (Cx) subunits (100) that provide a direct pathway for small molecules (<2 kDa) such as ions (Ca^{2+} and H^{+}), secondary messengers (phosphatidyl inositides, cAMP), and metabolic products (amino acids), between the coupled cells (101). Specificity of gap junction allows formation of communication compartments essential for tissue function and homeostasis. Melanocytes were able to form gap junctions with their natural neighbors, keratinocytes, whereas melanoma cells exhibited communication capabilities among themselves and with dermal fibroblasts and vascular endothelial cells (86) (Figure 1). The selective formation of heterotypic gap junctions between skin cells is likely dependent upon cell sorting dictated by specific cadherins. Intercellular communication via gap junctions may play a role in regulating cellular interactions during tumor invasion. For example, when B16 mouse melanoma cells were transfected with wild-type Cx26, the resulting cells became competent for coupling with endothelial cells and more competent for metastasis, suggesting that certain connexin(s) might play a role in intravasation and extravasation of tumor cells through heterologous gap junction formation with endothelial cells (102). Similarly, The switch of E- to N-cadherin expression during melanoma progression endows melanoma cells with new adhesive properties facilitating gap junction formation with adjacent N-cadherin-expressing melanoma cells or fibroblasts during dermal invasion and with vascular endothelial cells in the processes of intra- or extravasation. MelCAM expressed by melanoma cells may act as coreceptors with N-cadherin for gap junction formation (103). On the other hand, restoration of E-cadherin expression in melanoma cells resulted in the re-establishment of gap junctional communication with keratinocytes (86).

The mechanisms as to how the different heterotypic gap junctions serve to coordinate epidermal morphogenesis and melanocytic transformation remain at present unclear. There is emerging information showing that gap junctions serve to regulate cell growth and tissue morphogenesis. For example, GJIC can modulate gene expression, presumably via modulation of signals that diffuse from cell to cell (104). It is conceivable that alterations in gap junction signaling may directly stimulate melanomagenesis or simply allow tumor progression towards a more malignant phenotype through the loss of normal homeostatic growth regulation. Further elucidation of the molecular components of the partner-specific gap

junctional signaling pathways in skin cells may provide new insights into the pathogenesis of human melanoma.

8. CAMS IN MELANOMA

Cell adhesion molecules (CAM) mediating homophilic and heterophilic cell-cell interactions have been suggested to play important roles in organogenesis and maintenance of tissue integrity (105). These molecules do not simply function as a molecular glue sticking cells into static structures 18. Instead, they support and direct the dynamic interchange of information between two cells by actively transducing signals into the cells through interaction of their cytoplasmatic regions with kinases as well as through interaction with growth factor receptors, and other unidentified mechanisms. Aberrant expression of different CAM is a contributing factor to tumor development and progression (7, 106).

9. MELCAM

The cell-cell adhesion protein MelCAM (MUC18, CD146), a member of the immunoglobulin superfamily (15, 107), has been strongly implicated in melanoma progression and metastasis (108). MelCAM mediates melanoma cell interactions via heterotypic Ca2+-independent adhesion to a currently undefined ligand (109). Although not expressed on normal melanocytes in vivo and only rarely detected on benign nevus cells, MelCAM is highly expressed in advanced primary tumors and metastatic melanoma lesions and (110). In addition, MelCAM expression is up-regulated proportional to increasing vertical tumor thickness which is an established indicator of metastatic potential (106). MelCAM-negative melanoma cells with a non-metastatic and low tumorigenic profile became highly tumorigenic upon transfection with MelCAM in vivo (111, 112). On the other hand, inhibition of MelCAM in a highly metastatic cell line resulted in reduced invasion and increased apoptosis in artificial skin reconstructs (103), and decreased tumorigenicity of melanoma cells in mice (103).

MelCAM is not only a cell adhesion molecule but also a signaling molecule. It possesses several protein kinase recognition motifs in its cytoplasmic domain (113), suggesting a potential involvement of the cytoplasmic region in cell signaling (107). It has been shown that activated MelCAM forms complex with Fyn and activates FAK (114).

Our recent study has found new link between AKT activation and MelCAM expression in melanoma. AKT (also known as protein kinase B, PKB), the cellular homologue of the retroviral oncogene v-AKT, encodes a serine/threonine kinase. Upon activation, AKT acts to promote cell survival, proliferation and growth, and angiogenesis. Especially, AKT plays a critical role in survival when cells are exposed to pro-apoptotic stimuli such as growth factor withdrawal, irradiation, detachment, DNA damage and administration of apoptosis-inducing reagents (115). In addition to BRAF mutations and MAPK activation (116), constitutive AKT activation has been observed in melanoma cell lines and in primary tumors (23, 117). Pharmacological inhibition of AKT in human melanoma cell lines substantially reduced the expression of MelCAM. Overexpression of constitutively active AKT upregulated the levels of MelCAM in melanoma cell lines whereas expression of a dominant negative PI-3 kinase downregulated MelCAM. Overexpression of MelCAM activated endogenous AKT and inhibited pro-apoptotic protein BAD in melanoma cells, leading to increased survival under stress conditions.

10. L1 CAM (CD171)

The cell adhesion molecule L1 (L1-CAM) is a 200-220 kDa type I membrane glycoprotein of the immunoglobulin family with six immunoglobulin (Ig)-like and five fibronectin-type-III (FN III)-like domains in its extracellular segment. L1 can undergo homophilic L1-L1 binding involving immunoglobulin domains (118) and can form heterophilic binding to integrins including $\alpha5\beta1$, $\alpha v\beta1$, $\alpha v\beta3$ and $\alpha IIb\beta3$. It plays an important role in axon guidance and cell migration in the nervous

system (119). L1 is also expressed in tumors derived from neural crest and in certain carcinomas (120). An increase in L1 immunoreactivity in malignant melanomas as compared to melanocytic nevi was observed (120-122). L1 functions not only as an adhesive molecule but also as a signal transducing receptor (123) with its cytoplasmic domain interacting with the cytoskeleton [(124-126). L1-CAM mediates neuronal adhesion and fasciculation, and stimulation of fibroblast growth factor (FGF)-receptor-dependent neurite outgrowth by homophilic interaction. L1 may promote metastasis by facilitating tumor cell invasion or migration (127). However, the exact role of this molecule in invasion and metastasis in melanocytic transformation still needs to be determined.

11. ICAM-1

Expression of intracellular adhesion molecule-1 (ICAM-1) is stronger in malignant than benign melanocytic lesions and correlates with increasing vertical thickness in primary melanoma (128-130), a marker of poor prognosis. Interfering with its expression by antisense nucleotides inhibits experimental metastasis by melanomas in nude mice. Although the mechanisms of ICAM-1 in the progression of melanoma is not known, expression of this adhesion molecule may promote aggregate formation with leukocytes and therefore enhance survival of melanoma cells in the vascular system and encourage extravasation (18). TNF, IL-1 and γ-IFN cause the release of soluble ICAM-1 by the human melanoma cell line A375M, which is associated with tumor growth in nude mice. The release of soluble ICAM-1 from melanoma, in response to host-derived cytokines may, therefore, have relevance to immune recognition of the tumor. The serum of melanoma patients has been reported to contain elevated levels of soluble ICAM-1, correlating gradually with disease progression (31).

12. INTEGRINS

Integrins belong to a large group of heterodimeric cell surface receptors formed by non-covalent association of an α-chain with a β-chain, mediating cation-dependent adhesion to the ECM and to cell surface ligands (131, 132). At least 20 different α and 9 different β subunits have been identified to date, each with distinct ligand binding and signalling specificity, which is determined by the combination of α and β subunits. Integrins are important for tumor progression because of their ability to mediate physical interactions with ECMs and their ability to regulate signaling pathways that control actin dynamics and cell movement (133). Integrin engagement activates a battery of downstream molecules crucial for motility and survival. For example, focal adhesion kinase (FAK), whose phosphorylation is necessary for functional adhesion signaling and migration, was shown to be a component of pro-survival pathways. FAK also links integrin-mediated signals to the Ras-Raf-MAPK-ERK pathway (134-137).

The expression of integrins αvβ3 and α4β1 correlates with prognostic parameters and with poor clinical outcome in melanoma patients and can confer invasive and metastatic properties to human melanoma cells injected into nude mice (18, 138).

13. αVβ3 INTEGRIN (CD51/CD61)

Expression of the vitronectin receptor, αvβ3 integrin, is one of the best molecular markers correlating with the change from RGP to the metastatically competent VGP melanoma (75, 76). A series of studies have shown that αvβ3 is a good prognostic indicator of poor survival and short disease-free interval. In melanoma cells, activation of αvβ3 integrin has been shown to prevent apoptosis (139), to stimulate growth through association with growth factor receptors and to stimulate matrix invasion through a physical association with MMP-2 and urokinase-type plasminogen activator receptor. Introduction of β3-integrin into RGP melanoma cell lines converted them into VGP-like melanoma cells (8). A series of studies have shown that this integrin is a good prognostic indicator of poor survival and short disease-free interval. A number of potent small-molecule antagonists of the αvβ3-integrin have now been identified and are progressing in the clinic.

14. α4β1 INTEGRIN (CD49D/CD29/VLA-4)

The ligands of α4β1 integrin include thrombospondin, fibronectin and the vascular cell adhesion molecule (VCAM-1/CD106). α4β1 integrin expression is characteristic of advanced primary melanomas and may mediate interaction of the tumor cells with VCAM-1 on vascular endothelium (75, 129, 140). Adhesion mediated by α4β1 integrin expressed on lymphocyte subpopulations and VCAM-1 expressed on activated endothelial cells is one of the first steps in extravasation of these cells into tissue sites of inflammation. Therefore, expression of α4β1 integrin by melanoma cells could potentially enable the melanoma cells to emigrate from the vasculature by the same mechanism and metastasise into target tissues. Blocking α4β1 integrin/VCAM-1 interaction, using either specific antibodies (140) or the disintegrin eristostatin, inhibited metastasis formation by human and murine melanoma cells. Pretreatment of recipient mice with TNF-α or IL-1 leads to an increase in the number of pulmonary lesions, which is associated with the induction of VCAM-1 on vascular endothelia in the lung and is completely dependent on expression of α4β1 integrin by the tumor cells (49).

15. SHARED CELL ADHESION MOLECULES BETWEEN MELANOMA CELLS AND VASCULAR ENDOTHELIAL CELLS

It is generally believed that in order to metastasize to distant organ, melanoma cells have to invade lymph or blood vessels, circulate to and arrest within the vasculature of distant organs and finally extravasate and form metastatic foci. However, recent findings suggest that the latter stages of the cascade are more complex because the tumor cells can proliferate at the site of arrest within a vessel (141) thus bypassing the final extravasation step, or can form vessel-like channels on their own (142, 143) bypassing the need for true endothelium lined vessels. In a series of experiments, Mary Hendrix and co-workers described that tumor cells can have endothelial cell-like functions and form channels that allow fluid flow (142, 143). The group has identified some of the cell surface molecules, such as EphA2 and VE-cadherin, on aggressive melanoma cells that are shared with endothelial cells and that are likely involved in 'vasculogenic mimicry' (143). Vasculogenic mimicry is the ability of aggressive cancer cells to form de novo vessel-like networks in vitro in the absence of endothelial cells or fibroblasts, concomitant with their expression of vascular-associated cellular marker (144). These exciting new findings underscore the plasticity of malignant cells from advanced tumor progression stages.

Table 1 lists some of the shared cell adhesion molecules between melanoma cells and endothelial cells. These melanoma-endothelial associated markers are mostly involved in adhesion, invasion and growth. For example, On endothelial cells, β3 integrin is predominantly expressed by activated and not in resting cells, whereas MelCAM is the most widely distributed endothelial cell marker that is constitutively expressed by all vessels including precursor cells in blood (145, 146).

Table 1. Cell adhesion molecules shared between melanoma cells and vascular endothelial cells

Family	Molecule
Cadherin	VE-cadherin N-cadherin
CAM	MelCAM VCAM ICAM-1 CEA-1 CAM N-CAM ALCAM PECAM
Integrin	αvβ3

REFERENCES

1 Ries, L.A., et al., 2000, The annual report to the nation on the status of cancer, 1973-1997, with a special section on colorectal cancer. Cancer, 88:2398-2424.

2. Christofori, G. and Semb, H., 1999, The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. Trends Biochem Sci, 24:73-76.
3. Li, G., and Herlyn, M., 2000, Dynamics of intercellular communication during melanoma development. Mol Med Today, 6:163-169.
4. Clark, W.H., Jr. et al., 1984, A study of tumor progression: the precursor lesions of superficial spreading and nodular melanoma:Lack of association between intraocular melanoma and cutaneous dysplastic nevi. Human Pathology, 15:1147-1165.
5. Meier, F. et al., 1998, Molecular events in melanoma development and progression. Front Biosci, 3:D1005-1010.
6. Hsu, M.Y. et al., 2000, E-cadherin expression in melanoma cells restores keratinocyte-mediated growth control and down-regulates expression of invasion-related adhesion receptors. Am J Pathol, 156:1515-1525.
7. Li, G., Satyamoorthy, K., and Herlyn, M., 2002, Dynamics of cell interactions and communication during melanoma development. Crit Rev Oral Biol Med, 13:62-70.
8. Hsu, M.Y. et al., 1998, Adenoviral gene transfer of beta3 integrin subunit induces conversion from radial to vertical growth phase in primary human melanoma. Am J Pathol, 153:1435-1442.
9. Bissell, M.J. and Radisky, D., 2001, Putting tumours in context. Nature Reviews, 1:46-54.
10. Gumbiner, B.M., 2000, Regulation of cadherin adhesive activity. J Cell Biol, 148:399-404.
11. Kowalczyk, A.P., Bornslaeger, E.A., Norvell, S.M., Palka, H.L., and Green, K.J., 1999, Desmosomes: intercellular adhesive junctions specialized for attachment of intermediate filaments. Int Rev Cytol, 185:237-302.
12. Goodenough, D.A., Goliger, J.A., and Paul, D.L., 1996, Connexins, connexons, and intercellular communication. Annu Rev Biochem, 65:475-502.
13. Stevenson, B.R., and Keon, B.H., 1998, The tight junction: morphology to molecules. Annu Rev Cell Dev Biol, 14:89-109.
14. Morita, K., Furuse, M., Fujimoto, K., and Tsukita, S., 1999, Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. Proc Natl Acad Sci U S A, 96:511-516 ,1999,.
15. Lehmann, J.M., Riethmuller, G., and Johnson, J.P., 1989, MUC18, a marker of tumor progression in human melanoma, shows sequence similarity to the neural cell adhesion molecules of the immunoglobulin superfamily. Proc Natl Acad Sci U S A, 86:9891-9895.
16. Johnson, J.P., 1991, The role of ICAM-1 in tumor development. Chem Immunol, 50:143-163.
17. Hynes, R.O., 1992, Integrins: versatility, modulation, and signaling in cell adhesion. Cell, 69:11-25.
18. Johnson, J.P., Cell adhesion molecules in the development and progression of malignant melanoma. Cancer Metastasis Rev, 18:345-357.
19. Siliciano, J.D., and Goodenough, D.A., 1988, Localization of the tight junction protein, ZO-1, is modulated by extracellular calcium and cell-cell contact in Madin-Darby canine kidney epithelial cells. J Cell Biol, 107:2389-2399.
20. Pasdar, M., and Nelson, W.J., 1988, Kinetics of desmosome assembly in Madin-Darby canine kidney epithelial cells: temporal and spatial regulation of desmoplakin organization and stabilization upon cell-cell contact. I. Biochemical analysis. J Cell Biol, 106:677-685.
21. Frenzel, E.M. and Johnson, R.G. Gap junction formation between cultured embryonic lens cells is inhibited by antibody to N-cadherin. Dev Biol 179, 1-16 ,1996,.
22. Fujimoto, K., et al., 1997, Dynamics of connexins, E-cadherin and alpha-catenin on cell membranes during gap junction formation. J Cell Sci, 110:311-322.
23. Li, G., et al., 2003, Function and regulation of melanoma-stromal fibroblast interactions: when seeds meet soil Downregulation of E-cadherin and Desmoglein 1 by autocrine hepatocyte growth factor during melanoma development. Oncogene, 22:3162-3171.
24. Tang, A., et al., 1994, E-cadherin is the major mediator of human melanocyte adhesion to keratinocytes in vitro. J Cell Sci, 107:983-992.
25. Furukawa, F., et al., 1997, Roles of E- and P-cadherin in the human skin. Microsc Res Tech, 38:343-352.
26. Hsu, M.Y., Wheelock, M.J., Johnson, K.R., and Herlyn, M., 1996, Shifts in cadherin profiles between human normal melanocytes and melanomas. J Investig Dermatol Symp Proc, 1:188-194.
27. Gumbiner, B.M., 1996, Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell, 84:345-357.
28. Kikuchi, A., 2000, Regulation of beta-catenin signaling in the Wnt pathway. Biochemical and Biophysical Research Communications, 268:243-248.
29. Takeichi, M., 1995, Morphogenetic roles of classic cadherins. Curr Opin Cell Biol, 7:619-627.
30. Nishimura, E.K.,, Yoshida, H., Kunisada, T., and Nishikawa, S.I., 1999, Regulation of E- and P-cadherin expression correlated with melanocyte migration and diversification. Dev Biol, 215:155-166.

31. Hirai, Y., Nose, A., Kobayashi, S., and Takeichi, M., 1989, Expression and role of E- and P-cadherin adhesion molecules in embryonic histogenesis. II. Skin morphogenesis. Development, 105:271-277.
32. Takeichi, M., 1988, The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. Development, 102:639-655.
33. Garrod, D.R., 1993, Desmosomes and hemidesmosomes. Curr Opin Cell Biol, 5:30-40.
34. Koch, P.J., and Franke, W.W., 1994, Desmosomal cadherins: another growing multigene family of adhesion molecules. Curr Opin Cell Biol, 6:682-687.
35. North, A.J., et al., 1999, Molecular map of the desmosomal plaque. J Cell Sci, 112:4325-4336.
36. Bornslaeger, E.A., Corcoran, C.M., Stappenbeck, T.S., and Green, K.J., 1996, Breaking the connection: displacement of the desmosomal plaque protein desmoplakin from cell-cell interfaces disrupts anchorage of intermediate filament bundles and alters intercellular junction assembly. J Cell Biol, 134:985-1001.
37. Kouklis, P.D., Hutton, E., and Fuchs, E., 1994, Making a connection: direct binding between keratin intermediate filaments and desmosomal proteins. J Cell Biol, 127:1049-1060.
38. Smith, E.A. and Fuchs, E., 1998, Defining the interactions between intermediate filaments and desmosomes. J Cell Biol, 141:1229-1241.
39. Aberle, H. et al., 1994, Assembly of the cadherin-catenin complex in vitro with recombinant proteins. J Cell Sci, 107:3655-3663.
40. Butz, S., and Kemler, R., 1994, Distinct cadherin-catenin complexes in Ca,2+,-dependent cell-cell adhesion. FEBS Lett, 355:195-200.
41. Lewis, J.E. et al., 1997, Cross-talk between adherens junctions and desmosomes depends on plakoglobin. J Cell Biol, 136:919-934.
42. Conn, I.G., Vilela, M.J., Garrod, D.R., Crocker, J., and Wallace, D.M., 1990, Immunohistochemical staining with monoclonal antibody 32-2B to desmosomal glycoprotein 1. Its role in the histological assessment of urothelial carcinomas. Br J Urol, 65:176-180.
43. Depondt, J., Shabana, A.H., Florescu-Zorila, S., Gehanno, P., and Forest, N. 1999, Down-regulation of desmosomal molecules in oral and pharyngeal squamous cell carcinomas as a marker for tumour growth and distant metastasis. Eur J Oral Sci, 107:183-193.
44. Krunic, A.L., Garrod, D.R., Smith, N.P., Orchard, G.S., and Cvijetic, O.B., 1996, Differential expression of desmosomal glycoproteins in keratoacanthoma and squamous cell carcinoma of the skin: an immunohistochemical aid to diagnosis. Acta Derm Venereol, 76:394-398.
45. Shinohara, M., et al., 1998, Immunohistochemical study of desmosomes in oral squamous cell carcinoma: correlation with cytokeratin and E-cadherin staining, and with tumour behaviour. J Pathol, 184:369-381.
46. De Bruin, A., et al., 1999, Loss of invasiveness in squamous cell carcinoma cells overexpressing desmosomal cadherins. Cell Adhes Commun, 7:13-28.
47. Tada, H., Hatoko, M., Tanaka, A., Kuwahara, M., and Muramatsu, T., 2000, Expression of desmoglein I and plakoglobin in skin carcinomas. J Cutan Pathol, 27:24-29.
48. Silye, R., et al., 1998, E-cadherin/catenin complex in benign and malignant melanocytic lesions. J Pathol, 186:350-355.
49. Danen, E.H. et al., 1996, E-cadherin expression in human melanoma. Melanoma Res, 6:127-131.
50. Scott, G.A., and Cassidy, L., 1998, Rac1 mediates dendrite formation in response to melanocyte stimulating hormone and ultraviolet light in a murine melanoma model. J Invest Dermatol, 111:243-250.
51. Hirohashi, S., 1998, Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. Am J Pathol, 153:333-339.
52. Guilford, P., 1999, E-cadherin downregulation in cancer: fuel on the fire?. Mol Med Today, 5:172-177.
53. Graff, J.R., et al., 1995, E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res, 55:5195-5199.
54. Graff, J.R., Gabrielson, E., Fujii, H., Baylin, S.B., and Herman, J.G., 2000, Methylation patterns of the E-cadherin 5' CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. J Biol Chem, 275:2727-2732.
55. Yoshiura, K., et al., 1995, Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. Proc Natl Acad Sci U S A, 92:7416-7419.
56. Cano, A., et al., 2000, The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol, 2:76-83.
57. Nieto, M.A., Sargent, M.G., Wilkinson, D.G., and Cooke, J., 1994, Control of cell behavior during vertebrate development by Slug, a zinc finger gene. Science, 264:835-839.
58. Batlle, E., et al., 2000, The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol, 2:84-89.
59. Yokoyama, K., et al., 2001, Reverse correlation of E-cadherin and snail expression in oral squamous cell carcinoma cells in vitro. Oral Oncol, 37:65-71.
60. Lochter, A., et al., 1997, Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-

mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. J Cell Biol, 139:1861-1872.

61. Li, G., Satyamoorthy, K., and Herlyn, M., 2001, N-cadherin-mediated intercellular interactions promote survival and migration of melanoma cells. Cancer Res, 61:3819-3825.
62. Poser, I., et al., 2001, Loss of E-cadherin expression in melanoma cells involves upregulation of the transcriptional repressor Snail. J Biol Chem, 25:25.
63. Li, G., et al., 2001, Downregulation of E-cadherin and Desmoglein 1 by autocrine hepatocyte growth factor during melanoma development. Oncogene, 20:8125-8135.
64. Nakamura, T., et al., 1989, Molecular cloning and expression of human hepatocyte growth factor. Nature, 342:440-443.
65. Sonnenberg, E., Meyer, D., Weidner, K.M., and Birchmeier, C., 1993, Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. J Cell Biol, 123:223-235.
66. Bottaro, D.P., et al., 1991, Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. Science, 251:802-804.
67. Rosen, E.M., Nigam, S.K., and Goldberg, I.D., 1994, Scatter factor and the c-met receptor: a paradigm for mesenchymal/epithelial interaction. J Cell Biol, 127:1783-1787.
68. Bellusci, S., et al., 1994, Creation of an hepatocyte growth factor/scatter factor autocrine loop in carcinoma cells induces invasive properties associated with increased tumorigenicity. Oncogene, 9: 1091-1099.
69. Tamatani, T., Hattori, K., Iyer, A., Tamatani, K., and Oyasu, R., 1999, Hepatocyte growth factor is an invasion/migration factor of rat urothelial carcinoma cells in vitro. Carcinogenesis, 20:957-962.
70. To, C.T., and Tsao, M.S., 1998, The roles of hepatocyte growth factor/scatter factor and met receptor in human cancers, Review, Oncol Rep, 5:1013-1024.
71. Vande Woude, G.F., et al., 1997, Met-HGF/SF: tumorigenesis, invasion and metastasis. Ciba Found Symp, 212:119-130.
72. Moriyama, T., Kataoka, H., Koono, M., and Wakisaka, S., 1999, Expression of hepatocyte growth factor/scatter factor and its receptor c-Met in brain tumors: evidence for a role in progression of astrocytic tumors, Review, Int J Mol Med, 3:531-536.
73. DeLuca, S.M., et al., 1999, Hepatocyte growth factor/scatter factor promotes a switch from E- to N-cadherin in chick embryo epiblast cells. Exp Cell Res, 251: 3-15.
74. Trusolino, L., et al., 1998, Growth factor-dependent activation of alphavbeta3 integrin in normal epithelial cells: implications for tumor invasion. J Cell Biol, 142:1145-1156.
75. Albelda, S.M., et al., 1990, Integrin distribution in malignant melanoma: association of the beta 3 subunit with tumor progression. Cancer Res, 50:6757-6764.
76. Van Belle, P.A., et al., 1999, Progression-related expression of beta3 integrin in melanomas and nevi. Hum Pathol, 30:562-567.
77. Sefton, M., Sanchez, S., and Nieto, M.A., 1998, Conserved and divergent roles for members of the Snail family of transcription factors in the chick and mouse embryo. Development, 125:3111-3121.
78. Hajra, K.M., Chen, D.Y., and Fearon, E.R., 2002, The SLUG Zinc-Finger Protein Represses E-Cadherin in Breast Cancer. Cancer Res, 62:1613-1618.
79. Savagner, P., Yamada, K.M., and Thiery, J.P., 1997, The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial-mesenchymal transition. J Cell Biol, 137:1403-1419.
80. Hatta, K., and Takeichi, M., 1986, Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development. Nature, 320:447-449.
81. Edelman, G.M., Gallin, W.J., Delouvee, A., Cunningham, B.A.,and Thiery, J.P., 1983, Early epochal maps of two different cell adhesion molecules. Proc Natl Acad Sci U S A, 80:4384-4388.
82. Jouneau, A., Yu, Y.Q., Pasdar, M., and Larue, L., 2000, Plasticity of cadherin-catenin expression in the melanocyte lineage. Pigment Cell Res, 13:260-272.
83. Tran, N.L., Nagle, R.B., Cress, A.E., and Heimark, R.L., 1999, N-Cadherin expression in human prostate carcinoma cell lines. An epithelial-mesenchymal transformation mediating adhesion withStromal cells. Am J Pathol, 155:787-798.
84. Husmark, J., Heldin, N.E., and Nilsson, M. 1999, N-cadherin-mediated adhesion and aberrant catenin expression in anaplastic thyroid-carcinoma cell lines. Int J Cancer, 83:692-699.
85. Kawamura-Kodama, K., Tsutsui, J., Suzuki, S.T., Kanzaki, T., and Ozawa, M., N-cadherin expressed on malignant T cell lymphoma cells is functional, and promotes heterotypic adhesion between the lymphoma cells and mesenchymal cells expressing N-cadherin. J Invest Dermatol 112, 62-66 ,1999,.
86. Hsu, M., Andl, T., Li, G., Meinkoth, J.L. and Herlyn, M., 2000, Cadherin repertoire determines partner-specific gap junctional communication during melanoma progression. J Cell Sci, 113:1535-1542.

87. Navarro, P., Ruco, L., and Dejana, E., 1998, Differential localization of VE- and N-cadherins in human endothelial cells: VE-cadherin competes with N-cadherin for junctional localization. J Cell Biol, 140:1475-1484.
88. Jahroudi, N., and Greenberger, J.S., 1995, The role of endothelial cells in tumor invasion and metastasis. J Neurooncol, 23:99-108.
89. Sandig, M., Voura, E.B., Kalnins, V.I., and Siu, C.H., 1997, Role of cadherins in the transendothelial migration of melanoma cells in culture. Cell Motil Cytoskeleton, 38:351-364.
90. Voura, E.B., Sandig, M., and Siu, C.H., 1998, Cell-cell interactions during transendothelial migration of tumor cells. Microsc Res Tech, 43:265-275.
91. Ruoslahti, E., and Obrink, B., 1996, Common principles in cell adhesion. Exp Cell Res, 227:1-11.
92. Bixby, J.L.,and Zhang, R., 1990, Purified N-cadherin is a potent substrate for the rapid induction of neurite outgrowth. J Cell Biol, 110:1253-1260.
93. Doherty, P., and Walsh, F.S., 1991, The contrasting roles of N-CAM and N-cadherin as neurite outgrowth-promoting molecules. J Cell Sci Suppl, 15:13-21.
94. Riehl, R., et al., 1996, Cadherin function is required for axon outgrowth in retinal ganglion cells in vivo. Neuron, 17:837-848.
95. Hazan, R.B., Kang, L., Whooley, B.P., and Borgen, P.I., 1997, N-cadherin promotes adhesion between invasive breast cancer cells and the stroma. Cell Adhes Commun, 4:399-411.
96. Hazan, R.B., Phillips, G.R., Qiao, R.F., Norton, L., and Aaronson, S.A., 2000, Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis [published erratum appears in J Cell Biol, Apr 3:149,1, following 236]. J Cell Biol, 148:779-790.
97. Nabeshima, K., Inoue, T., Shimao, Y., Kataoka, H., and Koono, M., 1999, Cohort migration of carcinoma cells: differentiated colorectal carcinoma cells move as coherent cell clusters or sheets. Histol Histopathol, 14:1183-1197.
98. Nieman, M.T., Prudoff, R.S., Johnson, K.R., and Wheelock, M.J., 1999, N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. J Cell Biol, 147:631-644.
99. Williams, E.J., Furness, J., Walsh, F.S., and Doherty, P., 1994, Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin. Neuron, 13:583-594.
100. Evans, W.H., 1994, Assembly of gap junction intercellular communication channels. Biochem Soc Trans, 22:788-792.
101. Kuraoka, A., Inai, T., Ishibashi, T., and Shibata, Y., 2001, Diversity and molecular anatomy of gap junctions. Histochemistry and Cell Biology, 115:277-284.
102. Ito, A., et al., 2000, A role for heterologous gap junctions between melanoma and endothelial cells in metastasis. J Clin Invest, 105:1189-1197.
103. Satyamoorthy, K., Muyrers, J., Meier, F., Patel, D., and Herlyn, M., 2001, Mel-CAM-specific genetic suppressor elements inhibit melanoma growth and invasion through loss of gap junctional communication. Oncogene, 20:4676-4684.
104. Huang, R.P., et al., 1998, Reversion of the neoplastic phenotype of human glioblastoma cells by connexin 43, cx43. Cancer Res, 58:5089-5096.
105. Armstrong, D.K., et al., 1999, Haploinsufficiency of desmoplakin causes a striate subtype of palmoplantar keratoderma. Hum Mol Genet, 8:143-148.
106. Johnson, J.P., 1992, Cell adhesion molecules in neoplastic disease. Int J Clin Lab Res, 22:69-72.
107. Sers, C., Kirsch, K., Rothbacher, U., Riethmuller, G., and Johnson, J.P., 1993, Genomic organization of the melanoma-associated glycoprotein MUC18: implications for the evolution of the immunoglobulin domains. Proc Natl Acad Sci U S A, 90:8514-8518.
108. Lehmann, J.M., et al., 1987, Discrimination between benign and malignant cells of melanocytic lineage by two novel antigens, a glycoprotein with a molecular weight of 113,000 and a protein with a molecular weight of 76,000. Cancer Res, 47:841-845.
109. Shih, I.M., Speicher, D., Hsu, M.Y., Levine, E., and Herlyn, M., 1997, Melanoma cell-cell interactions are mediated through heterophilic Mel-CAM/ligand adhesion. Cancer Res, 57:3835-3840.
110. Shih, I.M., Elder, D.E., Speicher, D., Johnson, J.P., and Herlyn, M., 1994, Isolation and functional characterization of the A32 melanoma-associated antigen. Cancer Res, 54:2514-2520.
111. Luca, M., et al., 1993, Direct correlation between MUC18 expression and metastatic potential of human melanoma cells. Melanoma Res, 3:35-41.
112. Xie, S., et al., 1997, Expression of MCAM/MUC18 by human melanoma cells leads to increased tumor growth and metastasis. Cancer Res, 57:2295-2303.
113. Johnson, J.P., Bar-Eli, M., Jansen, B., and Markhof, E., 1997, Melanoma progression-associated glycoprotein MUC18/MCAM mediates homotypic cell adhesion through interaction with a heterophilic ligand. Int J Cancer, 73:769-774.
114. Anfosso, F., et al., 1998, Activation of human endothelial cells via S-endo-1 antigen ,CD146, stimulates the tyrosine phosphorylation of focal adhesion kinase p125,FAK. J Biol Chem, 273:26852-26856.
115. Datta, S.R., et al., 1997, Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell, 91:231-241.

116. Satyamoorthy, K., et al., 2003, Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation. Cancer Research, 63:756-759.
117. Singh, A.B., Ellis, D.L., Richmond, A., and Dhawan, P., 2002, Constitutive activation of Akt/protein kinase B in melanoma leads to up-regulation of nuclear factor-kappaB and tumor progression. Cancer Research, 62:7335-7342.
118. Sirim, P., et al., 1999, Pathological missense mutations of neural cell adhesion molecule L1 affect homophilic and heterophilic binding activities. Journal of Cell Biology, 145:865-876.
119. MacFarlane, J., et al., 1999, The L1 family of neural cell adhesion molecules: old proteins performing new tricks. EMBO Journal, 18:4744-4753.
120. Linnemann, D., Raz, A., and Bock, E., 1989, Differential expression of cell adhesion molecules in variants of K1735 melanoma cells differing in metastatic capacity. Int J Cancer, 43:709-712.
121. Fogel, M., et al., 2003, L1 adhesion molecule , CD 171, in development and progression of human malignant melanoma. Cancer Letters, 189:237-247.
122. Thies, A., et al., 2002, Overexpression of the cell adhesion molecule L1 is associated with metastasis in cutaneous malignant melanoma. European Journal of Cancer, 38:1708-1716.
123. Schuch, U., Lohse, M.J., and Schachner, M., 1989, Neural cell adhesion molecules influence second messenger systems. Neuron, 3:13-20.
124. Davis, J.Q., and Bennett, V., 1994, Ankyrin binding activity shared by the neurofascin/L1/NrCAM family of nervous system cell adhesion molecules. J Biol Chem, 269:27163-27166.
125. Wong, E.V., Schaefer, A.W., Landreth, G., and Lemmon, V., 1996, Involvement of p90rsk in neurite outgrowth mediated by the cell adhesion molecule L1. J Biol Chem, 271:18217-18223.
126. Dahlin-Huppe, K., Berglund, E.O., Ranscht, B., and Stallcup, W.B., 1997, Mutational analysis of the L1 neuronal cell adhesion molecule identifies membrane-proximal amino acids of the cytoplasmic domain that are required for cytoskeletal anchorage. Mol Cell Neurosci, 9:144-156.
127. Ohnishi, T., Matsumura, H., Izumoto, S., Hiraga, S., and Hayakawa, T., 1998, A novel model of glioma cell invasion using organotypic brain slice culture. Cancer Res, 58:2935-2940.
128. Schadendorf, D., et al., 1993, IL-8 produced by human malignant melanoma cells in vitro is an essential autocrine growth factor. J Immunol, 151:2667-2675.
129. Schadendorf, D., Heidel, J., Gawlik, C., Suter, L., and Czarnetzki, B.M., 1995, Association with clinical outcome of expression of VLA-4 in primary cutaneous malignant melanoma as well as P-selectin and E-selectin on intratumoral vessels. Journal of the National Cancer Institute, 87:366-371.
130. Natali, P.G., et al., 1993, Expression of the c-Met/HGF receptor in human melanocytic neoplasms: demonstration of the relationship to malignant melanoma tumour progression. Br J Cancer, 68:746-750.
131. van der Flier, A., Sonnenberg, A., and Favre, B., 2001, Function and interactions of integrins. The hemidesmosomal protein bullous pemphigoid antigen 1 and the integrin beta 4 subunit bind to ERBIN. Molecular cloning of multiple alternative splice variants of ERBIN and analysis of their tissue expression. Cell and Tissue Research, 305:285-298.
132. Eliceiri, B.P., and Cheresh, D.A., 2001, Adhesion events in angiogenesis. Circulation Research, 89:1104-1110.
133. Hynes, R.O., 2002, Integrins: bidirectional, allosteric signaling machines. A reevaluation of integrins as regulators of angiogenesis. Cell, 110:673-687.
134. Sudhakar, A., et al., 2002, New aspects of integrin signaling in cancer. Science, 295:140-143.
135. Liotta, L.A., and Kohn, E.C., 2001, The microenvironment of the tumour-host interface. Nature, 411:375-379.
136. Hutter, C., et al., 2002, Integrins in invasive growth. Development, 129:2303-2315.
137. Cheresh, D.A., and Eliceiri, B.P., 2002, Src-mediated coupling of focal adhesion kinase to integrin alpha,v,beta5 in vascular endothelial growth factor signaling. Journal of Cell Science, 115:3729-3738.
138. Sherman, L., et al., 1999, Molecular role,s, for integrins in human melanoma invasion. Human Molecular Genetics, 8:267-275.
139. Montgomery, A.M., et al., 1994, Integrin alpha v beta 3 rescues melanoma cells from apoptosis in three-dimensional dermal collagen. Cell, 79:1157-1164.
140. Okahara, H., Yagita, H., Miyake, K., and Okumura, K., 1994, Involvement of very late activation antigen 4 ,VLA-4, and vascular cell adhesion molecule 1 ,VCAM-1, in tumor necrosis factor alpha enhancement of experimental metastasis. Cancer Research, 54:3233-3236.
141. Al-Mehdi, A.B., et al., 1998, Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: a new model for metastasis. Nature Medicine, 6:100-102 ,2000.
142. Seftor, E.A., et al., 2000, Vasculogenic mimicry and tumor angiogenesis. Laboratory Investigation 78, 153-163 ,.
143. Hendrix, M.J. and Maniotis, A.J. Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry.[see comment]. American Journal of Pathology, 156:361-381.

144. Sood, A., et al., 2001, The clinical significance of tumor cell-lined vasculature in ovarian carcinoma: implications for anti-vasculogenic therapy. Indian Journal of Gastroenterology, 20:22-23.
145. Eliceiri, B.P., and Cheresh, D.A., 2000, Role of alpha v integrins during angiogenesis. Cancer Journal, 6: Suppl 3:S245-249.
146. Masuda, H., Kalka, C., and Asahara, T., 2000, Endothelial progenitor cells for regeneration. Human Cell, 13: 153-160.

Chapter 6

Matrix Metalloproteinases: Mediators of Tumour-Host Cell Interactions

Robert J. Diaz[*,1], Alexandra Eichten[*,1], Karin E. de Visser[*,1] and Lisa M. Coussens [1,2,3]
*[1]Cancer Research Institute, [2]Department of Pathology, [3]Comprehensive Cancer Center, University of California, San Francisco, CA, USA, * These authors contributed equally to this work*

Abstract: Matrix metalloproteinases (MMPs) are a family of metalloendopeptidases that induce remodelling of extracellular matrix (ECM) and differentially cleave many soluble mediators that regulate cell physiology. Due to their matrix-degrading capabilities and elevated expression levels in both neoplastic and host cells in human cancer, MMPs have acquired considerable attention as targets for anti-cancer therapy. This chapter summarizes two decades of research examining MMP biochemistry and biology utilizing *in vitro* cell-based and biochemical analyses, more recent examination of their functional significance in *de novo* mouse models of human cancer development and results from human clinical trials where MMP inhibitors were evaluated for efficacy as anti-cancer therapeutics.

Key words: Matrix metalloproteinases, tumour-host cell, microenvironment, integrins, tumor, angiogenesis, metastasis, angiogenesis inhibitors, neoplastic cell progressison, TIMP, RECK, tumour-host interactions, "cysteine switch", ECM, TNFL, MMP, Chemokines, carcinogenesis, ECM, TGFβ, EGF, bFGF, E-cadherin, tumstatin, integrin, protease inhibitors

1. INTRODUCTION

Cancers develop in a multistep manner and evolve through distinct histopathological stages characterized by significant changes in cellular and acellular organization and phenotype. While it is clear that initiating events involving activation of oncogenes and inactivation of tumour suppressor genes are essential for cancer development (1-3), extrinsic changes involving the neoplastic microenvironment fundamentally contribute to and aid progression to the tumour state. Thus, cancer development can be viewed as a collaboration between initiated neoplastic cells and activated/responding "host" cells (fibroblasts, inflammatory cells and cells composing the vasculature) and the dynamic microenvironment in which they live (4-10).

Autocrine and paracrine interactions between cellular and acellular components within developing tumours enable enhanced proliferative capacity, activation and persistence of angiogenesis and lymphangiogenesis, evasion of cell death programs and ectopic tissue growth capabilities (4). Many of these cellular programs are modulated by the actions of a family of secreted and cell surface enzymes, e.g., matrix metalloproteinases (MMPs), a family of zinc-dependent proteinases originally identified for their ability to cleave extracellular matrix (ECM) components *in vitro* (11). Since their original identification as ECM-degrading enzymes, the known biological activities of MMPs has expanded and now encompasses liberation of ECM-sequestered growth factors (12), activation of inflammatory chemoattractants (13) and ligands regulating apoptosis (14), and inactivation of ligand-

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 81-126.

binding proteins modulating proliferation (15). Consistent with these multiple roles for MMPs during neoplastic progression, correlative studies on human cancers have revealed that elevated MMP mRNA levels are associated with higher tumour staging and worse clinical outcome (16, 17). Moreover, MMP loss-of-function and gain-of-function studies utilizing mouse models of human cancer development have revealed that MMPs are functionally significant potentiators of carcinogenesis (12, 18-21). This chapter focuses on the complexity of interactions during cancer development involving MMPs and reviews recent findings where the functional significance of MMPs during neoplastic progression has been addressed experimentally.

2. MMP STRUCTURE AND FUNCTION

MMPs belong to the super-family of metzincins metalloendopeptidases (11, 22, 23). To date, ~ 26 human secreted or transmembrane MMPs have been identified (Figure 1) (24-26). Vertebrate MMPs each have distinct, but often overlapping, substrate specificities and collectively possess enzymatic activity against virtually all ECM components (24, 26, 27). In addition to their dependence on zinc and calcium, MMPs share several other common features. Individual MMPs have been variously named, grouped and subdivided based on their substrate specificities and the presence or absence of specific functional protein domains (Figure 1).

2.1 MMP Structure

Like many other classes of proteolytic enzymes, MMPs are first synthesized as inactive proenzymes or zymogens. They are found as either secreted or cell surface enzymes sharing several highly conserved domains, including a pre- and pro-peptide domain, a catalytic domain containing a zinc atom binding site, as well as several other structural domains believed to facilitate specific interactions with substrates and/or other target molecules (11, 24, 25, 28).

With the exception of MMP-7, -26 and the type II transmembrane MMP, MMP-23, all MMP family members contain the carboxyl-terminal hemopexin/vitronectin-like domain. Several functions have been ascribed to this domain depending upon the specific MMP family member. The hemopexin domain in proMMP-2 and -9 is thought to mediate interactions with specific proteinase inhibitors (28), while in MMP-1 and -8 this domain is associated with inhibitor as well as substrate binding (28). With regards to substrate specificity, the hinge region that links the hemopexin and catalytic domains, may play a major role. Whereas the hinge region is variable in length and composition among family members, MMPs that are able to degrade fibrillar collagens (MMP -1, -8, -13, -14) contain a hinge region of distinct size and composition (25). Structure-function studies have confirmed the substrate specificity dictated by this region (29). The catalytic domain for all MMP family members contains three conserved histidines that coordinate the zinc ion in the active site (30). While MMP-2 and -9 contain these conserved histidine amino acid residues within their catalytic domains, they also contain a 182 amino acid insertion in this domain homologous to the collagen-binding region of fibronectin. This region is required for gelatinolytic activity as well as the collagen binding properties of MMP-2 (31, 32).

The seven different membrane type MMPs (MT-MMPs) are anchored to the cell membrane either by a transmembrane type I domain, a glycosylphosphatidylinositol (GPI) domain or a type II N-terminal signal domain containing a unique C-terminal cysteine array and an Ig-like domain (33). These distinct membrane-anchoring domains are thought to regulate location and activity of MT-MMPs (34). In addition, several MMPs contain small domain inserts that contribute to specific functions. For example, MMP-11, 14-17, 21-25 and –28 harbor furin-like inserts within propeptide domains that enable activation intracellularly by pro-protein convertases, Ca^{2+}-dependent serine proteases of the subtilisin group (furin/PACE) (35). In summary, although MMPs share functional domains, structural differences exist such that MMPs can be classified into eight categories (Figure 1). These differences are responsible in part for the variety of biological processes that MMPs are involved in.

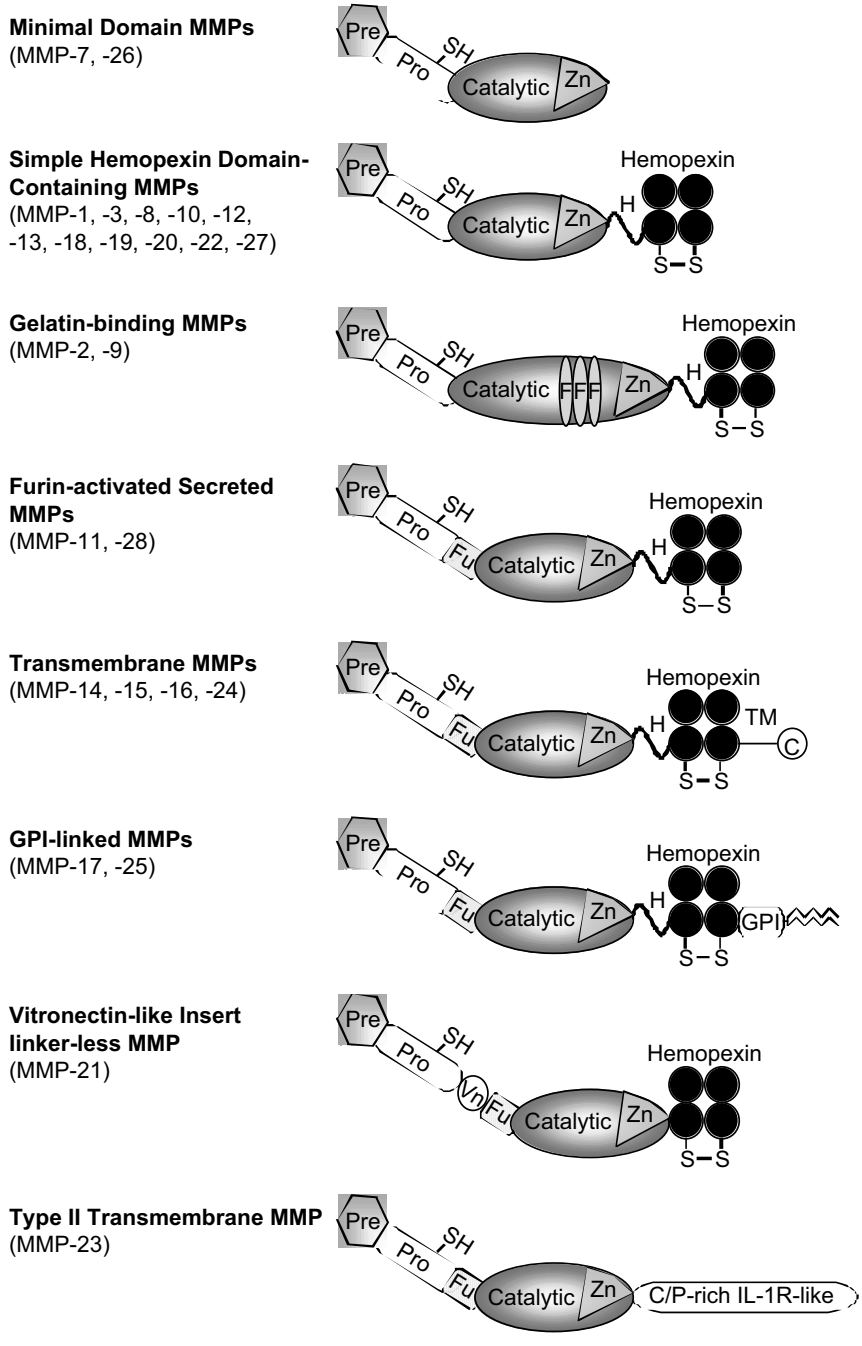


Figure 1. MMPs can be classified into eight groups based on their domain organization. Pre: signal sequence; Pro: zinc-interacting sulfydryl (SH) group containing propeptide; Fu: furin-susceptible site; Vn: virtonectin-like insert; zinc-binding site (Zn) containing catalytic domain; F: collagen binding fibronectin type II insert; H: hinge region; Hemopexin-like domain with the first and last repeat linked by a disulfide bond; TM: transmembrane domain; C: cytoplasmic tail; GPI: glycophosphatidyl inositol-anchoring domain; C/P-rich IL-1R-like: cysteine/proline-rich interleukin-1 receptor domain.

2.2 Regulation of MMP activity

The zymogen forms of MMPs are inactive. Crystallographic studies have confirmed that enzyme latency is due to coordinate bonding between the active site zinc atom with an unpaired cysteine thiol group located near the carboxyl end of MMP propeptides (36). Activation of zymogens is tightly controlled owing to cell-type specific expression characteristics, as well as post-translational regulation at the levels of zymogen activation, interaction with endogenous inhibitors and spatial constraints in pericellular microenvironments (25, 28, 37).

2.2.1 Transcriptional regulation of MMPs

In quiescent tissue, MMPs are typically expressed at low levels or more commonly transcriptionally silent. However, upon induction of tissue remodelling, MMP expression is rapidly induced by cytokines and polypeptide growth factors, e.g. interleukin (IL)-1, tumour necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β, epidermal growth factor (EGF), glucocorticoids, phorbol esters and collagen-induced signalling through receptor tyrosine kinases (discoidin domain receptors (DDR) 1 and 2) (38-42). A well-studied example of these processes is the induction of MMP-2 and -9 expression by TNF-α (43-46). Upon binding to its receptor, TNF-R55, TNF-α activates protein kinase R (PKR) to induce transcription of MMP-2 and -9 via phosphorylation of NF-κB, c-jun, c-fos and AP-1 (43-46). The importance of this pathway in regulating MMP expression is highlighted by the significant reduction in skin tumours induced by TPA (12-O-tetradecanoyolphorbol) in c-jun homozygous null (c-jun$^{-/-}$) mice (46). Alternatively, induction of MMP mRNA expression can be regulated as a result of varied interactions between ECM and pericellular collagens with plasma membrane spanning receptor tyrosine kinases, specifically discoidin domain receptor (DDR) 1 and 2 (47, 48). DDR1 is activated by types I, IV or V collagens, is expressed primarily in epithelial tissues and has been implicated in neoplasms such as breast cancer and glioblastomas (49). DDR1 regulates chemokine production in tissue infiltrating macrophages via p38 mitogen-activated protein kinase (50). In addition, studies using DDR1$^{-/-}$ transgenic mice indicate that DDR1 activation is required for MMP-2 mRNA expression in both invading macrophages and leukocytes (41, 51). DDR2 on the other hand is expressed in mesenchymal cells and in fibroblasts surrounding DDR1 positive tumour cells (52, 53). DDR2$^{-/-}$ transgenic mice and real time PCR studies have shown that DDR2 regulates MMP-1 and -2 expression in fibroblasts (42, 47). In summary, MMP mRNAs during neoplastic progression are regulated by diverse intracellular signalling pathways that reflect rapidly changing dynamic interactions between cells and their immediate microenvironments; thus, functionally linking MMP expression and tissue remodelling with the needs of expanding tumours.

Expression levels of MMP mRNAs can also be effected by single-nucleotide polymorphisms (SNPs) present within MMP promoter regions (54). These polymorphisms contribute to individual differences in MMP transcription and are associated with increased susceptibility to cancer (54). For instance, insertion of an additional guanine residue in the MMP-1 promoter results in significantly higher levels of MMP-1 mRNA (55). Clinical studies have shown that a significantly higher proportion of ovarian and colorectal cancer patients carry this polymorphism suggesting it as a risk factor for poor prognosis (56).

2.2.2 MMP activation by propeptide proteolysis

There are several distinct mechanisms by which MMP zymogens are activated. The first involves an inter-molecular proteolytic reaction known as the "cysteine switch" (57, 58). The consensus PRCGXPDV motif in MMP propeptide domains contains a cysteine-sulphydryl group that binds to Zn^{2+} ions in the active site of the N-terminal catalytic domain, thus preventing proteolytic activity (25). When interactions between the Zn^{2+} ion and the cysteine-suphydrl group are destabilized, either by chemical or physical means, proteolytic cleavage

occurs at the carboxy terminal side of the PRCGXPDV consensus motif (11, 59) resulting in irreversible loss of the cysteine residue allowing further intra/intermolecular proteolysis generating a fully active enzyme (60). In cell-free systems, the cysteine-zinc atom interaction can be interrupted by organomercurials and chaotropic agents. Alternatively, limited proteolysis of the propeptide destabilizes the cysteine-zinc bond. Interruption of the cysteine-zinc bond by any means, results in conformational changes rendering the "switch" open. Following opening, autocatalytic or proteolytic cleavage of the remainder of the propeptide yields a truncated and catalytically competent enzyme. In contrast to MMPs activated via the cysteine switch, MMP-23, a type II transmembrane MMP, is activated by a single cleavage site at Arg^{79} within the signal anchor domain (34, 61). Sharing only two common features with other MMP family members, a catalytic domain and the basic motif, MMP-23 is unique among the MMPs in that cleavage in the signal peptide at residue Arg^{79} is responsible for both secretion and activation (34, 61).

MMPs containing a furin-like recognition domain in their propeptides (MMP-11, -28 and MT-MMPs) are activated intracellularly by a group of calcium-dependent transmembrane serine proteinases of the subtilisin group termed furin/PACE/kex 2-like proteinases (Figure 1). MMPs without this recognition sequence are secreted in latent form (37). Proteolytic activation of latent secreted MMPs involves propeptide cleavage by other MMPs (62-64) or by serine proteases, such as those within the urokinase-type plasminogen activator (uPA)-plasminogen system (65-67) or serine proteases expressed by inflammatory cells such as mast cell chymase (68-70) and neutrophil elastase (71-74). Serine proteinase mediated cleavage of secreted MMP propeptide domains induces autocatalytic activation of MMP-1, -3 and –9, whereas proMMP-2 is resistant to activation by serine proteinases. Some activated MMPs can further activate other proMMPs. For example, MMP-3 activates proMMP-1 and proMMP-9; thus, serine and metalloproteinases also act as initiators for a complex array of proMMP activation cascades *in vivo*.

Cell-mediated activation mechanisms are also utilized as seen in the activation of proMMP-2 in complex with MMP-14 and TIMP-2. MMP-14 is associated with the plasma membrane where the N-terminal domain of TIMP-2 binds to active site residues in MMP-14 resulting in a dimeric complex that then serves as a receptor for proMMP-2 via the C-terminal domain of TIMP-2 interacting with the C-terminal domain of proMMP-2 (75). An adjacent free MMP-14 then cleaves proMMP-2 propeptides generating an intermediate MMP-2 species and the fully active MMP-2 is subsequently generated through an autocatalytic mechanism (67, 76). Recent data indicates that MMP-16 utilizes TIMP-2 and TIMP-3 to activate proMMP-2 by a similar process (77).

Several advantages for having proteolytic enzymes in a bound state at the cell surface have been proposed. First, bound proenzymes may be more readily activated, thus generating higher local levels of activity than what might be found in the soluble phase. Second, enzymes at the cell surface may be protected from activation by bound inhibitors. Third, the binding of an enzyme to the cell surface may provide a means of concentrating the components of a multistep pathway, thereby increasing the rate of reactions. Fourth, immobilizing enzymes on the surface of a cell or in the matrix may provide a means of restricting activity of the enzyme, so that only substrates in the vicinity of the cell or only adjacent matrix components are targeted. Hence, activation at the cell surface links MMP expression with proteolysis, and may actually provide the most significant control point in MMP activity.

2.2.3 Regulation of MMP activity by endogenous inhibitors

MMP activity is tightly regulated by several endogenous inhibitors including, tissue inhibitors of metalloproteinases (TIMPs), thrombospondins, α2-macroglobulin and RECK (Reversion Inducing Cysteine rich protein with Kazal motifs (Table 1 (78-82). The most thoroughly studied MMP inhibitors are the TIMPs. To date, four vertebrate TIMPS have been identified (TIMP-1 to -4). TIMPs are small proteins (21-28 kDa) that bind to MMPs in

a 1:1 stoichiometric ratio and reversibly block MMP activity (37). TIMP-1, -2 and –4 are secreted soluble proteins whereas TIMP-3 is matrix associated (83). TIMPs differ in both their expression patterns and affinities for MMPs. For example, TIMP-1 and TIMP-2 inhibit the activity of many MMPs. TIMP–3 on the other hand preferentially inhibits activity of MMP-1, -3, -7 and –13 (84), whereas TIMP-4 primarily inhibits MMP-2 and –7 and to a lesser extent MMP-1, -3 and –9 (85). Thrombospondin-2 binds MMP-2 and this complex results in scavenger receptor-mediated endocytosis and clearance of MMP-2 (86). Thrombospondin-1 on the other hand binds to proMMP-2 and –9 and thereby directly blocks their activation (79). The plasma protein α2-macroglobulin also regulates MMP activity by forming a complex resulting in scavenger receptor-mediated endocytosis (87); however, the inhibitory effect of α2-macroglobulin is more general in that it binds to the majority of MMPs (86). RECK is an endogenous inhibitor of MMP-2, -9 and -14 (82) and is abundant in adult tissues primarily found in vascular smooth muscle cells proximal to large blood vessels (82, 88). RECK is a secreted glycoprotein containing a serine-protease inhibitor-like domain, two epidermal growth factor-like repeats and a modified C-terminal GPI domain anchoring it to plasma membranes. RECK also inhibits secretion of proMMP-9 and the final processing step of proMMP-2 (82). The GPI anchor is thought to allow RECK access to regions of focal proteolysis along the cell surface thus enabling it to regulate proteolytic events during embryogenesis and angiogenesis (89).

Table 1. Characteristics of MMP inhibitors.
* Required for MMP-14 or MMP-16 mediated activation of MMP-2

MMP inhibitor	**MMP-2 activation***	**MMPs inhibited** (reference publication)
TIMP-1	No (365)	MMP-1 (366), -2 (367), -3 (366), -7 (368), -8 (369), -9 (367), -10 (366), -11 (370), -12 (118), -13 (371), -17 (372), -19 (373), -25 (374), -26 (375)
TIMP-2	Yes (67)	MMP-1 (376), -2 (376), -3 (366), -7 (377), -8 (378), -9 (376), -10 (366), -13 (371), -14 (379), -16 (380), -17 (372), -19 (373), -24 (381), -25 (374), -26 (375. 382)
TIMP-3	Yes (382)	MMP-1, (383), -2 (383), -9 (383), -13 (63), -14 (384), -16 (380, 382), -17 (372), -19 (373), -25 (374)
TIMP-4	No (385)	MMP-1 (386), -2 (374), -3 (374), -7 (374), -8 (385), -9 (374), -14 (374), -19 (373), -26 (375, 382)
RECK	No (82)	MMP-2 (82), -9 (88), -14 (82).
Thrombospondin-1	No (79)	MMP-2 (79), MMP-9 (79, 80)
Thrombospondin-2	No (86)	MMP-2 (86)
α-Macroglobulin	No (78)	Universally inhibits MMPs via receptor mediated endocytosis (86, 87)

2.2.4 MMP Localization

An increasing body of evidence suggests that cell surface localization of MMPs is critical for optimal MMP function (90). It has been shown that membrane bound MMPs and integrins are localized to invadopodia (91), whereas secreted MMPs transiently localize to cell surfaces by associating with cell surface proteoglycans, adhesion receptors or basement membrane components (92). Secreted MMPs like MMP-1 for example, associate with cell surfaces via integrin and EMMPRIN interactions (93-95). MMP-2 also associates with plasma membranes by interacting with αvβ3 integrin through its hemopexin-like domain (96), whereas MMP-7 binds to the hyaluronan receptor CD44 (97). MMP-9 associates with several plasma membrane spanning receptors (CD44, ICAM-1, integrins) as well as the basement membrane component type IV collagen (98-101).

The significance of MMP localization in regulating their effects on cell function has been

examined by inhibiting cell surface localization of MMP-9 in a mouse mammary carcinoma cell line (102). This resulted in loss of both invasive and metastatic capacity, properties that were restored by constitutive cell surface expression of an MMP-9 fusion protein (102), suggesting that for at least some cell types, migration through basement membrane structures may rely upon these interactions. Furthermore, disruption of CD44-MMP-7 interactions in lactating mammary epithelia resulted in relocation of MMP-7 from apical to basal cell surfaces and was associated with increased epithelial cell death and tissue remodelling (97), suggesting that whereas cell surface localization of some MMPs may impart a migratory phenotype, similar association of other family members may regulate cell proliferation and/or cell death. Taken together, MMP activity is regulated at four levels, e.g. transcriptional, post-translational propeptide cleavage, inhibition by endogenous inhibitors and differential cell surface localization. These processes are tightly regulated in normal homeostatic conditions; however, as will be discussed below, during neoplastic progression, MMP expression and activation is enhanced, a property that can stimulate and/or promote various aspects of neoplastic cell growth.

2.3 MMP Function

MMPs are thought to functionally contribute to physiological and pathological tissue remodelling, especially during embryonic and tumour development (17). It is believed that ECM remodelling is essential for maintaining tissue integrity and involves a tightly regulated balance between ECM synthesis and ECM degradation (103). During wound healing, MMPs secreted by epithelial cells, fibroblasts and inflammatory cells remodel pericellular ECM in the immediate area of tissue damage (104). In turn, fibroblasts and vascular cells synthesize appropriate amounts and composition of ECM components (type I collagen, fibronectin etc.) important for tissue repair (104). In contrast, in fibrotic environments (i.e. liver cirrhosis, lung fibrosis and scleroderma), the balance between ECM synthesis, accumulation and degradation is shifted favouring synthesis and accumulation resulting in the fibrotic phenotype, a phenotype that can also be caused by increased synthesis of ECM components independent of the degradative enzymes that remodel it (105, 106). In contrast, a shift in favour of ECM degradation is seen in degenerative pathologies such as arthritis (107) and tumour development (17, 108). During tissue remodelling, ECM components such as type I collagen and basement membrane components such as types IV, XV and XVIII collagen and laminin can be cleaved by various MMPs (17, 37). Cleavage of these larger macromolecules into smaller fragments can result in release of cryptic embedded bioactive fragments that regulate cell physiology in context-dependent manners, e.g., proliferation, angiogenesis, cell adhesion and migration (90, 109). The realization that ECM remodelling not only alters the organization and composition of physical barriers between tissue compartments potentially enabling migration, but also provides novel products that affect cell physiology, adds an additional level of functionality to MMP family members (110).

Another major function of MMPs is thought to be in their ability to regulate presence of bioactive mediators such as other proteinases, proteinase inhibitors, clotting factors, chemokines, growth factors, growth factor binding proteins, cell surface receptors, and cell-cell and cell-matrix adhesion molecules (108, 111). These MMP substrate molecules are found sequestered in ECM or attached to cell surfaces, or represent ECM components themselves, e.g., type I, IV, XV and XVIII collagen and laminin (91, 110, 112-116). For example, MMP-9 is known to target the proangiogenic growth factor vascular endothelial growth factor (VEGF) (12); however, VEGF itself is not believed to be a direct cleavage target of MMP-9 suggesting that an ECM molecule sequestering VEGF is the target. Both MMP-2 and MMP-9 activate latent transforming grwoth factor beta (TGFβ) residing in the matrix (97) and numerous MMPs can activate components of the plasma clotting system such as fibrinogen and plasminogen (112, 117-119), while MMP-2, -3, -7, -9 and 12 can cleave plasminogen generating the angiogenic inhibitor angiostatin (112, 118, 120). It has also been shown that MMP-14 derived from macrophages regulates neovascularization in tumours by degrading fibrinogen networks that serve

as a temporary scaffold for endothelial cells (117). In addition, multiple MMPs can modulate immune responses by processing of chemokines (121, 122), a property important for resolution of acute inflammation and possibly also during tumour development.

MMPs are also thought to promote tumour cell survival by conferring protection against apoptotic cell death. For example, MMP-7 sheds membrane bound Fas ligand (FasL) resulting in production of soluble FasL that significantly lowers the ability to trigger apoptosis via the Fas receptor pathway (123). MMP-7 cleaves the heparin–binding EGF precursor (HB-EGF) from the cell surface resulting in generation of signals conferring protection from apoptosis by binding of mature active form of HB-EGF to both the ErbB1 and ErbB2 tyrosine kinase cell spanning receptors (97). MMPs, besides promoting tumour progression via these diverse mechanisms, also exhibit anti-tumour functions. For example, male homozygous null MMP-8 mice (MMP-$8^{-/-}$) exhibit a significant increase in skin tumour incidence upon chemically induced carcinogenesis (124). Tumour susceptibility is sex hormone dependent since removal of ovaries in MMP-$8^{-/-}$ females also results in a similar enhanced susceptibility to chemically induced skin carcinogenesis (124). Moreover, treatment of MMP-$8^{-/-}$ mice with tamoxifen, an estrogen receptor antagonist, also results in increased skin carcinogenesis in MMP-$8^{-/-}$ females (124), suggesting that loss of MMP-8 function, by either homozygous loss or MMP inhibition (natural or synthetic), enhances rather than reduces tumour susceptibility. Taken together, it is clear that MMP function extends well beyond ECM remodelling and, as a consequence of their diverse activities toward substrates, MMPs participate in many biological (e.g. embryogensis, angiogenesis, endometrial cycling and wound healing) and pathological (e.g. cancer, arthritis and cardiovascular disease) processes by both positive and negative mechanisms.

3. CELL-TYPE SPECIFIC MMP EXPRESSION

The association of MMP expression with neoplastic progression is well documented *in vivo* and *in vitro* (17). MMPs have been associated with the malignant phenotype in a wide variety of human tissues, including breast, colon, lung, ovary, pancreas, prostate, stomach and squamous cell carcinomas of the head, neck and skin (Table 2; reviewed in: (17, 125-128). MMP-2, -3, -7, -9, -10, -13, -14 and 17 were first cloned from tumour cells lines and MMP-11 was cloned as a metastasis-specific gene from metatstatic tumours (reviewed in (17)). In fact, whether constitutively expressed or induced by oncogenes, growth factors or cytokines, expression of all members of the MMP family has been documented in cultured neoplastic cells derived from diverse developmental lineages (125). Although this characteristic led investigators to speculate that expression of MMPs by epithelial tumour cells was a critical step in the transformation and/or invasive process, it is not representative of MMP expression observed in *in vivo* situations (17). *In situ* hybridization and immunodetection studies have revealed that whereas neoplastic cells express a limited repertoire of MMPs, MMP expression more frequently originates from tumour-associated stromal cells, i.e. activated fibroblasts, macrophages, neutrophils, mast cells, endothelial cells and pericytes (Table 2). These expression patterns are indicative of distinct processes at a particular stage in neoplastic progression that either neoplastic or stromal cells are involved in. For example, during mammary carcinogenesis, mammary epithelial cells express MMP-3, -7, -9 and -13 (129-136), whereas epithelial cells undergoing an epithelial to mesenchymal transition express MMP-11 (137). Differential expression of MMPs is also observed in stromal fibroblasts. An early step in neoplastic progression is marked by myofibroblast expression of MMP-13 (138). In contrast, at a later stage in neoplastic progression, myofibroblasts at the invasive front of a mammary carcinomas express MMP-1, -2, -11 and -14 (129, 130, 131).

Cells of the immune system recruited to tumour sites also express a variety of MMPs. Macrophages express MMP-9 and -12 (131, 139), neutrophils

express MMP-8 and -9 and lymphocytes express MMP-3 and -9 (129, 140). During angiogenesis, endothelial cells express MMP-2, -3 and -9 while pericytes express MMP-9 (131, 133). Additional evidence that MMP expression is stage and cell type-dependent comes from studies showing that MMP-3 expression in squamous cell carcinomas switches from stromal fibroblast to neoplastic cells during epithelial to mesenchymal transitions (141). In addition, transgenic mouse models of human cancer have proven useful tools to examine expression characteristics of MMP mRNAs in various organs as well as for determining the role of particular MMPs during neoplastic development (21). Excellent examples of this are represented by the Rip1-Tag2 model of pancreatic islet cell carcinogenesis and the K14-HPV16 model of squamous epithelial carcinogenesis (12, 20, 142). Data from both models have indicated that MMP-9 regulates activation of the angiogenic switch and that the sources of MMP-9 are predominantly inflammatory cells recruited to the neoplastic site (12, 20, 142). Taken together, these studies have several implications. MMP-expression during neoplastic progression is stage and cell type-dependent and the expression of MMPs observed in cultured cells may have to do with the fact that most culture environments fail to recapitulate the microenvironmental complexities present *in vivo*. Most notably, spatial restrictions of MMP mRNA expression are maintained where they are either expressed by neoplastic epithelial cells or stromal cells but not typically both, implying that mechanisms regulating cell-type specificity, across tumour types, are maintained during neoplastic transformation.

Table 2. Expression of MMPs in most common human cancers*
*Based on 2004 estimated US cancer cases (American Cancer Society). Adapted from (17, 125). ISH: detection of mRNA expression as demonstrated by in situ hybridization; RT-PCR: detection of mRNA expression by RT-PCR; IHC: detection of protein expression by immunohistochemistry.

Neoplasia	MMP	Localization in tumour
Lung	MMP-1	Neoplastic cells (IHC)(387), Stromal cells (IHC) (387-390)
	MMP-2	Neoplastic cells (ISH) (387), Fibroblast (ISH) (391) (392-395), Endothelial cells (ISH: (393, 394)
	MMP-3	Neoplastic cells (IHC) (391, 393), Stromal cells (ISH) (393, 396), ECM near blood vessels (IHC) (396)
	MMP-7	Neoplastic cells (ISH) (396, 397), Endothelial cells (IHC) (397)
	MMP-9	Neoplastic cells (ISH) (391, 398-401), Stromal cells (ISH) (391, 393, 396, 402)
	MMP-10	Neoplastic cells (IHC) (403), ECM near blood vessels (IHC) (404)
	MMP-11	Neoplastic cells (IHC) (391, 398), Stromal cells (IHC) (391)
	MMP-13	Neoplastic cells (IHC) (391, 398), Stromal cells (IHC) (391)
	MMP-14	Neoplastic cells (ISH) (391, 393, 398), Fibroblast (ISH) (391, 393, 405), Endothelial cells (IHC) (393)
	MMP-26	Neoplastic cells (ISH) (406)
Breast	MMP-1	Neoplastic cells (ISH) (129, 130), Stromal cells (ISH) (129-131)
	MMP-2	Neoplastic cells (ISH) (129, 133, 134, 407), Stromal cells (ISH) (129-131), Endothelial cells (ISH) (133)
	MMP-3	Neoplastic cells (ISH) (130, 131, 133, 408), Stromal cells (ISH) (130, 131, 133, 408), Lymphocytes (IHC) (129), Endothelial cells (IHC) (133), ECM near blood vessels (IHC) (409)
	MMP-7	Neoplastic cells (ISH) (131)
	MMP-9	Neoplastic cells (ISH) (129, 133, 134, 408), Stromal cells (ISH) (129), Fibroblast (IHC) (133, 134, 136) , Macrophages (ISH) (139), Neutrophils (IHC) (139) Endothelial cells (ISH) (131, 133), Pericytes (ISH) (139)
	MMP-10	ECM near blood vessels (IHC) (409)
	MMP-11	Neoplastic cells (ISH) (137), Stromal cells (ISH) (131, 137, 410)
	MMP-12	Macrophages (IHC) (131)
	MMP-13	Neoplastic cells (IHC) (131), Myofibroblast (IHC) (138)
	MMP-14	Neoplastic cells (IHC) (134, 411), Myofibroblast (IHC) (412)
	MMP-19	Neoplastic cells (IHC) (413), Endothelial cells (IHC) (413)

Neoplasia	MMP	Localization in tumour
	MMP-26	Neoplastic cells (IHC) (382, 406)
Neoplasia	MMP	Localization in tumour
Prostate	MMP-2	Neoplastic cells (ISH) (414-417), Stromal cells (ISH) (417)
	MMP-7	Neoplastic cells (ISH) (414, 417)
	MMP-9	Macrophages (ISH) (417)
	MMP-10	ECM near blood vessels (IHC) (418)
	MMP-14	Neoplastic cells (IHC) (416)
	MMP-26	Neoplastic cells (IHC) (406, 419)
Colon	MMP-1	Neoplastic cells (ISH) (420), Stromal cells (ISH) (420, 421)
	MMP-2	Neoplastic cells (ISH) (422, 423), Fibroblast (ISH) (421-423), Endothelial cells (ISH) (424), Myofibroblast (ISH) (424), ECM (IHC) (425)
	MMP-3	ECM near blood vessels (IHC) (425)
	MMP-7	Neoplastic cells (ISH) (397, 426, 427), Endothelial cells (ISH) (397)
	MMP-9	Macrophages (ISH) (140, 175, 428), Neutrophils (ISH) (140), ECM (IHC) (425)
	MMP-10	ECM near blood vessels (IHC) (425)
	MMP-11	Fibroblast (ISH) (429)
	MMP-12	Neoplastic cells (ISH) (430)
	MMP-14	Neoplastic cells (ISH) (423, 431), Stromal cells (ISH) (423, 431), Macrophages (ISH) (431)
	MMP-21	Neoplastic cells (IHC) (432)
Ovary	MMP-1	Neoplastic cells (IHC) (433)
	MMP-2	Neoplastic cells (ISH) (433-436), Stromal cells (ISH) (433, 434, 436, 437), Fibroblast (ISH) (435)
	MMP-7	Neoplastic cells (IHC) (438)
	MMP-9	Neoplastic cells (ISH) (434, 436, 439), Stromal cells (ISH) (436, 439, 440), Macrophages (ISH) (434, 437), Neutrophils (IHC) (434)
	MMP-11	Fibroblast (ISH) (441)
	MMP-14	Neoplastic cells (ISH) (436, 441)
	MMP-21	Neoplastic cells (ISH) (432)
Squamous cell carcinoma of the skin	MMP-1	Neoplastic cells (ISH) (442) Stromal cells (IHC) (442)
	MMP-2	Fibroblast (ISH) (442-444)
	MMP-3	Neoplastic cells (ISH) (443), Stromal cells (ISH) (443, 445)
	MMP-7	Neoplastic cells (ISH) (446), Macrophages (ISH) (444), Neutrophils (ISH) (447) , Eosinophils (ISH) (447)
	MMP-10	Neoplastic cells (IHC) (448)
	MMP-11	Fibroblast (IHC) (449)
	MMP-12	Neoplastic cells (ISH) (450), Macrophages (ISH) (450)
	MMP-13	Neoplastic cells (ISH) (445), Stromal cells (ISH) (445)
	MMP-14	Neoplastic cells (IHC) (448), Fibroblast (IHC) (448)
	MMP-19	Neoplastic cells (RT-PCR) (451)
	MMP-21	Neoplastic cells (RT-PCR) (432)

4. MMPS AND NEOPLASTIC PROGRESSION: PRO AND ANTI-TUMOUR FUNCTIONS

Various members of the MMP family are present and active in tumour microenvironments where they are thought to participate in many aspects of neoplastic progression including inflammation, angiogenesis, neoplastic cell proliferation, migration, invasion into ectopic compartments and metastasis formation (Figure 2). Our understanding of the molecular and cellular mechanisms regulated by MMPs that influence these processes in different tumour types has expanded greatly in recent years, however many outstanding questions remain. Understanding these mechanisms and elucidating

how MMPs exert pro- and/or anti-tumour affects, may reveal novel drug targets for development of rational anti-cancer therapeutics.

4.1 Inflammation and MMPs during tumour development

Based on the characteristics of activation and the specificity of target recognition, the immune system can be divided into two subsets - innate and adaptive (143). The innate immune system, also called the first line of immune defence, comprises macrophages, neutrophils, granulocytes, mast cells, eosinophils, basophils and natural killer (NK) cells. The innate immune system is characterized by its ability to respond to foreign antigens in a non-specific manner and is not intrinsically affected by prior contact with pathogens. The adaptive immune system on the other hand is composed of T and B lymphocytes and antibodies, is very specific in its capacity to recognize antigens and is characterized by immunological memory (143). In order to provide sufficient protection against all kinds of infectious agents, the innate and adaptive immune systems are closely linked by influencing each others recruitment and activation pathways (144).

The immune system plays a dual role in tumour development and progression (145). Several studies have reported that the immune system, in particular the adaptive immune system, can suppress tumour development. Studies supporting this concept of immune-surveillance have shown that infiltration of tumours with T lymphocytes is beneficial for cancer patients (146-150). In addition, an increase in the incidence of spontaneous and chemically induced tumours has been observed in immune-deficient mouse models of tumour development (151). Based on the idea that a 'tumour' can be a recognizable target for the immune system, many groups have attempted to activate the immune system in order to obtain successful anti-tumour immune responses (152).

In contrast to the immune-surveillance theory, accumulating clinical and experimental data suggest that the other arm of the immune system, the innate immune system, plays a promoting role during neoplastic progression (6, 153). Extensive analysis of human tumour samples has revealed abundance of innate immune cells, in particular mast cells and macrophages, that correlates with angiogenesis and poor prognosis (154-160). Another indication that inflammatory cells play a promoting role in carcinogenesis is the observation that chronic inflammation often predisposes patients to the development of cancer (161, 162). Well-known examples are the association of inflammatory bowel syndrome with development of colon cancer (163) and the increased risk to develop gastric cancer in patients with chronic helicobacter pylori infection (164). Consistent with these clinical observations are experimental findings that development of colon cancer in TGFβ1-deficient mice is eliminated by maintaining mice in germ-free environments, thus reducing risk of inflammation (165). In addition, long-term use of aspirin and non-steroidal anti-inflammatory drugs has been shown to diminish cancers; colon cancer risk by ~ 50%, gastric and oesophageal cancer risk by ~ 40%, and breast cancer by ~ 20% (166-171). Thus, clinical data clearly suggest a promoting role of inflammatory cells during neoplastic progression; however, they do not provide any mechanisms by which inflammatory cells contribute to the tumour development process. Many investigators now believe that elucidating the mechanisms by which inflammatory cells participate in carcinogenesis will eventually facilitate development of novel therapeutic agents against human cancer (6, 153). As described above, inflammatory cells are important sources of MMPs in tissues engaged in either physiologic or pathologic remodelling. In the next paragraphs, we will focus on the role of inflammatory cell- and other host cell-derived MMPs in neoplastic progression.

Expression of MMPs in human cancer often correlates with poor prognosis (154-158), suggesting that MMPs promote carcinogenesis via either direct and/or indirect pathways. In human carcinomas, the majority of MMPs present are not expressed by neoplastic cells, although exceptions do exist, but instead are predominantly expressed by activated stromal cells, e.g., fibroblasts, vascular cells and a diverse assortment of inflammatory cells (Table 2) (6, 172-175). Since inflammatory cells are often strongly associated with cancer progression, several studies have investigated whether MMPs are

involved as mediators linking inflammation with malignancy.

Compelling evidence that inflammatory cells promote carcinogenesis via secretion of MMPs has been provided by experimental mouse models of *de novo* carcinogenesis harboring homozygous null mutations in various MMPs (12, 18-20). The role of MMP-9 during tumorigenesis was addressed in a transgenic mouse model of squamous carcinogenesis of the skin (176) by studying the phenotypic consequences of genetic deletion of MMP-9 (20, 177). In this tumour model, the appearance of activated MMP-9 in premalignant dysplastic lesions coincides with extensive mast cell infiltration of dermal stroma and when transgenic mice are rendered deficient for either mast cells (142) or MMP-9 (20), tumour-prone mice display significantly reduced epithelial proliferative indices, altered differentiation characteristics and attenuated angiogenesis. Importantly, MMP-9 deficiency results in 50% reduction in incidence of carcinomas as compared to MMP-9 proficient controls (20). Importantly, the characteristics of neoplastic progression in this model were restored by reconstitution with wild type MMP-9 sufficient bone marrow-derived cells (20), thus providing compelling data suggesting that inflammatory cells contribute to neoplastic progression, in part, by their production of MMP-9 in the neoplastic microenvironment. In a different *de novo* mouse tumour model, e.g., pancreatic islet cell carcinogenesis, MMP-9 is also only detected in infiltrating inflammatory cells, not in neoplastic cells (12). In this mouse model, genetic ablation of MMP-9 also results in suppression of angiogenesis and tumour growth (12). Likewise, growth and activation of angiogenesis in xenografted MMP-9-expressing human ovarian carcinomas is significantly attenuated in MMP-9-deficient/immune-deficient mice (178) that can be "rescued" by MMP-9 proficient splenocytes that induce MMP-9^+ macrophage infiltration into the tumour microenvironment, resulting in increased vascularization and tumorigenicity (178). These data provide compelling support for the contention that inflammatory cell-derived MMP-9 contributes to tumorigenesis in multiple organ environments. Recently it has also become clear that inflammatory cell-derived MMPs also play a contributing role during metastasis formation (179, 180). Utilizing a mouse model system of experimental lung metastasis, MMP-9 expression in macrophages and endothelial cells of lungs of tumor-bearing hosts positively regulated metastasis formation to the pulmonary site (179). Correlating with this, human cancer patients with metastatic pulmonary disease similarly exhibit significantly elevated MMP-9 levels in diseased lung tissue as compared to those from tumour-free patients or disease-free lungs (179) suggesting that inflammatory cell-derived MMP-9 promotes metastatic tumour formation. What are the mechanisms by which activated stromal cells regulate MMP expression in neoplastic microenvironments and affect cancer development? MMPs are potent mediators with many different functional capacities and their biological activities greatly depend on the microenvironment in which they are deposited. Consequently, MMPs participate in many aspects of neoplastic progression, including proliferation of neoplastic cells, extracellular matrix remodelling, angiogenesis, lymphangiogenesis and metastasis formation.

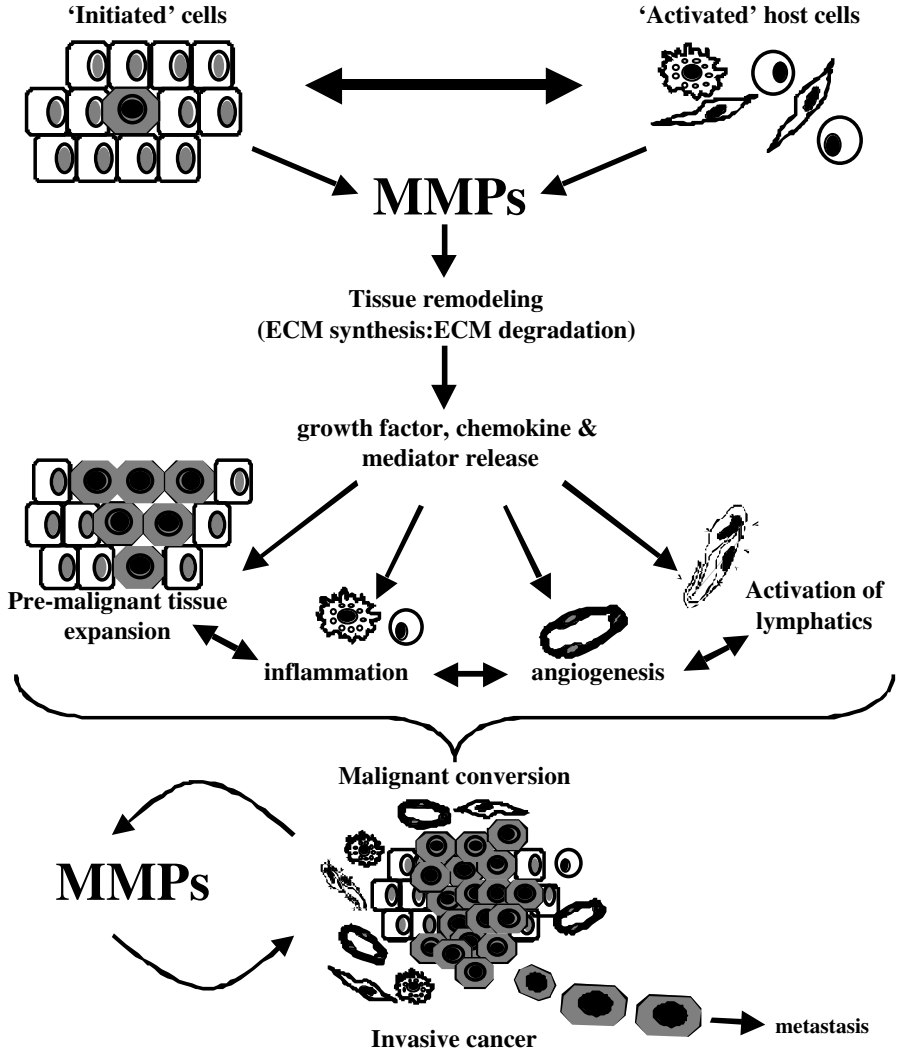


Figure 2. MMPs and tumor-host cell interactions: Cancer development results from the interplay of genetically altered neoplastic cells with activated stromal cells and the dynamic microenvironment in which they live. The presence of genetically altered cells in otherwise healthy tissue activates a "host response", in particular activation of fibroblasts and immune cells. Both genetically altered cells and activated host cells present in early pre-malignant lesions secrete diverse factors, including MMPs. MMPs can initiate remodeling of virtually all ECM components, resulting in release of mediators sequestered in the ECM and activation of latent growth factors. Altered bioavailability of these mediators triggers proliferation of neoplastic cells and angiogenesis. In addition, MMPs, produced by neoplastic and activated host cells, regulate various aspects of tumour development and facilitate many collaborative interactions between diverse cells types present in the neoplastic microenvironment. Known regulatory mechanisms involving MMPs include: stimulating neoplastic cell hyperproliferation, activation of angiogenesis, stimulating inflammatory cell recruitment and function via modulation of chemotactic mediators, as well as inducing tissue remodelling resulting in both the synthesis as well as degradation of matrix components. Following malignant conversion and development of bona fide invasive cancers, MMP activity can further influence the malignant phenotype of emerging tumours as well as the viability of metastatic cells in distant tissue compartments.

4.2 MMPs and neoplastic cell proliferation

The balance between neoplastic cell proliferation and cell death is a critical determinant of tumour outgrowth. Multiple paracrine and autocrine growth factors have been identified that modulate the mitogenic activity and/or survival capacity of various cell types within tumours. Since inhibition of growth factor-induced signalling cascades can block expansion of neoplastic cells in some contexts, and delay or inhibit growth in others, (181-187), there has been great interest in characterizing the mechanisms regulating growth factor bioavailability in neoplastic microenvironments.

It has become clear that ECM remodelling by stromal- and/or neoplastic cell-derived MMPs results in release of a variety of growth factors sequestered in the ECM and in proteolytic shedding and activation of multiple latent ECM and membrane-anchored growth factors (108, 188-190). The increase in bio-available growth factors regulated by MMP-mediated proteolytic cleavage directly impacts proliferative capacity of diverse cell populations, including neoplastic cells (108). The role of MMPs in modulating the proliferative activity of neoplastic cells has been underscored by the observation that neoplastic keratinocytes in MMP-9 deficient/HPV16 transgenic mice exhibit a suppressed proliferative index (20). Likewise, collagenase expression in transgenic mouse skin promotes hyperproliferative changes in the epidermis (191) and transgenic overexpression of TIMP-1 inhibits SV40 T antigen-induced hepatocyte proliferation (192, 193).

Several growth factors are produced as membrane anchored precursors requiring conversion to soluble forms for biological activity (97, 194-196). Great effort has been placed in identification of enzymes responsible for proteolytic conversion of insoluble mitogenic precursors into diffusible active growth factors, as this is an important post-translational event regulating growth location, activity and bioavailability. MMPs play a crucial role in proteolytic release of mitogenic precursors from the cell surface membrane, a process frequently referred to as 'ectodomain shedding' (197, 198). For example, EGF family members, including EGF, heparin binding EGF-like growth factor (HB-EGF) and TGFα, are synthesized as latent membrane spanning proteins requiring cleavage and release by MMPs in order to obtain a conformation suitable for binding to their plasma membrane receptors (194-196). Soluble EGF family ligands stimulate many biological responses, in particular proliferation and migration in cells expressing EGF receptors, altered expression of which has been reported in various human cancers. MMP-3 releases HB-EGF from the cell surface whereas an MMP related proteinase ADAM17, releases soluble TGFα (195). HB-EGF and MMP-7 form a complex with CD44, a heparin sulphate proteoglycan found on the surface of normal and neoplastic cells (97, 199). Formation of this complex allows cleavage of HB-EGF by MMP-7, thus generating mature HB-EGF, which in turn enhances cell proliferation and cell survival (97). The importance of CD44 in neoplastic cell proliferation has been underscored by the observation that transgenic mice expressing antisense CD44 cDNA in skin keratinocytes display impaired keratinocyte proliferation and fail to undergo hyperproliferative growth in response to carcinogen exposure (200).

Proteolytic release of membrane-anchored growth factor precursors can be inhibited by TIMPs (84, 196, 201) and by synthetic metalloprotease inhibitors (MPIs) (194, 202). For example, blocking proteolytic shedding of membrane-anchored EGF family member precursors by treatment with MPIs almost completely abolished proliferation of human mammary epithelial cells and colon cancer cell lines (194). Thus, proliferation of neoplastic cells can be manipulated by MMP-mediated regulation of ectodomain shedding suggesting that MPIs might be applied therapeutically to regulate bioavailability of growth factors in proliferating tissues.

Other growth factors are maintained in a latent form by complex formation with soluble or cell-surface bound proteins. For example, activity of insulin-like growth factors IGF-I and IGF-II is controlled by binding to various soluble IGF-binding proteins (IGF-BP) (203-205). Proteolytic cleavage of IGF-BP by several MPs, including MMP-1, -2, -3, -9 and -11, releases IGF that subsequently exerts mitogenic effects (206-211). Expression of IGFs is often upregulated in hyperproliferative tissues, including cancer tissues where they correlate with

poor prognosis (204, 212-215). The importance of MMPs in promoting neoplastic cell proliferation via increasing bioavailability of IGF has been demonstrated in a transgenic mouse model of hepatic carcinogenesis (211). Transgenic overexpression of TIMP-1 in SV40 T antigen-induced hepatocytes inhibited proliferation (211) due to inhibition of MMP-mediated proteolysis of IGF-BP-3 resulting in reduced levels of bioavailable IGF-II (211). Similar to IGF, basic FGF (bFGF), a mitogenic growth factor linked to angiogenesis and fibroblast activation is sequestered in the ECM by specific binding to various proteins (216). Several heparin sulfates, including perlecan, regulate bioavailability of FGF by sequestering latent FGF at cell surfaces and within basement membranes (217). MMP-1 and -3 have been reported to degrade perlecan resulting in FGF release (216). However, MMP activity does not always result in enhancement of proliferation. MMP-2 has been reported to cleave FGF receptor 1, which in turn prevents mitogenic signalling (218). Another protein regulating FGF activity is FGF-BP. In contrast to perlecan, FGF-BP does not limit bioavailability of FGF, but instead mobilizes and activates FGF (219). Whether MMPs also degrade FGF-BP and thus negatively modulate FGF bioavailability remains to be established. Likewise, bioavailability of TGFβ, a multi-potent polypeptide growth factor, is regulated by MMPs (220). The role of TGFβ during tumor progression and development is very complex and depends on the type and progression stage of neoplastic cells (221-224). In general, activated stromal and neoplastic cells in early tumour stages are sensitive to TGFβ-mediated growth inhibition (225, 226), whereas neoplastic cells in later stages often escape TGFβ-mediated growth inhibition (222, 223). TGFβ is produced as a latent protein activated in part by proteolytic mechanisms (220, 227). The TGFβ prodomain, also referred to as β-latency associated peptide (β-LAP), binds non-covalently to mature TGFβ thus forming an inactive latent complex (220). Latent TGFβ-binding proteins link to this complex stabilizing and maintaining TGFβ sequestered within ECM in an inactive state (227-230). TGFβ can be activated by proteolytic degradation of LAP by MMP-9 and MMP-2, resulting in release of active TGFβ (102). Likewise, several TGFβ binding proteins that sequester active TGFβ in ECM, including membrane-anchored proteoglycan betaglycan and the ECM proteoglycan decorin, are cleaved by various MMPs (231-234), where upon release from latent complexes, TGFβ exerts its tumor suppressive and/or promoting functions (222).

In conclusion, the function of stromal cell- and neoplastic cell- derived MMPs is not limited to degradation and remodelling of ECM. An additional function, one that has implications for therapeutic anti-cancer strategies, is the shedding of various potent growth factors from cell surfaces and release of mitogens sequestered by ECM; thus, by regulating bioavailability of growth factors, MMPs deposited in tumor microenvironments can drive neoplastic progression and cancer development.

4.3 MMP regulation of neoplastic cell adhesion, migration and invasion

Tumours are characterized by their phenotype, cell of origin and whether they exhibit either benign or malignant characteristics, with malignancy directly inferring neoplastic cell invasion across basement membranes and ectopic tissue growth. In order for neoplastic cells to invade surrounding tissue, they must exit the primary tumour site, cross tissue boundaries and migrate into ectopic tissue. Based upon their collective ability to degrade structural components of basement membranes and ECM *in vitro*, MMPs have long been viewed as key regulators of neoplastic cell migration and invasion (17). However, examination of MMP functions in *de novo* mouse models of tumour development have challenged these viewpoints and revealed new mechanisms for MMP action that functionally contribute to tumour development.

Substrate targets for MMPs have been extensively studied *in vitro* (reviewed in (17, 111) which has generated a large body of literature describing ECM as well as non-ECM substrates for MMP family members, suggesting a role for MMPs in tissue remodelling and other physiological and pathological processes, including cancer. These studies have revealed tremendous overlap and functional redundancy among MMP family members (Table 3).

Table 3. MMP Substrates. Adapted from (17, 125). *Only few MMP substrates have been verified as in vivo substrates. ADAMTS, A disintegrin and metalloproteinase with thromobospondin type 1 motifs; C1q, complement component 1q; FGFR, fibroblast growth factor receptor; HB-EGF, heparin-binding epidermal growth factor; IGF-BP, insulin-like growth-factor-binding-protein; IL-1β interleukin-1β; IL-2Rα, interleukin-2 receptor α; MMP, matrix metalloproteinase; NC1, non-collagenous 1 region; PA1, plasminogen activator inhibitor; TGF-β transforming growth factor-β; TNF-α, tumour necrosis factor-α; uPA, urokinase-type plasminogen activator.

MMP	**ECM Substrates**		**Non ECM substrates**			
	Collagen	**Other ECM components**	**Chemokines and Cytokines**	**Growth Factors**	**MMPs**	**Other**
MMP-1	Type, I, II, III, VII, X (24), XI (59) collagen	Gelatins (24), aggrecan (24), brevican (452) entatctin/nidogen (24), fibronectin (59) laminin (59), tenascin (24), vitronectin (59)	CXCL12 (4), IL-1β (59) proTNF-α (59)	TGFβ (59)	ProMMP-2 (24)	IGFBP-2, -3, link protein (24), α1-antichymotypsin (59), α2-macroglobulin (59), α1-proteinase inhibitor (59), C1q (59), casein (59), myelin basic protein (59), L-selectin (5), fibrin (59) link protein link protein (24), link protein (24)
MMP-2	Type, I (24), III (59), IV (24), V (24), VII (24), X (24) XI (24) collagen	Gelatins (24), aggrecan (24), brevican (452), decorin (59), elastin (24), entactin/nidogen (59), fibronectin (24), fibulins (453), laminin (24), osteonecin (59), tenascin (59), vitronectin (59), ADAMTS-1 (454)	CXCL12 (4), IL-1β(59) TNF-α (59)	Pro-HB-EGF (195)	ProMMP-9 (24), -13 (63)	IGF-BP (24), link protein (59), C1q (59), α1-antichymotypsin (59), α1-proteinase inhibitor (59) FGFR1 (218), substance P (59) plasminogen (59), myelin basic protein (59)
MMP-3	Type III (59), IV (24), V (24), VII (24), IX (24), X (24), X1 (24) collagen	Gelatins (24), aggrecan (452), decorin (59), elastin (59), brevican (59), decorin (59), elastin (59), entactin. nidogen (24), fibronectin (59), laminin (24), osteonectin (59), osteopontin (455), perlecan (216), tenascin (24), vitronectin (24), NC1 fragment of collagen XVIII (289)	CXCL12 (121), pro-TNF-α (59)	Pro-HB-EGF (97)	ProMMP-1 (24), -7 (195), -8 (369), -9 (456), -13 (24)	PAI-1 (457), plasminogen (59), substance P (59), T kininogen(59), α1-antichymotrypsin (24), α2-macroglobulin (24), α1-proteinse inhibitor (24), uPA (458), link protein (24), myelin basic protein (59) C1q (59), casein (59), E-cadherin (24), fibrin (24), fibrinogen (59), L-selectin (459), fibrillin (59)

MMP	ECM Substrates		Non ECM substrates			
	Collagen	Other ECM components	Chemokines and Cytokines	Growth Factors	MMPs	Other
MMP-7	Type I (59), IV (59) collagen	Gelatins (24), aggrecan (59), brevican (452) decorin (59) elastin (24), entactin/nidogen (24), fibronectin (24), fibulins (24), laminin (24), vitronectin (24), osteonectin (59), tenascin, (24),β4 integrin (246)	Pro-TNF-α (59)		ProMMP-1 (460), -2 (24), -9 (460)	Link protein (24), myelinbasic protein (59), osteopontin (461), α1-proteinase inhibitor (24), casein (59), E-cadherin, (59), FAS ligand (14), fibrinogen (59), plasminogen (59),
MMP-8	Type I (24), II (24), III (24) collagen	Aggrecan (24), brevican (452), ADAMTS-1 (454)		Latent TGF-β (102)		α2-macroglobulin (fibrillin (59), C1q (59), fibrinogen (59), substance P (59)
MMP-9	Type IV (24), V (24), XI (24), XIV (24) collagen	Gelatins (24), aggrecan (24), decorin (59), elastin (24), laminin (59), NC1 fragment of collagen XVIII (289), osteonectin (59), vitronectin (24)	CXCL1 (322), CXCL4 (322), CXCL7-precursor (322), CXCL12 (121), IL-1β (59), IL-1β (59), IL-8/CXCL8 (322), IL-2Rα (123), pro-TNF-α (59)		ProMMP-2 (24)	Link protein (24), myelin basic protein (24), α2-macroglobulin (59), α1-proteinase inhibitor (59), casein (59), C1q (59), endothelin (462), fibrin (59), fibrillin (59), fibrinogen (59), galectin-3 (463), plasminogen (59), substance P (59), IGF-BP3 (r29), fibrillin (59)
MMP-10	Type III (464), IV (464), V (464), collagen	Gelatins (24), aggrecan (59), brevican (452), elastin (24), fibronectin (464)			ProMMP-1 (24), -7 (465), -8 (466), -9 (465)	Link protein (24), casein (59), fibrinogen (59)
MMP-11						IGF-BP (2), α2-macroglobulin (24), α1-proteinase inhibitor (24)

MMP	ECM Substrates		Non ECM substrates			
	Collagen	**Other ECM components**	**Chemokines and Cytokines**	**Growth Factors**	**MMPs**	**Other**
MMP-12	Type I, (2), IV (24) collagen	Gelatins (24), aggrecan (59), elastin (24), fibronectin (24), entactin/ nidogen (24), fibrillin (59), laminin (24), vitronectin (59), NC1 fragment of collagen XVIII (289)	Pro-TNF-α (59)			Myelin basic protein (24), α2-macroglobulin (59), α1-proteinase inhibitor (24), factor X11 (59), fibrinogen (24), IgG (467), plasminogen (24)
MMP-13	Type I (24)II (24), III (24), IV (468), VI (59), IX (468), X (468), XIV (468)	Gelatins (59), aggrecan (59), brevican (452), fibronectin (468), osteonectin (468), tenascin (468)				Fibrillin., α2-macroglobulin (59)
MMP-14	Type I (469), II, (469), III (469) collagen	Gelatins (24), aggrecan (469), entactin/nidogen (469), fibrillin (59), fibronectin (469), perlecan (469), vitronectin (24), tenascin (469),	CXCL12 (59), pro-TNF-α (469)		ProMMP-2 (59), -13 (63)	α2-macroglobulin (1), α1-proteinase inhibitor (24), CD44 (237), factor XII (59), fibrin (59), fibrinogen (59), αv integrin (470) tissue transglutaminase (248)
MMP-15		Aggrecan (471), entactin/nidogen (471), fibronectin (471), perlecan (471), laminin (471), tenascin (471), tissue, transglutaminase (248), ADAMTS-1 (472)			ProMMP-2 (471)	
MMP-16	Type III (59) collagen	Gelatin (473), fibronectin (59)			ProMMP-2 (382)	Casein (473), tissue transglutaminase (248)
MMP-17		Gelatin (474)	TNF -α (471)		ProMMP-2 (474)	Fibrin (372), fibrinogen (372)

MMP	ECM Substrates		Non ECM substrates			
	Collagen	**Other ECM components**	**Chemokines and Cytokines**	**Growth Factors**	**MMPs**	**Other**
MMP-19	Type I, (59), IV (373) collagen	Gelatins (373), aggrecan (475), entactin/nidogen (373), fibronectin (373), laminin (373), tenascin (373), Cartilage oligomeric matrix protein (475)				Casein (59)
MMP-20		Aggrecan (475), cartilage oligomeric matrix protein (475) NC1 fragment of collagen XVIII (289)				Amelogenin (19)
MMP-22		Gelatin (476)				Casein (476)
MMP-24		Gelatin (474), fibronectin (474),			ProMMP-2 (381)	
MMP-25	Type IV (374) collagen	Gelatin (374), fibronectin (477)			ProMMP-2 (375)	Fibrinogen (374), fibrin (374).
MMP-26	Type IV (375) collagen	Gelatin (478), fibronectin (375), vitronectin (406)			ProMMP-9 (375)	α1-proteinase inhibitor (59), casein (478), fibrinogen (406)
MMP-28						Casein (479)
Mcol-A	Type I (480), II (480) collagen					
75-kDa chicken gelatinase	Gelatins (481), fibronectin (481)					

To date however, only a few MMP substrates have been verified as bone fide *in vivo* substrates (17), validation of which in appropriate *in vivo* contexts is necessary to fully understand the multitude of molecular and cellular events regulated by MMPs.

Cell surface expression of cell-cell and cell-ECM adhesion molecules are tightly regulated (235) with expression varying to accommodate changes in pericellular microenvironments and differential regulation of stationary versus migratory growth characteristics. Besides impacting migration and invasive capacities of neoplastic cells by remodelling key ECM molecules, MMPs also act in concert with diverse cell surface molecules implicated in adhesion (236-238). One family of cell surface adhesion molecules differentially affected by MMPs are integrins. These consist of dimeric membrane spanning cell-ECM adhesion molecules containing one α and β subunits (239). Integrins are important mediators of cell migration in part due to the diversity of complexes formed by α and β subunits forming ~ 24 different cell-ECM receptors in humans (240). Integrins engage ECM molecules pericellularly, whereas intracellulary they interact with signalling molecules and cytoskeletal components and regulate cell shape, polarity, differentiation and various aspects of intracellular signal transduction (240). When cells are at rest and tissues are homeostatic, integrin expression reflects cell-ECM interaction favouring structural integrity and polarized cell growth (241). In contrast, when tissues are engaged in either physiological or pathological remodelling, integrin expression and repertoires change in a manner consistent with a cells need to 'move' within the microenvironment (242). While MMPs are known to target components of ECM to facilitate migration, they also are known to associate with various integrin receptors on cell surfaces where pericellular proteolysis is concentrated (111, 189). Several MMPs have been reported to co-localize with integrins at attachment and detachment sites on migrating cells, specifically MMP-2 and MMP-14 co-localize with αvβ3 integrins on migrating epithelial cells (243, 244). Co-localization of MMP-2 with αvβ3 integrin, in combination with the observation that MMP-2 triggers cell migration by cleaving laminin 5, a component of basement membranes, suggests a mechanism by which MMPs promote cell migration and invasion (91). Moreover, it has been reported that type I collagen binding to integrin α2β1 results in increased expression of MMP-1, suggesting that interaction of integrins with ECM ligands regulates MMP expression (245). However, all MMP-integrin interactions are not merely mechanisms favouring membrane co-localization. This fact is highlighted by the observation that MMP-7 cleaves (or sheds) the extracellular domain of β4 integrins on prostatic carcinoma cells resulting in downregulation of β4 integrin-ECM adhesion – a scenario that favours a more migratory phenotype (246). Taken together, these observations articulate the diversity of interactions MMPs are involved in that can either favour a migratory phenotype or differentially regulate cellular response by inducing gene expression of proteins that themselves regulate stationary versus migratory cell growth.

Tissue transglutaminase (tTG) is a ubiquitous cell surface receptor that promotes attachment of fibronectin via its association with β1 and β2 integrins and thereby impacts cell migration (247). Membrane-bound MT-MMPs have been shown to cleave and inactivate tTG resulting in decreased adhesion and migration of cells on fibronectin *in vitro* suggesting that tumour cells can adjust their adhesion and locomotion dependening upon matrix substrates (248).

The transmembrane cell adhesion molecule E-cadherin regulates homotypic interactions between epithelial cells via pericellular ectodomain engagement on opposing cells and intracellular engagement with catenins and components of cytoskeleton (249). It is thought that homotypic E-cadherin-mediated interactions are significant for epithelial cell migration based on the observation that E-cadherin expression is downregulated or lost in many carcinomas (249-253), suggesting that E-cadherin acts, in part, as a tumour suppressor (254). Based on these observations, Christofori and colleagues tested this hypothesis using a mouse model of pancreatic islet cell carcinogenesis, e.g., Rip1-Tag2 mice (254-256). To test whether loss of E-cadherin-mediated cell adhesion is a cause or a consequence of tumour cell migration, either full length E-cadherin or a dominant-negative E-

cadherin mutant was overexpressed in Rip1-Tag2 pancreatic β cells. Expression of E-cadherin arrested tumour development at an early stage, while expression of the dominant negative E-cadherin mutant induced early invasion and metastasis (254-256). These results suggest that loss of E-cadherin mediated cell-cell adhesion is a rate-limiting step during carcinogenic progression. Ectodomain shedding of E-cadherin has been demonstrated downstream of MMP-3 and -7 *in vitro*, cleavage of which parallels onset of migration in some cell types (236, 257). In human carcinomas, elevated MMP-3 expression correlates with late-stage tumour development and overall prognosis (141, 258), suggesting a possible cell-cell regulatory mechanism important for invasive growth capacity. The significance of MMP-3 in regulating cell-cell and cell-ECM interactions is underscored by the observation that transgenic mice expressing an autoactivated form of MMP-3 in mammary epithelial cells develop reactive stroma and mammary tumours independent of carcinogenic initiation (259-261), suggesting that active MMP-3 exhibits strong tumor promoting effects. The overexpression of MMP-7 in the mouse mammary gland promotes mammary hyperplasia and accelerates the onset of mammary tumours (262), which is thought to be mediated by the selection for apoptosis resistant cells during this chronic exposure to MMP-7 (263) as well as by the shedding of FasL by MMP-7 (123). In contrast, deletion of MMP-7 in the *Min* mouse model of colorectal cancer resulted in suppression of intestinal tumourigenesis (18). MMP-7 also mediates E-cadherin shedding in injured lung epithelium (264) suggesting that MMP-7 regulates cell migration and invasion via differential regulation of E-cadherin.

The hyaluronan receptor CD44 is a broadly distributed transmembrane glycoprotein expressed by many cell types and is involved in a variety of physiological cell functions such as adhesion, migration, invasion and survival (237, 265-267). CD44 mediates cell-cell and cell-matrix interactions mainly via its affinity for hyaluronan, a glycosaminoglycan constituent of the ECM, but also to a lesser extend via its affinity for other ligands such as osteopontin (268). Histochemical evaluations of human carcinomas suggest that expression levels of CD44 positively correlate with poor prognosis implying a role for CD44 in tumour progression (269). Stamenkovic and colleagues have shown that CD44 serves as a docking molecule for MMP-9, retaining MMP-9 proteolytic activity at the cell surface (98). In addition, CD44 was reported to complex MMP-7 as well as MMP-14 at the cell surface of neoplastic cells and localize them to lamellipodia where they might be involved in migratory processes (97, 270). Taken together, these data suggest that CD44 mediated tumor cell migration and invasion is mediated by the targeted retention of MMPs at the tumor cell surface, thus directing ECM degradation to facilitate tumour cell migration through ECM.

Taken together, there is an overwhelming body of experimental evidence supporting the concept that MMPs play a critical role in the invasion and metastatic potential of neoplastic cells. However, transgenic mouse models of *de novo* tumour formation harbouring homozygous null mutations in individual MMP genes, while generally demonstrating a decreased incidence of malignant tumours, have not revealed a significant role for any one MMP in regulating cellular invasion *in vivo* (12, 20, 262). Why this disparity? One possible explanation is that although the two- and three-dimensional *in vitro* culture conditions mimic microenvironmental conditions *in vivo*, they are not an exact recapitulation and do not include the alterations seen *in vivo*; thus, *in vitro* experiments can only provide clues about MMP-mediated events such as invasion and metastasis of tumour cells.

4.4 MMPs and Tumour-associated Angiogenesis

When any tissue expands or a primary tumour develops, in order to grow beyond ~2-4 mm^3, influx of oxygen and nutrition and efflux of waste products must be ensured (272). In order to meet these metabolic needs of a rapidly growing tumour mass, development of a new blood vasculature is required and accomplished by activation of pre-existing vascular beds, e.g., angiogenesis (273-277). During angiogenesis, a well-orchestrated series of events encompassing initiation of endothelial cell proliferation and directional migration of endothelial

cells through remodelled basement membrane and perivascular stroma towards angiogenic stimuli (developing neoplasms) occurs (8, 278, 279). Once endothelial cells are enticed into a proliferative and migratory state, recruitment of perivascular support cells enables stabilization of nascent vessels, functional lumen formation and appropriate blood flow; however, while all these regulatory programs (cellular and molecular) are common to physiologic angiogenesis, tumour-associated angiogenesis possess a distinctly tortuous and chaotic organization that is inherently leaky (reviewed in (37, 280-283). Activation of pro-angiogenic molecular and cellular programs in a neoplastic context are regulated at many levels and controlled by a diverse assortment of positive and negative-acting soluble and insoluble mediators whose balanced equilibrium is kept tightly in check under homeostatic conditions; however, under conditions of tissue stress, such as occurs during premalignancy, their balance is rapidly upset favouring the pro-angiogenic phenotype (4, 8, 278, 284).

MMPs have been functionally implicated as mediators of tumour angiogenesis at several discrete steps, based upon bioactivity of their effector substrates that regulate angiogenesis by both positive and negative mechanisms. For example, using a modified chick chorioallantoic angiogenesis assay (CAM) that quantifies new blood vessel development into fibrillar collagen implants, it was revealed that helical domain cleavage of fibrillar type I collagen is required for growth factor stimulated angiogenesis (285). New vessel growth was significantly reduced by TIMP-1, a synthetic MPI BB3103 or when collagen implants were composed of collagenase-resistant type I collagen (286) suggesting that MMP mediated cleavage of type I collagen is a rate limiting step in growth factor-stimulated angiogenesis *in vivo*. In addition to cleavage products of type I collagen, a cleavage product of type IV collagen has been shown to promote angiogenesis *in vivo* (287). Proteolytic cleavage of type IV collagen by MMP-2 results in exposure of a cryptic epitope, designated HUIV26, within the triple helical domain that is required for angiogenesis and tumour growth (287). Inhibition of interactions between endothelial cells and the HUIV26 site by a monoclonal antibody directed to this site (Mab HUVI 26) decreased basic fibroblast growth factor (bFGF) and/or VEGF-induced angiogenesis by 70% compared to controls in both a rat corneal micropocket assay (288) and chick CAM angiogenic assay (287). Furthermore, Mab HUVI26 inhibited tumour growth in nude mice injected with M21 human melanoma cells and chick embryos injected with HT1080 human fibrosarcoma cells by 80% - 90% when compared to controls (287). Interestingly, the exposure of the HUVI26 epitope was associated with a loss of endothelial cell $\alpha 1\beta 1$ integrin binding and a gain in $\alpha v\beta 3$ binding suggesting that this shift in endothelial cell-integrin binding initiates a signaling cascade required for angiogenesis *in vivo* (287).

In contrast to the angiogenic promoting activity of ECM cleavage products, the C-terminal globular non-collagenous (NC1) domains of the basement membrane collagens types IV, XV and XVIII have been shown to be potent inhibitors of angiogenesis. One of the first angiogenic inhibitors discovered was endostatin, a 20-kDa NC1 fragment from type XVIII collagen (112). Endostatin can be produced by cleavage of collagen type XVIII by MMP-3, -7, -9, -12, -13 and –20 (289) and acts by reducing endothelial cell proliferation (112, 290). In addition, restin, a 22-kDa NC1 fragment from type XV collagen inhibits migration, but not proliferation, of endothelial cells *in vitro* and suppresses tumour induced angiogenesis in a renal xenograft carcinoma model (116). All three chains of type IV collagen ($\alpha 1$, $\alpha 2$ and $\alpha 3$) are potent inhibitors of angiogenesis and tumour growth (110, 113, 114, 291). For instance, the 24-kDa NC1 fragment of the $\alpha 1$ chain of type IV collagen, termed arrestin, inhibits the growth of human xenograft tumours in nude mice by significantly inhibiting growth factor mediated angiogenesis (110). Furthermore, its anti-angiogenic activity is mediated by binding to endothelial $\alpha 1\beta 1$ integrins (110). Likewise, canstatin, the 24-kDa NC1 fragment of the $\alpha 2$ chain of type IV collagen, suppressed growth of human xenograft tumours in nude mice by inhibiting angiogenesis (113). *In vitro* studies indicate that canstatin specifically inhibits proliferation, migration and tube formation of endothelial cells (113). Lastly, the 24-kDa NC1 fragment of the $\alpha 3$

chain of type IV collagen, termed tumstatin, acts as an angiogenesis inhibitor, inhibiting both endothelial cell proliferation and blood vessel formation (114, 115, 291, 292) Studies using transgenic mouse models indicate that tumstatin is generated by MMP-9 and suppresses angiogenesis via αvβ3 integrin interactions (293). Other MMP substrates identified as possessing anti-angiogenic activities include angiostatin, a cleavage product of plasminogen, that is a potent inhibitor of endothelial cell proliferation (118, 294). Pozzi et al. demonstrated that treatment of mice with doxycycline, which preferentially inhibits MMP-9 activity (295) results in reduced MMP-9 plasma levels and consequently in reduced angiostatin generation, that in turn results in decreased angiogenesis (296). Taken together these studies indicate that MMP-generated cleavage products of ECM, basement membrane proteins and other soluble molecules act as suppressors or activators of pathological angiogenesis in tissue-dependent and stage-dependent manners and implicate MMPs as important mediators of tumour-associated angiogenesis by pro-tumour and anti-tumour mechanisms.

4.5 MMPs and metastasis

Metastases arise upon the spread of malignant cells from primary tumour sites to distant organs and are commonly found in the first capillary bed encountered by metastasizing malignant cells (10, 297, 298). Tumour cells spread via three routes, e.g., hematogenous spread, dissemination via lymphatic vessels and direct migration along facial planes (10, 299-305). To spread via a hematogenous route, malignant cells must leave the primary tumour, intravasate into blood vasculature, survive and extravasate at a distal site where once present, reinitiate proliferation, induce local angiogenesis, resist local cell death programs and grow to form a secondary tumour – a multi-step process where tissue remodelling is a prerequisite and thus implicating MMPs.

MMPs were first implicated in hematogenous spread of tumour metastasis based on clinical observations correlating increased MMP expression in primary tumours with metastasis at distant sites (17, 127). For example, MMP-1 expression in primary cervical carcinomas is associated with lymph node (306) and peritoneal gastric metastases (307), while increased expression of MMP-7 in gastric carcinomas correlates with liver and lymph node metastases (308). It has also been observed that expression levels of MMP-2 and -9 are especially high in metastatic lung carcinomas and melanomas (309). In the case of MMP-2, high serum levels were reported to correlate with the presence of metastases in lung cancer patients (310). To address the significance of these clinical correlates, several groups variably altered MMP expression/activity in experimental immune-deficient models of metastasis (311-317). While results from these studies were compelling, and in part fuelled by use of MPIs in human clinical trials (128, 318-321), to date experimental evidence definitively demonstrating that MMPs regulate *de novo* metastasis formation *in vivo* is minimal. One study has however provided a functional role for MMP-9 as a regulator of metastatic growth (179). In this study, 3LL-LLC cells spontaneously metastasize to lung in a VEGF receptor 1 (VEGFR1)-dependent manner. Increased MMP-9 expression in lungs of tumour-bearing animals was demonstrated to be essential for distal tumour formation, suggesting that MMP-9 was not utilized for travel to the secondary site, but instead was essential for establishing vascular support and/or tissue remodelling in the metastatic microenvironment (179, 180). Taken together, these studies suggest that MMPs are involved in metastasis formation; however, it is not clear, which MMPs promote or prevent metastasis development and what the underlying mechanisms they regulate are.

Chemokines have also been identified as important protein substrates of MMPs *in vivo* and as a consequence variably regulate infiltration and migration of leukocytes into or out of tissue compartments (13, 322) and by similar mechanisms, variably regulate neoplastic cell movements. For example, MMP-1, -3, -9, –13 and –14 target and inactivate CXCL12, the ligand for CXCR4 on leukocytes (121). The observation that expression of CXCR4 on breast carcinoma cells and its binding to CXCL12 is implicated in metastasis development (323), in combination with CXCL12 being reported to be a MMP target, suggest that MMPs might be

involved in regulating CXCR4/CXCL12 mediated metastasis development. A study by van den Steen *et al.* suggested that MMP-9-targetted CXCL8 increased chemokine activity tenfold (324). Since signalling via the two CXCL8 receptors CXCR1 and CXCR2 is required for the invasive potential of melanoma cells *in vitro* (325, 326), MMP-9 might be involved in metastasis of melanoma by regulating the binding activity of CXCL8 to its receptors. These studies suggest that MMPs directly impact chemokines by cleavage resulting in either inactivation or activation of the respective chemokine. These modifications change the binding capacities of chemokines to their receptors and thus impact metastasis of tumour cells.

5. CLINICAL IMPLICATIONS

The studies discussed above indicate that complex interactions between neoplastic cells and their surrounding microenvironment regulate MMP expression, localization, activation and biological effect. Furthermore, these studies indicate that MMPs play diverse roles in tissue remodelling essential for tumour growth and maintenance. Based on compelling data supporting a pro-tumour role for MMPs in cancer development, in combination with data suggesting anti-cancer roles for TIMPs (192, 211, 327-339), synthetic MPIs were developed (340) and evaluated in both *in vitro* and *in vivo* cancer models (318-321, 341-345). To date, over 150 US patents have been issued for MPIs (16, 346) that can be categorized into five groups, e.g., collagen peptidomimetics, collagen non-peptidomimetics, tetracycline derivatives, small peptides and unconventional MPIs (16, 17, 344). Peptidomimetic MPIs were designed to mimic cleavage sites of MMP substrates where the zinc binding group is positioned at the cleavage site, resulting in blockage of the active site zinc upon binding to the target MMP and are exemplified by Batimastat and Marimastat (16, 17, 344). Collagen non-peptidomimetics, also known as deep pocket MPIs, were designed based on the crystal structure of MMP catalytic sites (16, 17, 344) and includes Prinomastat/AG3340 and tanomastat/BAY 12-9566 (344) among others. Tetracycline derivatives, such as Metastat, act by inhibiting both the synthesis and activity of MMPs (342). Finally, the small peptide class was generated by screening phage display peptide libraries where peptides demonstrating high specificity for individual MMPs were amplified (347). For example, a class of cyclin peptides containing a HWGF motif specifically inhibits MMP-2 and -9 activity and inhibits tumour growth in mouse models (347). Finally, unconventional MPIs include an extract from shark cartilage (Neovastat/AE-941) and a component of green tea (348, 349).

Initial efficacy of a broad spectrum MPI (SC-44463) was first reported in an experimental mouse model of metastasis formation (350). Many studies followed testing individual MPIs in more complex and clinically relevant models (16, 321, 351-355). For example, treatment of immune-deficient mice with batimastat, a broad-spectrum hydroxamate inhibitor, following resection of human breast cancer xenografts reduced metastasis and inhibited local re-growth of tumours (356). In addition, in the *Min* mouse model of intestinal tumorigenesis, batimastat reduced tumour multiplicity by 48% when administered between 6 and 14 weeks of age (354) and A-177430, a broadspectrum MPI, reduced tumour multiplicity by 69% when administered between 5 and 12 weeks of age (357). Furthermore, MMI-166, a selective MPI for MMP-2, -3 and -9, significantly decreased the number of metastases of TK-4 human colon cancer cells injected in nude mice (358). Similar results were observed when CT1746, a selective inhibitor for MMP-2, -3, -7 and -9 was administered to nude mice injected with the human colon cancer cell line CO-3 (359). Taken together, MPI studies in tumour xenograft mouse models strongly supported MPIs as promising anticancer therapeutics. More compelling and biologically relevant studies with MPIs involved efficacy testing in mouse models of *de novo* tumour formation (354, 355). MPI treatment in these models indicated that efficacy was best achieved if the MPI was administered during premalignant progression and prior to overt tumour development (354, 355) suggesting that tumor stage is a critical determinant of MPI efficacy.

In spite of encouraging results with MPI in numerous mouse models of cancer development,

human clinical trials with MPIs were discouraging (128, 318-321, 346, 360, 361). While some MPIs elicited adverse patient effects in early trials, others entered Phase III clinical trials either alone or in combination with conventional chemotherapy (gemcitabine) as compared to chemotherapy alone where no significant survival advantage was found (128, 321, 360, 362). In advanced gastric cancer, advanced glioblastoma, small lung cell carcinoma (SCLC), non-small cell carcinoma (NSCLC) and ovarian cancer Phase III trials, no significant increase in survival was observed in Marimastat treated cohorts when compared to patients receiving placebo (128, 362). However, a significant improvement in survival was observed in patients that either received chemotherapy prior to entering trial or did not have metastases at time of diagnosis when compared to placebo treated patients (128, 362) implying that Marimastat, if administered at earlier stages of cancer development represented an efficacious therapy (128, 321). In trials evaluating Prinomastat in advanced SCLC, no significant survival benefits were observed in patients treated with conventional chemotherapy (either cisplatin + gemcitabine or cisplatin + paclitaxel) plus Prinomastat and similar results were observed in patients with metastatic hormone refractory prostate cancer treated with chemotherapy (mitoxantrone + prednisone) plus Prinomastat (128). The studies involving Tanomastat were even more disappointing and were terminated prematurely when patients demonstrated significantly poorer survival rates than patients receiving placebo (363).

Given our current knowledge of MMP biology and retrospective analysis of their mechanisms of action in developing tumours, the failure of MPIs in human clinical trials was not surprising. While human clinical trials were conducted according to currently accepted criteria, they failed to consider many facets of MMP biology and largely did not consider MMP expression differences between tumour types. Trials were conducted in patients harbouring large tumour burdens where efficacy would only have been possible if tumour regression or enhanced survival was achieved - unlikely endpoints for non-cytotoxic agents and improbable given results obtained with *de novo* models of tumour development where best efficacy was achieved when MPIs were administered during early tumour development. Failure of MPIs in clinical trials was in part attributed to limited understanding and appreciation for the diversity of cellular and mechanisms regulated by MMPs *in vivo* as exemplified by the fact that spatial and temporal expression and activity differences between MMPs during neoplastic progression of diverse cancer types was not taken into consideration. Use of broad spectrum MPIs that, amongst other MMPs, inhibit MMP-8 activity, results in a significant increase rather than a decrease in tumour incidence (124). Given the observation that MMP-8 homozygous null mice exhibit an increased tumour incidence following carcinogen exposure (124) suggest that a sophisticated understanding of MMP biology is crucial for effective targeting of MMPs during carcinogenesis.

6. CONCLUDING REMARKS

MMPs have been found to promote or inhibit neoplastic progression by a multitude of mechanisms that not only include remodelling of ECM components, but also by regulating bioavailability and/or activity of cell adhesion molecules, growth factors, other proteases, chemokines, cytokines and proteins involved in the clotting cascade. A more thorough understanding of the underlying mechanisms of MMP mediated molecular and cellular pathways important during carcinogenesis, as well as elucidating what MMPs are active at which tumour stage and type, will be crucial to insure that future MPI anti-cancer therapies will be effective.

ACKNOWLEGEMENTS

We thank Evelyn Galenski for administrative assistance. AE is supported by a fellowship from the Serono Foundation for the Advancement of Medical Sciences. KEdV is supported by a fellowship from the Dutch Cancer Society. LMC is supported by the National Institutes of Health, the National Cancer Institute and the Department of Defense.

REFERENCES

1. Knudson, A.G.J., 1977, Genetic predisposition to cancer. In Origins of Human Cancer, J.D.W. H.H. Hiatt, J.A. Wiunsted eds, Vol. 4:45-52. Cold Spring Harbor Lab, Cold Spring Harbor, NY.
2. Fearon, E. R., and Vogelstein, B., 1990, A genetic model for colorectal tumorigenesis. Cell, 61:759-767.
3. Knudson, A. G., 2001, Two genetic hits (more or less) to cancer. Nat Rev Cancer 1:157-162.
4. Hanahan, D., and Weinberg, R. A., 2000, The hallmarks of cancer. Cell, 100:57-70.
5. Bissell, M. J., and Radisky, D., 2001, Putting tumours in context. Nat Rev Cancer, 1:46-54.
6. Coussens, L. M., and Werb, Z., 2002, Inflammation and cancer. Nature, 420:860-867.
7. Hahn, W. C., and Weinberg, R. A., 2002, Modelling the molecular circuitry of cancer, Nat Rev Cancer, 2:331-341.
8. Bergers, G., and Benjamin, L. E., 2003, Angiogenesis: Tumorigenesis and the angiogenic switch. Nat Rev Cancer, 3:401-410.
9. Hussain, S. P., Hofseth, L. J., and Harris, C. C., 2003, Radical causes of cancer. Nat Rev Cancer, 3:275-286.
10. Fidler, I. J., 2003, Timeline: The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat Rev Cancer, 3:453-458.
11. Nagase, H., and Woessner, J. F., 1999, Matrix metalloproteinases. J Biol Chem, 274:21491-21494.
12. Bergers, G., Brekken, R., McMahon, G., Vu, T. H., Itoh, T., Tamaki, K., Tanzawa, K., Thorpe, P., Itohara, S., Werb, Z., and Hanahan, D., 2000, Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol, 2:737-744.
13. McQuibban, G. A., Gong, J. H., Tam, E. M., McCulloch, C. A., Clark-Lewis, I., and Overall, C. M., 2000, Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. Science, 289:1202-1206.
14. Powell, W. C., Fingleton, B., Wilson, C. L., Boothby, M., and Matrisian, L. M., 1999, The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis. Curr Biol, 9:1441-7.
15. Manes, S., Llorente, M., Lacalle, R. A., Gomez-Mouton, C., Kremer, L., Mira, E., and Martinez, A. C., 1999, The matrix metalloproteinase-9 regulates the insulin-like growth factor-triggered autocrine response in DU-145 carcinoma cells. J Biol Chem, 274:6935-6945.
16. Sternlicht, M. D., and Bergers, G., 2000, Matrix metalloproteinases as emerging targets in anticancer therapy: status and prospects. Emerg Theurpeut Targets, 4:609-633.
17. Egeblad, M., and Werb, Z., 2002, New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer, 2:161-174.
18. Wilson, C. L., Heppner, K. J., Labosky, P. A., Hogan, B. L., and Matrisian, L. M., 1997, Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. Proc Natl Acad Sci USA, 94:1402-7.
19. Sternlicht, M. D., Lochter, A., Sympson, C. J., Huey, B., Rougier, J. P., Gray, J. W., Pinkel, D., Bissell, M. J., and Werb, Z., 1999, The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. Cell, 98:137-146.
20. Coussens, L. M., Tinkle, C. L., Hanahan, D., and Werb, Z., 2000, MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. Cell, 103:481-490.
21. Coussens, L. M., Shapiro, S. D., Soloway, P. D., and Werb, Z., 2001, Models for gain-of-function and loss-of-function of MMPs. Transgenic and gene targeted mice. Methods Mol Biol, 151:149-179.
22. Rawlings, N. D., and Barrett, A. J., 1995, Evolutionary families of metallopeptidases. Methods Enzymol, 248:183-228.
23. Stocker, W., Grams, F., Baumann, U., Reinemer, P., Gomis-Ruth, F. X., McKay, D. B., and Bode, W., 1995, The metzincins--topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptidases. Protein Sci, 4:823-40.
24. Sternlicht, M.D., and Werb, Z., 1999, ECM Proteinases. In Guidebook to the Extracellular Matrix, Kries, T., and Vale, R., eds, 503-562. Oxford University Press, Oxford, UK.
25. Overall, C. M., 2002, Molecular determinants of metalloproteinase substrate specificity: matrix metalloproteinase substrate binding domains, modules, and exosites. Mol Biotechnol, 22:51-86.
26. Puente, X. S., Sanchez, L. M., Overall, C. M., and Lopez-Otin, C., 2003, Human and mouse proteases: a comparative genomic approach. Nat Rev Genet, 4:544-558.
27. Lopez-Otin, C., and Overall, C. M., 2002, Protease degradomics: a new challenge for proteomics. Nat Rev Mol Cell Biol, 3:509-519.
28. Woessner, J. F., and Nagase, H., 2000, Matrix metalloproteinases and TIMPs. Oxford University Press, Oxford, UK.
29. Hirose, T., Patterson, C., Pourmotabbed, T., Mainardi, C. L., and Hasty, K. A., 1993, Structure-function relationship of human neutrophil collagenase: identification of regions responsible for substrate specificity and general proteinase activity. Proc Natl Acad Sci USA, 90:2569-2573.
30. Li, J., Brick, P., O'Hare, M. C., Skarzynski, T., Lloyd, L. F., Curry, V. A., Clark, I. M., Bigg, H. F.,

Hazleman, B. L., Cawston, T. E., and et al., 1995, Structure of full-length porcine synovial collagenase reveals a C- terminal domain containing a calcium-linked, four-bladed beta-propeller. Structure, 3:541-9.

31. Murphy, G., Nguyen, Q., Cockett, M. I., Atkinson, S. J., Allan, J. A., Knight, C. G., Willenbrock, F., and Docherty, A. J., 1994, Assessment of the role of the fibronectin-like domain of gelatinase A by analysis of a deletion mutant. J Biol Chem, 269:6632-6.
32. Banyai, L., Tordai, H., and Patthy, L., 1994, The gelatin-binding site of human 72 kDa type IV collagenase (gelatinase A). Biochem J, 298(Pt 2):403-407.
33. Itoh, Y., Kajita, M., Kinoh, H., Mori, H., Okada, A., and Seiki, M., 1999, Membrane type 4 matrix metalloproteinase (MT4-MMP, MMP-17) is a glycosylphosphatidylinositol-anchored proteinase. J Biol Chem, 274:34260-34266.
34. Pei, D., Kang, T., and Qi, H., 2000, Cysteine array matrix metalloproteinase (CA-MMP)/MMP-23 is a type II transmembrane matrix metalloproteinase regulated by a single cleavage for both secretion and activation. J Biol Chem, 275:33988-97.
35. Seidah, N. G., and Chretien, M., 1997, Eukaryotic protein processing: endoproteolysis of precursor proteins. Curr Opin Biotechnol, 8:602-607.
36. Nagase, H., 1997, Activation mechanisms of matrix metalloproteinases. Biol Chem, 378:151-160.
37. Lafleur, M. A., Handsley, M. M., and Edwards, D. R., 2003, Metalloproteinases and their inhibitors in angiogenesis. Expert Rev Mol Med, 5:1-39.
38. Esteve, P. O., Chicoine, E., Robledo, O., Aoudjit, F., Descoteaux, A., Potworowski, E. F., and St Pierre, Y., 2002, Protein kinase C-zeta regulates transcription of the matrix metalloproteinase-9 gene induced by IL-1 and TNF-alpha in glioma cells via NF-kappa B. J Biol Chem, 277:35150-35155.
39. Troussard, A. A., Costello, P., Yoganathan, T. N., Kumagai, S., Roskelley, C. D., and Dedhar, S., 2000, The integrin linked kinase (ILK) induces an invasive phenotype via AP-1 transcription factor-dependent upregulation of matrix metalloproteinase 9 (MMP-9). Oncogene, 19:5444-5452.
40. Fini, M.E., Cook, J.R., Mohan, R., and Brinckerhoff, C.E., 1998, Regulation of matrix metalloproteinase gene expression. In Matrix Metalloproteinases, Parks, W.C., and Mecham, R.P., eds, 299-356. Academic Press, New York.
41. Hou, G., Vogel, W. F., and Bendeck, M. P., 2002, Tyrosine kinase activity of discoidin domain receptor 1 is necessary for smooth muscle cell migration and matrix metalloproteinase expression. Circ Res, 90:1147-1149.
42. Olaso, E., Labrador, J. P., Wang, L., Ikeda, K., Eng, F. J., Klein, R., Lovett, D. H., Lin, H. C., and Friedman, S. L., 2002, Discoidin domain receptor 2 regulates fibroblast proliferation and migration through the extracellular matrix in association with transcriptional activation of matrix metalloproteinase-2. J Biol Chem, 277:3606-3613.
43. Arnott, C. H., Scott, K. A., Moore, R. J., Hewer, A., Phillips, D. H., Parker, P., Balkwill, F. R., and Owens, D. M., 2002, Tumour necrosis factor-alpha mediates tumour promotion via a PKC alpha- and AP-1-dependent pathway. Oncogene, 21:4728-4738.
44. Ventura, J. J., Kennedy, N. J., Lamb, J. A., Flavell, R. A., and Davis, R. J., 2003, c-Jun NH(2)-terminal kinase is essential for the regulation of AP-1 by tumor necrosis factor. Mol Cell Biol, 23:2871-2882.
45. Gilbert, S. J., Duance, V. C., and Mason, D. J., 2004, Does protein kinase R mediate TNF-alpha- and ceramide-induced increases in expression and activation of matrix metalloproteinases in articular cartilage by a novel mechanism?. Arthritis Res Ther, 6:R46-R55.
46. Chen, N., Nomura, M., She, Q. B., Ma, W. Y., Bode, A. M., Wang, L., Flavell, R. A., and Dong, Z., 2001, Suppression of skin tumorigenesis in c-Jun NH(2)-terminal kinase-2-deficient mice. Cancer Res, 61:3908-3912.
47. Vogel, W., Gish, G. D., Alves, F., and Pawson, T., 1997, The discoidin domain receptor tyrosine kinases are activated by collagen. Mol Cell, 1:13-23.
48. Vogel, W., 1999, Discoidin domain receptors: structural relations and functional implications. Faseb J, 13 Suppl:S77-82.
49. Johnson, J. D., Edman, J. C., and Rutter, W. J., 1993, A receptor tyrosine kinase found in breast carcinoma cells has an extracellular discoidin I-like domain. Proc Natl Acad Sci USA, 90:10891.
50. Matsuyama, W., Wang, L., Farrar, W. L., Faure, M., and Yoshimura, T., 2004, Activation of discoidin domain receptor 1 isoform b with collagen up-regulates chemokine production in human macrophages: role of p38 mitogen-activated protein kinase and NF-kappaB. J Immunol, 172:2332-2340.
51. Kamohara, H., Yamashiro, S., Galligan, C., and Yoshimura, T., 2001, Discoidin domain receptor 1 isoform-a (DDR1alpha) promotes migration of leukocytes in three-dimensional collagen lattices. Faseb J, 15:2724-2726.
52. Alves, F., Vogel, W., Mossie, K., Millauer, B., Hofler, H., and Ullrich, A., 1995, Distinct structural characteristics of discoidin I subfamily receptor tyrosine kinases and complementary expression in human cancer. Oncogene, 10:609-618.
53. Barker, K. T., Martindale, J. E., Mitchell, P. J., Kamalati, T., Page, M. J., Phippard, D. J., Dale, T. C., Gusterson, B. A., and Crompton, M. R., 1995, Expression patterns of the novel receptor-like tyrosine kinase, DDR, in human breast tumours. Oncogene, 10:569-575.

54. Ye, S., 2000, Polymorphism in matrix metalloproteinase gene promoters: implication in regulation of gene expression and susceptibility of various diseases. Matrix Biol, 19:623-629.
55. Kanamori, Y., Matsushima, M., Minaguchi, T., Kobayashi, K., Sagae, S., Kudo, R., Terakawa, N., and Nakamura, Y., 1999, Correlation between expression of the matrix metalloproteinase-1 gene in ovarian cancers and an insertion/deletion polymorphism in its promoter region. Cancer Res, 59:4225-7.
56. Ghilardi, G., Biondi, M. L., Mangoni, J., Leviti, S., DeMonti, M., Guagnellini, E., and Scorza, R., 2001, Matrix metalloproteinase-1 promoter polymorphism 1G/2G is correlated with colorectal cancer invasiveness. Clin Cancer Res, 7:2344-2346.
57. Van Wart, H. E., and Birkedal-Hansen, H., 1990, The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. Proc Natl Acad Sci USA, 87:5578-82.
58. Springman, E. B., Angleton, E. L., Birkedal-Hansen, H., and Van Wart, H. E., 1990, Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of a Cys73 active-site zinc complex in latency and a "cysteine switch" mechanism for activation. Proc Natl Acad Sci USA, 87:364-8.
59. Sternlicht, M. D., and Werb, Z., 2001, How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol, 17:463-516.
60. O'Connell, J. P., Willenbrock, F., Docherty, A. J., Eaton, D., and Murphy, G., 1994, Analysis of the role of the COOH-terminal domain in the activation, proteolytic activity, and tissue inhibitor of metalloproteinase interactions of gelatinase B. J Biol Chem, 269:14967-73.
61. Pei, D., 1999, CA-MMP: a matrix metalloproteinase with a novel cysteine array, but without the classic cysteine switch. FEBS Lett, 457:262-70.
62. Fridman, R., Toth, M., Pena, D., and Mobashery, S., 1995, Activation of progelatinase B (MMP-9) by gelatinase A (MMP-2). Cancer Res, 55:2548-2555.
63. Knauper, V., Will, H., Lopez-Otin, C., Smith, B., Atkinson, S. J., Stanton, H., Hembry, R. M., and Murphy, G., 1996, Cellular mechanisms for human procollagenase-3 (MMP-13) activation. Evidence that MT1-MMP (MMP-14) and gelatinase a (MMP-2) are able to generate active enzyme. J Biol Chem, 271:17124-31.
64. Itoh, Y., Takamura, A., Ito, N., Maru, Y., Sato, H., Suenaga, N., Aoki, T., and Seiki, M., 2001, Homophilic complex formation of MT1-MMP facilitates proMMP-2 activation on the cell surface and promotes tumor cell invasion. Embo J, 20:4782-4793.
65. Ramos-DeSimone, N., Hahn-Dantona, E., Sipley, J., Nagase, H., French, D. L., and Quigley, J. P., 1999, Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion. J Biol Chem, 274:13066-76.
66. Overall, C. M., and Sodek, J., 1990, Concanavalin A produces a matrix-degradative phenotype in human fibroblasts. Induction and endogenous activation of collagenase, 72-kDa gelatinase, and Pump-1 is accompanied by the suppression of the tissue inhibitor of matrix metalloproteinases. J Biol Chem, 265:21141-21151.
67. Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A., and Goldberg, G. I., 1995, Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. J Biol Chem, 270:5331-5338.
68. Fang, K. C., Raymond, W. W., Lazarus, S. C., and Caughey, G. H., 1996, Dog mastocytoma cells secrete a 92-kD gelatinase activated extracellularly by mast cell chymase. J Clin Invest 97:1589-1596.
69. Fang, K. C., Raymond, W. W., Blount, J. L., and Caughey, G. H., 1997, Dog mast cell alpha-chymase activates progelatinase B by cleaving the Phe88-Gln89 and Phe91-Glu92 bonds of the catalytic domain. J Biol Chem, 272:25628-25635.
70. Saarinen, J., Kalkkinen, N., Welgus, H. G., and Kovanen, P. T., 1994, Activation of human interstitial procollagenase through direct cleavage of the Leu83-Thr84 bond by mast cell chymase. J Biol Chem, 269:18134-18140.
71. Okada, Y., and Nakanishi, I., 1989, Activation of matrix metalloproteinase 3 (stromelysin) and matrix metalloproteinase 2 ('gelatinase') by human neutrophil elastase and cathepsin G. FEBS Lett, 249:353-356.
72. Rice, A., and Banda, M. J., 1995, Neutrophil elastase processing of gelatinase A is mediated by extracellular matrix. Biochemistry, 34:9249-56.
73. Ferry, G., Lonchampt, M., Pennel, L., de Nanteuil, G., Canet, E., and Tucker, G. C., 1997, Activation of MMP-9 by neutrophil elastase in an in vivo model of acute lung injury. FEBS Lett, 402:111-115.
74. Shamamian, P., Schwartz, J. D., Pocock, B. J., Monea, S., Whiting, D., Marcus, S. G., and Mignatti, P., 2001, Activation of progelatinase A (MMP-2) by neutrophil elastase, cathepsin G, and proteinase-3: a role for inflammatory cells in tumor invasion and angiogenesis. J Cell Physiol, 189:197-206.
75. Butler, G. S., Hutton, M., Wattam, B. A., Williamson, R. A., Knauper, V., Willenbrock, F., and Murphy, G., 1999, The specificity of TIMP-2 for matrix metalloproteinases can be modified by single amino acid mutations. J Biol Chem, 274:20391-20396.

76. Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M., 1994, A matrix metalloproteinase expressed on the surface of invasive tumour cells. Nature, 370:61-5.
77. Zhao, H., Bernardo, M. M., Osenkowski, P., Sohail, A., Pei, D., Nagase, H., Kashiwagi, M., Soloway, P. D., DeClerck, Y. A., and Fridman, R., 2004, Differential Inhibition of Membrane Type 3 (MT3)-Matrix Metalloproteinase (MMP) and MT1-MMP by Tissue Inhibitor of Metalloproteinase (TIMP)-2 and TIMP-3 Regulates Pro-MMP-2 Activation. J Biol Chem, 279:8592-8601.
78. Baker, A. H., Edwards, D. R., and Murphy, G., 2002, Metalloproteinase inhibitors: biological actions and therapeutic opportunities. J Cell Sci, 115:3719-3727.
79. Bein, K., and Simons, M., 2000, Thrombospondin type 1 repeats interact with matrix metalloproteinase 2. Regulation of metalloproteinase activity. J Biol Chem, 275:32167-32173.
80. Rodriguez-Manzaneque, J. C., Lane, T. F., Ortega, M. A., Hynes, R. O., Lawler, J., and Iruela-Arispe, M. L., 2001, Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor. Proc Natl Acad Sci USA, 98:12485-12490.
81. Rhee, J., and Coussens, L., 2002, RECKing MMP function: implications for cancer development. Trends in Cell Bio, 12:209-211.
82. Oh, J., Takahashi, R., Kondo, S., Mizoguchi, A., Adachi, E., Sasahara, R. M., Nishimura, S., Imamura, Y., Kitayama, H., Alexander, D. B., Ide, C., Horan, T. P., Arakawa, T., Yoshida, H., Nishikawa, S., Itoh, Y., Seiki, M., Itohara, S., Takahashi, C., and Noda, M., 2001, The Membrane-Anchored MMP Inhibitor RECK Is a Key Regulator of Extracellular Matrix Integrity and Angiogenesis. Cell, 107:789-800.
83. Brew, K., Dinakarpandian, D., and Nagase, H., 2000, Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochim Biophys Acta, 1477:267-283.
84. Amour, A., Slocombe, P. M., Webster, A., Butler, M., Knight, C. G., Smith, B. J., Stephens, P. E., Shelley, C., Hutton, M., Knauper, V., Docherty, A. J., and Murphy, G., 1998, TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. FEBS Lett, 435:39-44.
85. Liu, L., Rich, B. E., Inobe, J., Chen, W., and Weiner, H. L., 1997, A potential pathway of Th2 development during primary immune response. IL-10 pretreated dendritic cells can prime naive CD4+ T cells to secrete IL-4. Adv Exp Med Biol, 417:375-81.
86. Yang, Z., Strickland, D. K., and Bornstein, P., 2001, Extracellular matrix metalloproteinase 2 levels are regulated by the low density lipoprotein-related scavenger receptor and thrombospondin 2. J Biol Chem, 276:8403-8408.
87. Sottrup-Jensen, L., Sand, O., Kristensen, L., and Fey, G. H., 1989, The alpha-macroglobulin bait region. Sequence diversity and localization of cleavage sites for proteinases in five mammalian alpha- macroglobulins. J Biol Chem, 264:15781-9.
88. Takahashi, C., Sheng, Z., Horan, T. P., Kitayama, H., Maki, M., Hitomi, K., Kitaura, Y., Sasahara, R. M., Horimoto, A., and al., e., 1998, Regulation of matrix metalloproteinase-9 and inhibition of tumor invasion by the membrane-anchored glycoprotein RECK. Proc Nat Acad Sci USA, 95:13221-13226.
89. Welm, B., Mott, J. D., and Werb, Z., 2002, Vasculogenesis is a wreck without Reck: Regulating matrix metalloproteinase activity during embryogenesis is critical for development. Curr Biol, 12:209-211.
90. Stamenkovic, I., 2003, Extracellular matrix remodelling: the role of matrix metalloproteinases. J Pathol, 200:448-464.
91. Giannelli, G., Falk-Marzillier, J., Schiraldi, O., Stetler-Stevenson, W. G., and Quaranta, V., 1997, Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. Science, 277:225-228.
92. Bosman, F. T., and Stamenkovic, I., 2003, Functional structure and composition of the extracellular matrix. J Pathol, 200:423-428.
93. Dumin, J. A., Dickeson, S. K., Stricker, T. P., Bhattacharyya-Pakrasi, M., Roby, J. D., Santoro, S. A., and Parks, W. C., 2001, Pro-collagenase-1 (matrix metalloproteinase-1) binds the alpha(2)beta(1) integrin upon release from keratinocytes migrating on type I collagen. Journal of Biological Chemistry, 276:29368-29374.
94. Guo, H., Zucker, S., Gordon, M. K., Toole, B. P., and Biswas, C., 1997, Stimulation of matrix metalloproteinase production by recombinant extracellular matrix metalloproteinase inducer from transfected Chinese hamster ovary cells. J Biol Chem, 272:24-27.
95. Guo, H., Li, R., Zucker, S., and Toole, B. P., 2000, EMMPRIN (CD147), an inducer of matrix metalloproteinase synthesis, also binds interstitial collagenase to the tumor cell surface. Cancer Res, 60:888-891.
96. Brooks, P. C., Strömblad, S., Sanders, L. C., von Schalscha, T. L., Aimes, R. T., Stetler-Stevenson, W. G., Quigley, J. P., and Cheresh, D. A., 1996, Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. Cell, 85:683-693.
97. Yu, W. H., Woessner, J. F., Jr., McNeish, J. D., and Stamenkovic, I., 2002, CD44 anchors the assembly of matrilysin/MMP-7 with heparin-binding

epidermal growth factor precursor and ErbB4 and regulates female reproductive organ remodeling. Genes Dev, 16:307-323.

98. Yu, Q., and Stamenkovic, I., 1999, Localization of matrix metalloproteinase 9 to the cell surface provides a mechanism for CD44-mediated tumor invasion. Genes Dev, 13:35-48.
99. Bourguignon, L. Y., Gunja-Smith, Z., Iida, N., Zhu, H. B., Young, L. J., Muller, W. J., and Cardiff, R. D., 1998, CD44v(3,8-10) is involved in cytoskeleton-mediated tumor cell migration and matrix metalloproteinase (MMP-9) association in metastatic breast cancer cells. J Cell Physiol, 176:206-215.
100. Fiore, E., Fusco, C., Romero, P., and Stamenkovic, I., 2002, Matrix metalloproteinase 9 (MMP-9/gelatinase B) proteolytically cleaves ICAM-1 and participates in tumor cell resistance to natural killer cell- mediated cytotoxicity. Oncogene, 21:5213-5223.
101. Olson, M. W., Toth, M., Gervasi, D. C., Sado, Y., Ninomiya, Y., and Fridman, R., 1998, High affinity binding of latent matrix metalloproteinase-9 to the alpha2(IV) chain of collagen IV. J Biol Chem, 273:10672-81.
102. Yu, Q., and Stamenkovic, I., 2000, Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev, 14:163-176.
103. Ruiter, D., Bogenrieder, T., Elder, D., and Herlyn, M., 2002, Melanoma-stroma interactions: structural and functional aspects, Lancet Oncol, 3:35-43.
104. Martin, P., 1997, Wound healing--aiming for perfect skin regeneration. Science, 276:75-81.
105. Uitto, J., and Kouba, D., 2000, Cytokine modulation of extracellular matrix gene expression: relevance to fibrotic skin diseases. J Dermatol Sci, 24 Suppl 1:S60-S69.
106. van Kempen, L. C., Ruiter, D. J., van Muijen, G. N., and Coussens, L. M., 2003, The tumor microenvironment: a critical determinant of neoplastic evolution. Eur J Cell Biol, 82:539-548.
107. Gheree-Kermani, M., and Phan, M., 2001, Role of Cytokines and cytokine therapy in wound healing and fibrotic disease. Curr Pharm Des:1083-1103.
108. Bergers, G., and Coussens, L. M., 2000, Extrinsic regulators of epithelial tumor progression: metalloproteinases. Curr Opin Genet Dev, 10:120-127.
109. Engbring, J. A., and Kleinman, H. K., 2003, The basement membrane matrix in malignancy. J Pathol, 200:465-470.
110. Colorado, P. C., Torre, A., Kamphaus, G., Maeshima, Y., Hopfer, H., Takahashi, K., Volk, R., Zamborsky, E. D., Herman, S., Sarkar, P. K., Ericksen, M. B., Dhanabal, M., Simons, M., Post, M., Kufe, D. W., Weichselbaum, R. R., Sukhatme, V. P., and Kalluri, R., 2000, Anti-angiogenic cues from vascular basement membrane collagen. Cancer Res, 60:2520-2526.
111. McCawley, L. J., and Matrisian, L. M., 2001, Matrix metalloproteinases: they're not just for matrix anymore!. Curr Opin Cell Biol, 13:534-40.
112. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J., 1997, Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell, 88:277-285.
113. Kamphaus, G. D., Colorado, P. C., Panka, D. J., Hopfer, H., Ramchandran, R., Torre, A., Maeshima, Y., Mier, J. W., Sukhatme, V. P., and Kalluri, R., 2000, Canstatin, a novel matrix-derived inhibitor of angiogenesis and tumor growth. J Biol Chem, 275:1209-1215.
114. Maeshima, Y., Colorado, P. C., and Kalluri, R., 2000, Two RGD-independent αvβ3 integrin binding sites on tumstatin regulate distinct anti-tumor properties. J Biol Chem, 275:23745-50.
115. Petitclerc, E., Boutaud, A., Prestayko, A., Xu, J., Sado, Y., Ninomiya, Y., Sarras, M. P., Jr., Hudson, B. G., and Brooks, P. C., 2000, New functions for non-collagenous domains of human collagen type IV. Novel integrin ligands inhibiting angiogenesis and tumor growth in vivo. J Biol Chem, 275:8051-8061.
116. Ramchandran, R., Dhanabal, M., Volk, R., Waterman, M. J., Segal, M., Lu, H., Knebelmann, B., and Sukhatme, V. P., 1999, Antiangiogenic activity of restin, NC10 domain of human collagen XV: comparison to endostatin. Biochem Biophys Res Commun, 255:735-739.
117. Hiraoka, N., Allen, E., Apel, I. J., Gyetko, M. R., and Weiss, S. J., 1998, Matrix metalloproteinases regulate neovascularization by acting as pericellular fibrinolysins. Cell, 95:365-377.
118. Cornelius, L. A., Nehring, L. C., Harding, E., Bolanowski, M., Welgus, H. G., Kobayashi, D. K., Pierce, R. A., and Shapiro, S. D., 1998, Matrix metalloproteinases generate angiostatin: effects on neovascularization. J Immunol, 161:6845-6852.
119. Hiller, O., Lichte, A., Oberpichler, A., Kocourek, A., and Tschesche, H., 2000, Matrix metalloproteinases collagenase-2, macrophage elastase, collagenase-3, and membrane type 1-matrix metalloproteinase impair clotting by degradation of fibrinogen and factor XII, J Biol Chem 275:33008-33013.
120. Vaisanen, A., Kallioinen, M., Taskinen, P. J., and Turpeenniemi-Hujanen, T., 1998, Prognostic value of MMP-2 immunoreactive protein (72 kD type IV collagenase) in primary skin melanoma. J Pathol, 186:51-58.
121. McQuibban, G. A., Butler, G. S., Gong, J. H., Bendall, L., Power, C., Clark-Lewis, I., and Overall, C. M., 2001, Matrix metalloproteinase activity

inactivates the CXC chemokine stromal cell-derived factor-1. J Biol Chem, 276:43503-8.

122. McQuibban, G. A., Gong, J. H., Wong, J. P., Wallace, J. L., Clark-Lewis, I., and Overall, C. M., 2002, Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. Blood, 100:1160-1167.
123. Sheu, B. C., Hsu, S. M., Ho, H. N., Lien, H. C., Huang, S. C., and Lin, R. H., 2001, A novel role of metalloproteinase in cancer-mediated immunosuppression. Cancer Res, 61:237-242.
124. Balbin, M., Fueyo, A., Tester, A. M., Pendas, A. M., Pitiot, A. S., Astudillo, A., Overall, C. M., Shapiro, S. D., and Lopez-Otin, C., 2003, Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. Nat Genet, 35:252-257.
125. Coussens, L. M., and Werb, Z., 1996, Matrix metalloproteinases and the development of cancer. Chem Biol, 3:895-904.
126. Nelson, A. R., Fingleton, B., Rothenberg, M. L., and Matrisian, L. M., 2000, Matrix metalloproteinases: biologic activity and clinical implications, J Clin Oncol 18:1135-1149.
127. McCawley, L. J., and Matrisian, L. M., 2001, Tumor progression: defining the soil round the tumor seed. Curr Biol, 11:R25-R27.
128. Coussens, L. M., Fingleton, B., and Matrisian, L. M., 2002, Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science, 295:2387-2392.
129. Iwata, H., Kobayashi, S., Iwase, H., Masaoka, A., Fujimoto, N., and Okada, Y., 1996, Production of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human breast carcinomas, Jpn J Cancer Res 87:602-611.
130. Brummer, O., Athar, S., Riethdorf, L., Loning, T., and Herbst, H., 1999, Matrix-metalloproteinases 1, 2, and 3 and their tissue inhibitors 1 and 2 in benign and malignant breast lesions: an in situ hybridization study. Virchows Arch, 435:566-573.
131. Heppner, K. J., Matrisian, L. M., Jensen, R. A., and Rodgers, W. H., 1996, Expression of most matrix metalloproteinase family members in breast cancer represents a tumor-induced host response. Am J Pathol, 149:273-282.
132. Visscher, D. W., Hoyhtya, M., Ottosen, S. K., Liang, C. M., Sarkar, F. H., Crissman, J. D., and Fridman, R., 1994, Enhanced expression of tissue inhibitor of metalloproteinase-2 (TIMP-2) in the stroma of breast carcinomas correlates with tumor recurrence. Int J Cancer, 59:339-344.
133. Lebeau, A., Nerlich, A. G., Sauer, U., Lichtinghagen, R., and Lohrs, U., 1999, Tissue distribution of major matrix metalloproteinases and their transcripts in human breast carcinomas. Anticancer Res, 19:4257-64.
134. Jones, J. L., Glynn, P., and Walker, R. A., 1999, Expression of MMP-2 and MMP-9, their inhibitors, and the activator MT1- MMP in primary breast carcinomas. J Pathol, 189:161-168.
135. Remacle, A. G., Noel, A., Duggan, C., McDermott, E., O'Higgins, N., Foidart, J. M., and Duffy, M. J., 1998, Assay of matrix metalloproteinases types 1, 2, 3 and 9 in breast cancer. Br J Cancer, 77:926-31.
136. Scorilas, A., Karameris, A., Arnogiannaki, N., Ardavanis, A., Bassilopoulos, P., Trangas, T., and Talieri, M., 2001, Overexpression of matrix-metalloproteinase-9 in human breast cancer: a potential favourable indicator in node-negative patients. Br J Cancer, 84:1488-1496.
137. Ahmad, A., Hanby, A., Dublin, E., Poulsom, R., Smith, P., Barnes, D., Rubens, R., Anglard, P., and Hart, I., 1998, Stromelysin 3: an independent prognostic factor for relapse-free survival in node-positive breast cancer and demonstration of novel breast carcinoma cell expression. Am J Pathol, 152:721-728.
138. Nielsen, B. S., Rank, F., Lopez, J. M., Balbin, M., Vizoso, F., Lund, L. R., Dano, K., and Lopez-Otin, C., 2001, Collagenase-3 expression in breast myofibroblasts as a molecular marker of transition of ductal carcinoma in situ lesions to invasive ductal carcinomas. Cancer Res, 61:7091-7100.
139. Nielsen, B. S., Sehested, M., Kjeldsen, L., Borregaard, N., Rygaard, J., and Dano, K., 1997, Expression of matrix metalloprotease-9 in vascular pericytes in human breast cancer. Lab Invest, 77:345-55.
140. Nielsen, B. S., Timshel, S., Kjeldsen, L., Sehested, M., Pyke, C., Borregaard, N., and Dano, K., 1996, 92 kDa type IV collagenase (MMP-9) is expressed in neutrophils and macrophages but not in malignant epithelial cells in human colon cancer. Int J Cancer, 65:57-62.
141. Wright, J. H., McDonnell, S., Portella, G., Bowden, G. T., Balmain, A., and Matrisian, L. M., 1994, A switch from stromal to tumor cell expression of stromelysin-1 mRNA associated with the conversion of squamous to spindle carcinomas during mouse skin tumor progression. Mol Carcinog, 10:207-215.
142. Coussens, L. M., Raymond, W. W., Bergers, G., Laig-Webster, M., Behrendtsen, O., Werb, Z., Caughey, G. H., and Hanahan, D., 1999, Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. Genes Dev, 13:1382-1397.
143. Janeway, C. A., Travers, P., Walport, M., and Shlomchik, M., 2001, Immunobiology, 5th ed. Garland Publishing, New York and London.

144. Dranoff, G., 2002, Tumour immunology: Immune recognition and tumor protection. Curr Opin in Immunology, 14:161-164.
145. Dranoff, G., 2003, Coordinated tumor immunity. J Clin Invest, 111:1116-1118.
146. Oshikiri, T., Miyamoto, M., Shichinohe, T., Suzuoki, M., Hiraoka, K., Nakakubo, Y., Shinohara, T., Itoh, T., Kondo, S., and Katoh, H., 2003, Prognostic value of intratumoral CD8+ T lymphocyte in extrahepatic bile duct carcinoma as essential immune response. J Surg Oncol, 84:224-228.
147. Abe, M., Kondo, S., Hirano, S., Ambo, Y., Tanaka, E., Morikawa, T., Okushiba, S., and Katoh, H., 2003, Long-term survival after radical resection of advanced pancreatic cancer: a case report with special reference to CD8+ T-cell infiltration. Int J Gastrointest Cancer, 33:107-110.
148. Wakabayashi, O., Yamazaki, K., Oizumi, S., Hommura, F., Kinoshita, I., Ogura, S., Dosaka-Akita, H., and Nishimura, M., 2003, CD4(+) T cells in cancer stroma, not CD8(+) T cells in cancer cell nests, are associated with favorable prognosis in human non-small cell lung cancers. Cancer Sci, 94:1003-1009.
149. Nakakubo, Y., Miyamoto, M., Cho, Y., Hida, Y., Oshikiri, T., Suzuoki, M., Hiraoka, K., Itoh, T., Kondo, S., and Katoh, H., 2003, Clinical significance of immune cell infiltration within gallbladder cancer. Br J Cancer, 89:1736-1742.
150. Funada, Y., Noguchi, T., Kikuchi, R., Takeno, S., Uchida, Y., and Gabbert, H. E., 2003, Prognostic significance of CD8+ T cell and macrophage peritumoral infiltration in colorectal cancer. Oncol Rep, 10:309-313.
151. Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J., and Schreiber, R. D., 2002, Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol, 3:991-998.
152. Dudley, M. E., and Rosenberg, S. A., 2003, Adoptive-cell-transfer therapy for the treatment of patients with cancer. Nat Rev Cancer, 3:666-675.
153. Balkwill, F., and Mantovani, A., 2001, Inflammation and cancer: back to Virchow?. Lancet, 357:539-545.
154. Duncan, L. M., Richards, L. A., and Mihm, M. C., Jr., 1998, Increased mast cell density in invasive melanoma. J Cutan Pathol, 25:11-15.
155. Imada, A., Shijubo, N., Kojima, H., and Abe, S., 2000, Mast cells correlate with angiogenesis and poor outcome in stage I lung adenocarcinoma. Eur Respir J, 15:1087-1093.
156. Takanami, I., Takeuchi, K., and Naruke, M., 2000, Mast cell density is associated with angiogenesis and poor prognosis in pulmonary adenocarcinoma. Cancer, 88:2686-2692.
157. Tomita, M., Matsuzaki, Y., and Onitsuka, T., 2000, Effect of mast cells on tumor angiogenesis in lung cancer. Ann Thorac Surg, 69:1686-1690.
158. Toth-Jakatics, R., Jimi, S., Takebayashi, S., and Kawamoto, N., 2000, Cutaneous malignant melanoma: correlation between neovascularization and peritumor accumulation of mast cells overexpressing vascular endothelial growth factor. Hum Pathol, 31:955-960.
159. Shea, C. R., and Prieto, V. G., 1994, Mast cells in angiolipomas and hemangiomas of human skin: are they important for angiogenesis?. J Cutan Pathol, 21:247-251.
160. Benitez-Bribiesca, L., Wong, A., Utrera, D., and Castellanos, E., 2001, The role of mast cell tryptase in neoangiogenesis of premalignant and malignant lesions of the uterine cervix. J Histochem Cytochem, 49:1061-1062.
161. Ness, R. B., and Cottreau, C., 1999, Possible role of ovarian epithelial inflammation in ovarian cancer. J Natl Cancer Inst, 91:1459-67.
162. Weitzman, S. A., and Gordon, L. I., 1990, Inflammation and cancer: role of phagocyte-generated oxidants in carcinogenesis. Blood, 76:655-663.
163. Shacter, E., and Weitzman, S. A., 2002, Chronic inflammation and cancer. Oncology, 16:217-226.
164. Ernst, P. B., and Gold, B. D., 2000, The disease spectrum of Helicobacter pylori: the immunopathogenesis of gastroduodenal ulcer and gastric cancer. Annu Rev Microbiol, 54:615-640.
165. Engle, S. J., Ormsby, I., Pawlowski, S., Boivin, G. P., Croft, J., Balish, E., and Doetschman, T., 2002, Elimination of Colon Cancer in Germ-free Transforming Growth Factor Beta 1-deficient Mice. Cancer Res, 62:6362-6366.
166. Williams, C. S., Mann, M., and DuBois, R. N., 1999, The role of cyclooxygenases in inflammation, cancer, and development. Oncogene, 18:7908-7916.
167. Garcia-Rodriguez, L. A., and Huerta-Alvarez, C., 2001, Reduced risk of colorectal cancer among long-term users of aspirin and nonaspirin nonsteroidal antiinflammatory drugs. Epidemiology, 12:88-93.
168. Meier, C. R., Schmitz, S., and Jick, H., 2002, Association between acetaminophen or nonsteroidal antiinflammatory drugs and risk of developing ovarian, breast, or colon cancer. Pharmacotherapy, 22:303-309.
169. Sharpe, C. R., Collet, J. P., McNutt, M., Belzile, E., Boivin, J. F., and Hanley, J. A., 2000, Nested case-control study of the effects of non-steroidal anti-inflammatory drugs on breast cancer risk and stage. Br J Cancer, 83:112-120.
170. Cotterchio, M., Kreiger, N., Sloan, M., and Steingart, A., 2001, Nonsteroidal anti-inflammatory drug use and breast cancer risk. Cancer Epidemiol Biomarkers Prev, 10:1213-1217.

171. Akre, K., Ekstrom, A. M., Signorello, L. B., Hansson, L. E., and Nyren, O., 2001, Aspirin and risk for gastric cancer: a population-based case-control study in Sweden. Br J Cancer, 84:965-968.
172. Bashkin, P., Razin, E., Eldor, A., and Vlodavsky, I., 1990, Degranulating mast cells secrete an endoglycosidase that degrades heparan sulfate in subendothelial extracellular matrix. Blood, 75:2204-2212.
173. Inuzuka, K., Ogata, Y., Nagase, H., and Shirouzu, K., 2000, Significance of coexpression of urokinase-type plasminogen activator, and matrix metalloproteinase 3 (stromelysin) and 9 (gelatinase B) in colorectal carcinoma. J Surg Res, 93:211-218.
174. Stahle-Backdahl, M., Sudbeck, B. D., Eisen, A. Z., Welgus, H. G., and Parks, W. C., 1992, Expression of 92-kDa type IV collagenase mRNA by eosinophils associated with basal cell carcinoma. J Invest Dermatol, 99:497-503.
175. Zeng, Z. S., and Guillem, J. G., 1996, Colocalisation of matrix metalloproteinase-9-mRNA and protein in human colorectal cancer stromal cells. Br J Cancer, 74:1161-1167.
176. Coussens, L. M., Hanahan, D., and Arbeit, J., 1996, Genetic predisposition and parameters of malignant progression in K14-HPV16 transgenic mice. Am J Path, 149:1899-1917.
177. van Kempen, L. C. L., Rhee, J. S., Dehne, K., Lee, J., Edwards, D. R., and Coussens, L. M., 2002, Epithelial carcinogenesis: dynamic interplay between neoplastic cells and their microenvironment. Differentiation, 70:501-623.
178. Huang, S., Van Arsdall, M., Tedjarati, S., McCarty, M., Wu, W., Langley, R., and Fidler, I. J., 2002, Contributions of stromal metalloproteinase-9 to angiogenesis and growth of human ovarian carcinoma in mice. J Natl Cancer Inst, 94:1134-1142.
179. Hiratsuka, S., Nakamura, K., Iwai, S., Murakami, M., Itoh, T., Kijima, H., Shipley, J. M., Senior, R. M., and Shibuya, M., 2002, MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. Cancer Cell, 2:289-300.
180. van Kempen, L. C., and Coussens, L. M., 2002, MMP9 potentiates pulmonary metastasis formation. Cancer Cell, 2:251-252.
181. Biggs, J. R., and Kraft, A. S., 1995, Inhibitors of cyclin-dependent kinase and cancer. J Mol Med, 73:509-614.
182. Lin, P., Buxton, J. A., Acheson, A., Radziejewski, C., Maisonpierre, P. C., Yancopoulos, G. D., Channon, K. M., Hale, L. P., Dewhirst, M. W., George, S. E., and Peters, K. G., 1998, Antiangiogenic gene therapy targeting the endothelium-specific receptor tyrosine kinase Tie2. Proc Natl Acad Sci USA, 95:8829-8834.
183. Fong, T. A., Shawver, L. K., Sun, L., Tang, C., App, H., Powell, T. J., Kim, Y. H., Schreck, R., Wang, X., Risau, W., Ullrich, A., Hirth, K. P., and McMahon, G., 1999, SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. Cancer Res, 59:99-106.
184. Noonberg, S. B., and Benz, C. C., 2000, Tyrosine kinase inhibitors targeted to the epidermal growth factor receptor subfamily: role as anticancer agents. Drugs, 59:753-67.
185. Attoub, S., Rivat, C., Rodrigues, S., Van Bocxlaer, S., Bedin, M., Bruyneel, E., Louvet, C., Kornprobst, M., Andre, T., Mareel, M., Mester, J., and Gespach, C., 2002, The c-kit Tyrosine Kinase Inhibitor STI571 for Colorectal Cancer Therapy. Cancer Res, 62:4879-4883.
186. Somlyo, A. V., Phelps, C., Dipierro, C., Eto, M., Read, P., Barrett, M., Gibson, J. J., Burnitz, M. C., Myers, C., and Somlyo, A. P., 2003, Rho kinase and matrix metalloproteinase inhibitors cooperate to inhibit angiogenesis and growth of human prostate cancer xenotransplants. Faseb J, 17:223-234.
187. Bergers, G., Song, S., Meyer-Morse, N., Bergsland, E., and Hanahan, D., 2003, Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest, 111:1287-1295.
188. Fowlkes, J. L., and Winkler, M. K., 2002, Exploring the interface between metallo-proteinase activity and growth factor and cytokine bioavailability. Cytokine Growth Factor Rev, 13:277-287.
189. Stamenkovic, I., 2000, Matrix metalloproteinases in tumor invasion and metastasis. Semin Cancer Biol, 10:415-33.
190. Lynch, C. C., and Matrisian, L. M., 2002, Matrix metalloproteinases in tumor-host cell communication. Differentiation, 70:561-573.
191. D'Armiento, J., DiColandrea, T., Dalal, S. S., Okada, Y., Huang, M. T., Conney, A. H., and Chada, K., 1995, Collagenase expression in transgenic mouse skin causes hyperkeratosis and acanthosis and increases susceptibility to tumorigenesis. Mol Cell Biol, 15:5732-5739.
192. Martin, D. C., Sanchez-Sweatman, O. H., Ho, A. T., Inderdeo, D. S., Tsao, M. S., and Khokha, R., 1999, Transgenic TIMP-1 inhibits simian virus 40 T antigen-induced hepatocarcinogenesis by impairment of hepatocellular proliferation and tumor angiogenesis. Lab Invest, 79:225-234.
193. Martin, D. C., Ruther, U., Sanchez-Sweatman, O. H., Orr, F. W., and Khokha, R., 1996, Inhibition of SV40 T antigen-induced hepatocellular carcinoma in TIMP-1 transgenic mice. Oncogene, 13:569-576.
194. Dong, J., Opresko, L. K., Dempsey, P. J., Lauffenburger, D. A., Coffey, R. J., and Wiley, H.

S., 1999, Metalloprotease-mediated ligand release regulates autocrine signaling through the epidermal growth factor receptor. Proc Natl Acad Sci USA, 96:6235-6240.

195. Suzuki, M., Raab, G., Moses, M. A., Fernandez, C. A., and Klagsbrun, M., 1997, Matrix metalloproteinase-3 releases active heparin-binding EGF-like growth factor by cleavage at a specific juxtamembrane site. J Biol Chem, 272:31730-31737.
196. Arribas, J., Coodly, L., Vollmer, P., Kishimoto, T. K., Rose-John, S., and Massague, J., 1996, Diverse cell surface protein ectodomains are shed by a system sensitive to metalloprotease inhibitors. J Biol Chem, 271:11376-11382.
197. Werb, Z., 1997, ECM and cell surface proteolysis: regulating cellular ecology. Cell, 91:439-442.
198. Werb, Z., and Yan, Y., 1998, A cellular striptease act. Science, 282:1279-1280.
199. Yu, W. H., and Woessner, J. F., Jr., 2000, Heparan sulfate proteoglycans as extracellular docking molecules for matrilysin (matrix metalloproteinase 7). J Biol Chem, 275:4183-4191.
200. Kaya, G., Rodriguez, I., Jorcano, J. L., Vassalli, P., and Stamenkovic, I., 1997, Selective suppression of CD44 in keratinocytes of mice bearing an antisense CD44 transgene driven by a tissue-specific promoter disrupts hyaluronate metabolism in the skin and impairs keratinocyte proliferation. Genes Dev, 11:996-1007.
201. Gallea-Robache, S., Morand, V., Millet, S., Bruneau, J. M., Bhatnagar, N., Chouaib, S., and Roman-Roman, S., 1997, A metalloproteinase inhibitor blocks the shedding of soluble cytokine receptors and processing of transmembrane cytokine precursors in human monocytic cells. Cytokine, 9:340-346.
202. Lombard, M. A., Wallace, T. L., Kubicek, M. F., Petzold, G. L., Mitchell, M. A., Hendges, S. K., and Wilks, J. W., 1998, Synthetic matrix metalloproteinase inhibitors and tissue inhibitor of metalloproteinase (TIMP)-2, but not TIMP-1, inhibit shedding of tumor necrosis factor-alpha receptors in a human colon adenocarcinoma (Colo 205) cell line. Cancer Res, 58:4001-7.
203. McCusker, R. H., Busby, W. H., Dehoff, M. H., Camacho-Hubner, C., and Clemmons, D. R., 1991, Insulin-like growth factor (IGF) binding to cell monolayers is directly modulated by the addition of IGF-binding proteins. Endocrinology, 129:939-949.
204. Osborne, C. K., Coronado, E. B., Kitten, L. J., Arteaga, C. I., Fuqua, S. A., and Ramaharma, K., 1989, Insulin-like growth factor-II (IGF-II): a potential autocrine/paracrine growth factor for human breast cancer acting via the IGF-I receptor. Mol Endocrinol, 3:1701-1709.
205. Shimasaki, S., Shimonaka, M., Zhang, H. P., and Ling, N., 1991, Identification of five different insulin-like growth factor binding proteins (IGFBPs) from adult rat serum and molecular cloning of a novel IGFBP-5 in rat and human. J Biol Chem, 266:10646-10653.
206. Fowlkes, J. L., Enghild, J. J., Suzuki, K., and Nagase, H., 1994, Matrix metalloproteinases degrade insulin-like growth factor-binding protein-3 in dermal fibroblast cultures. J Biol Chem, 269:25742-25746.
207. Fowlkes, J. L., Serra, D. M., Nagase, H., and Thrailkill, K. M., 1999, MMPs are IGFBP-degrading proteinases: implications for cell proliferation and tissue growth. Ann N Y Acad Sci, 878:696-699.
208. Fowlkes, J. L., Serra, D. M., Bunn, R. C., Thrailkill, K. M., Enghild, J. J., and Nagase, H., 2003, Regulation of Insulin-Like Growth Factor-I (Igf-I) Action by Matrix Metalloproteinase-3 (Mmp-3) Involves Selective Disruption of Igf-I/Igf-Binding Protein-3 (Igfbp-3) Complexes. Endocrinology.
209. Conover, C. A., Durham, S. K., Zapf, J., Masiarz, F. R., and Kiefer, M. C., 1995, Cleavage analysis of insulin-like growth factor (IGF)-dependent IGF-binding protein-4 proteolysis and expression of protease-resistant IGF-binding protein-4 mutants. J Biol Chem, 270:4395-4400.
210. Manes, S., Mira, E., Barbacid, M. M., Cipres, A., Fernandez-Resa, P., Buesa, J. M., Merida, I., Aracil, M., Marquez, G., and Martinez, A. C., 1997, Identification of insulin-like growth factor-binding protein-1 as a potential physiological substrate for human stromelysin-3. J Biol Chem, 272:25706-12.
211. Martin, D. C., Fowlkes, J.L., Babic, B., and Khokha, R., 1999, Insulin-like growth factor II signaling in neoplastic proliferation is blocked by transgenic expression of the metalloproteinase inhibitor TIMP-1. J Cell Biol, 146:881-892.
212. Tennant, M. K., Thrasher, J. B., Twomey, P. A., Drivdahl, R. H., Birnbaum, R. S., and Plymate, S. R., 1996, Protein and messenger ribonucleic acid (mRNA) for the type 1 insulin-like growth factor (IGF) receptor is decreased and IGF-II mRNA is increased in human prostate carcinoma compared to benign prostate epithelium. J Clin Endocrinol Metab, 81:3774-3782.
213. D'Errico, A., Grigioni, W. F., Fiorentino, M., Baccarini, P., Lamas, E., De Mitri, S., Gozzetti, G., Mancini, A. M., and Brechot, C., 1994, Expression of insulin-like growth factor II (IGF-II) in human hepatocellular carcinomas: an immunohistochemical study. Pathol Int, 44:131-137.
214. Chan, J. M., Stampfer, M. J., Giovannucci, E., Gann, P. H., Ma, J., Wilkinson, P., Hennekens, C. H., and Pollak, M., 1998, Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. Science, 279:563-566.
215. Hankinson, S. E., Willett, W. C., Colditz, G. A., Hunter, D. J., Michaud, D. S., Deroo, B., Rosner, B.,

Speizer, F. E., and Pollak, M., 1998, Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. Lancet, 351:1393-1396.

216. Whitelock, J. M., Murdoch, A. D., Iozzo, R. V., and Underwood, P. A., 1996, The degradation of human endothelial cell-derived perlecan and release of bound basic fibroblast growth factor by stromelysin, collagenase, plasmin, and heparanases. J Biol Chem, 271:10079-10086.
217. Friedl, A., Chang, Z., Tierney, A., and Rapraeger, A. C., 1997, Differential binding of fibroblast growth factor-2 and -7 to basement membrane heparan sulfate: comparison of normal and abnormal human tissues. Am J Pathol, 150:1443-1455.
218. Levi, E., Fridman, R., Miao, H. Q., Ma, Y. S., Yayon, A., and Vlodavsky, I., 1996, Matrix metalloproteinase 2 releases active soluble ectodomain of fibroblast growth factor receptor 1, Proc Natl Acad Sci U S A 93:7069-74.
219. Czubayko, F., Liaudet-Coopman, E. D., Aigner, A., Tuveson, A. T., Berchem, G. J., and Wellstein, A., 1997, A secreted FGF-binding protein can serve as the angiogenic switch in human cancer. Nat Med, 3:1137-1140.
220. Annes, J. P., Munger, J. S., and Rifkin, D. B., 2003, Making sense of latent TGFbeta activation. J Cell Sci, 116:217-224.
221. Massague, J., Blain, S. W., and Lo, R. S., 2000, TGFbeta signaling in growth control, cancer, and heritable disorders. Cell, 103:295-309.
222. Derynck, R., Akhurst, R. J., and Balmain, A., 2001, TGF-beta signaling in tumor suppression and cancer progression. Nat Genet, 29:117-129.
223. Akhurst, R. J., 2002, TGF-beta antagonists: why suppress a tumor suppressor?. J Clin Invest, 109:1533-1536.
224. Moustakas, A., Pardali, K., Gaal, A., and Heldin, C. H., 2002, Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. Immunol Lett, 82:85-91.
225. Markowitz, S. D., and Roberts, A. B., 1996, Tumor suppressor activity of the TGF-beta pathway in human cancers. Cytokine Growth Factor Rev, 7:93-102.
226. Alexandrow, M. G., and Moses, H. L., 1995, Transforming growth factor beta 1 inhibits mouse keratinocytes late in G1 independent of effects on gene transcription. Cancer Res, 55:3928-3932.
227. Taipale, J., Saharinen, J., and Keski-Oja, J., 1998, Extracellular matrix-associated transforming growth factor-beta: role in cancer cell growth and invasion. Adv Cancer Res, 75:87-134.
228. Miyazono, K., Ichijo, H., and Heldin, C. H., 1993, Transforming growth factor-beta: latent forms, binding proteins and receptors. Growth Factors, 8:11-22.
229. Munger, J. S., Harpel, J. G., Gleizes, P. E., Mazzieri, R., Nunes, I., and Rifkin, D. B., 1997, Latent transforming growth factor-beta: structural features and mechanisms of activation. Kidney Int, 51:1376-1382.
230. Oklu, R., and Hesketh, R., 2000, The latent transforming growth factor beta binding protein (LTBP) family. Biochem J, 352 Pt 3:601-610.
231. Imai, K., Hiramatsu, A., Fukushima, D., Pierschbacher, M. D., and Okada, Y., 1997, Degradation of decorin by matrix metalloproteinases: identification of the cleavage sites, kinetic analyses and transforming growth factor-beta1 release. Biochem J, 322:809-814.
232. Hildebrand, A., Romaris, M., Rasmussen, L. M., Heinegard, D., Twardzik, D. R., Border, W. A., and Ruoslahti, E., 1994, Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta. Biochem J, 302(Pt 2):527-534.
233. Kresse, H., Hausser, H., Schonherr, E., and Bittner, K., 1994, Biosynthesis and interactions of small chondroitin/dermatan sulphate proteoglycans. Eur J Clin Chem Clin Biochem, 32:259-264.
234. Velasco-Loyden, G., Arribas, J., and Lopez-Casillas, F., 2003, The shedding of betaglycan is regulated by pervanadate and mediated by MT1-MMP. J Biol Chem.
235. Damsky, C., 2002, Cell-cell and cell-extracellular matrix adhesion receptors. Ann NY Acad Sci, 961:154-155.
236. Noe, V., Fingleton, B., Jacobs, K., Crawford, H. C., Vermeulen, S., Steelant, W., Bruyneel, E., Matrisian, L. M., and Mareel, M., 2001, Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. J Cell Sci, 114:111-118.
237. Kajita, M., Itoh, Y., Chiba, T., Mori, H., Okada, A., Kinoh, H., and Seiki, M., 2001, Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. J Cell Biol, 153:893-904.
238. Deryugina, E. I., Soroceanu, L., and Strongin, A. Y., 2002, Up-regulation of vascular endothelial growth factor by membrane-type 1 matrix metalloproteinase stimulates human glioma xenograft growth and angiogenesis. Cancer Res, 62:580-588.
239. Hynes, R. O., 2002, A reevaluation of integrins as regulators of angiogenesis. Nat Med, 8:918-921.
240. Hynes, R. O., 2002, Integrins: bidirectional, allosteric signaling machines. Cell, 110:673-687.
241. Zamir, E., and Geiger, B., 2001, Molecular complexity and dynamics of cell-matrix adhesions. J Cell Sci, 114:3583-3590.
242. Kassis, J., Lauffenburger, D. A., Turner, T., and Wells, A., 2001, Tumor invasion as dysregulated cell motility. Semin Cancer Biol, 11:105-117.

243. Monsky, W. L., Kelly, T., Lin, C. Y., Yeh, Y., Stetler-Stevenson, W. G., Mueller, S. C., and Chen, W. T., 1993, Binding and localization of M(r) 72,000 matrix metalloproteinase at cell surface invadopodia. Cancer Res, 53:3159-3164.
244. Chen, W. T., and Wang, J. Y., 1999, Specialized surface protrusions of invasive cells, invadopodia and lamellipodia, have differential MT1-MMP, MMP-2, and TIMP-2 localization. Ann NY Acad Sci, 878:361-371.
245. Riikonen, T., Westermarck, J., Koivisto, L., Broberg, A., Kahari, V. M., and Heino, J., 1995, Integrin alpha 2 beta 1 is a positive regulator of collagenase (MMP-1) and collagen alpha 1(I) gene expression. J Biol Chem, 270:13548-13552.
246. von Bredow, D. C., Nagle, R. B., Bowden, G. T., and Cress, A. E., 1997, Cleavage of beta 4 integrin by matrilysin. Exp Cell Res, 236:341-345.
247. Akimov, S. S., Krylov, D., Fleischman, L. F., and Belkin, A. M., 2000, Tissue transglutaminase is an integrin-binding adhesion coreceptor for fibronectin. J Cell Biol, 148:825-838.
248. Belkin, A. M., Akimov, S. S., Zaritskaya, L. S., Ratnikov, B. I., Deryugina, E. I., and Strongin, A. Y., 2001, Matrix-dependent proteolysis of surface transglutaminase by membrane-type metalloproteinase regulates cancer cell adhesion and locomotion. J Biol Chem, 276:18415-18422.
249. Van Aken, E., De Wever, O., Correia da Rocha, A., and Mareel, M., 2001, Defective E-cadherin/catenin complexes in human cancer. Virchows Arch, 439:725-751.
250. Bracke, M. E., Van Roy, F. M., and Mareel, M. M., 1996, The E-cadherin/catenin complex in invasion and metastasis. Curr Top Microbiol Immunol, 213:123-161.
251. De Leeuw, W. J., Berx, G., Vos, C. B., Peterse, J. L., Van de Vijver, M. J., Litvinov, S., Van Roy, F., Cornelisse, C. J., and Cleton-Jansen, A. M., 1997, Simultaneous loss of E-cadherin and catenins in invasive lobular breast cancer and lobular carcinoma in situ. J Pathol, 183:404-411.
252. Llorens, A., Rodrigo, I., Lopez-Barcons, L., Gonzalez-Garrigues, M., Lozano, E., Vinyals, A., Quintanilla, M., Cano, A., and Fabra, A., 1998, Down-regulation of E-cadherin in mouse skin carcinoma cells enhances a migratory and invasive phenotype linked to matrix metalloproteinase-9 gelatinase expression. Lab Invest, 78:1131-42.
253. Beavon, I. R., 2000, The E-cadherin-catenin complex in tumour metastasis: structure, function and regulation. Eur J Cancer, 36:1607-1620.
254. Christofori, G., and Semb, H., 1999, The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. Trends Biochem Sci, 24:73-76.
255. Perl, A. K., Wilgenbus, P., Dahl, U., Semb, H., and Christofori, G., 1998, A causal role for E-cadherin in the transition from adenoma to carcinoma. Nature, 392:190-3.
256. Perl, A. K., Dahl, U., Wilgenbus, P., Cremer, H., Semb, H., and Christofori, G., 1999, Reduced expression of neural cell adhesion molecule induces metastatic dissemination of pancreatic beta tumor cells. Nat Med, 5:286-91.
257. Lochter, A., Galosy, S., Muschler, J., Freedman, N., Werb, Z., and Bissell, M. J., 1997, Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. J Cell Biol, 139:1861-1872.
258. Nikkola, J., Vihinen, P., Vlaykova, T., Hahka-Kemppinen, M., Kahari, V. M., and Pyrhonen, S., 2002, High expression levels of collagenase-1 and stromelysin-1 correlate with shorter disease-free survival in human metastatic melanoma. Int J Cancer. 97:432-438.
259. Sympson, C. J., Bissell, M. J., and Werb, Z., 1995, Mammary gland tumor formation in transgenic mice overexpressing stromelysin-1. Semin Cancer Biol, 6:159-163.
260. Thomasset, N., Lochter, A., Sympson, C. J., Lund, L. R., Williams, D. R., Behrendtsen, O., Werb, Z., and Bissell, M. J., 1998, Expression of autoactivated stromelysin-1 in mammary glands of transgenic mice leads to a reactive stroma during early development. Am J Pathol, 153:457-467.
261. Lochter, A., Werb, Z., and Bissell, M. J., 1999, Transcriptional regulation of stromelysin-1 gene expression is altered during progression of mouse mammary epithelial cells from functionally normal to malignant. Matrix Biol, 18:455-467.
262. Rudolph-Owen, L. A., Chan, R., Muller, W. J., and Matrisian, L. M., 1998, The matrix metalloproteinase matrilysin influences early-stage mammary tumorigenesis. Cancer Res, 58:5500-5506.
263. Vargo-Gogola, T., Fingleton, B., Crawford, H. C., and Matrisian, L. M., 2002, Matrilysin (matrix metalloproteinase-7) selects for apoptosis-resistant mammary cells in vivo. Cancer Res, 62:5559-5563.
264. McGuire, J. K., Li, Q., and Parks, W. C., 2003, Matrilysin (matrix metalloproteinase-7) mediates E-cadherin ectodomain shedding in injured lung epithelium. Am J Pathol, 162:1831-1843.
265. Naot, D., Sionov, R. V., and Ish-Shalom, D., 1997, CD44: structure, function, and association with the malignant process. Adv Cancer Res, 71:241-319.
266. Pohl, M., Sakurai, H., Stuart, R. O., and Nigam, S. K., 2000, Role of hyaluronan and CD44 in in vitro branching morphogenesis of ureteric bud cells. Dev Biol, 224:312-325.
267. Okamoto, I., Kawano, Y., Murakami, D., Sasayama, T., Araki, N., Miki, T., Wong, A. J., and Saya, H.,

2001, Proteolytic release of CD44 intracellular domain and its role in the CD44 signaling pathway. J Cell Biol, 155:755-62.
268. Cichy, J., and Pure, E., 2003, The liberation of CD44. J Cell Biol, 161:839-843.
269. Jalkanen, S., Joensuu, H., Soderstrom, K. O., and Klemi, P., 1991, Lymphocyte homing and clinical behavior of non-Hodgkin's lymphoma. J Clin Invest, 87:1835-1840.
270. Mori, H., Tomari, T., Koshikawa, N., Kajita, M., Itoh, Y., Sato, H., Tojo, H., Yana, I., and Seiki, M., 2002, CD44 directs membrane-type 1 matrix metalloproteinase to lamellipodia by associating with its hemopexin-like domain. Embo J, 21:3949-3959.
271. Li, R., Huang, L., Guo, H., and Toole, B. P., 2001, Basigin (murine EMMPRIN) stimulates matrix metalloproteinase production by fibroblasts. J Cell Physiol, 186:371-9.
272. Folkman, J., 1990, What is the evidence that tumors are angiogenesis dependent?. J Nat Cancer Inst, 82:4-6.
273. Folkman, J., and Shing, Y., 1992, Angiogenesis. J Biol Chem, 267:10931-10934.
274. Weidner, N., Folkman, J., Pozza, F., Bevilacqua, P., Allred, E. N., Moore, D. H., Meli, S., and Gasparini, G., 1992, Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. J Natl Cancer Inst, 84:1875-1887.
275. Folkman, J., 1994, Tumor angiogenesis. Nature Medicine, 1:206-232.
276. Folkman, J., 1995, Tumor Angiogenesis. In The Molecular Basis of Cancer, Mendelsohn, J., Howley, P. M., Israel, M. A., and Liotta L. A., eds, Vol. 9:206-232. W. B. Saunders Company, Philadelphia.
277. Folkman, J., and D'Amore, P. A., 1996, Blood vessel formation: what is its molecular basis. Cell, 87:1153-1155.
278. Carmeliet, P., and Jain, R. K., 2000, Angiogenesis in cancer and other diseases. Nature, 407:249-257.
279. Carmeliet, P., 2003, Angiogenesis in health and disease. Nat Med, 9:653-60.
280. Hanahan, D., and Folkman, J., 1996, Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell, 86:353-364.
281. Nagy, J. A., Brown, L. F., Senger, D. R., Lanir, N., Van de Water, L., Dvorak, A. M., and Dvorak, H. F., 1989, Pathogenesis of tumor stroma generation: a critical role for leaky blood vessels and fibrin deposition. Biochim Biophys Acta, 948:305-326.
282. Lindahl, P., Johansson, B. R., Leveen, P., and Betsholtz, C., 1997, Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science, 277:242-245.
283. Paku, S., and Paweletz, N., 1991, First steps of tumor-related angiogenesis. Lab Invest, 65:334-346.
284. Giordano, F. J., and Johnson, R. S., 2001, Angiogenesis: the role of the microenvironment in flipping the switch. Curr Opin Genet Dev, 11:35-40.
285. Seandel, M., Noack-Kunnmann, K., Zhu, D., Aimes, R. T., and Quigley, J. P., 2001, Growth factor-induced angiogenesis in vivo requires specific cleavage of fibrillar type I collagen. Blood, 97:2323-2332.
286. Liu, X., Wu, H., Byrne, M., Jeffrey, J., Krane, S., and Jaenisch, R., 1995, A targeted mutation at the known collagenase cleavage site in mouse type I collagen impairs tissue remodeling. J Cell Biol, 130:227-237.
287. Xu, J., Rodriguez, D., Petitclerc, E., Kim, J. J., Hangai, M., Moon, Y. S., Davis, G. E., Brooks, P. C., and Yuen, S. M., 2001, Proteolytic exposure of a cryptic site within collagen type IV is required for angiogenesis and tumor growth in vivo. J Cell Biol, 154:1069-1079.
288. DiPietro, L. A., Burdick, M., Low, Q. E., Kunkel, S. L., and Strieter, R. M., 1998, MIP-1alpha as a critical macrophage chemoattractant in murine wound repair. J Clin Invest, 101:1693-1698.
289. Ferreras, M., Felbor, U., Lenhard, T., Olsen, B. R., and Delaisse, J., 2000, Generation and degradation of human endostatin proteins by various proteinases. FEBS Lett, 486:247-251.
290. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J., 1994, Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell, 79:315-328.
291. Maeshima, Y., Colorado, P. C., Torre, A., Holthaus, K. A., Grunkemeyer, J. A., Ericksen, M. B., Hopfer, H., Xiao, Y., Stillman, I. E., and Kalluri, R., 2000, Distinct antitumor properties of a type IV collagen domain derived from basement membrane. J Biol Chem, 275:21340-8.
292. Petitclerc, E., Stromblad, S., von Schalscha, T. L., Mitjans, F., Piulats, J., Montgomery, A. M., Cheresh, D. A., and Brooks, P. C., 1999, Integrin $\alpha v\beta 3$ promotes M21 melanoma growth in human skin by regulating tumor cell survival. Cancer Res, 59:2724-30.
293. Hamano, Y., Zeisberg, M., Sugimoto, H., Lively, J. C., Maeshima, Y., Changqing, Y., R.O., H., Werb, Z., Sudhakar, A., and Kalluri, R., 2003, Physiological levels of tumstatin, a fragment of collagen IV alpha3 chain, are generated by MMP-9 protelysis and suppress angioenesis via alphaVbeta3 integrin. Cancer Cell, 3:589-601.
294. Patterson, B. C., and Sang, Q. A., 1997, Angiostatin-converting enzyme activities of human matrilysin (MMP-7) and gelatinase B/type IV collagenase (MMP-9). J Biol Chem, 272:28823-5.

295. Sobrin, L., Liu, Z., Monroy, D. C., Solomon, A., Selzer, M. G., Lokeshwar, B. L., and Pflugfelder, S. C., 2000, Regulation of MMP-9 activity in human tear fluid and corneal epithelial culture supernatant. Invest Ophthalmol Vis Sci, 41:1703-1709.
296. Pozzi, A., LeVine, W. F., and Gardner, H. A., 2002, Low plasma levels of matrix metalloproteinase 9 permit increased tumor angiogenesis. Oncogene, 21:272-281.
297. Grunert, S., Jechlinger, M., and Beug, H., 2003, Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. Mol Cell Biol, 4:657-665.
298. Birchmeier, C., Birchmeier, W., Gherardi, E., and Vande Woude, G., 2003, Met, metastasis, motility and more. Nat Med, 4:915-925.
299. Woodhouse, E. C., Chuaqui, R. F., and Liotta, L. A., 1997, General mechanisms of metastasis. Cancer, 80:1529-37.
300. Yokota, J., 2000, Tumor progression and metastasis. Carcinogenesis, 21:497-503.
301. Fidler, I. J., 2001, Seed and soil revisited: contribution of the organ microenvironment to cancer metastasis. Surg Oncol Clin N Am, 10:257-269.
302. Fidler, I. J., 2002, Critical determinants of metastasis. Semin Cancer Biol, 12:89-96.
303. Comoglio, P. M., and Trusolino, L., 2002, Invasive growth: from development to metastasis, J Clin Invest 109:857-62.
304. Jussila, L., and Alitalo, K., 2002, Vascular growth factors and lymphangiogenesis. Physiol Rev, 82:673-700.
305. Engers, R., and Gabbert, H. E., 2000, Mechanisms of tumor metastasis: cell biological aspects and clinical implications. J Cancer Res Clin Oncol, 126:682-692.
306. Moser, P. L., Kieback, D. G., Hefler, L., Tempfer, C., Neunteufel, W., and Gitsch, G., 1999, Immunohistochemical detection of matrix metalloproteinases (MMP) 1 and 2, and tissue inhibitor of metalloproteinase 2 (TIMP 2) in stage IB cervical cancer. Anticancer Res, 19:4391-4393.
307. Inoue, T., Yashiro, M., Nishimura, S., Maeda, K., Sawada, T., Ogawa, Y., Sowa, M., and Chung, K. H., 1999, Matrix metalloproteinase-1 expression is a prognostic factor for patients with advanced gastric cancer. Int J Mol Med, 4:73-77.
308. Yamashita, K., Azumano, I., Mai, M., and Okada, Y., 1998, Expression and tissue localization of matrix metalloproteinase 7 (matrilysin) in human gastric carcinomas. Implications for vessel invasion and metastasis. Int J Cancer, 79:187-194.
309. Gokaslan, Z. L., Chintala, S. K., York, J. E., Boyapati, V., Jasti, S., Sawaya, R., Fuller, G., Wildrick, D. M., Nicolson, G. L., and Rao, J. S., 1998, Expression and role of matrix metalloproteinases MMP-2 and MMP-9 in human spinal column tumors. Clin Exp Metastasis, 16:721-728.
310. Garbisa, S., Scagliotti, G., Masiero, L., Di Francesco, C., Caenazzo, C., Onisto, M., Micela, M., Stetler-Stevenson, W. G., and Liotta, L. A., 1992, Correlation of serum metalloproteinase levels with lung cancer metastasis and response to therapy. Cancer Res, 52:4548-4549.
311. Nyormoi, O., Mills, L., and Bar-Eli, M., 2003, An MMP-2/MMP-9 inhibitor, 5a, enhances apoptosis induced by ligands of the TNF receptor superfamily in cancer cells. Cell Death Differ, 10:558-569.
312. Hotz, H. G., Hines, O. J., Hotz, B., Foitzik, T., Buhr, H. J., and Reber, H. A., 2003, Evaluation of vascular endothelial growth factor blockade and matrix metalloproteinase inhibition as a combination therapy for experimental human pancreatic cancer. J Gastrointest Surg, 7:220-227.
313. Shinoda, K., Shibuya, M., Hibino, S., Ono, Y., Matsuda, K., Takemura, A., Zou, D., Kokubo, Y., Takechi, A., and Kudoh, S., 2003, A novel matrix metalloproteinase inhibitor, FYK-1388 suppresses tumor growth, metastasis and angiogenesis by human fibrosarcoma cell line. Int J Oncol, 22:281-288.
314. Winding, B., NicAmhlaoibh, R., Misander, H., Hoegh-Andersen, P., Andersen, T. L., Holst-Hansen, C., Heegaard, A. M., Foged, N. T., Brunner, N., and Delaisse, J. M., 2002, Synthetic matrix metalloproteinase inhibitors inhibit growth of established breast cancer osteolytic lesions and prolong survival in mice. Clin Cancer Res, 8:1932-1939.
315. Katori, H., Baba, Y., Imagawa, Y., Nishimura, G., Kagesato, Y., Takagi, E., Ishii, A., Yanoma, S., Maekawa, R., Yoshioka, T., Nagashima, Y., Kato, Y., and Tsukuda, M., 2002, Reduction of in vivo tumor growth by MMI-166, a selective matrix metalloproteinase inhibitor, through inhibition of tumor angiogenesis in squamous cell carcinoma cell lines of head and neck. Cancer Lett, 178:151-159.
316. Weber, M. H., Lee, J., and Orr, F. W., 2002, The effect of Neovastat (AE-941) on an experimental metastatic bone tumor model. Int J Oncol, 20:299-303.
317. Naglich, J. G., Jure-Kunkel, M., Gupta, E., Fargnoli, J., Henderson, A. J., Lewin, A. C., Talbott, R., Baxter, A., Bird, J., Savopoulos, R., Wills, R., Kramer, R. A., and Trail, P. A., 2001, Inhibition of angiogenesis and metastasis in two murine models by the matrix metalloproteinase inhibitor, BMS-275291. Cancer Res, 61:8480-8485.
318. Brown, P. D., 1999, Clinical studies with matrix metalloproteinase inhibitors. APMIS, 107:174-180.
319. Drummond, A. H., Beckett, P., Brown, P. D., Bone, E. A., Davidson, A. H., Galloway, W. A., Gearing,

A. J., Huxley, P., Laber, D., McCourt, M., Whittaker, M., Wood, L. M., and Wright, A., 1999, Preclinical and clinical studies of MMP inhibitors in cancer. Ann N Y Acad Sci, 878:228-235.

320. Whittaker, M., Floyd, C. D., Brown, P., and Gearing, A. J., 1999, Design and therapeutic application of matrix metalloproteinase inhibitors. Chem Rev, 99:2735-76.
321. Brown, P. D., 2000, Ongoing trials with matrix metalloproteinase inhibitors. Expert Opin Investig Drugs, 9:2167-2177.
322. Opdenakker, G., Van den Steen, P. E., and Van Damme, J., 2001, Gelatinase B: a tuner and amplifier of immune functions. Trends Immunol, 22:571-579.
323. Müller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N., Barrera, J. L., Mohar, A., Verastegui, E., and Zlotnik, A., 2001, Involvement of chemokine receptors in breast cancer metastasis. Nature, 410:50-56.
324. Van den Steen, P. E., Proost, P., Wuyts, A., Van Damme, J., and Opdenakker, G., 2000, Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. Blood, 96:2673-2681.
325. Ramjeesingh, R., Leung, R., and Siu, C. H., 2003, Interleukin-8 secreted by endothelial cells induces chemotaxis of melanoma cells through the chemokine receptor CXCR1. Faseb J, 17:1292-1294.
326. Varney, M. L., Li, A., Dave, B. J., Bucana, C. D., Johansson, S. L., and Singh, R. K., 2003, Expression of CXCR1 and CXCR2 receptors in malignant melanoma with different metastatic potential and their role in interleukin-8 (CXCL-8)-mediated modulation of metastatic phenotype. Clin Exp Metastasis, 20:723-731.
327. Kawamata, H., Kawai, K., Kameyama, S., Johnson, M. D., Stetler-Stevenson, W. G., and Oyasu, R., 1995, Over-expression of tissue inhibitor of matrix metalloproteinases (TIMP1 and TIMP2) suppresses extravasation of pulmonary metastasis of a rat bladder carcinoma. Int J Cancer, 63:680-687.
328. Onisto, M., Riccio, M. P., Scannapieco, P., Caenazzo, C., Griggio, L., Spina, M., Stetler-Stevenson, W. G., and Garbisa, S., 1995, Gelatinase A/TIMP-2 imbalance in lymph-node-positive breast carcinomas, as measured by RT-PCR. Int J Cancer, 63:621-6.
329. Bian, J., Wang, Y., Smith, M. R., Kim, H., Jacobs, C., Jackman, J., Kung, H. F., Colburn, N. H., and Sun, Y., 1996, Suppression of in vivo tumor growth and induction of suspension cell death by tissue inhibitor of metalloproteinases (TIMP)-3. Carcinogenesis, 17:1805-1811.
330. Fong, K. M., Kida, Y., Zimmerman, P. V., and Smith, P. J., 1996, TIMP1 and adverse prognosis in non-small cell lung cancer. Clin Cancer Res, 2:1369-1372.
331. Grignon, D. J., Sakr, W., Toth, M., Ravery, V., Angulo, J., Shamsa, F., Pontes, J. E., Crissman, J. C., and Fridman, R., 1996, High levels of tissue inhibitor of metalloproteinase-2 (TIMP-2) expression are associated with poor outcome in invasive bladder cancer. Cancer Res, 56:1654-1659.
332. Martin, D. C., Rüther, U., Sanchez-Sweatman, O. H., Orr, F. W., and Khokha, R., 1996, Inhibition of SV40 T antigen-induced hepatocellular carcinoma in TIMP-1 transgenic mice. Oncogene, 13:569-576.
333. Matsuzawa, K., Fukuyama, K., Hubbard, S. L., Dirks, P. B., and Rutka, J. T., 1996, Transfection of an invasive human astrocytoma cell line with a TIMP-1 cDNA: modulation of astrocytoma invasive potential. J Neuropathol Exp Neurol, 55:88-96.
334. Soloway, P. D., Alexander, C. M., Werb, Z., and Jaenisch, R., 1996, Targeted mutagenesis of Timp-1 reveals that lung tumor invasion is influenced by Timp-1 genotype of the tumor but not by that of the host. Oncogene, 13:2307-14.
335. Thorgeirsson, U. P., Yoshiji, H., Sinha, C. C., and Gomez, D. E., 1996, Breast cancer; tumor neovasculature and the effect of tissue inhibitor of metalloproteinases-1 (TIMP-1) on angiogenesis. In Vivo, 10:137-144.
336. Kruger, A., Fata, J. E., and Khokha, R., 1997, Altered tumor growth and metastasis of a T-cell lymphoma in Timp-1 transgenic mice. Blood, 90:1993-2000.
337. Shoji, A., Sakamoto, Y., Tsuchiya, T., Moriyama, K., Kaneko, T., Okubo, T., Umeda, M., and Miyazaki, K., 1997, Inhibition of tumor promoter activity toward mouse fibroblasts and their in vitro transformation by tissue inhibitor of metalloproteinases- 1 (TIMP-1). Carcinogenesis, 18:2093-100.
338. Kruger, A., Sanchez-Sweatman, O. H., Martin, D. C., Fata, J. E., Ho, A. T., Orr, F. W., Ruther, U., and Khokha, R., 1998, Host TIMP-1 overexpression confers resistance to experimental brain metastasis of a fibrosarcoma cell line. Oncogene, 16:2419-23.
339. Baker, A. H., George, S. J., Zaltsman, A. B., Murphy, G., and Newby, A. C., 1999, Inhibition of invasion and induction of apoptotic cell death of cancer cell lines by overexpression of TIMP-3. Br J Cancer, 79:1347-1355.
340. Brown, P. D., 1995, Matrix metalloproteinase inhibitors: a novel class of anticancer agents. Adv Enzyme Regul, 35:293-301.
341. Brown, P. D., 1997, Matrix metalloproteinase inhibitors in the treatment of cancer. Med Oncol, 14:1-10.

342. Hidalgo, M., and Eckhardt, S. G., 2001, Development of matrix metalloproteinase inhibitors in cancer therapy. J Natl Cancer Inst, 93:178-193.
343. Bernardo, M. M., Brown, S., Li, Z. H., Fridman, R., and Mobashery, S., 2002, Design, Synthesis, and Characterization of Potent, Slow-binding Inhibitors That Are Selective for Gelatinases. J Biol Chem, 277:11201-11207.
344. Coussens, L. M., B. Fingleton, B., and Matrisian, L. M., 2002, Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science, 295:2387-2392.
345. Brown, S., Bernardo, M. M., Li, Z. H., Kotra, L. P., Tanaka, Y., Fridman, R., and Mobashery, S., 2000, Potent and Selective Mechanism-Based Inhibition of Gelatinases. Journal of American Chemical Society, 122:6799-6800.
346. Brown, P. D., 2001, New hope for matrix metalloproteinase inhibitors in cancer therapy. Drug Discov Today, 6:615.
347. Koivunen, E., Arap, W., Valtanen, H., Rainisalo, A., Medina, O. P., Heikkila, P., Kantor, C., Gahmberg, C. G., Salo, T., Konttinen, Y. T., Sorsa, T., Ruoslahti, E., and Pasqualini, R., 1999, Tumor targeting with a selective gelatinase inhibitor. Nat Biotechnol, 17:768-774.
348. Garbisa, S., Biggin, S., Cavallarin, N., Sartor, L., Benelli, R., and Albini, A., 1999, Tumor invasion: molecular shears blunted by green tea. Nat Med, 5:1216.
349. Falardeau, P., Champagne, P., Poyet, P., Hariton, C., and Dupont, E., 2001, Neovastat, a naturally occurring multifunctional antiangiogenic drug, in phase III clinical trials. Semin Oncol, 28:620-625.
350. Reich, R., Thompson, E. W., Iwamoto, Y., Martin, G. R., Deason, J. R., Fuller, G. C., and Miskin, R., 1988, Effects of inhibitors of plasminogen activator, serine proteinases, and collagenase IV on the invasion of basement membranes by metastatic cells. Cancer Res, 48:3307-12.
351. Davies, B., Brown, P. D., East, N., Crimmin, M. J., and Balkwill, F. R., 1993, A synthetic matrix metalloproteinase inhibitor decreases tumor burden and prolongs survival of mice bearing human ovarian carcinoma xenografts. Cancer Res, 53:2087-2091.
352. Wang, X., Fu, X., Brown, P. D., Crimmin, M. J., and Hoffman, R. M., 1994, Matrix metalloproteinase inhibitor BB-94 (batimastat) inhibits human colon tumor growth and spread in a patient-like orthotopic model in nude mice. Cancer Res, 54:4726-8.
353. Eccles, S. A., Box, G. M., Court, W. J., Bone, E. A., Thomas, W., and Brown, P. D., 1996, Control of lymphatic and hematogenous metastasis of a rat mammary carcinoma by the matrix metalloproteinase inhibitor batimastat (BB-94). Cancer Res, 56:2815-2822.
354. Goss, K. J., Brown, P. D., and Matrisian, L. M., 1998, Differing effects of endogenous and synthetic inhibitors of metalloproteinases on intestinal tumorigenesis. Int J Cancer, 78:629-635.
355. Bergers, G., Javaherian, K., Lo, K. M., Folkman, J., and Hanahan, D., 1999, Effects of angiogenesis inhibitors on multistage carcinogenesis in mice. Science, 284:808-812.
356. Sledge, G. W., Jr., Qulali, M., Goulet, R., Bone, E. A., and Fife, R., 1995, Effect of matrix metalloproteinase inhibitor batimastat on breast cancer regrowth and metastasis in athymic mice. J Natl Cancer Inst, 87:1546-50.
357. Wagenaar-Miller, R. A., Gorden, L., and Matrisian, L. M., 2004, Matrix metalloproteinases in colorectal cancer: is it worth talking about?. Cancer Metastasis Rev, 23:119-135.
358. Oba, K., Konno, H., Tanaka, T., Baba, M., Kamiya, K., Ohta, M., Kaneko, T., Shouji, T., Igarashi, A., and Nakamura, S., 2002, Prevention of liver metastasis of human colon cancer by selective matrix metalloproteinase inhibitor MMI-166. Cancer Lett, 175:45-51.
359. An, Z., Wang, X., Willmott, N., Chander, S. K., Tickle, S., Docherty, A. J., Mountain, A., Millican, A. T., Morphy, R., Porter, J. R., Epemolu, R. O., Kubota, T., Moossa, A. R., and Hoffman, R. M., 1997, Conversion of highly malignant colon cancer from an aggressive to a controlled disease by oral administration of a metalloproteinase inhibitor. Clin Exp Metastasis, 15:184-195.
360. Bramhall, S. R., Rosemurgy, A., Brown, P. D., Bowry, C., and Buckels, J. A., 2001, Marimastat as first-line therapy for patients with unresectable pancreatic cancer: a randomized trial. J Clin Oncol, 19:3447-3455.
361. Rao, J. S., 2003, Molecular mechanisms of glioma invasiveness: the role of proteases. Nat Rev Cancer, 3:489-501.
362. Fielding, J., Scholefield, J., Stuart, R., Hawkins, R., McCulloch, P., Maughan, T., Seymour, M., Cutsem, E.V., Thorlacius-Ussing, O., and Hovendal, C., 2000, Presented at the American Society of Clinical Oncology (ASCO)'s 36th Annual Meeting, New Orleans.
363. Moore, M.J., Eisenberg, J.H., Dagenais, M., Hagan, K., Fields, A., Greenberg, B., Schwartz, B., Ottaway, J., Zee, B., and Seymour, L., posting date 2000, A Comparison Between Gemcitabine (GEM) and the Matrix Metalloproteinase (MMP) Inhibitor BAY12-9566 (9566) in Patients (PTS) with Advanced Pancreatic Cancer. Published Online.
364. McIntyre, J. O., Fingleton, B., Wells, K. S., Piston, D. W., Lynch, C. C., Gautam, S., and Matrisian, L. M., 2004, Development of a novel fluorogenic proteolytic beacon for in vivo detection and imaging

of tumour-associated matrix metalloproteinase-7 activity. Biochem J, 377:617-628.
365. Wang, Z., Juttermann, R., and Soloway, P. D., 2000, TIMP-2 is required for efficient activation of proMMP-2 in vivo. J Biol Chem, 275:26411-5.
366. Ward, R. V., Hembry, R. M., Reynolds, J. J., and Murphy, G., 1991, The purification of tissue inhibitor of metalloproteinases-2 from its 72 kDa progelatinase complex. Demonstration of the biochemical similarities of tissue inhibitor of metalloproteinases-2 and tissue inhibitor of metalloproteinases-1. Biochem J, 278:179-87.
367. Howard, E. W., Bullen, E. C., and Banda, M. J., 1991, Preferential inhibition of 72- and 92-kDa gelatinases by tissue inhibitor of metalloproteinases-2. J Biol Chem, 266:13070-13075.
368. Quantin, B., Murphy, G., and Breathnach, R., 1989, Pump-1 cDNA codes for a protein with characteristics similar to those of classical collagenase family members. Biochemistry, 28:5327-34.
369. Knauper, V., Wilhelm, S. M., Seperack, P. K., DeClerck, Y. A., Langley, K. E., Osthues, A., and Tschesche, H., 1993, Direct activation of human neutrophil procollagenase by recombinant stromelysin. Biochem J, 295:581-6.
370. Murphy, G., Segain, J. P., O'Shea, M., Cockett, M., Ioannou, C., Lefebvre, O., Chambon, P., and Basset, P., 1993, The 28-kDa N-terminal domain of mouse stromelysin-3 has the general properties of a weak metalloproteinase. J Biol Chem, 268:15435-41.
371. Knauper, V., Lopez-Otin, C., Smith, B., Knight, G., and Murphy, G., 1996, Biochemical characterization of human collagenase-3. J Biol Chem, 271:1544-50.
372. English, W. R., Puente, X. S., Freije, J. M., Knauper, V., Amour, A., Merryweather, A., Lopez-Otin, C., and Murphy, G., 2000, Membrane type 4 matrix metalloproteinase (MMP17) has tumor necrosis factor-alpha convertase activity but does not activate pro-MMP2. J Biol Chem, 275:14046-14055.
373. Stracke, J. O., Hutton, M., Stewart, M., Pendas, A. M., Smith, B., Lopez-Otin, C., Murphy, G., and Knauper, V., 2000, Biochemical characterization of the catalytic domain of human matrix metalloproteinase 19. Evidence for a role as a potent basement membrane degrading enzyme. J Biol Chem, 275:14809-14816.
374. English, W. R., Velasco, G., Stracke, J. O., Knauper, V., and Murphy, G., 2001, Catalytic activities of membrane-type 6 matrix metalloproteinase (MMP25). FEBS Lett, 491:137-142.
375. Uria, J. A., and Lopez-Otin, C., 2000, Matrilysin-2, a new matrix metalloproteinase expressed in human tumors and showing the minimal domain organization required for secretion, latency, and activity. Cancer Res, 60:4745-4751.
376. DeClerck, Y. A., Yean, T. D., Chan, D., Shimada, H., and Langley, K. E., 1991, Inhibition of tumor invasion of smooth muscle cell layers by recombinant human metalloproteinase inhibitor. Cancer Res, 51:2151-2157.
377. Quesada, A. R., Barbacid, M. M., Mira, E., Fernandez-Resa, P., Marquez, G., and Aracil, M., 1997, Evaluation of fluorometric and zymographic methods as activity assays for stromelysins and gelatinases. Clin Exp Metastasis. 15:26-32.
378. Knauper, V., Osthues, A., DeClerck, Y. A., Langley, K. E., Blaser, J., and Tschesche, H., 1993, Fragmentation of human polymorphonuclear-leucocyte collagenase. Biochem J, 291(Pt 3):847-854.
379. Will, H., Atkinson, S. J., Butler, G. S., Smith, B., and Murphy, G., 1996, The soluble catalytic domain of membrane type 1 matrix metalloproteinase cleaves the propeptide of progelatinase A and initiates autoproteolytic activation. Regulation by TIMP-2 and TIMP-3. J Biol Chem, 271:17119-17123.
380. Shimada, T., Nakamura, H., Ohuchi, E., Fujii, Y., Murakami, Y., Sato, H., Seiki, M., and Okada, Y., 1999, Characterization of a truncated recombinant form of human membrane type 3 matrix metalloproteinase. Eur J Biochem, 262:907-914.
381. Llano, E., Pendas, A. M., Freije, J. P., Nakano, A., Knauper, V., Murphy, G., and Lopez-Otin, C., 1999, Identification and characterization of human MT5-MMP, a new membrane-bound activator of progelatinase a overexpressed in brain tumors. Cancer Res, 59:2570-2576.
382. Zhao, Y. G., Xiao, A. Z., Park, H. I., Newcomer, R. G., Yan, M., Man, Y. G., Heffelfinger, S. C., and Sang, Q. X., 2004, Endometase/matrilysin-2 in human breast ductal carcinoma in situ and its inhibition by tissue inhibitors of metalloproteinases-2 and -4: a putative role in the initiation of breast cancer invasion. Cancer Res, 64:590-598.
383. Apte, S. S., Olsen, B. R., and Murphy, G., 1995, The gene structure of tissue inhibitor of metalloproteinases (TIMP)-3 and its inhibitory activities define the distinct TIMP gene family. J Biol Chem, 270:14313-14318.
384. Will, H., and Hinzmann, B., 1995, cDNA sequence and mRNA tissue distribution of a novel human matrix metalloproteinase with a potential transmembrane segment. Eur J Biochem, 231:602-8.
385. Bigg, H. F., Morrison, C. J., Butler, G. S., Bogoyevitch, M. A., Wang, Z., Soloway, P. D., and Overall, C. M., 2001, Tissue inhibitor of metalloproteinases-4 inhibits but does not support the activation of gelatinase A via efficient inhibition of membrane type 1-matrix metalloproteinase. Cancer Res, 61:3610-3608.

386. Liu, Y. E., Wang, M., Greene, J., Su, J., Ullrich, S., Li, H., Sheng, S., Alexander, P., Sang, Q. A., and Shi, Y. E., 1997, Preparation and characterization of recombinant tissue inhibitor of metalloproteinase 4 (TIMP-4). J Biol Chem, 272:20479-20483.
387. Nomura, H., Fujimoto, N., Seiki, M., Mai, M., and Okada, Y., 1996, Enhanced production of matrix metalloproteinases and activation of matrix metalloproteinase 2 (gelatinase A) in human gastric carcinomas. Int J Cancer, 69:9-16.
388. Ko, B. K., Cho, H. R., Choi, D. W., Nam, C. W., Park, C. J., Kim, G. Y., Kim, S. S., Woo, Y. J., Huh, J., and Kim, M. Y., 1998, Reduced expression of tissue inhibitor of metalloproteinase in nodal metastasis of stomach cancer. J Korean Med Sci, 13:286-90.
389. Joo, Y. E., Seo, K. S., Kim, H. S., Rew, J. S., Park, C. S., and Kim, S. J., 2000, Expression of tissue inhibitors of metalloproteinases (TIMPs) in gastric cancer. Dig Dis Sci, 45:114-121.
390. Hong, S. I., Park, I. C., Hong, W. S., Son, Y. S., Lee, S. H., Lee, J. I., Choi, D. W., Moon, N. M., Choe, T. B., and Jang, J. J., 1996, Overexpression of tissue inhibitors of metalloproteinase-1 and -2 in the stroma of gastric cancer. J Korean Med Sci, 11:474-479.
391. Thomas, P., Khokha, R., Shepherd, F. A., Feld, R., and Tsao, M. S., 2000, Differential expression of matrix metalloproteinases and their inhibitors in non-small cell lung cancer. J Pathol, 190:150-156.
392. Bolon, I., Gouyer, V., Devouassoux, M., Vandenbunder, B., Wernert, N., Moro, D., Brambilla, C., and Brambilla, E., 1995, Expression of c-ets-1, collagenase 1, and urokinase-type plasminogen activator genes in lung carcinomas. Am J Pathol, 147:1298-1310.
393. Nawrocki, B., Polette, M., Marchand, V., Monteau, M., Gillery, P., Tournier, J. M., and Birembaut, P., 1997, Expression of matrix metalloproteinases and their inhibitors in human bronchopulmonary carcinomas: quantificative and morphological analyses. Int J Cancer, 72:556-64.
394. Soini, Y., Paakko, P., and Autio-Harmainen, H., 1993, Genes of laminin B1 chain, alpha 1 (IV) chain of type IV collagen, and 72-kd type IV collagenase are mainly expressed by the stromal cells of lung carcinomas. Am J Pathol, 142:1622-1630.
395. Suzuki, M., Iizasa, T., Fujisawa, T., Baba, M., Yamaguchi, Y., Kimura, H., and Suzuki, H., 1998, Expression of matrix metalloproteinases and tissue inhibitor of matrix metalloproteinases in non-small-cell lung cancer. Invasion Metastasis, 18:134-141.
396. Bolon, I., Devouassoux, M., Robert, C., Moro, D., Brambilla, C., and Brambilla, E., 1997, Expression of urokinase-type plasminogen activator, stromelysin 1, stromelysin 3, and matrilysin genes in lung carcinomas. Am J Pathol, 150:1619-1629.
397. Nagashima, Y., Hasegawa, S., Koshikawa, N., Taki, A., Ichikawa, Y., Kitamura, H., Misugi, K., Kihira, Y., Matuo, Y., Yasumitsu, H., and Miyazaki, K., 1997, Expression of matrilysin in vascular endothelial cells adjacent to matrilysin-producing tumors. Int J Cancer, 72:441-5.
398. Michael, M., Babic, B., Khokha, R., Tsao, M., Ho, J., Pintilie, M., Leco, K., Chamberlain, D., and Shepherd, F. A., 1999, Expression and prognostic significance of metalloproteinases and their tissue inhibitors in patients with small-cell lung cancer. J Clin Oncol, 17:1802-8.
399. Kodate, M., Kasai, T., Hashimoto, H., Yasumoto, K., Iwata, Y., and Manabe, H., 1997, Expression of matrix metalloproteinase (gelatinase) in T1 adenocarcinoma of the lung. Pathol Int, 47:461-9.
400. Canete-Soler, R., Litzky, L., Lubensky, I., and Muschel, R. J., 1994, Localization of the 92 kd gelatinase mRNA in squamous cell and adenocarcinomas of the lung using in situ hybridization. Am J Pathol, 144:518-527.
401. Cox, G., Jones, J. L., and O'Byrne, K. J., 2000, Matrix metalloproteinase 9 and the epidermal growth factor signal pathway in operable non-small cell lung cancer. Clin Cancer Res, 6:2349-2355.
402. Anderson, I. C., Sugarbaker, D. J., Ganju, R. K., Tsarwhas, D. G., Richards, W. G., Sunday, M., Kobzik, L., and Shipp, M. A., 1995, Stromelysin-3 is overexpressed by stromal elements in primary non-small cell lung cancers and regulated by retinoic acid in pulmonary fibroblasts. Cancer Res, 55:4120-4126.
403. Cho, N. H., Hong, K. P., Hong, S. H., Kang, S., Chung, K. Y., and Cho, S. H., 2004, MMP expression profiling in recurred stage IB lung cancer. Oncogene, 23:845-851.
404. Bodey, B., Bodey, B., Jr., Groger, A. M., Siegel, S. E., and Kaiser, H. E., 2001, Invasion and metastasis: the expression and significance of matrix metalloproteinases in carcinomas of the lung. In Vivo, 15:175-180.
405. Polette, M., Nawrocki, B., Gilles, C., Sato, H., Seiki, M., Tournier, J. M., and Birembaut, P., 1996, MT-MMP expression and localisation in human lung and breast cancers. Virchows Arch, 428:29-35.
406. Marchenko, G. N., Ratnikov, B. I., Rozanov, D. V., Godzik, A., Deryugina, E. I., and Strongin, A. Y., 2001, Characterization of matrix metalloproteinase-26, a novel metalloproteinase widely expressed in cancer cells of epithelial origin. Biochem J, 356:705-718.
407. Polette, M., Gilbert, N., Stas, I., Nawrocki, B., Noel, A., Remacle, A., Stetler-Stevenson, W. G., Birembaut, P., and Foidart, M., 1994, Gelatinase A expression and localization in human breast cancers. An in situ hybridization study and immunohistochemical detection using confocal microscopy. Virchows Arch, 424:641-5.

408. Ioachim, E. E., Athanassiadou, S. E., Kamina, S., Carassavoglou, K., and Agnantis, N. J., 1998, Matrix metalloproteinase expression in human breast cancer: an immunohistochemical study including correlation with cathepsin D, type IV collagen, laminin, fibronectin, EGFR, c-erbB-2 oncoprotein, p53, steroid receptors status and proliferative indices. Anticancer Res, 18:1665-1670.
409. Bodey, B., Bodey, B., Jr., Siegel, S. E., and Kaiser, H. E., 2001, Matrix metalloproteinases in neoplasm-induced extracellular matrix remodeling in breast carcinomas, Anticancer Res 21:2021-2028.
410. Basset, P., Wolf, C., Rouyer, N., Bellocq, J. P., Rio, M. C., and Chambon, P., 1994, Stromelysin-3 in stromal tissue as a control factor in breast cancer behavior. Cancer, 74:1045-1049.
411. Ueno, H., Nakamura, H., Inoue, M., Imai, K., Noguchi, M., Sato, H., Seiki, M., and Okada, Y., 1997, Expression and tissue localization of membrane-types 1, 2, and 3 matrix metalloproteinases in human invasive breast carcinomas. Cancer Res, 57:2055-2060.
412. Bisson, C., Blacher, S., Polette, M., Blanc, J. F., Kebers, F., Desreux, J., Tetu, B., Rosenbaum, J., Foidart, J. M., Birembaut, P., and Noel, A., 2003, Restricted expression of membrane type 1-matrix metalloproteinase by myofibroblasts adjacent to human breast cancer cells. Int J Cancer, 105:7-13.
413. Djonov, V., Hogger, K., Sedlacek, R., Laissue, J., and Draeger, A., 2001, MMP-19: cellular localization of a novel metalloproteinase within normal breast tissue and mammary gland tumours. J Pathol, 195:147-155.
414. Boag, A. H., and Young, I. D., 1994, Increased expression of the 72-kd type IV collagenase in prostatic adenocarcinoma. Demonstration by immunohistochemistry and in situ hybridization. Am J Pathol, 144:585-591.
415. Stearns, M. E., and Wang, M., 1993, Type IV collagenase (M(r) 72,000) expression in human prostate: benign and malignant tissue. Cancer Res, 53:878-883.
416. Upadhyay, J., Shekarriz, B., Nemeth, J. A., Dong, Z., Cummings, G. D., Fridman, R., Sakr, W., Grignon, D. J., and Cher, M. L., 1999, Membrane type 1-matrix metalloproteinase (MT1-MMP) and MMP-2 immunolocalization in human prostate: change in cellular localization associated with high-grade prostatic intraepithelial neoplasia. Clin Cancer Res, 5:4105-4110.
417. Knox, J. D., Wolf, C., McDaniel, K., Clark, V., Loriot, M., Bowden, G. T., and Nagle, R. B., 1996, Matrilysin expression in human prostate carcinoma. Mol Carcinog, 15:57-63.
418. Kuniyasu, H., Ellis, L. M., Evans, D. B., Abbruzzese, J. L., Fenoglio, C. J., Bucana, C. D., Cleary, K. R., Tahara, E., and Fidler, I. J., 1999, Relative expression of E-cadherin and type IV collagenase genes predicts disease outcome in patients with resectable pancreatic carcinoma. Clin Cancer Res, 5:25-33.
419. Zhao, Y. G., Xiao, A. Z., Newcomer, R. G., Park, H. I., Kang, T., Chung, L. W., Swanson, M. G., Zhau, H. E., Kurhanewicz, J., and Sang, Q. X., 2003, Activation of pro-gelatinase B by endometase/matrilysin-2 promotes invasion of human prostate cancer cells. J Biol Chem, 278:15056-15064.
420. Shiozawa, J., Ito, M., Nakayama, T., Nakashima, M., Kohno, S., and Sekine, I., 2000, Expression of matrix metalloproteinase-1 in human colorectal carcinoma. Mod Pathol, 13:925-933.
421. Otani, Y., Okazaki, I., Arai, M., Kameyama, K., Wada, N., Maruyama, K., Yoshino, K., Kitajima, M., Hosoda, Y., and Tsuchiya, M., 1994, Gene expression of interstitial collagenase (matrix metalloproteinase 1) in gastrointestinal tract cancers. J Gastroenterol, 29:391-7.
422. Grigioni, W. F., D'Errico, A., Fiorentino, M., Baccarini, P., Onisto, M., Caenazzo, C., Stetler-Stevenson, W. G., Garbisa, S., and Mancini, A. M., 1994, Gelatinase A (MMP-2) and its mRNA detected in both neoplastic and stromal cells of tumors with different invasive and metastatic properties. Diagn Mol Pathol, 3:163-169.
423. Kikuchi, R., Noguchi, T., Takeno, S., Kubo, N., and Uchida, Y., 2000, Immunohistochemical detection of membrane-type-1-matrix metalloproteinase in colorectal carcinoma. Br J Cancer, 83:215-8.
424. Poulsom, R., Pignatelli, M., Stetler-Stevenson, W. G., Liotta, L. A., Wright, P. A., Jeffery, R. E., Longcroft, J. M., Rogers, L., and Stamp, G. W., 1992, Stromal expression of 72 kda type IV collagenase (MMP-2) and TIMP-2 mRNAs in colorectal neoplasia. Am J Pathol, 141:389-96.
425. Bodey, B., Bodey, B., Jr., Siegel, S. E., and Kaiser, H. E., 2000, Prognostic significance of matrix metalloproteinase expression in colorectal carcinomas. In Vivo, 14:659-666.
426. McDonnell, S., Navre, M., Coffey, R. J., Jr., and Matrisian, L. M., 1991, Expression and localization of the matrix metalloproteinase pump-1 (MMP- 7) in human gastric and colon carcinomas. Mol Carcinog, 4:527-33.
427. Adachi, Y., Yamamoto, H., Itoh, F., Arimura, Y., Nishi, M., Endo, T., and Imai, K., 2001, Clinicopathologic and prognostic significance of matrilysin expression at the invasive front in human colorectal cancers. Int J Cancer, 95:290-294.
428. Ring, P., Johansson, K., Hoyhtya, M., Rubin, K., and Lindmark, G., 1997, Expression of tissue inhibitor of metalloproteinases TIMP-2 in human colorectal cancer--a predictor of tumour stage. Br J Cancer, 76:805-811.

429. Porte, H., Chastre, E., Prevot, S., Nordlinger, B., Empereur, S., Basset, P., Chambon, P., and Gespach, C., 1995, Neoplastic progression of human colorectal cancer is associated with overexpression of the stromelysin-3 and BM-40/SPARC genes. Int J Cancer, 64:70-5.
430. Yang, W., Arii, S., Gorrin-Rivas, M. J., Mori, A., Onodera, H., and Imamura, M., 2001, Human macrophage metalloelastase gene expression in colorectal carcinoma and its clinicopathologic significance. Cancer, 91:1277-1283.
431. Ohtani, H., Motohashi, H., Sato, H., Seiki, M., and Nagura, H., 1996, Dual over-expression pattern of membrane-type metalloproteinase-1 in cancer and stromal cells in human gastrointestinal carcinoma revealed by in situ hybridization and immunoelectron microscopy. Int J Cancer, 68:565-70.
432. Ahokas, K., Lohi, J., Lohi, H., Elomaa, O., Karjalainen-Lindsberg, M. L., Kere, J., and Saarialho-Kere, U., 2002, Matrix metalloproteinase-21, the human orthologue for XMMP, is expressed during fetal development and in cancer. Gene, 301:31-41.
433. Davidson, B., Reich, R., Berner, A., Givant-Horwitz, V., Goldberg, I., Risberg, B., Kristensen, G. B., Trope, C. G., Bryne, M., Kopolovic, J., and Nesland, J. M., 2001, Ovarian carcinoma cells in serous effusions show altered MMP-2 and TIMP- 2 mRNA levels. Eur J Cancer, 37:2040-2049.
434. Paju, A., Sorsa, T., Tervahartiala, T., Koivunen, E., Haglund, C., Leminen, A., Wahlstrom, T., Salo, T., and Stenman, U. H., 2001, The levels of trypsinogen isoenzymes in ovarian tumour cyst fluids are associated with promatrix metalloproteinase-9 but not promatrix metalloproteinase-2 activation. Br J Cancer, 84:1363-1371.
435. Westerlund, A., Apaja-Sarkkinen, M., Hoyhtya, M., Puistola, U., and Turpeenniemi-Hujanen, T., 1999, Gelatinase A-immunoreactive protein in ovarian lesions- prognostic value in epithelial ovarian cancer. Gynecol Oncol, 75:91-98.
436. Davidson, B., Goldberg, I., Gotlieb, W. H., Kopolovic, J., Ben-Baruch, G., Nesland, J. M., Berner, A., Bryne, M., and Reich, R., 1999, High levels of MMP-2, MMP-9, MT1-MMP and TIMP-2 mRNA correlate with poor survival in ovarian carcinoma. Clin Exp Metastasis, 17:799-808.
437. Naylor, M. S., Stamp, G. W., Davies, B. D., and Balkwill, F. R., 1994, Expression and activity of MMPS and their regulators in ovarian cancer. Int J Cancer, 58:50-56.
438. Tanimoto, H., Underwood, L. J., Shigemasa, K., Parmley, T. H., Wang, Y., Yan, Y., Clarke, J., and O'Brien, T. J., 1999, The matrix metalloprotease pump-1 (MMP-7, Matrilysin): A candidate marker/target for ovarian cancer detection and treatment. Tumour Biol, 20:88-98.
439. Huang, L. W., Garrett, A. P., Bell, D. A., Welch, W. R., Berkowitz, R. S., and Mok, S. C., 2000, Differential expression of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 protein and mRNA in epithelial ovarian tumors. Gynecol Oncol, 77:369-376.
440. Davidson, B., Goldberg, I., Berner, A., Nesland, J. M., Givant-Horwitz, V., Bryne, M., Risberg, B., Kristensen, G. B., Trope, C. G., Kopolovic, J., and Reich, R., 2001, Expression of membrane-type 1, 2, and 3 matrix metalloproteinases messenger RNA in ovarian carcinoma cells in serous effusions. Am J Clin Pathol, 115:517-524.
441. Mueller, J., Brebeck, B., Schmalfeldt, B., Kuhn, W., Graeff, H., and Hofler, H., 2000, Stromelysin-3 expression in invasive ovarian carcinomas and tumours of low malignant potential. Virchows Arch, 437:618-24.
442. Tsukifuji, R., Tagawa, K., Hatamochi, A., and Shinkai, H., 1999, Expression of matrix metalloproteinase-1, -2 and -3 in squamous cell carcinoma and actinic keratosis. Br J Cancer, 80:1087-91.
443. Shimada, T., Nakamura, H., Yamashita, K., Kawata, R., Murakami, Y., Fujimoto, N., Sato, H., Seiki, M., and Okada, Y., 2000, Enhanced production and activation of progelatinase A mediated by membrane-type 1 matrix metalloproteinase in human oral squamous cell carcinomas: implications for lymph node metastasis. Clin Exp Metastasis, 18:179-188.
444. Pyke, C., Ralfkiaer, E., Huhtala, P., Hurskainen, T., Dano, K., and Tryggvason, K., 1992, Localization of messenger RNA for Mr 72,000 and 92,000 type IV collagenases in human skin cancers by in situ hybridization. Cancer Res, 52:1336-41.
445. Airola, K., Johansson, N., Kariniemi, A. L., Kahari, V. M., and Saarialho-Kere, U. K., 1997, Human collagenase-3 is expressed in malignant squamous epithelium of the skin. J Invest Dermatol, 109:225-231.
446. Karelina, T. V., Goldberg, G. I., and Eisen, A. Z., 1994, Matrilysin (PUMP) correlates with dermal invasion during appendageal development and cutaneous neoplasia. J Invest Dermatol, 103:482-7.
447. Stahle-Backdahl, M., and Parks, W. C., 1993, 92-kd gelatinase is actively expressed by eosinophils and stored by neutrophils in squamous cell carcinoma. Am J Pathol, 142:995-1000.
448. Kerkela, E., Ala-aho, R., Lohi, J., Grenman, R., V, M. K., and Saarialho-Kere, U., 2001, Differential patterns of stromelysin-2 (MMP-10) and MT1-MMP (MMP-14) expression in epithelial skin cancers. Br J Cancer, 84:659-69.

449. Thewes, M., Worret, W. I., Engst, R., and Ring, J., 1999, Stromelysin-3 (ST-3): immunohistochemical characterization of the matrix metalloproteinase (MMP)-11 in benign and malignant skin tumours and other skin disorders. Clin Exp Dermatol, 24:122-126.
450. Kerkela, E., Ala-Aho, R., Jeskanen, L., Rechardt, O., Grenman, R., Shapiro, S. D., Kahari, V. M., and Saarialho-Kere, U., 2000, Expression of human macrophage metalloelastase (MMP-12) by tumor cells in skin cancer. J Invest Dermatol, 114:1113-9.
451. Impola, U., Toriseva, M., Suomela, S., Jeskanen, L., Hieta, N., Jahkola, T., Grenman, R., Kahari, V. M., and Saarialho-Kere, U., 2003, Matrix metalloproteinase-19 is expressed by proliferating epithelium but disappears with neoplastic dedifferentiation. Int J Cancer, 103:709-716.
452. Nakamura, H., Fujii, Y., Inoki, I., Sugimoto, K., Tanzawa, K., Matsuki, H., Miura, R., Yamaguchi, Y., and Okada, Y., 2000, Brevican is degraded by matrix metalloproteinases and aggrecanase-1 (ADAMTS4) at different sites. J Biol Chem, 275:38885-90.
453. Fernandez-Patron, C., Radomski, M. W., and Davidge, S. T., 1999, Vascular matrix metalloproteinase-2 cleaves big endothelin-1 yielding a novel vasoconstrictor. Circ Res, 85:906-911.
454. Rodriguez-Manzaneque, J. C., Milchanowski, A. B., Dufour, E. K., Leduc, R., and Iruela-Arispe, M. L., 2000, Characterization of METH-1/ADAMTS1 processing reveals two distinct active forms. J Biol Chem, 275:33471-9.
455. Agostini, C., Trentin, L., Facco, M., Sancetta, R., Cerutti, A., Tassinari, C., Cimarosto, L., Adami, F., Cipriani, A., Zambello, R., and Semenzato, G., 1996, Role of IL-15, IL-2, and their receptors in the development of T cell alveolitis in pulmonary sarcoidosis. J Immunol, 157:910-918.
456. Ogata, Y., Enghild, J. J., and Nagase, H., 1992, Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. J Biol Chem, 267:3581-3584.
457. Lijnen, H. R., and Collen, D., 1990, Serine proteases and their serpin inhibitors in the nervous system. Plenum Press, New York.
458. Ugwu, F., Van Hoef, B., Bini, A., Collen, D., and Lijnen, H. R., 1998, Proteolytic cleavage of urokinase-type plasminogen activator by stromelysin-1 (MMP-3). Biochemistry, 37:7231-7236.
459. Preece, G., Murphy, G., and Ager, A., 1996, Metalloproteinase-mediated regulation of L-selectin levels on leucocytes. J Biol Chem, 271:11634-40.
460. Imai, K., Yokohama, Y., Nakanishi, I., Ohuchi, E., Fujii, Y., Nakai, N., and Okada, Y., 1995, Matrix metalloproteinase 7 (matrilysin) from human rectal carcinoma cells. Activation of the precursor, interaction with other matrix metalloproteinases and enzymic properties. J Biol Chem, 270:6691-6697.
461. Agnihotri, R., Crawford, H. C., Haro, H., Matrisian, L. M., Havrda, M. C., and Liaw, L., 2001, Osteopontin, a novel substrate for matrix metalloproteinase-3 (stromelysin-1) and matrix metalloproteinase-7 (matrilysin). J Biol Chem, 276:28261-28267.
462. Fernandez-Patron, C., Zouki, C., Whittal, R., Chan, J. S., Davidge, S. T., and Filep, J. G., 2001, Matrix metalloproteinases regulate neutrophil-endothelial cell adhesion through generation of endothelin-1. Faseb J, 15:2230-2240.
463. Ochieng, J., Fridman, R., Nangia-Makker, P., Kleiner, D. E., Liotta, L. A., Stetler-Stevenson, W. G., and Raz, A., 1994, Galectin-3 is a novel substrate for human matrix metalloproteinases-2 and -9. Biochemistry, 33:14109-14114.
464. Nicholson, R., Murphy, G., and Breathnach, R., 1989, Human and rat malignant-tumor-associated mRNAs encode stromelysin-like metalloproteinases. Biochemistry, 28:5195-203.
465. Nakamura, H., Fujii, Y., Ohuchi, E., Yamamoto, E., and Okada, Y., 1998, Activation of the precursor of human stromelysin 2 and its interactions with other matrix metalloproteinases. Eur J Biochem, 253:67-75.
466. Knauper, V., Smith, B., Lopez-Otin, C., and Murphy, G., 1997, Activation of progelatinase B (proMMP-9) by active collagenase-3 (MMP- 13). Eur J Biochem, 248:369-73.
467. Banda, M. J., Clark, E. J., and Werb, Z., 1983, Selective proteolysis of immunoglobulins by mouse macrophage elastase. J Exp Med, 157:1184-1196.
468. Knauper, V., Cowell, S., Smith, B., Lopez-Otin, C., O'Shea, M., Morris, H., Zardi, L., and Murphy, G., 1997, The role of the C-terminal domain of human collagenase-3 (MMP-13) in the activation of procollagenase-3, substrate specificity, and tissue inhibitor of metalloproteinase interaction. J Biol Chem, 272:7608-16.
469. d'Ortho, M. P., Clerici, C., Yao, P. M., Delacourt, C., Delclaux, C., Franco-Montoya, M. L., Harf, A., and Lafuma, C., 1997, Alveolar epithelial cells in vitro produce gelatinases and tissue inhibitor of matrix metalloproteinase-2. Am J Physiol, 273:663-675.
470. Deryugina, E. I., Ratnikov, B. I., Postnova, T. I., Rozanov, D. V., and Strongin, A. Y., 2002, Processing of integrin alpha(v) subunit by membrane type 1 matrix metalloproteinase stimulates migration of breast carcinoma cells on vitronectin and enhances tyrosine phosphorylation of focal adhesion kinase. J Biol Chem, 277:9749-56.
471. d'Ortho, M. P., Will, H., Atkinson, S., Butler, G., Messent, A., Gavrilovic, J., Smith, B., Timpl, R.,

Zardi, L., and Murphy, G., 1997, Membrane-type matrix metalloproteinases 1 and 2 exhibit broad-spectrum proteolytic capacities comparable to many matrix metalloproteinases. Eur J Biochem, 250:751-757.

472. Morrison, C. J., Butler, G. S., Bigg, H. F., Roberts, C. R., Soloway, P. D., and Overall, C. M., 2001, Cellular activation of MMP-2 (gelatinase A) by MT2-MMP occurs via a TIMP-2-independent pathway. J Biol Chem, 276:47402-47410.
473. Shofuda, K., Yasumitsu, H., Nishihashi, A., Miki, K., and Miyazaki, K., 1997, Expression of three membrane-type matrix metalloproteinases (MT-MMPs) in rat vascular smooth muscle cells and characterization of MT3-MMPs with and without transmembrane domain. J Biol Chem, 272:9749-9754.
474. Wang, Y., Johnson, A. R., Ye, Q. Z., and Dyer, R. D., 1999, Catalytic activities and substrate specificity of the human membrane type 4 matrix metalloproteinase catalytic domain. J Biol Chem, 274:33043-33049.
475. Stracke, J. O., Fosang, A. J., Last, K., Mercuri, F. A., Pendas, A. M., Llano, E., Perris, R., Di Cesare, P. E., Murphy, G., and Knauper, V., 2000, Matrix metalloproteinases 19 and 20 cleave aggrecan and cartilage oligomeric matrix protein (COMP). FEBS Lett, 478:52-56.
476. Yang, M., and Kurkinen, M., 1998, Cloning and characterization of a novel matrix metalloproteinase (MMP), CMMP, from chicken embryo fibroblasts. CMMP, Xenopus XMMP, and human MMP19 have a conserved unique cysteine in the catalytic domain. J Biol Chem, 273:17893-17900.
477. Velasco, G., Cal, S., Merlos-Suarez, A., Ferrando, A. A., Alvarez, S., Nakano, A., Arribas, J., and Lopez-Otin, C., 2000, Human MT6-matrix metalloproteinase: identification, progelatinase A activation, and expression in brain tumors. Cancer Res, 60:877-882.
478. de Coignac, A. B., Elson, G., Delneste, Y., Magistrelli, G., Jeannin, P., Aubry, J. P., Berthier, O., Schmitt, D., Bonnefoy, J. Y., and Gauchat, J. F., 2000, Cloning of MMP-26. A novel matrilysin-like proteinase. Eur J Biochem, 267:3323-3329.
479. Lohi, J., Wilson, C. L., Roby, J. D., and Parks, W. C., 2001, Epilysin, a novel human matrix metalloproteinase (MMP-28) expressed in testis and keratinocytes and in response to injury. J Biol Chem, 276:10134-44.
480. Balbin, M., Fueyo, A., Knauper, V., Lopez, J. M., Alvarez, J., Sanchez, L. M., Quesada, V., Bordallo, J., Murphy, G., and Lopez-Otin, C., 2001, Identification and enzymatic characterization of two diverging murine counterparts of human interstitial collagenase (MMP-1) expressed at sites of embryo implantation. J Biol Chem, 276:10253-10262.
481. Hahn-Dantona, E. A., Aimes, R. T., and Quigley, J. P., 2000, The isolation, characterization, and molecular cloning of a 75-kDa gelatinase B-like enzyme, a member of the matrix metalloproteinase (MMP) family. An avian enzyme that is MMP-9-like in its cell expression pattern but diverges from mammalian gelatinase B in sequence and biochemical properties. J Biol Chem, 275:40827-40838.

Chapter 7

Role of Brain Microenvironment in Brain Metastases

J. Grunfeld and V.K. Puduvalli
Department of Neuro-Oncology, University of Texas M.D. Anderson Cancer Center, Houston, TX, USA

Abstract: The development of brain metastasis portends a grave prognosis for patients with systemic cancer. Efforts to alter the course of this disease have been hampered by a poor understanding of the biology of the metastatic process. Recent insights into the biologic determinants of this process aided by advances in molecular biology and biotechnology have altered the basic concepts of our understanding of how cancer cells metastasize to distant organs. These findings have validated and extended the "seed and soil" hypothesis emphasizing a critical role for the microenvironment of the target organ in the development of metastatic lesions. The brain microenvironment has unique characteristics that distinguish it from other organs of the body. Hence, therapeutic strategies to target the interaction between the metastatic tumor cell and the brain require a clear understanding of the molecular and anatomic features that influence this process. Recent studies have revealed an intricate and often facilitatory interaction between these elements of the brain metastatic process. These findings may allow the development of targeted therapies that in combination with therapeutic strategies against systemic malignancies hold promise to improve the prognosis of patients with brain metastases.

Key words: Brain, metastasis, microenvironment, molecular mediators

1. INTRODUCTION

Brain metastases are the most common malignancies affecting the nervous system, and their incidence far outnumbers the incidence of primary brain tumors (1). In autopsy series, intracranial metastases (symptomatic or undetected) have been demonstrated in 24% of all cancer patients examined (2). The disease confers significant mortality and morbidity. Median survival from the time of detection is 4 weeks in the absence of therapeutic intervention(s), with death resulting from intracranial disease progression. (3, 4) Even with advances in current treatments, the overall median survival remains in the range of 3-6 months. (5-7) The morbidity associated with brain metastases results from the progressive development of neurologic and systemic symptoms (8). Many therapeutic approaches have attempted to alter the course of the disease, but they have only minimally affected the overall course of the malignancy and the prognosis of the patient. The privileged status of the brain created by the blood–brain barrier (BBB), the co-existence of progressive systemic and intracranial disease that can obscure morbidity due to brain disease, and the limited understanding of the biology of metastatic disease processes have hindered the development of meaningful therapeutic advances. Understanding the biology of the metastatic process has in part been limited by difficulties in obtaining brain metastatic tumor tissue, which would enable researchers to study the determinants underlying the biologic behavior of brain metastases. There is also a paucity of investigators whose preclinical and translational studies are predominantly focused on understanding the brain metastatic process. In

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 127-147.

addition, most clinical trials of new anticancer agents in humans exclude from enrollment patients with brain metastases, which precludes appreciation of the effects of the agents being assessed against this disease process. Although in some malignancies the occurrence of brain metastasis is an early event, possibly due to intrinsic biologic characteristics of the primary tumor, in most cases, the appearance of metastatic lesions in the brain occurs only in the late stages of disease. The progressive increase in overall tumor burden overwhelms natural biologic boundaries that normally insulate the brain from such events. Because of the overlapping effects of systemic and intracranial disease, clinical trial designs are required to be increasingly complex and the outcomes are difficult to measure. Furthermore, recent advances in molecular pharmacotherapeutics and biotechnology have been translated into improved control of the underlying systemic disease, making it increasingly likely that disease sheltered in protected sites such as the brain could become a more relevant determinant of patient prognosis than the primary disease. It is thus imperative to gain insights into the biology of brain metastasis so that new and rational therapeutic approaches can be developed for controlling this disease.

2. EVOLUTION IN THE CONCEPTS OF THE METASTATIC PROCESS

The initial concept was that metastasis develops from tumor cells that are shed from a primary lesion into the circulation, followed by passive transfer of the cells until they are arrested in the capillaries of target organs where they establish new disease foci (9). It is possible that in the past the lack of effective treatments resulted in uncontrolled disease progression, which rapidly increased the overall disease burden. In such a setting, widespread metastases are common and organ selectivity may be less apparent. Generally, these lesions localize to the gray-white matter junction, most frequently in watershed regions of the brain's blood supply (10, 11). This pathologic pattern of distribution is invoked to support the common notion that the spread of brain metastases is primarily hematogenic. However, more than a century ago, it was observed that the occurrence of metastases did not follow simple rules based on anatomy or blood supply. The inference was that factors critical to the development of metastases were related to the tissue of origin as well as to the target tissue (12). For brain metastases, this idea evolved into the intriguing theory that not only are specific cells in the primary tumor primed to metastasize to the brain but that there may also be cooperation between metastatic tumor cells and the brain microenvironment that helps to establish metastatic tumor foci in the brain. This concept has been strengthened by the observation that some malignancies have a higher predilection than others to metastasize to the brain. It is now well accepted that brain metastatic disease is the result of several combined factors, including the tissue of origin of the primary tumor, biologic factors related to the phenotype of the involved tumor cells, and the brain microenvironment. Together, these factors strongly influence host tissue-tumor cell interactions, and anatomic and physiologic mediators that regulate the transport and physical arrest of metastatic cells. A better understanding of the biology of this process has opened the door for developing targeted therapeutic interventions, an area of interest that has been intensively investigated in recent years. This chapter is an overview of some of the recent advances in the field of brain tumor metastasis, with reference to the various molecular factors relevant to this process, and it also examines how a better understanding of these factors is helping in the effort to conceptualize and develop novel therapeutic approaches for more effectively managing brain metastases.

3. THE BRAIN MICROENVIRONMENT – RELEVANCE TO METASTASIS

Based on the concept that "the distribution of the secondary growths is not a matter of chance", Stephen Paget proposed a "seed and soil" hypothesis, which suggested that intrinsic characteristics of both the metastasizing cells and the host tissue were critical to the establishment and advancement of metastatic disease (12). Clinical

observation supports the concept that malignancies have a predilection to metastasize to specific target organs and that the number and frequency of occurrence of such lesions vary widely among individuals. Importantly, the distribution of metastatic lesions in various organs in the body is not proportional either to their total vasculature or total endothelial surface area. These observations suggest the existence of specific intercellular interactions due to the biology of the involved tumor cells as well as from the "readiness" of the microenvironment in the target organ to "receive" these cells. Preclinical studies of the interactions between tumor cells and their microenvironment support this mutual dependence between individual cells and individual physiologic environments. Such early concepts evolved into a more comprehensive view of the metastatic process, which now incorporates *mechanical factors* such as blood flow sludging, platelet-related interactions, *physiologic factors* such as hemodynamic changes, pH regulation, oxygen concentration, and metabolic demand, *biologic factors* such as expression of adhesion molecules and receptors in the target organ, and *molecular characteristics* of metastatic tumor cells related to their intrinsic biology and their tissue of origin. Despite the apparently insurmountable nature of these complex interactions for developing therapeutic approaches, insights into the biology of the metastatic process are facilitating the development of targeted approaches to treatment.

4. THE BRAIN MICROENVIRONMENT-STRUCTURAL AND FUNCTIONAL CONSIDERATIONS

Overview. As is true for metastatic disease in general, metastases to the brain are influenced by the anatomy and physiology of the target organ. The lymphatic system that places a significant role in metastasis in other organs is absent in the brain. Direct extension of tumor into the brain from adjacent structures is an unusual occurrence. When it occurs, it often results in compression and displacement of the brain rather than infiltration. Thus, most metastatic cells reach the brain through its rich blood supply via extensive capillary beds that serve areas of high metabolic activity. The subset of these cells that form tumor foci in the brain traverse the microvasculature and eventually arrest in the terminal branches of the small capillaries supplying the brain by various physiologic and molecular mechanisms discussed in detail later in this section. The cells traverse the vascular endothelium, cross the blood–brain barrier, and migrate by following specific microenvironmental cues that determine the final site of tumor growth. In brain, tumor cells proliferate to form a nidus that continuously interacts with the brain microenvironment. The small metastatic mass continues to proliferate until it reaches a critical mass beyond which its oxygen requirement cannot be sustained by diffusion and leads to progressive hypoxia. Cells susceptible to these insults may perish whereas others resort to anaerobic metabolism, resulting in the generation of acidosis and, subsequently, necrosis. These events generate angiogenic signals that promote the growth and establishment of a fresh network of collateral blood vessels, which then supply the mass. This event triggers renewed proliferation in the tumor and changes in vascular permeability. The resultant extensive edema eventually causes displacement, infiltration and local destruction of the brain tissue. Clinical symptoms ensue because of these local effects, declaring the presence of the metastatic lesion.

Vasculature of the Brain. The blood supply to the brain is derived from the "anterior'" circulation comprised of the two internal carotid arteries, and the "posterior" circulation formed by the two vertebral arteries that communicate at the base of the brain via the circle of Willis and divide into numerous branches within the brain. The middle cerebral arteries supplies the frontoparietotemporal regions and the anterior cerebral arteries supply the medial frontoparietal of the brain above the tentorium; the vertebral arteries, on the other hand, enter the posterior fossa, supplying the brainstem and cerebellum as well as parts of the parieto-occipital and medial temporal regions of the brain. The main arteries enter the subarachnoid space and

branch extensively, forming rich anastamoses at the pial surface before forming pial vessels and arterioles that penetrate the brain substance. These long and medullary arteries traverse through the cortex and penetrate the subjacent white matter without inter-communication, thus forming small independent vascular systems within the cortex. The terminal branches of these blood vessels are the capillaries (< 10μm in diameter) that form a rich network of anastamoses in the white matter (13). It has been estimated that the surface area of the brain microvasculature is approximately 100 cm^2 per gram of tissue (14). Subcortical arteries that enter the white matter coil, loop and spiral in large adventitious spaces, giving off few neocortical branches, and dispersing within the white matter. These loops may have areas of turbid flow and potentially function as mechanical traps for circulating tumor cells (15). After numerous anastamoses become established among capillaries within the substance of the brain, draining venules and veins form. These subsequently converge into cerebral venous sinuses that exit the brain via the jugular veins. In addition to the anatomic features described above, the brain microvasculature is subject to elaborate physiologic controls based on local metabolic demands and is capable of modulating flow in response to such stimuli.

The Blood–Brain Barrier. The endothelium lining the blood vessels forms the first, and possibly the most significant, barrier that a metastatic cell encounters upon entering the nervous system. The BBB refers to the highly specialized boundary between blood and the brain substance and is composed primarily of nonleaky-type tight junctions between capillary endothelial cells. These junctions are reinforced by pericytes, astrocytic foot processes, and joint basal laminae. These components function together as a complex filtering mechanism that mechanically restricts large molecules, infectious agents, and cells from infiltrating the substance of the brain. In addition, this system dynamically controls the entry of diverse molecules, drugs or toxins through receptor systems, specialized channels, and via other poorly understood active filtering processes.

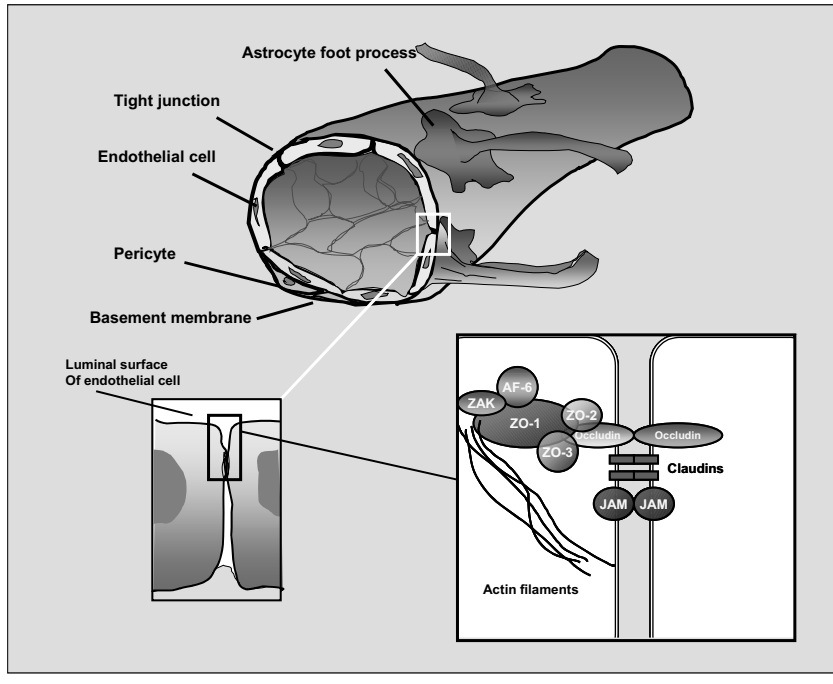


Figure 1. Ultrastructure of the Blood-Brain Barrier.

The molecular composition of the BBB is not fully understood but recent studies have provided insights into the specialized nature of the molecular architecture and dynamics of this unique barrier (Figure 1). The endothelial cells, which are the main cellular component of the BBB, have characteristic intercellular regions of apposing contact called tight junctions (zona occludens) that are relatively specific to the BBB and provide regions of high electric resistance that result in low permeability (16). By their presence in the apical (luminal) regions of the endothelial cell-cell contact zone, they form a continuous paracellular barrier, sealing the endothelial surface and forming the most restrictive element of the BBB. They are composed of a complex combination of proteins, including several transmembrane proteins such as claudin, occludin, and junctional adhesion molecules (JAM) that are organized around an actin cytoskeletal matrix (17, 18). Being located in the apical regions of the intercellular clefts towards the luminal surface of the endothelial cell, these proteins can interact with various adhesion molecules present in circulating cells, and regulate adhesion and migration of leukocytes, platelets and possibly tumor cells. On the intracellular side, the transmembrane proteins in turn intimately interact with the cytoskeletal proteins, including actin filaments and several accessory cytoplasmic proteins such as zona occludens-1and 2 (ZO-1 and ZO-2) proteins, cingulin, 7H6, Rap and AF-6 proteins, which are organized around scaffolding proteins (19). In addition to these structural considerations, it is known that phosphorylation of the transmembrane and accessory proteins can rapidly regulate tight junction function and hence affect permeability across the BBB. For example, phosphorylation and de-phosphorylation of claudin changes the structural integrity of the tight junction, usually improving the assembly of the junction. It has also been noted that tyrosine phosphorylation of existing tight junction proteins can decrease occludin expression, leading to increased permeability (20). Conversely, in mature junctions and well-formed cell-cell contacts, there is reduced tyrosine phosphorylation of proteins involved in cell-to-cell contact (21). These regulatory processes are important in modifying the integrity of the BBB and putatively influence its interaction with components in the blood such as metastatic tumor cells.

Under physiologic conditions, cellular components of the blood cannot traverse the brain microvascular endothelium. However, pathologic alterations of the BBB can break down its barrier functions, allowing proinflammatory mediators, such as reactive oxygen species and cytokines, to induce upregulation of surface adhesion molecules such as PECAM-1, E-selectin, and ICAM-1. More adhesive properties are thus activated on the endothelial surface so that circulating cells, including malignant cells, are able to adhere to these surfaces. The concept that cancer triggers an inflammatory reaction in the brain in response to injury is also pertinent to this issue (22). If this idea is correct, cell complexes composed of tumor cells and activated platelets can arrest in the brain microvasculature and induce the release of cytokines. This, in turn, can initiate an injury reaction, which can facilitate the entry of tumor cells into the brain. In addition, endothelial cell growth factors can regulate tight junction components, causing alterations in BBB permeability. The vascular endothelial growth factor, fibroblast growth factor, tumor necrosis factor, interleukins, and interferons are some of the proteins that are commonly associated with malignancies and that can affect the BBB. These observations highlight the fact that the BBB is an intricate and tightly regulated structure that can be influenced by various factors that may be involved in the malignant process. Of particular relevance, malignant cells have to adhere to and transgress the endothelium of the brain before they can establish a metastatic focus, making this event a critical step in the metastatic process.

The Brain Interstitium and Initial Growth of the Metastatic Focus. Metastatic brain lesions have a predilection to localize to the junction between the gray and white matter (11). This region of the brain coincides with the vascular border zone where blood vessels form whorls and loops that are believed to produce hemodynamic circumstances that favor the adhesion of metastatic cells. Although there is evidence of migration and invasion of tumor cells once the initial nidus is formed, radiologic and histopathologic data support the fact that most

metastatic lesions occur as spherical masses that grow locally at the initial site of tumor foci arrest in the brain. Recent studies also support the theory that tumor cells can adhere to the vascular endothelium and proliferate within the blood vessel, forming tumor masses even prior to entry into the parenchyma of target organs, including the brain (23). Following this event, the tumor cells physically disrupt the BBB, allowing cellular entry into the parenchyma where a larger tumor focus is established through secondary growth. Conjecturally, such a mechanism could also activate cell signaling pathways associated with injury and inflammation. This activated cascade induces degradation of local tissue and expression of molecules that promote the processes of migration of tumor cells into the brain substance, subsequent local invasion, and the initiation of angiogenesis. It is also known that tumor cells can interact with platelets, forming cellular aggregates that can adhere to the endothelium of capillaries (24, 25). If such aggregates induce regional ischemia is uncertain, but plausible. Metastatic lesions often present with high signal on diffusion-weighted MRI images, a fact that is attributed to edema and related "T2 shine-through." However, these regions could also potentially indicate the existence of small areas of local ischemia at the point where tumor cells lodge within the small capillaries. If aggregates such as these are able to enter the deep, penetrating branches of the cerebral circulation and induce ischemia, yet another mechanism is available for creating alterations in the BBB and producing a route of entry for tumor cells into the brain. Supporting this possibility, Doi et al. showed that experimentally induced ischemia can increase the number of metastatic lesions in the liver from colon cancer in association with an increase in E-selectin expression (26). Other studies using the same ischemia model have shown that tumor cells overexpressing Galectin-3, a β-galactoside binding protein, efficiently form metastatic liver lesions compared with control tumor cells (27). Although these mechanisms are theoretically plausible as influencing the formation of brain metastases, a survey of the literature reveals few studies directed towards explicating these putative mechanisms. Hence, their relevance to the establishment of brain metastases remains to be determined.

Once the metastatic cell traverses the endothelium, it enters the brain interstitium, a complex but poorly understood environment in which subsequent tumor growth occurs. Sulfated matrix proteoglycans, composed predominantly of heparan sulfate and to a lesser extent, chondroitin and dermatan sulfates, form a major constituent of the brain extracellular matrix (ECM). Proteoglycans intimately interact with and are subject to degradation by invading metastatic tumor cells. In vitro studies using brain metastatic melanoma cells and brain endothelial cells have shown that these two cell populations can cooperate in producing heparanase, a degradative enzyme which cleaves heparan sulfate, and in concert foster local break down of the architecture of the brain matrix (28). Similarly, astrocytes can interact with metastatic melanoma cells and induce heparanase production, again promoting matrix degradation (29). Marchetti et al. showed that the increased production of heparanase was mediated by the interaction between neurotrophins such as NGF and NT-3 produced by normal cells within the brain and the low affinity neurotrophin receptor, p75NTR, which is expressed by invading tumor cells (30). Other important matrix-degrading proteins such as metalloproteinases have also been strongly implicated as participating in local invasion of metastatic lesions. In a study by Okada, MMP-2 and MT1-MMP expression was localized to the tumor cells and gelatinolytic activity was seen within nests of metastatic carcinoma cells by in situ zymography, strongly suggesting a role for these processes in local degradation of the ECM (31).

In addition to remodeling of the brain ECM by invading metastatic cells, the establishment of the initial tumor focus requires the recruitment of autocrine and paracrine signals, including various growth factors, into the regional environment. Our knowledge of these events is mainly derived from studies of brain metastases from melanoma in which tumor cells were shown to elaborate various factors such as TGF-α, TGF-β, βFGF and IL-1β. These factors are postulated not only to keep tumor cells alive by autocrine and paracrine mechanisms but also to induce the production of heparanase, which

contributes to matrix degradation (32). It has also been shown that a paracrine form of transferrin may play a role in establishing brain metastases, particularly because brain-metastatic cells express high levels of transferrin receptors, which can bind low levels of transferrin in the brain parenchyma and initiate biologic changes such as increased invasion and proliferation (33). Given that in experimental models, most tumor cells extravasate but only a few cells are able to survive and establish larger tumor foci (34), factors that promote tumor survival become highly significant in the development of brain metastases.

5. THE RELEVANCE OF TUMOR SPECIFIC FACTORS

There is sufficient evidence to show that the process of metastasis occurs in distinct stages (Table 1), each of which presents a substantial barrier for the metastatic cell that it must sequentially overcome before establishing itself in the target tissue (35). The complexity of this process is marked by discrete hurdles that must be overcome by metastatic cells before they can survive and grow in the host tissue. This process highlights the important fact that cells that are destined to survive form a special subpopulation within the primary tumor that possesses intrinsic properties used to facilitate their survival (36). Accordingly, some tumors are believed to incorporate cells with intrinsic characteristics that allow them to metastasize to the brain, whereas others do not possess cells with these characteristics. Several elegant studies have shown that the metastatic process is governed at each step by pathologic molecular interactions. To establish a metastatic focus, these interactions mimic normally occurring physiologic contacts, resulting in the abnormal recruitment of molecular mediators that are normally involved in physiologic cell-to-cell interaction and that generate cell survival and proliferation signals. The degree of production and recruitment of such molecules is likely a defining characteristic of tumor cells with metastatic potential. By analyzing the rate-limiting steps in the various stages of metastases, several molecules have been identified that appear to be indispensable to tumor cells for establishing a remote malignant focus. The role of molecules necessary to promulgate metastasis may be conveniently considered in relation to the various stages of the metastatic process and may be categorized based on their normal physiologic functions in the body, such as adhesion, invasion, angiogenesis, and proliferation. The following sections outline the mediators of these molecular events that are critical for the metastatic process once a tumor cell has reached the brain.

Table 1. Brain Metastasis: Stages in development.

Stage	Role of Host tissue
Intravasation from primary site	
Transit via blood circulation	Adhesion to platelets
Host Tissue phase	
Adherence to brain endothelium	Facilitation of adhesion
Extravasation	Production of degradative enzymes
Primary growth phase	Neurotrophin interaction
Recruitment of blood supply	Response of brain vasculature
Secondary growth phase	Blood supply

6. MOLECULAR MEDIATORS OF METASTASIS

6.1 Mediators of Adhesion

Overview. A circulating tumor cell exhibits its organ specificity when it adheres to the endothelium of a target organ (37). Thus, the arrival of a tumor cell into the brain via the cerebral vasculature and its adherence to the vessel walls signals the first step of a direct interaction between the metastatic cell and the brain. This step is partly due to a *physical* arrest of the cell governed by mechanical and hemodynamic factors operant in the microvasculature of the target organ (38). Equally important in metastatic localization is the adherence of the metastatic cell to an endothelial cell via *molecular* interactions between the tumor cell and the subendothelial ECM (39). Continuous blood flow in the blood vessels of the central nervous system generates considerable shear forces and is a potent inhibitor of the adhesion of cells in the vascular component, including those derived from malignancies. To overcome these forces, a metastatic cell utilizes specific and robust molecular mechanisms involving adhesion molecules (Figure 2 A). In vivo studies using endothelial cell monolayers in mice demonstrated the specificity of interaction between tumor cells and the capillary endothelium. In this context it has been seen that tumor cells express cell adhesion molecules that are involved in normal physiologic adhesive interactions. Several such molecules have been implicated in the metastatic process, including intercellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), epithelial cell adhesion molecule (EP-CAM) (40) and selectins, which constitute a family of cell-adhesion molecules including L-selectin/CD62L, E-selectin (CD62E), and P-selectin (CD62P) (41). These molecules are known to be involved in the interaction between cellular components of the blood stream such as leukocytes and the endothelial cells lining the vasculature, but have also attracted interest into the molecular mechanisms of the malignant process. Studies of these interactions not only provide a better understanding of tumor biology, but also are of particular interest from a therapeutic standpoint.

In addition to the interaction between tumor cells and the endothelium, recent reports provide evidence that some metastatic cells may overexpress cell surface integrin receptors such as $\alpha V\beta 3$ integrin that enables them to interact with integrins on platelet surfaces such as $\alpha IIb\beta 3$ (42). This association results in the formation of microthrombi that promote cell stasis in regions of slower blood flow, enabling the tumor cell to establish contact with mediators of adhesion on the endothelium of the target organ (Figure 2 B). The interaction between cell surface integrin receptors and activated platelets requires a functionally activated subtype of $\alpha V\beta 3$ integrin. The parental tumors in one study contained a subpopulation of cells which constitutively expressed activated $\alpha V\beta 3$ integrin, suggesting that parental cells may be primed for the metastatic process if they achieve anchorage independence from the primary tumor (43, 44). A similar interaction has been described between tumor cell surface heparin sulfate proteoglycans (HSPGs) and P-selectin on platelets, resulting in adhesion of platelets to the tumor cells (45). In addition, an increased serum concentration of VCAM-1 was shown to be associated with locally advanced metastatic gastric cancer (46). Patients with these advanced cancers also had a significantly poor survival compared with patients who had normal levels of these molecules.

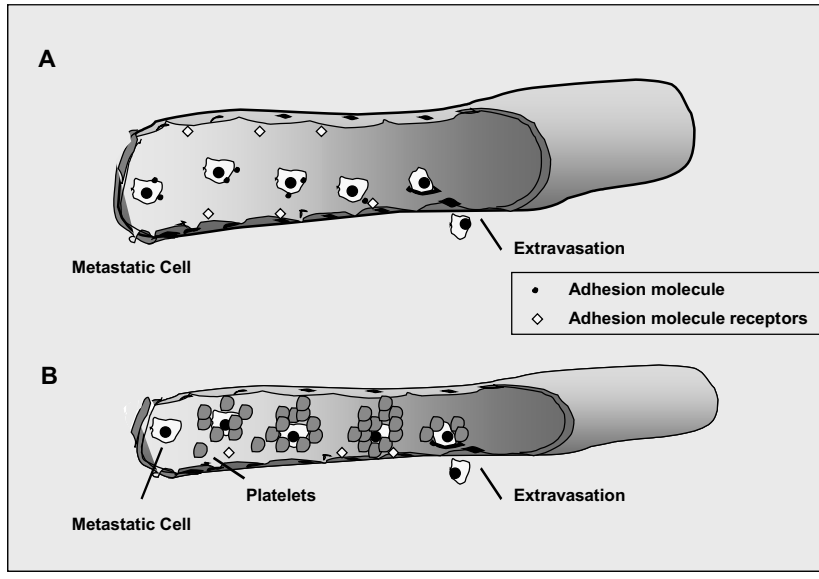


Figure 2. Mechanisms of adhesion of metastatic tumor cells to brain endothelium. A. Tumor cells express adhesion molecules on their surface, which interact with their respective receptors, B. tumor cells express molecules that promote adhesion to platelets and activate them to form microthrombi. The tumor-platelet complexes may lodge physically in the endothelium and the activated platelets may trigger changes in the blood-brain barrier that facilitate extravasation.

The specificity of interaction between tumor cells and specific organs such as the brain cannot be explained on the basis of general adhesion interactions alone because such interactions are likely to be physiologically active in several organs in a nonspecific way. As such, it is likely that molecules are expressed in the brain endothelium that specifically interact with adhesion molecules on the tumor cell surface and enable target tissue-specific adhesion of the cell as the first step in the process of metastasis. Whether such adhesion is required for metastasis to occur is controversial. A capacity for the adhesion and extravasation of tumor cells does not necessarily correlate with metastatic potential (47). However, it is clear that without adhesion and extravasation, even those cells destined to become metastatic will be unable to reach the target site. Adhesion interactions between tumor cells might, however, contribute to the overall load of tumor cells congregating in a specific metastatic site. Although the adhesion interactions between endothelial cells and tumor cells are not the sole regulators of metastasis, the interaction is an important first step that allows entry of metastatic cells into the brain.

Immunoglobulin-Like Cell Adhesion Molecules (CAMs). This family of adhesion molecules is characterized by their similarity to and evolutionary relationship with the immunoglobulin family (48). Several of these molecules have been postulated as playing a part in normal adhesion functions in the brain as well as in pathologic processes such as metastasis. Of these, the role of the neural cell adhesion molecule (NCAM) has been implicated in axonal growth and cell-cell interactions in the brain and the retina. NCAM is relevant to brain metastasis by virtue of the observation that it was constitutively expressed in melanoma cells isolated from brain metastatic lesions but not in tumors from other organs. Its expression in melanoma cells suggested that NCAM has a role as an immunoregulatory molecule during the formation of brain metastasis (49). NCAM is able to modulate metastasis by regulating tumor cell-matrix adhesion interactions; inducing FGFR-4 mediated signaling, which is responsible for

producing neurite outgrowth and matrix adhesion of tumor cells (50). Similarly, another adhesion molecule, intercellular cell adhesion molecule-1 (ICAM-1) is found to be selectively expressed in metastatic melanoma cells (not by other malignant cell types) but not in the primary lesion (51). Under physiologic conditions, ICAM-1 is expressed at low levels in the endothelial cells of the brain microvasculature (52) and the molecule plays a significant role in cell migration across the BBB, particularly leukocyte infiltration associated with inflammatory processes. Elevated levels of soluble ICAM-1 have been found in several malignancies and are related to development of angiogenesis (53). One report showed that the levels of ICAM-1 increased rapidly on the luminal surface of the endothelium when cell adhesion occurred and demonstrated an increased interaction with integrins as well as changes in protein phosphorylation and cytoskeletal reorganization Anti-CAM antibodies blocked the interaction between the tumor cell and endothelial cell. VEGF can upregulate ICAM-1 expression through the PI-3 kinase/AKT pathway (54). Blockage of the PI-3 kinase/Akt pathway with cell permeable inhibitors abolished this effect, suggesting that migratory events that might be controlled by this pathway, including interactions between metastatic cancer cells and the BBB, can potentially be disrupted. The role of CAMs in brain metastases is being further investigated and results of such studies will define the possibilities of therapeutically targeting these molecules.

Integrins. Integrins are a large family of cell surface adhesion receptors that interact with diverse intra- and extracellular stimuli to promote cell-cell interactions and related biological processes (55). They occur as heterodimers consisting of α and β subunits and exhibit a range of overlapping interactions with their ligands, which depend on the particular combination of subunits recruited (56). By virtue of their transmembrane position, they are capable of interacting externally with the ECM and internally with the cytoskeleton, thus providing a dynamic bridge for transmembrane communication between the cell and its environment. Upon interaction with ECM proteins via the Arg-Gly-Asp (RGD) motif, integrins cluster together at the point of contact and assemble cellular actin filaments, which results in the progressive, lateral recruitment of additional integrin molecules that combine to form the focal adhesions (56). In addition, integrins recruit several adaptor and signaling molecules, including focal adhesion kinase (FAK), src, Fyn, Talin, Vinculin and Paxillin (57). This activity results in the activation of the Ras, Rho and MAPK pathways, partly through the phosphatidyl inositol 3-kinase pathway, resulting in a spectrum of signals that can impact motility, cell cycle control, cell survival and proliferation (58-60). Of the integrin family members, the αv heterodimeric receptors form a distinct sub-family, which serve as vitronectin receptors (except $\alpha v\beta 6$), share the property of recognition of the RGD-motif in their ligands, and are implicated in malignancies (61). The most studied of these molecules is the $\alpha v\beta 3$ integrin, whose participation has been implicated in metastatic disease, and whose expression occurs in late stages of specific malignancies, including primary brain tumors (62). The $\alpha v\beta 3$ integrin interacts with various substrates, thus enabling tumor cells that express it to adhere to different substrates and interact with them in diverse environments (63). Cells overexpressing $\alpha v\beta 3$ integrins have an increased capacity to invade in Matrigel assays. Inducing the expression of this integrin in poorly invading cell lines increases their ability to invade. In addition, the interaction between $\alpha v\beta 3$ integrin and the ECM has been identified as an important factor for the survival of endothelial cells in newly formed blood vessels (64). VEGF-A can induce the expression and activation of $\alpha v\beta 3$ integrins, thus providing one mechanism whereby tumor cells might recruit a blood supply locally and ensure the integrity of newly formed blood vessels (65). Wang et al. (66) demonstrated that circulating tumor cells express $\alpha 3\beta 1$ integrins on their surface. These can interact with its ligand, LN-5, which in turn is expressed in areas of exposed basement membrane in the pulmonary vasculature, providing a molecular basis for occurrence of lung metastasis (66). Based on these data, small molecule inhibitors of integrins are currently in preclinical and early clinical testing against malignancies, including metastatic disease. One such agent is the cyclic RGD-motif peptide, Cilengitide (EMD121974),

which is a αvβ3 and αvβ5 integrin inhibitor (67). Cilengitide has recently completed phase I trials in humans and is entering phase II trials (68). Other agents of interest include Vitaxin, an anti-integrin humanized antibody that has entered clinical trials (69) and the RGD-peptidomimetic agents, S137 and S247, which can inhibit αvβ3, α5β1and α2β3integrins and decreased colon cancer metastasis in animal models (70).

Selectins. Selectins are a family of CAMs, which includes L-selectin (CD62L), E-selectin (CD62E) and P-selectin (CD62P), whose activities include mediating the capture of leukocytes from the blood stream as they reach the cerebrovascular endothelium. Selectins interact with vascular glycoproteins in the context of a carbohydrate structure called Sialyl Lewis x (SialylLex) (71). In the setting of metastasis, breast and lung carcinoma cells express glycoprotein molecules that can function as ligands for P-selectin such as P-selectin glycoprotein ligand-1 (PSGL-1), and CD24. Also SialylLex is abundantly expressed on the surface of epithelial malignant cells. Similarly, epithelial cell cancers express heparan sulfate-like proteoglycans, which can also function as ligands for P- and E-selectins, thus suggesting their role in metastasis (72). It has been suggested that these ligands interact with P-selectin that is expressed on circulating platelets, promoting the formation of a platelet aggregate around the tumor cell which not only protects it from the immune system but also facilitates impaction of the cells in small microcapillaries allowing adhesion to occur in the target tissue (44, 45, 73). Such selectins might also permit interaction between the tumor cells and an activated endothelium that expresses E- or P-selectins. The ability of heparin to inhibit metastasis in rodent models has been linked to its ability to inhibit P-selectin (74, 75). Several selectin inhibitors are currently in development against pathologic states other than cancer but are likely to be studied in the milieu of malignancies, especially metastases (76).

Tetraspanins. Tetraspanins constitute a superfamily of an evolutionarily conserved group of transmembrane proteins with four transmembrane domains and with surface domains that interact with various integrins and are implicated in the metastatic process (77, 78). α3β1 integrins can form complexes with tetraspanins that can control elongation of invading pseudopodia of tumor cells. These complexes have also been implicated in matrix metalloproteinase-2 (MMP-2) production, which is associated with tumor invasiveness (79). Tetraspanins act by modulating the actin cytoskeleton and assisting in degrading the surrounding ECM as the cancer cell advances through metastatic progression. By their prominent interactions with adhesion molecules such as integrins and with each other, they are involved in diverse processes such as cell activation and proliferation, adhesion and motility, differentiation, and tumorigenesis. However, their role in malignancy and metastasis is complex. Animal experiments have shown that expression of the tetraspanins CD9, CD63, or CD82 in tumor cells suppresses their metastatic potential (80, 81). In contrast, expression of CD151, which is expressed by cells with an epithelial and mesenchymal origin, increases invasion and the metastatic potential of tumor cells (82). CD151 forms stable complexes with the laminin-binding integrins α3β1, α6β1, α6β4 and α7β1 and can also associate with intracellular signaling molecules such as PKC-α and PKCβII, and the type II PI-4-kinase. The formation of such complexes may be required for the coordination of signals that regulate cell adhesion and migration. They may hence have a postulated role in brain metastasis, which remains to be defined. Lee et al. recently identified KITENIN, a novel tetraspanin, which when overexpressed, resulted in increased invasiveness and early metastasis (83). Given that tetraspanins are also widely expressed in the central nervous system, additional studies are warranted to determine the relevance of these molecules to brain metastases.

Focal adhesion kinase (FAK). FAK is a non-receptor tyrosine kinase and is detected in molecular complexes associated with focal contacts in the process of cell-cell adhesion. It is activated by tyrosine phosphorylation when ligands like vitronectin or other matrix proteins bind to integrin receptors, and is frequently associated with an

invasive phenotype (84). FAK binds to specific motifs in the cytoplasmic tail of β integrins and undergoes tyrosine phosphorylation, an interaction that is facilitated by a docking protein, Cas, which is itself activated by tyrosine phosphorylation (85). Recently, increased tyrosine phosphorylation of Cas has been shown to correlate with increased integrin-mediated cell migration in Cos cells (which are derived from the CV-1 cell line by transformation with a replication origin defective mutant of the SV40 virus). FAK is overexpressed in metastatic cells and is believed to contribute to the metastatic process by modulating invasion and motility (86). FAK is also expressed in cerebral metastases and has been found to interact with VEGF and nitric oxide signaling systems (87). VEGF increases the tyrosine phosphorylation of FAK and increases its localization to focal adhesions in endothelial cells (88), suggesting a complex interaction between angiogenesis signals and those that facilitate metastasis. In a recent study, Lu et al. reported that FAK is dephosphorylated in response to EGF treatment in human carcinomas that overexpress EGFR (89). This causes decreased activity of FAK, leading to the breakdown of focal adhesions and resulting in cells that become less adherent, more motile, and more prone to metastasis. However, following the re-adhesion of cells, FAK activity is restored via the integrin receptor pathway and the cells lose their sensitivity to EGF. This could provide a mechanism for intravasation, reattachment, and extravasation of metastatic cells.

6.2 Molecular mediators of Invasion and Angiogenesis

Overview. Once the tumor cell has adhered to the endothelium, it activates various mechanisms to enable it to traverse the endothelium and enter the brain parenchyma. Subsequently, after initial proliferation, the cells invade the brain parenchyma locally and activate angiogenic signals to form the metastatic focus. Although they are distinct biologic processes, invasion and angiogenesis share several common features and recruit the same molecular mediators. Molecules that participate in the process of angiogenesis include VEGF and its receptors, mediators of invasion such as matrix metalloproteinases (MMPs), urokinase plasminogen activator (uPA) and its receptor, and molecules involved in remodeling of ECM such as heparanase. A more detailed discussion regarding these molecules is presented in several reviews on this topic to which the reader is referred (90-93).

Matrix metalloproteinases (MMPs): Matrix metalloproteinases are a family of endopeptidases that predominantly exist in an inactive zymogen form and contain an active domain and a catalytic domain, the activity of which is zinc-dependent (91). They are elaborated by tumor cells in response to extracellular stimuli, including those from the ECM. Based on the ECM proteins that they preferentially degrade, MMPs are classified into three large groups – collagenases, stromelysins, and gelatinases (94). *Collagenases* are MMPs that act against several specific types of collagen, cleaving the proteins at defined sites into simpler products, which undergo further processing by other MMPs. S*tromelysins* form the second group of MMPs and are active in degrading various ECM substrates, including elastin, laminin, collagen and fibronectin. *Gelatinases,* the third class of MMPS, (also known as MMP-2 and MMP-9) are collagenases that have important roles in primary and metastatic brain tumor invasion, particularly because of their ability to induce degradation of the basement membrane (95).

Urokinase-like plasminogen activator (uPA) and receptor (uPAR). The uPA/uPAR system has a significant role in degrading the ECM by plasminogen activation at the cell-surface and is hence highly relevant to the malignant process especially with respect to invasion and angiogenesis. Upon binding to the uPAR, uPA initiates cleavage of plasminogen to plasmin focusing the proteolytic activity to regions of the cell that highly express the receptor such as the leading edge of migrating cells (96). This helps focal degradation of the ECM in specific locations aiding directional migration and invasion of the tumor cell. The uPA/uPAR system also cooperates with MMPs in inducing target tissue remodeling and permitting migration and invasion of metastatic cells. In addition to its extracellular tissue effects, αVβ3 integrin, the vitronectin receptor, and uPAR influence each other's expression and can

cooperate to induce adhesion and invasion (97). In glioma cells, downregulation of uPA resulted in reduced levels of phosphorylated PI3K and Akt, which are associated with decreased migration and invasion (98); this finding may also be relevant to brain metastatic cells. Similarly, down-regulation of uPAR expression in human colon carcinoma cells results in disrupted interactions with integrins and inhibiting the Erk-MAPK pathway (99). These events result in decreased invasion and migration of tumor cells, suggesting that uPAR participates in mediating an intracellular signaling pathway in invasion and, hence, metastasis. Thus, several lines of evidence suggest that the uPA/uPAR system is highly relevant to metastasis; structural analysis of the components of this system may allow targeted inhibition of this system as a therapeutic strategy against metastasis (100,101).

Heparanase and Heparan Sulfate Proteoglycans. HSPGs are important components of the endothelial basement membrane but also associate with the ECM and the cell surface. They are glycosaminoglycans composed of a core protein with multiple covalently linked heparan sulfate chains (102). The breakdown of HSPGs in the basement membrane is a critical step in the extravasation of tumor cell into the target organs. Heparanase is an endoglycosidase that degrades the heparin sulfate chains of HSPGs, thus breaching the basement membrane (BM) (103). Heparanase activity is normally seen in platelets, leukocytes, and placental trophoblasts but has also been described in melanoma, lymphoma, and prostate cancer (104). Recently, Marchetti et al. reported that astrocytes produce heparanase and potentiate the invasion of metastatic melanoma cells derived from brain metastasis (29). It is likely that heparanases and HSPGs also play an important role in brain metastasis from other cancers and in a similar manner.

Hypoxia-inducible factor 1 (HIF-1). HIF-1 is a basic-helix-loop-helix transcription factor that has essential roles in mammalian development and physiology. In its functional state, HIF-1 forms a heterodimer composed of HIF-1alpha and HIF-1beta subunits. The expression of HIF-1alpha is closely linked to cellular hypoxia and is regulated by tissue oxygen concentration (105). When the tissue growth reaches a state in which the cellular consumption of oxygen outstrips its supply, HIF-1 levels are upregulated. Under such hypoxic conditions, HIF-1-regulated genes, including those for VEGF, erythropoietin, and enzymes of the glycolytic cycles, are actively transcribed. This facilitates improved oxygen delivery or adaptation of the cell to hypoxic conditions. The hypoxic environment in growing metastatic tumors induces the overexpression of HIF-1alpha, which activates adaptive mechanisms in the tumor and induces angiogenesis (106). Interestingly, HIF-1 is degraded by a mdm2-mediated mechanism, which is regulated by p35. Loss of p53 (commonly seen in malignancies) results in the inability of the cell to degrade HIF-1 through mdm2 (43). HIF-1 is essential for neovascularization in several metastatic cancers and plays an important role in tumor growth and survival.

Vascular Endothelial Growth Factor (VEGF). VEGF has been of particular interest in regard to BBB functions and, by extension, to the metastatic process. In a mouse model of brain metastasis from breast cancer, Kim et al. isolated a population of cells from brain metastatic lesions, which demonstrated an increased propensity for metastasizing to this organ (107). They observed that these cells secreted high levels of VEGF and that chemical inhibitors of VEGF caused a decrease in brain metastasis. The authors concluded that high VEGF levels contribute to the development of brain metastasis by tumor cells. VEGF also induces reorganization of vascular endothelial cadherin, an effect that is antagonized by the inhibition of PKC, Erk or eNOS. VEGF also upregulates levels of ICAM-1 and the chemokine MIP-1 α in association with decreased association between astrocytic foot processes and the vascular endothelium, thus weakening the integrity of the BBB and increasing vascular permeability (108). Recent studies showed that the permeability of the BBB is increased in response to VEGF, a relationship that is mediated by eNOS (109). Relevant to these findings is the observation by Martinez-Estrada et al. that systemically administered erythropoetin can protect

against VEGF-induced increased permeability of the BBB by reducing the levels of eNOS and restoring the structural integrity of the tight junctions (110). Approaches similar to this may potentially provide a mechanism to inhibit the adhesion of metastatic cells to the brain endothelium, thus preventing development of brain metastases.

Chemokines and their receptors. Tumor cell metastasis shares many characteristics of leukocyte trafficking in response to inflammatory and injury-related signals. Among the molecules involved in this process, chemokines have emerged as key mediators of cell-cell interactions, which also appear to play a role in metastasis. Chemokines are a family of small, secreted molecules that function significantly in leukocyte trafficking, particularly in response to injury and inflammation, and also function as ligands to a set of chemokine receptors. They are divided into several families on the basis of their specific structures and the cysteine residue-motifs in their peptide sequence as well as the specific receptors that they engage. Chemokine receptors are seven-transmembrane domain proteins belonging to the superfamily of G protein-coupled receptors, which are highly expressed on migrating cells. Upon ligand binding, the receptors signal integrins via protein kinase C and activate migration by modulating cytoskeletal components. Of the various families of chemokines the CXC family has been specifically associated with metastases and angiogenesis. In breast cancer, SDF-1 (CXCL12), which serves as a ligand to the chemokine receptor CXCR4, is overexpressed compared with its expression in normal breast tissue (111). In vitro studies using breast cancer cells showed that SDF-1 stimulation caused PI3K activation, promoting survival signals and increased vascular permeability accompanying vascular instability. Interestingly, SDF-1-stimulated cells also showed increased migration and ability to penetrate brain microvascular endothelial cells; treatment with CXCR4-inhibiting antibodies or PI3K inhibitors abrogated this effect, suggesting a role for this chemokine receptor and the PI3K pathway in brain metastasis from breast cancer. Small molecule inhibitors against CXCR4 are currently in development in the hope that they will have the potential to be used as therapeutic agents against brain metastasis from breast cancer (112).

7. MOLECULAR PROFILING OF THE PRIMARY TUMORS AND ITS RELEVANCE TO METASTASIS

The theory that clonal selection is an underlying mechanism of tumorigenesis as well as of the evolution of tumor heterogeneity is now well accepted. Prevalent concepts that are thought to be relevant to metastasis propose that metastasis represents an overall process of genetic selection in which cells that eventually metastasize evolve during the later stages of the malignant process in a highly selective process (113). More recent evidence has suggested that the primary malignancy may contain cells that have the potential to metastasize to specific organs because of their inherent biologic characteristics rather than genetic selection. Presumably, such biologic characteristics would have to be predestined early in the evolution of the tumor so that biologic characteristics established in specific cells are triggered and allow the cells to interact with and survive in the target organ when metastasis occurs. Clearly, several other factors likely determine if these cells eventually reach target organs, including survival through primary therapies, detachment from the primary tumor focus, and ability to traverse the vascular compartment. It is important to emphasize that of the cells that are released from the primary malignancy and reach the brain, only those endowed with specific biologic characteristics can form metastases in that organ. Accordingly, it has been postulated that profiling the tumor as it exists in the primary site, either at diagnosis or at recurrence, could potentially prognosticate the potential of a given tumor to form brain metastases.

Comparing the molecular profiles of matched primary with brain-metastatic tumor tissue might reveal “signatures” in the latter that can provide clues to biologic characteristics that determine metastatic behavior. Weigelt et al. showed that the gene expression profiles of matched samples from primary breast cancer and metastatic lesions from

the breast, even if these were spawned or became apparent later in the course of the disease, were similar. The authors contended that this finding supported the concept that an inherent capacity to metastasize is a driving force behind metastasis rather than metastasis reflecting a process of individual genetic selection (114). Interestingly, these authors found that differences in microenvironment did not appear to affect this tissue similarity, pointing to a primary characteristic inherent in the tumor cell that causes it to metastasize and grow in a distant organ. The authors did not address the possibility that the cells could have metastasized early, remained dormant until favorable circumstances arose and thus established secondary foci later in the disease course. In another interesting study using infrared DNA spectra, Malins et al. compared the DNA base and backbone structure of histologically normal prostate tissue with matched prostate cancer tissue from patients with and without metastatic disease (115). Based on similarities between the DNA structure of histologically normal tissue and metastasizing primary tumor in matched samples, they suggested that the metastatic and primary phenotypes evolve independently, again suggesting the early emergence of cells with metastatic characteristics. They also found that histologically normal tissue from patients with metastasizing tumor had similar DNA structures and proposed that the metastatic potential was in progenitor cells, with metastatic features "hardwired" into the DNA.

Other investigators also demonstrated that cells that metastasized to specific organs bear characteristics that facilitate their localization to those specific organs and that these physiologic traits are distinct from the non-metastatic components of the primary tissue. In breast cancer cells metastatic to the brain (but not in cells from primary tissue), Nishizuka et al. found that several cytokine receptors were upregulated that could respond to astrocyte-derived cytokines and hypothesized that the metastatic cells would thus be better suited to respond to paracrine signals from the brain microenvironment (116). A similar role has been suggested for neurotrophins expressed by metastatic cells in promoting invasion and responding to astrocyte-derived signals in the brain by autocrine and paracrine mechanisms (30). It should be recognized that some of the differences in profiling studies could be due to the effect of the brain microenvironment on the tumor cell and not due to intrinsic properties of the tumor cell. Thus, it would be equally important to identify molecular features in subpopulations of the primary tumor cells that are "destined" to metastasize; such features would also be present in the metastatic cells at all stages of the metastatic process. Target organ-specific features present in metastatic cells would be absent in those primary tumors and metastatic cells that do not metastasize to the brain. Thus, comparative profiling between tumors that metastasize to the brain or those that fail to do so may help identify early molecular signatures that could guide patient selection as well as subsequent treatment. Significant efforts are currently ongoing to systematically study the biologic profile and molecular alterations of brain metastasis that potentially dictate their clinical behavior. (22, 117-119)

8. PREVENTION OF BRAIN METASTASIS BY MODIFICATION OF BIOLOGIC FACTORS – FROM BENCH TO BEDSIDE

A better understanding of the multistep process of brain metastasis will allow the identification of rate limiting steps in this disease that may permit therapeutic intervention. For malignancies that manifest with brain metastases early in the course of the disease, such as lung cancer, primary prevention of brain involvement may be challenging because these cancers may often present with brain lesions. In such cases, inhibition of angiogenesis, invasion, and disruption of signals that arise from the brain microenvironment to facilitate tumor growth in association with treatment of the primary malignancies could be a reasonable strategy. Identification of biologic 'Achilles heels' common to both the primary and metastatic lesions may facilitate using the same agent or combination of agents to treat the disease in its different locations. The limitations of drug delivery to the brain and

variations in tissue pharmacology between the primary site and the metastatic lesions could, however, potentially heighten the challenge of this approach. Combining such techniques with radiation therapy (stereotactic or whole brain) could allow the dose of radiation used in radiation therapy to be reduced, reducing the risk of toxicity, while increasing targeted activity against the brain metastases.

Malignancies that are associated with brain metastases late in the course of the disease will likely afford a better opportunity for primary prevention than earlier-occurring lesions. In such situations, clinical experience suggests that successful therapy of the primary disease does not ensure prevention of brain metastases, which may nevertheless appear later in the disease course in the absence of activity at the primary site. Preventing metastases from occurring in this setting would presumably require continuous suppression of a combination of factors responsible not only for brain metastases, but also for other systemic metastases along with treatment of the primary disease. Hence, identification of biologic factors that are universally common to metastases (such as those described in the sections above) but that are not involved in normal physiologic processes in adults may afford the best opportunity for this chemoprevention strategy. When brain metastases occur in the face of widespread metastases and a high tumor burden, treatments that target biologic characteristics common to the entire disease process or those that impact the components of the disease process that are most relevant to patient prognosis may be appropriate targets for intervention.

If it is true that a subpopulation of cells in the primary tumor is destined to metastasize and that the other cells do not evolve into such a metastatic phenotype, treatments that can target and eliminate such cells in the primary tumor early in the course of the disease may eliminate the possibility of metastases and obviate the need for chronic therapy. These approaches require a precise and comprehensive understanding of the molecular factors that determine biologic characteristics in the primary and metastatic tumors. In this context, ongoing studies using preclinical models, translational approaches and comprehensive profiling of primary and metastatic tissue will undoubtedly provide the basis for rational therapeutic approaches; in addition, active collaboration between industry, academia and government will be needed to focus attention on metastatic disease process as a priority area in the fight against cancer.

REFERENCES

1. Johnson, J. D., and Young, B., 1996, Demographics of brain metastasis. Neurosurg Clin N Am, 337-344.
2. Posner, J. B., and Chernik, N. L., 1978, Intracranial metastases from systemic cancer. Adv Neurol, 579-592.
3. Patchell, R. A., 1991, Brain metastases. Neurol Clin, 817-824.
4. Klos, K. J., and O'Neill, B. P., 2004, Brain metastases. Neurologist, 31-46.
5. Nussbaum, E. S., Djalilian, H. R., Cho, K. H., and Hall, W. A., 1996, Brain metastases. Histology, multiplicity, surgery, and survival. Cancer, 1781-1788.
6. Mehta, M. P., Rodrigus, P., Terhaard, C.H.J., Rao, A., Suh, J., Roa, W., Souhami, L., Bezjak, A., Leibenhaut, M., Komaki, R., Schultz, C., Timmerman, R., Curran, W., Smith, J., Phan, S. C., Miller, R. A., and Renschler, M. F., 2003, Survival and Neurologic Outcomes in a Randomized Trial of Motexafin Gadolinium and Whole-Brain Radiation Therapy in Brain Metastases. Journal of Clinical Oncology, 2529-2536.
7. Fleckenstein, K., Hof, H., Lohr, F., Wenz, F., and Wannenmacher, M., 2004, Prognostic factors for brain metastases after whole brain radiotherapy. Data from a single institution. Strahlenther Onkol 268-273.
8. Puduvalli, V., and Armstrong, T,, 2004, Management of patients with brain metastasis, In Palliative Care Consultations in Primary and Metastatic Brain Tumours, Booth, S., Bruera, E., and Oliver D., eds, Oxford University Press, NY.
9. Coman, D., deLong, R.P., and McCutcheon, M., 1951, Studies on the mechanisms of metastasis; the distribution of tumors in various organs in relation to the distribution of arterial emboli. Cancer Research, 648-651.
10. Delattre, J. Y., Krol, G., Thaler, H. T., and Posner, J. B., 1988, Distribution of brain metastases. Arch Neurol, 741-744.
11. Hwang, T. L., Close, T. P., Grego, J. M., Brannon, W. L., and Gonzales, F., 1996, Predilection of brain metastasis in gray and white matter junction and vascular border zones. Cancer, 1551-1555.

12. Paget, S., 1889, The distribution of secondary growths in cancer of the breast. Lancet, 571-573.
13. Duvernoy, H., Delon, S., and Vannson, J. L., 1983, The vascularization of the human cerebellar cortex. Brain Res Bull, 419-480.
14. Pardridge, W. M., Triguero, D., and Farrell, C. R., 1990, Downregulation of blood-brain barrier glucose transporter in experimental diabetes. Diabetes, 1040-1044.
15. Nonaka, H., Akima, M., Hatori, T., Nagayama, T., Zhang, Z., and Ihara, F., 2003, The microvasculature of the cerebral white matter: arteries of the subcortical white matter. J Neuropathol Exp Neurol 154-161.
16. Smith, Q.R., and Rapoport, S.I., 1986, Cerebrovascular permeability coefficients to sodium, potassium, and chloride. J Neurochem, 1732-1742.
17. Martin-Padura, I., Lostaglio, S., Schneemann, M., Williams, L., Romano, M., Fruscella, P., Panzeri, C., Stoppacciaro, A., Ruco, L., Villa, A., Simmons, D., and Dejana, E., 1998, Junctional Adhesion Molecule, a Novel Member of the Immunoglobulin Superfamily That Distributes at Intercellular Junctions and Modulates Monocyte Transmigration. The Journal of Cell Biology, 117-127.
18. Citi, S., 1993, The molecular organization of tight junctions. The Journal of Cell Biology, 485-489.
19. Bazzoni, G., and Dejana, E., 2004, Endothelial Cell-to-Cell Junctions: Molecular Organization and Role in Vascular Homeostasis. Physiological Reviews, 869-901.
20. Wachtel, M., Frei, K., Ehler, E., Fontana, A., Winterhalter, K., and Gloor, S. M., 1999, Occludin proteolysis and increased permeability in endothelial cells through tyrosine phosphatase inhibition. Journal of Cell Science, 4347-4356.
21. Lampugnani, M. G., Corada, M., Andriopoulou, P., Esser, S., Risau, W., and Dejana, E., 1997, Cell confluence regulates tyrosine phosphorylation of adherens junction components in endothelial cells. Journal of Cell Science, 2065-2077.
22. Kozaki, K., Koshikawa, K., Tatematsu, Y., Miyaishi, O., Saito, H., Hida, T., Osada, H., and Takahashi, T., 2001, Multi-faceted analyses of a highly metastatic human lung cancer cell line NCI-H460-LNM35 suggest mimicry of inflammatory cells in metastasis. Oncogene, 4228-4234.
23. Glinsky, V.V., Glinsky, G.V., Glinskii, O.V., Huxley, V.H., Turk, J.R., Mossine, V.V., Deutscher, S.L., Pienta, K.J., and Quinn, T.P., 2003, Intravascular Metastatic Cancer Cell Homotypic Aggregation at the Sites of Primary Attachment to the Endothelium. Cancer Research, 3805-3811.
24. Karpatkin, S., and Pearlstein, E., 1981, Role of platelets in tumor cell metastases. Annals of Internal Medicine, 636-641.
25. Honn, K. V., Tang, D. G., and Crissman, J. D., 1992, Platelets and cancer metastasis: a causal relationship?. Cancer Metastasis Review, 325-351.
26. Doi, K., Horiuchi, T., Uchinami, M., Tabo, T., Kimura, N, Yokomachi, J, Yoshida, M, and Tanaka, K., 2002, Hepatic ischemia-reperfusion promotes liver metastasis of colon cancer. Journal of SurgicalResearch, 243-247.
27. Moon, B. K., Lee, Y. J., Battle, P., Jessup, J. M., Raz, A., and Kim, H. R., 2001, Galectin-3 protects human breast carcinoma cells against nitric oxide-induced apoptosis: implication of galectin-3 function during metastasis. American Journal of Pathology, 1055-1060.
28. Marchetti, D., 1997, Specific degradation of subendothelial matrix proteoglycans by brain-metastatic melanoma and brain endothelial cell heparanases. Journal of Cell Physiology, 334- 342.
29. Marchetti, D., Li, J., and Shen, R., 2000, Astrocytes contribute to the brain-metastatic specificity of melanoma cells by producing heparanase. Cancer Research, 4767-4770.
30. Marchetti, D., McQuillan, D. J., Spohn, W. C., Carson, D. D., and Nicolson, G. L., 1996, Neurotrophin stimulation of human melanoma cell invasion: selected enhancement of heparanase activity and heparanase degradation of specific heparan sulfate subpopulations. Cancer Research, 2856-2856.
31. Okada, Y., 2000, Tumor cell-matrix interaction: pericellular matrix degradation and metastasis. Verh Dtsch Ges Pathol, 33-42.
32. Menter, D. G., Herrmann, J. L., and Nicolson, G. L., 1995, The role of trophic factors and autocrine/paracrine growth factors in brain metastasis. Clinical and Experimental Metastasis, 67-88.
33. Nicolson, G. L., and Menter, D. G., 1995, Trophic factors and central nervous system metastasis. Cancer Metastasis Review, 303-321.
34. Luzzi, K. J., MacDonald, I. C., Schmidt, E. E., Kerkvliet, N., Morris, V. L., Chambers, A. F., and Groom, A. C., 1998, Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. American Journal of Pathology, 865-873.
35. Price, J. E., Aukerman, S. L., and Fidler, I. J., 1986, Evidence that the process of murine melanoma metastasis is sequential and selective and contains stochastic elements. Cancer Research, 5172-5178.
36. Nicolson, G. L., and Custead, S. E., 1982, Tumor metastasis is not due to adaptation of cells to a new organ environment. Science, 176-178.
37. Pauli, B. U., Augustin-Voss, H. G., el Sabban, M. E., Johnson, R. C., and Hammer, D. A., 1990, Organ-preference of metastasis. The role of

endothelial cell adhesion molecules. Cancer Metastasis Review, 175-189.

38. Ito, S., Nakanishi, H., Ikehara, Y., Kato, T., Kasai, Y., Ito, K., Akiyama, S,. Nakao, A., and Tatematsu, M., 2001, Real-time observation of micrometastasis formation in the living mouse liver using a green fluorescent protein gene-tagged rat tongue carcinoma cell line. International Journal of Cancer, 212-217.
39. Kawaguchi, T., Kawaguchi, M., Dulski, K. M., and Nicolson, G. L., 1985, Cellular behavior of metastatic B16 melanoma in experimental blood-borne implantation and cerebral invasion. An electron microscopic study. Invasion Metastasis, 16-30.
40. Tang, D. G., and Honn, K. V., 1994, Adhesion molecules and tumor metastasis: an update. Invasion Metastasis, 109-122.
41. Orr, F. W., Wang, H. H., Lafrenie, R. M., Scherbarth, S., and Nance, D. M., 2000, Interactions between cancer cells and the endothelium in metastasis. Journal of Pathology, 310-329.
42. Felding-Habermann, B., Habermann, R., SaldÆvar, E., and Ruggeri, Z. M., 1996, Role of beta3 Integrins in Melanoma Cell Adhesion to Activated Platelets under Flow. Journal of Biological Chemistry, 5892-5900.
43. Ravi, R., Mookerjee, B., Bhujwalla, Z. M., Sutter, C. H., Artemov, D., Zeng, Q., Dillehay, L. E., Madan, A., Semenza, G. L., and Bedi, A., 2000, Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes and Development, 33-44.
44. Tsuruo, T., Kawabata, H., Iida, H., and Yamori, T., 1986, Tumor-induced platelet aggregation and growth promoting factors as determinants for successful tumor metastasis. Clinical and Experimental Metastasis, 25 -33.
45. Varki, A., Varki, N. M., Borsig, L., Wong, R., Feramisco, J., Nadeau, D. R., 2001, P-selectin, carcinoma metastasis and heparin: novel mechanistic connections with therapeutic implications. Brazilian Journal of Medical Biology Research, 711-717.
46. Velikova, G., Banks, R. E., Gearing, A., Hemingway, I., Forbes, M. A., Preston, S. R., Jones, M., Wyatt, J., Miller, K., Ward, U., Al Maskatti, J., Singh, S. M., Ambrose, N. S., Primrose, J. N., and Selby, P. J., 1997, Circulating soluble adhesion molecules E-cadherin, E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in patients with gastric cancer. British.Journal of Cancer, 1398-1404.
47. Koop, S., MacDonald, I. C., Luzzi, K., Schmidt, E. E., Morris, V. L., Grattan, M., Khokha, R., Chambers, A. F., and Groom, A. C., 1995, Fate of melanoma cells entering the microcirculation: over 80% survive and extravasate. Cancer Research, 2520 –2523.
48. Brummendorf, T. and Rathjen, F. G., 1995, Cell adhesion molecules 1: immunoglobulin superfamily. Protein Profile, 963-1108.
49. Geertsen, R., Zenklusen, R., Kamarashev, J., Burg, G., and Dummer, R., 1999, Inverse regulation of neuronal cellular adhesion molecule (NCAM) by IFN-gamma in melanoma cell cultures established from CNS lesions. International Journal of Cancer, 135-140.
50. Cavallaro, U., Niedermeyer, J., Fuxa, M., and Christofori, G., 2001, N-CAM modulates tumour-cell adhesion to matrix by inducing FGF-receptor signalling. Nature Cell Biology, 650-657.
51. Natali, P., Nicotra, M. R., Cavaliere, R., Bigotti, A., Romano, G., Temponi, M., and Ferrone, S., 1990, Differential expression of intercellular adhesion molecule 1 in primary and metastatic melanoma lesions. Cancer Research, 1271-1278.
52. Staykova, M., Maxwell, L., and Willenborg, D., 2000, Kinetics and polarization of the membrane expression of cytokine-induced ICAM-1 on rat brain endothelial cells. J Neuropathol Exp Neurol, 120-128.
53. Hunter, T., 1987, A thousand and one protein kinases. Cell, 823-829.
54. Radisavljevic, Z., Avraham, H., and Avraham, S., 2000, Vascular Endothelial Growth Factor Up-regulates ICAM-1 Expression via the Phosphatidylinositol 3 OH-kinase/AKT/Nitric Oxide Pathway and Modulates Migration of Brain Microvascular Endothelial Cells. Journal of Biological Chemistry, 20770-20774.
55. Diamond, M. S., and Springer, T. A., The dynamic regulation of integrin adhesiveness. 1994, Current Biology, 506-517.
56. Giancotti, F. G., and Ruoslahti, E., 1999, Integrin Signaling. Science, 1028-1033.
57. Lin, T. H., Rosales, C., Mondal, K., Bolen, J. B., Haskill, S., and Juliano, R. L., 1995, Integrin-mediated Tyrosine Phosphorylation and Cytokine Message Induction in Monocytic Cells. Journal of Biological Chemistry, 16189-16197.
58. Reyes-Reyes, M., Mora, N., Zentella, A., and Rosales, C., 2001, Phosphatidylinositol 3-kinase mediates integrin-dependent NF-kappaB and MAPK activation through separate signaling pathways. Journal of Cell Science, 1579-1589.
59. Aplin, A. E., and Juliano, R. L., Integrin and cytoskeletal regulation of growth factor signaling to the MAP kinase pathway. 1999, Journal of Cell Science, 695-706.
60. Cary, L. A., Han, D. C., and Guan, J. L., 1999, Integrin-mediated signal transduction pathways. Histology and Histopathology, 1001-1009.

61. Marshall, J. F., and Hart, I. R., 1996, The role of alpha v-integrins in tumour progression and metastasis. Seminars of Cancer Biology, 129-138.
62. Felding-Habermann, B., O'Toole, T. E., Smith, J. W., Fransvea, E., Ruggeri, Z. M., Ginsberg, M. H., Hughes, P. E., Pampori, N., Shattil, S. J., Saven, A., and Mueller, B. M., 2001, Integrin activation controls metastasis in human breast cancer. Proceedings of the National Academy of Sciences, 1853-1858.
63. Horton, M. A., 1997, The alpha v beta 3 integrin "vitronectin receptor". International Journal of Biochemistry and Cell Biology, 21-725.
64. Brooks, P. C., Clark, R. A., and Cheresh, D. A., 1994, Requirement of vascular integrin alpha v beta 3 for angiogenesis. Science, 569-571.
65. Byzova, T. V., Goldman, C. K., Pampori, N., Thomas, K. A., Bett, A., Shattil, S. J., and Plow, E. F., 2000, A mechanism for modulation of cellular responses to VEGF: activation of the integrins. Molecular Cell, 851-860.
66. Wang, H., Fu, W., Im, J. H., Zhou, Z., Santoro, S. A., Iyer, V., DiPersio, C. M., Yu, Q. C., Quaranta, V., Al Mehdi, A., and Muschel, R. J., 2004, Tumor cell {alpha}3{beta}1 integrin and vascular laminin-5 mediate pulmonary arrest and metastasis. The Journal of Cell Biology, 935-941.
67. Smith, J. W., 2003, Cilengitide Merck. Current Opinions Investigational Drugs, 741-745.
68. Eskens, F. A., Dumez, H., Hoekstra, R., Perschl, A., Brindley, C., Bottcher, S., Wynendaele, W., Drevs, J., Verweij, J., and van Oosterom, A. T., 2003, Phase I and pharmacokinetic study of continuous twice weekly intravenous administration of Cilengitide (EMD 121974), a novel inhibitor of the integrins alphavbeta3 and alphavbeta5 in patients with advanced solid tumours. European Journal of Cancer, 917-926.
69. Posey, J. A., Khazaeli, M. B., DelGrosso, A., Saleh, M. N., Lin, C. Y., Huse, W., and LoBuglio, A. F., 2001, A pilot trial of Vitaxin, a humanized anti-vitronectin receptor (anti alpha v beta 3) antibody in patients with metastatic cancer. Cancer Biotherapy and Radiopharamacology, 125-132.
70. Reinmuth, N., Liu, W., Ahmad, S. A., Fan, F., Stoeltzing, O., Parikh, A. A., Bucana, C. D., Gallick, G. E., Nickols, M. A., Westlin, W. F., and Ellis, L. M., 2003, {alpha}v{beta}3 Integrin Antagonist S247 Decreases Colon Cancer Metastasis and Angiogenesis and Improves Survival in Mice. Cancer Research, 2079-2087.
71. McEver, R. P., 1997, Selectin-carbohydrate interactions during inflammation and metastasis. Glycoconjugation Journal, 585-591.
72. Aigner, S., Sthoeger, Z. M., Fogel, M., Weber, E., Zarn, J., Ruppert, M., Zeller, Y., Vestweber, D., Stahel, R., Sammar, M., and Altevogt, P., 1997, CD24, a mucin-type glycoprotein, is a ligand for P-selectin on human tumor cells. Blood, 3385-95.
73. Stone, J. P., and Wagner, D. D., 1993, P-selectin mediates adhesion of platelets to neuroblastoma and small cell lung cancer. Journal of Clinical Investigation, 804-813.
74. Borsig, L., Wong, R., Feramisco, J., Nadeau, D. R., Varki, N. M., and Varki, A., 2001, Heparin and cancer revisited: Mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. Proceedings of the National Academy of Sciences, USA, 3352-3357.
75. Wei, M., Tai, G., Gao, Y., Li, N., Huang, B., Zhou, Y., Hao, S., and Zeng, X., 2004, Modified Heparin Inhibits P-selectin-mediated Cell Adhesion of Human Colon Carcinoma Cells to Immobilized Platelets under Dynamic Flow Conditions. Journal of Biological Chemistry, 29202-29210.
76. Lefer, D. J., 2000, Pharmacology of selectin inhibitors in ischemia/reperfusion states. Annual Review of Pharmacology and Toxicology, 283-294.
77. Berditchevski, F., 2001, Complexes of tetraspanins with integrins: more than meets the eye. Journal of Cell Science, 4143-4151.
78. Yanez-Mo, M., Tejedor, R., Rousselle, P., Sanchez-Madrid, F., 2001, Tetraspanins in intercellular adhesion of polarized epithelial cells: spatial and functional relationship to integrins and cadherins. Journal of Cell Science, 577-587.
79. Sugiura, T., and Berditchevski, F., 1999, Function of alpha3beta1-tetraspanin protein complexes in tumor cell invasion. Evidence for the role of the complexes in production of matrix metalloproteinase 2 (MMP-2). Journal of Cell Biology, 1375-1389.
80. Ikeyama, S., Koyama, M., Yamaoko, M., Sasada, R., and Miyake, M., 1993, Suppression of cell motility and metastasis by transfection with human motility-related protein (MRP-1/CD9) DNA. The Journal of Experimental Medicine, 1231-1237.
81. Radford, K. J., Mallesch, J., and Hersey, P., 1995, Suppression of human melanoma cell growth and metastasis by the melanoma-associated antigen CD63 (ME491). International Journal of Cancer, 631-635.
82. Testa, J. E., Brooks, P. C., Lin, J. M., and Quigley, J. P., 1999, Eukaryotic Expression Cloning with an Antimetastatic Monoclonal Antibody Identifies a Tetraspanin (PETA-3/CD151) as an Effector of Human Tumor Cell Migration and Metastasis. Cancer Research, 3812-3820.
83. Lee, J. H., Park, S. R., Chay, K. O., Seo, Y. W., Kook, H., Ahn, K. Y., Kim, Y. J., and Kim, K. K., 2004, KAI1 COOH-Terminal Interacting Tetraspanin (KITENIN), a Member of the Tetraspanin Family, Interacts with KAI1, a Tumor Metastasis Suppressor, and Enhances Metastasis of Cancer. Cancer Research, 4235-4243.

84. Parsons, J. T., Martin, K. H., Slack, J. K., Taylor, J. M., and Weed, S. A., 2000, Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. Oncogene, 5606-5613.
85. Bruce-Staskal, P. J., and Bouton, A. H., 2001, PKC-dependent activation of FAK and src induces tyrosine phosphorylation of Cas and formation of Cas-Crk complexes. Experimental Cell Research, 296-306.
86. Nikolopoulos, S. N., and Turner, C. E., 2001, Integrin-Linked Kinase (ILK) Binding to Paxillin LD1 Motif Regulates ILK Localization to Focal Adhesions. Journal of Biological Chemistry, 23499-23505.
87. Ludwig, H. C., Akhavan-Shigari, R., Rausch, S., Schallock, K., Quentin, C., Bockermann, V., and Kolenda, H., 2000, Expression of focal adhesion kinase (p125 FAK) and proline-rich tyrosine kinase 2 (PYK2/CAKb) in cerebral metastases, correlation with VEGF-R, ecNOS III-labelling and morphometric data. Anticancer Research, 1419-1424.
88. Abedi, H., and Zachary, I., 1997, Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. Journal of Biological Chemistry, 15442-15451.
89. Lu, Z., Jiang, G., Blume-Jensen, P., and Hunter, T., 2001, Epidermal growth factor-induced tumor cell invasion and metastasis initiated by dephosphorylation and downregulation of focal adhesion kinase. Molecular and Cell Biology, 4016-4031.
90. Zhang, M., and Olsson, Y., 1995, Reactions of astrocytes and microglial cells around hematogenous metastases of the human brain. Expression of endothelin-like immunoreactivity in reactive astrocytes and activation of microglial cells. Journal of Neurological Sciences, 26-32.
91. Yong, V. W., Power, C., Forsyth, P., and Edwards, D. R., 2001, Metalloproteinases in biology and pathology of the nervous system. Nature Reviews Neuroscience, 502-511.
92. Saaristo, A., Karpanen, T., and Alitalo, K., 2000, Mechanisms of angiogenesis and their use in the inhibition of tumor growth and metastasis. Oncogene, 6122-6129.
93. Cavallaro, U., and Christofori, G., 2000, Molecular mechanisms of tumor angiogenesis and tumor progression. Journal of Neurooncology, 63-70.
94. Stamenkovic, I., 2000, Matrix metalloproteinases in tumor invasion and metastasis. Seminars in Cancer Biology, 415-433.
95. Stetler-Stevenson, W. G., and Yu, A. E., 2001, Proteases in invasion: matrix metalloproteinases. Seminars in Cancer Biology, 143-154.
96. Collen, D., 1999, The plasminogen (fibrinolytic) system. Thrombosis and Haemostasis, 259-270.
97. Khatib, A. M., Nip, J., Fallavollita, L., Lehmann, M., Jensen, G., and Brodt, P., 2001, Regulation of urokinase plasminogen activator/plasmin-mediated invasion of melanoma cells by the integrin vitronectin receptor alphaVbeta3. International Journal of Cancer, 300-308.
98. Chandrasekar, N., Mohanam, S., Gujrati, M., Olivero, W. C., Dinh, D. H., and Rao, J. S., 2003, Downregulation of uPA inhibits migration and PI3k/Akt signaling in glioblastoma cells. Oncogene, 392-400.
99. Ahmed, N., Oliva, K., Wang, Y., Quinn, M., and Rice, G., 2003, Downregulation of urokinase plasminogen activator receptor expression inhibits Erk signalling with concomitant suppression of invasiveness due to loss of uPAR-beta1 integrin complex in colon cancer cells. British Journal of Cancer, 374-384.
100. Crowley, C. W., Cohen, R. L., Lucas, B. K., Liu, G., Shuman, M. A., and Levinson, A. D., 1993, Prevention of Metastasis by Inhibition of the Urokinase Receptor. Proceedings of the National Academy of Sciences, 5021-5025.
101. Ploug, M., Gardsvoll, H., Jorgensen, T. J., Lonborg, H. L., and Dano, K., 2002, Structural analysis of the interaction between urokinase-type plasminogen activator and its receptor: a potential target for anti-invasive cancer therapy. Biochemistry Society Transactions, 177-183.
102. Sanderson, R. D., 2001, Heparan sulfate proteoglycans in invasion and metastasis. Seminars in Cell Developmental Biology, 89-98.
103. Koliopanos, A., Friess, H., Kleeff, J., Shi, X., Liao, Q., Pecker, I., Vlodavsky, I., Zimmermann, A., Buchler, M. W., and Sanderson, R. D., 2001, Heparanase expression in primary and metastatic pancreatic cancer. Cancer Research, 4655-4659.
104. Kosir, M. A., Wang, W., Zukowski, K. L., Tromp, G., and Barber, J., 1999, Degradation of basement membrane by prostate tumor heparanase. Journal of Surgical Research, 42-47.
105. Semenza, G. L., 2000, Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. Critical Reviews in Biochemistry and Molecular Biology, 71-103.
106. Zhong, H., De Marzo, A. M., Laughner, E., Lim, M., Hilton, D. A., Zagzag, D., Buechler, P., Isaacs, W. B., Semenza, G. L., and Simons, J. W., 1999, Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Research, 5830-5835.
107. Kim, L. S., Huang, S., Lu, W., Lev, D. C., and Price, J. E., 2004, Vascular endothelial growth factor expression promotes the growth of breast cancer

brain metastases in nude mice. Clinical and Experimental Metastasis, 107-118.

108. Croll, S. D., Ransohoff, R. M., Cai, N., Zhang, Q., Martin, F. J., Wei, T., Kasselman, L. J., Kintner, J., Murphy, A. J., Yancopoulos, G. D., and Wiegand, S. J., 2004, VEGF-mediated inflammation precedes angiogenesis in adult brain. Experimental Neurology, 388-402.
109. Aramoto, H., Breslin, J. W., Pappas, P. J., Hobson II, R. W., and Duran, W. N., 2004, Vascular endothelial growth factor stimulates differential signaling pathways in the in vivo microcirculation. AJP-Heart and Circulatory Physiology (epub ahead of publication).
110. Martinez-Estrada, O. M., Rodriguez-Millan, E., Gonzalez-de Vicente, E., Reina, M., Vilaro, S., and Fabre, M., 2003, Erythropoietin protects the in vitro blood-brain barrier against VEGF-induced permeability. European Journal of Neuroscience, 2538-2544.
111. Lee, B. C., Lee, T. H., Avraham, S., and Avraham, H. K., 2004, Involvement of the Chemokine Receptor CXCR4 and Its Ligand Stromal Cell-Derived Factor 1{alpha} in Breast Cancer Cell Migration Through Human Brain Microvascular Endothelial Cells. Molecular Cancer Research, 327-338.
112. Seibert, C. and Sakmar, T. P., 2004, Small-molecule antagonists of CCR5 and CXCR4: a promising new class of anti-HIV-1 drugs. Current Pharmacologic Design, 2041-2062.
113. Fidler, I. J., and Kripke, M. L., 1977, Metastasis results from preexisting variant cells within a malignant tumor. Science, 893-895.
114. Weigelt, B., Glas, A. M., Wessels, L. F. A., Witteveen, A. T., Peterse, J. L., and van't Veer, L. J., 2003, Gene expression profiles of primary breast tumors maintained in distant metastases. Proceedings of the National Academy of Sciences, 15901-15905.
115. Malins, D. C., Gilman, N. K., Green, V. M., Wheeler, T. M., Barker, E. A., Vinson, M. A., Sayeeduddin, M., Hellstrom, K. E., and Anderson, K. M., 2004, Metastatic cancer DNA phenotype identified in normal tissues surrounding metastasizing prostate carcinomas. Proceedings of the National Academy of Sciences, 11428-11431.
116. Nishizuka, I., Ishikawa, T., Hamaguchi, Y., Kamiyama, M., Ichikawa, Y., Kadota, K., Miki, R., Tomaru, Y., Mizuno, Y., Tominaga, N., Yano, R., Goto, H., Nitanda, H., Togo, S., Okazaki, Y., Hayashizaki, Y., and Shimada, H., 2002, Analysis of gene expression involved in brain metastasis from breast cancer using cDNA microarray. Breast Cancer, 26-32.
117. Yu, Y., Khan, J., Khanna, C., Helman, L., Meltzer, P. S., and Merlino, G., 2004, Expression profiling identifies the cytoskeletal organizer ezrin and the developmental homeoprotein Six-1 as key metastatic regulators. Nature Medicine, 175-181.
118. Chen, Z., Zhang, K., Zhang, X., Yuan, X. H., Yuan, Z., Jin, L., and Xiong, M., 2003, Comparison of gene expression between metastatic derivatives and their poorly metastatic parental cells implicates crucial tumor-environment interaction in metastasis of head and neck squamous cell carcinoma. Clinical and Experimental Metastasis, 335-342.
119. Clark, E. A., Golub, T. R., Lander, E. S., and Hynes, R. O., 2000, Genomic analysis of metastasis reveals an essential role for RhoC. Nature, 532-535.

Chapter 8

Influence of the Bone Microenvironment on Breast Cancer Metastasis to Bone

B.L. Eckhardt, N. Pouliot and R.L. Anderson
Trescowthick Research Laboratories, Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia, 3002

Abstract: Cancer patients do not generally die as a direct consequence of the primary tumour, but due to the formation of secondary tumours – metastases – that arise during tumour progression. Bone metastases are a common complication in patients with advanced breast and prostate cancer. Once established, bone metastases cause intractable pain, hypocalcaemia, spinal cord compression and bone frailty. The mechanisms regulating site-specific metastasis are not well understood despite being the focus of research for over a century. However, it is becoming clear that the microenvironment at the secondary tumour site contributes to metastatic progression by regulating the properties of metastatic cells. The stromal microenvironment provides an opportunistic niche in which circulating tumour cells can evade the immune system and be refractory to conventional therapies. A better understanding of tumour-stroma interactions may identify critical factors regulating metastatic progression and lead to the development of stromal therapies for breast and other malignancies. Here, the evidence implicating stromal factors in the metastasis of breast tumours to bone will be reviewed.

Key words: Bone metastasis, breast cancer, animal model, microenvironment, TGF-β, stromal therapy

1. METASTATIC PROGRESSION

Metastasis is a dynamic process consisting of a series of interrelated events, each involving interactions between the tumour cell and the tissue specific microenvironment of the host. Each stage needs to be completed to produce a secondary tumour. A primary tumour cannot grow beyond 1mm^3 without an adequate blood supply (1). In hypoxic conditions, tumour cells hijack normal growth processes by inducing the expression of several cytokines, pro-angiogenic factors and growth factors within the microenvironment (2, 3). Having established a vascular network, tumour cells invade the surrounding stroma and intravasate into the circulation. This requires the recruitment of active proteolytic enzymes including matrix metalloproteinases (MMPs), the serine proteases urokinase (uPA) and tissue plasminogen activator (tPA), plasmin and thrombin into the invading front of the tumour (4, 5). Most proteases are actually expressed by the host and are activated by the presence of tumour cells (6). Proteolytic degradation of the extracellular matrix (ECM) enhances tumour progression by releasing entrapped growth factors and revealing cryptic adhesive binding sites. Integrins can bind to these adhesive sites and activate intracellular signalling cascades that promote cellular division, motility and invasion (7, 8). Whilst in circulation, tumour cells must survive anoikis and vasculature turbulence, prior to arresting in the capillary bed of a distant organ. The tumour cells must then extravasate from the circulation and successfully colonize the secondary organ, an event

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 149-164.

that requires the cell to survive and re-initiate proliferative activity. If these pre-metastatic lesions successfully evade the immune response of the host and attract a new blood supply, they will establish as clinically relevant metastatic lesions. Further, growing metastases can shed tumour cells into the circulation and initiate the development of other metastatic lesions, a phenomenon known as metastasis of metastases (9, 10).

Metastasis is a highly inefficient process. A tumour cell that is incapable of completing any of one of these events will fail to produce a clinically relevant lesion. Hence, each step of the metastatic process is a potential therapeutic target, with some steps being more rate limiting than others (11). For instance, studies using *in vivo* video microscopy of tumour cells have shown that 80% of melanoma cells arrest in the liver after injection into the superior mesenteric vein but only a fraction (0.02%) of them form overt metastases (12). Similarly, RT-PCR based assays for tumour cell markers have been utilized to detect micrometastases in the bone marrow of 25-70% of patients with common malignancies, including those tumour types that do not generally form bone metastases (reviewed in (13)). Collectively, these data suggest that although metastatic spread to specific sites such as bone is relatively efficient, it is the ability of the tumour cell to survive, proliferate and establish in the secondary site that is rate limiting. As with earlier steps in metastasis, factors within the microenvironment of the secondary site play a dominant role in subsequent growth of the metastatic nodule (11).

It is known that different tumour types have tissue specific metastatic patterns. Breast cancer cells commonly metastasize to the lymph nodes, lung, liver and bone. Such specific spread of tumour cells was originally observed by Steven Paget in 1889, who coined the “seed and the soil” hypothesis (14). This hypothesis stated that for breast tumour cells (the “seed”) to spread to distant tissues (the “soils”) the microenvironment of the organ must be congenial to their growth. More recent studies have shown that bone complications arising from breast cancer occur in approximately 70% of patients (15). Current hypotheses point to the function, structural composition and stromal-tumour cell interactions within bone that aid colonization by metastatic cells (13, 16, 17).

2. REGULATION OF NORMAL BONE REMODELLING

Bone is a dynamic organ, undergoing constant remodelling involving active destruction and re-synthesis of the bone matrix. Within normal adult bone, homeostatic mechanisms maintain the balance between the bone forming osteoblasts and the bone resorbing osteoclasts (Figure 1).

Osteoblasts arise from mesenchymal osteoprogenitor cells (reviewed in (18)). During development, these cells secrete a complex mixture of growth factors and ECM proteins into the surrounding bone microenvironment (bone matrix) before they either apoptose or terminally differentiate into osteocytes (the cellular component of hardened bone). The majority of bone matrix protein consists of type I collagen fibres (85-90%), which provide structural support for the mineralisation of bone (19). The remaining 10-15% consists of proteoglycans, γ-carboxylated (gla) proteins, cell adhesive proteins and growth factors. A large number of adhesive proteins found in bone contain RGD (Arg-Gly-Asp) motifs (17); examples of these are type I collagen, bone sialoprotein, fibronectin, laminin-10, osteopontin, thrombospondin and vitronectin. The RGD motif is a well-characterized binding site for several adhesion receptors of the integrin family and, depending on substrate- receptor context, can regulate cellular motility, invasion and growth (20). Osteoblasts also secrete growth factors into the bone matrix, including transforming growth factor-β (TGF-β), insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), interleukins, platelet derived growth factor (PDGF) and bone morphogenic proteins (BMPs) (21), (22).

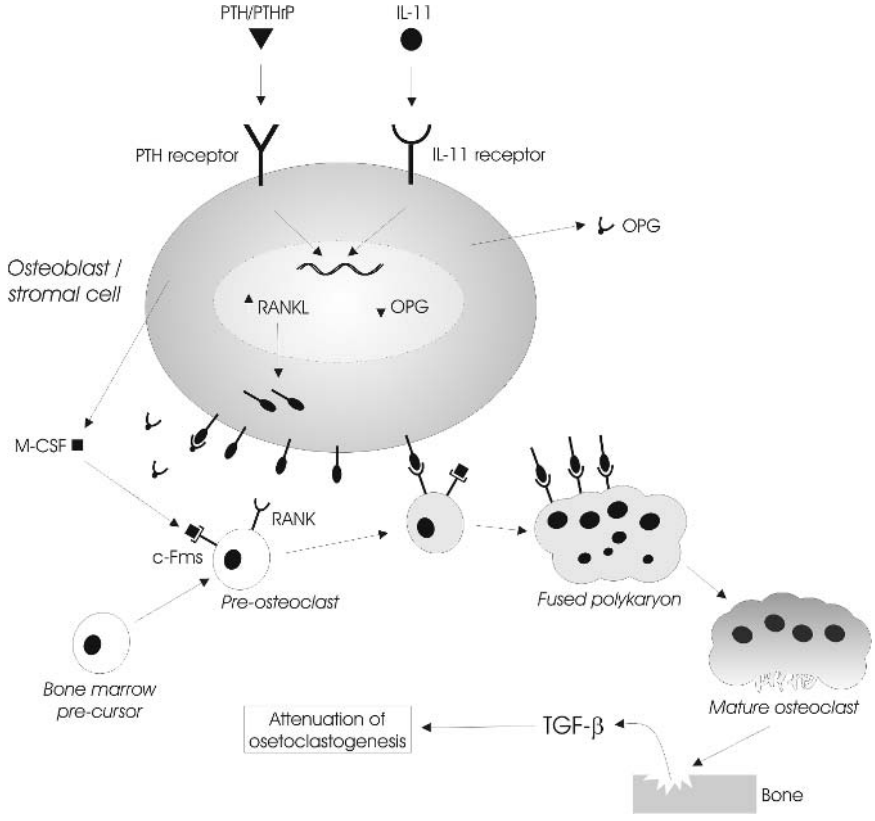


Figure 1. A model of normal bone remodelling. Bone is continually being remodelled, a process that requires interactions of bone forming osteoblasts with bone resorbing osteoclasts. Osteoclastogenesis occurs as a result of the interaction of bone marrow precursor cells, osteoblasts and bone marrow stromal cells with a multitude of growth factors, hormones and cytokines that alter the expression of key osteoclastogeneic factors RANK, RANKL, OPG and M-CSF. Active osteoclasts resorb bone and subsequently release growth factors including TGF-β, which attenuates osteoclastogenesis by increasing osteoblast proliferation and decreasing osteoclast activity.

These growth factors remain latent in the bone matrix but can be released and activated upon proteolytic degradation of the bone. FGF, PDGF, TGF-β and several BMPs have been reported to enhance the differentiation and growth of osteoblasts (reviewed in (23)). Thus, release of these factors from the bone matrix provides a feedback mechanism to promote bone formation and attenuate bone resorption.

Osteoclasts are differentiated cells arising from the monocyte-macrophage lineage. The primary role of the osteoclast is to resorb bone. Activated osteoclasts are recruited to the bone surface and attach through interactions with the $\alpha_v\beta_3$ integrin receptor (24). This interaction is crucial in the bone remodelling process as β3 integrin knockout mice develop osteosclerosis due to the lack of functional osteoclasts (25). Osteoclasts acidify the local microenvironment at the bone-osteoclast interface ("resorption zone") and secrete several proteases such as MMPs and cathepsins B, L, K and S, which are used to degrade components of the ECM. The most abundant protease expressed by osteoclasts is cathepsin K which targets type I collagen (26, 27). Whilst cathepsin K seems to be the prevalent protease in solubilisation of the bone matrix, several MMPs have also been implicated in the proteolysis of bone (reviewed in (28)). Interestingly, osteoclast secreted MMPs – MMP-9, MMP-10, MMP-12 and MMP-14 – do not contribute significantly to bone degradation, whilst MMP-13, an osteoblast secreted MMP with collagenase activity, can be recruited into the resorption zone and degrade bone (29, 30). In addition to bone proteolysis, several MMPs have

been implicated in the regulation of osteoclast signalling, migration and invasion (28).

Osteoclastogenesis, the development of mature osteoclasts, is a process that is tightly regulated through a complex network of cytokines and receptor interactions within the bone stroma (Figure 1). In particular, stromal expression of macrophage colony-stimulating factor (M-CSF) and the receptor activator of NFκB ligand (RANKL) are necessary and sufficient to induce osteoclastogenesis *in vivo* and *in vitro* (31). M-CSF, through binding to its receptor c-Fms, acts as a survival factor for osteoclast precursor cells allowing them to respond to inducers of osteoclastogenesis. Expression of membrane bound RANKL is induced in stromal cells and osteoblasts by various stimuli, including parathyroid hormone (PTH), PTH related protein (PTHrP), calcitriol, tumour necrosis factor-α (TNF-α), glucocorticoids, prostaglandin E_2 (PGE_2), interleukin-1 (IL-1), interleukin-11 (IL-11), thyroid hormone, fibroblast growth factor-2 (FGF-2) and insulin like growth factor-1 (IGF-1) (32). Binding of RANKL to its membrane receptor RANK on osteoclast precursors activates inhibitor of NFκB kinase (IKK), c-Jun N-terminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK) and Src signalling pathways that cooperate to induce the differentiation of haematopoietic progenitors into mature osteoclasts (31). Mice with homozygous deletions in either RANKL or RANK have no functional osteoclasts and develop severe osteopetrosis (33, 34), demonstrating the critical importance of the RANKL/RANK interaction in osteoclastogenesis.

Osteoprotegerin (OPG), a member of the tumour necrosis factor receptor superfamily, is secreted by osteoblasts and other bone stromal cells and suppresses osteoclastogenesis by competing with RANK for RANKL binding (35, 36). Consistent with this, OPG deficient mice exhibit decreased bone density due to increased osteoclast activity (37). The regulation of RANKL and OPG are intertwined as evidenced by factors such as IL-11, PTHrP and PGE_2 that increase RANKL but suppress OPG expression (32). Conversely, active TGF-β released during osteolysis stimulates osteoblastogenesis and attenuates osteoclastogenesis by increasing OPG and suppressing RANKL expression (38, 39).

Any perturbation of the delicate balance between osteoblast mediated bone formation and osteoclast mediated bone resorption is likely to impinge on normal bone turnover, resulting in enhanced bone degradation or formation. Tumour cells homing to bone cause an imbalance in osteoblast-osteoclast regulation to promote their survival and proliferation in this organ.

3. MODELLING THE PROCESS OF BREAST CANCER METASTASIS TO BONE

The development of improved animal models of metastasis has increased our understanding of the molecular mechanisms that regulate the colonization of breast cancer cells in bone. An excellent review on the current models of tumour metastasis to bone is available (40). In 1988, a mouse model was described in which melanoma established in bone following the inoculation of cells into the arterial circulation of immunocompromised mice (41). Since its conception, this model has been extensively used and has provided much insight in the mechanisms of metastatic colonization of bone by several tumour lines including breast (42-46). A further advance came from the development of a syngeneic mouse model that can spontaneously metastasize to bone following inoculation into the mammary fat pad (43, 47). In this model, the 4T1.2 tumour line produces spontaneous lung and osteolytic bone lesions following the inoculation of as few as 1000 cells into the mammary gland (Figure 2). The model is invaluable for studies of metastatic progression as it mimics both early and late stages of human breast cancer metastasis to bone. We are currently utilizing cDNA array profiling of this model and functional analysis to identify genes that are associated with metastatic progression. The model allows the contribution of both the stromal and tumour cell compartments in bone to be assessed.

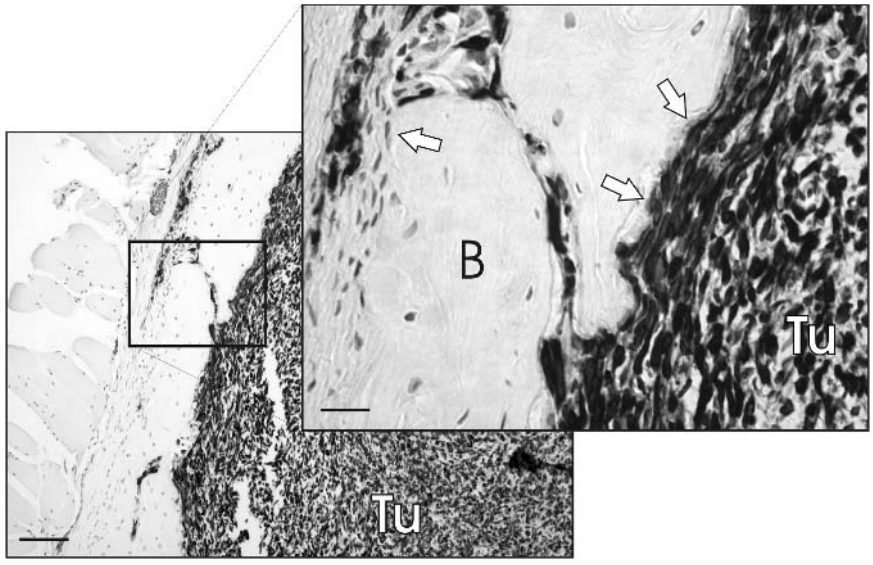


Figure 2. Cytokeratin 18 expression identifies bone metastases following growth of the 4T1.2 primary tumour in the mammary fat pad. These bone lesions are highly osteolytic, as indicated by the presence of osteoclasts (arrows;TRAP positive cells identified on an adjacent section) and the fracturing of the cortical bone. B = cortical bone, Tu = tumour cells. Scale bar; 100μm or 20μm (inset).

4. CHEMOKINE MEDIATED TUMOUR CELL HOMING TO BONE

The selectivity of metastatic cells for certain tissues is dictated, in part, by the spectrum of surface receptor molecules expressed on the cancer cell and by the presence of complementary ligands at the secondary site. When disseminating tumour cells arrive in bone they arrest in the small endothelium-lined venous sinusoids. These sinusoids are fenestrated and lack a basement membrane, making them highly permeable and permissive for the removal of metabolic waste or cellular migration into the organ. In fact, Muller et al. (2001) proposed that the metastatic dissemination of breast tumour cells is akin to the normal trafficking of leukocytes from the bloodstream into and out of target organs – a process that is critically regulated by chemokines and their receptors. Metastatic breast cancer cells, malignant tumours and metastatic nodules express high levels of the chemokine receptors CXCR4 and CCR7 compared to normal mammary epithelium. The ligands for these receptors, SDF-1α (CXCR4) and 6Ckine (CCR7), are expressed abundantly in tissues to which breast cancer metastasizes most avidly, namely lung, lymph node, liver and bone. The role of these receptors in breast tumour homing to bone is supported by the observation that neutralizing antibodies directed against CXCR4 inhibit *in vitro* migration of MDA-MB-231 cells towards a chemotactic gradient of SDF-1α. Similarly, neutralizing antibodies to CXCR4 inhibit experimental and spontaneous lung metastases derived from MDA-MB-231 tumours in SCID mice (48). Although the role of CXCR4/SDF-1α in metastasis to bone was not analysed, other studies have shown that SDF-1α signalling through CXCR4 stimulates transendothelial migration of prostate cancer cells (49). Collectively, these results implicate a potential 'homing' mechanism for the attraction of metastatic breast tumours to bone.

5. INTEGRIN MEDIATED TUMOUR CELL ADHESION IN BONE

Attachment to the vasculature and subsequent extravasation from the blood stream requires integrin-mediated tumour cell adhesion to

endothelial ECM components. Integrins are membrane bound receptors that function as heterodimers of α and β subunits. Combinations of α and β subunits confer substrate and signalling specificity (50) and several pairings including $\alpha_2\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$ and $\alpha_v\beta_3$ have been extensively studied in breast cancer metastasis (see reviews (51-53)). The $\alpha_v\beta_3$ integrin is of particular interest, as it is frequently upregulated during metastatic progression and is a receptor for several ECM proteins commonly found in bone including fibronectin, osteopontin, bone sialoprotein and vitronectin (8, 50). Engagement of integrin $\alpha_v\beta_3$ to fibronectin or vitronectin *in vitro* modulates several intracellular signalling pathways involving Rho GTPases, FAK, Src and PKC, leading to cytoskeletal changes and enhanced motility (54-56). Clinically, high $\alpha_v\beta_3$ integrin expression in primary breast cancers is correlated with a greater metastatic potential and the development of skeletal metastases (57). Further evidence for the role of tumour-associated $\alpha_v\beta_3$ integrin in metastasis comes from studies demonstrating that MDA-MB-435 cells selected for high levels of activated $\alpha_v\beta_3$ integrin display an enhanced ability to form spontaneous bone and lung metastases following orthotopic injection in mice (58). Integrin $\alpha_v\beta_3$ may play multiple roles during the metastatic spread of breast tumours to bone and appears to be required for the interaction of tumour cells with platelets and subsequent aggregation in thrombi and arrest in distant capillaries (58, 59). It has been demonstrated that $\alpha_v\beta_3$ integrin can recruit and activate local MMPs (MMP-2 and MMP-9, for instance) thereby facilitating ECM degradation and cellular migration (60, 61) and potentially enhancing the extravasation of tumour cells into bone.

6. TYPES OF BONE METASTASES

Bone metastases can be categorized into three distinct phenotypes: osteolytic (bone resorbing), osteoblastic (bone forming) and mixed lesions containing elements of both (62). In patients with advanced breast cancer, the majority of bone lesions are osteolytic, while approximately 15-30% are osteoblastic, and 5% have mixed lesions (62, 63). In contrast, patients with advanced prostate cancer generally develop osteoblastic lesions. These phenotypes reflect the perturbation of normal bone remodelling processes by the presence of tumour cells. Interestingly, secondary bone formation is observed in osteolytic lesions formed by breast tumour cells and some bone resorption occurs in osteoblastic metastases (62, 64). This suggests that the pathology of each type of lesion is not static, rather, the observed phenotype in each metastatic lesion results from a shift in the dynamic equilibrium of normal bone remodelling.

7. OSTEOLYTIC BONE METASTASIS

Breast tumour lines such as MDA-MB-231, MDA-MB-435 and 4T1.2 are responsive to growth factors found in bone and promote their release from bone by activating osteolytic mechanisms. The 'vicious cycle' theory describes the special predilection of breast tumours to metastasize to bone (65) by proposing that dual paracrine feedback mechanisms operate between the tumour and bone stromal cells, leading to the uncoupling of osteoblast-osteoclast signalling, resorption of bone and amplification of metastatic tumour growth (Figure 3).

Although tumour cells have been implicated in the direct resorption of bone *in vitro* (66), the majority of bone degradation *in vivo* is mediated by activated osteoclasts (67). Osteoclasts regulate the activity of several proteolytic factors, including cathepsins, uPA and MMPs, which degrade the bone matrix and release and activate several growth factors from mineralised bone such as TGF-β (68, 69). TGF-β has been shown to elicit diverse responses in bone including cellular proliferation, ECM deposition, protease production, angiogenesis and suppression of immune surveillance (70-72). Although TGF-β inhibits the proliferation of normal mammary epithelium and delays the development of primary breast tumours (73, 74), it appears to promote the establishment of epithelial tumour cells in bone. Experimental MDA-MB-231 metastasis to bone is reduced when cells are made insensitive to the action of TGF-β by transfection of a dominant-negative TGF-β type II receptor (TβRII) (75). Furthermore, metastases derived from this TGF-β

insensitive cell line are less osteolytic and fewer activated osteoclasts are observed compared to the parental cell line. Transfection of a constitutively active TGF-β type I receptor in these cells restored the osteolytic phenotype.

Further reports have shown that TGF-β leads to altered gene expression in breast cancer cells by activating SMAD and p38 MAPK pathways (76). Several TGF-β responsive genes have been implicated in the development of osteolytic metastases. These include TNF-α, PTHrP, IL-11 and IL-6 (77-79). As described above, PTHrP and IL-11 stimulate osteoclastogenesis in normal bone by elevating RANKL and suppressing OPG expression in osteoblasts. This leads to the release of matrix-associated growth factors that further enhance osteoclastogenesis and tumour cell growth (Figure 3).

PTHrP is expressed in 50-70% of primary breast carcinomas (80, 81) but its expression is markedly elevated in bone metastases (82). Whilst PTHrP expression in the primary tumour is associated with improved survival and reduced metastasis (81), in bone it has the potential to be induced in tumour cells by the bone microenvironment and thereby promote tumour growth in bone. This has been demonstrated in an experimental bone metastasis assay using MDA-MB-231 cells (44). Furthermore, increased expression of PTHrP in MCF7 cells (which were weakly osteolytic in this study), promoted experimental bone metastatic lesions with an enhanced osteolytic phenotype (83). The use of neutralizing PTHrP antibodies for the treatment of osteolytic bone disease is currently under clinical investigation (67).

IL-6 and IL-11 are multifunctional cytokines that can enhance osteoclastogenesis and bone resorption in bone organ cultures (84), through mechanisms that increase RANKL/RANK signalling and inhibit osteoblast calcification (79, 84). Although IL-6 and IL-11 bind to separate receptors, both cytokines transduce signals through the gp130 receptor. Signalling through the gp130 receptor in osteoblasts is critical for the induction of osteoclastogenesis, as neutralizing antibodies to this receptor inhibit the formation of active osteoclasts in bone organ cultures (85). The expression of IL-11 is upregulated in tumour cells upon TGF-β stimulation and in osteoblasts upon PTHrP or TGF-β stimulation (77, 79). IL-11 also acts in an autocrine manner to induce PGE_2 expression in osteoblasts (77). PGE_2 potentiates osteoclast activation by further increasing RANKL expression, while suppressing the inhibitory factors OPG and granulocyte-macrophage colony stimulating factor (GM-CSF) in stromal cells (77, 86).

A recent study utilized microarray profiling to identify genes that are causal to the establishment of breast tumours in bone (46). Several genes relating to bone colonization efficiency were identified by expression profiling of parental MDA-MB-231 cells and bone metastatic variants isolated from bone after intracardiac inoculation. Among the genes identified were osteopontin (OPN), CXCR4, IL-11 and connective tissue growth factor (CTGF), all of which were expressed at higher levels in the bone metastatic variants. Although expression of any one of these four genes in parental MDA-MB-231 cells produced little, if any change in metastatic potential, co-expression of two or more enhanced the ability of the cells to colonize bone. Using chromatin immunoprecipitation assays, they demonstrated that induction of IL-11 and CTGF expression resulted from activation of the TGF-β/Smad signalling pathway in the tumour cells. Consistent with this, the expression of IL-11 and CTGF could be induced by treatment of MDA-MB-231 cells with TGF-β (46).

Genes identified in this study may constitute novel therapeutic targets for metastatic bone disease. CTGF is an extracellular matrix protein that has been implicated in bone remodelling and angiogenesis (reviewed in (87)). By binding to cytokines in the bone matrix, CTGF can modulate cellular signalling. CTGF binds to both BMP4 (a known inducer of bone formation) and TGF-β and antagonizes the former but promotes the signalling of the latter (88). Through suppression of BMP4 and induction of TGF-β signalling, CTGF could potentially be involved in the vicious cycle of osteolytic bone metastases. Collectively, these studies show that successful bone metastasis requires the coordinated action of multiple paracrine pathways, in which TGF-β signalling plays a central role by altering the bone microenvironment and promoting the growth of the metastatic lesion.

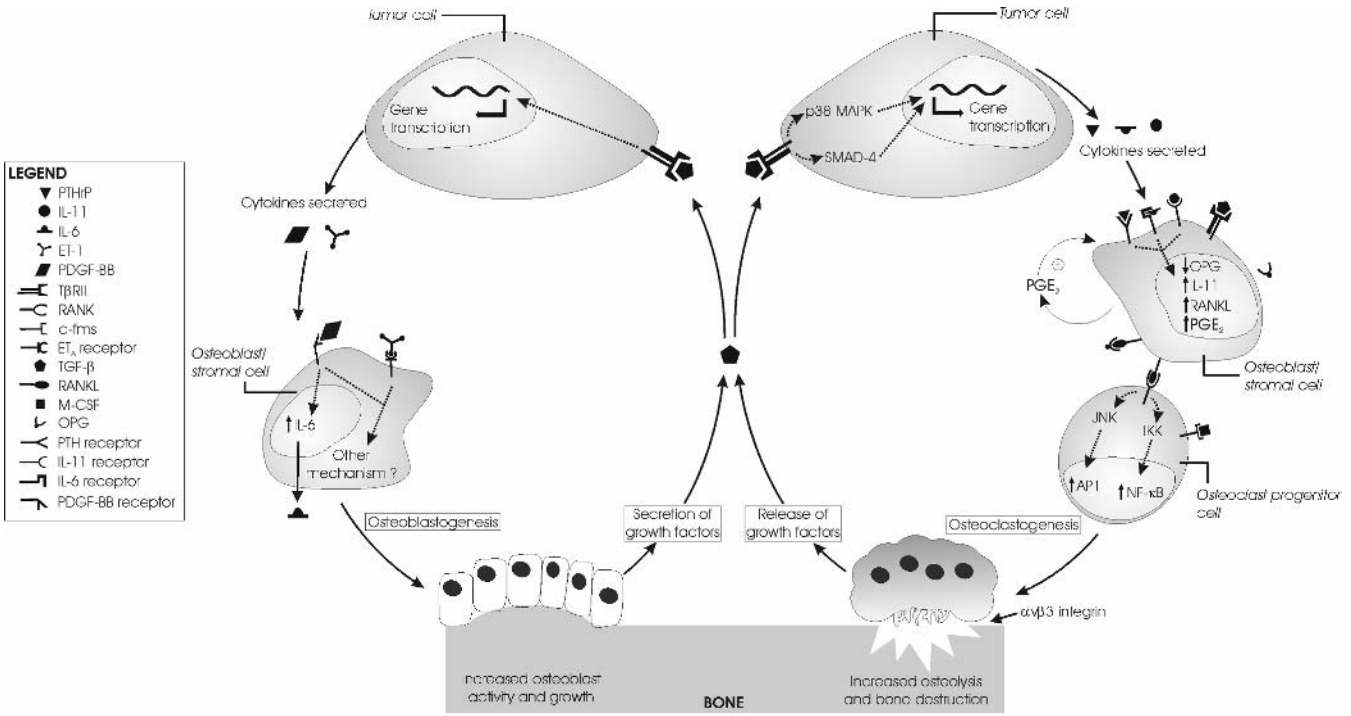


Figure 3. Interactions between tumour cells and the bone microenvironment. Breast tumour cells disrupt the homeostatic mechanisms that regulate normal bone remodelling, leading to morphological changes in bone structure and enhanced release of bone derived cytokines that aid the growth and establishment of the tumour cell. Lesions can be osteoblastic, leading to increased bone deposition (left side of diagram) or osteolytic, leading to bone loss (right side of diagram).

8. OSTEOBLASTIC METASTASIS

Osteoblastic metastases are less common in breast cancer but are well documented in metastatic prostate cancer. The mechanisms responsible for the formation of osteoblastic metastases in both types of cancers are poorly understood. Recently, this area of research has been strengthened by the development of new models of breast cancer with associated osteoblastic metastases.

MCF-7 cells expressing the *Neu* oncogene (MCF-7/*Neu*) produce overt osteoblastic bone metastases (with ectopic sites of active osteolysis) and high plasma levels of PDGF-BB after arterial inoculation of cells into nude mice (64). PDGF-BB is a potent osteotrophic factor expressed by osteoblasts, osteoclasts and aggregated platelets (89). Introduction of antisense DNA or neutralizing antibodies reduced PDGF-BB levels *in vivo* and subsequently decreased metastatic burden in bone. Furthermore, overexpression of PDGF-BB in osteolytic MDA-MB-231 cells resulted in the formation of mixed osteolytic/osteoblastic lesions *in vivo*. Comparisons of PDGF-BB transfected MDA-MB-231 cells and control cells showed no difference in PTHrP levels, which may account for the observed mixed phenotype. Interestingly, PDGF-BB can induce IL-6 expression in osteoblasts (90) and can directly activate osteoclasts *in vitro* (91), which could potentially explain the partial-osteolytic nature of the MCF-7/*Neu* cells. A mechanism for the formation of osteoblastic metastases by PDGF-BB expressing tumour cells has not been elucidated *in vivo*, however bone stromal cells express PDGF-BB receptors and signalling through these receptors could disrupt osteoblast-osteoclast homeostasis in favour of enhanced bone growth.

Three breast tumour lines (MCF-7, T47D and ZR-75-1) have been reported recently to produce osteoblastic metastases after intracardiac inoculation in *nude* mice (92). In these tumour lines, endothelin-1 (ET-1) was found to be the secreted factor that was causal for the formation of osteoblastic metastases. Endothelin-1 regulates angiogenesis, osteoblast proliferation and activity *in vitro* and is elevated in the serum of patients with advanced prostate cancer (93-95). ET-1 can act via two receptors, ET_A or ET_B, which are expressed in bone stromal cells. *In vivo* studies in the model described by Yin et al., demonstrated that blockade of the ET_A receptor prevented the ability of ZR-75-1 but not MDA-MB-231 tumour cells to colonize bone (92).

Several bone-derived growth factors including IL-1β, TNF-α and TGF-β can increase ET-1 in PC3 cells *in vitro* (96). Hence, it is plausible that the 'vicious cycle' theory proposed for the mechanism of osteolytic bone destruction by breast cancer cells may also hold true for the establishment of osteoblastic bone lesions. In this case, ET-1 stimulates osteoblast activity, which enriches the local microenvironment with growth factors that induce tumour growth and subsequent expression of ET-1 (Figure 3). A dual role for TGF-β for the establishment of either osteolytic or osteoblastic metastases would be most intriguing, however a causal role for TGF-β in the formation of ET-1 induced osteoblastic breast cancer metastases remains to be established.

9. BONE STROMAL THERAPY FOR METASTATIC BREAST CANCER

The interaction between breast tumour cells and the host stroma is critical for the successful formation of bone lesions. Since the events that lead to bone resorption and to the release of factors from bone can contribute to survival and establishment of tumour cells in bone, therapies designed to target the mechanisms of osteoclastogenesis may prove to be effective. Over the last decade, several therapeutic approaches that target stromal-tumour interactions including proteolytic inhibitors, inhibitors of osteoclastogenesis and compounds that disrupt the action of breast tumours in bone have been developed (Table 1).

Table 4 . Bone stroma targeted compounds currently in use or under investigation as inhibitors of bone metastases.

Target	Mechanism	Stage	Ref.
Bisphosphonates	Bone resorption; tumor cell growth and reduced bone pain	In clinical use	102
Osteoprotegerin	Prevents RANKL binding to RANK to activate osteoclasts	Phase I	106
RANKL antibody	Prevents RANKL binding to RANK to activate osteoclasts	Phase I	104
PTHrP antibody	Neutralizing PTHrP effects	Phase III	67
Vitamin-D analogues	Inhibition of PTHrP expression	Phase III	67
MMP inhibitors	Inhibition of proteolysis	Phase III	113
integrin beta3 inhibitors	Osteoclast adhesion, tumor migration	preclinical	108

Bisphosphonates, based on their affinity for calcium ions, have a high avidity for mineralised bone (97). When released by osteolysis, bisphosphonates are readily absorbed by the osteoclast, resulting in altered metabolism and induction of apoptosis (98). In Phase III clinical trials zoledronic acid, a nitrogen containing bisphosphonate, has shown efficacy in reducing skeletal morbidity in patients with bone metastatic tumours (99, 100). Zoledronic acid inhibits the formation of mature osteoclasts by preventing the fusion of osteoclast precursors, most likely through the disruption of RANKL/RANK signalling (98, 101). It also displays direct anti-tumour effects by inducing apoptosis, inhibiting breast cancer cell invasion and reducing angiogenesis *in vitro* (102).

Reduced tumour-induced bone destruction can also be achieved by disruption of RANKL/RANK signalling through the administration of monoclonal antibodies to RANKL, recombinant OPG or RANK-Fc fusion proteins. These proteins compete with RANK for RANKL binding, effectively reducing osteoclastic bone resorption in several pre-clinical models (103-105). Initial clinical trials of recombinant OPG administration in patients with advanced breast cancer have produced promising results. The agent is well tolerated and suppresses bone resorption to a similar extent as pamidronate (106). Although shown to be an effective suppressor of tumour induced osteolysis in experimental models of myeloma (ARH-77 cells) and prostate cancer (LNCap cells) (105, 107), RANK-Fc is yet to be tested in models of breast cancer.

Tumour expressed integrin receptors that interact with components of the bone extracellular matrix offer another therapeutic target. Using β_3 knockout mice Bakewell et al., demonstrated that β_3 integrin is crucial for tumour cell adhesion to platelets and entry into the bone marrow and suggest that drugs designed to target platelet $\alpha_{II}\beta_3$ integrin may be a promising antimetastatic therapy (108). The $\alpha_v\beta_3$ integrin or downstream components of its signalling pathway are also attractive targets, since $\alpha_v\beta_3$ integrin expression is not widespread but is elevated in bone metastatic tumours, activated osteoclasts and angiogenic vessels (17). Soluble collagen type I fragments effectively inhibit the adhesion of tumour cells to bone (109). Similarly, neutralizing antibodies to α_2, α_3, α_5, α_V, β_1, β_3, and β_5 integrin subunits inhibit the *in vitro* adhesion of breast tumour cells to bone matrix (109, 110). Furthermore, neutralizing antibodies to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins disrupt tumour angiogenesis, migration and proliferation of breast cancer cells (8, 111). Specific peptide inhibitors to $\alpha_v\beta_3$ integrin prevent osteoclast mediated bone resorption *in vitro* and bone loss in an *in vivo* model of osteoporosis (112). Collectively, these studies support the use of integrin inhibitors to block the development of osteolytic lesions, however this strategy is yet to be tested in metastasis-associated bone resorption *in vivo*.

The recent development of pre-clinical models of osteoblastic metastasis have already made an impact on therapeutic interventions that target the bone stroma (92). As described previously, blocking the interaction of the osteoblastic factor such as ET-1 through the use of drugs targeting the ET_A receptor, reduced osteopetrosis and metastatic burden. Interestingly, targeting of the ET_B receptor did not inhibit osteoblastic bone metastases in this model, suggesting that the action of ET-1 to induce osteoblastic bone lesions is specific to the ET_A receptor.

In summary, the successful establishment of metastatic bone tumours requires complementary interactions between the tumour cells and the local microenvironment. Bone is a rich source of several stimulatory factors that are released after proteolysis of the bone matrix. In particular, TGF-β appears to play a critical role in the establishment of a vicious cycle of tumour growth in bone (Figure 3). Current therapeutics aim to inhibit the known interactions between the bone stroma and tumour cells that induce osteotrophic bone lesions. To date, only a handful of genes that drive tumour growth in bone have been identified. Studies on the TGF-β signalling axis in breast cancer metastasis clearly demonstrate how a locally produced cytokine can aid in the establishment of bone metastases. However, it is likely that several other cytokines including BMPs, interleukins and IGFs are also important but their role in breast cancer metastasis to bone will require further investigation. Implementation of gene expression profiling on clinically relevant models of breast cancer metastasis to bone will aid in the identification of novel genes required for the formation of bone metastatic lesions. These may prove to be relevant therapeutic targets and lead to the development of improved treatments for metastatic breast cancer.

ACKNOWLEDGEMENTS

Support from the NIH (CA90291) and the Department of the Army Breast Cancer Research Program (DAMD17-98-1-8144 and DAMD17-01-1-0371) is gratefully acknowledged.

REFERENCES

1. Fidler, I. J. 2002, The organ microenvironment and cancer metastasis. Differentiation, 70:498-505.
2. Boudreau, N., and Myers, C., 2003, Breast cancer-induced angiogenesis: multiple mechanisms and the role of the microenvironment. Breast Cancer Res, 5:140-146.
3. Brown, L. F., Guidi, A. J., Schnitt, S. J., Van De Water, L., Iruela-Arispe, M. L., Yeo, T. K., Tognazzi, K., and Dvorak, H. F., 1999, Vascular stroma formation in carcinoma in situ, invasive carcinoma, and metastatic carcinoma of the breast. Clin Cancer Res, 5:1041-1056.
4. Chang, C. and Werb, Z., 2001, The many faces of metalloproteases: cell growth, invasion, angiogenesis and metastasis. Trends Cell Biol, 11: S37-43.
5. Liotta, L. A., and Kohn, E. C., 2001, The microenvironment of the tumour-host interface. Nature, 411:375-379.
6. Sternlicht, M. D., Bissell, M. J., and Werb, Z., 2000, The matrix metalloproteinase stromelysin-1 acts as a natural mammary tumour promoter. Oncogene, 19: 1102-1113.
7. Maheshwari, G., Brown, G., Lauffenburger, D. A., Wells, A., and Griffith, L. G., 2000, Cell adhesion and motility depend on nanoscale RGD clustering. J Cell Sci, 113 (Pt 10): 1677-1686.
8. Sung, V., Stubbs, J. T., 3rd, Fisher, L., Aaron, A. D., and Thompson, E. W., 1998, Bone sialoprotein supports breast cancer cell adhesion proliferation and migration through differential usage of the alpha(v)beta3 and alpha(v)beta5 integrins. J Cell Physiol, 176:482-494.
9. Engers, R., and Gabbert, H. E., 2000, Mechanisms of tumour metastasis: cell biological aspects and clinical implications. J Cancer Res Clin Oncol, 126: 682-692.
10. Liotta, L. A., Steeg, P. S., and Stetler-Stevenson, W. G., 1991, Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell, 64:327-336.
11. Kauffman, E. C., Robinson, V. L., Stadler, W. M., Sokoloff, M. H., and Rinker-Schaeffer, C. W., 2003, Metastasis suppression: the evolving role of metastasis suppressor genes for regulating cancer cell growth at the secondary site. J Urol, 169:1122-1133.
12. Luzzi, K. J., MacDonald, I. C., Schmidt, E. E., Kerkvliet, N., Morris, V. L., Chambers, A. F., and Groom, A. C. , 1998, Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. Am J Pathol, 153: 865-873.
13. Weber, M. H., Goltzman, D., Kostenuik, P., Rabbani, S., Singh, G., Duivenvoorden, W. C., and Orr, F. W., 2000, Mechanisms of tumour metastasis to bone. Crit Rev Eukaryot Gene Expr, 10:281-302.
14. Paget, S., 1889, The distribution of secondary growths in cancer of the breast. 1889. Lancet, 1: 571-573.
15. Coleman, R. E. and Rubens, R. D., 1987, The clinical course of bone metastases from breast cancer. Br J Cancer, 55:61-66.
16. Reddi, A. H., Roodman, D., Freeman, C., and Mohla, S., 2003, Mechanisms of tumour metastasis

to the bone: challenges and opportunities. J Bone Miner Res, 18:190-194.
17. van der Pluijm, G., Lowik, C., and Papapoulos, S., 2000, Tumour progression and angiogenesis in bone metastasis from breast cancer: new approaches to an old problem. Cancer Treat Rev, 26:11-27.
18. Aubin, J. E., 1998, Advances in the osteoblast lineage. Biochem Cell Biol, 76:899-910.
19. Mackie, E. J., 2003, Osteoblasts: novel roles in orchestration of skeletal architecture. Int J Biochem Cell Biol, 35:1301-1305.
20. Jin, H., and Varner, J., 2004, Integrins: roles in cancer development and as treatment targets. Br J Cancer, 90:561-565.
21. Linkhart, T. A., Mohan, S., and Baylink, D. J., 1996, Growth factors for bone growth and repair: IGF, TGF beta and BMP. Bone, 19:1S-12S.
22. Solheim, E., 1998, Growth factors in bone. Int Orthop, 22:410-416.
23. Mundy, G. R., Chen, D., Zhao, M., Dallas, S., Xu, C., and Harris, S., 2001, Growth regulatory factors and bone. Rev Endocr Metab Disord, 2:105-115.
24. Nakamura, I., Pilkington, M. F., Lakkakorpi, P. T., Lipfert, L., Sims, S. M., Dixon, S. J., Rodan, G. A., and Duong, L. T., 1999, Role of alpha(v)beta(3) integrin in osteoclast migration and formation of the sealing zone. J Cell Sci, 112(Pt 22):3985-3993.
25. McHugh, K. P., Hodivala-Dilke, K., Zheng, M. H., Namba, N., Lam, J., Novack, D., Feng, X., Ross, F. P., Hynes, R. O., and Teitelbaum, S. L., 2000, Mice lacking beta3 integrins are osteosclerotic because of dysfunctional osteoclasts. J Clin Invest, 105:433-440.
26. Drake, F. H., Dodds, R. A., James, I. E., Connor, J. R., Debouck, C., Richardson, S., Lee-Rykaczewski, E., Coleman, L., Rieman, D., Barthlow, R., Hastings, G., and Gowen, M., 1996, Cathepsin K, but not cathepsins B, L, or S, is abundantly expressed in human osteoclasts. J Biol Chem, 271: 12511-12516.
27. Atley, L. M., Mort, J. S., Lalumiere, M., and Eyre, D. R., 2000, Proteolysis of human bone collagen by cathepsin K: characterization of the cleavage sites generating by cross-linked N-telopeptide neoepitope. Bone, 26:241-247.
28. Delaisse, J. M., Andersen, T. L., Engsig, M. T., Henriksen, K., Troen, T., and Blavier, L., 2003, Matrix metalloproteinases (MMP) and cathepsin K contribute differently to osteoclastic activities. Microsc Res Tech, 61:504-513.
29. Dew, G., Murphy, G., Stanton, H., Vallon, R., Angel, P., Reynolds, J. J., and Hembry, R. M., 2000, Localisation of matrix metalloproteinases and TIMP-2 in resorbing mouse bone. Cell Tissue Res, 299:385-394.
30. Yamagiwa, H., Tokunaga, K., Hayami, T., Hatano, H., Uchida, M., Endo, N., and Takahashi, H., 1999, E. Expression of metalloproteinase-13 (Collagenase-3) is induced during fracture healing in mice. Bone, 25:197-203.
31. Boyle, W. J., Simonet, W. S., and Lacey, D. L., 2003, Osteoclast differentiation and activation. Nature, 423:337-342.
32. Troen, B. R., 2003, Molecular mechanisms underlying osteoclast formation and activation. Exp Gerontol, 38:605-614.
33. Kong, Y. Y., Yoshida, H., Sarosi, I., Tan, H. L., Timms, E., Capparelli, C., Morony, S., Oliveira-dos-Santos, A. J., Van, G., Itie, A., Khoo, W., Wakeham, A., Dunstan, C. R., Lacey, D. L., Mak, T. W., Boyle, W. J., and Penninger, J. M., 1999, OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature, 397:315-323.
34. Li, J., Sarosi, I., Yan, X. Q., Morony, S., Capparelli, C., Tan, H. L., McCabe, S., Elliott, R., Scully, S., Van, G., Kaufman, S., Juan, S. C., Sun, Y., Tarpley, J., Martin, L., Christensen, K., McCabe, J., Kostenuik, P., Hsu, H., Fletcher, F., Dunstan, C. R., Lacey, D. L., and Boyle, W. J., 2000, RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. Proc Natl Acad Sci U S A, 97:1566-1571.
35. Lacey, D. L., Timms, E., Tan, H. L., Kelley, M. J., Dunstan, C. R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E., Capparelli, C., Eli, A., Qian, Y. X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., Guo, J., Delaney, J., and Boyle, W. J., 1998, Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell, 93:165-176.
36. Simonet, W. S., Lacey, D. L., Dunstan, C. R., Kelley, M., Chang, M. S., Luthy, R., Nguyen, H. Q., Wooden, S., Bennett, L., Boone, T., Shimamoto, G., DeRose, M., Elliott, R., Colombero, A., Tan, H. L., Trail, G., Sullivan, J., Davy, E., Bucay, N., Renshaw-Gegg, L., Hughes, T. M., Hill, D., Pattison, W., Campbell, P., Boyle, W. J., and et al., 1997, Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell, 89: 309-319.
37. Bucay, N., Sarosi, I., Dunstan, C. R., Morony, S., Tarpley, J., Capparelli, C., Scully, S., Tan, H. L., Xu, W., Lacey, D. L., Boyle, W. J., and Simonet, W. S., 1998, Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev, 12:1260-1268.
38. Takai, H., Kanematsu, M., Yano, K., Tsuda, E., Higashio, K., Ikeda, K., Watanabe, K., and Yamada, Y., 1998, Transforming growth factor-beta stimulates the production of osteoprotegerin/

osteoclastogenesis inhibitory factor by bone marrow stromal cells. J Biol Chem, 273:27091-27096.

39. Quinn, J. M., Itoh, K., Udagawa, N., Hausler, K., Yasuda, H., Shima, N., Mizuno, A., Higashio, K., Takahashi, N., Suda, T., Martin, T. J., and Gillespie, M. T., 2001, Transforming growth factor beta affects osteoclast differentiation via direct and indirect actions. J Bone Miner Res, 16:1787-1794.
40. Rosol, T. J., Tannehill-Gregg, S. H., LeRoy, B. E., Mandl, S., and Contag, C. H., 2003, Animal models of bone metastasis. Cancer, 97:748-757.
41. Arguello, F., Baggs, R. B., and Frantz, C. N., 1988, A murine model of experimental metastasis to bone and bone marrow. Cancer Res, 48:6876-6881.
42. Sasaki, A., Boyce, B. F., Story, B., Wright, K. R., Chapman, M., Boyce, R., Mundy, G. R., and Yoneda, T., 1995, Bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice. Cancer Res, 55:3551-3557.
43. Lelekakis, M., Moseley, J. M., Martin, T. J., Hards, D., Williams, E., Ho, P., Lowen, D., Javni, J., Miller, F. R., Slavin, J., and Anderson, R. L., 1999, A novel orthotopic model of breast cancer metastasis to bone. Clin Exp Metastasis, 17:163-170.
44. Guise, T. A., Yin, J. J., Taylor, S. D., Kumagai, Y., Dallas, M., Boyce, B. F., Yoneda, T., and Mundy, G. R., 1996, Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. J Clin Invest, 98:1544-1549.
45. Sung, V., Cattell, D. A., Bueno, J. M., Murray, A., Zwiebel, J. A., Aaron, A. D., and Thompson, E. W., 1997, Human breast cancer cell metastasis to long bone and soft organs of nude mice: a quantitative assay. Clin Exp Metastasis, 15:173-183.
46. Kang, Y., Siegel, P. M., Shu, W., Drobnjak, M., Kakonen, S. M., Cordon-Cardo, C., Guise, T. A., and Massague, J., 2003, A multigenic program mediating breast cancer metastasis to bone. Cancer Cell, 3:537-549.
47. Parker, B. S., Eckhardt, B. L., and Anderson, R. L., 2004, Models of breast cancer metastasis to bone: characterization of a clinically relevant model. In Bone Metastasis, G. Singh and F. W. Orr (eds.). Kluwer Press, The Netherlands.
48. Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N., Barrera, J. L., Mohar, A., Verastegui, E., and Zlotnik, A., 2001, Involvement of chemokine receptors in breast cancer metastasis. Nature, 410:50-56.
49. Taichman, R. S., Cooper, C., Keller, E. T., Pienta, K. J., Taichman, N. S., and McCauley, L. K., 2002, Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. Cancer Res, 62:1832-1837.
50. Hood, J. D. and Cheresh, D. A., 2002, Role of integrins in cell invasion and migration. Nat Rev Cancer, 2:91-100.
51. Mercurio, A. M., Bachelder, R. E., Chung, J., O'Connor, K. L., Rabinovitz, I., Shaw, L. M., and Tani, T., 2001, Integrin laminin receptors and breast carcinoma progression. J Mammary Gland Biol Neoplasia, 6:299-309.
52. Sloan, E. K. and Anderson, R. L., 2002, Genes involved in breast cancer metastasis to bone. Cell Mol Life Sci, 59:1491-1502.
53. Shaw, L. M., 1999, Integrin function in breast carcinoma progression. J Mammary Gland Biol Neoplasia, 4:367-376.
54. Boudreau, N. J. and Jones, P. L.. 1999, Extracellular matrix and integrin signalling: the shape of things to come. Biochem J, 339(Pt 3):481-488.
55. Li, X., Regezi, J., Ross, F. P., Blystone, S., Ilic, D., Leong, S. P., and Ramos, D. M., 2001, Integrin alphavbeta3 mediates K1735 murine melanoma cell motility in vivo and in vitro. J Cell Sci, 114:2665-2672.
56. Butler, B., Williams, M. P., and Blystone, S. D., 2003, Ligand-dependent activation of integrin alpha vbeta 3. J Biol Chem, 278:5264-5270.
57. Liapis, H., Flath, A., and Kitazawa, S., 1996, Integrin alpha V beta 3 expression by bone-residing breast cancer metastases. Diagn Mol Pathol, 5:127-135.
58. Felding-Habermann, B., O'Toole, T. E., Smith, J. W., Fransvea, E., Ruggeri, Z. M., Ginsberg, M. H., Hughes, P. E., Pampori, N., Shattil, S. J., Saven, A., and Mueller, B. M., 2001, Integrin activation controls metastasis in human breast cancer. Proc Natl Acad Sci U S A, 98:1853-1858.
59. Felding-Habermann, B., Habermann, R., Saldivar, E., and Ruggeri, Z. M., 1996, Role of beta3 integrins in melanoma cell adhesion to activated platelets under flow. J Biol Chem, 271:5892-5900.
60. Brooks, P. C., Stromblad, S., Sanders, L. C., von Schalscha, T. L., Aimes, R. T., Stetler-Stevenson, W. G., Quigley, J. P., and Cheresh, D. A., 1996, Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. Cell, 85:683-693.
61. Rolli, M., Fransvea, E., Pilch, J., Saven, A., and Felding-Habermann, B., 2003, Activated integrin alphavbeta3 cooperates with metalloproteinase MMP-9 in regulating migration of metastatic breast cancer cells. Proc Natl Acad Sci U S A, 100:9482-9487.
62. Roodman, G. D., 2004, Mechanisms of bone metastasis. N Engl J Med, 350:1655-1664.
63. DeMartini, A. L., Buzdar, A. U., and Blumenschein, G. R., 1983, Osteoblastic metastatic disease as a

therapeutic response to adjuvant chemotherapy in breast cancer. J Surg Oncol, 23:32-34.
64. Yi, B., Williams, P. J., Niewolna, M., Wang, Y., and Yoneda, T., 2002, Tumour-derived platelet-derived growth factor-BB plays a critical role in osteosclerotic bone metastasis in an animal model of human breast cancer. Cancer Res, 62:917-923.
65. Mundy, G. R., 1997, Mechanisms of bone metastasis. Cancer, 80:1546-1556.
66. Eilon, G. and Mundy, G. R., 1978, Direct resorption of bone by human breast cancer cells in vitro. Nature, 276:726-728.
67. Mundy, G. R., 2002, Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer, 2:584-593.
68. Martin, T. J., Allan, E. H., and Fukumoto, S., 1993, The plasminogen activator and inhibitor system in bone remodelling. Growth Regul 3:209-214.
69. Maeda, S., Dean, D. D., Gomez, R., Schwartz, Z., and Boyan, B. D., 2002, The first stage of transforming growth factor beta1 activation is release of the large latent complex from the extracellular matrix of growth plate chondrocytes by matrix vesicle stromelysin-1 (MMP-3). Calcif Tissue Int, 70:54-65.
70. Wrana, J. L., Maeno, M., Hawrylyshyn, B., Yao, K. L., Domenicucci, C., and Sodek, J., 1988, Differential effects of transforming growth factor-beta on the synthesis of extracellular matrix proteins by normal fetal rat calvarial bone cell populations. J Cell Biol, 106:915-924.
71. Festuccia, C., Angelucci, A., Gravina, G. L., Villanova, I., Teti, A., Albini, A., Bologna, M., and Abini, A., 2000, Osteoblast-derived TGF-beta1 modulates matrix degrading protease expression and activity in prostate cancer cells. Int J Cancer, 85: 407-415.
72. Huang, X., and Lee, C., 2003, From TGF-beta to cancer therapy. Curr Drug Targets, 4:243-250.
73. Siegel, P. M., Shu, W., Cardiff, R. D., Muller, W. J., and Massague, J., 2003, Transforming growth factor beta signaling impairs Neu-induced mammary tumourigenesis while promoting pulmonary metastasis. Proc Natl Acad Sci U S A, 100:8430-8435.
74. Benson, J. R., 2004, Role of transforming growth factor beta in breast carcinogenesis. Lancet Oncol, 5: 229-239.
75. Yin, J. J., Selander, K., Chirgwin, J. M., Dallas, M., Grubbs, B. G., Wieser, R., Massague, J., Mundy, G. R., and Guise, T. A., 1999, TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. J Clin Invest, 103:197-206.
76. Kakonen, S. M., Selander, K. S., Chirgwin, J. M., Yin, J. J., Burns, S., Rankin, W. A., Grubbs, B. G., Dallas, M., Cui, Y., and Guise, T. A., 2002, Transforming growth factor-beta stimulates parathyroid hormone-related protein and osteolytic metastases via Smad and mitogen-activated protein kinase signaling pathways. J Biol Chem, 277: 24571-24578.
77. Morgan, H., Tumber, A., and Hill, P. A., 2004, Breast cancer cells induce osteoclast formation by stimulating host IL-11 production and downregulating granulocyte/macrophage colony-stimulating factor. Int J Cancer, 109:653-660.
78. Suarez-Cuervo, C., Harris, K. W., Kallman, L., Vaananen, H. K., and Selander, K. S., 2003, Tumour necrosis factor-alpha induces interleukin-6 production via extracellular-regulated kinase 1 activation in breast cancer cells. Breast Cancer Res Treat, 80:71-78.
79. Morinaga, Y., Fujita, N., Ohishi, K., and Tsuruo, T., 1997, Stimulation of interleukin-11 production from osteoblast-like cells by transforming growth factor-beta and tumour cell factors. Int J Cancer, 71:422-428.
80. Southby, J., Kissin, M. W., Danks, J. A., Hayman, J. A., Moseley, J. M., Henderson, M. A., Bennett, R. C., and Martin, T. J., 1990, Immunohistochemical localization of parathyroid hormone-related protein in human breast cancer. Cancer Res, 50:7710-7716.
81. Henderson, M., Danks, J., Moseley, J., Slavin, J., Harris, T., McKinlay, M., Hopper, J., and Martin, T., 2001, Parathyroid hormone-related protein production by breast cancers, improved survival, and reduced bone metastases. J Natl Cancer Inst, 93: 234-237.
82. Powell, G. J., Southby, J., Danks, J. A., Stillwell, R. G., Hayman, J. A., Henderson, M. A., Bennett, R. C., and Martin, T. J., 1991, Localization of parathyroid hormone-related protein in breast cancer metastases: increased incidence in bone compared with other sites. Cancer Res, 51:3059-3061.
83. Thomas, R. J., Guise, T. A., Yin, J. J., Elliott, J., Horwood, N. J., Martin, T. J., and Gillespie, M. T., 1999, Breast cancer cells interact with osteoblasts to support osteoclast formation. Endocrinology, 140: 4451-4458.
84. Tamura, T., Udagawa, N., Takahashi, N., Miyaura, C., Tanaka, S., Yamada, Y., Koishihara, Y., Ohsugi, Y., Kumaki, K., Taga, T., and et al., 1993, Soluble interleukin-6 receptor triggers osteoclast formation by interleukin 6. Proc Natl Acad Sci U S A, 90: 11924-11928.
85. Kudo, O., Sabokbar, A., Pocock, A., Itonaga, I., Fujikawa, Y., and Athanasou, N. A., 2003, Interleukin-6 and interleukin-11 support human osteoclast formation by a RANKL-independent mechanism. Bone, 32:1-7.
86. Suda, K., Udagawa, N., Sato, N., Takami, M., Itoh, K., Woo, J. T., Takahashi, N., and Nagai, K., 2004, Suppression of osteoprotegerin expression by

prostaglandin E(2) is crucially involved in lipopolysaccharide-induced osteoclast formation. J Immunol, 172:2504-2510.

87. Moussad, E. E. and Brigstock, D. R., 2000, Connective tissue growth factor: what's in a name? Mol Genet Metab, 71:276-292.
88. Abreu, J. G., Ketpura, N. I., Reversade, B., and De Robertis, E. M., 2002, Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-beta. Nat Cell Biol, 4:599-604.
89. Heldin, C. H. and Westermark, B., 1999, Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev, 79:1283-1316.
90. Franchimont, N., Durant, D., Rydziel, S., and Canalis, E., 1999, Platelet-derived growth factor induces interleukin-6 transcription in osteoblasts through the activator protein-1 complex and activating transcription factor-2. J Biol Chem, 274: 6783-6789.
91. Zhang, Z., Chen, J., and Jin, D., 1998, Platelet-derived growth factor (PDGF)-BB stimulates osteoclastic bone resorption directly: the role of receptor beta. Biochem Biophys Res Commun, 251: 190-194.
92. Yin, J. J., Mohammad, K. S., Kakonen, S. M., Harris, S., Wu-Wong, J. R., Wessale, J. L., Padley, R. J., Garrett, I. R., Chirgwin, J. M., and Guise, T. A., 2003, A causal role for endothelin-1 in the pathogenesis of osteoblastic bone metastases. Proc Natl Acad Sci U S A, 100:10954-10959.
93. Nelson, J. B., Hedican, S. P., George, D. J., Reddi, A. H., Piantadosi, S., Eisenberger, M. A., and Simons, J. W., 1995, Identification of endothelin-1 in the pathophysiology of metastatic adeno-carcinoma of the prostate. Nat Med, 1:944-949.
94. Medinger, M., Adler, C. P., Schmidt-Gersbach, C., Soltau, J., Droll, A., Unger, C., and Drevs, J., 2003, Angiogenesis and the ET-1/ETA receptor system: immunohistochemical expression analysis in bone metastases from patients with different primary tumours. Angiogenesis, 6:225-231.
95. Kasperk, C. H., Borcsok, I., Schairer, H. U., Schneider, U., Nawroth, P. P., Niethard, F. U., and Ziegler, R., 1997, Endothelin-1 is a potent regulator of human bone cell metabolism in vitro. Calcif Tissue Int, 60:368-374.
96. Le Brun, G., Aubin, P., Soliman, H., Ropiquet, F., Villette, J. M., Berthon, P., Creminon, C., Cussenot, O., and Fiet, J., 1999, Upregulation of endothelin 1 and its precursor by IL-1beta, TNF-alpha, and TGF-beta in the PC3 human prostate cancer cell line. Cytokine, 11:157-162.
97. Rogers, M. J., Gordon, S., Benford, H. L., Coxon, F. P., Luckman, S. P., Monkkonen, J., and Frith, J. C., 2000, Cellular and molecular mechanisms of action of bisphosphonates. Cancer, 88:2961-2978.
98. Russell, R. G. and Rogers, M. J., 1999, Bisphosphonates: from the laboratory to the clinic and back again. Bone, 25:97-106.
99. Cameron, D., 2003, Proven efficacy of zoledronic acid in the treatment of bone metastases in patients with breast cancer and other malignancies. Breast, 12Suppl2:S22-29.
100. Rosen, L. S., Gordon, D., Tchekmedyian, S., Yanagihara, R., Hirsh, V., Krzakowski, M., Pawlicki, M., de Souza, P., Zheng, M., Urbanowitz, G., Reitsma, D., and Seaman, J. J., 2003, Zoledronic acid versus placebo in the treatment of skeletal metastases in patients with lung cancer and other solid tumours: a phase III, double-blind, randomized trial--the Zoledronic Acid Lung Cancer and Other Solid Tumours Study Group. J Clin Oncol, 21: 3150-3157.
101. Croucher, P., Jagdev, S., and Coleman, R., 2003, The anti-tumour potential of zoledronic acid. Breast, 12Suppl2:S30-36.
102. Green, J. R., 2002, Bisphosphonates in cancer therapy. Curr Opin Oncol, 14:609-615.
103. Morony, S., Capparelli, C., Sarosi, I., Lacey, D. L., Dunstan, C. R., and Kostenuik, P. J., 2001, Osteoprotegerin inhibits osteolysis and decreases skeletal tumour burden in syngeneic and nude mouse models of experimental bone metastasis. Cancer Res, 61:4432-4436.
104. Body, J. J., Coleman, R. E., Lipton, A., Murphy, R., Holloway, D. L., Bekker, P. J., and DePaoli, A. M., 2003, Rapid, profound and prolonged suppression of bone turnover with a single subcutaneous dose of AMG-162 in women with breast cancer metastasis to bone. In: The 4th International Conference on cancer-induced bone diseases, San Antonio, Texas 76.
105. Sordillo, E. M., and Pearse, R. N., 2003, RANK-Fc: a therapeutic antagonist for RANK-L in myeloma. Cancer, 97:802-812.
106. Body, J. J., Greipp, P., Coleman, R. E., Facon, T., Geurs, F., Fermand, J. P., Harousseau, J. L., Lipton, A., Mariette, X., Williams, C. D., Nakanishi, A., Holloway, D., Martin, S. W., Dunstan, C. R., and Bekker, P. J., 2003, A phase I study of AMGN-0007, a recombinant osteoprotegerin construct, in patients with multiple myeloma or breast carcinoma related bone metastases. Cancer, 97:887-892.
107. Zhang, J., Dai, J., Yao, Z., Lu, Y., Dougall, W., and Keller, E. T., 2003, Soluble receptor activator of nuclear factor kappaB Fc diminishes prostate cancer progression in bone. Cancer Res, 63:7883-7890.
108. Bakewell, S. J., Nestor, P., Prasad, S., Tomasson, M. H., Dowland, N., Mehrotra, M., Scarborough, R., Kanter, J., Abe, K., Phillips, D., and Weilbaecher, K., 2003, N. Platelet and osteoclast beta3 integrins

are critical for bone metastasis. Proc Natl Acad Sci U S A, 100:14205-14210.

109. Lundstrom, A., Holmbom, J., Lindqvist, C., and Nordstrom, T., 1998, The role of alpha2 beta1 and alpha3 beta1 integrin receptors in the initial anchoring of MDA-MB-231 human breast cancer cells to cortical bone matrix. Biochem Biophys Res Commun, 250:735-740.
110. van der Pluijm, G., Vloedgraven, H., Papapoulos, S., Lowick, C., Grzesik, W., Kerr, J., and Robey, P. G., 1997, Attachment characteristics and involvement of integrins in adhesion of breast cancer cell lines to extracellular bone matrix components. Lab Invest, 77:665-675.
111. Kumar, C. C., 2003, Integrin alpha v beta 3 as a therapeutic target for blocking tumour-induced angiogenesis. Curr Drug Targets, 4:123-131.
112. Engleman, V. W., Nickols, G. A., Ross, F. P., Horton, M. A., Griggs, D. W., Settle, S. L., Ruminski, P. G., and Teitelbaum, S. L., 1997, A peptidomimetic antagonist of the alpha(v)beta3 integrin inhibits bone resorption in vitro and prevents osteoporosis in vivo. J Clin Invest, 99: 2284-2292.
113. Coussens, L. M., Fingleton, B., and Matrisian, L. M., 2002, Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science, 295:2387-2392.

Chapter 9

Interaction of Tumour with Host Stroma in Hepatocellular Carcinoma

D.S.R. Lockwood and D.C. Gotley
University of Queensland, Brisbane, Australia

Abstract: Hepatocellular cancer has been observed to progress in distinct morphological patterns: expanding or infiltrating, and these are ultimately associated with clinical outcomes. Tumor encapsulation is a particular morphological phenomenon that has attracted attention because of potential insights to be gained about tumor and stromal interactions. Much of the current knowledge of the biology of liver stroma comes from the study of cirrhosis, which is a form of chronic wound healing, and most hepatocellular cancers develop on a background of hepatic cirrhosis. Hepatic stellate cells and myofibroblasts are responsible for extracellular matrix turnover in the liver, and are responsible for the collagen in and around encapsulated tumors. Since encapsulated tumors have a better prognosis than non-encapsulated tumors, it has been proposed that encapsulation is a host response to the presence of the tumor, a kind of host defense mechanism. It is unlikely to be as simple as this, since the presence of the capsule may reflect inherently attenuated invasive properties of the tumor. This chapter reviews the interactions between hepatocellular cancer and hepatic stroma, and reveals a complex model of tumor-host stromal interactions, that may yet translate to opportunities for therapeutic benefit.

Key words: Hepatocellular cancer, cirrhosis, hepatic stellate cells, myofibroblasts, Encapsulation, Liver ECM, MMPs, collogen, Fibronectin, Integrins, tumour-host interactions, vitamin A

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is of interest not only as a major cause of cancer mortality worldwide, but because it provides a unique model to study tumor growth in a symmetrical three dimensional matrix. Since Eggel's morphological classification in 1901, it has been recognised that HCC progresses in one of several different patterns, two of the most important being an expanding mode or an infiltrating mode (1). Not surprisingly, the mode of progression has a bearing on the clinical outcome, including the prognosis without treatment, and the likelihood and speed of recurrence after surgical resection. Advances in laboratory techniques have given insights to the cellular and molecular basis of these grossly visible variations in tumour behaviour. Interactions between tumor cells and host stroma, both cellular and extracellular matrix (ECM) appear to play a central role in determining tumor behaviour in HCC. Although considered in isolation here, interactions between tumor and stroma also impinge on pathways controlling angiogenesis, apoptosis, and other processes considered elsewhere in this volume.

One morphological pattern, encapsulation, has attracted particular attention. Okuda *et al* (2) originally described a subset of tumors (10% of patients) with capsules ranging in thickness from 2 to 10mm, associated exclusively in that series with

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 165-176.

advanced cirrhosis. Later series have generally broadened the definition to include expanding tumors with any discrete fibrous capsule discernible on light microscopy, and virtually all have linked the presence of a tumor capsule with either decreased recurrence following resection, better disease-free survival, or improved overall survival (3-15).

Two interrelated questions immediately follow: how is the substance of the tumor capsule formed, and why is its presence associated with a less aggressive phenotype? Some answers are to be found in the biology of ECM and its cellular sources in the liver.

2. EXTRACELLULAR MATRIX OF THE LIVER

As in most tissues, ECM in the liver is much more than a simple framework for parenchymal cells. ECM components, arranged in precisely defined three-dimensional relationships, act as signposts for cell adhesion, migration, differentiation, polarisation and apoptosis of cells (16). A simple illustration of this is the behaviour of hepatocytes grown *in vitro*. Hepatocytes cultured on typical polystyrene adhere and proliferate poorly, undergoing apoptosis within a few days. If the culture surface is pre-coated with type I collagen, hepatocytes attach well and proliferate, but expression of albumin and other liver-specific proteins is quickly lost. Retained expression of these proteins in cell culture is dependent on attachment to basement membrane-type ECM components.

A detailed description of the distribution of ECM within the liver is beyond the scope of this chapter, and the reader is referred to reviews of this subject and of ECM biology in general (17, 18). Much of the current knowledge of liver ECM is derived from work on liver fibrosis and cirrhosis. Within normal liver parenchyma, ECM is found in three main distributions: portal tracts, central veins/venules, and the perisinusoidal space of Disse. The composition of portal tracts and central vein supporting stroma is similar to that of large vascular structures elsewhere. The supporting interstitium consists mostly of fibrillar collagens (mostly I and III (19)), with smaller amounts of collagen V, VI, fibronectin, elastin, and many others (20). Type IV (basement membrane) collagen is found in its typical subendothelial location along with associated basement membrane glycoproteins including laminin, nidogen/entactin and fibulin (18).

In contrast, the perisinusoidal space of Disse has ultrastructural and molecular characteristics unique to the liver. Lying between the sinusoidal endothelial cells and hepatocytes, it is not empty, but consists of a loose conglomeration of various ECM components typically 1μm thick (21). Hepatic stellate cells (HSC), Kupffer cells, and pit cells are found within the space of Disse, with HSC and their processes surrounding all sinusoids. Although collagen IV and other basement membrane proteins (laminin, perlecan, nidogen/entactin) are identifiable on immunohistochemistry, a continuous basement membrane as seen lining capillaries of most other organs is not seen (in common with spleen, lymph nodes, and bone marrow) (20). Fibrillar collagens (I, III and V) are also important components, forming a loose but cohesive “cable” system continuous with both portal tract and central space at either end of the sinusoid (20). Various other ECM molecules are present in smaller amounts (collagens VI, XIV, fibronectin, decorin, tenascin-C among them (17)). This loose matrix, in combination with discontinuous endothelium (up to 40% of the sinusoid is devoid of endothelium), facilitates exchange of macromolecules between sinusoidal blood and hepatocytes.

The integrin family of cell surface molecules is central to many aspects of cell behaviour in the liver (17). Through these (and other) transmembrane receptors, the ECM binding of all liver cells feeds into pathways controlling polarisation, proliferation, apoptosis, differentiation, and normal function. Thus, the cells that determine the ECM environment have a central place in maintaining normal homeostasis, and in disease processes including neoplasia.

2.1 Hepatic fibrosis and cirrhosis

Our understanding of the biology of hepatic ECM comes mostly from research into the development of hepatic fibrosis and its end result, cirrhosis. The great majority of HCC arise in the

context of cirrhosis, and conversely HCC is a significant mode of death in patients with cirrhosis. Globally, the predominant cause of cirrhosis (and hence HCC) is viral hepatitis (chiefly hepatitis B and C). Regardless of the causative agent, cirrhosis develops as a result of repeated or continuous injury to hepatocytes, resulting in a chronic wound-healing process and ultimately scar formation. The most obvious end result of persistent injury on light microscopy is greatly increased stable fibrillar collagen, seen as broad tracts of fibrous tissue between regenerative nodules. In health, collagen makes up only 5-10% of total liver protein (compared to about one third of total body protein). In cirrhosis this proportion is dramatically increased to as much as 50%, with a proportionately greater increase in fibrillar collagen (types I and III). Less obvious on light microscopy is the process of "capillarisation" - increased density of both collagen I and basement membrane components (including collagen IV and laminin) within the perisinusoidal space of Disse, together with loss of endothelial fenestration. However, this change is enough to significantly impair macromolecule exchange and may have direct effects on hepatocyte gene expression and differentiation.

2.2 Cellular sources of hepatic ECM: Myofibroblasts and hepatic stellate cells

The main ECM-producing cells in both the normal and cirrhotic liver are hepatic stellate cells (HSC), and myofibroblasts (MFB). Constituting 5-8% of the cell population in the normal liver, "resting" HSC form a continuous network with a mean internuclear distance of 40μm, and are in contact via numerous elongated processes with endothelial cells, hepatocytes, and nerve endings. They have generally been considered mesenchymal cells, on the basis of intermediate filament expression including vimentin (21). Interestingly though, HSC express several intermediate filaments typical of neural cells, and a variety of nerve growth factors. In normal liver they are characterised by the presence of perinuclear vitamin-A rich droplets, dendritic morphology, minimal proliferation (prolonged G0), and continuous low levels of both ECM synthesis (mainly collagens III, IV, I and laminin) and proteinase production, consistent with normal "maintenance" turnover.

The vitamin A content of HSC allows highly-enriched cell isolates to be obtained by centrifugation on a density gradient, and cultured *in vitro*. When cultured on polystyrene, HSC undergo a remarkable transformation of morphology and gene expression (designated "activation") to a myofibroblast (MFB) phenotype with a flat spreading morphology (on 2-dimensional culture), greatly increased ECM synthesis, proliferation, expression of other intermediate filaments (notably α-smooth muscle actin (α-SMA)), capacity for migration (22), and numerous other changes. The stimulus for activation *in vitro* appears to relate in part to alterations in mechanical stresses on the cell (17), although the absence of normal contact with ECM components (particularly basement membrane), and possibly also other liver cells (21) also plays a role. *In vitro* culture of HSC also provides a striking example of how the very shape of the surrounding ECM can determine cell behaviour, morphology and function, even before the bewildering *in vivo* complexity of ECM molecules is introduced. HSC suspended within a collagen gel do not undergo the same activation process seen in routine two-dimensional culture, (even if the culture surface is coated with collagen I) (23). A similar phenomenon has been described in hepatocytes (24). This and many other factors complicate the extrapolation of *in vitro* results beyond the culture dish.

Nevertheless, many features of *in vitro* activation can be reproduced *in vivo*, including expression of contractile filaments (α-SMA), and up-regulated ECM synthesis (especially collagen). This is a constant feature in diverse animal models of hepatic injury, from carbon tetrachloride administration to ligation of the bile duct. It has been conclusively demonstrated that myofibroblasts are the chief source of the dramatic increase in collagen which characterises fibrotic liver injury, and ultimately cirrhosis if the stimulus is repeated or perpetuated.

The original (and still widespread) supposition was that HSC and essentially all hepatic MFB represent a single cell lineage, with expression

phenotype determined by microenvironment within normal liver (ECM binding, cell-cell contact, and position along the portal-to-central gradient of blood flow), and various pathological stimuli in disease. There is a competing view that not all heterogeneity of expression seen in MFB can be explained this way, that portal MFB seen in normal liver have a different embryologic derivation to HSC, and that the important ECM-producing cells in fibrotic liver disease represent migrated portal MFB. According to this theory, α-SMA positive cells proliferating in culture after repeated passages (on which much *in vitro* work is based) represent outgrown portal MFB rather than activated HSC (which undergo apoptosis). This may be particularly relevant to biliary causes of liver disease, where the early pathological events occur within the portal triad rather than the lobule. The outcome of this debate is probably not imminent, but is of great interest to those researching stromal interactions in HCC, and further developments are awaited. If the main ECM-producing cell type in or around HCC was shown not to derive from HSC, many experimental results would require re-interpretation. However in this chapter, no distinction is made between activated HSC and MFB unless otherwise specified.

Other liver cells play a role in hepatic ECM production. There is evidence that endothelial cells are a significant source of the basement membrane components typically found subjacent (25). Hepatocytes themselves, once thought to be the main source of liver ECM, do not synthesise significant amounts of the principal collagens, but do have an important role in synthesising both cellular and plasma fibronectin (26).

2.3 Matrix metalloproteinases

The composition of the ECM even in normal liver is not static, but reflects a dynamic balance of synthesis and degradation. The main agents of ECM degradation in the liver belong to the matrix metalloproteinase (MMP) family. MMPs are subject to complex regulatory mechanisms after secretion including the requirement for activation, and binding by tissue inhibitors of matrix metalloproteinase (TIMPs). These mechanisms also serve in part to localise the active form of the enzyme at the desired site. For instance, TIMP-2 inhibits MMP-2 activity, except when a third component, membrane-type 1 MMP (MT-1 MMP) is available. This three-molecule complex results in MMP-2 activity, which degrades a wide variety of ECM substrates. Direct interaction between integrins and MMPs may also serve to localise activity to the cell surface (27).

Immunohistochemistry of normal liver demonstrates that MFB are also the chief cellular source of most MMPs (notably -2, -3, -7 and -9), with most activity found within portal tracts. Importantly, MFB can manufacture all three elements required for active MMP-2 (28). Hepatocytes appear to manufacture small amounts of MMP-1 (a collagenase) (29) which is not expressed in significant amounts by HSC/MFB (30).

In cirrhosis, degradation of normal ECM is recognised to play a key role in pathogenesis, despite the fact that cirrhosis is characterised by a dramatic net gain in ECM. A sequence of changes of MMP and TIMP expression has been proposed: initially, a combination capable of degrading normal liver ECM is found (MMP-3, MMP-13, uPA), followed by an increase in MT1-MMP/MMP-2 activity with TIMP-1. This latter combination favours degradation of basement membrane but accumulation of fibrillar collagen (31) (similar to that seen at the margin of HCC). MMPs are active in other processes in the extracellular domain, including activation of cytokines (TGF-β) (32), directly promoting proliferation of HSC (MMP-2) (33), releasing matrix-bound growth factors (34), and mediating FAS shedding from the cell surface, (role in apoptosis) (35). They may also modify cell behaviour by revealing cryptic attachment sites within ECM (36), including sequential up-regulation of other MMPs.

3. STROMA IN AND AROUND HEPATOCELLULAR CARCINOMA

3.1 Stromal elements within HCC

The stromal component of HCC has attracted attention partly because it differs so dramatically

between HCC and normal liver parenchyma. Most HCC preserve a degree of trabecular architecture, with islands of tumor cells lined by sinusoidal endothelium. There is a striking overall paucity of collagen, particularly type III which is virtually absent from tumor sinusoids (37,38) giving a characteristic soft consistency (unlike that of metastatic lesions to the liver). Septa composed mainly of fibrillar collagen are seen between tumor nodules, and are identical in composition to the peritumoral capsule if present. Occasionally, rows of compressed hepatocytes (or perhaps better-differentiated tumor cells) can be identified within these septa, suggesting that plates of normal liver or well-differentiated tumor have become entrapped between expanding tumor nodules (38).

The amount of type IV and type VI collagens varies from scant fibrils to thick irregular bundles. Unlike fibrillar collagens, collagens IV and VI may be present to a greater extent within tumor than in the surrounding liver (38,39). No clinical correlate has been reported with the variable content of these collagens, except for reduced collagen VI within tumors arising from cirrhotic parenchyma (38). The explanation may relate to the differing aetiologies found in cirrhotic *versus* non-cirrhotic cases; the pathological significance (if any) is unclear.

Fibronectin is found within HCC, and unlike many other tumors is not confined to stroma but is found in close association with (and within) tumor cells. Since fibronectin synthesis is a function of normal hepatocytes this is not unexpected, and expression is seen most strongly in well-differentiated tumors (40). Fibronectin is a key orchestrating molecule in the early phase of wound healing, and its role in progression of HCC deserves further study.

3.2 The peritumoural capsule

At the interface between HCC and surrounding liver a wide range of patterns are seen. As alluded to previously, one of these patterns is characterised by a discrete layer of connective tissue and stromal cells interposed between tumor cells and surrounding parenchymal cells, known as encapsulation. Encapsulation is a well-defined pathological term, but unfortunately is not a simple "on or off" state. Few tumors are entirely devoid of connective tissue interposition between tumor and host, and equally few have a completely intact capsule if a thorough inspection of the host-tumor interface is undertaken. To further confuse the issue, some authors require a minimum thickness of capsular connective tissue, while others have included all tumors with an expanding growth pattern. The completeness of the capsule is probably more important than its thickness (3). While most studies have concluded that the tendency of a tumor to develop a capsule at the interface with host tissue has favourable implications, it is generally assumed to be self-evident which tumors should be classified as encapsulated. The marked geographical variation in aetiology, behaviour and morphology of HCC makes such assumptions precarious. A four point scale for quantifying the completeness of encapsulation has therefore been proposed based on the proportion of interface displaying a zone of capsule (38). While no conclusion was reached on which subset(s) should be defined as encapsulated, it is hoped that such descriptors may allow more meaningful comparison of results.

When present, the capsule consists mostly of dense fibrillar collagen, with stable collagen I on its internal aspect adjacent to tumor, and a higher proportion of collagen III on the outer aspect. While the outer layer is continuous with a transitional zone of increased collagen deposition in adjacent compressed parenchyma, the demarcation of the inner aspect from the tumor is very sharp.

3.3 Alterations in ECM of surrounding host liver

Regardless of the presence of a discrete peritumoral capsule, immunohistochemistry reveals increased concentration of collagen to a variable degree around all HCC. One element of this is the loss of intervening hepatocytes and the apposition of portal tract structures, but increased density of staining can also be seen within the sinusoids of adjacent parenchyma. This increased collagen content within peritumoral sinusoids, extending a variable distance (2-15mm) from the interface, is more easily appreciated than the "capillarisation" of cirrhosis on collagen immunohistochemistry.

The changes in ECM of the surrounding liver share many features with cirrhosis. The major difference is that while these changes occur as a temporal sequence in cirrhosis, a histological section of the host-tumor interface reveals these phases occurring simultaneously (with the exception of hepatocyte regeneration). Within the same section, the processes can be traced from histologically normal liver, to hepatocyte injury, apoptosis and necrosis, inflammation, HSC activation and MFB proliferation (41), MMP degradation of normal matrix components, increased sinusoidal ECM density, deposition of confluent collagen III, and finally stable collagen I (37, 38).

3.4 Cellular source of stroma in HCC

Staining for α-SMA and electron microscopy (19) within the substance of HCC reveals a population of MFB, which appear to be the principal source of collagen within tumor (42). Tumor cells manufacture variable amounts of fibronectin, but this tends to be lost in invasive tumors and its significance is unclear (26,40,43). The other important non-transformed cell type within HCC is endothelial cells. There is some evidence that tumor endothelial cells also manufacture ECM (collagen I, III, and basement membrane materials) and this is an important topic for further research (44).

MFB are also seen in high concentrations at the tumor-host interface, including metastatic tumors (45, 46). They are most evident within the peritumoral capsule if present, where *in situ* hybridisation reveals active synthesis of type I collagen by MFB within the capsule (41). The profusion of peritumoral MFB comes about through induced proliferation and possibly also through chemotaxis of locally-derived HSC (22). Platelet-derived growth factor (PDGF) has been studied at the host-tumor interface in HCC, since it is the most potent proliferative and chemotactic cytokine for HSC/MFB (47). A potential mechanism has been identified for a self-sustaining autocrine loop, since MFB express both PDGF-BB and its receptor.

3.5 Mechanism and significance of tumour encapsulation

The pathological significance of encapsulation was not always universally accepted, due in part to discrepancies in definition criteria and marked geographical variation in the incidence and thickness of the tumor capsule. Using the original criteria of Okuda *et al.* (2), the incidence of encapsulation in Western series is much lower (2-3%), and virtually zero in South Africa, suggesting that the mechanism of carcinogenesis and/or host genetics help determine capsule formation.

The association between tumor encapsulation, less frequent vascular invasion, and reduced recurrence, have prompted researchers to consider how the peritumoral capsule might be formed. A section taken from the host-tumor interface of an expanding HCC reveals a zone of compressed hepatocytes, with increasing density of ECM nearer the tumor. It seems plausible that native parenchymal ECM accumulates around the edge of such a tumor to some degree. This is consistent with Okuda's original observation that thick capsules only occurred in cirrhotic liver, with denser parenchymal ECM (2), and in our series there is also a trend towards increased capsule thickness in cirrhotic livers.

However, capsules are sometimes observed around smaller tumors which have not displaced enough parenchyma to develop a tumor capsule by passive accumulation alone. More importantly, active collagen synthesis by MFBs has been demonstrated within the capsule surrounding HCC (41). It has been proposed that the peritumoral capsule represents a host response designed to wall off the tumor, providing a physical barrier to tumor growth (48).

The intensity of staining for MFBs decreases with increasing distance from the host-tumor interface along with the density of collagen deposition. One explanation for this pattern would be a diffusible factor secreted by tumor cells, promoting either activation of resting HSC, recruitment by chemotaxis of MFB, or proliferation of MFB already present. In keeping with this, tumors which display increased collagen content in the surrounding host liver have relatively more

collagen with the stroma of the tumor (38). A tumor-secreted factor has been shown to activate HSC *in vitro* (49), and indeed, altered HSC phenotype, proliferation and increased ECM synthesis have been described in parenchymal tissue far remote from the tumor (50). The variable amount of ECM deposition might then be explained by differing levels of secretion of one or more fibrogenic soluble factors. This presents something of a paradox, since *in vitro* results generally show that tumor cell invasiveness is *enhanced* by increased availability of ECM substrate, particularly collagen I.

Closer examination of the tumor-host interface in encapsulated *versus* invasive tumors using immunohistochemistry for collagen I reveals the explanation. Tumors do indeed vary in their capacity to incite a fibrogenic response in the surrounding parenchyma, and this response corresponds closely with the extent of collagen deposition within the stroma of the tumor (although still scant compared to other tumors such as colorectal metastases). However those tumors which incite the greatest fibrogenic response in the surrounding tissue do not tend to form a more intact peritumoral capsule, but instead display enhanced invasiveness, more frequent effraction of the tumor-host interface, and an increased likelihood of vascular invasion. Similar results have been identified at the level of mRNA expression; higher levels of collagen I mRNA expression within tumor (not necessarily by tumor cells) were positively associated with invasion, and such tumors were *less* likely to develop a peritumoral capsule (51). Therefore, while the substance of the capsule does arise from an active fibrotic process, the associated attenuation of invasion is not a result of being "fenced in" by more intense fibrosis. Instead, the presence of a capsule reflects inherently attenuated invasive properties of that tumor. Coincidentally, the ability to induce active fibrogenesis in the surrounding parenchyma, and perhaps within the tumor, is itself implicated as an important determinant of tumor invasiveness.

Consider a hypothetical tumor with completely uninhibited cell proliferation, but no inherent ability to degrade parenchymal ECM or basement membranes. It would be predicted to displace parenchyma by pressure necrosis, regional ischaemia and hypoxia, oxidative stress and other injurious stimuli, with secondary inflammation, release of fibrogenic cytokines including PDGF and TGF-β, and a wound healing response. Activation, chemotaxis and local proliferation of HSC/MFB would be expected in compressed nearby parenchyma, along with MMP activity. As in cirrhosis, we might predict a sequence where normal parenchymal ECM is degraded by MMPs from HSC/MFB and replaced with fibrillar collagen. The end result would be very similar to what we see in a well-encapsulated HCC.

However something different must occur at the interface of invasive tumors, where invasion through parenchyma outpaces the deposition and accumulation of fibrous ECM. The obvious difference between simple expansion (as in benign tumors or non-aggressive HCC) and an invasive tumor is the ability to invade through the capsule and advance actively through parenchyma and blood vessels. A rapidly expanding tumor might achieve this partly through physical attenuation and disruption of capsule and parenchyma. However it has been well demonstrated for many tumors that the ability to invade and metastasize is dependent on their ability to actively degrade ECM, specifically by elaborating MMPs (52).

3.6 MMPs in hepatocellular carcinoma

Perhaps predictably, there are numerous studies which show a relationship between invasiveness and tumor cell expression of various MMPs, both of tumor cell lines *in vitro* and in analysis of resected human and animal model tumors. Since metastasis inevitably requires disruption of vascular or lymphatic basement membrane, the gelatinases (MMP-2 (53-56) and MMP-9 (54, 56-60)) have received most attention, although MMP-1 (53, 54, 61), MMP-3 (62, 63), and MMP-7 (53) have also been implicated. Unfortunately the evidence regarding the relative importance of these MMPs is conflicting. Mechanistic studies tend to attribute invasive potential to a single MMP, but the MMP of importance varies between cell lines, and may naturally vary between human tumors. One member of this family, membrane-type I MMP (MT1-MMP) may carry more weight as an independent determinant of invasiveness (53, 59, 64, 65), and has

also been identified in DNA array analysis in a "signature" of invasive HCC (66). This is presumed to be related to its role in directed pericellular activation of MMP-2 (67, 68).

In general, the level of MMP expression and activity in HCC is lower than that seen in cholangiocarcinoma (69, 70) hepatic colorectal metastasis (71), or pancreatic carcinoma. It may be relevant that these tumors also have a more prominent stromal element than HCC. In HCC, the enhanced invasiveness associated with collagen I has been attributed to parallel increases in expression of MMP-2, TIMP-2 and MT1-MMP (51), and designated "high matrix turnover" tumors. Binding to collagen I via β1 integrins can increase MMP expression and *in vitro* invasiveness (72), and promote cell dissociation (73). While this makes a neat connection with known molecular events, one group of tumors reminds us that there is always another layer of complexity. Fibrolamellar HCC is characterised by a much higher ECM content than other HCC, and in keeping with the "high matrix turnover" model displays increased expression of MMP-2 (74). However in fibrolamellar HCC this profile of stroma and MMP activity does not translate into increased invasiveness (it is generally less invasive), and the primary determinant of invasiveness in this group is clearly another (as yet unidentified) factor.

While MMP-mediated degradation of parenchymal stroma obviously requires elaboration of MMP protein, the total availability of pro-enzyme may not be the main factor determining invasiveness. In our hypothetical model of non-invasive HCC, it was predicted that MMP activity would be seen consistent with secondary hepatic injury as part of a wound healing response in the compressed and stressed adjacent parenchyma. Immunohistochemistry confirms that much of the demonstrable MMP protein is found some distance from the invasive front, even in invasive tumors (unpublished data).

3.7 Role of stromal cells in HCC invasion

We have already seen that the stromal environment within and around HCC is dictated mainly by HSC/MFB, and that this environment helps determine the invasive properties of the tumor, but the role of host stromal cells may extend further still. Since normal hepatocytes rely on HSC for matrix degradation in normal physiology and benign fibrotic disease, it is feasible that HSC/MFB are "recruited" by tumor cells to provide the matrix-degrading activity required for invasion. *In situ* hybridisation suggests that HSC, and not tumor cells, are the main source of MMP-2 and TIMP-2 (71) at the host-tumor interface. Other *in vitro* evidence suggests that inducing migration of HSC also leads to increased expression of MMP-2 (22).

A possible pathway of communication between HCC tumor cells and stromal cells is through one or more tumor-secreted factors, but there is also evidence suggesting that diffusible factors synthesized by stromal cells conversely influence tumor cell behaviour. MFB have been shown to manufacture hepatocyte growth factor (HGF) and induce increased invasiveness ins HCC cell lines *in vitro* (which express the receptor for HGF, c-met). This was proposed by one group to be mediated by increased expression of MT1-MMP (65), and by another group either urokinase (75), or MMP-3 (62).

3.8 Cirrhosis and tumour progression in HCC

Since increased availability of collagen I promotes invasive behaviour, and increased collagen I is a key feature of cirrhosis, it would provide useful confirmatory evidence to demonstrate increased invasiveness of HCC arising in cirrhosis. One study reports exactly this association, describing a correlation between cirrhosis, increased MMP-2 expression, increased vascular invasion, and earlier tumor recurrence following resection (51). In another study, cirrhosis was reported to enhance metastasis in a mouse model (76). However, it would also be expected that this would result in a significantly lower incidence of encapsulated tumors in the presence of cirrhosis, and this is not a feature of most clinical series (2, 3). Peritumoral fibrosis developing as the tumor advances could outweigh the presence or absence of cirrhosis in the host liver, which could explain why the expected association between cirrhosis and invasiveness is not observed (38).

4. SUMMARY AND FUTURE DIRECTIONS

Like any malignancy, HCC is not a homogeneous disease. In a clinical series, there may be no two tumors which "achieve" invasive behaviour through exactly the same mechanisms. Inevitably, many of the clues to the molecular details come from research focussed on a single molecule or pathway, and extrapolation to clinical relevance is always uncertain. Nevertheless, the weight of evidence suggests that the relationship between HCC and host stroma (both ECM and stromal cells) is an important part of the equation determining the growth pattern of the tumor, and thereby, the clinical outcome.

1. *The ECM environment is an important determinant of tumour cell behaviour.*

Many of the ECM changes around the tumor are shared with non-malignant processes, and are consistent with a non-specific response to injury and wound healing, but certain features are positively linked with invasive behaviour. In particular, collagen I is implicated as a promoter of invasive tumor cell behaviour, probably by providing a substrate for attachment *via* integrins.

2. *Stromal cell behaviour influences tumour progression.*

Since HSC/MFB are the main source of ECM in normal liver, cirrhosis, and HCC, this is already evident from point 1, but stromal cells may play other, more direct roles in determining growth patterns. This might be as the source of matrix-degrading enzymes, or by synthesizing factors such as HGF which in turn alter behaviour of HCC cells. Stromal cells are also the source of ECM which forms a capsule around less invasive tumors. The exact relationship between tumor and stroma which results in encapsulation is not known, but it is not as simple as a pervasive fibrogenic stimulus. It is likely that for a given tumor phenotype, host stromal cell phenotype also plays a role in determining tumor progression.

The improved understanding of the tumor/stroma relationship in HCC is yet to translate into any therapeutic benefit, but there are real prospects that it may. As a genetically stable population, non-transformed host stromal cells are likely to have a more predictable response than tumor cells, and will not develop resistance. For obvious reasons, HSC/MFB have been targeted in the treatment of cirrhosis, both to prevent progression and perhaps even to reverse the accumulation of abnormal ECM77. The many parallels between pathological ECM accumulation in cirrhosis and HCC suggest that compounds with demonstrated effectiveness in cirrhosis should also be evaluated for their effects on tumor progression.

REFERENCES

1. Okuda, K., 2000, Hepatocellular carcinoma. J Hepatol 32(1 Suppl):225-37.
2. Okuda, K., et al., 1977, Clinicopathologic features of encapsulated hepatocellular carcinoma: a study of 26 cases. Cancer, 40(3):1240-5.
3. Ng, I.O., et al., 1992, Tumor encapsulation in hepatocellular carcinoma. A pathologic study of 189 cases. Cancer, 70(1):45-9.
4. Franco, D., et al., 1990, Resection of hepatocellular carcinomas. Results in 72 European patients with cirrhosis. Gastroenterology, 98(3):733-8.
5. Nakashima, T., et al., 1983, Pathology of hepatocellular carcinoma in Japan. 232 Consecutive cases autopsied in ten years. Cancer, 51(5):863-77.
6. Kanai, T., et al., 1987, Pathology of small hepatocellular carcinoma. A proposal for a new gross classification. Cancer, 60(4):810-9.
7. Akashi, Y., et al., 1991, Prognosis of unresectable hepatocellular carcinoma: an evaluation based on multivariate analysis of 90 cases. Hepatology, 14(2): 262-8.
8. Regalia, E., et al., 1998, Pattern and management of recurrent hepatocellular carcinoma after liver transplantation. J Hepatobiliary Pancreat Surg, 5(1): 29-34.
9. Gozzetti, G., et al., 1988, Clinical experience with hepatic resections for hepatocellular carcinoma in patients with cirrhosis. Surg Gynecol Obstet, 166(6): 503-10.
10. Sonoyama, T., et al., 2003, Predictors of postoperative diffuse intrahepatic recurrence of hepatocellular carcinoma. Hepatogastroenterology, 50(52): 1078-84.
11. Sheen, I.S., Jeng, K.S., and Wu, J.Y., 2003, Is p53 gene mutation an indicatior of the biological behaviors of recurrence of hepatocellular carcinoma? World J Gastroenterol, 9(6):1202-7.
12. Ker, C.G., et al., 2003, Clinical significance of cell differentiation in hepatocellular carcinoma. Hepatogastroenterology, 50(50):475-9.

13. Ercolani, G., et al., 2003, Liver resection for hepatocellular carcinoma on cirrhosis: univariate and multivariate analysis of risk factors for intrahepatic recurrence. Ann Surg, 237(4):536-43.
14. De Carlis, L., et al., 2003, Surgical treatment of hepatocellular cancer in the era of hepatic transplantation. J Am Coll Surg, 196(6):887-97.
15. Qin, L.X., and Tang, Z.Y., 2002, The prognostic significance of clinical and pathological features in hepatocellular carcinoma. World J Gastroenterol, 8(2):193-9.
16. Schuppan, D., 1990, Structure of the extracellular matrix in normal and fibrotic liver: collagens and glycoproteins. Semin Liver Dis, 10(1):1-10.
17. Schuppan, D., et al., 2001, Matrix as a modulator of hepatic fibrogenesis. Semin Liver Dis, 21(3):351-72.
18. Aumailley, M., and Gayraud B., 1998, Structure and biological activity of the extracellular matrix. J Mol Med, 76(3-4):253-65.
19. Yamamoto, M., et al., 1984, Distribution of collagen types I, III, and V in fibrotic and neoplastic human liver. Acta Pathol Jpn, 34(1):77-86.
20. Martinez Hernandez, A., and Amenta, S., 1993, The hepatic extracellular matrix. I. Components and distribution in normal liver [editorial]. Virchows Arch A Pathol Anat Histopathol, 423(1):1-11.
21. Pinzani, M., 1995, Novel Insights into the Biology and Physiology of the Ito Cell. Pharmacology & Therapeutics, 66(2):387-412.
22. Ikeda, K., et al., 1999, In vitro migratory potential of rat quiescent hepatic stellate cells and its augmentation by cell activation. Hepatology, 29(6): 1760-7.
23. Senoo, H., et al., 1996, Three-dimensional structure of extracellular matrix reversibly regulates morphology, proliferation and collagen metabolism of perisinusoidal stellate cells (vitamin A-storing cells). Cell Biol Int, 20(7):501-12.
24. Hodgkinson, C., Wright, M.C., and Paine, A.J., 2000, Fibronectin-mediated hepatocyte shape change reprograms cytochrome P450 2C11 gene expression via an integrin-signaled induction of ribonuclease activity. Mol Pharmacol, 58(5):976-81.
25. Neubauer, K., et al., 1999, Transforming growth factor-beta1 stimulates the synthesis of basement membrane proteins laminin, collagen type IV and entactin in rat liver sinusoidal endothelial cells. J Hepatol, 31(4):692-702.
26. Matsui, S., et al., 1997, Expression, localization and alternative splicing pattern of fibronectin messenger RNA in fibrotic human liver and hepatocellular carcinoma. J Hepatol, 27(5):843-53.
27. Brooks, C., et al., 1996, Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. Cell, 85(5):683-93.
28. Theret, N., et al., 1999, MMP2 activation by collagen I and concanavalin A in cultured human hepatic stellate cells. Hepatology, 30(2):462-8.
29. Johansson, N., Ahonen, M., and Kahari, V.M., 2000, Matrix metalloproteinases in tumor invasion. Cell Mol Life Sci, 57(1):5-15.
30. Iredale, J., et al., 1995, Tissue inhibitor of metalloproteinase-I and interstitial collagenase expression in autoimmune chronic active hepatitis and activated human hepatic lipocytes. Clin Sci (Colch), 89(1):75-81.
31. Benyon, R.C., and Arthur, M.J., 2001, Extracellular matrix degradation and the role of hepatic stellate cells. Semin Liver Dis, 21(3):373-84.
32. Yu, Q., and Stamenkovic, I., 2000, Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev, 14(2):163-76.
33. Benyon, R.C., et al., 1999, Progelatinase A is produced and activated by rat hepatic stellate cells and promotes their proliferation. Hepatology, 30(4): 977-86.
34. Whitelock, J.M., et al., 1996, The degradation of human endothelial cell-derived perlecan and release of bound basic fibroblast growth factor by stromelysin, collagenase, plasmin, and heparanases. J Biol Chem, 271(17):10079-86.
35. Tanaka, M., et al., 1996, Fas ligand in human serum. Nat Med, 2(3):317-22.
36. Kleiner, D.E., and Stetler-Stevenson, W.G., 1999, Matrix metalloproteinases and metastasis. Cancer Chemother Pharmacol, 43 Suppl:S42-51.
37. Torimura, T., et al., 1991, Mechanism of fibrous capsule formation surrounding hepatocellular carcinoma. Immunohistochemical study. Arch Pathol Lab Med, 115(4):365-71.
38. Lockwood, D.S., et al., 2003, Tumor progression in hepatocellular carcinoma: relationship with tumor stroma and parenchymal disease. J Gastroenterol Hepatol, 18(6):666-72.
39. Le Bail, B., et al., 1997, Extracellular matrix composition and integrin expression in early hepatocarcinogenesis in human cirrhotic liver. J Pathol, 181(3):330-7.
40. Jagirdar, J., et al., 1985, Fibronectin patterns in hepatocellular carcinoma and its clinical significance. Cancer, 56(7):1643-8.
41. Ooi, L., et al., 1997, Evidence that "myofibroblast-like" cells are the cellular source of capsular collagen in hepatocellular carcinoma. J Hepatol, 26(4):798-807.
42. Faouzi, S., et al., 1999, Myofibroblasts are responsible for collagen synthesis in the stroma of human hepatocellular carcinoma: an in vivo and in vitro study. J Hepatol, 30(2):275-84.
43. Nejjari, M., et al., 2002, Expression, regulation, and function of alpha V integrins in hepatocellular

carcinoma: an in vivo and in vitro study. Hepatology, 36(2):418-26.

44. Maher, J.J., and McGuire, R.F., 1990, Extracellular matrix gene expression increases preferentially in rat lipocytes and sinusoidal endothelial cells during hepatic fibrosis in vivo. J Clin Invest, 86(5):1641-8.
45. Gulubova, M., and Popov, A., 1995, Sinusoidal changes in extratumoral liver tissue. Report of a case of metastatic liver small cell carcinoma with collagen IV detection and ultrastructural studies. Zentralbl Pathol, 140(6):443-7.
46. Lunevicius, R., et al., 2001, Clinicopathological significance of fibrotic capsule formation around liver metastasis from colorectal cancer. J Cancer Res Clin Oncol, 127(3):193-9.
47. Pinzani, M., and Marra, F., 2001, Cytokine receptors and signaling in hepatic stellate cells. Semin Liver Dis, 21(3):397-416.
48. Gulubova, M.V., 1997, Carcinoma-associated collagen type III and type IV immune localization and Ito cell transformation indicate tumor-related changes in sinusoids of the human liver. Acta Histochem, 99(3):325-44.
49. Faouzi, S., et al., 1999, Activation of cultured rat hepatic stellate cells by tumoral hepatocytes. Lab Invest, 79(4):485-93.
50. Gulubova, M.V., 1997, Collagen type III and type IV detection in and around human hepatocellular carcinoma. Gen Diagn Pathol, 142(3-4):155-63.
51. Theret, N., et al., 2001, Increased extracellular matrix remodeling is associated with tumor progression in human hepatocellular carcinomas. Hepatology, 34(1):82-8.
52. Nakajima, M., et al., 1990, Influence of organ environment on extracellular matrix degradative activity and metastasis of human colon carcinoma cells. J Natl Cancer Inst, 82(24):1890-8.
53. Miyoshi, A., et al., 2004, Snail and SIP1 increase cancer invasion by upregulating MMP family in hepatocellular carcinoma cells. Br J Cancer, 90(6): 1265-73.
54. Kaneko, F., et al., 2004, Down-regulation of matrix-invasive potential of human liver cancer cells by type I interferon and a histone deacetylase inhibitor sodium butyrate. Int J Oncol, 24(4):837-45.
55. Yang, C., et al., 2003, Integrin alpha1beta1 and alpha2beta1 are the key regulators of hepatocarcinoma cell invasion across the fibrotic matrix microenvironment. Cancer Res, 63(23): 8312-7.
56. Li, Y., et al., 2003, Inhibitory effects of antisense RNA of HAb18G/CD147 on invasion of hepatocellular carcinoma cells in vitro. World J Gastroenterol, 9(10):2174-7.
57. Matsuo, M., Sakurai H., and Saiki, I., 2003, ZD1839, a selective epidermal growth factor receptor tyrosine kinase inhibitor, shows antimetastatic activity using a hepatocellular carcinoma model. Mol Cancer Ther, 2(6):557-61.
58. Hah, N., and Lee, S.T., 2003, An absolute role of the PKC-dependent NF-kappaB activation for induction of MMP-9 in hepatocellular carcinoma cells. Biochem Biophys Res Commun, 305(2):428-33.
59. Tsuchiya, Y., et al., 2002, A new pseudo-peptide of Arg-Gly-Asp (RGD) inhibits intrahepatic metastasis of orthotopically implanted murine hepatocellular carcinoma. Int J Oncol, 20(2):319-24.
60. Chung, T.W., et al., 2002, Enhanced expression of matrix metalloproteinase-9 by hepatitis B virus infection in liver cells. Arch Biochem Biophys. 408(2): 147-54.
61. Kamochi, J., et al., 2002, Ribozyme mediated suppression of vascular endothelial growth factor gene expression enhances matrix metalloproteinase 1 expression in a human hepatocellular carcinoma cell line. Int J Oncol, 21(1):81-4.
62. Monvoisin, A., et al., 2002, Involvement of matrix metalloproteinase type-3 in hepatocyte growth factor-induced invasion of human hepatocellular carcinoma cells. Int J Cancer, 97(2):157-62.
63. Yu, F.L., et al., 2004, Induction of hepatoma cells migration by phosphoglucose isomerase/autocrine motility factor through the upregulation of matrix metalloproteinase-3. Biochem Biophys Res Commun, 314(1):76-82.
64. Lara Pezzi, E., et al., 2002, The hepatitis B virus X protein promotes tumor cell invasion by inducing membrane-type matrix metalloproteinase-1 and cyclooxygenase-2 expression. J Clin Invest, 110(12):1831-8.
65. Murakami, K., et al., 1999, Invasiveness of hepatocellular carcinoma cell lines: contribution of membrane-type 1 matrix metalloproteinase. Neoplasia, 1(5):424-30.
66. Goldenberg, D., et al., 2002, Analysis of differentially expressed genes in hepatocellular carcinoma using cDNA arrays. Mol Carcinog, 33(2): 113-24.
67. Harada, T., et al., 1998, Membrane-type matrix metalloproteinase-1(MT1-MTP) gene is overexpressed in highly invasive hepatocellular carcinomas. J Hepatol, 28(2):231-9.
68. Ogata, R., et al., 1999, Increased expression of membrane type 1 matrix metalloproteinase and matrix metalloproteinase-2 with tumor dedifferentiation in hepatocellular carcinomas. Hum Pathol, 30(4):443-50.
69. Lichtinghagen, R., et al., 1995, Expression pattern of matrix metalloproteinases in human liver. Eur J Clin Chem Clin Biochem, 33(2):65-71.
70. Terada, T., Okada, Y., and Nakanuma, Y., 1996, Expression of immunoreactive matrix metalloproteinases and tissue inhibitors of matrix

metalloproteinases in human normal livers and primary liver tumors. Hepatology, 23(6):1341-4.

71. Musso, O., et al., 1997, In situ detection of matrix metalloproteinase-2 (MMP2) and the metalloproteinase inhibitor TIMP2 transcripts in human primary hepatocellular carcinoma and in liver metastasis. J Hepatol, 26(3):593-605.
72. Masumoto, A., Arao, S., and Otsuki, M., 1999, Role of beta1 integrins in adhesion and invasion of hepatocellular carcinoma cells. Hepatology, 29(1): 68-74.
73. Genda, T., et al., 2000, Loss of cell-cell contact is induced by integrin-mediated cell- substratum adhesion in highly-motile and highly-metastatic hepatocellular carcinoma cells. Lab Invest, 80(3): 387-94.
74. Schoedel, K.E., et al., 2003, HGF, MET, and matrix-related proteases in hepatocellular carcinoma, fibrolamellar variant, cirrhotic and normal liver. Mod Pathol, 16(1):14-21.
75. Monvoisin, A., et al., 1999, Direct evidence that hepatocyte growth factor-induced invasion of hepatocellular carcinoma cells is mediated by urokinase. J Hepatol, 30(3):511-8.
76. Kuriyama, S., et al., 1999, Hepatocellular carcinoma in an orthotopic mouse model metastasizes intrahepatically in cirrhotic but not in normal liver. Int J Cancer, 80(3):471-6.
77. Bataller, R., and Brenner, D.A., 2001, Hepatic stellate cells as a target for the treatment of liver fibrosis. Semin Liver Dis, 21(3):437-51.

Chapter 10

Dynamic Nature of Tumour-Host Interactions Within the Tumor Microenvironment

D. Nagorsen[1] and F. M. Marincola[2]
[1]*Department of Haematology, Oncology and Transfusion Medicine, Charité - Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany,* [2]*Immunogenetics Section, Department of Transfusion Medicine, Clinical Center, National Institutes of Health, Bethesda, MD, USA*

Abstract: The recent progress in tumor immunology exemplifies the successful application of modern biotechnology for the understanding of the complex natural or therapy-induced phenomenon of immune-mediated rejection of cancer. Tumor antigens recognized by T cells were identified and successfully utilized in active immunization trials for the induction of tumor-antigen specific T cells. This achievement has left, however, the clinicians and researchers perplexed by the paradoxical observation of the immunization-induced T cells can recognize tumor cells in standard assays but most often cannot induce tumor regression. In this presentation, we will argue that successful immunization is one of several steps required for tumor clearance but more work needs to be done to understand how T cells can localize and be effective at the receiving end within a tumor microenvironment in most cases not conducive to the execution of their effector function. In fact, metastatic melanoma stands out among human cancers because of its immune responsiveness. Yet, the reason(s) remain(s) unclear. We believe that the key to the understanding of this complex phenomenon relies on the real-time study of tumor/host interactions in the tumor microenvironment. Most likely, T cells induced by immunization can reach the tumor site but they are not capable of performing their effector function because they encounter a tumor microenvironment not conducive to T cell activation. In this chapter, we will review some of the basic approaches that may help solve this puzzle by studying directly human disease and its adaptation to treatment.

Key words: Immunotherapy, melanoma, functional genomics, microenvironment, chemokine receptor

1. IMMUNE SURVEILLANCE

The natural history of tumours is determined by interactions between four main components of the tumour microenvironment: tumour cells, stroma, blood vessels, and infiltrating immune cells. Relationships within this tetragon are responsible for development or containment of tumour cell growth. In the present review, we will focus on the interaction between tumour and immune system that are commonly covered by the general term: immune surveillance. Whether immune surveillance truly occurs in humans and the degree of its relevance in determining the final outcome of cancer remains a central focus of discussion (1, 2). The true role of immune surveillance, particularly in humans, is far from being validated although experimental models suggest that both the innate and the acquired immune response can control tumour growth. While the question remains, research has made progress recently by developing new methods that allow direct and accurate investigation of the interaction between host and tumour particularly in the context of human disease where pristine experimental

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 177-187.

conditions are often unachievable. The concept of immune surveillance against cancer is supported by several animal studies and various observations in humans (3,4). These include increased prevalence of tumour development following immune suppression and the observation that the extent of intra-tumoural T cells is correlated with improved clinical outcome in various solid tumours (3, 5-8). Therefore, we are increasingly induced to believe that tumour-host interactions occur along a two-way-road on which tumour and immune system shape each other. To emphasize the dual role of host-protection and tumour-sculpting immune surveillance processes have also been called "cancer immunoediting" (3).

2. TUMOUR ASSOCIATED ANTIGENS AND T CELLS

Since the identification of tumour associated antigens (TAA) in the early 1990s (9, 10), tumour immunology has become a rapidly growing field of research and clinical investigation. Within a decade, more than 60 major histocompatibility (MHC) class I-associated TAAs had been identified (11) and new ones are continuously described. The appearance of tumour-specific reactions against tumour cells is dependent on a complex system. As for all antigens recognized by T cells, TAA are cleaved within the tumour cells by proteasomes into short peptides of 8-12 amino acids. These peptides are transported through the transporter associated with antigen presentation (TAP) into the endoplasmatic reticulum. Here, peptides with good binding affinity for the MHC class I molecules (in humans called Human Leukocyte Antigens or HLA) naturally associate with them. The peptide/HLA complexes, stabilized by a further protein (β_2-microglobulin) migrate then to the cell surface where they become exposed to the potential interaction with HLA class I-restricted T cells. In an HLA-dependent fashion, TAA-derived epitopes are detected by T cells through HLA/epitope-T cell receptor (TCR) engagement. An effective T cell response can only be generated after the T cells have been primed by presentation of TAA by professional antigen-presenting cells (APC), such as dendritic cells. Therefore, APC within tumours (resident APC) may play a major role for the development of specific T cell responses.

3. CHANGES IN TUMOUR AND IMMUNE CELLS

It is likely that immune cells influence the tumour cell phenotype and changes in tumour cells shape in return the immune response. Through a selective process, tumour cells likely adapt to immune pressure by changing their phenotype and escaping immune surveillance. Due to genetic instability tumours continuously re-program their genotype and thus their phenotype. Several mechanisms may be involved. Tumour cells often are defective in TAAs (12) or HLA molecule expression (13, 14), and/or have malfunctioning antigen-processing (15). A loss of expression of the HLA–epitope complex on the surface of tumour cells renders impossible their recognition by TAA-specific T cells. Furthermore, HLA-epitope complex down-regulation on tumour cell membranes correlates with decreased T-cell-triggering capability (16). Dysfunction of antigen-processing machinery might have a strong role for the coexistence of TAA-specific T cells with cancer cells expressing the target components necessary for their recognition (17-19). Complete TAA loss obviously eliminates one of the most important pre-conditions for a targeted T cell function. All given escape mechanisms happen under an assumed pressure by the immune system. Tumor/immune cell interactions are bidirectional. Tumour cells can modulate T cell function. For example, systemic and intra-tumour T cell dysfunction including anergic T cells and T cells with down-regulated CD3-zeta chain has been described in tumour patients (20,21). This anergic status of T cells has been proposed as the cause of their ineffectiveness in containing tumour growth (22). The cause of malfunction of TAA-specific T cells is not known but it is known that tumour cells can secrete immune suppressive factors, such as TGF-β (23). Several additional variables might influence T cells effector function and, consequently, their clinical effectiveness (discussed in 24). These include the activation of regulatory T cells, the development of death resistance by tumour

cells, involvement of natural killer cells and their receptors, and the potential contribution of Fas expression. Additionally, new data shed light on the role of transcription factors in tumour's defence against immune system. STAT3 expression in tumour cells leads to inhibition of production of pro-inflammatory cytokines and chemokines (25). These variables might have an important role in modulating the immune response at both the systemic level and the tumour site; and their significance for immune-system/tumour-interaction warrants further exploration.

4. THE SYSTEMIC IMMUNE RESPONSE

Several studies have demonstrated the presence of TAA-specific T cells in peripheral blood of immunized patients, proving that the primary goal of vaccination - inducing a systemic TAA-specific immune response - can reproducibly be achieved (26). Additionally, systemic tumour-directed T cell responses can evolve spontaneously in various malignant diseases without prior immunotherapy (27). Although these spontaneously occurring TAA-specific T cells have been characterized as CD3+CD8+IFNγ+CD45RA+ (28), a phenotype supposedly representative of cytotoxic effector T cells (29), their actual function in clinical settings remains unknown. Clinically, although rare clinical responses can be achieved using peptide vaccination (30), no conclusive correlation between systemic T cell response and clinical cancer regression has been convincingly demonstrated, so far. Thus, investigations of the systemic immune response do not provide sufficient information about the interaction between host and cancer cells at the actual site of "conflict" - in the tumour microenvironment.

5. METHODS TO ANALYZE THE TUMOUR MICROENVIRONMENT

Interactions between tumour and immune system lead to changes within the tumour microenvironment. These changes can affect different levels of biologic process, such as cell phenotypes, function, protein expression, and gene regulation; various methods have been utilized to investigate these alterations. Immunohistopathology remains a basic method for cell analysis in tumour immunology. Main applications for this method lie in the evaluation of antigen expression on tumour cells and in the detection/enumeration of tumour-infiltrating immune cells. From an immunological point of view, melanoma is one of the best investigated malignant diseases. Infiltration of melanomas with T cells occurs frequently and can be related to a favourable prognosis (31). In another example, we have shown that most melanoma lesions are infiltrated by CD14 positive mononuclear phagocytes (32). Taking a further example, for colorectal cancer, several independent studies have linked CD8+ infiltration of tumours or CD8+ infiltration patterns to a favourable prognosis (5,33-36). However, the antigen-specificity was not determined in those studies. Intralesional staining with fluorescence-labelled tetrameric HLA–peptide complexes (tHLA) represents an important extension of conventional antibody-based histopathology. tHLA enable direct enumeration and characterization of TAA-specific T cells (22,37,38). However, *in situ* staining with tetramers remains technically difficult (39). Culturing tumour cells and in particular tumour infiltrating cells, such as tumour infiltrating lymphocytes (TIL), is crucial for adoptive T cell transfer and for *ex vivo* and *in vitro* analyses. However, one always has to be aware of the fact that cells underlie complex changes during *in vitro* expansion. Tumour cells show an altered antigen repertoire and adhesion molecule profile. Cultured TAA-specific CD8+ T cells do not accurately reflect *in vivo* immune responsiveness because *in vitro* expansion leads to changes in the T cell function and characteristics (40). T cells can be further analyzed for their T cell receptor repertoire (spectratyping, immunoscope; 41, 42). These methods can provide important information about breadth and flexibility of TAA-specific T-cell responses. TCR analysis indicates, e.g., that the T-cell response in regressing melanoma lesions after cytokine therapy is dominated by T cells directed toward a limited number of epitopes and that

epitope-specific T cells frequently use a highly restricted TCR repertoire (43). Protein-based assays include ELISA assays to investigate, e.g., cytokines or chemokines, and staining with specific antibodies against cell markers, such as tumour antigens or T cell subset characteristics. To investigate gene regulation within the tumour microenvironment, two main techniques are used: microarrays to analyze a broad variety of transcriptomal changes and quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) to analyze single genes more detailed.

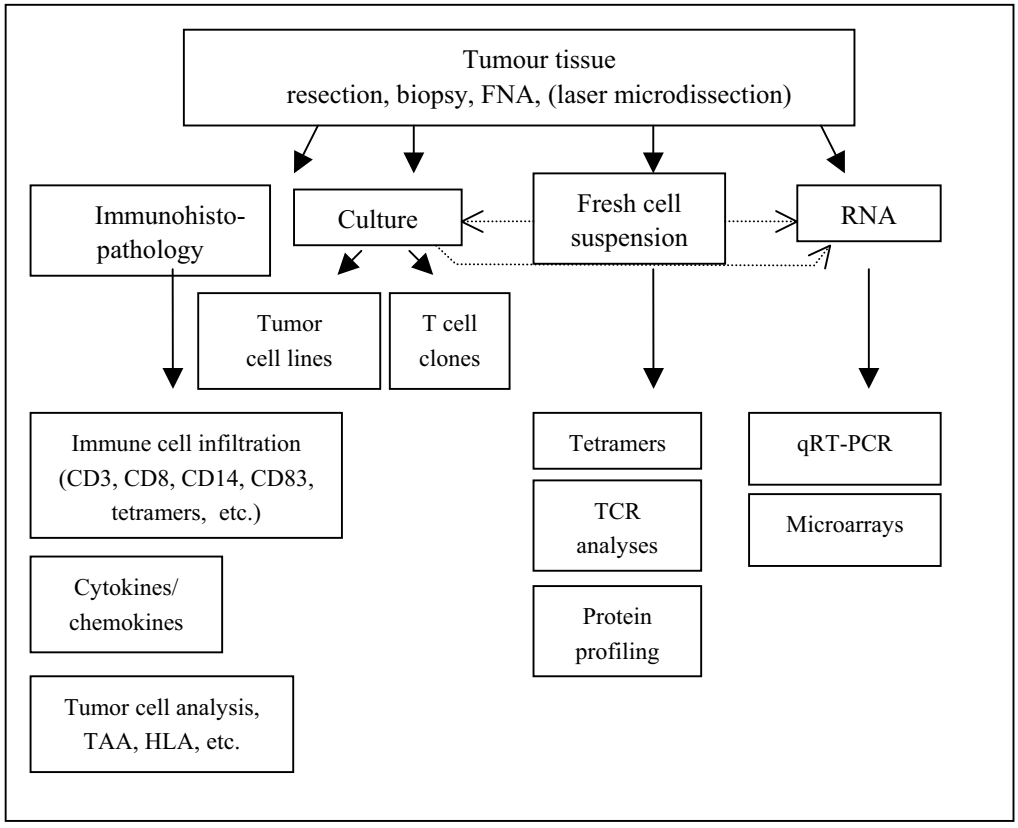


Figure 1. Several ways to analyze the tumour microenvironment.

6. IMMUNOTHERAPY

Most studies analyzing the dynamic interaction between tumour and immune system in the microenvironment have been performed as part of an immune monitoring in association with immunotherapy. During the last years, various partially efficient, immunotherapeutic approaches including peptide vaccine, DC vaccine, cytokines, viral vectors, passive transfer of antibodies or adoptive antigen-specific T cell transfer have been developed (reviewed in 30, 44, 45). The aim of an immunotherapy is the initiation of an immune response at the tumour site or site of immunization that is followed by a stepwise expansion of the immune response to loco-regional, systemic and peripheral sites, which finally causes rejection of tumour cells by the immune system (46). Although some clinical success has been achieved by immunotherapy, the major break-through has not been reached yet. Nevertheless, valuable information about the dynamic processes within the tumour microenvironment and the systemic immune response has been obtained during these therapies.

7. ANALYSES OF THE TUMOUR MICROENVIRONMENT

For several malignancies, it has been shown that a T cell infiltration or a specific pattern of T cell infiltration of the tumour is correlated with better survival (5-8). However, in these studies, the Ag-specificity of infiltrating T cells was not analyzed. For most tumours it is unknown whether TAA-specific T cells – spontaneously or vaccine induced– do migrate to the tumour site. A possible mechanism causing the variable behaviour of tumours of individual patients or lesions might be found in defects in the localization of TAA-specific T cells at the tumour site. Localization at a tumour site of adoptively transferred tumour-infiltrating lymphocytes (TILs) is a prerequisite for a clinical response (47). By analogy, we can assume that immune responses elicited by immunization might not work because they do not migrate to the tumour site. Few studies have addressed this question in humans because of technical difficulties to analyze TAA-specific responses *in situ* within tumours (39). Comparison of pre- and post-immunization samples obtained from melanoma lesions through fine-needle aspiration (FNA) biopsies suggests that immune responses elicited by vaccination can localize at a tumour site. Expansion of tumour–TIL pairs from repeated FNA biopsies of identical lesions in patients undergoing epitope-specific immunotherapy demonstrated that immunotherapy-induced T cells can be expanded more readily from melanoma metastases after treatment (18). Furthermore, quantitative real-time PCR of cytokine gene expression in FNA samples before and after vaccination demonstrated a post-immunization enhancement of IFN-γ transcripts in lesions that maintained expression of the targeted TAA and of the restricting HLA class I allospecificity (19). The relationship between IFN-γ and TAA expression suggests that vaccine-induced T cells interacted with tumour cells and/or APCs in the tumour microenvironment. Thus, vaccine-induced T cells can migrate to melanoma metastasis. Furthermore, the lack of clinical responses in this study demonstrate that tumour localization of T cells and TAA expression are not the sole factors required for an effective immunotherapy.

Cancer–host interactions within the tumour microenvironment might not be sufficient to elicit and maintain an effective T cell response because the danger signal required for full activation is not present (48). This might enable tumours to survive and grow in an “ignorant” immune environment, which is not sustaining and promoting the function of immunization-induced T cells that have localized within the tumour (49, 50). Local cytokines might promote such activation and proliferation of TAA-specific T cells. However, the required levels for interleukin-2 (IL-2) and other cytokines are relatively high (51, 52). Furthermore, CD4+ helper T cells might be necessary to provide an additional stimulus, which could lead to survival and amplification of CTL responses at the tumour site. Interestingly, for vaccination purposes, an additional foreign helper protein aiming at the support by CD4+ T cells increases the frequency of CD8+ T cell responses (53). On the other hand, e.g., in colorectal cancer, about 15% of tumour-infiltrating T cells express CD25 (36). These CD4+ CD25+ regulatory T cells play an important role for the suppression of tumour-directed T cell responses (54, 55). It is not understood to which degree these regulatory T cells influence the immunological “ignorance” systemically and at the tumour site.

Slowly, we might begin to understand homing processes of T cells. Apparently, one important component is the interaction between chemokine expression within a tissue, such as the tumour, and expression of chemokine receptors on the surface of antigen-specific T cells. First experience has been gained from analysis of chemokine receptor expression in antigen-specific T cells in viral diseases and melanoma. Expression of CCR7, a chemokine receptor important for homing into lymphatic tissue, is associated with memory subsets of antigen-specific T-cells (56), CXCR4, the receptor mediating migration to its ligand SDF-1 (highly expressed in bone marrow), is functionally expressed on melanoma-specific T-cells in peripheral blood and in bone marrow (57). The importance of chemokine receptors for cellular homing is also supported by data from tumour cells. Only tumour cells from the intestine were activated by CCR9 ligand Teck, a chemokine which is only expressed in thymus and intestine (58).

Leading cells into a specific tissue based on chemokine receptor expression, such as T cells into tumour, would require that corresponding chemokines are expressed within the target tissue. Tumour cells, stroma, and infiltrating immune cells create a complex chemokine milieu within the tumour microenvironment. In murine models, it has been shown, that expression of certain chemokines leads to a hampered tumour growth (59, 60). For human ovarian carcinoma, it was shown that T cell infiltration is an important survival marker. Furthermore, T cell infiltration is – in a preliminary study - closely correlated to mRNA levels of monokine induced by interferon-gamma (MIG) (61). Using radiolabelling several cytokines and chemokines were analyzed *in vivo* and found in several tumours. This technique is also very promising, however beyond the scope of this review (more information on this methodology can be found in the excellent review 62).

Besides changes in the immune response within the tumour microenvironment, changes in the tumour cells themselves influence the relationship between host and malignant tumour. Malignant cells are genetically unstable. The resulting tumour-cell heterogeneity during the course of disease represents a major challenge for cancer immunotherapy (24,63). Comparison of the antigenic profile of autologous melanoma lesions surgically removed at different time points of the natural course of the disease or following immunotherapy has demonstrated that temporal changes often are associated with the specificity of the immunization (16). In particular, antigen expression by tumour cells can be lost after TAA-specific immunization (12, 64). Such a loss of antigen is an important component of tumour immune escape mechanisms (16) and represents a possible explanation for the paradoxical co-existence of cancer cells with TAA-specific immune cells in the same host (65).

However, the antigenic heterogeneity of synchronous metastases raises the question whether differences in the expression of various markers among distinct lesions might simply reflect the intrinsic heterogeneity of metastases rather than time- or treatment-induced changes (15, 66). This possibility can be tested using a series of FNA biopsies (17, 67), which allow the study of the kinetics of gene expression within the same tumour lesion at several time points relevant to the disease process or its treatment. In FNAs serially obtained from 52 melanoma metastases before and after immunization with a gp100-derived peptide showed a rapid decrease in gp100 expression in metastases that regressed following immunization but detected no change in lesions that did not regress (68). This finding suggests that a successful immunization primarily induces killing of cells expressing the target TAA. It is not known whether this process is able to initiate a broader immune response. Frequently, however, the immune selection induced by the originally successful localization of TAA-specific T cells might lead to immune escape in recurring lesions by loss of complexes of HLA with TAA epitope from cancer cells (12). Thus, it is likely that tumour escape variants will emerge most frequently during or after effective immunotherapy (24).

Tumour cells potentially can revert to a stage in which they can function like stem cells with strong modulatory effects on the surrounding environment. A growing number of mechanisms that might mediate tumour–host interactions are known (summarized in 16). The identification of individual mechanisms capable of modulating tumour–host interactions has reached its limits because of genetic polymorphism of humans and heterogeneity of their diseases. The complexity of the several molecular pathways responsible for the natural and/or treatment-induced behaviour of tumour cells can be analyzed with the microarray or genechip technology, which can portray a whole gene expression pattern by measuring the expression of thousands of genes (69). Combining FNA and microarray technique, two subsets of molecular phenotypes that underlie the extent of instability of cancer over time were found in serial analysis of melanoma lesions (70). One subset of metastases containing mainly early tumour samples showed a transcriptional repertoire associated with normal human melanocytes, whereas a second subset portrayed a distinct, late-progression expression profile. Ranking of individual genes identified 30 transcripts whose gene transcription pattern was predictive of responsiveness to immunotherapy in malignant melanoma (70). Approximately half of

these genes were related to T cell regulation suggesting that responsiveness of melanoma metastases to systemic immune stimulation is pre-determined within an environment conducive to immune recognition. In a further microarray study, a specific pattern of cytokines and chemokines, including T cell attracting chemokines PARC and MIG, was shown to be up-regulated during systemic immune therapy with interleukin-2 (71). Molecular methods cannot identify the source cells of the found cytokines; they could have been expressed by tumour cells as well as immune cells. Subsequent protein profiling data suggest that DC maturation at the tumour site might possibly play a role in mediating the systemic immunotherapy at the tumour site (72).

For now, these transcriptional profiling analyses may raise more questions than they answer. Most importantly, as already pointed out, it remains unclear which cell population produces which molecules, and what is their bioactivity and its functional consequence. Nevertheless, transcriptional profiling gives an important impression on the whole picture of the tumour microenvironment and will stay an important hypothesis-generating tool in the future. Gene profiling technology has already entered clinical oncology as tool for prediction of clinical outcome of breast cancer patients (73, 74).

We have developed a hypothesis which suggests that there are some tumours more `immunogenic' than others, such as melanoma and renal-cell cancer. They spontaneously express and secrete cytokines and chemokines which induce an inflammatory reaction within the tumour microenvironment (46). When this local inflammatory reaction is strong enough, tumours regress spontaneously. However, most commonly, this inflammatory reaction is not sufficient to induce tumour regression (see figure 2). A response to therapy might occur in these cases when an additional inflammatory stimulus is brought to the tumour site by antigen-specific and/or non-specific therapy, such as IL-2 therapy. By contrast, response to therapy does not occur when a certain threshold of inflammation is not reached by the sum of therapeutic and spontaneous response. Another possible way getting an inflammatory response to the tumour site might possibly be a bacterial infection in few cases (75).

8. CONCLUSION

Tumours represent complex, individual microenvironments with four main components: tumour cells, stroma cells, blood vessels, and infiltrating immune cells. Tumour and immune system underlie permanent changes during the course of a malignant disease or during immunotherapy of cancer. Tumour cells change their antigen repertoire or develop sophisticated defence mechanisms; over time of disease T cells are primed and altered in their characteristics, and various cytokines/chemokines are produced within the tumour environment. All these mechanisms seem to be insufficient to cause tumour regression in more than a vast minority of patients. The immune system is usually only capable of keeping the tumour at bay for a limited time. An external trigger, like a systemic immunotherapy, might change the balance in favour of an effective immune response in some cases by causing a pro-inflammatory environment at the tumour site.

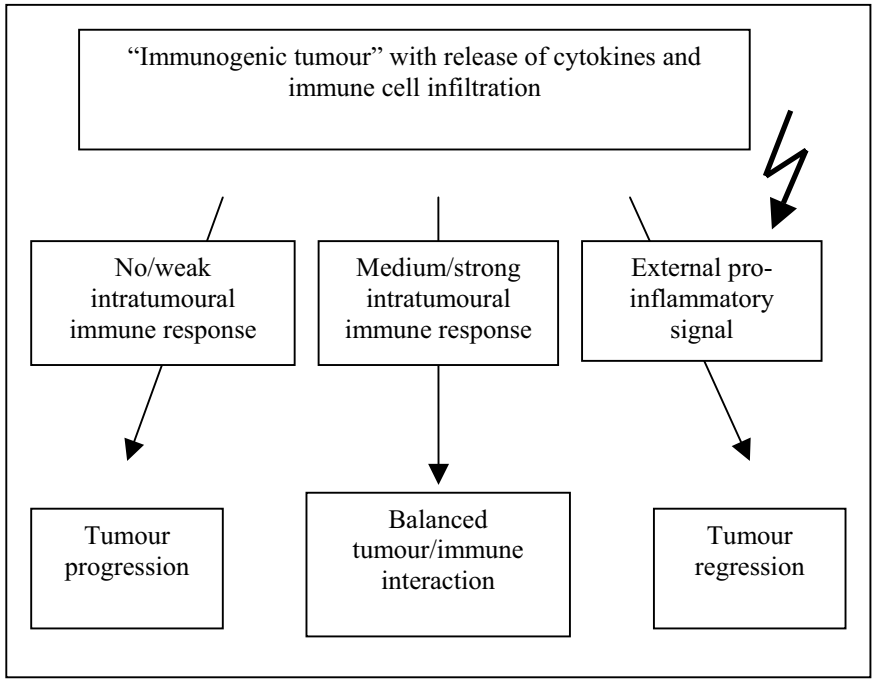


Figure 2. Balance between tumour and immune system within the tumour microenvironment.

REFERENCES

1 Qin, Z., and Blankenstein, T., 2004, A cancer immunosurveillance controversy. Nat Immunol, 5:3-4.

2 Schreiber, R.D., Old, L.J., Hayda,y A.C., and Smyth, M.J., 2004, Response to 'A cancer immuno-surveillance controversy'. Nat Immunol, 5:4-5 .

3 Dunn, G.P., Bruce, A.T., Ikeda, H., Old, L.J., and Schreibe,r R.D., 2002, Cancer immunoediting: from immunosurveillance to tumour escape. Nat Immunol, 3:991-8.

4 Boon, T., van Baren, N., 2003, Immunosurveillance against cancer and immunotherapy--synergy or antagonism?. N Engl J Med, 348:252-4.

5 Naito Y., Saito K., Shiiba K., Ohuchi A., Saigenji K., Nagura H., and Ohtani H., 1998, CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. Cancer Res, 58:3491-3494.

6 Zhang, L., Conejo-Garcia, J. R., Katsaros, D., Gimotty, P.A., Massobrio, M., Regnani, G., Makrigiannakis, A., Gray, H., Schlienger, K., 2003, Liebman, M. N., Rubin, S.C., and Coukos, G., Intratumoural T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med, 348:203-213.

7 Mihm, M., Clemente, C., and Cascinelli ,N., 1996, Tumour infiltrating lymphocytes in lymph node melanoma metastases: a histopathologic prognostic indicator and an expression of local immune response. Lab Investig, 74:43-47.

8 Schumacher, K., Haensch, W., Roefzaad, C., and Schlag, P.M., 2001, Prognostic significance of activated CD8(+) T cell infiltrations within esophageal carcinomas. Cancer Res, 61:3932-3936.

9 van der Bruggen, P., Traversari, C., Chomez, P., Lurquin, C., De Plaen, E., Van den Eynde, B., Knuth, A., and Boon, T., 1991, A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science, 254:1643-1647.

10 Boon, T., and van der Bruggen, P., 1996, Human tumour antigens recognized by T lymphocytes. J Exp Med, 183:725-729.

11 Renkvist, N., Castelli, C., Robbins P. F., and Parmiani, G., 2001, A listing of human tumour antigens recognized by T cells. Cancer Immunol Immunother, 50:3-15.

12 Jager, E., Ringhoffer, M., Altmannsberger, M., Arand, M., Karbach, J., Jager D., Oesch, F., and Knuth, A., 1997, Immunoselection in vivo: independent loss of MHC class I and melanocyte differentiation antigen expression in metastatic melanoma. Int J Cancer, 71:142–147.

13 Ferrone, S., and Marincola, F.M., 1995, Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance. Immunol Today, 16:487–494.

14 Garrido, F., Ruiz-Cabello, F., Cabrera, T., Perez-Villar, J.J., Lopez-Botet, M., Duggan-Keen, M., and Stern, P.L., 1997, Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. Immunol Today, 18:89–95.

15 Hicklin, D.J., Marincola, F.M., and Ferrone, S., 1999, HLA class I antigen downregulation in human cancers: T-cell immunotherapy revives an old story. Mol Med Today, 5:178–186.

16 Marincola, F.M., Jaffee, E.M., Hicklin, D.J., and Ferrone, S., 2000, Escape of human solid tumours from T cell recognition: molecular mechanisms and functional significance. Adv Immunol, 74:181-273.

17 Lee, K.H., Panelli, M.C., Kim, C.J., Riker, A.I., Bettinotti, M.P., Roden, M.M., Fetsch, P., Abati, A., Rosenberg, S.A., and Marincola, F.M., 1998, Functional dissociation between local and systemic immune response following peptide vaccination. J Immunol, 161:4183–4194.

18 Kammula, U.S., Lee, K.H., Riker, A.I., Wang, E., Ohnmacht, G.A., Rosenberg, S.A., and Marincola, F.M., 1999, Functional analysis of antigen-specific T lymphocytes by serial measurement of gene expression in peripheral blood mononuclear cells and tumour specimens. J Immunol, 163:6867–6879.

19 Panelli, M.C., Riker, A., Kammula, U., Wang, E., Lee, K.H., Rosenberg, S.A., and Marincola, F.M., 2000, Expansion of tumour/T cell pairs from fine needle aspirates (FNA) of melanoma metastases. J Immunol, 164:495–504.

20 Pellegrini P., Berghella A.M., Del Beato T., Cicia S., Adorno D., and Casciani, C.U., 1996, Disregulation in TH1 and TH2 subsets of CD4+ T cells in peripheral blood of colorectal cancer patients and involvement in cancer establishment and progression. Cancer Immunol Immunother, 42:1-8.

21 Yoong, K.F., and Adams, D.H., 1998, Interleukin 2 restores CD3-ζ chain expression but fails to generate tumour-specific lytic activity in tumour-infiltrating lymphocytes derived from human colorectal hepatic metastases. Br J Cancer, 77:1072-1081.

22 Lee, P.P., Yee, C., Savage, P.A., Fong, L., Brockstedt, D., Weber, J.S., Johnson, D., Swetter, S., Thompson, J., Greenberg, P.D., Roederer, M., and Davis, M.M., 1999, Characterization of circulating T cells specific for tumour-associated antigens in melanoma patients. Nat Med, 5:677–685.

23 Pasche, B., 2001, Role of transforming growth factor in cancer. J Cell Physiol. 186:153.

24 Khong, H.T., and Restifo, N.P., 2002, Natural selection of tumour variants in the generation of `tumour escape' phenotypes. Nat Immunol, 3:999–1005.

25 Wang, T., Niu, G., Kortylewski, M., Burdelya, L., Shain, K., Zhang, S., Bhattacharya, R., Gabrilovich, D., Heller, R., Coppola, D., Dalton, W., Jove, R., Pardoll, D., and Yu, H., 2004, Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumour cells. Nat Med, 10:48-54. Correction in: Nat Med, 10:209.

26 Parmiani, G., Castelli, C., Dalerba, P., Mortarini, R., Rivoltini, L., Marincola, F.M., and Anichini, A., 2002, Cancer immunotherapy with peptide-based vaccines: What have we achieved? Where are we going?. J Natl Cancer Inst, 94:805–18.

27 Nagorsen, D., Scheibenbogen, C., Marincola, F.M., Letsch, A., and Keilholz, U., Natural T cell immunity against cancer. Clin Cancer Res, 9:4296-4303.

28 Nagorsen, D., Keilholz, U., Rivoltini, L., Schmittel, A., Letsch, A., Asemissen, A. M., Berger, G., Buhr, H. J., Thiel, E., and Scheibenbogen, C., 2000, Natural T cell response against MHC class I epitopes of epithelial cell adhesion molecule, her-2/neu, and carcinoembryonic antigen in patients with colorectal cancer. Cancer Res, 60:4850-4854.

29 Hamann, D., Baars, P. A., Rep, M.H.G., Hooibrink, B., Kerkhof-Garde, S.R., Klein, M.R., and van Lier, R.A.W., 1997, Phenotypical and functional separation of memory and effector human CD8+ T cells. J Exp Med, 186:1407-1418.

30 Scheibenbogen, C., Letsch, A., Schmittel, A., Asemissen, A.M., Thiel, E., and Keilholz, U., 2003, Rational peptide-based tumour vaccine development and T cell monitoring. Semin Cancer Biol, 13(6):423-429.

31 thor Straten, P., Becker, J.C., Guldberg, P., and Zeuthen, J., 1999, In situ T cells in melanoma. Cancer Immunol Immunother, 48(7):386-95.

32 Nagorsen, D., Fetsch, P.A., Abati, A., Marincola, F.M., and Panelli, M.C., 2004, Degree of CD14 expression in melanoma infiltrating mononuclear phagocytes, submitted.

33 Ropponen, K.M., Eskelinen, M.J., Lipponen, P.K., Alhava, E., and Kosma, V.M., 1997, Prognostic value of tumour-infiltrating lymphocytes (TILs) in colorectal cancer. J Pathol, 182:318-24.

34 Oberg, A., Samii, S., Stenling, R., and Lindmark, G., 2002, Different occurrence of CD8+, CD45R0+, and CD68+ immune cells in regional lymph node metastases from colorectal cancer as potential prognostic predictors. Int J Colorectal Dis, 17:25-9.

35 Funada, Y., Noguchi, T., Kikuchi, R., Takeno, S., Uchida, Y., and Gabbert, H.E., 2003, Prognostic significance of CD8+ T cell and macrophage peritumoural infiltration in colorectal cancer. Oncol Rep, 10:309-13.

36 Diederichsen, AC, Hjelmborg, JB, Christensen, PB, Zeuthen, J, and Fenger, C., 2003, Prognostic value of the CD4+/CD8+ ratio of tumour infiltrating lymphocytes in colorectal cancer and HLA-DR expression on tumour cells. Cancer Immunol Immunother, 52:423-8.

37 Romero, P., Dunbar, P.R., Valmori, D., Pittet, M., Ogg, G.S., Rimoldi, D., Chen, J.L., Lienard, D., Cerottini, J.C., and Cerundolo, V., 1998, Ex vivo staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen-experienced tumour-specific cytolytic T lymphocytes. J Exp Med, 188:1641–1650.

38 Pittet, M.J., Valmori, D., Dunbar, P.R., Speiser, D.E., Lienard, D., Lejeune, F., Fleischhauer, K., Cerundolo, V., Cerottini, J.C., and Romero, P., 1999, High frequencies of naive Melan-A/MART-1-specific CD8(+) T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2 individuals. J Exp Med, 190:705–715.

39 Skinner, P.J., Daniels, M.A., Schmidt, C.S., Jameson, S.C., and Haase, A.T., 2000, Cutting edge: In situ tetramer staining of antigen-specific T cells in tissues. J Immunol, 165:613-7.

40 Monsurro, V., Nagorsen, D., Wang, E., Provenzano, M., Dudley, M.E., Rosenberg, S.A., and Marincola, F.M., 2002, Functional heterogeneity of vaccine-induced CD8(+) T cells. J Immunol, 168:5933-42.

41 Gorski, J., Yassai, M., Zhu, X., Kissela, B., Kissella, B., corrected to Kissela, B., Keever, C., and Flomenberg, N., 1994, Circulating T cell repertoire complexity in normal individuals and bone marrow recipients analyzed by CDR3 size spectratyping. Correlation with immune status. J Immunol, 152:5109-19.

42 Pannetier, C., Even, J., and Kourilsky, P., 1995, T-cell repertoire diversity and clonal expansions in normal and clinical samples. Immunol Today, 16:176-81.

43 Willhauck, M., Scheibenbogen, C., Moehler, T., Pawlita, M., Thiel, E., and Keilholz, U., 2003, Restricted T cell repertoire in melanoma metastases regressing after cytokine therapy. Cancer Research, 63, 3483-5

44 Cranmer, L.D., Trevor, K.T., and Hersh, E.M., 2004, Clinical applications of dendritic cell vaccination in the treatment of cancer. Cancer Immunol Immunother, 53:275-306.

45 Dudley, M.E., and Rosenberg, S.A., 2003, Adoptive-cell-transfer therapy for the treatment of patients with cancer. Nat Rev Cancer, 3:666-75.

46 Marincola, F.M., Wang, E., Herlyn, M., Seliger, B., and Ferrone, S., 2003, Tumours as elusive targets of T-cell-based active immunotherapy. Trends Immunol, 24:335-42.

47 Pockaj, B.A., Sherry, R.M., Wei, J.P., Yannelli, J.R., Carter, C.S., Leitman, S.F., Carasquillo, J.A., Steinberg, S.M., Rosenberg, S.A., and Yang, J.C., 1994, Localization of 111indium-labeled tumour infiltrating lymphocytes to tumour in patients receiving adoptive immunotherapy. augmentation with cyclophosphamide and correlation with response. Cancer, 73:1731–1737.

48 Matzinger, P., 1994, Tolerance, danger, and the extended family. Annu Rev Immunol, 12:991–1045.

49 Fuchs, E.J., and Matzinger, P., 1996, Is cancer dangerous to the immune system?. Semin Immunol, 8:271–280.

50 Ochsenbein, A.F., Klenerman, P., Karrer, U., Ludewig, B., Pericin, M., Hengartner, H., and Zinkernagel, R.M., 1999, Immune surveillance against a solid tumour fails because of immunological ignorance. Proc Natl Acad Sci USA, 96:2233–2238.

51 Gervois, N., Guilloux, Y., Diez, E., and Jotereau, F., 1996, Suboptimal activation of melanoma infiltrating lymphocytes (TIL) due to low avidity of TCR/MHC-tumour peptide interactions. J. Exp. Med, 183:2403–2407.

52 Valitutti, S., Muller, S., Dessing, M., and Lanzavecchia, A., 1996, Different responses are elicited in cytotoxic T lymphocytes by different levels of T cell receptor occupancy. J Exp Med, 183:1917–1921.

53 Scheibenbogen, C., Schadendorf, D., Bechrakis, N.E., Nagorsen, D., Hofmann, U., Servetopoulou, F., Letsch, A., Philipp, A., Foerster, M.H., Schmittel, A., Thiel, E., and Keilholz, U., 2003, Effects of granulocyte-macrophage colony-stimulating factor and foreign helper protein as immunologic adjuvants on the T-cell response to vaccination with tyrosinase peptides. Int J Cancer, 104:188-94.

54 Somasundaram, R., Jacob, L., Swoboda, R., Caputo, L., Song, H., Basak, S., Monos, D., Peritt, D., Marincola, F., Cai, D., Birebent, B., Bloome, E., Kim, J., Berencsi, K., Mastrangelo, M., and Herlyn, D., 2002, Inhibition of cytolytic T lymphocyte proliferation by autologous CD4+/ CD25+ regulatory T cells in a colorectal carcinoma patient is mediated by transforming growth factor-beta. Cancer Res, 62:5267-72.

55 Wolf, A.M., Wolf, D., Steurer, M., Gastl, G., Gunsilius, E., and Grubeck-Loebenstein, B., 2003, Increase of regulatory T cells in the peripheral blood of cancer patients. Clin Cancer Res, 9(2):606-12.

56 Sallusto, F., Lenig, D., Forster, R., Lipp, M., and Lanzavecchia, A., 1999, Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature, 401:708-12.

57 Letsch, A., Keilholz, U., Assfalg, G., Mailander, V., Thiel, E., and Scheibenbogen, C., 2003, Bone marrow contains melanoma-reactive CD8+ effector T cells and, compared with peripheral blood, enriched numbers of melanoma-reactive CD8+ memory T cells. Cancer Res, 63:5582-6.

58 Letsch, A., Keilholz, U., Schadendorf, D., Assfalg, G., Asemissen, A.M., Thiel, E., and Scheibenbogen, C., 2004, Functional CCR9 Expression is Associated with Small Intestinal Metastasis. J Invest Dermatol, in press, 2004.

59 Hu, J.Y., Li, G.C., Wang, W.M., Zhu, J.G., Li, Y.F., Zhou, G.H., and Sun, Q.B., 2002, Transfection of colorectal cancer cells with chemokine MCP-3 (monocyte chemotactic protein-3) gene retards tumour growth and inhibits tumour metastasis. World J Gastroenterol 8:1067-72.

60 Crittenden, M., Gough, M., Harrington, K., Olivier, K., Thompson, J., and Vile, R.G., 2003, Expression of inflammatory chemokines combined with local tumour destruction enhances tumour regression and long-term immunity. Cancer Res, 63(17):5505-12.

61 Coukos, G., 2003, Immunohistochemistry of Tumours for Immune cells. Plenary Session Abstracts, SBT workshop on Cancer Biometrics, Bethesda.

62 Signore, A., Capriotti, G., Scopinaro, F., Bonanno, E., and Modesti, A., 2003, Radiolabelled lymphokines and growth factors for in vivo imaging of inflammation, infection and cancer. Trends Immunol, 24:395-402.

63 Lollini, P.L., and Forni, G., 2003, Cancer immunoprevention: tracking down persistent tumour antigens. Trends Immunol, 24:62–66.

64 Riker, A., Cormier, J., Panelli, M., Kammula, U., Wang, E., Abati, A., Fetsch, P., Lee, K.H., Steinberg, S., Rosenberg, S., and Marincola, F., 1999, Immune selection following antigen specific immunotherapy of melanoma. Surgery, 126:112–120.

65 Nielsen, M.B., and Marincola, F.M., 2000, Melanoma vaccines: the paradox of T cell activation without clinical response. Cancer Chemother Pharmacol, 46 Suppl:S62-6.

66 Cormier, J.N., Hijazi, Y.M., Abati, A., Fetsch, P., Bettinotti, M., Steinberg, S.M., Rosenberg, S.A., and Marincola, F.M., 1998, Heterogeneous expression of melanoma-associated antigens and HLA-A2 in metastatic melanoma in vivo. Int J Cancer, 75:517–524.

67 Wang, E., and Marincola, F.M., 2000, A natural history of melanoma: serial gene expression analysis. Immunol Today, 21:619–623.

68 Ohnmacht, G.A., Wang, E., Mocellin, S., Abati, A., Filie, A., Fetsch, P., Riker, A.I., Kammula, U.S., Rosenberg, S.A., and Marincola, F.M., 2001, Short term kinetics of tumour antigen expression in response to vaccination. J. Immunol, 167:1809–1820.

69 Brown, P.O., and Botstein, D., 1999, Exploring the new world of the genome with DNA microarrays. Nat Genet, 21:33–37.

70 Wang, E., Miller, L.D., Ohnmacht, G.A., Mocellin, S., Perez-Diez, A., Petersen, D., Zhao, Y., Simon, R., Powell, J.I., Asaki, E., Alexander, H.R., Duray, P.H., Herlyn, M., Restifo, N.P., Liu, E.T., Rosenberg, S.A., and Marincola, F.M., 2002, Prospective molecular profiling of subcutaneous melanoma metastases suggests classifiers of immune responsiveness. Cancer Res, 62:3581–3586.

71 Panelli, M.C., Wang, E., Phan, G., Puhlmann, M., Miller, L., Ohnmacht, G.A., Klein, H.G., and Marincola, F.M., 2002, Gene-expression profiling of the response of peripheral blood mononuclear cells and melanoma metastases to systemic IL-2 administration. Genome Biol ,3:RESEARCH0035.

72 Nagorsen, D., Marincola, F.M., and Panelli, M.C., 2004, Cytokine and chemokine expression profiles of maturing dendritic cells using multiprotein platform arrays. Cytokine, 25:31-5.

73 van 't Veer, L.J., Dai, H., van de Vijver, M.J., He, Y.D., Hart, A.A., Mao, M., Peterse, H.L., van der Kooy, K., Marton, M.J., Witteveen, A.T., Schreiber, G.J., Kerkhoven, R.M., Roberts, C., Linsley, P.S., Bernards, R., and Friend, S.H., 2002, Gene expression profiling predicts clinical outcome of breast cancer. Nature, 415:530-6.

74 van de Vijver M.J., He Y.D., van't Veer L.J., Dai H., Hart A.A., Voskuil D.W., Schreiber G.J., Peterse J.L., Roberts C., Marton M.J., Parrish M., Atsma D., Witteveen A., Glas A., Delahaye L., van der Velde T., Bartelink H., Rodenhuis S., Rutgers E.T., Friend S.H., and Bernards R., 2002, A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 347:1999-2009.

75 Nagorsen D., and Marincola F.M., Kaiser H.E., 2002, Bacteria-related spontaneous and therapeutic remission of human malignancies. In Vivo 16:551-556.

Chapter 11

Breast Cancer Progression: A "Vicious Cycle" of Pro-Malignancy Activities is Mediated by Inflammatory Cells, Chemokines and Cytokines

A. Ben-Baruch
Dept. Cell Research and Immunology, George S. Wise Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv, Israel

Abstract: Breast cancer progression is a multi-step process, affected by intrinsic, as well as by microenvironmental factors. The inflammatory milieu of breast tumors, comprising of cells, chemokines and cytokines, was recently suggested to have a major role in this process. The current chapter addresses the presence of these elements in breast tumors, and their roles in the malignancy and metastatic fate of breast cancer. First, the presence of Tumor-Associated Macrophages (TAM), and the array of tumor-promoting activities that they exert in breast tumors, is described. Thereafter, the inflammatory chemokines CCL2 (MCP-1), CCL5 (RANTES) and CXCL8 (Interleukin 8) are addressed, followed by description of the expression and roles of inflammatory cytokines, which are monocyte/macrophage-derived, namely Interleukin 1 (IL-1), Tumor Necrosis Factor α (TNFα) and Interleukin 6 (IL-6). Throughout the chapter, major emphasis is put on the manner by which the different inflammatory mediators cross-interact with each other, as well as with the tumor cells, together establishing an inflammatory microenvironment that consists of an extensive network of pro-malignancy activities.

Key words: Breast Cancer, CCL2, CCL5, CXCL8, IL-1, IL-6, inflammatory chemokines, inflammatory cytokines, TNFα, tumor-associated macrophages, "vicious cycle" theory

1. INTRODUCTION

Breast cancer is a leading cause of death for women in the Western Hemisphere. The processes of breast cancer development and progression are attributed to genetic, hormonal, environmental and nutritional factors. In addition, a key role in breast cancer was recently suggested to the tumor microenvironment. In general, the tumor microenvironment consists of stroma cells (e.g. endothelial cells and fibroblasts), host-derived leukocyte infiltrates, soluble mediators, extracellular proteins and more (1-5). Microenvironmental cells and factors may affect the outcome of the cancerous process in many different manners, depending on the tumor type, the organ and the disease stage.

Of the different microenvironmental factors that may affect tumor progression, including breast cancer, major roles were attributed to inflammatory components: cells, chemokines and cytokines [Representative reviews on the subject: (5-18)]. As described below, these components establish a network of effectors that jointly regulates breast cancer progression. Inflammatory chemokines induce angiogenesis, as well as the recruitment of inflammatory cells to breast tumors, primarily of monocytes that differentiate in the tumor to Tumor-Associated Macrophages (TAM). Breast tumor cells, as well as TAM and stroma cells secrete a large variety of factors, including inflammatory

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 189-217.

chemokines and cytokines. These, in turn, affect in paracrine and autocrine manners breast cancer progression, in most cases supporting the process.

The current discussion is focused on the inflammatory milieu of breast tumors, and as such it addresses the presence and activity of TAM at breast tumors. This is followed by description of the roles of inflammatory chemokines in breast cancer, primarily as mediators of monocyte recruitment and angiogenesis. Thereafter, specific inflammatory cytokines that are classified as monocyte /macrophage-derived factors and may therefore be secreted by TAM, are discussed. Emphasis is put on the interplay between the different mediators – cells, chemokines and cytokines – together establishing an array of interactions that possibly supports breast cancer progression.

2. TUMOR-ASSOCIATED MACROPHAGES IN BREAST CANCER

High presence of host-derived inflammatory cells, primarily T cells and TAM, was observed in breast tumors (11, 19-38). At first, the existence of these immune cells in proximity to the tumor cells raised hopes that immune components may combat the developing tumor, and that manipulation of these cells could be exploited to limit disease progression. To date, much uncertainty exists regarding the roles of T lymphocytes in breast tumors, and their ability to raise effective immunity against the developing tumor (21-23, 25, 26, 29, 39-41). In contrast, there is a consensus regarding the roles of TAM in breast cancer, primarily in invasive carcinoma, suggesting that this cell population supports breast cancer development and progression (5, 21, 26-32, 34, 36, 38, 42, 43).

TAM originate from blood monocytes that migrated into the tumor (10, 11, 44, 45). In breast tumors, the resulting differentiated TAM were shown to be diffusely distributed throughout the tumor, and to reside in tumor zones and tumor edges, around ductal areas and in the tumor stromal areas (28, 29, 31, 35, 36). The association of macrophages with prognosis in breast cancer was extensively studied, mainly in biopsies of patients that were diagnosed with invasive carcinoma of the breast. The resulting reports indicated that elevated levels of macrophages were associated with parameters of bad prognosis, such as erbB2 expression (21, 29, 31). Moreover, the reports on this issue demonstrated that high presence of TAM was significantly correlated with high tumor grade, reduced relapse-free survival, clinical aggressiveness, poor prognosis and recurrence (5, 21, 26-32, 34, 36, 38, 42, 43).

Macrophages are versatile cells that can, under specific conditions, exert tumor-inhibiting functions, such as antigen presentation, cytotoxicity, phagocytosis, and the secretion of Th1-stimulating cytokines (11, 13, 46). However, the studies on TAM in a variety of malignant diseases in general, and in breast cancer in particular suggest that TAM in specific tumors may be skewed to the tumor-supporting phenotype [General reviews on TAM: (6-11, 13, 18, 45-47)]. The current evidence indicates that in breast tumors, TAM exert a large variety of tumor-promoting functions, including the secretion of angiogenic factors, the promotion of breast cancer cell growth, the release of proteases that support the metastatic process, and the secretion of tumor-promoting inflammatory chemokines and inflammatory cytokines, as indicated below.

Breast TAM were demonstrated to express, or to be associated with several angiogenesis-supporting factors, including Vascular Endothelial Growth Factor (VEGF), Thymidine Phosphorylase (TP), and a secreted form of basic Fibroblast Growth Factor (bFGF) (35, 37, 43, 48). In accordance with these findings, a significant correlation was observed between increased macrophage counts, high vascular grade and increased angiogenesis in biopsies of breast cancer patients and in animal models (29, 32, 34, 43, 49). Of interest were studies by Leek and Lewis, indicating that the macrophages accumulate in poorly vascularized hypoxic areas within the tumor, where the hypoxia stimulates the secretion of VEGF by TAM and by the tumor cells. These results suggest that in aggressive breast tumors, the lack of vascular supply results in hypoxia, followed by monocyte recruitment and VEGF secretion by TAM, giving rise to elevated angiogenesis at surrounding tumor areas (11, 34, 48).

Additional studies suggested the existence of another TAM sub-population in breast tumors, associated with low VEGF expression in the tumors, but with high expression of Epidermal Growth Factor Receptor (EGFR) by the tumor cells (37). Since TAM consist a major source of Epidermal Growth Factor (EGF) in breast tumors (50), these findings suggest that TAM-derived EGF acts on tumor cell-expressed EGFR, promoting tumor cell growth, and leading to increased aggressiveness. Further promotion of breast tumor growth may be mediated by the ability of TAM to secrete estradiol (51), which is associated with increased risk for breast cancer (52), in quantities that are sufficient for tumor growth enhancement (51).

Moreover, TAM at breast tumor sites were suggested to release a variety of proteases that may degrade and remodel the extracellular matrix, therefore facilitating the metastatic process. Indeed, breast TAM were shown to express matrix metalloproteinases (MMP) and urokinase plasminogen activator (uPA), and uPA immunostaining was correlated with macrophage infiltrates in breast tumors (27, 53, 54).

The above TAM-derived tumor-promoting mechanisms may be complemented by the ability of TAM to secrete inflammatory mediators. The inflammatory components that are released by TAM at breast tumor sites include not only inflammatory, tumor-promoting chemokines such as CCL2 and possibly CCL5 and CXCL8, but also inflammatory cytokines, e.g. Tumor Necrosis Factor α (TNFα) [(35, 36, 55-60); For additional references, see specific sections]. TNFα is one of several inflammatory cytokines that are characterized as monocyte/macrophage-derived components, including also Interleukin 1 (IL-1) and Interleukin 6 (IL-6). The expression and the roles of the inflammatory chemokines and cytokines in breast cancer are discussed further below.

3. INFLAMMATORY CHEMOKINES IN BREAST CANCER

The chemokine superfamily consists of a large number of small molecular weight proteins that induce chemotaxis of leukocytes to sites of inflammation, as well as to hematopoietic organs. The chemokines are sub-classified according to two criteria: structural and functional. Structurally, the classification is based on the arrangement of conserved cysteine residues in the chemokine molecule. The two larger chemokine sub-classes include the CC and the CXC groups, consisting of many chemokines with specific, as well as redundant activities. Making a simplistic generalization, the members of the CC chemokine group induce the migration of mainly monocytes and T lymphocytes. The CXC group is further sub-divided to those that contain an ELR (Glu-Leu-Arg) motif (ELR^+-CXC chemokines) and those that do not (ELR^--CXC chemokines). The general outlines of the CXC group suggest that members of the ELR^+-CXC sub-group attract mainly neutrophils, and they are considered as highly potent angiogenic factors. The ELR^--CXC chemokines are powerful chemoattractants of monocytes and T cells, and are considered as angiostatic factors (a clear exception is CXCL12, known also as Stromal Cell Derived Factor-1, SDF-1) (61-67).

The other, less stringent classification of chemokines is based on the context of their functionality. The "inflammatory chemokines" induce leukocyte recruitment to sites of acute and chronic inflammation. Following stimulation, many cell types can secrete inflammatory chemokines, therefore these chemokines are inducible in nature. On the other hand, the "homeostatic chemokines" are secreted in a constitutive manner at hematopoietic sites, regulating the directed migration of leukocytes in processes of haematopoiesis and lymphoid organ development (61-63).

Chemokines, inflammatory and also homeostatic, were recently suggested to affect breast cancer progression. As the present discussion is focused on the inflammatory milieu of breast tumors, major emphasis is put on the roles of the

inflammatory chemokines in breast cancer progression. However, it should be mentioned that an ample number of studies has been recently published on the roles of homeostatic chemokines, primarily CXCL12, in breast cancer. This chemokine was suggested to contribute to breast cancer progression by virtue of its secretion in metastatic organs of breast cancer, thereby inducing the homing of breast cancer metastatic cells that express the corresponding CXCR4 receptor, to these sites. In the scope of the present discussion, the roles of CXCL12 and other homeostatic chemokines will not be described, and the readers are referred to reviews and specific studies on this issue (68-81).

The research on inflammatory chemokines in breast cancer provides evidence mainly for their pro-malignancy roles in this disease. Three inflammatory chemokines were studied thus far extensively, including CCL2, CCL5 and CXCL8. In addition to these studies, investigations start emerging, suggesting that other inflammatory chemokines, primarily the ELR$^-$-CXC chemokines CXCL10 (γ-Interferon Inducible Protein-10, IP-10) and CXCL9 (Monokine Induced by IFNγ, Mig) may regulate breast cancer progression. The majority of these studies suggest that the two chemokines are potential inhibitors of breast cancer (82-93). However, thus far, the studies were performed only in animal models - mainly using chemokine or cytokine over-expression - and in *in vitro* systems. Several of these studies provided evidence for the ability of these chemokines to inhibit breast cancer growth, and others only indirectly supported their tumor-suppressing effects. Currently, the expression of CXCL10 and CXCL9, and their possible roles were not analysed in tumor-derived samples of breast cancer patients. Therefore, it is not known if they are endogenously expressed in breast tumors, or whether they could be only used as potential externally-applied or over-expressed substances that may limit breast cancer. In view of the above, CXCL10 and CXCL9 will not be discussed in depth, however, related references were mentioned above.

3.1 CCL2 and CCL5

CCL2 (Monocyte Chemoattractant Protein-1, MCP-1) and CCL5 (Regulated on Activation T Cell Expressed and Secreted, RANTES) are CC chemokines that potently induce monocyte migration (63, 94, 95), and as such were suggested to induce the recruitment of monocytes from the circulation to breast tumors, giving rise to the high prevalence of TAM in breast tumors (see references below). Investigations that were performed on clinical samples from breast cancer patients and healthy individuals, as well as studies in animal models and *in vitro* analyses, strongly support the pro-malignancy roles played by these two chemokines in breast cancer progression.

3.1.1 CCL2

In breast cancer, CCL2 expression was detected in breast tumor cells, mostly by *in situ* immunohistochemistry (IHC) analysis (36, 57, 58, 96, 97). Normal mammary epithelial cells in proximity to the tumor cells did not express CCL2 (36, 58). CCL2 is generally characterized as an inducible chemokine (61, 63), and its expression by breast tumor cells was indeed up-regulated *in vitro* by the inflammatory cytokines IL-1α and TNFα (83, 98, 99). However, CCL2 expression was also demonstrated to be constitutive, as it was produced by unstimulated human and murine breast cancer cells in culture (58, 83, 84, 100-102). Moreover, expression of the chemokine at breast tumors was detected also in TAM, lymphocytes, smooth muscle cells, endothelial cells and fibroblasts (36, 57, 58, 96, 103).

The studies on CCL2 expression in breast tumors indicated that elevated levels of CCL2 expression were associated with markers of bad prognosis, poorly differentiated tumor cells, early relapse and poor prognosis (36, 57, 58, 96). A study by Salcedo *et al.* provided a proof-of-concept for the role of CCL2 in breast cancer progression, showing that neutralizing antibodies against the chemokine increased survival, and reduced lung metastasis in a xenograft model of breast cancer (104). The pro-malignancy role of CCL2 in breast cancer was also supported by the use of two independent murine mammary carcinoma cell systems, each consisting of variants of high *versus* low malignancy potential, showing that the highly tumorigenic variants

secreted considerably elevated levels of CCL2 than the low malignancy variants (83, 101, 102).

As expected, a key mechanism for CCL2-mediated pro-malignancy effects is its ability to induce the recruitment of monocytes into breast tumors, giving rise to elevated levels of TAM. High levels of CCL2 in breast tumors were highly correlated with macrophage counts in the tumor (36, 57, 58). In accordance, very low numbers of macrophages were observed in areas of normal mammary epithelial cells, in which CCL2 was not expressed (36). The role of CCL2 in monocyte recruitment is further supported by *in vitro* studies showing that CCL2, derived directly from breast cancer cells, induced monocytic cell migration (100). Concomitantly with increased monocyte migration, it was recently indicated that CCL2 down-regulated T cell-mediated effector functions (yet not the overall immunogenicity of the tumor) (105) in a murine mammary carcinoma model.

In addition, a large body of evidence suggests that CCL2 is a potent angiogenic chemokine, supporting angiogenesis by acting directly on endothelial cells, as well as indirectly by promoting inflammatory cell recruitment, followed by the secretion of macrophage-derived angiogenic factors (36, 104, 106-110). In accordance with these observations, CCL2 expression in breast tumors was significantly correlated with microvessel density, and with the levels of the angiogenic factors VEGF and TP, as well as with the angiogenic chemokine CXCL8 (57, 58).

CCL2 expression in breast tumors was associated with that of MMP (58), suggesting that interactions between CCL2 and MMP, either direct or indirect, may up-regulate the expression of proteases at breast tumor sites, therefore facilitating the metastatic process. The activities of CCL2 may be exerted on breast TAM, as suggested by the fact that CCL2 promoted MMP expression by monocytic cells (111). In addition to its MMP-related activities on TAM, it is possible that the chemokine may affect directly the tumor cells, signaling through specific binding sites or the CCR2 receptor, recently demonstrated to be expressed by the tumor cells (84, 112, 113). In this regard, it is of interest that elevated expression of CCL2 was associated with increased levels of MMP in high malignancy variants of mammary carcinoma cells, as compared to low levels of both CCL2 and MMP in the low malignancy variants of these cells (83, 102). The ability of CCL2 to promote MMP expression at the tumor site may facilitate the establishment of metastases at remote sites, and may be further supported by the ability of the chemokine to induce migration of breast cancer cells (112).

Further analysis indicated that CCL2 expression was associated with elevated expression levels of inflammatory cytokines. Studies in breast cancer patients demonstrated that the *in situ* expression of CCL2 was highly correlated with that of the potentially tumor-promoting cytokine TNFα (57). A cross-regulatory circuit was suggested between tumor cell-derived CCL2 and TAM-produced TNFα, as based on findings showing that CCL2 up-regulated the secretion of TNFα by monocytic cells, and that monocytic cell-derived TNFα promoted CCL2 secretion by mammary carcinoma cells (83).

In the context of CCL2 interactions with inflammatory cytokines, it is possible that CCL2 may cooperate or cross-interact also with the potential pro-malignancy cytokine IL-6. It was shown in two unrelated mammary carcinoma cell systems that promoted levels of CCL2 secretion were associated with elevated IL-6 release in high malignancy variants, as compared to low tumorigenicity variants (83, 102). Furthermore, the secretion of CCL2 by fibroblasts derived from breast tumors was dependent on IL-6 activity (103). Another intriguing possibility for tumor-supporting functions of CCL2 is that cross-regulatory pathways may exist in TAM not only between CCL2 and inflammatory cytokines, but also between CCL2 and other tumor-supporting chemokines, such as CCL5. Such a mechanism was suggested by Locati's study, demonstrating that CCL5 promoted CCL2 secretion in monocytic cells (114).

Overall, the results of the above-described investigations suggest that one of the initial events in breast cancer development is the ability of tumor cell-derived CCL2 to chemoattract monocytes to the tumor. The secretion of tumor-promoting factors by the resulting TAM, joined by the pro-malignancy activities of tumor cell- and TAM-derived CCL2, as well as by other tumor-promoting factors, may

further support establishment of metastases and disease progression.

3.1.2 CCL5

Analyses of CCL5 expression, performed by IHC on biopsies of breast cancer patients indicated that CCL5 was expressed by infiltrating leukocytes, as well as by breast tumor cells (59). Although classified as an inflammatory chemokine, constitutive secretion of CCL5 was detected by breast tumor cells of human and murine origins, in culture (59, 100, 115, 116). The secretion of CCL5 could be further induced by the inflammatory cytokine TNFα (100, 117), synergising with Interferon γ in this regulatory activity (100). CCL5 expression was hardly detected in normal duct epithelial cells, or in epithelial cells that constitute benign breast lesions in proximity to tumor cells (as determined mainly in stages I and II of disease) (59).

Analysis of CCL5 expression in breast cancer patients demonstrated prevalent chemokine expression (59). High incidence and intensity of CCL5 expression were detected by IHC in sections of most of the patients with advanced disease (stages II and III) as compared to stage I breast cancer patients (59). Determination of CCL5 expression in plasma also indicated that CCL5 expression was elevated in advanced disease (118), supporting the pro-malignancy roles of CCL5 in breast cancer. Moreover, CCL5 immunoreactivity was detected in only a small percentage of patients diagnosed with benign breast lesions or patients that underwent reduction mammoplasty (59). As CCL5 may have pro-malignancy roles in breast cancer, its expression by healthy individuals may represent a population-at-risk, in which CCL5 expression may be indicative of an ongoing, but as yet undetectable, malignancy process.

Experimental models support the role of CCL5 in breast cancer progression, as indicated by the fact that inhibition of CCL5 activities, by a receptor antagonist or by direct down regulation of CCL5, decreased the volume and weight of mammary tumors, and significantly inhibited tumor growth (115, 116). *In vitro* invasion tests also supported the role of CCL5 in promoting breast cancer (119).

Although CCL5 was shown to support vascularization (100), the major pathway in which it supports tumor growth is probably by changing the equilibrium between different leukocyte subsets at the tumor site. A recent publication by Adler *et al.* indicated that down-regulation of CCL5 expression by breast tumor cells resulted in reduced *in vivo* growth, associated with increased infiltration of T cells and greater tumor-specific T cell responses (115). CCL5 may therefore have the ability to down regulate T cell migration (by unidentified manners) to breast tumors, thereby escaping potential immune activities against the developing tumor (115).

In contrast to CCL5-induced inhibition of T cell recruitment to breast tumors, CCL5 strongly potentiates the migration of monocytes to these tumors, therefore promoting TAM presence in breast carcinomas. Studies in murine systems indicated that breast tumor-derived CCL5 was directly responsible for monocyte recruitment to breast tumors (115, 116). A role for breast tumor cell-derived CCL5 in inducing monocyte migration and increasing TAM presence was also indicated by *in vitro* analyses using conditioned medium of breast tumor cells that secrete the chemokine (100). Evidence was also provided regarding the ability of breast tumor cell-derived CCL5 to induce eosinophil migration, suggesting its contribution to eosinophil recruitment to breast tumors (117), however the role of such an infiltrate in breast cancer was not defined as yet.

Therefore, a major mechanism by which CCL5 may promote breast cancer progression is induction of monocyte infiltration to breast tumors. Following their recruitment, the resulting TAM, as well as the tumor cells, may be exposed to CCL5-induced tumor-promoting activities. One such pathway may be mediated by the ability of CCL5 to increase CCL2 secretion by monocytic cells (114), implying that cross-regulatory mechanisms may exist between the two pro-malignancy chemokines, thus supporting breast cancer progression. Another possibility for CCL5-mediated pro-tumorigenic effects is its potential role in facilitating metastasis formation by up-regulating MMP9 transcription and expression, not only by monocytic cells, but also directly by breast tumor cells (100, 111, 115, 120), as well as by inducing migratory responses of breast cancer cells (112, 113).

The above-mentioned functions of CCL5 manifest, between others, direct activities of the chemokine on breast tumor cells. Since CCL5 is secreted by breast tumor cells, it is possible that the chemokine affects the tumor cells in autocrine manners *in situ*. Such a mechanism necessitates the expression of CCL5 receptors by the tumor cells. Several receptors are known for CCL5, including CCR1, CCR3 and CCR5 (63, 64, 66). The possibility that CCL5 may act by autocrine manners on breast tumor cells is supported by the fact that CCR5 expression was noted by IHC on breast tumor cells in biopsies of breast cancer patients, as well as by detection of CCL5 binding sites and receptors in human breast cancer cell lines (100, 112, 113).

However, with regard to the expression of CCR5 by breast cancer cells, it is of interest to note that a report by Manes *et al.* suggested that the expression of functional CCR5 was involved in reduced breast tumor cell proliferation, in a mechanism requiring p53-mediated activity (121). The results of this study implied that a mutated dysfunctional CCR5 is associated with shorter disease-free survival in breast cancer patients, suggesting a protective role for CCR5, and possibly its ligands, in breast cancer. The basis for the inconsistency between this observation and all the other findings on the pro-malignancy roles of CCL5, potentially mediated by CCR5 but also by other CCL5 receptors, is not defined. However, it should be noted that the p53 status of the patients in the other reports was not determined. Furthermore, the other studies did not analyse the proportions of wild type CCR5-expressing *versus* mutated CCR5-expressing patients, nor did they determine the expression of other CCL5 receptors, such as CCR1 and CCR3, in breast tumors.

Altogether, the current information on the roles of CCL5 suggests that it supports breast cancer progression. As with CCL2, it is possible that CCL5 secretion by breast tumor cells is followed by CCL5-induced monocyte migration and by the expression of tumor-promoting functions by the resulting TAM. In addition, CCL5 may further stimulate TAM and breast tumor cells to express pro-malignancy mediators, favouring tumor growth and metastasis formation in this disease.

3.2 CXCL8

CXCL8 (Interleukin 8, IL-8) is an ELR^+-CXC chemokine, and is characterized mainly as a neutrophil chemoattractant, inducibly secreted by several cell types, including mainly monocytic cells (63, 94, 95). However, in the malignancy context, it is believed that CXCL8 exerts several pro-malignancy functions which may not be related to its chemotactic activities.

At breast tumors, CXCL8 is expressed primarily by the tumor cells. Constitutive secretion of CXCL8 by breast tumor cell lines was demonstrated, however in line with the inducible nature of CXCL8, its expression was up-regulated in breast tumor cells by the inflammatory pro-malignancy cytokines IL-1α, IL-1β and TNFα (60, 97, 122-135). CXCL8 expression was detected in breast tumors also in Small Vessel Endothelial Cells (SVEC) and Large Vessel Endothelial Cells (LVEC), as well as by stroma cells, probably fibroblasts (60, 124, 126). The fact that monocytes constitute a prime source for CXCL8 (63, 94, 95) raises the possibility that CXCL8 at breast tumor sites may be secreted also by TAM.

In contrast to CCL2 and CCL5, CXCL8 is expressed not only be breast tumor cells, but also by cells adjacent to the carcinoma (122). CXCL8 expression was noted in normal mammary epithelial cells in a large proportion of healthy individuals (60, 125, 136, 137), suggesting that this chemokine may serve as an essential defence mechanism against breast infections by induction of neutrophil migration (137-140).

Since CXCL8 is expressed by breast epithelial cells of healthy women, it is expected that dramatic elevations in the proportions of CXCL8-expressing individuals will not be observed in the breast cancer population as compared to healthy individuals. Indeed, determination of the proportions of patients expressing CXCL8, performed by several methods – by IHC or at the mRNA level *in situ,* or on isolated breast epithelial cells – suggests that the percentages of breast cancer patients that express CXCL8 are similar to normal individuals (60, 124, 126). Moreover, determination of CXCL8 expression by epithelial cells indicated that the proportions of CXCL8-expressing patients are similar in breast

cancer patients and in patients diagnosed with Ductal Carcinoma *In Situ* (DCIS) (125).

The studies determining the incidence of patients expressing CXCL8 are joined by other reports, analysing the levels of CXCL8 expression. Some of these investigations compared between breast cancer patients and healthy individuals by using cultures of patients' cells, plasma samples and tissue homogenates, but not by IHC. These analyses indicated that the levels of CXCL8 in patients and healthy individuals were similar, and did not have prognostic values in breast cancer (57, 122, 141). Also, in a recent study using variants of GI101A human breast cancer cells differing in their metastatic potential, the metastasis-selected variants did not show increases in the expression of CXCL8 (130). Together, these results suggest that CXCL8 expression is not elevated in breast cancer, but they also do not provide evidence for any protective roles for the chemokine in breast cancer.

However, side by side with these observations, there are reports indicating that the proportions of CXCL8 expression and its levels, as determined *in situ* and by the use of serum samples, are higher in breast cancer patients than in healthy individuals (125, 126, 142, 143). Importantly, IHC-based analysis of CXCL8 expression in breast cancer patients and DCIS patients indicated that the staining intensity of CXCL8 was significantly higher in samples of these two groups of patients than in normal ductal epithelial cells in benign tissues (125). It is possible that direct analysis of CXCL8 expression in breast tumors by IHC provides the most clinically relevant information on CXCL8 expression in breast tumors.

The results of these latter studies suggest that the proportion of CXCL8-expressing individuals are not correlated with breast cancer, but it is possible that elevated levels of CXCL8 expression are associated with disease development and progression. High CXCL8 secretion levels were also noted in human MDA-231 breast cancer cell variants characterized by an increased metastatic phenotype (131). As previously noted, another study using GI101A breast cancer cells demonstrated no correlation between CXCL8 and the metastatic potential of the cells (130). It is possible that the differences between the two studies reflect cell line-specific effects. Nevertheless, the potential contribution of CXCL8 to determination of the metastatic potential of breast cancer cells was further supported by several studies demonstrating a strong correlation between the metastatic potential of different human breast cancer cell lines and elevated levels of CXCL8 expression (127, 132, 134).

Support for the potential roles of CXCL8 in breast cancer is provided also by studies performed on patients that had received several courses of therapy before entering the study. It was indicated that the levels of CXCL8 were higher in patients with a progressive disease, as compared to patients demonstrating partial response or no change in clinical parameters following treatment (144, 145). In a model system imitating chemotherapy resistance, elevated levels of CXCL8 were detected in cells that survived sequential treatment with chemotherapeutic agents, associated with enhanced tumor growth in an orthotopic tumor model (129).

It is possible that the discrepancies between the different reports on CXCL8's role in breast cancer are the result of the heterogeneity of the breast cancer patient population, and the differential expression of other tumor-related factors in these patients. For example, recent evidence indicated that high levels of CXCL8 expression in breast cancer cells were associated with negative expression of estrogen receptor (ER) (125, 132, 134). Since the populations of breast cancer patients in the different studies were not divided according to the ER status, the proportion of ER+ *versus* ER- patients in the surveys could have affected the results obtained in studies on CXCL8 contribution to breast cancer.

The implications of ER- association with CXCL8 elevated expression in breast cancer are not fully understood as yet. Of note in this respect is the fact that the levels of CXCL8 expression by breast tumor cells were regulated directly by the expression of ER, primarily ERα (132, 134), and not by estradiol. Treatment with 17β-estradiol (E_2) did not affect the expression of CXCL8 in ER+ or ER- breast cancer cells, nor did it down-regulate CXCL8 expression by ER- breast cancer cells that were transfected by ERα (132, 134). In contrast, CXCL8 was shown to potently increase the activity of 17β-hydroxysteroid dehydrogenase type I (146), a key enzyme in the synthesis of E_2, which is considered

as a prime hormone in the promotion of breast cancer cell growth (52). Together, the above results suggest that not only the expression of CXCL8 *per se*, but also the manner it associates with estrogen and its receptors, may be important for the control of breast cancer development and progression.

Overall, the above findings suggest that CXCL8 may exert tumor-promoting functions in breast cancer; nevertheless, its full-blown capacity may necessitate joint activity with other pro-malignancy factors. This possibility is supported by a recent report, indicating that antibodies against CXCL8 could not reduce the *in vivo* malignancy potential of breast tumor cells, but they synergised potently with antibodies neutralizing EGFR, resulting in decreased metastatic tumor burden (128). These results suggest that the activities of CXCL8 and EGF could converge, jointly supporting specific pathways in the metastatic cascade. Indeed, the study by Schraufstatter *et al*. suggested that following CXCL8 binding to its receptors on endothelial cells, EGFR was activated, inducing migration of the endothelial cells. One of the mechanisms that may be involved in this process is CXCL8-induced activation of proteolytic enzymes, resulting in cleavage of pro-HB-EGF to HB-EGF, thereby activating EGFR (147). These results have major implications for the angiogenic process, suggesting that CXCL8-induced angiogenicity may occur *via* the activation of EGF-related pathways. Such combined and inter-related activities of CXCL8 and EGF may elevate the pro-malignancy potential of CXCL8, and the two factors can thus jointly contribute to disease progression.

The above mentioned observations are in line with the potent angiogenic capabilities of CXCL8 [Only a small number of references is cited: (65, 128, 148, 149)]. The angiogenic role of CXCL8 in breast cancer is highly plausible, as indicated by its coordinated expression with the angiogenic chemokine CCL2 in breast tumors (57), and by the fact that endothelial cells in breast tumors expressed the CXCL8 receptors, CXCR1 and CXCR2 (124). Since CXCR2 is the major CXCL8 receptor mediating the angiogenesis-related activities of this chemokine (149-151), these results suggest that CXCL8 may act on the endothelial cells to increase angiogenesis in breast cancer. Indeed, it was illustrated that breast tumor cell-derived CXCL8 promoted in endothelial cells the formation of elongated tube-like structures, as well as endothelial cell proliferation (132), both important for angiogenicity.

The expression of CXCL8 binding sites, and more specifically of the CXCL8 receptors CXCR1 and CXCR2 was also detected by breast tumor cells in culture and *in situ* (112, 124, 128). Of interest is the fact that breast tumor cells expressed CXCR1 and CXCR2 in all breast cancer specimens analysed, whereas only 50% of benign epithelial cell samples expressed the receptors (124). These observations suggest that autocrine pro-malignancy activities of CXCL8 in breast cancer may occur, possibly contributing to breast cancer progression. Although binding sites for CXCL8 were detected in breast tumor cells (112, 124, 128), direct evidence for such autocrine activities of CXCL8 on breast tumor cells was not provided as yet, and the ability of CXCL8 to support breast tumor cell proliferation was thus far ruled out (128, 132, 133).

Additional modes of action were suggested to contribute to the pro-malignancy activities of CXCL8. First, interactions between CXCL8 and other potentially pro-malignancy factors, such as the cytokine IL-1 may exist, as suggested by observations showing correlations between CXCL8 and IL-1 expression in breast tumors (125, 152). The existence of cross-regulatory circuits between CXCL8 and IL-1 is further reinforced by the ability of IL-1 to promote CXCL8 expression by breast tumor cell lines (123, 125, 127, 129, 153). Additional interactions between CXCL8 and pro-malignancy cytokines are suggested by the ability of TNFα to up-regulate CXCL8 expression by breast tumor cells (123, 127).

CXCL8 was also suggested to directly support the metastatic process in breast cancer by promoting tumor cell motility, and by inducing the invasion of breast tumor cells (112, 128, 131, 132, 154). Together with the fact the CXCL8-related malignancy of breast tumor cells was also associated with elevated expression levels of MMP (128), it is possible that CXCL8-induced MMP expression may facilitate tumor cell release from the primary tumor site, and that CXCL8 may promote invasion and metastasis formation. Moreover, reports by Bendre

et al. indicated that CXCL8 was involved in skeleton metastasis, and that this chemokine stimulated osteoclastogenesis and bone resorption, suggesting that CXCL8-induced osteolysis may be associated with metastatic processes in breast cancer (131, 155, 156).

Taken together, the above-described results suggest that CXCL8 may be a key mediator of breast cancer, possibly inducing angiogenesis and acting *via* a broad array of additional mechanisms. However, the overall impression is that cooperation between CXCL8 and other tumor-supporting components is required for maximal pro-malignancy activities of CXCL8 at breast tumors, raising the need for further investigation of its ability to jointly act with other malignancy-enhancing factors in breast cancer.

4. INFLAMMATORY CYTOKINES IN BREAST CANCER

The three inflammatory cytokines mentioned above, IL-1, TNFα and IL-6 are produced by monocytic cells, but also by other cell types. IL-1 and TNFα share many lines of similarities, and show cross-interactions with each other, also affecting the secretion of IL-6. All three cytokines have major roles in the regulation of immune activities, and are also involved in a variety of clinical manifestations (157-163).

IL-1, TNFα and IL-6 are secreted at breast tumor sites by tumor cells, TAM, endothelial cells and/or other stroma cells, as described below. The current evidence strongly suggests that IL-1 supports breast cancer development and progression, and that TNFα is a potential pro-malignancy factor in this disease. With regard to IL-6, there are conflicting reports concerning its role in breast cancer, however its ability to promote breast cancer development and progression is plausible, as indicated herein.

4.1 INTERLEUKIN 1

The expression of IL-1, in its IL-1α and/or IL-1β forms, was detected in breast tumor cells, endothelial cells, smooth muscle cells, fibroblasts and uncharacterised stroma cells (60, 125, 152, 164-168). Direct demonstration of IL-1 expression by TAM was not provided. However, the possibility that TAM produce this cytokine in breast cancer is supported by the observation that IL-1 is secreted by monocytic cells in the immunological context, and by the fact that elevated IL-1β levels were correlated with high macrophage counts in this disease (165).

Studies detecting IL-1 expression *in situ* by IHC in tissue biopsies, and analyses of tumor cell extracts strongly support the role of both IL-1α and IL-1β in breast cancer progression. Determination of the expression levels of IL-1α and IL-1β by IHC indicated that the proportion of IL-1-positive patients was similar in breast cancer patients, DCIS patients and healthy individuals (125); however, the expression levels of the two cytokines were increased in invasive breast carcinoma and in DCIS patients, as compared to patients diagnosed with benign lesions of the breast (125). Moreover, the results of a study by Jin *et al*., analysing IL-1β expression in tissue extracts demonstrated that the cytokine expression levels in invasive carcinoma patients were significantly higher not only in comparison to control healthy patients, but also as compared to DCIS patients (165). High IL-1β expression levels in this study were correlated with parameters characterizing aggressive tumors, as well as with high tumor grade.

The above results were further confirmed by another IHC study demonstrating that high IL-1α levels were correlated with poor differentiation (167). Of interest were the observations of this study, indicating that elevated levels of IL-1α expression were correlated with the lack of ER expression, primarily of ERα (167). In line with these findings, other studies also demonstrated inverse correlation between elevated IL-1 expression and ER presence (125, 166), suggesting that the tumor-promoting activities of IL-1, in similarity to those of CXCL8, may depend on the hormonal setting of the tumor microenvironment.

Overall, the results of these studies support the role of IL-1 in breast cancer progression, as was also suggested by the analysis of breast tumor cell lines (167), and by the determination of IL-1 activities in animal model systems (169, 170). Thus far, one

report only, determining the expression of IL-1β in serum and not in tumor-related clinical samples, suggested that the expression of IL-1 did not correlate with clinicopathological parameters (171).

In breast carcinomas, the receptors for IL-1 are expressed by endothelial cells (SVEC, LVEC), smooth muscle cells and fibroblasts, as well as by the tumor cells and normal duct cells (125). Significant elevations in IL-1 receptor expression (both IL-1RI and IL-1RII) were observed in biopsies of invasive carcinoma and DCIS patients as compared to individuals with fibrocystic breast diseases (125). This pattern of IL-1 receptor expression suggests that not only the elevated levels of IL-1, but also of its receptors, may contribute to the pro-malignancy effects of IL-1 in breast cancer.

Paracrine activities of IL-1 may give rise to increased angiogenesis, as supported by IL-1 receptor expression by the endothelial cells (IL-1 may act to increase the expression of angiogenesis-promoting functions), and by the fact that IL-1β expression was significantly correlated with the endothelial marker von Willebrand's factor (165). In addition, pro-angiogenic activities of IL-1 may be mediated by its cross-interactions with the angiogenic chemokine CXCL8. Analyses of samples from breast cancer patients indicated that the expression levels of IL-1α and IL-1β were associated with those of CXCL8 (125, 152). IL-1 may act in paracrine manners to promote CXCL8 secretion, as suggested by the observation that IL-1α up-regulated CXCL8 expression by fibroblasts (153). IL-1-induced autocrine activities that are related to CXCL8 secretion are also possible, as supported by the fact that breast tumor cells express both IL-1α and IL-1β and their receptors, and that the two cytokines stimulate in these cells the expression of CXCL8, mainly in ER negative cell lines (123, 125, 127, 129, 153).

The angiogenic activities of IL-1 may be further mediated by its interactions with CCL2 and its receptors. IL-1α was shown to up-regulate the expression of CCL2 by breast tumor cells (98, 99) while IL-1β promoted the expression of CCR2, the CCL2 receptor, by endothelial cells (110). The joint activities of both IL-1 forms may result in increased CCL2-induced angiogenic processes. The interactions between IL-1, CCL2 and CXCL8 support the possibility that cross-interactions between the inflammatory cytokines and inflammatory chemokines may contribute to breast cancer progression.

Additional analyses were performed *in vitro* on breast cancer cell lines, some demonstrating tumor-inhibiting functions of IL-1, but the majority providing evidence for its tumor-supporting roles. The tumor-inhibiting effects of IL-1 consisted primarily of growth arrest, including its ability to antagonize the mitogenic effects of Insulin Growth Factor-1 (IGF-1) (172-174). However, these analyses were performed only on the MCF-7 cells, and as mentioned in the TNFα section below, may raise concerns regarding the generality of these findings.

The reports suggesting tumor-suppressing roles for IL-1 are outnumbered by studies implying that IL-1α and IL-1β potently elevate tumor-promoting activities by breast cancer cells and by cells in the tumor microenvironment. Some of these activities were mentioned above, consisting of interactions between IL-1 and the pro-malignancy chemokines CXCL8 and CCL2. IL-1α and IL-1β may also increase the expression and activities of other inflammatory and potentially pro-malignancy cytokines, TNFα and IL-6, by breast tumor cells and/or fibroblasts (153, 172, 175, 176). The associations between IL-1 and IL-6 were further validated by the correlation between IL-1α and IL-6 expression noted in breast tumor biopsies (164), and also by the fact that IL-1 additively acted with IL-6 to induce breast cancer motility and spreading (177). In addition to the IL-1 and TNFα associations mentioned above, other interactions between the two cytokines may occur, as manifested by their joint abilities to stimulate the activity of estradiol 17β-hydroxysteroid dehydrogenase in breast tumor cells (178), an enzyme which is involved in E_2 synthesis and may thus promote the growth of hormone-dependent breast tumor cells (52).

IL-1 may also induce the expression of additional mediators that could contribute to breast cancer progression. IL-1β was shown to stimulate in breast fibroblasts the release of fibroblast growth factor 7, a growth factor for mammary cells (179), while IL-1α up-regulated MMP3 expression in fibroblasts, suggesting that it may support indirectly

the metastatic process by facilitating tumor cell spreading (153).

As mentioned above, key effects of IL-1 are related to its ability to induce estrogen expression by breast tumor cells and by fibroblasts. Estrogen, as an important mitogenic stimulant in breast cancer, may contribute to the growth of estrogen-dependent breast tumor cells (52). Estrogen-related activities of IL-1 are indicated by its ability to up-regulate estradiol 17β-hydroxysteroid dehydrogenase and aromatase activities in breast cancer cells, and in breast fibroblasts (178, 180, 181), thereby supporting estrogen synthesis (182, 183). Moreover, the production of prostaglandin E_2, a stimulator of aromatase and estrogen synthesis, and an immunosuppressive agent in breast cancer (182-188), was up-regulated by IL-1β in breast tumor cells and in breast fibroblasts (189).

In all, the current data suggest that IL-1 secretion by breast tumor cells and possibly also by TAM, may up-regulate a variety of pro-malignancy mechanisms that enhance processes of breast tumor growth and metastasis formation, including cross-interactions between inflammatory cytokines and chemokines.

4.2 TUMOR NECROSIS FACTOR α

The immune-related activities of TNFα and its identification as the active component in crude bacterial preparations that contained tumor-necrotizing toxins, raised the possibility that this cytokine may prove as a potential tumor-inhibiting factor (10, 15, 16, 158, 190). However, the current use of TNFα in therapy of malignant human diseases is limited, owing to relatively minimal efficacy in clinical trials and high toxicity. In parallel, increasing amount of evidence emerged, showing that TNFα is correlated with advanced stages of malignancy, and that it supports tumor progression in a variety of malignant diseases. As based on these observations, it was suggested that exposure to TNFα at very high concentrations may indeed have anti-tumor activities, whereas its chronic expression at tumor sites plays a key role in tumor progression (10, 15, 16, 190).

In breast cancer, TNFα is expressed primarily by breast TAM, while its receptors are expressed by the tumor cells and by stroma cells (35, 55, 56, 100, 135, 191, 192). TNFα expression by breast tumor cells was also noted, however it was speculated that this expression resulted of TAM-derived TNFα, binding to its receptors which were expressed by the tumor cells (192). The expression of the cytokine and its receptors at breast tumor sites raised questions regarding its role in disease inhibition or progression. To date, the results are inconclusive, however they support the possibility that TNFα may function as a tumor-promoting factor in breast cancer, as indicated below.

4.2.1 Can TNFα limit breast cancer development and progression?

A number of studies indicated that there were no significant differences in TNFα expression between breast cancer patients and healthy individuals, and no associations with advanced disease (126, 141, 165, 171, 193). These investigations determined the proportion of TNFα-expressing individuals and its levels, at the mRNA or protein levels, using tumor homogenates/extracts or plasma samples. Since the proportions and levels of TNFα expression in patients and in healthy individuals were similar, these studies did not provide any direct evidence for tumor-inhibiting functions of the cytokine in breast cancer.

Straightforward indications that TNFα may act as a tumor-inhibitory inflammatory cytokine in breast cancer were suggested by two animal model systems. A study by Sabel and colleagues indicted that intratumoral injection of TNFα-loaded microspheres suppressed the growth of mammary carcinoma cells. The TNFα treatment was combined with interleukin-12, resulting in resistance to tumor rechallenge (194). The effect of the cytokines in this case was attributed to the induction of polymorphonuclear (PMN) and CD8+ T cell infiltration, and to the presence of tumor-specific reactive T cells in the nodes and spleens of treated mice. In another study, in which a breast cancer xenograft model was used, recombinant TNFα arrested the growth of established tumors (195).

As a tumor-necrotizing factor, it is possible that the tumor-inhibiting functions of TNFα are also mediated by its ability to induce tumor cell lysis, either indirectly or directly. TNFα was shown to increase the expression of the adhesion molecule ICAM (intercellular adhesion molecule) by the tumor cells, thereby facilitating tumor cell lysis by lymphokine-stimulated killer (LAK) cells (196). TNFα was also shown to suppress the ability of IGF-1 to stimulate DNA synthesis in human breast carcinoma MCF-7 cells, significantly inhibiting the anti-apoptotic effects of IGF-1 (173). With regard to direct activities of TNFα in cell lysis, the evidence is somewhat problematic. Inhibition of tumor cell growth, as well as lysis by TNFα was demonstrated in several breast tumor cell lines, showing different sensitivities of the various lines. However, sensitivity to TNFα was noted mainly in the MCF-7 cells, raising concerns regarding the generality of the phenomenon (see below). These cells indeed showed sensitivity to TNFα, however TNFα-resistant MCF-7 cells were described as well (172, 173, 196-208).

4.2.2 TNFα may act as a tumor-promoting cytokine in breast cancer

The above findings are strongly contrasted by studies suggesting a tumor-supporting role for TNFα in breast cancer. These investigations, unlike the previously mentioned ones, were performed primarily by IHC analyses of patient biopsies. First, it was demonstrated that TNFα was expressed in breast tumors, but not in normal breast tissues, or in non-malignant breast tissue adjacent to the tumor (55). This report was followed by a study by Miles *et al*., showing that the proportion of TNFα-expressing TAM was elevated with increased tumor grade (56). A strong support for the potential role of TNFα in breast cancer was provided by Leek and his coworkers, showing by IHC that TNFα levels were associated with a more advanced axillary lymph node status (192). However, TNFα expression in this study was not correlated with poor survival, suggesting that although the cytokine may be involved in controlling nodal metastasis, its effects are not sufficient to independently affect prognosis directly (192).

An additional study, performed on serum samples of patients further supported the pro-malignancy roles of TNFα in breast cancer. It was found that breast cancer patients expressed significantly higher levels of the cytokine as compared to healthy individuals, and that elevated levels of the cytokine were significantly associated with increased tumor size, as well as with more advanced stage and lymph node status (209).

As based on studies in other tumor systems (16, 190), TNFα may act as a tumor-supporting factor by virtue of its ability to promote the expression of angiogenic factors. Such a role for TNFα in breast cancer is supported by the fact that its expression was correlated with angiogenic factors in breast tumors, such as VEGF, TP and bFGF (35, 192). It was suggested that TNFα receptors, expressed by the tumor cells, bind TAM-derived TNFα which induces TP expression in breast tumor cells (192). Furthermore, TNFα expression was also correlated with the pro-malignancy chemokine CCL2 which exerts potent angiogenic activities (57). *In vitro* studies indicated that TNFα promoted the expression of the two angiogenic chemokines, CCL2 and CXCL8 by breast tumor cells (83, 123, 127), as well as the expression of the CCL2 receptor CCR2 by endothelial cells (110). The combined effects of these TNFα activities may result in increased angiogenic effects, contributing to metastasis formation.

The ability of TNFα to promote CCL2 and CXCL8 expression is important not only from the angiogenesis point of view, but also because it provides evidence for cross-interactions between this inflammatory cytokine and inflammatory chemokines, that may possibly advance breast cancer progression. Such inter-relationships were also demonstrated by the ability of TNFα to up-regulate the secretion of the pro-malignancy chemokine CCL5 by breast tumor cells (100, 117).

Cross-interactions may also exist between one inflammatory cytokine and other such cytokines, as suggested by the fact that TNFα increased in breast tumor cells the secretion of the inflammatory cytokine IL-6 (83, 175), and synergised with IL-1β in tumor-promoting activities (178). Further positive feedback events between the different inflammatory cytokines may be mediated by IL-1α-induced

elevation of TNFα expression by breast cancer cells (172, 176).

The above-mentioned activities of TNFα on breast tumor cells may exemplify the direct activities of TAM-derived TNFα on the tumor cells, shown to express TNFα receptors (55, 100, 191). Additional possible activities of TNFα on breast tumor cells may include stimulation of tumor cell growth, as indicated by the fact that it elevated the growth of human breast and rat mammary epithelial cells (normal and transformed) (210). In addition, TNFα was also suggested to promote breast cancer progression by acting on breast tumor cells and fibroblasts, stimulating estradiol 17β-hydroxysteroid dehydrogenase and aromatase activity (178, 180, 188, 211), possibly increasing the expression of estrogen which supports breast cancer cell growth.

Another level of TNFα-mediated pro-malignancy activities may be exerted by the induction of MMP expression by breast tumor cells (83, 100), thereby possibly increasing their metastatic potential. Similar effects of TNFα on TAM were also suggested, as based on the ability of the cytokine to up-regulate MMP9 expression by monocytic cells (111, 212). The increased levels of MMP expression may enhance metastasis formation, and this process may be further up-regulated by the motility-inducing activities of TNFα on breast tumor cells (191). It was demonstrated that the ability of leukocytes to induce the motility of MCF-7 cells was dependent on TNFα, in a process that was independent of its toxic effects (191), suggesting that TNFα-induced motility assists the tumor cells escape immune mechanisms.

4.2.3 What is the actual effect of TNFα in breast cancer?

As described above, there is no consensus regarding the roles of TNFα in breast cancer. While some of the analyses that were detecting TNFα expression in breast cancer patients did not suggest any role for TNFα in breast cancer, others provided direct and strong evidence indicating that elevated expression of TNFα is correlated with disease, and in certain cases with more advanced malignancy. Similar to CXCL8, it is possible that TNFα pro-malignancy activities require cross-interactions with as yet unidentified factors, whose differential expression by the patients included in the cohorts, affected the results of the surveys, and thus resulted in discrepancies. Also, the possibility exists that the methods employed for TNFα detection contributed to the inconsistencies observed. In this respect, it is important to note that all the analyses using direct detection by IHC of TNFα expression in biopsies of patients provided evidence for the tumor-supporting roles of the cytokine. In contrast, analyses that did not show any differences in TNFα expression between breast cancer patients and healthy individuals, or at different stages of disease, used tissue homogenates/extracts or serum samples. In this regard, one has to remember that tissue homogenates/extracts contain a mixture of contents derived from many cell types, and that circulating TNFα may be derived from many different sources, and may represent immune activities. This suggests that measurement of TNFα in biopsies may be advantageous over the other methods of its detection, implying that TNFα may indeed have pro-malignancy roles in breast cancer.

In vivo studies in animal model systems suggested tumor-inhibiting effects for TNFα in breast cancer, while the results of *in vitro* analyses provided controversial results. Inhibitory effects of TNFα were shown mainly in the MCF-7 cells, their generality is questionable and such roles were outnumbered by opposite observations suggesting numerous pro-malignancy activities of the cytokine in breast cancer.

Overall, it is suggested that a tumor-supporting role of TNFα in breast cancer cannot be overlooked or ignored, and moreover should be considered as a highly plausible mechanism. The possibility thus exists that TNFα, derived primarily from TAM following CCL2- and CCL5-induced monocyte recruitment, contributes to promotion of breast cancer development and metastasis formation. TNFα may up-regulate angiogenesis and breast tumor cell motility, and may exert additional tumor-supporting functions, including cross-interactions with inflammatory chemokines and inflammatory cytokines.

4.3 INTERLEUKIN 6

The current available data indicates that IL-6 is expressed by breast tumor cells, with contradicting reports on its expression by breast stroma cells (60, 122, 135, 164, 168, 213-215). IL-6 expression in tissue adjacent to carcinoma was also detected (122). Similar to TNFα, the results on IL-6 in breast cancer patients are inconclusive. However, in contrast to the TNFα studies, an actual controversy exists regarding the role of this cytokine in breast cancer. A number of studies strongly argue that IL-6 may have protective effects in breast cancer. In contrast, a very large number of investigations, performed mainly on serum samples from breast cancer patients, complemented by *in vivo* animal models and *in vitro* studies, suggest an important role for IL-6 in disease progression, as indicated below.

4.3.1 IL-6 may have protective effects against breast cancer

As mentioned above, several of the studies performed on IL-6 expression in breast cancer patients suggest that the cytokine may be protective in breast cancer. The study by Karczewska *et al.* indicated that the expression of IL-6 and its receptor subunits, detected at the mRNA levels in breast cancer biopsies, was a significant positive prognostic factor for overall survival and disease free survival (215). Further evaluation of IL-6 expression is breast carcinomas, using IHC, indicated that IL-6 expression was inversely correlated with histological tumor grade, although not with tumor size or nodal status (214).

In additional studies, in which IL-6 was detected in patient biopsies, in tumor extracts, in isolated tumor cells and in serum samples, the comparison between breast cancer patients and normal control group indicated that IL-6 expression was either similar in the two groups or reduced in breast cancer patients (60, 122, 126, 135, 141). However, the reduced expression of IL-6 was dependent on tumor histotype, and was evident in invasive ductal carcinoma (122). In contrast, increased IL-6 levels were detected in patients diagnosed with invasive lobular carcinoma (122).

Of the many studies performed on the *in vitro* effects of IL-6 on breast cancer, only a relatively small number of investigations support a potential role for IL-6 in preventing breast cancer. Of interest is the fact that these studies described mainly the ability of IL-6 to inhibit DNA synthesis and the growth of normal mammary cells and breast tumor cells (173, 216-218). It is important to note that side by side with the growth inhibitory effects of IL-6, the cytokine induced cell characteristics that could actually support the metastatic process, namely reduced adhesiveness and expression of E-cadherin, and increased motility of the cells (216-218).

4.3.2 IL-6 may have tumor-stimulating effects in breast cancer

In contrast to the observations mentioned above, a large number of studies suggests a tumor-promoting role for IL-6 in breast cancer. The large majority of these studies used serum samples, although one report analysed IL-6 expression at the mRNA level in tumor extracts. These studies clearly indicated that the levels of IL-6 were elevated in breast cancer patients *versus* the control group, and were significantly associated with higher grade, shorter survival, poor prognosis, as well as worse survival in patients with a metastatic disease (14, 143, 144, 171, 216, 219-223).

Also, a recent study by DeMichele *et al.* indicated that polymorphism in the IL-6 gene, associated with its reduced transcription and therefore low expression, was significantly correlated with improved outcome in high-risk breast cancer patients, suggesting that IL-6 contributes to a more aggressive phenotype of breast cancer (224). However, the study of Iacopetta *et al.* contradicted these results, showing the opposite (225). The reasons for the discrepancies between the two studies are not determined as yet, but it is possible that medications, as well as other factors that differ between the patients included in the cohorts, affected the findings of these two studies.

Additional support for the role of IL-6 in promoting breast cancer was provided by the observation that patients unresponsive to chemo-endocrine therapy showed significantly higher serum

IL-6 levels than those who responded (171). Further investigation analysed the expression of IL-6 in breast cancer patients following treatment with anthracyclines. The results indicated that in patients showing no or partial response, the levels of IL-6 remained stable or decreased following treatment, whereas the levels of IL-6 in progressive disease patients were higher and gradually increased until the time of patient death (145). Furthermore, it was demonstrated that following vaccination of breast cancer patients with tumor-associated antigens and biological modifiers, the concentration of IL-6 decreased significantly (223). From the therapeutic perspective, it is interesting to note that IL-6 promoted the resistance of breast tumor cells to chemotherapy, being associated with increased expression of multidrug resistance genes (226).

The potential role of IL-6 in promoting breast cancer progression is also suggested by a comparison performed between high and low metastatic variants of murine mammary carcinoma cells, indicating that the high malignancy phenotype is associated with elevated levels of IL-6, in conjunction with increased expression of CCL2 and MMP (83, 102). Further illustration of the potential pro-malignancy role of IL-6 in breast cancer was provided by a study in which IL-6 was over-expressed in murine mammary carcinoma cells. The IL-6-expressing cells, and control IL-6 non-expressing cells were compared, indicating that following **intravenous** challenge of the tumor cells, IL-6 expression favoured the final stages of the metastatic process when the tumor cells have entered the circulation and spread to metastatic sites (227). However, additional results which were included in this investigation reflect the ambiguity regarding the role of IL-6 in breast cancer, since **subcutaneous** administration of the two cell types suggested that IL-6 reduced tumor cell growth and metastasis formation (227).

The mechanisms by which IL-6 may enhance breast cancer development and progression are many, and may include paracrine and autocrine modes of activity. As based on other tumor types, paracrine activities may include induction of osteolysis and angiogenesis (14), however direct proofs for a role for IL-6 in osteolysis in breast cancer were not provided thus far. The role of IL-6 in stimulating angiogenesis is supported by the fact that IL-6 was shown to be correlated with high VEGF serum levels and increased vascularity in breast cancer (221, 227).

A key role in the ability of IL-6 to stimulate breast cancer progression may be induction of expression and/or activity of enzymes that participate in the estrogen-synthesis pathway. Expression of IL-6 was associated with breast tumors that expressed three or more steroid, E_2-related, enzymes (222). IL-6 is considered as a major stimulator of aromatase activity, as well as of other estrogen-producing enzymes in breast tumor cells and in breast-derived fibroblasts (180-182, 188, 211, 228), thereby possibly increasing estrogen-mediated growth support of hormone-dependent breast tumor cells. With regard to the associations of IL-6 with the ER status, it was shown that IL-6 regulated differently the proliferation of ER+ and ER- mammary tumor cells. The study by Chiu *et al.* indicated that ER+ mammary carcinoma cells were sensitive, whereas ER- cells were resistant to IL-6-mediated growth inhibition. Furthermore, inhibition of ER+ cell growth was induced by supernatants of IL-6- secreting ER- cells (213). The results of this investigation also suggested that IL-6 secretion by the ER- cells did not stimulate the proliferation of these cells in autocrine manners.

Another potential mode of pro-malignancy activity of IL-6 in breast cancer may be related to its ability to reduce E-cadherin expression, to increase cell scattering/spreading and to induce anti-adhesive effects (216-218). These functions of IL-6 may facilitate the release of the cancer cells from the primary site, to be further complemented by the ability of the cytokine to induce tumor cell motility, eventually supporting the metastatic spread of breast cancer cells (154, 217, 218).

Additional pro-malignancy activities of IL-6 in breast cancer may be related to its cross-interactions with other potentially tumor-supporting inflammatory cytokines, such as IL-1. As indicated in the IL-1 section, associations and additive effects between the two cytokines were shown (164, 175, 177), suggesting that interactions between them may support the malignancy process in breast cancer. Positive feedback loop between IL-6 and TNFα may also exist, as TNFα induced up-regulation of IL-6 in

human and murine breast cancer cells (83, 153, 175). Furthermore, associations between IL-6 and pro-malignancy chemokines may occur, as indicated by the elevated expression of both factors in highly malignant variants of mammary tumor cells (83, 102), and by the ability of IL-6 to stimulate CCL2 secretion by breast fibroblasts (103).

4.3.3 What is the actual effect of IL-6 in breast cancer?

As indicated previously, opposing evidence was provided by the different studies on the possible roles of IL-6 in breast cancer. The data suggest that there is a marked difference between the analyses that were performed. Studies using clinical material from the tumor itself (biopsies, homogenates /extracts, isolated cells) suggested that IL-6 may be protective, whereas reports analysing circulating IL-6 levels implied that IL-6 may support breast cancer progression. In this regard, it should be noted that it is not clear whether circulating IL-6 is derived directly from the tumor cells, or whether its production manifests immune activities that may take place in breast cancer patients. Studies on IL-6 expression in serum of colon cancer patients suggested that tumor cells are the actual source for the circulating cytokine (229). However, it is not known whether this is also the case in breast cancer.

The thus far available data leaves us with uncertainty concerning the roles of IL-6 in breast cancer. As of now, the reasons for the discrepancies between the different reports are not defined, and the possibility that other, IL-6 unrelated factors affect the roles of IL-6 in breast cancer is highly plausible. Moreover, the source of clinical material, being from the tumor or alternatively a serum sample, may affect the results of the different studies. Nevertheless, one has to bear in mind the fact that a large number of studies on breast cancer patients, in animal model systems and *in vitro*, do support the contribution of IL-6 to breast cancer development and progression. Therefore, in the limits of the current information that is on hand, the roles of IL-6 as a tumor-supporting factor cannot be excluded; nevertheless, its classification as a breast cancer-promoting cytokine should be critically considered.

5. A "VICIOUS CYCLE" OF PRO-MALIGNANCY ACTIVITIES IS MEDIATED BY INFLAMMATORY CELLS, CHEMOKINES AND CYTOKINES IN BREAST CANCER

Breast cancer is affected by a large variety of intrinsic mechanisms and microenvironmental factors. The findings described above suggest that the inflammatory milieu of breast tumors has a key role in the initiation, development and progression of breast cancer. The inflammatory mediators in breast cancer, including cells, chemokines and cytokines, set an array of interactions that possibly facilitates these processes, as described in the herein-proposed model (see Figure 1).

It is suggested that a fundamental event in the initiation and development of breast cancer is the secretion of CCL2 and CCL5 by breast tumor cells. Upon transformation, breast tumor cells start secreting the chemokines constitutively, or alternatively the release of the chemokines by breast epithelial cells is induced by microenvironmental stimuli. In response to CCL2 and CCL5, monocytic cells are recruited from the circulation to the breast tissue, differentiating into macrophages. The resulting TAM secrete a variety of pro-malignancy factors, including growth factors for breast epithelial cells, angiogenic factors, proteolytic enzymes and inflammatory chemokines and cytokines, together supporting breast cancer development and progression. Moreover, the angiogenic properties of CCL2 may act together with those of the highly angiogenic chemokine CXCL8, jointly inducing angiogenesis and vascularization at the primary site, further promoting the development of the primary tumor, its growth and metastasis formation.

The malignancy process may be further driven by the secretion of inflammatory cytokines at breast tumor sites, acting at the initial stages of disease, as well as in more advanced stages. These cytokines include IL-1, TNFα and possibly IL-6, secreted by a variety of cells at the tumor area. While IL-1 and IL-6 are released primarily by the tumor cells, TNFα is

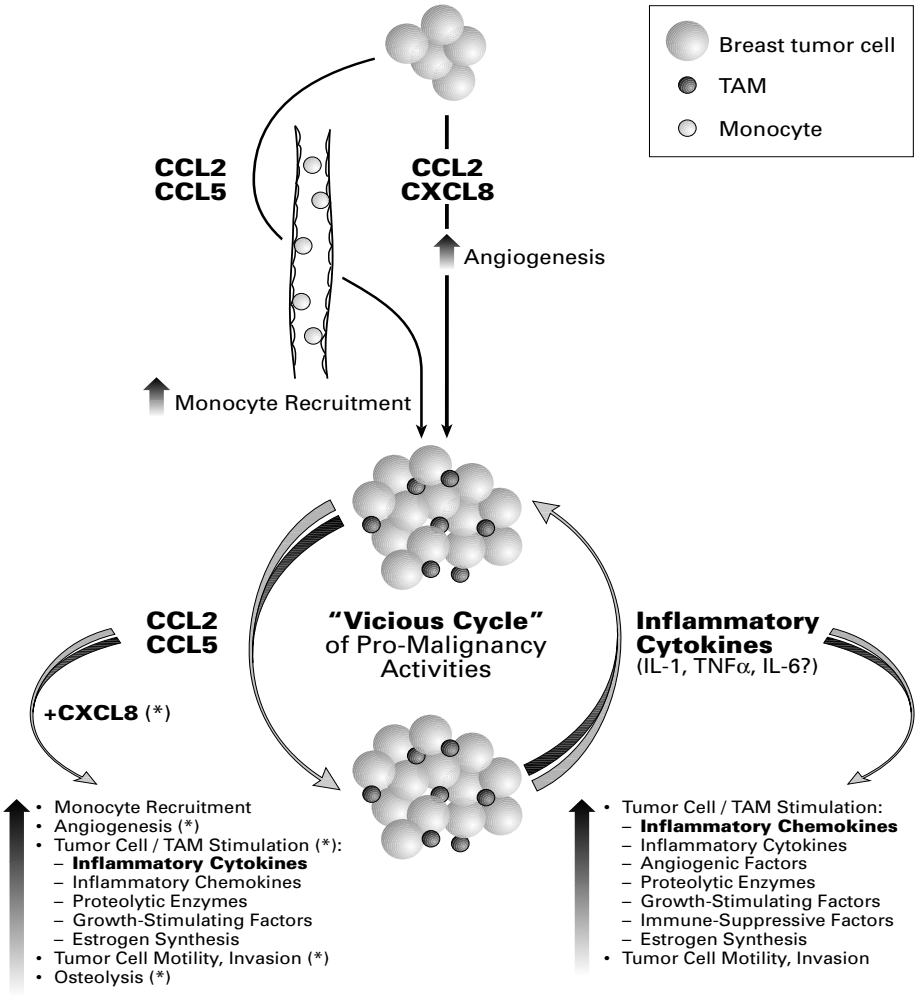


Figure 1. A "vicious cycle" of pro-malignancy activities is mediated by inflammatory cells, chemokines and cytokines in breast cancer. Herein is a proposed model for the interplay between inflammatory mediators and breast tumor cells in breast cancer development and progression. The model suggests that one of the key events in the development of breast cancer is the secretion of inflammatory chemokines by breast tumor cells, inducing angiogenesis and monocyte recruitment to the breast tissue. The resulting tumor-associated macrophages (TAM) release a large variety of tumor-promoting factors, including proteases, angiogenic factors, growth-stimulating factors and inflammatory chemokines and cytokines. This process is assisted by the release of inflammatory cytokines by breast tumor cells, by TAM and by other cells in the tumor microenvironment. The secretion of inflammatory mediators, chemokines and cytokines by these different cell types up-regulates the expression of a large variety of effectors that support the development of breast cancer and metastasis formation. Furthermore, the ability of inflammatory chemokines and inflammatory cytokines to stimulate each other's expression by breast tumor cells and/or by TAM results in repeated cycles of activities of these pro-malignancy factors, thereby giving rise to a "vicious cycle" of pro-malignancy events that exacerbate tumor growth and the metastatic spread of breast tumor cells (For further details, see **Section 5**). The designation "IL-6?" represents the current inconsistencies regarding the roles of IL-6 in breast cancer development and progression. The designation (*) represents tumor-promoting activities in which CXCL8 may also be involved, alone or in addition to CCL2 and CCL5.

secreted mainly by TAM. The three inflammatory cytokines may exert a large variety of pro-malignancy activities. Joined by the tumor-supporting functions of the inflammatory chemokines and of TAM products, the tumor microenvironment becomes overloaded by

numerous factors that facilitate the metastatic process: proteolytic enzymes, angiogenic and growth-stimulating factors, immune-suppressive components, estrogen and inflammatory mediators. Furthermore, the inflammatory chemokines and cytokines can also facilitate the metastatic process by reducing the expression of specific adhesionmolecules and by inducing tumor cell motility. Such functions may support the release of the tumor cells from the primary site and increase their ability to establish metastases at distant sites.

While at the remote metastatic site, the inflammatory mediators may further assist the metastatic process by the large variety of direct pro-malignancy activities that they exert, as well as by stimulating osteoclastogenesis and bone resorption.

Importantly, the activities of inflammatory chemokines on breast tumor cells and/or TAM may up-regulate the secretion of inflammatory cytokines by these cells. The inflammatory cytokines, in turn, may further increase the secretion of inflammatory chemokines by tumor cells and/or TAM, therefore resulting in repeated cycles of interactions. Importantly, this process may be further exacerbated by reciprocal interactions between the tumor cells and TAM *via* the activities of the inflammatory chemokines and cytokines. Thus, a "**vicious cycle**" of pro-malignancy activities is established, which is combined by the tumor-promoting activities of all the inflammatory mediators, pushing forward breast cancer development and progression.

6. CONCLUSIONS

Breast cancer is a highly heterogeneous disease. The reasons for its development and progression are numerous; therefore the interactions that occur between microenvironmental factors and the tumor cells may be affected by the genetic background of the patient, by the hormonal setting and by life style. The research on the activities and regulation of inflammatory cells, chemokines and cytokines has provided information on a major pathway that may contribute to breast cancer initiation, development and progression. However, it is obvious that much is still unknown regarding this pathway, about the overall network of interactions that exists between the different mediators, and about the contribution of other inflammatory components that are not characterized as yet.

Moreover, many other factors, non-immune and immune in their nature, may affect breast cancer. In the context of immune factors and chemokines, it is important to note that the metastatic process may be further facilitated by homeostatic chemokines that direct the homing of the tumor cells to specific metastatic sites. In contrast, other chemokines may potentially act as tumor-limiting factors, for example by inducing the recruitment of T lymphocytes or NK cells to breast tumor sites, or by acting as powerful angiostatic factors. Outside the "chemokine world", cytokines, antigen presenting cells and other components that have the potential to stimulate immune functions, may act as tumor-inhibiting factors.

However, the variety of components that potentially inhibit breast cancer development and progression are probably dominated in the course of the malignancy process by elements that have opposing effects, resulting in an uncontrolled growth of tumor cells and metastasis formation. This situation emphasizes the ultimate need for further research of the different mediators, those that promote the devastating malignancy process, and those that may inhibit it.

Further investigations of the mediators that regulate breast cancer progression may enable the development of novel manoeuvres that limit the activity of the pro-malignancy factors, side by side with manipulations that promote the function of the tumor-limiting ones. Much care should be taken while modulating immune factors and activities, of any kind, as this may result in impairment of normal immune functions. Nevertheless, the potential of such an attitude could be promising if a striking shift in the deleterious equilibrium between the different elements will skew the imbalance in favour of beneficial components, thereby limiting breast cancer development and progression.

ABBREVIATIONS

bFGF: Basic Fibroblast Growth Factor.
DCIS: Ductal Carcinoma *In Situ.*

E_2: 17β-Estradiol.
EGF: Epidermal Growth Factor.
EGFR: Epidermal Growth Factor Receptor.
ER: Estrogen Receptor.
IHC: Immunohistochemistry.
IGF-1: Insulin Growth Factor 1.
IL-1: Interleukin 1.
IL-6: Interleukin 6.
LVEC: Large Vessel Endothelial Cells.
MMP: Matrix Metalloproteinases.
SVEC: Small Vessel Endothelial Cells.
TAM: Tumor-Associated Macrophages.
TP: Thymidine Phosphorylase.
VEGF: Vascular Endothelial Growth Factor.

ACKNOWLEDGEMENTS

The author thanks Prof. Keydar, Prof. Witz, Ms. Azenshtein, Ms. Luboshitz, Dr. Neumark, Ms. Shina, Ms. Meshel, Dr. Wigler and Prof. Chaitchik for their contribution.

REFERENCES

1. Roskelley, C. D., and Bissell, M. J., 2002, The dominance of the microenvironment in breast and ovarian cancer. Semin Cancer Biol, 12:97.
2. Liotta, L. A., and Kohn, E. C., 2001, The microenvironment of the tumour-host interface. Nature, 411:375.
3. Witz, I. P., 2001, Presence and functions of immune components in the tumor microenvironment. Adv Exp Med Biol, 495:317.
4. DeClerck, Y. A., 2000, Interactions between tumour cells and stromal cells and proteolytic modification of the extracellular matrix by metalloproteinases in cancer. Eur J Cancer, 36:1258.
5. Yu, J. L., and Rak, J. W., 2003, Host microenvironment in breast cancer development: inflammatory and immune cells in tumour angiogenesis and arteriogenesis. Breast Cancer Res, 5:83.
6. Coussens, L. M., and Werb, Z., 2001, Inflammatory cells and cancer: think different!. J Exp Med, 193:F23.
7. Brigati, C., Noonan, D. M., Albini, A., and Benelli, R., 2002, Tumors and inflammatory infiltrates: friends or foes?. Clin Exp Metastasis, 19:247.
8. Coussens, L. M., and Werb, Z., 2002, Inflammation and cancer. Nature, 420:860.
9. Sica, A., Saccani, A., and Mantovani, A., 2002, Tumor-associated macrophages: a molecular perspective. Int Immunopharmacol, 2:1045.
10. Balkwill, F., and Mantovani, A., 2001, Inflammation and cancer: back to Virchow?. Lancet 357:539.
11. Leek, R. D., and Harris, A. L., 2002, Tumor-associated macrophages in breast cancer. J Mammary Gland Biol Neoplasia, 7:177.
12. Ben-Baruch, A., 2002, Host microenvironment in breast cancer development: inflammatory cells, cytokines and chemokines in breast cancer progression: reciprocal tumor-microenvironment interactions. Breast Cancer Res, 5:31.
13. Bingle, L., Brown, N. J., and Lewis, C. E., 2002, The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. J Pathol, 196:254.
14. Kurebayashi, J., 2000, Regulation of interleukin-6 secretion from breast cancer cells and its clinical implications. Breast Cancer, 7:124.
15. Wilson, J., and Balkwill, F., 2002, The role of cytokines in the epithelial cancer microenvironment. Semin Cancer Biol, 12:113.
16. Balkwill, F., 2002, Tumor necrosis factor or tumor promoting factor?. Cytokine Growth Factor Rev, 13:135.
17. Wang, J. M., Deng, X., Gong, W., and Su, S., 1998, Chemokines and their role in tumor growth and metastasis. J Immunol Methods, 220:1.
18. Vicari, A. P., and Caux. C., 2002, Chemokines in cancer. Cytokine Growth Factor Rev, 13:143.
19. van Ravenswaay Claasen, H. H., Kluin, P. M.,and Fleuren. G. J., 1992, Tumor infiltrating cells in human cancer. On the possible role of CD16+ macrophages in antitumor cytotoxicity. Lab Invest, 67:166.
20. Tang, R., Beuvon, F., Ojeda, M., Mosseri, V., Pouillart, P., and Scholl S., 1992, M-CSF (monocyte colony stimulating factor) and M-CSF receptor expression by breast tumour cells: M-CSF mediated recruitment of tumour infiltrating monocytes?. J Cell Biochem, 50:350.
21. Pupa, S. M.., Bufalino, R., Invernizzi, A. M., Andreola, S., Rilke, F., Lombardi, L., Colnaghi, M. I., and Menard. S., 1996, Macrophage infiltrate and prognosis in c-erbB-2-overexpressing breast carcinomas. J Clin Oncol, 14:85.
22. Ben-Hur, H., Cohen, O., Schneider, D., Gurevich, P., Halperin, R., Bala, U., Mozes, M., and Zusman I., 2002, The role of lymphocytes and macrophages in human breast tumorigenesis: an immunohistochemical and morphometric study. Anticancer Res, 22:1231.
23. An, T., Sood, U., Pietruk, T., Cummings, G., Hashimoto, K., and Crissman J. D., 1987, In situ quantitation of inflammatory mononuclear cells in

ductal infiltrating breast carcinoma. Relation to prognostic parameters. Am J Pathol, 128:52.

24. Lee, A. H., Happerfield, L. C., Millis, R. R., and Bobrow, L. G., 1996, Inflammatory infiltrate in invasive lobular and ductal carcinoma of the breast. Br J Cancer, 74:796.
25. O'Sullivan, C., and Lewis, C. E., 1994, Tumour-associated leucocytes: friends or foes in breast carcinoma. J Pathol, 172:229.
26. Stewart, T. H., and Heppner, G. H., 1997, Immunological enhancement of breast cancer. Parasitology, 115Suppl:S141.
27. Visscher, D. W., Tabaczka, P., Long, D., and Crissman, J. D., 1995, Clinicopathologic analysis of macrophage infiltrates in breast carcinoma. Pathol Res Pract, 191:1133.
28. van Netten, J. P., Ashmed, B. J., Cavers, D., Fletcher, C., Thornton, I. G., Antonsen, B. L., Coy, P., and Brigden, M. L., 1992, 'Macrophages' and their putative significance in human breast cancer. Br J Cancer, 66:220.
29. Lee, A. H., Happerfield, L. C., Bobrow, L. G., and Millis, R. R., 1997, Angiogenesis and inflammation in invasive carcinoma of the breast. J Clin Pathol, 50:669.
30. van Netten, J. P., Ashmead, B. J., Parker, R. L., Thornton, I. G., Fletcher, C., Cavers, D., Coy, P., and Brigden, M. L., 1993, Macrophage-tumor cell associations: a factor in metastasis of breast cancer? J Leukoc Biol, 54:360.
31. Volodko, N., Reiner, A., Rudas, M.,and Jakesz R.,. 2004, Tumour-associated macrophages in breast cancer and their prognostic correlations. The Breast, 7:99.
32. Leek, R. D., Lewis, C. E., Whitehouse, R., Greenall, M., Clarke, J., and Harris A. L., 1996, Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. Cancer Res, 56:4625.
33. Lee, A. H., Happerfield, L. C., Bobrow, L. G., and Millis, R. R., 1997, Angiogenesis and inflammation in ductal carcinoma in situ of the breast. J Pathol, 181:200.
34. Leek, R. D., Landers, R. J., Harris, A. L., and Lewis, C. E., 1999, Necrosis correlates with high vascular density and focal macrophage infiltration in invasive carcinoma of the breast. Br J Cancer, 79:991.
35. Lewis, C. E., Leek, R., Harris, A., and McGee, J. O., 1995, Cytokine regulation of angiogenesis in breast cancer: the role of tumor-associated macrophages. J Leukoc Biol, 57:747.
36. Goede, V., Brogelli, L., Ziche, M., and Augustin, H. G., 1999, Induction of inflammatory angiogenesis by monocyte chemoattractant protein-1. Int J Cancer, 82:765.
37. Leek, R. D., Hunt, N. C., Landers, R. J., Lewis, C. E., Royds, J. A., and Harris, A. L., 2000, Macrophage infiltration is associated with VEGF and EGFR expression in breast cancer. J Pathol, 190:430.
38. van Netten, J. P., George, E. J., Ashmead, B. J., Fletcher, C., Thornton, I. G., and Coy, P., 1993, Macrophage-tumour cell associations in breast cancer. Lancet, 342:872.
39. Hadden, J. W., 1999, The immunology and immunotherapy of breast cancer: an update. Int J Immunopharmacol, 21:79.
40. Wei, W. Z., and Heppner, G. H., 1996, Breast cancer immunology. Cancer Treat Res, 83:395.
41. Schirrmacher, V., Feuerer, M., Beckhove, P., Ahlert, T., and Umansky, V., 2002, T cell memory, anergy and immunotherapy in breast cancer. J Mammary Gland Biol Neoplasia, 7:201.
42. Jonjic, N., Valkovic, T., Lucin, K., Iternicka, Z., Krstulja, M., Mustac, E., Dobi-Babic, R., Sasso, F., and Melato, M., 1998, Comparison of microvessel density with tumor associated macrophages in invasive breast carcinoma. Anticancer Res, 18:3767.
43. Toi, M., Ueno, T., Matsumoto, H., Saji, H., Funata, N., Koike, M., and Tominaga, T., 1999, Significance of thymidine phosphorylase as a marker of protumor monocytes in breast cancer. Clin Cancer Res, 5:1131.
44. Crowther, M., Brown, N. J., Bishop, E. T., and Lewis, C. E., 2001, Microenvironmental influence on macrophage regulation of angiogenesis in wounds and malignant tumors. J Leukoc Biol, 70:478.
45. Mantovani, A., Ming, W. J., Balotta, C., ,Abdeljalil, B. and Bottazzi, B., 1986, Origin and regulation of tumor-associated macrophages: the role of tumor-derived chemotactic factor. Biochim Biophys Acta, 865:59.
46. Mantovani, A., Sozzani, S., Locati, M., Allavena, P., and Sica, A., 2002, Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol, 23:549.
47. Mantovani, A., Bottazzi, B., Colotta, F., Sozzani, S., and Ruco, L., 1992, The origin and function of tumor-associated macrophages. Immunol Today, 13:265.
48. Lewis, J. S., Landers, R. J., Underwood, J. C., Harris, A. L., and Lewis, C. E., 2000, Expression of vascular endothelial growth factor by macrophages is up-regulated in poorly vascularized areas of breast carcinomas. J Pathol, 192:150.
49. Barbera-Guillem, E., Nyhus, J. K., Wolford, C. C., Friece, C. R., and Sampsel, J. W., 2002, Vascular endothelial growth factor secretion by tumor-infiltrating macrophages essentially supports tumor angiogenesis, and IgG immune complexes potentiate the process. Cancer Res, 62:7042.

50. O'Sullivan, C., Lewis, C. E., Harris, A. L, and McGee, J. O., 1993, Secretion of epidermal growth factor by macrophages associated with breast carcinoma. Lancet, 342:148.
51. Mor, G., Yue, W., Santen, R. J., Gutierrez, L., Eliza, M., Berstein, L. M., Harada, N., Wang, J., Lysiak, J., Diano, S., and Naftolin, F., 1998, Macrophages, estrogen and the microenvironment of breast cancer. J Steroid Biochem Mol Biol, 67:403.
52. Travis, R. C., and Key, T. J., 2003, Oestrogen exposure and breast cancer risk. Breast Cancer Res, 5:239.
53. Kennedy, S., Duffy, M. J., Duggan, C., Barnes, C., Rafferty, R., and Kramer, M. D., 1998, Semi-quantitation of urokinase plasminogen activator and its receptor in breast carcinomas by immunocytochemistry. Br J Cancer, 77:1638.
54. Heppner, K. J.,., Matrisian, L. M Jensen, R. A., and Rodgers, W. H., 1996, Expression of most matrix metalloproteinase family members in breast cancer represents a tumor-induced host response. Am J Pathol, 149:273.
55. Pusztai, L., Clover, L. M., Cooper, K., Starkey, P. M., Lewis, C. E., and McGee, J. O., 1994, Expression of tumour necrosis factor alpha and its receptors in carcinoma of the breast. Br J Cancer, 70:289.
56. Miles, D. W., Happerfield, L. C., Naylor, M. S., Bobrow, L. G., Rubens, R. D., and Balkwill, F. R., 1994, Expression of tumour necrosis factor (TNF alpha) and its receptors in benign and malignant breast tissue. Int J Cancer, 56:777.
57. Ueno, T., Toi, M., Saji, H., Muta, M., Bando, H., Kuroi, K., Koike, M., Inadera, H., and Matsushima, K., 2000, Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. Clin Cancer Res, 6:3282.
58. Saji, H., Koike, M., Yamori, T., Saji, S., Seiki, M., Matsushima, K., and Toi, M., 2001, Significant correlation of monocyte chemoattractant protein-1 expression with neovascularization and progression of breast carcinoma. Cancer, 92:1085.
59. Luboshits, G., Shina, S., Kaplan, O., Engelberg, S., Nass, D., Lifshitz-Mercer, B., Chaitchik, S., Keydar, I., and Ben-Baruch, A., 1999, Elevated expression of the CC chemokine regulated on activation, normal T cell expressed and secreted (RANTES) in advanced breast carcinoma. Cancer Res, 59:4681.
60. Speirs, V., Green, A. R., and White, M. C., 1996, A comparative study of cytokine gene transcripts in normal and malignant breast tissue and primary cell cultures derived from the same tissue samples. Int J Cancer, 66:551.
61. Sallusto, F., Mackay, C. R., and Lanzavecchia, A., 2000, The role of chemokine receptors in primary, effector, and memory immune responses. Annu Rev Immunol, 18:593.
62. Kim, C. H., and Broxmeyer, H. E., 1999, Chemokines: signal lamps for trafficking of T and B cells for development and effector function. J Leukoc Biol, 65:6.
63. Locati, M., Otero, K., Schioppa, T., Signorelli, P., Perrier, P., Baviera, S., Sozzani, S., and Mantovani, A., 2002, The chemokine system: tuning and shaping by regulation of receptor expression and coupling in polarized responses. Allergy, 57:972.
64. Broxmeyer, H. E., and Kim, C. H., 1999, Regulation of hematopoiesis in a sea of chemokine family members with a plethora of redundant activities. Exp Hematol, 27:1113.
65. Belperio, J. A., Keane, M. P., Arenberg, D. A., Addison, C. L., Ehlert, J. E., Burdick, M. D., and Strieter, R. M., 2000, CXC chemokines in angiogenesis. J Leukoc Biol, 68:1.
66. Murphy, P. M., Baggiolini, M., Charo, I. F., Hebert, C. A., Horuk, R., Matsushima, K., Miller, L. H., Oppenheim, J. J., and Power, C. A., 2000, International union of pharmacology. XXII. Nomenclature for chemokine receptors. Pharmacol Rev, 52:145.
67. Salcedo, R., and Oppenheim, J. J., 2003, Role of chemokines in angiogenesis: CXCL12/SDF-1 and CXCR4 interaction, a key regulator of endothelial cell responses. Microcirculation, 10:359.
68. Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N., Barrera, J. L., Mohar, A., Verastegui, E., and Zlotnik, A., 2001, Involvement of chemokine receptors in breast cancer metastasis. Nature, 410:50.
69. Schmid, B. C., Rudas, M., Rezniczek, G. A., Leodolter, S., and Zeillinger, R., 2004, CXCR4 is Expressed in Ductal Carcinoma in situ of the Breast and in Atypical Ductal Hyperplasia. Breast Cancer Res Treat, 84:247.
70. Kato, M., Kitayama, J., Kazama, S., and Nagawa, H., 2003, Expression pattern of CXC chemokine receptor-4 is correlated with lymph node metastasis in human invasive ductal carcinoma. Breast Cancer Res, 5:R144.
71. Helbig, G., Christopherson, II, K. W., Bhat-Nakshatri, P., Kumar, S., Kishimoto, H., Miller, K. D., Broxmeyer, H. E., and Nakshatri, H., 2003, NF-kappaB promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4. J Biol Chem, 278:21631.
72. Chen, Y., Stamatoyannopoulos, G., and Song, C. Z., 2003, Down-regulation of CXCR4 by inducible small interfering RNA inhibits breast cancer cell invasion in vitro. Cancer Res, 63:4801.

73. Bachelder, R. E., Wendt, M. A., and Mercurio, A. M., 2002, Vascular endothelial growth factor promotes breast carcinoma invasion in an autocrine manner by regulating the chemokine receptor CXCR4. Cancer Res, 62:7203.
74. Fernandis, A. Z., Prasad, A., Band, H., Klosel, R., and Ganju, R. K., 2004, Regulation of CXCR4-mediated chemotaxis and chemoinvasion of breast cancer cells. Oncogene, 23:157.
75. Prasad, A., Fernandis, A. Z., Rao, Y., and Ganju, R. K., 2004, Slit protein-mediated inhibition of CXCR4-induced chemotactic and chemoinvasive signaling pathways in breast cancer cells. J Biol Chem, 279:9115.
76. Hall, J. M., and Korach, K. S., 2003, Stromal cell-derived factor 1, a novel target of estrogen receptor action, mediates the mitogenic effects of estradiol in ovarian and breast cancer cells. Mol Endocrinol, 17:792.
77. Liotta, L. A., 2001, An attractive force in metastasis. Nature, 410:24.
78. Murphy, P. M., 2001, Chemokines and the molecular basis of cancer metastasis. N Engl J Med, 345:833.
79. Strieter, R. M., 2001, Chemokines: not just leukocyte chemoattractants in the promotion of cancer. Nat Immunol, 2:285.
80. Wolf, M., Clark-Lewis, I., Buri, C., Langen, H., Lis, M., and Mazzucchelli, L., 2003, Cathepsin D specifically cleaves the chemokines macrophage inflammatory protein-1 alpha, macrophage inflammatory protein-1 beta, and SLC that are expressed in human breast cancer. Am J Pathol, 162:1183.
81. Braun, S. E., Chen, K., Foster, R. G., Kim, C. H., Hromas, R., Kaplan, M. H., Broxmeyer, H. E., and Cornetta, K., 2000, The CC chemokine CK beta-11/MIP-3 beta/ELC/Exodus 3 mediates tumor rejection of murine breast cancer cells through NK cells. J Immunol, 164:4025.
82. Lee, T. V., Kim, D. K., Peoples, G. E., Castilleja, A., Murray, J. L., Gershenson, D. M., and Ioannides, C. G., 2000, Secretion of CXC chemokine IP-10 by peripheral blood mononuclear cells from healthy donors and breast cancer patients stimulated with HER-2 peptides. J Interferon Cytokine Res, 20:391.
83. Neumark, E., Sagi-Assif, O., Shalmon, B., Ben-Baruch, A., and Witz, I. P., 2003, Progression of mouse mammary tumors: MCP-1-TNFalpha cross-regulatory pathway and clonal expression of promalignancy and antimalignancy factors. Int J Cancer, 106:879.
84. Aronica, S. M., Fanti, P., Kaminskaya, K., Gibbs, K., Raiber, L., Nazareth, M., Bucelli, R., Mineo, M., Grzybek, K., Kumin, M., Poppenberg, K., Schwach, C.,and Janis, K., 2004, Estrogen Disrupts Chemokine-Mediated Chemokine Release from Mammary Cells: Implications for the Interplay between Estrogen and IP-10 in the Regulation of Mammary Tumor Formation. Breast Cancer Res Treat, 84:235.
85. Dorsey, R., Kundu, N., Yang, Q., Tannenbaum, C. S., Sun, H., Hamilton, T. A., and Fulton, A. M., 2002, Immunotherapy with interleukin-10 depends on the CXC chemokines inducible protein-10 and monokine induced by IFN-gamma. Cancer Res, 62:2606.
86. Pulaski, B. A., Clements, V. K. , Pipeling, M. R., and Ostrand-Rosenberg, S., 2000, Immunotherapy with vaccines combining MHC class II/CD80+ tumor cells with interleukin-12 reduces established metastatic disease and stimulates immune effectors and monokine induced by interferon gamma. Cancer Immunol Immunother, 49:34.
87. Coughlin, C. M., Salhany, K. E., Gee, M. S., LaTemple, D. C., Kotenko, S., Ma, X., Gri, G., Wysocka, M., Kim, J. E., Liu, L., Liao, F., Farber, J. M., Pestka, S., Trinchieri, G., and Lee, W. M.,1998, Tumor cell responses to IFNgamma affect tumorigenicity and response to IL-12 therapy and antiangiogenesis. Immunity, 9:25.
88. Dias, S., Thomas, H., and Balkwill, F., 1998, Multiple molecular and cellular changes associated with tumour stasis and regression during IL-12 therapy of a murine breast cancer model. Int J Cancer, 75:151.
89. Palmer, K., Hitt, M., Emtage, P. C., Gyorffy, S., and Gauldie, J., 2001, Combined CXC chemokine and interleukin-12 gene transfer enhances antitumor immunity. Gene Ther, 8:282.
90. Luster, A. D., and Leder, P., 1993, IP-10, a -C-X-C- chemokine, elicits a potent thymus-dependent antitumor response in vivo. J Exp Med, 178:1057.
91. Sun, H., Jackson, M. J., Kundu, N., and Fulton, A. M., 1999, Interleukin-10 gene transfer activates interferon-gamma and the interferon-gamma-inducible genes Gbp-1/Mag-1 and Mig-1 in mammary tumors. Int J Cancer, 80:624.
92. Sun, H., Kundu, N., Dorsey, R., Jackson, M. J., and Fulton, A. M., 2001, Expression of the Chemokines IP-10 and Mig in IL-10 Transduced Tumors. J Immunother, 24:138.
93. Goldberg-Bittman, E., Neumark, E., Sagi-Assif, O., Azenshtein, E., Meshel, T., Witz, I. P., Ben-Baruch, A., 2004, The expression of the chemokine receptor CXCR3 and its ligand, CXCL10, in human breast adenocarcinoma cell lines. Immunol Lett, 92:171.
94. Taub, D. D., and Oppenheim, J. J., 1994, Chemokines, inflammation and the immune system. Ther Immunol, 1:229.
95. Rollins, B. J., 1997, Chemokines. Blood, 90:909.
96. Valkovic, T., Lucin, K., Krstulja, M., Dobi-Babic, R., and Jonjic, N., 1998, Expression of monocyte

chemotactic protein-1 in human invasive ductal breast cancer. Pathol Res Pract, 194:335.

97. Luciani, M. G., Stoppacciaro, A., Peri, G., Mantovani, A., and Ruco, L. P., 1998, The monocyte chemotactic protein a (MCP-1) and interleukin 8 (IL-8) in Hodgkin's disease and in solid tumours. Mol Pathol, 51:273.
98. Inadera, H., Sekiya, T., Yoshimura, T., and Matsushima, K., 2000, Molecular analysis of the inhibition of monocyte chemoattractant protein-1 gene expression by estrogens and xenoestrogens in MCF-7 cells. Endocrinology, 141:50.
99. Kelly, R. W., Carr, G. G., and Riley, S. C., 1997, The inhibition of synthesis of a beta-chemokine, monocyte chemotactic protein-1 (MCP-1) by progesterone. Biochem Biophys Res Commun, 239:557.
100. Azenshtein, E., Luboshits, G., Shina, S., Neumark, E., Shahbazian, D., Weil, M., Wigler, N., Keydar, I., and Ben-Baruch, A., 2002, The CC chemokine RANTES in breast carcinoma progression: regulation of expression and potential mechanisms of promalignant activity. Cancer Res, 62:1093.
101. Neumark, E., Anavi, R., Witz, I. P., and Ben-Baruch, A., 1999, MCP-1 expression as a potential contributor to the high malignancy phenotype of murine mammary adenocarcinoma cells. Immunol Lett, 68:141.
102. Neumark, E., Cohn, M. A., Lukanidin, E., Witz, I. P., and Ben-Baruch, A., 2002, Possible co-regulation of genes associated with enhanced progression of mammary adenocarcinomas. Immunol Lett, 82:111.
103. Silzle, T., Kreutz, M,. Dobler, M. A., Brockhoff, G., Knuechel, R., and Kunz-Schughart, L. A., 2003, Tumor-associated fibroblasts recruit blood monocytes into tumor tissue. Eur J Immunol, 33:1311.
104. Salcedo, R., Ponce, M. L., Young, H. A., Wasserman, K., Ward, J. M., Kleinman, H. K., Oppenheim, J. J., and Murphy, W. J., 2000, Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. Blood, 96:34.
105. Vitiello, P. F., Shainheit, M. G., Allison, E. M., Adler, E. P., and Kurt, R. A., 2004, Impact of tumor-derived CCL2 on T cell effector function. Immunol Lett, 91:239.
106. Yamada, M., Kim, S., Egashira, K., Takeya, M., Ikeda, T., Mimura, O., and Iwao, H., 2003, Molecular mechanism and role of endothelial monocyte chemoattractant protein-1 induction by vascular endothelial growth factor. Arterioscler Thromb Vasc Biol, 23:1996.
107. Ohta, M., Kitadai, Y., Tanaka, S., Yoshihara, M., Yasui, W., Mukaida, N., Haruma, K., and Chayama, K., 2002, Monocyte chemoattractant protein-1 expression correlates with macrophage infiltration and tumor vascularity in human esophageal squamous cell carcinomas. Int J Cancer, 102:220.
108. Ohta, M., Kitadai, Y., Tanaka, S., Yoshihara, M., Yasui, W., Mukaida, N., Haruma, K., and Chayama, K., 2003, Monocyte chemoattractant protein-1 expression correlates with macrophage infiltration and tumor vascularity in human gastric carcinomas. Int J Oncol, 22:773.
109. Ambati, B. K., Joussen, A. M., Kuziel, W. A., Adamis, A. P., and Ambati, J., 2003, Inhibition of corneal neovascularization by genetic ablation of CCR2. Cornea, 22:465.
110. Weber, K. S., Nelson, P. J., Grone, H. J., and Weber, C., 1999, Expression of CCR2 by endothelial cells: implications for MCP-1 mediated wound injury repair and In vivo inflammatory activation of endothelium. Arterioscler Thromb Vasc Biol, 19:2085.
111. Robinson, S. C., Scott, K. A., and Balkwill, F. R., 2002, Chemokine stimulation of monocyte matrix metalloproteinase-9 requires endogenous TNF-alpha. Eur J Immunol, 32:404.
112. Youngs, S. J., Ali, S. A., Taub, D. D., and Rees, R. C., 1997, Chemokines induce migrational responses in human breast carcinoma cell lines. Int J Cancer, 71:257.
113. Prest, S. J., Rees, R. C., Murdoch, C., Marshall, J. F., Cooper, P. A., Bibby, M., Li, G., and Ali, S. A., 1999, Chemokines induce the cellular migration of MCF-7 human breast carcinoma cells: subpopulations of tumour cells display positive and negative chemotaxis and differential in vivo growth potentials. Clin Exp Metastasis, 17:389.
114. Locati, M., Deuschle, U., Massardi, M. L., Martinez, F. O., Sironi, M., Sozzani, S., Bartfai, T., and Mantovani, A., 2002, Analysis of the gene expression profile activated by the CC chemokine ligand 5/RANTES and by lipopolysaccharide in human monocytes. J Immunol, 168:3557.
115. Adler, E. P., Lemken, C. A., Katchen, N. S., and Kurt, R. A., 2003, A dual role for tumor-derived chemokine RANTES (CCL5). Immunol Lett, 90:187.
116. Robinson, S. C., Scott, K. A., Wilson, J. L., Thompson, R. G., Proudfoot, A. E., and Balkwill, F. R., 2003, A chemokine receptor antagonist inhibits experimental breast tumor growth. Cancer Res, 63:8360.
117. Ali, S., Kaur, J., and Patel, K. D., 2000, Intercellular cell adhesion molecule-1, vascular cell adhesion molecule-1, and regulated on activation normal T cell expressed and secreted are expressed by human breast carcinoma cells and support eosinophil adhesion and activation. Am J Pathol, 157:313.
118. Niwa, Y., Akamatsu, H., Niwa, H., Sumi, H., Ozaki, Y.,and Abe, A., 2001, Correlation of tissue and plasma RANTES levels with disease course in

patients with breast or cervical cancer. Clin Cancer Res, 7:285.

119. Mira, E., Lacalle, R. A., Gonzalez, M. A., Gomez-Mouton, C., Abad, J. L., Bernad, A., Martinez, A. C., and Manes, S., 2001, A role for chemokine receptor transactivation in growth factor signaling. EMBO Rep, 2:151.
120. Klier, C. M., Nelson, E. L., Cohen, C. D., Horuk, R., Schlondorff, D., and Nelson, P. J., 2001, Chemokine-Induced secretion of gelatinase B in primary human monocytes. Biol Chem, 382:1405.
121. Manes, S., Mira, E., Colomer, R., Montero, S., Real, L. M., Gomez-Mouton, C., Jimenez-Baranda, S., Garzon, A., Lacalle, R. A., Harshman, K., Ruiz, A., and Martinez, A. C., 2003, CCR5 expression influences the progression of human breast cancer in a p53-dependent manner. J Exp Med, 198:1381.
122. Basolo, F., Fiore, L., Fontanini, G., Conaldi, P. G., Calvo, S., Falcone, V., and Toniolo, A., 1996, Expression of and response to interleukin 6 (IL6) in human mammary tumors. Cancer Res, 56:3118.
123. Pantschenko, A. G., Pushkar, I., Miller, L. J., Wang, Y. P., Anderson, K., Peled, Z., Kurtzman, S. H., and Kreutzer, D. L., 2003, In vitro demonstration of breast cancer tumor cell sub-populations based on interleukin-1/tumor necrosis factor induction of interleukin-8 expression. Oncol Rep, 10:1011.
124. Miller, L. J., Kurtzman, S. H., Wang, Y., Anderson, K. H., Lindquist, R. R., and Kreutzer, D. L., 1998, Expression of interleukin-8 receptors on tumor cells and vascular endothelial cells in human breast cancer tissue. Anticancer Res, 18:77.
125. Pantschenko, A. G., Pushkar, I., Anderson, K. H., Wang, Y., Miller, L. J., Kurtzman, S. H., Barrows, G.,and Kreutzer, D. L., 2003, The interleukin-1 family of cytokines and receptors in human breast cancer: implications for tumor progression. Int J Oncol, 23:269.
126. Green, A. R., Green, V. L., White, M. C., and Speirs, V., 1997, Expression of cytokine messenger RNA in normal and neoplastic human breast tissue: identification of interleukin-8 as a potential regulatory factor in breast tumours. Int J Cancer, 72:937.
127. De Larco, J. E., Wuertz, B. R., Rosner, K. A., Erickson, S. A., Gamache, D. E., Manivel, J. C., and Furcht, L. T., 2001, A potential role for interleukin-8 in the metastatic phenotype of breast carcinoma cells. Am J Pathol, 158:639.
128. Salcedo, R., Martins-Green, M., Gertz, B., Oppenheim, J. J., and Murphy, W. J., 2002, Combined administration of antibodies to human interleukin 8 and epidermal growth factor receptor results in increased antimetastatic effects on human breast carcinoma xenografts. Clin Cancer Res, 8:2655.
129. De Larco, J. E., Wuertz, B. R., Manivel, J. C., and Furcht, L. T., 2001, Progression and enhancement of metastatic potential after exposure of tumor cells to chemotherapeutic agents. Cancer Res, 61:2857.
130. Lev, D. C., Kiriakova, G., and Price, J. E., 2003, Selection of more aggressive variants of the gI101A human breast cancer cell line: a model for analyzing the metastatic phenotype of breast cancer. Clin Exp Metastasis, 20:515.
131. Bendre, M. S., Gaddy-Kurten, D., Mon-Foote, T., Akel, N. S., Skinner, R. A., Nicholas, R. W., and Suva, L. J., 2002, Expression of interleukin 8 and not parathyroid hormone-related protein by human breast cancer cells correlates with bone metastasis in vivo. Cancer Res, 62:5571.
132. Lin, Y., Huang, R., Chen, L., Li, S., Shi, Q. Jordan, C., and Huang, R. P., 2004, Identification of interleukin-8 as estrogen receptor-regulated factor involved in breast cancer invasion and angiogenesis by protein arrays. Int J Cancer, 109:507.
133. Fujisawa, N., Sakao, Y., Hayashi, S., Hadden, III, W. A., Harmon, C. L., and Miller, E. J., 2000, alpha-Chemokine growth factors for adenocarcinomas; a synthetic peptide inhibitor for alpha-chemokines inhibits the growth of adenocarcinoma cell lines. J Cancer Res Clin Oncol, 126:19.
134. Freund, A., Chauveau, C., Brouillet, J. P., Lucas, A., Lacroix, M., Licznar, A., Vignon, F., and Lazennec, G., 2003, IL-8 expression and its possible relationship with estrogen-receptor-negative status of breast cancer cells. Oncogene, 22:256.
135. Basolo, F., Conaldi, P. G., Fiore, L., Calvo, S., and Toniolo, A., 1993, Normal breast epithelial cells produce interleukins 6 and 8 together with tumor-necrosis factor: defective IL6 expression in mammary carcinoma. Int J Cancer, 55:926.
136. Palkowetz, K. H., Royer, C. L., Garofalo, R., Rudloff, H. E., Schmalstieg, Jr., F. C., and Goldman, A. S., 1994, Production of interleukin-6 and interleukin-8 by human mammary gland epithelial cells. J Reprod Immunol, 26:57.
137. Maheshwari, A., Christensen, R. D., and Calhoun, D. A., 2003, ELR+ CXC chemokines in human milk. Cytokine, 24:91.
138. Barber, M. R., Pantschenko, A. G., Hinckley, L. S., and Yang, T. J., 1999, Inducible and constitutive in vitro neutrophil chemokine expression by mammary epithelial and myoepithelial cells. Clin Diagn Lab Immunol, 6:791.
139. Barber, M. R., and Yang, T. J., 1998, Chemotactic activities in nonmastitic and mastitic mammary secretions: presence of interleukin-8 in mastitic but not nonmastitic secretions. Clin Diagn Lab Immunol, 5:82.
140. Nishimura, T., 2003, Expression of potential lymphocyte trafficking mediator molecules in the mammary gland. Vet Res, 34:3.

141. Pusztai, L., Mendoza, T. R., Reuben, J. M., Martinez, M. M., Willey, J. S., Lara, J., Syed, A., Fritsche, H. A., Bruera, E., Booser, D., Valero, V., Arun, B., Ibrahim, N., Rivera, E., Royce, M., Cleeland, C. S. ,and Hortobagyi, G. N., 2004, Changes in plasma levels of inflammatory cytokines in response to paclitaxel chemotherapy. Cytokine, 25:94.
142. Zakrzewska, I., Kozlowski, L., and Wojtukiewicz, M., 2002, Value of interleukin-8 determination in diagnosis of benign and malignant breast tumor. Pol Merkuriusz Lek, 13:302.
143. Kozlowski, L., Zakrzewska, I., Tokajuk, P., and Wojtukiewicz, M. Z., 2003, Concentration of interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-10 (IL-10) in blood serum of breast cancer patients. Rocz Akad Med Bialymst, 48:82.
144. Yokoe, T., Iino, Y., Takei, H., Horiguchi, J., Koibuchi, Y., Maemura, M., Ohwada, S., and Morishita, Y., 1997, Changes of cytokines and thyroid function in patients with recurrent breast cancer. Anticancer Res, 17:695.
145. Yokoe, T., Iino, Y., and Morishita, Y., 2000, Trends of IL-6 and IL-8 levels in patients with recurrent breast cancer: preliminary report. Breast Cancer, 7:187.
146. Speirs, V., Green, A. R., and Atkin, S. L., 1998, Activity and gene expression of 17beta-hydroxysteroid dehydrogenase type I in primary cultures of epithelial and stromal cells derived from normal and tumourous human breast tissue: the role of IL-8. J Steroid Biochem Mol Biol, 67:267.
147. Schraufstatter, I. U., Trieu, K., Zhao, M., Rose, D. M., Terkeltaub, R. A., and Burger, M., 2003, IL-8-mediated cell migration in endothelial cells depends on cathepsin B activity and transactivation of the epidermal growth factor receptor. J Immunol, 171:6714.
148. Xie, K., 2001, Interleukin-8 and human cancer biology. Cytokine Growth Factor Rev, 12:375.
149. Li, A., Dubey, S., Varney, M. L., and Singh, R. K., 2002, Interleukin-8-induced proliferation, survival, and MMP production in CXCR1 and CXCR2 expressing human umbilical vein endothelial cells. Microvasc Res, 64:476.
150. Heidemann, J., Ogawa, H., Dwinell, M. B., Rafiee, P., Maaser, C., Gockel, H. R., Otterson, M. F., Ota, D. M., Lugering, N., Domschke, W., and Binion, D. G., 2003, Angiogenic effects of interleukin 8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2. J Biol Chem, 278:8508.
151. Addison, C. L., Daniel, T. O., Burdick, M. D., Liu, H., Ehlert, J. E., Xue, Y. Y., Buechi, L., Walz, A., Richmond, A., andStrieter, R. M., 2000, The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity. J Immunol, 165:5269.
152. Kurtzman, S. H., Anderson, K. H., Wang, Y., Miller, L. J., Renna, M., Stankus, M., Lindquist, R. R., Barrows, G., and Kreutzer, D. L., 1999, Cytokines in human breast cancer: IL-1alpha and IL-1beta expression. Oncol Rep, 6:65.
153. Nozaki, S., Sledge, Jr., G. W., and Nakshatri, H., 2000, Cancer cell-derived interleukin 1alpha contributes to autocrine and paracrine induction of pro-metastatic genes in breast cancer. Biochem Biophys Res Commun, 275:60.
154. Arihiro, K., Oda, H., Kaneko, M., and Inai, K., 2000, Cytokines facilitate chemotactic motility of breast carcinoma cells. Breast Cancer, 7:221.
155. Bendre, M. S., Montague, D. C., Peery, T., Akel, N. S., Gaddy, D., and Suva, L. J., 2003, Interleukin-8 stimulation of osteoclastogenesis and bone resorption is a mechanism for the increased osteolysis of metastatic bone disease. Bone, 33:28.
156. Bendre, M., Gaddy, D., Nicholas, R. W., and Suva, L. J., 2003, Breast cancer metastasis to bone: it is not all about PTHrP. Clin Orthop, S39.
157. Rankin, J. A., 2004, Biological mediators of acute inflammation. AACN Clin Issues, 15:3.
158. Palladino, M. A., Bahjat, F. R., Theodorakis, E. A., and Moldawer, L. L., 2003, Anti-TNF-alpha therapies: the next generation. Nat Rev Drug Discov, 2:736.
159. Dinarello, C. A., 2002, The IL-1 family and inflammatory diseases. Clin Exp Rheumatol, 20:S1.
160. Yudkin, J. S., Kumari, M., Humphries, S. E., and Mohamed-Ali, V., 2000, Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link?. Atherosclerosis, 148:209.
161. Kamimura, D., Ishihara, K., and Hirano, T., 2003, IL-6 signal transduction and its physiological roles: the signal orchestration model. Rev Physiol Biochem Pharmacol, 149:1.
162. Streetz, K. L., Wustefeld, T., Klein, C., Manns, M. P., and Trautwein, C., 2001, Mediators of inflammation and acute phase response in the liver. Cell Mol Biol (Noisy-le-grand), 47:661.
163. 1994, The cytokine handbook. Thomson A. Academic Press, London.
164. Robinson, E. K., Sneige, N., and Grimm, E. A., 1998, Correlation of interleukin 6 with interleukin 1alpha in human mammary tumours, but not with oestrogen receptor expression. Cytokine, 10:970.
165. Jin, L., Yuan, R. Q., Fuchs, A., Yao, Y., Joseph, A., Schwall, R., Schnitt, S. J., Guida, A., Hastings, H. M., Andres, J., Turkel, G., Polverini, P. J., Goldberg, I. D.,and Rosen, E. M.,1997, Expression of interleukin-1beta in human breast carcinoma. Cancer, 80:421.
166. Miller, L. J., Kurtzman, S. H., Anderson, K., Wang, Y., Stankus, M., Renna, M., Lindquist, R., Barrows,

G., and Kreutzer, D. L., 2000, Interleukin-1 family expression in human breast cancer: interleukin-1 receptor antagonist. Cancer Invest, 18:293.

167. Singer, C. F., Kronsteiner, N., Hudelist, G., Marton, E., Walter, I., Kubista, M., Czerwenka, K., Schreiber, M., Seifert, M., and Kubista, E., 2003, Interleukin 1 system and sex steroid receptor expression in human breast cancer: interleukin 1alpha protein secretion is correlated with malignant phenotype. Clin Cancer Res, 9:4877.
168. Bhat-Nakshatri, P., Newton, T. R., Goulet, Jr., R., and Nakshatri, H., 1998, NF-kappaB activation and interleukin 6 production in fibroblasts by estrogen receptor-negative breast cancer cell-derived interleukin 1alpha. Proc Natl Acad Sci USA, 95:6971.
169. Kumar, S., Kishimoto, H., Chua, H. L., Badve, S., Miller, K. D., Bigsby, R. M., and Nakshatri, H., 2003, Interleukin-1 alpha promotes tumor growth and cachexia in MCF-7 xenograft model of breast cancer. Am J Pathol, 163:2531.
170. Voronov, E., Shouval, D. S., Krelin, Y., Cagnano, E., Benharroch, D., Iwakura, Y., Dinarello, C. A., and Apte, R. N., 2003, IL-1 is required for tumor invasiveness and angiogenesis. Proc Natl Acad Sci USA, 100:2645.
171. Zhang, G. J., and I. Adachi. 1999. Serum interleukin-6 levels correlate to tumor progression and prognosis in metastatic breast carcinoma. Anticancer Res 19:1427.
172. Sgagias, M. K., Kasid, A., and Danforth, Jr., D. N., 1991, Interleukin-1 alpha and tumor necrosis factor-alpha (TNF alpha) inhibit growth and induce TNF messenger RNA in MCF-7 human breast cancer cells. Mol Endocrinol, 5:1740.
173. Shen, W. H., Zhou, J. H., Broussard, S. R., Freund, G. G., Dantzer, R., and Kelley, K. W., 2002, Proinflammatory cytokines block growth of breast cancer cells by impairing signals from a growth factor receptor. Cancer Res, 62:4746.
174. Costantino, A., Vinci, C., Mineo, R., Frasca, F., Pandini, G., Milazzo, G., Vigneri, R., and Belfiore, A., 1996, Interleukin-1 blocks insulin and insulin-like growth factor-stimulated growth in MCF-7 human breast cancer cells by inhibiting receptor tyrosine kinase activity. Endocrinology, 137:4100.
175. Suarez-Cuervo, C., Harris, K. W., Kallman, L., Vaananen, H. K. and Selander, K. S., 2003, Tumor necrosis factor-alpha induces interleukin-6 production via extracellular-regulated kinase 1 activation in breast cancer cells. Breast Cancer Res Treat, 80:71.
176. Speiser, P., Zeillinger, R., Wiltschke, C., Sedlak, J., and Chorvath, B., 1993, IL-1 alpha induced, TNF alpha mediated HLA class II (DR) antigen up-regulation in a human ductal breast carcinoma cell line ZR-75-1. Neoplasma, 40:137.
177. Verhasselt, B., Van Damme, J., van Larebeke, N., Put, W., Bracke, M., De Potter, C., and Mareel, M., 1992, Interleukin-1 is a motility factor for human breast carcinoma cells in vitro: additive effect with interleukin-6. Eur J Cell Biol, 59:449.
178. Duncan, L. J., Coldham, N. G., and Reed, M. J., 1994, The interaction of cytokines in regulating oestradiol 17 beta-hydroxysteroid dehydrogenase activity in MCF-7 cells. J Steroid Biochem Mol Biol, 49:63.
179. Palmieri, C., Roberts-Clark, D., Assadi-Sabet, A., Coope, R. C., O'Hare, M., Sunters, A., Hanby, A., Slade, M. J., Gomm, J. J., Lam, E. W., and Coombes, R. C., 2003, Fibroblast growth factor 7, secreted by breast fibroblasts, is an interleukin-1beta-induced paracrine growth factor for human breast cells. J Endocrinol, 177:65.
180. Macdiarmid, F., Wang, D., Duncan, L. J., Purohit, A., Ghilchick, M. W., and Reed, M. J., 1994, Stimulation of aromatase activity in breast fibroblasts by tumor necrosis factor alpha. Mol Cell Endocrinol, 106:17.
181. Honma, S., Shimodaira, K., Shimizu, Y., Tsuchiya, N., Saito, H., Yanaihara, T., and Okai, T., 2002, The influence of inflammatory cytokines on estrogen production and cell proliferation in human breast cancer cells. Endocr J, 49:371.
182. de Jong, P. C., Blankenstein, M. A., van de Ven, J., Nortier, J. W., Blijham, G. H., and Thijssen, J. H., 2001, Importance of local aromatase activity in hormone-dependent breast cancer: a review. Breast, 10:91.
183. Simpson, E. R., 2000, Biology of aromatase in the mammary gland. J Mammary Gland Biol Neoplasia, 5:251.
184. Brueggemeier, R. W., J. A. Richards, and T. A. Petrel. 2003. Aromatase and cyclooxygenases: enzymes in breast cancer. J Steroid Biochem Mol Biol 86:501.
185. Cameron, D. J., and O'Brien, P., 1982, Relationship of the suppression of macrophage mediated tumor cytotoxicity in conjunction with secretion of prostaglandin from the macrophages of breast cancer patients. Int J Immunopharmacol, 4:445.
186. Sombroek, C. C., Stam, A. G., Masterson, A. J., Lougheed, S. M., Schakel, M. J., Meijer, C. J., Pinedo, H. M., van den Eertwegh, A. J., Scheper, R. J., and de Gruijl, T. D., 2002, Prostanoids play a major role in the primary tumor-induced inhibition of dendritic cell differentiation. J Immunol, 168:4333.
187. Pockaj, B. A., Basu, G. D., Pathangey, L. B., Gray, R. J., Hernandez, J. L., Gendler, S. J., and Mukherjee, P., 2004, Reduced T-cell and dendritic cell function is related to cyclooxygenase-2 overexpression and prostaglandin E2 secretion in patients with breast cancer. Ann Surg Oncol, 11:328.

188. Purohit, A., Singh, A., Ghilchik, M. W., and Reed, M. J., 1999, Inhibition of tumor necrosis factor alpha-stimulated aromatase activity by microtubule-stabilizing agents, paclitaxel and 2-methoxyestradiol. Biochem Biophys Res Commun, 261:214.
189. Schrey, M. P., and Patel, K. V., 1995, Prostaglandin E2 production and metabolism in human breast cancer cells and breast fibroblasts. Regulation by inflammatory mediators. Br J Cancer, 72:1412.
190. Szlosarek, P. W., and Balkwill, F. R., 2003, Tumour necrosis factor alpha: a potential target for the therapy of solid tumours. Lancet Oncol, 4:565.
191. Carpenter, P. M., Gatanaga, T., Nguyen, H. P., and Hiserodt, J. C., 1997, Lymphocyte and monocyte-induced motility of MCF-7 cells by tumor necrosis factor-alpha. Int J Cancer, 71:64.
192. Leek, R. D., Landers, R., Fox, S. B., Ng, F., Harris, A. L., and Lewis, C. E., 1998, Association of tumour necrosis factor alpha and its receptors with thymidine phosphorylase expression in invasive breast carcinoma. Br J Cancer ,77:2246.
193. Krajcik, R. A., Massardo, S., and Orentreich, N., 2003, No association between serum levels of tumor necrosis factor-alpha (TNF-alpha) or the soluble receptors sTNFR1 and sTNFR2 and breast cancer risk. Cancer Epidemiol Biomarkers Prev, 12:945.
194. Sabel, M. S., Skitzki, J., Stoolman, L., Egilmez, N. K., Mathiowitz, E., Bailey, N., Chang, W. J., and Chang, A. E., 2004, Intratumoral IL-12 and TNF-alpha-loaded microspheres lead to regression of breast cancer and systemic antitumor immunity. Ann Surg Oncol, 11:147.
195. de Kossodo, S., Moore, R., Gschmeissner, S., East, N., Upton, C., andBalkwill, F. R., 1995, Changes in endogenous cytokines, adhesion molecules and platelets during cytokine-induced tumour necrosis. Br J Cancer, 72:1165.
196. Budinsky, A. C., Brodowicz, T., Wiltschke, C., Czerwenka, K., Michl, I., Krainer, M., and Zielinski, C. C., 1997, Decreased expression of ICAM-1 and its induction by tumor necrosis factor on breast-cancer cells in vitro. Int J Cancer, 71:1086.
197. Basu, A., Mohanty, S., and Sun, B., 2001, Differential sensitivity of breast cancer cells to tumor necrosis factor-alpha: involvement of protein kinase C. Biochem Biophys Res Commun, 280:883.
198. Weitsman, G. E., Ravid, A., Liberman, U. A., and Koren, R., 2003, Vitamin D enhances caspase-dependent and -independent TNFalpha-induced breast cancer cell death: The role of reactive oxygen species and mitochondria. Int J Cancer, 106:178.
199. Diarra-Mehrpour, M., Arrabal, S., Jalil, A., Pinson, X., Gaudin, C., Pietu, G., Pitaval, A., Ripoche, H., Eloit, M., Dormont, D., and Chouaib, S., 2004, Prion protein prevents human breast carcinoma cell line from tumor necrosis factor alpha-induced cell death. Cancer Res, 64:719.
200. Basu, A., 1998, The involvement of novel protein kinase C isozymes in influencing sensitivity of breast cancer MCF-7 cells to tumor necrosis factor-alpha. Mol Pharmacol, 53:105.
201. Cai, Z., Capoulade, C., Moyret-Lalle, C., Amor-Gueret, M., Feunteun, J., Larsen, A. K., Paillerets, B. B., and Chouaib, S., 1997, Resistance of MCF7 human breast carcinoma cells to TNF-induced cell death is associated with loss of p53 function. Oncogene, 15:2817.
202. Burow, M. E., Weldon, C. B., Tang, Y., McLachlan, J. A., and Beckman, B. S., 2001, Oestrogen-mediated suppression of tumour necrosis factor alpha-induced apoptosis in MCF-7 cells: subversion of Bcl-2 by anti-oestrogens. J Steroid Biochem Mol Biol, 78:409.
203. Egeblad, M., and Jaattela, M., 2000, Cell death induced by TNF or serum starvation is independent of ErbB receptor signaling in MCF-7 breast carcinoma cells. Int J Cancer, 86:617.
204. Hoffmann, M., Schmidt, M., and Wels, W., 1998, Activation of EGF receptor family members suppresses the cytotoxic effects of tumor necrosis factor-alpha. Cancer Immunol Immunother, 47:167.
205. Burow, M. E., Weldon, C. B., Tang, Y., Navar, G. L., Krajewski, S., Reed, J. C., Hammond, T. G., Clejan, S., and Beckman, B. S., 1998, Differences in susceptibility to tumor necrosis factor alpha-induced apoptosis among MCF-7 breast cancer cell variants. Cancer Res, 58:4940.
206. Rozen, F., Zhang, J., and Pollak, M., 1998, Antiproliferative action of tumor necrosis factor-alpha on MCF-7 breastcancer cells is associated with increased insulin-like growth factor binding protein-3 accumulation. Int J Oncol, 13:865.
207. Amin, F., Bowen, I. D., Szegedi, Z., Mihalik, R., and Szende, B., 2000, Apoptotic and non-apoptotic modes of programmed cell death in MCF-7 human breast carcinoma cells. Cell Biol Int, 24:253.
208. Burow, M. E., Tang, Y., Collins-Burow, B. M., Krajewski, S., Reed, J. C., McLachlan, J. A., and Beckman, B. S., 1999, Effects of environmental estrogens on tumor necrosis factor alpha-mediated apoptosis in MCF-7 cells. Carcinogenesis, 20:2057.
209. Sheen-Chen, S. M., Chen, W. J., Eng, H. L., and Chou, F. F., 1997, Serum concentration of tumor necrosis factor in patients with breast cancer. Breast Cancer Res Treat, 43:211.
210. Varela, L. M., Stangle-Castor, N. C., Shoemaker, S. F., Shea-Eaton, W. K., and Ip, M. M., 2001, TNFalpha induces NFkappaB/p50 in association with the growth and morphogenesis of normal and transformed rat mammary epithelial cells. J Cell Physiol, 188:120.

211. Purohit, A., Newman, S. P., and Reed, M. J., 2002, The role of cytokines in regulating estrogen synthesis: implications for the etiology of breast cancer. Breast Cancer Res, 4:65.
212. Vaday, G. G., Hershkoviz, R., Rahat, M. A., Lahat, N., Cahalon, L., and Lider, O., 2000, Fibronectin-bound TNF-alpha stimulates monocyte matrix metalloproteinase-9 expression and regulates chemotaxis. J Leukoc Biol, 68:737.
213. Chiu, J. J., Sgagias, M. K., and Cowan, K. H., 1996, Interleukin 6 acts as a paracrine growth factor in human mammary carcinoma cell lines. Clin Cancer Res, 2:215.
214. Fontanini, G., Campani, D., Roncella, M., Cecchetti, D., Calvo, S., Toniolo, A., and Basolo, F., 1999, Expression of interleukin 6 (IL-6) correlates with oestrogen receptor in human breast carcinoma. Br J Cancer 80:579.
215. Karczewska, A., Nawrocki, S., Breborowicz, D. Filas, V., and Mackiewicz, A., 2000, Expression of interleukin-6, interleukin-6 receptor, and glycoprotein 130 correlates with good prognoses for patients with breast carcinoma. Cancer, 88:2061.
216. Asgeirsson, K. S., Olafsdottir, K., Jonasson, J. G., and Ogmundsdottir, H. M., 1998, The effects of IL-6 on cell adhesion and e-cadherin expression in breast cancer. Cytokine, 10:720.
217. Krueger, J., Ray, A., Tamm, I., and Sehgal, P. B., 1991, Expression and function of interleukin-6 in epithelial cells. J Cell Biochem, 45:327.
218. Sehgal, P. B., and Tamm, I., 1991, Interleukin-6 enhances motility of breast carcinoma cells. Exs, 59:178.
219. Salgado, R., Junius, S., Benoy, I., Van Dam, P., Vermeulen, P., Van Marck, E., Huget, P., and Dirix, L. Y., 2003, Circulating interleukin-6 predicts survival in patients with metastatic breast cancer. Int J Cancer, 103:642.
220. Bachelot, T., Ray-Coquard, I., Menetrier-Caux, C., Rastkha, M., Duc, A., and Blay, J. Y., 2003, Prognostic value of serum levels of interleukin 6 and of serum and plasma levels of vascular endothelial growth factor in hormone-refractory metastatic breast cancer patients. Br J Cancer, 88:1721.
221. Benoy, I., Salgado, R., Colpaert, C., Weytjens, R., Vermeulen, P. B., and Dirix, L. Y., 2002, Serum interleukin 6, plasma VEGF, serum VEGF, and VEGF platelet load in breast cancer patients. Clin Breast Cancer, 2:311.
222. Speirs, V., Walton, D. S., Hall, M. C., and Atkin, S. L., 1999, In vivo and in vitro expression of steroid-converting enzymes in human breast tumours: associations with interleukin-6. Br J Cancer, 81:690.
223. Jiang, X. P., Yang, D. C., Elliott, R. L., and Head, J. F., 2000, Reduction in serum IL-6 after vacination of breast cancer patients with tumour-associated antigens is related to estrogen receptor status. Cytokine, 12:458.
224. DeMichele, A., Martin, A. M., Mick, R., Gor, P., Wray, L., Klein-Cabral, M., Athanasiadis, G., Colligan, T., Stadtmauer, E., and Weber, B., 2003, Interleukin-6 -174G-->C polymorphism is associated with improved outcome in high-risk breast cancer. Cancer Res, 63:8051.
225. Iacopetta, B., Grieu, F., and Joseph, D., 2004, The -174 G/C gene polymorphism in interleukin-6 is associated with an aggressive breast cancer phenotype. Br J Cancer, 90:419.
226. Conze, D., Weiss, L., Regen, P. S., Bhushan, A., Weaver, D., Johnson, P., and Rincon, M., 2001, Autocrine production of interleukin 6 causes multidrug resistance in breast cancer cells. Cancer Res, 61:8851.
227. Di Carlo, E., Modesti, A., Castrilli, G., Landuzzi, L., Allione, A., de Giovanni, C., Musso, T., and Musiani, P., 1997, Interleukin 6 gene-transfected mouse mammary adenocarcinoma: tumour cell growth and metastatic potential. J Pathol, 182:76.
228. Purohit, A., Singh, A., Ghilchik, M. W., Serlupi-Crescenzi, O., and Reed, M. J., 2003, Inhibition of IL-6+IL-6 soluble receptor-stimulated aromatase activity by the IL-6 antagonist, Sant 7, in breast tissue-derived fibroblasts. Br J Cancer, 88:630.
229. Utsumi, K., Takai, Y., Tada, T., Ohzeki, S., Fujiwara, H., and Hamaoka, T., 1990, Enhanced production of IL-6 in tumor-bearing mice and determination of cells responsible for its augmented production. J Immunol, 145:397.

Chapter 12

Tumor and Host Endothelial Cell Selective Interactions and Modulation by Microenvironmental Chemokines: Tumor-Endothelial Cell Cross Talk Specificity

N. Lamerant-Fayel, C. Crola da Silva and C. Kieda
Centre de Biophysique Moléculaire, UPR 4301 CNRS, Glycobiology of Cell Recognition, 45071, Orléans, France

Abstract: The variability and adaptability of tumor cells allow their tissue escape despite the efficient body defence. Tumor cells use vessels 1) for nutrition 2) for routing through blood and lymph circulation 3) to invade selected secondary sites. The molecular mechanisms that regulate both 1) endothelial cell growth under tumor induction and 2) reciprocal recognition between endothelial cells and blood borne tumor cells are crucial to elucidate..Tumor cells use normal cells properties to be recognized by normal endothelial cells but endothelium is highly organo specific; it is further modulated by the microenvironmental factors, as it reflects the biological state. This will be particularly illustrated by the adhesion/invasion molecules and the early signals of recruitment as achieved by the local expression and the presentation's ways of chemokines to actively attract circulating partners. Refined cell and molecular interactions are insured by sugar-mediated recognitions since glycosylation is the post-translational transformation that confers to glycoconjugates their structural specific identity as recognitions signals. Advantage could be taken of such specific characters as a way to design targeted anti tumor therapy strategies.

Key words: Chemokine, endothelial cell, organospecificity, homing, cancer, cell recognition, glycoconjugates, angiogenesis

1. INTRODUCTION

Vascular endothelial cells (ECs) play a key role in numerous physiological processes as lymphocyte trafficking, angiogenesis but also in pathological processes as inflammation (1), autoimmune diseases (2) or metastasis (3). Tumour cell migration and metastasis share many similarities with leukocyte trafficking: blood circulating cells are recognised by endothelial cells which allow them to enter in the surrounding tissue; this is achieved in a tissue-specific manner. For example, naive T cells extravasate better in peripheral lymph nodes and naive B cells, in Peyer's patches (4). In the same way, metastasis is not a random process and some tumors show specific metastatic sites (5). Breast cancer secondary localisation favours regional lymph nodes, bone marrow, lung and liver. Malignant melanoma has a similar pattern but also has a high incidence in skin metastasis. Prostate cancer metastases also favour the bone marrow.

A better understanding of the mechanisms which control the organo-selectivity would give keys to immunotherapy (6, 7), targeted drug therapy (8) and cell therapy designs.

Although all vascular endothelial cells share certain common functions, it has become clear that considerable heterogeneity exists both structurally and functionally (9, 10) along the length of the

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 219-231.

vascular tree and in the microvascular beds of various organs (11-14). The structural heterogeneity of ECs is a perfect example of their adaptation to the unique demands of the surrounding tissue (15). ECs can form a tight continuous monolayer in organs such as the brain or the lungs, where they perform important barrier functions. Alternatively, they can form a discontinuous layer with intercellular gaps or fenestrae in organs such as kidney, spleen, or bone marrow, where rapid exchange of fluid, particles, and cells takes place. According to this, secondary lymphoid organs (with the exception of spleen) have high endothelial cells (HECs) walled venules that are specialized postcapillaries supporting high levels of lymphocyte extravasation from the blood (16).

ECs from diverse tissues are also heterogeneous with respect to their surface phenotype and protein expression (13, 17-19). However, one of the clearest examples of EC heterogeneity lies in the expression of homing receptors involved in cell trafficking. as these properties are used in a comparable way in pathological situations and, in particular, for the cancer cells dissemination. This chapter will review the normal homing selectivity confered by the endothelium organospecificity and its microenvironment dependency provided by the chemokine modulation, presentation and activities.

2. THE MULTIPLE STEPS OF THE HOMING PROCESS COOPERATE TO PROVIDE SPECIFICITY

Successful defense of the body against foreign invasion is critically dependent on the presence of lymphocyte clones with the right antigen specificity at the right position at the right time. For this, naive and mature T and B cells recirculate continuously through the body, going from blood to tissue and back to the bloodstream again (20). In contrast to naive lymphocytes, memory effector lymphocytes can access to non-lymphoid effector sites and display restricted, often tissue-selective, migration behaviour (21). Distinct subsets of memory-effector cells exist with tissue-selective patterns of migration (22, 23). The recirculation is guided by lymphocyte-endothelial cells recognition and subsequent diapedesis, directing lymphocyte homing (4, 21, 24). Thus, lymphocyte- endothelial cells interaction is a central regulatory point in the immune system, controlling the access of specialized lymphocyte subsets to particular tissues and influencing the nature of local immune and inflammatory responses. At the molecular level, this process is regulated by spatio-temporarily expressed adhesion molecules (25), present on both circulating leukocytes and on endothelial cells and in concert with chemokines.

A multi-step model of leukocyte adhesion to vascular endothelium was first provided (21, 26, 27) and is broadly applicable in different tissues, although the details of the signals involved differ. Recirculation begins with blood lymphocytes interacting transiently and reversibly with the vascular endothelium through villous-expressed adhesion receptors in a process called rolling. These receptors can be either selectins (28), which interact with carbohydrate epitopes of addressins, or, less commonly, members of the immunoglobulin (Ig) superfamily, which bind leukocyte integrins (29-31). Rolling brings lymphocytes into contact with the endothelium where they can sample the surface for activating factors. Activating factors (often chemokines for lymphocytes) bind to specific G protein-coupled receptors on the rolling lymphocytes (32,33) and trigger rapid activation of integrin receptors on the leukocyte surface. These activated integrins promote the leukocytes arrest and firm adhesion by binding their Ig-family ligands on the endothelium (34, 35). In the presence of the appropriate migratory signals, the leukocyte will then migrate across the endothelium into tissue, where tissue-associated chemokine gradients may also direct localisation.

As illustrated by the following examples, the homing organo-selectivity is due to the expression of specific molecules.

2.1 Homing organo-selectivity

2.1.1 Adhesion molecules, selectins and addressins

For blood lymphocytes early interaction with the vascular endothelium, a decisive contact between circulating cell and microvascular endothelial cell, is described to be mediated by both inducible E-selectin and P-selectin on the endothelial cells (36-43) and L-selectin on circulating cell (44).

Selectins. E-selectin and P-selectin are quite generally distributed among endothelial cells and consequently can participate to a regio selective reaction through the modulation of their degree and time of expression. This prompted the search for distinctive signals that were evidenced by help of an *in vitro* model for endothelial cell comparisons (9, 45).

A particular E-selectin ligand is the cutaneous lymphocyte associated antigen (CLA) (43, 46). The CLA ligand defines a skin-associated population of memory T cells that preferentially extravasate at normal (47) and chronically inflamed cutaneous sites (48) and has been shown to be involved in local immunity and inflammatory cutaneous reactions (49). The first evidence that a skin-homing subset exists came with the observation that a majority (40–90%) of the T lymphocytes in normal and inflammatory skin lesions express the CLA (47, 48, 50) In contrast, CLA T cells constitute only 10–15% of the pool of circulating T cells and never exceed 5% of lymphocytes within non-cutaneous inflamed sites (48, 51) CLA is a carbohydrate antigen that is closely related to the sialyl Lewis x antigen (sLex) (4, 48, 52) Interestingly, mycosis fungoides and other subtypes of cutaneous lymphomas express CLA (48, 53, 54) but not the mucosal homing receptor $\alpha 4\beta 7$. The highly selective expression of CLA on cutaneous T-cell lymphomas suggests that CLA mediates the skin-specific dissemination of these lymphomas in vivo. However, CLA E-selectin binding cannot fully explain the skin-specific homing of CLA memory T cells. Neutrophils express CLA (55) but they do not preferentially migrate to skin and E-selectin is induced on inflamed endothelium in both cutaneous and non-cutaneous sites (28). As we will see later, another step in the homing cascade is necessary to reach the specificity of skin homing and implies the chemokines CTACK and TARC.

Specific recognitions due to the leukocyte L-selectin (56-58) gets to be regio-selective despite its large distribution. This is attributed to its ligands (vascular addressins) of which expression is restricted among endothelial cells according to the tissues and the vessels. Addressins are mucin-type or mixed mucin/immunoglobulin-type glycoproteins (59-64).

Addressins. These molecules provide the regio-selectivity to selectin recognition because they are differentially expressed on the endothelium, depending upon its tissue origin (62, 65-67) Furthermore, their structure is highly modulated by the microenvironnement at the post-translational level (15). To become high affinity L-selectin ligands, they must undergo proper glycosylation, especially the most common sugar epitopes recognized by L-selectin : sialyl Lewis x (CD15s) and its sulphated form, presented by appropriate mucin-type proteins (GlyCAM-1, MadCAM-1 or CD34). In the mouse, typical peripheral lymph node addressins (PNAds) are GlyCAM-1 (66, 68), CD34 and Sgp200 (sulphated glycoprotein of 200 kD) (25, 66, 69). Typical mucosal addressin is MAdCAM-1, which has been found first in mouse (15, 25, 70 71) and further in human endothelial cells (72). MAdCAM-1 is almost completely confined to mucosal and related vessels and is thus one of the few examples of endothelial adhesion molecules that are restricted to a particular tissue. In addition to being recognized by L-selectin through its sialomucin sugar residues (60, 72, 73), MAdCAM-1 molecule possesses an immunoglobulin-like domain which interacts with the $\alpha 4\beta 7$-integrin homing receptor of lymphocytes (30) and thus can mediate both rolling and stable adhesion. Consequently, lymphocytes homing to gut is critically dependent on interaction between the lymphocyte $\alpha 4\beta 7$-integrin and its receptor MAdCAM-1 expressed on mucosal vessels (30). Lymphocytes interactions with MAdCAM-1 are particularly complex,

because they depend upon its glycosylation state. Thus, Peyer's patches HEC- MAdCAM-1 is decorated with unique oligosaccharide determinants that allow it to serve as a ligand for L-selectin and thereby to mediate rolling of naive lymphocytes. In contrast, in the lamina propria, initial rolling interactions between activated lymphocytes and endothelial cells are apparently mediated by binding of the α4β7-integrin to immunoglobulin-like domains of MAdCAM-1. At both locations, α4β7-integrin-MAdCAM1 interaction and possibly LFA-1 secure firm lymphocyte adhesion. The predominant role proposed for α4β7-integrin in mucosal homing has gained further support from the observation that cell trafficking to mucosal lymphatic tissues is dramatically reduced in β7-knockout animals (74). Moreover, gastrointestinal T-cell lymphomas express the mucosal homing receptor α4β7 (75) but not CLA.

2.1.2 Chemokines

In the second step of the cascade, chemokines and their receptors also help to control the homing specificity. The chemokines (*chemo*tactic cyto*kines*) are a large and growing family of nearly 50 6–14-kD proteins that mediate a wide range of biological functions (76). The chemokines are divided into four families **(CXC, CC, C and CX_3C)** based on the position of four cysteine residues that form two disulfide bonds. Chemokine receptors are consequently divided into four families based on the type of chemokine they bind, although no clear structural differences could be identified that would distinguish the receptor subfamilies (77). Chemokines play a vital role in leukocyte adhesion and extravasation. In various in vitro assays, chemokines support the chemotaxis or transendothelial migration of leukocytes (76), while *in vivo* injection (78) or over-expression of chemokines (79) results in leukocyte accumulation at the site of chemokine expression. Antagonists of chemokines prevent leukocyte trafficking (80) and have beneficial effects in several acute and chronic inflammatory models (81, 82). Chemokines also modulate angiogenesis (83), hematopoiesis (76), and T lymphocyte activation (84, 85), and several act as co-receptors with CD4 for entry of M-tropic and T-tropic HIV-1 (86, 87).

Interestingly, apart of their receptors, chemokines also interact with **glycosaminoglycans (GAGs)** on cell surfaces. Chemokines have been shown to bind to purified subfractions of heparin in vitro (88), as well as to naturally occurring GAGs such as heparan sulfate and chondroitin sulfate on the surface of endothelial cells both *in vitro* (89-91) and *in vivo* (92). The ability of chemokines to bind to GAGs is thought to be critical for chemokine biology. It has been proposed that the immobilization of chemokines by GAGs forms stable, solid-phase chemokine foci and gradients necessary to direct leukocyte trafficking *in vivo* (93, 94). Binding of chemokines to cell surface GAGs may also serve to increase their effective local concentration, and consequently increase their binding to cell surface receptors (91). Additionally, GAG binding could potentially influence chemokine *t*1/2 in vivo. Chemokines display specific affinity according to GAGs families (88), consequently, a detailed understanding of chemokine-GAG interactions may be critical to appreciate functional distinctions among chemokines.

Recent studies have shown that certain constitutively expressed chemokines play, in conjunction with adhesion receptors, an important role in regulating homeostatic lymphocyte recirculation through secondary lymphoid organs, whereas others may help control tissue-specific targeting of lymphocytes to extralymphoid organs (34, 95-100).

3. CHEMOKINES AS REGIO-SPECIFIC CHEMOATTRACTANTS

3.1.1 The CC chemokines and their receptors interplay

One example is the ***secondary lymphoid-tissue chemokine*** **SLC, (6-CKine, CCL21)** which is expressed in the high endothelial venules (HEV) of

lymph nodes and Peyer's patches, in the T cell areas of spleen, lymph nodes, and Peyer's patches, and in the lymphatic endothelium of multiple organs (101, 102). SLC acts through the receptor **CCR7** (103). SLC is a highly efficacious chemoattractant for lymphocytes with preferential activity toward naive T cells (101). Moreover, SLC induces firm adhesion of naive T lymphocytes via β2-integrin binding to the counter receptor, intercellular adhesion molecule-1 (ICAM-1), a necessary step for lymphocyte recruitment. The only other molecule that has been demonstrated to activate lymphocyte β2 integrin is GlyCAM-1, the L-selectin ligand expressed by endothelial cells in HEVs and secreted (104). It is possible that SLC and GlyCAM-1 act cooperatively to stimulate the firm adhesion of lymphocytes to HEV. The importance of SLC in T cell homing to secondary lymphoid organs is supported by the relative absence of T cells in the lymph nodes and Peyer's patches of *plt* mice (105) a spontaneous mutant strain deficient in the SLC gene expressed in high endothelium (106, 107). Interestingly, when SLC is injected subcutaneously into *plt* mice, afferent lymph apparently carries it into the draining node, where it is ultimately presented on the luminal surface of HEVs. In such reconstituted *plt* mice, the ability of T cells to adhere to HEVs and enter the organ is restored (96). Additionally, T cells homing *in vivo* in normal mice adhere almost exclusively to HEV sites presenting SLC protein (95).

Moreover as described in the later paragraphs, this chemokine is highly significant in cancer because CCR7 and its ligands CCL21 and CCL19 were shown recently to be involved in the metastasis process (108-110).

Another ***tissue-selective chemokine is the thymus-expressed chemokine*** **–TECK (CCL25).** TECK is highly expressed in the small intestine (111-113) but not in other epithelia such as the digestive tract (including stomach and colon), skin, lung, or salivary gland (98).The TECK receptor, CCR9 is expressed by discrete subsets of circulating memory $CD4^+$ and $CD8^+$ lymphocytes expressing the intestinal homing receptor α4β7, but not by other systemic memory lymphocyte subsets (111). This suggesting that CCR9 may be a receptor used preferentially by lymphocytes involved in aspects of gut immunity. Moreover, CCR9 is expressed on lymphocytes isolated from human small intestine (111). Together, this suggests a role for TECK and CCR9 in the intestinal immune compartment, but the extent to which this chemokine receptor–ligand pair is specifically associated with the intestinal versus other mucosal and epithelial sites remains to be deeper explored.

In the skin, two different chemokines play a critical part in homing specificity, ***Thymus and activation-regulated chemokine*** **TARC (CCL17)** and ***Cutaneous T cell–attracting chemokine*** **CTACK (CCL27)**. The CC-chemokine receptor **CCR4** on T lymphocytes and its ligand, TARC are implicated in lymphocyte–endothelial cells interactions during lymphocyte recruitment to normal and inflamed cutaneous sites (99). Immunohistochemistry suggests that TARC is constitutively expressed and inducible on cutaneous venules and some other systemic venules, but is absent from vessels at sites of lymphocyte recruitment into the intestines. Moreover, its receptor CCR4 is highly expressed by CLA^+ circulating skin memory lymphocytes, but not by the other memory cells, consistent with a role in tissue selective trafficking. In addition to lymphocyte arrest on endothelium, CCR4 supports diapedesis and chemotaxis.

The CTACK chemokine is constitutively expressed by skin keratinocytes, also attracts cutaneous memory T cells (100). CTACK binds to the receptor CCR10 which is also specifically expressed on circulating skin-homing CLA^+ T lymphocytes. CTACK may be transcytosed and presented on the endothelium (92). CTACK can be induced by the proinflammatory cytokines TNF-α and IL-1β. CTACK and TARC can both support homing of T cells to skin, and either one or the other is required for lymphocyte recruitment in cutaneous delayed type hypersensitivity (114).

Malignancies (see below) as melanoma, which has a similar metastatic pattern as breast cancer but also a high incidence of skin metastases, show high expression levels of CCR10 in addition to CXCR4 and CCR7 (109).

The **mucosa-associated epithelial chemokine MEC (CCL28)** seems to have an important role in lymphocyte homing in extracutaneous epithelial tissues, including diverse mucosal organs. MEC mRNA is most abundant in salivary gland, with strong expression in other mucosal sites (colon, trachea, and mammary gland). Constitutively expressed by epithelial cells, MEC supports lymphocyte chemotaxis through CCR10 (97, 115), a known CTACK receptor but also through CCR3 (97).

Consequently, CC chemokines are a family of locally produced molecules that together with their receptors expression and modulation and due to the various presentations modes they use to anchor themselves to the cell surface can participate to define local specific characteristics.

3.2 Fractalkine (CX3CL1)

Compared to other chemokines, fractalkine has at least three unique features that make it to function as a cell adhesion molecule : unique CX3C chemokine to date, its three-dimensional structure differs from other chemokines (116), it is a transmembrane molecule with a cytoplasmic tail that may transduce signal and has a mucin domain. Fractalkine and its receptor CX3CR1 are currently the only known members of the CX3C family (117-120).

Only **CXCL16** (121) and fractalkine are membrane-anchored protein. The chemokine module is attached to the cell membrane via a mucin-like stalk as a presentation vehicle (122) which extends the CX3C chemokine domain to present it to flowing leukocytes.

Fractalkine induces chemotaxis of monocytes, T cells and natural killer cells (123) where the CX3CR1 receptor is expressed (119, 120). Fractalkine and CX3CR1 function as cell adhesion molecules under both static and dynamic conditions (120, 123).

Unlike other chemokine/G-protein coupled receptor interactions that require signal transduction and integrin activation for cell adhesion to occur, the adhesive interaction between fractalkine and CX3CR1 is independent of signal transduction or integrin function (123, 124). Therefore, ***fractalkine and CX3CR1 provide an integrin-independent mechanism for leukocyte migration***.

Constitutively expressed in several lymphoid or peripheral tissues, mainly by endothelial cells (125-127) it is mainly induced in reactive lymph nodes in the high endothelial venule (HEV) cells, dendritic cells (DCs), follicular DCs, and a few germinal center lymphocytes (127). Fractalkine was expressed also in the skin (128) and the brain (129) where it participate to the intercellular communication.

Fractalkine mediates natural killer-dependent **antitumor** responses in vivo (130) and induces T-cell-dependent antitumor immunity through chemoattraction and activation of *in vivo* dendritic cells (131) (see below).

4. CHEMOKINE-MEDIATED ENDOTHELIUM ACTIVATION PARTICIPATE TO TUMOR CELL DISSEMINATION

4.1 Angiogenic response

The formation of vascular system is controlled by vasculogenesis and angiogenesis. Vasculogenesis is the vessel formation from angioblast , the progenitor of endothelial cell lineage (132). Angiogenesis is the development of a vascular system which requires degradation of basement membrane by proteases and invasion of the surrounding extracellular matrix by proliferation, migration and differentiation of ECs (133). In normal conditions, angiogenesis is a restricted phenomenon such as wound healing and menstruation. This process depends on the balance of proangiogenic and antiangiogenic factors (134, 135). If the regulation of this balance happens to be disrupted, angiogenesis can become a pathological process as it appears in cancers and/or other diseases (136).

Some chemokines are angiogenic factors for ECs. Together with their receptors, chemokines regulate angiogenesis in physiopathological

processes (137) such as cancer progression (138), atherosclerosis (139) and ischemia. Tumoral cells produce angiogenic factors like VEGF (140, 141) which is able to activate ECs by VEGF-receptor (142) to induce angiogenesis. The formation of new vessels to bring oxygen and nutrients is required for tumor progression. Chemokines and their receptors act in cancer progression at several levels: by the regulation of tumor invasion and cancer metastasis, by activation/suppression of a host tumor-specific immunological response, by the stimulation of tumor cell proliferation in an autocrine fashion and by regulation of tumor-associated angiogenesis.

4.2 Chemokine/chemokine receptors are determinants for tumor invasion and cancer metastasis

The angiogenesis/metastasis relationship and the importance of the balance of angiostatic/angiogenic factors were well documented (115, 143, 144).

In lymphocyte homing, chemokines partly control the good destination and non-random recirculation of lymphocytes (145). Tumor cells also use chemokines and their receptors to determine metastatic destination. The **CXCR4 and CCR7 receptors** are involved in breast cancer metastasis (109). Their respective ligands, **CXCL12/SDF-1α (Stromal derived factor 1α)** and **CCL21/SLC** are highly expressed in organs representing the main destinations of breast cancer metastases CCR7 and its ligands CCL21 and CCL19/MIP-3β were shown recently to be involved in metastasis (108-110). The chemokine receptor CCR7 was found highly expressed in human breast cancer cells, malignant breast tumours and metastases (109). Breast cancer is characterized by a distinct metastatic pattern involving the regional lymph nodes, bone marrow, lung and liver. The CCR7 ligands, MIP-3β and SLC, exhibit peak levels of expression in organs representing the first destinations of breast cancer metastasis. Wiley et al., (110) showed that expression of CCR7 enhanced metastasis of murine melanoma cells to draining lymph nodes in mouse models and that inhibition of SLC blocked the metastasis to draining lymph nodes. Malignant melanoma, which have a similar metastatic pattern as breast cancer but also a high incidence of skin metastases, show high expression levels of CCR10 in addition to CXCR4 and CCR7 (109).

The **IL8** chemokine is up-regulated by inflammatory stimuli or by reduced microenvironmental oxygen pressure in glioblastomas and modulate different metastatic phenotypes associated with metastasis progression of colon carcinoma (146). The CXCR4 receptor is up-regulated by microenvironment of colon carcinoma and participate to proliferation and migration of micrometastases (147).

Taking into account their organospecificity (9, 148), ECs express distinct chemokine receptors according to the line or organ origin (149). The combination of chemokine/chemokine receptors distribution, and cross-talk could in part explain the organo-specificity and open new ways for antitumor therapy.

4.3 Chemokines act as autocrine tumor growth factors

Some chemokines are able to stimulate proliferation of tumor cells by binding to cell surface specific receptors. IL8 is an autocrine factor in numerous types of cancers: malignant melanoma (150) colon carcinoma (151) neuroblastoma (152). It was shown *in vitro* that the CXC ***chemokine Growth regulated-oncogene*** **GRO** is mitogenic for malignant melanoma cell lines (153, 154). Furthermore, they can act as angiogenic factors interacting with specific receptors on ECs or by recruiting cells that produce angiogenic and mitogenic factors.

4.4 Chemokines in tumor angiogenesis

The presence of physiologic and pathological angiogenesis is usually associated with inflammatory cell infiltrate. The relationship between angiogenesis and leukocyte infiltration in tumor growth, cardiovascular disease, chronic inflammation and wound healing is largely

documented. The coordination of angiogenesis and inflammation is achieved by the ability of both ECs and leukocytes to respond to chemokines.

Chemokines are especially expressed by ECs in a timely and spatially restricted fashion (145) in order to regulate the recruitment of specific leukocytes and cell homing. Moreover, ECs express chemokine receptors. The role of a specific chemokine/chemokine receptor couple in the modulation of angiogenesis has been illustrated (155).

Among the chemokine families, some CXC chemokines are identified as regulators of angiogenesis, acting either as angiogenic or angiostatic factors (156). It seems that the presence of ELR motif correlates with an angiogenic activity (157, 158). The CXC chemokines are divided in two groups: ELR+ and ELR- (Glu-Leu-Arg). The involvement of this motif in angiogenesis was shown by mutagenesis experiments with an ELR+ chemokine, IL8 and an ELR- chemokine, Mig.

The Pro-angiogenic chemokines:

The CXC chemokines:

- -ELR+ chemokines. IL, was the first chemokine shown to stimulate endothelial cell chemotaxis, proliferation and *in vivo* angiogenesis (159).
- -ELR– chemokines. SDF-1α (CXCL12) and GRO-β (CXCL2) (160) are the few exceptions which possess angiogenic activity without the ELR motif.

The CC chemokines. CCL2 (MCP-1) is a positive mediator of angiogenesis and can directly contribute to tumor progression (161).

The CX3C chemokines. Fractalkine has a positive effect on angiogenesis in rheumatoid arthritis (162).

The anti-angiogenic chemokines:

The IP-10, Mig, PF4 chemokines inhibit endothelial cell proliferation and angiogenesis, by specific inhibition of growth factor-stimulated endothelial cell proliferation (163). SLC has an anti-tumoral effect by its angiostatic activity and chemoattraction of $CD8^+$ T and NK cells.

Hypoxia is a prevalent parameter in many tumors, it contributes to aggressiveness and is one of the major reasons for treatment failure. In the hypoxic tumoral environment, cells must adapt a strategy to activate expression of genes involved in oxygen delivery (angiogenesis) and metabolic adaptation. The cellular response to hypoxia is controlled in part by the hypoxia inducible factor-1 (HIF-1) transcription factor (164) so this factor is an important target for cancer chemotherapy (165). In this environment, the balance of angiogenic and angiostatic factors is disturbed.

Chemokine receptors and chemokines act in order to restore locally angiogenesis because low oxygen level induces high expression of the receptors.

This was described for CXCL12 receptor: CXCR4 (166) and RANTES contribution to the growth of murine mammary carcinoma (167).

5. CONCLUSION: PERSPECTIVES FOR CANCER THERAPY DESIGNS

5.1 Activation/suppression of tumor-specific immunological response

Immunotherapy research is developing of tumor vaccines. Some chemokines, especially from the CC family are able to activate a tumor-specific immune response because of their chemotactic properties towards monocytes, dendritic cells and T-cells, the specific cells of immunity. Immuno–modulating factors produced in the microenvironment of a tumor could potentially determine whether or not an immune response can take place and what kind of immune cells will be recruited. Dendritic cells according to their maturation state, respond to different chemokines (168). So, the local production of one chemokine can influence the class of the immune response initiated as for example, CCL21/SLC was shown to mediate T-cell-dependent antitumor responses *in vivo* (169).

5.2 Inhibition of angiogenesis

A major focus of angiogenesis research has been the control of tumor growth in order to develop new therapeutic tools (170). One strategy consists of asphyxiating the tumor by inhibiting the role of angiogenic factors. Some reviews discuss applications of inhibitors of angiogenesis (171,172). An anti-cancer therapy is illustrated by the anti-tumor effect of some chemokines , acting as anti-angiogenic factors and attracting immune effector cells to the tumor site such as SLC-ELC (173,174).

5.3 Block interaction chemokine-chemokine receptor

A way is to inhibit the interaction of a chemokine receptor with its ligand with antibody or antagonist peptides. As such, CXCR4 antagonists inhibit primary brain tumor growth (175). Recent cancer immunotherapy uses cytokine-and chemokine–based gene therapy (176). Chemokine-cytokine fusion proteins are new therapeutic tools, designed to suppress growth and dissemination of metastases (177, 178).

REFERENCES

1. Duijvestijn, A. M., Kerkhove, M., Bargatze, R. F., and Butcher, E. C., 1987, J Immunol, 138:713-719.
2. Jalkanen, S., Steere, A. C., Fox, R. I., and Butcher, E. C., 1986, Science, 233:556-558.
3. Sher, B. T., Bargatze, R., Holzmann, B., Gallatin, W. M., Matthews, D., Wu, N., Picker, L., Butcher, E. C., and Weissman, I. L., 1988, Adv Cancer Res, 51:361-390.
4. Picker, L. J., and Butcher, E. C., 1992, Annu Rev Immunol, 10:561-591.
5. Nicolson, G. L., 1993, Cancer Metastasis Rev, 12:325-343.
6. Li, Y., Wang, M. N., Li, H., King, K. D., Bassi, R., Sun, H., Santiago, A., Hooper, A. T., Bohlen, P., and Hicklin, D. J., 2002, J Exp Med, 195:1575-1584.
7. Wei, Y. Q., Wang, Q. R., Zhao, X., Yang, L., Tian, L., Lu, Y., Kang, B., Lu, C. J., Huang, M. J., Lou, Y. Y., Xiao, F., He, Q. M., Shu, J. M., Xie, X. J., Mao, Y. Q., Lei, S., Luo, F., Zhou, L. Q., Liu, C. E., Zhou, H., Jiang, Y., Peng, F., Yuan, L. P., Li, Q., Wu, Y., and Liu, J. Y., 2000, Nat Med, 6:1160-1166.
8. Staroselsky, A. N., Mahlin, T., Savion, N., Klein, O., Nordenberg, J., Donin, N., Michowitz, M., and Leibovici, J., 1996, J Exp Ther Oncol, 1:251-259.
9. Kieda, C., Paprocka, M., Krawczenko, A., Zalecki, P., Dupuis, P., Monsigny, M., Radzikowski, C., and Dus, D., 2002, Endothelium, 9:247-261.
10. Bizouarne, N., Mitterrand, M., Monsigny, M., and Kieda, C., 1993, Biol Cell, 79:27-35.
11. Simionescu, M., Simionescu, N., and Palade, G. E., 1975, J Cell Biol, 67:863-885.
12. Risau, W., 1995, Faseb J, 9:926-933.
13. Garlanda, C., and Dejana, E., 1997, Arterioscler Thromb Vasc Biol 17:1193-1202.
14. Cines, D. B., Pollak, E. S., Buck, C. A., Loscalzo, J., Zimmerman, G. A., McEver, R. P., Pober, J. S., Wick, T. M., Konkle, B. A., Schwartz, B. S., Barnathan, E. S., McCrae, K. R., Hug, B. A., Schmidt, A. M., and Stern, D. M., 1998, Blood, 91:3527-3561.
15. Denis, V., Dupuis, P., Bizouarne, N., de, O. S. S., Hong, L., Lebret, M., Monsigny, M., Nakache, M., and Kieda, C., 1996, J Leukoc Biol, 60:744-752.
16. Girard, J. P., and Springer, T. A., 1995, Immunol Today, 16:449-457.
17. Augustin, H. G., Kozian, D. H., and Johnson, R. C., 1994, Bioessays, 16:901-906.
18. Rajotte, D., Arap, W., Hagedorn, M., Koivunen, E., Pasqualini, R., and Ruoslahti, E., 1998, J Clin Invest, 102:430-437.
19. Pasqualini, R., and Ruoslahti, E., 1996, Nature, 380:364-366.
20. Gowans, J., and Knight, E., 1964, Proc R Soc Lond B Biol Sci, 159:257-282.
21. Butcher, E. C., and Picker, L. J., 1996, Science, 272:60-66.
22. Picker, L. J., Martin, R. J., Trumble, A., Newman, L. S., Collins, P. A., Bergstresser, P. R., and Leung, D. Y., 1994, Eur J Immunol, 24:1269-1277.
23. Picker, L. J., 1994, Curr Opin Immunol, 6:394-406.
24. Salmi, M., and Jalkanen, S., 1997, Adv Immunol, 64:139-218.
25. Berg, E. L., Goldstein, L. A., Jutila, M. A., Nakache, M., Picker, L. J., Streeter, P. R., Wu, N. W., Zhou, D., and Butcher, E. C., 1989, Immunol Rev, 108:5-18.
26. Adams, D. H., and Shaw, S., 1994, Lancet, 343:831-836.
27. Springer, T. A., 1994, Cell, 76:301-314.
28. Tedder, T. F., Steeber, D. A., Chen, A., and Engel, P., 1995, Faseb J, 9:866-873.
29. Alon, R., Kassner, P. D., Carr, M. W., Finger, E. B., Hemler, M. E., and Springer, T. A., 1995, J Cell Biol, 128:1243-1253.

30. Berlin, C., Bargatze, R. F., Campbell, J. J., von Andrian, U. H., Szabo, M. C., Hasslen, S. R., Nelson, R. D., Berg, E. L., Erlandsen, S. L., and Butcher, E. C., 1995, Cell, 80:413-422.
31. Lalor, P. F., Clements, J. M., Pigott, R., Humphries, M. J., Spragg, J. H., and Nash, G. B., 1997, Eur J Immunol, 27:1422-1426.
32. Campbell, J. J., Qin, S., Bacon, K. B., Mackay, C. R., and Butcher, E. C., 1996, J Cell Biol, 134:255-266.
33. Luster, A. D., 1998, N Engl J Med, 338:436-445.
34. Campbell, J. J., and Butcher, E. C., 2000, Curr Opin Immunol, 12:336-341.
35. Campbell, J. J., Hedrick, J., Zlotnik, A., Siani, M. A., Thompson, D. A., and Butcher, E. C., 1998, Science, 279:381-384.
36. Berg, E. L., Magnani, J., Warnock, R. A., Robinson, M. K., and Butcher, E. C., 1992, Biochem Biophys Res Commun, 184:1048-1055.
37. Bevilacqua, M. P., Stengelin, S., Gimbrone, M. A., and Seed, B., 1989, Science, 243:1160-1165.
38. Butcher, E. C., Williams, M., Youngman, K., Rott, L., and Briskin, M., 1999, Adv Immunol, 72:209-253.
39. Cummings, R. D., and Smith, D. F., 1992, Bioessays, 14:849-856.
40. Fieger, C. B., Emig-Vollmer, S., Petri, T., Grafe, M., Gohlke, M., Debus, N., Semmler, W., Tauber, R., and Volz, B., 2001, Biochim Biophys Acta 1524, 75-85.
41. Gulubova, M. V., 2002, Histochem J, 34:67-77.
42. Li, L., Short, H. J., Qian, K. X., Elhammer, A. P., and Geng, J. G., 2001, Biochem Biophys Res Commun, 288:637-644.
43. Picker, L. J., Kishimoto, T. K., Smith, C. W., Warnock, R. A., and Butcher, E. C., 1991, Nature, 349:796-799.
44. Tedder, T. F., Penta, A. C., Levine, H. B., and Freedman, A. S., 1990, J Immunol, 144:532-540.
45. Bizouarne, N., Denis, V., Legrand, A., Monsigny, M., and Kieda, C., 1993, Biol Cell, 79:209-218.
46. Berg, E. L., Yoshino, T., Rott, L. S., Robinson, M. K., Warnock, R. A., Kishimoto, T. K., Picker, L. J., and Butcher, E. C., 1991, J Exp Med, 174:1461-1466.
47. Bos, J. D., de Boer, O. J., Tibosch, E., Das, P. K., and Pals, S.T., 1993, Arch Dermatol Res, 85:179-183.
48. Picker, L. J., Michie, S. A., Rott, L. S., and Butcher, E. C., 1990, Am J Pathol, 136:1053-1068.
49. Santamaria Babi, L. F., Perez Soler, M. T., Hauser, C., and Blaser, K., 1995, Immunol Res, 14:317-324.
50. de Boer, O. J., Wakelkamp, I. M., Pals, S. T., Claessen, N., Bos, J. D., and Das, P. K., 1994, Arch Dermatol Res, 286:304-311.
51. Schweighoffer, T., Tanaka, Y., Tidswell, M., Erle, D. J., Horgan, K. J., Luce, G. E., Lazarovits, A. I., Buck, D., and Shaw, S., 1993, J Immunol, 151:717-729.
52. Duijvestijn, A. M., Horst, E., Pals, S. T., Rouse, B. N., Steere, A. C., Picker, L. J., Meijer, C. J., and Butcher, E. C., 1988, Am J Pathol, 130:147-155.
53. Meijer, C., Beljaards, F., Horst, E., Willemze, R., Van der Valk, P., and Pals, S., 1989, J Invest Dermatol, 92:479.
54. Noorduyn, L. A., Beljaards, R. C., Pals, S. T., van Heerde, P., Radaszkiewicz, T., Willemze, R., and Meijer, C. J., 1992, Histopathology, 21:59-64.
55. De Boer, O. J., Horst, E., Pals, S. T., Bos, J. D., and Das, P. K., 1994, Immunology, 81:359-365.
56. Bargatze, R. F., Kurk, S., Butcher, E. C., and Jutila, M. A., 1994, J Exp Med, 180:1785-1792.
57. Berg, E. L., McEvoy, L. M., Berlin, C., Bargatze, R. F., and Butcher, E. C., 1993, Nature, 366:695-698.
58. Hasslen, S. R., von Andrian, U. H., Butcher, E. C., Nelson, R. D., and Erlandsen, S. L., 1995, Histochem J, 27:547-554.
59. Shimizu, Y., and Shaw, S., 1993, Nature, 366:630-631.
60. Berg, E. L., Mullowney, A. T., Andrew, D. P., Goldberg, J. E., and Butcher, E. C., 1998, Am J Pathol, 152:469-477.
61. Hemmerich, S., Butcher, E. C., and Rosen, S. D., 1994, J Exp Med, 180:2219-2226.
62. Michie, S. A., Streeter, P. R., Butcher, E. C., and Rouse, R. V., 1995, Am J Pathol, 147:412-421.
63. von Andrian, U. H., Chambers, J. D., Berg, E. L., Michie, S. A., Brown, D. A., Karolak, D., Ramezani, L., Berger, E. M., Arfors, K. E., and Butcher, E. C., 1993, Blood, 82:182-191.
64. Yednock, T. A., Butcher, E. C., Stoolman, L. M., and Rosen, S. D., 1987, J Cell Biol, 104:725-731.
65. Michie, S. A., Streeter, P. R., Bolt, P. A., Butcher, E. C., and Picker, L. J., 1993, Am J Pathol, 143:1688-1698.
66. Streeter, P. R., Berg, E. L., Rouse, B. T., Bargatze, R. F., and Butcher, E. C., 1988, Nature, 331:41-46.
67. Vestweber, D., and Blanks, J. E., 1999, Physiol Rev, 79:181-213
68. Imai, Y., Lasky, L. A., and Rosen, S. D., 1992, Glycobiology, 2:373-381.
69. Berg, E. L., Robinson, M. K., Mansson, O., Butcher, E. C., and Magnani, J. L., 1991, J Biol Chem, 266:14869-14872.
70. Nakache, M., Berg, E. L., Streeter, P. R., and Butcher, E. C., 1989, Nature, 337:179-181.
71. Picker, L. J., Nakache, M., and Butcher, E. C., 1989, J Cell Biol, 109:927-937.
72. Briskin, M., Winsor-Hines, D., Shyjan, A., Cochran, N., Bloom, S., Wilson, J., McEvoy, L. M., Butcher, E. C., Kassam, N., Mackay, C. R.,

Newman, W., and Ringler, D. J., 1997, Am J Pathol, 151:97-110.
73. Morgan, S. M., Samulowitz, U., Darley, L., Simmons, D. L., and Vestweber, D., 1999, Blood, 93:165-175.
74. Wagner, N., Lohler, J., Kunkel, E. J., Ley, K., Leung, E., Krissansen, G., Rajewsky, K., and Muller, W., 1996, Nature, 382:366-370.
75. Drillenburg, P., van der Voort, R., Koopman, G., Dragosics, B., van Krieken, J. H., Kluin, P., Meenan, J., Lazarovits, A. I., Radaszkiewicz, T., and Pals, S. T., 1997, Am J Pathol, 150:919-927.
76. Taub, D. D., and Oppenheim, J. J., 1994, Ther Immunol, 1:229-246.
77. Mackay, C. R., 1996, J Exp Med, 184:799-802.
78. Taub, D. D., Anver, M., Oppenheim, J. J., Longo, D. L., and Murphy, W. J., 1996, J Clin Invest, 97:1931-1941.
79. Fuentes, M. E., Durham, S. K., Swerdel, M. R., Lewin, A. C., Barton, D. S., Megill, J. R., Bravo, R., and Lira, S. A., 1995, J Immunol, 155:5769-5776..
80. Bargatze, R. F., and Butcher, E. C., 1993, J Exp Med, 178:367-372.
81. Sekido, N., Mukaida, N., Harada, A., Nakanishi, I., Watanabe, Y., and Matsushima, K., 1993, Nature, 365:654-657.
82. Karpus, W. J., Lukacs, N. W., McRae, B. L., Strieter, R. M., Kunkel, S. L., and Miller, S. D., 1995, J Immunol, 155:5003-5010.
83. Gupta, S. K., Hassel, T., and Singh, J. P., 1995, Proc Natl Acad Sci U S A, 92:7799-7803.
84. Zhou, Z., Kim, Y. J., Pollok, K., Hurtado, J., Lee, J. K., Broxmeyer, H. E., and Kwon, B. S., 1993, J Immunol, 151:4333-4341.
85. Taub, D. D., Turcovski-Corrales, S. M., Key, M. L., Longo, D. L., and Murphy, W. J., 1996, J Immunol, 156:2095-2103.
86. Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P. D., Wu, L., Mackay, C. R., LaRosa, G., Newman, W., Gerard, N., Gerard, C., and Sodroski, J., 1996, Cell, 85:1135-1148.
87. Feng, Y., Broder, C. C., Kennedy, P. E., and Berger, E. A., 1996, Science, 272:872-877.
88. Witt, D. P., and Lander, A. D., 1994, Curr Biol, 4:394-400.
89. Rybak, M. E., Gimbrone, M. A., Jr., Davies, P. F., and Handin, R. I., 1989, Blood, 73:1534-1539.
90. Luster, A. D., Greenberg, S. M., and Leder, P., 1995, J Exp Med, 182:219-231.
91. Hoogewerf, A. J., Kuschert, G. S., Proudfoot, A. E., Borlat, F., Clark-Lewis, I., Power, C. A., and Wells, T. N., 1997, Biochemistry, 36:13570-13578.
92. Middleton, J., Neil, S., Wintle, J., Clark-Lewis, I., Moore, H., Lam, C., Auer, M., Hub, E., and Rot, A., 1997, Cell, 91:385-395.
93. Rot, A., 1992, Immunol Today, 13:291-294.
94. Tanaka, Y., Adams, D. H., Hubscher, S., Hirano, H., Siebenlist, U., and Shaw, S., 1993, Nature, 361:79-82.
95. Warnock, R. A., Campbell, J. J., Dorf, M. E., Matsuzawa, A., McEvoy, L. M., and Butcher, E. C., 2000, J Exp Med, 191:77-88.
96. Stein, J. V., Rot, A., Luo, Y., Narasimhaswamy, M., Nakano, H., Gunn, M. D., Matsuzawa, A., Quackenbush, E. J., Dorf, M. E., and von Andrian, U. H., 2000, J Exp Med, 191:61-76.
97. Pan, J., Kunkel, E. J., Gosslar, U., Lazarus, N., Langdon, P., Broadwell, K., Vierra, M. A., Genovese, M. C., Butcher, E. C., and Soler, D., 2000, J Immunol, 165:2943-2949.
98. Kunkel, E. J., Campbell, J. J., Haraldsen, G., Pan, J., Boisvert, J., Roberts, A. I., Ebert, E. C., Vierra, M. A., Goodman, S. B., Genovese, M. C., Wardlaw, A. J., Greenberg, H. B., Parker, C. M., Butcher, E. C., Andrew, D. P., and Agace, W. W., 2000, J Exp Med, 192:761-768.
99. Campbell, J. J., Haraldsen, G., Pan, J., Rottman, J., Qin, S., Ponath, P., Andrew, D. P., Warnke, R., Ruffing, N., Kassam, N., Wu, L., and Butcher, E. C., 1999, Nature, 400:776-780.
100. Morales, J., Homey, B., Vicari, A. P., Hudak, S., Oldham, E., Hedrick, J., Orozco, R., Copeland, N. G., Jenkins, N. A., McEvoy, L. M., and Zlotnik, A., 1999, Proc Natl Acad Sci U S A, 96:14470-14475.
101. Gunn, M. D., Tangemann, K., Tam, C., Cyster, J. G., Rosen, S. D., and Williams, L. T., 1998, Proc Natl Acad Sci U S A, 95:258-263.
102. Nagira, M., Imai, T., Hieshima, K., Kusuda, J., Ridanpaa, M., Takagi, S., Nishimura, M., Kakizaki, M., Nomiyama, H., and Yoshie, O., 1997, J Biol Chem, 272:19518-19524.
103. Yoshida, R., Nagira, M., Kitaura, M., Imagawa, N., Imai, T., and Yoshie, O., 1998, J Biol Chem, 273:7118-7122.
104. Hwang, S. T., Singer, M. S., Giblin, P. A., Yednock, T. A., Bacon, K. B., Simon, S. I., and Rosen, S. D., 1996, J Exp Med, 184:1343-1348.
105. Nakano, H., Tamura, T., Yoshimoto, T., Yagita, H., Miyasaka, M., Butcher, E. C., Nariuchi, H., Kakiuchi, T., and Matsuzawa, A., 1997, Eur J Immunol, 27:215-221.
106. Gunn, M. D., Kyuwa, S., Tam, C., Kakiuchi, T., Matsuzawa, A., Williams, L. T., and Nakano, H., 1999, J Exp Med, 189:451-460.
107. Vassileva, G., Soto, H., Zlotnik, A., Nakano, H., Kakiuchi, T., Hedrick, J. A., and Lira, S. A., 1999, J Exp Med, 190:1183-1188.
108. Mashino, K., Sadanaga, N., Yamaguchi, H., Tanaka, F., Ohta, M., Shibuta, K., Inoue, H., and Mori, M., 2002, Cancer Res, 62:2937-2941.

109. Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N., Barrera, J. L., Mohar, A., Verastegui, E., and Zlotnik, A., 2001, Nature, 410:50-56.
110. Wiley, H. E., Gonzalez, E. B., Maki, W., Wu, M. T., and Hwang, S. T., 2001, J Natl Cancer Inst, 93:1638-1643.
111. Zabel, B. A., Agace, W. W., Campbell, J. J., Heath, H. M., Parent, D., Roberts, A. I., Ebert, E. C., Kassam, N., Qin, S., Zovko, M., LaRosa, G. J., Yang, L. L., Soler, D., Butcher, E. C., Ponath, P. D., Parker, C. M., and Andrew, D. P., 1999, J Exp Med, 190:1241-1256.
112. Vicari, A. P., Figueroa, D. J., Hedrick, J. A., Foster, J. S., Singh, K. P., Menon, S., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Bacon, K. B., and Zlotnik, A., 1997, Immunity, 7:291-301.
113. Wurbel, M. A., Philippe, J. M., Nguyen, C., Victorero, G., Freeman, T., Wooding, P., Miazek, A., Mattei, M. G., Malissen, M., Jordan, B. R., Malissen, B., Carrier, A., and Naquet, P., 2000, Eur J Immunol, 30:262-271.
114. Reiss, Y., Proudfoot, A. E., Power, C. A., Campbell, J. J., and Butcher, E. C., 2001, J Exp Med, 194:1541-1547.
115. Wang, J. M., Deng, X., Gong, W., and Su, S., 1998, J Immunol Methods, 220:1-17.
116. Mizoue, L. S., Bazan, J. F., Johnson, E. C., and Handel, T. M., 1999, Biochemistry, 38:1402-1414.
117. Bazan, J. F., Bacon, K. B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D. R., Zlotnik, A., and Schall, T. J., 1997, Nature, 385:640-644.
118. Pan, Y., Lloyd, C., Zhou, H., Dolich, S., Deeds, J., Gonzalo, J. A., Vath, J., Gosselin, M., Ma, J., Dussault, B., Woolf, E., Alperin, G., Culpepper, J., Gutierrez-Ramos, J. C., and Gearing, D., 1997, Nature, 387:611-617.
119. Combadiere, C., Salzwedel, K., Smith, E. D., Tiffany, H. L., Berger, E. A., and Murphy, P. M., 1998, J Biol Chem, 273:23799-23804.
120. Imai, T., Hieshima, K., Haskell, C., Baba, M., Nagira, M., Nishimura, M., Kakizaki, M., Takagi, S., Nomiyama, H., Schall, T. J., and Yoshie, O., 1997, Cell, 91:521-530.
121. Wilbanks, A., Zondlo, S. C., Murphy, K., Mak, S., Soler, D., Langdon, P., Andrew, D. P., Wu, L., and Briskin, M., 2001, J Immunol, 166:5145-5154.
122. Fong, A. M., Erickson, H. P., Zachariah, J. P., Poon, S., Schamberg, N. J., Imai, T., and Patel, D. D., 2000, J Biol Chem, 275:3781-3786.
123. Fong, A. M., Robinson, L. A., Steeber, D. A., Tedder, T. F., Yoshie, O., Imai, T., and Patel, D. D., 1998, J Exp Med, 188:1413-1419.
124. Haskell, C. A., Cleary, M. D., and Charo, I. F., 1999, J Biol Chem, 274:10053-10058.
125. Papadopoulos, E. J., Sassetti, C., Saeki, H., Yamada, N., Kawamura, T., Fitzhugh, D. J., Saraf, M. A., Schall, T., Blauvelt, A., Rosen, S. D., and Hwang, S. T., 1999, Eur J Immunol, 29:2551-2559.
126. Harrison, J. K., Jiang, Y., Wees, E. A., Salafranca, M. N., Liang, H. X., Feng, L., and Belardinelli, L., 1999, J Leukoc Biol, 66:937-944.
127. Foussat, A., Coulomb-L'Hermine, A., Gosling, J., Krzysiek, R., Durand-Gasselin, I., Schall, T., Balian, A., Richard, Y., Galanaud, P., and Emilie, D., .2000, Eur J Immunol, 30:87-97
128. Sugaya, M., Nakamura, K., Mitsui, H., Takekoshi, T., Saeki, H., and Tamaki, K., 2003, J Dermatol Sci, 31:179-187.
129. Harrison, J. K., Jiang, Y., Chen, S., Xia, Y., Maciejewski, D., McNamara, R. K., Streit, W. J., Salafranca, M. N., Adhikari, S., Thompson, D. A., Botti, P., Bacon, K. B., and Feng, L., 1998, Proc Natl Acad Sci U S A, 95:10896-10901.
130. Lavergne, E., Combadiere, B., Bonduelle, O., Iga, M., Gao, J. L., Maho, M., Boissonnas, A., Murphy, P. M., Debre, P., and Combadiere, C., 2003, Cancer Res, 63:7468-7474.
131. Guo, J., Zhang, M., Wang, B., Yuan, Z., Guo, Z., Chen, T., Yu, Y., Qin, Z., and Cao, X., 2003, Int J Cancer, 103:212-220.
132. Flamme, I., Frolich, T., and Risau, W., 1997, J Cell Physiol, 173:206-210.
133. Risau, W., 1997, Nature, 386:671-674.
134. Keane, M. P., Arenberg, D. A., Moore, B. B., Addison, C. L., and Strieter, R. M., 1998, Proc Assoc Am Physicians, 110:288-296.
135. Moore, B. B., Keane, M. P., Addison, C. L., Arenberg, D. A., and Strieter, R. M., 1998, J Investig Med, 46:113-120.
136. Folkman, J., 1995, Nat Med, 1:27-31.
137. Murdoch, C., and Finn, A., 2000, Blood, 95:3032-3043.
138. Frederick, M. J., and Clayman, G. L., 2001, Expert Rev Mol Med, 2001:1-18.
139. Lucas, A. D., and Greaves, D. R., 2001, Expert Rev Mol Med, 2001:1-18.
140. Fidler, I. J., and Ellis, L. M., 1994, Cell, 79:185-188.
141. Yoshiji, H., Gomez, D. E., Shibuya, M., and Thorgeirsson, U. P., 1996, Cancer Res, 56:2013-2016.
142. Millauer, B., Wizigmann-Voos, S., Schnurch, H., Martinez, R., Moller, N. P., Risau, W., and Ullrich, A., 1993, Cell, 72:835-846.
143. Ellis, L. M., and Fidler, I. J., 1996, Eur J Cancer, 32A:2451-2460.
144. Moore, B. B., Arenberg, D. A., Addison, C. L., Keane, M. P., and Strieter, R. M., 1998, J Lab Clin Med, 132:97-103.

145. Kunkel, E., and Butcher, E., 2002, Adv Exp Med Biol, 512:65-72.
146. Li, A., Varney, M. L., and Singh, R. K., 2001, Clin Cancer Res, 7:3298-3304.
147. Zeelenberg, I. S., Ruuls-Van Stalle, L., and Roos, E., 2003, Cancer Res, 63:3833-3839.
148. Kieda, C., and Dus, D., 2003, Adv Exp Med Biol, 535:79-94.
149. Horuk, R., 2001, Cytokine Growth Factor Rev, 12:313-335.
150. Schadendorf, D., Moller, A., Algermissen, B., Worm, M., Sticherling, M., and Czarnetzki, B. M. ,1993, J Immunol, 151:2667-2675
151. Brew, R., Erikson, J. S., West, D. C., Kinsella, A. R., Slavin, J., and Christmas, S. E., 2000, Cytokine, 12:78-85.
152. Ferrer, F. A., Pantschenko, A. G., Miller, L. J., Anderson, K., Grunnet, M., McKenna, P. H., and Kreutzer, D., 2000, J Urol, 164:1016-1020.
153. Luan, J., Shattuck-Brandt, R., Haghnegahdar, H., Owen, J. D., Strieter, R., Burdick, M., Nirodi, C., Beauchamp, D., Johnson, K. N., and Richmond, A., 1997, J Leukoc Biol, 62:588-597.
154. Fujisawa, N., Sakao, Y., Hayashi, S., Hadden, W. A., 3rd, Harmon, C. L., and Miller, E. J., 2000, J Cancer Res Clin Oncol, 126:19-26.
155. Bernardini, G., Ribatti, D., Spinetti, G., Morbidelli, L., Ziche, M., Santoni, A., Capogrossi, M. C., and Napolitano, M., 2003, J Immunol Methods, 273:83-101.
156. Belperio, J. A., Keane, M. P., Arenberg, D. A., Addison, C. L., Ehlert, J. E., Burdick, M. D., and Strieter, R. M., 2000, J Leukoc Biol, 68:1-8.
157. Strieter, R. M., Polverini, P. J., Arenberg, D. A., and Kunkel, S. L., 1995, Shock, 4:155-160.
158. Strieter, R. M., Polverini, P. J., Kunkel, S. L., Arenberg, D. A., Burdick, M. D., Kasper, J., Dzuiba, J., Van Damme, J., Walz, A., Marriott, D., and et al., 1995, J Biol Chem, 270:27348-27357.
159. Koch, A. E., Polverini, P. J., Kunkel, S. L., Harlow, L. A., DiPietro, L. A., Elner, V. M., Elner, S. G., and Strieter, R. M., 1992, Science, 258:1798-1801.
160. Salcedo, R., and Oppenheim, J. J., 2003, Microcirculation, 10:359-370.
161. Salcedo, R., Ponce, M. L., Young, H. A., Wasserman, K., Ward, J. M., Kleinman, H. K., Oppenheim, J. J., and Murphy, W. J., 2000, Blood, 96:34-40.
162. Volin, M. V., Woods, J. M., Amin, M. A., Connors, M. A., Harlow, L. A., and Koch, A. E., 2001, Am J Pathol, 159:1521-1530.
163. Maione, T. E., Gray, G. S., Petro, J., Hunt, A. J., Donner, A. L., Bauer, S. I., Carson, H. F., and Sharpe, R. J., 1990, Science, 247:77-79.
164. Pugh, C. W., 2003, Ann Med 35, 380-390.
165. Welsh, S. J., and Powis, G., 2003, Curr Cancer Drug Targets, 3:391-405.
166. Schioppa, T., Uranchimeg, B., Saccani, A., Biswas, S. K., Doni, A., Rapisarda, A., Bernasconi, S., Saccani, S., Nebuloni, M., Vago, L., Mantovani, A., Melillo, G., and Sica, A., 2003, J Exp Med, 198:1391-1402.
167. Adler, E. P., Lemken, C. A., Katchen, N. S., and Kurt, R. A., 2003, Immunol Lett, 90:187-194.
168. Caux, C., Ait-Yahia, S., Chemin, K., de Bouteiller, O., Dieu-Nosjean, M. C., Homey, B., Massacrier, C., Vanbervliet, B., Zlotnik, A., and Vicari, A., 2000, Springer Semin Immunopathol, 22:345-369.
169. Sharma, S., Stolina, M., Luo, J., Strieter, R. M., Burdick, M., Zhu, L. X., Batra, R. K., and Dubinett, S. M., 2000, J Immunol, 164:4558-4563.
170. Van Der Bilt, J. D., and Borel Rinkes, I. H., 2004, Biochim Biophys Acta, 1654:95-104.
171. Voest, E. E., 1996, Anticancer Drugs, 7:723-727.
172. Folkman, J., and Ingber, D., 1992, Semin Cancer Biol, 3:89-96.
173. Nomura, T., and Hasegawa, H., 2000, Anticancer Res, 20:4073-4080.
174. Arenberg, D. A., Zlotnick, A., Strom, S. R., Burdick, M. D., and Strieter, R. M., 2001, Cancer Immunol Immunother, 49:587-592.
175. Rubin, J. B., Kung, A. L., Klein, R. S., Chan, J. A., Sun, Y., Schmidt, K., Kieran, M. W., Luster, A. D., and Segal, R. A., 2003, Proc Natl Acad Sci U S A, 100:13513-13518.
176. Chada, S., Ramesh, R., and Mhashilkar, A. M., 2003, Curr Opin Mol Ther, 5:463-474.
177. Ruehlmann, J. M., Xiang, R., Niethammer, A. G., Ba, Y., Pertl, U., Dolman, C. S., Gillies, S. D., and Reisfeld, R. A., 2001, Cancer Res, 61:8498-8503.
178. Nakahara, K., and Sakata, T., 2003, J Gene Med, 5:463-471.

Chapter 13

The Role of Chemokine Receptors, in Particular CXCR4, in Lymphoma and Carcinoma Metastasis

I.S. Zeelenberg and E. Roos
Division of Cell Biology, The Netherlands Cancer Institute, 121 Plesmanlaan, 1066 CX, Amsterdam

Abstract: Chemokines are small proteins that regulate leukocyte trafficking. They are important for development of tumors, because many chemokines are produced by tumor cells and attract leukocytes that contribute to malignancy. In addition, chemokines also act directly on tumor cells. In particular CXCL12 (SDF-1), and its receptor CXCR4, are required for metastasis of lymphoma and carcinoma cells. CXCL12 triggers invasion of lymphoma cells into tissues, in line with its role in leukocyte migration. Surprisingly, we found that CXCR4 is not required for invasion by carcinoma cells, but instead is essential at later stages, to promote outgrowth of micrometastases.

Key Words Chemokines, lymphoma, carcinoma, metastasis, melanoma, cell proliferation, CXCR4, CXCL12/SDF-1, cell signalling, glioblastoma, lymphoma

1. INTRODUCTION

Tumor formation is due to an accumulation of oncogenic mutations in a tumor progenitor cell, and cancer research has understandably been focused on the events in this cell and its progeny. It is, however, increasingly recognized that tumor cells do not act in isolation, and that tumor development depends on complex interactions with surrounding normal cells. An obvious example is angiogenesis, the formation of tumor blood vessels by normal endothelial cells, which is required for expansion of the tumor to a macroscopic size. These endothelial cells are attracted and activated by proteins released from the tumor cells. Other tumor-derived factors attract and activate leukocytes and fibroblasts, which in turn produce proteins that affect the tumor cell phenotype and often enhance its aggressiveness. The behavior of a tumor can therefore only be understood if the contributions of its non-tumor cell constituents are taken into account.

The normal function of many proteins that are produced by tumor cells is in wound healing. In wounds, they are released by epithelial cells, leukocytes and fibroblasts that are stimulated by factors secreted by activated platelets. Indeed, when fibroblasts in culture are stimulated with serum, which contains the same platelet factors, they produce a multitude of such "wound" proteins (1). Serum activates signal transducers such as Ras that induce their synthesis. In tumors, these signal transducers are often constitutively active and stimulate production of wound proteins, in the absence of the normal stimuli such as the platelet factors. Thus, tumor formation is to some extent comparable to wound healing. In fact, tumors may be regarded as "wounds that do not heal" (2).

Here, we will discuss the role of chemokines, a subset of factors that are produced by both tumor cells and normal cells in the tumor. Chemokines are mediators of leukocyte trafficking (3), but were recently found to play a major role in cancer as well.

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 233-244.

In part they are responsible for the migration of both normal and tumor cells, i.e. for the influx of normal cells into the tumor and for the invasion of tumor cells into normal tissues. Surprisingly, and perhaps more importantly, some chemokines promote growth of tumor cells and especially the outgrowth of tumor metastases. The emphasis in this chapter will be on these chemokines and in particular on CXCL12 (SDF-1) and its receptor CXCR4.

2. CHEMOKINES AND CHEMOKINE RECEPTORS

The *chemo*attractant cyto*kines* (chemokines) are small (8-14 kDa) proteins (4, 5). The main subfamilies are the CC-chemokines, with two adjacent cysteines in the N-terminal domain, and the CXC-chemokines, in which the cysteines are separated by a single other amino acid (6). CC- and CXC-chemokines bind to seven-transmembrane G-protein-coupled receptors, designated CCR and CXCR, respectively. The interactions are quite specific for CXCRs and often more promiscuous for CCRs, with some CC-chemokines binding multiple receptors and some CCRs binding multiple chemokines. Chemokines are often produced at sites of inflammation and are involved in host defense and immune reactions (7). However, some chemokines are present in normal non-inflamed tissues and play a major role in development and normal tissue homeostasis. This is particularly true for CXCL12 (SDF-1) and CXCL13 (BCA-1). CXCL12, the ligand of CXCR4, is present in many tissues (8,9) and may play a role in the recirculation of leukocytes through extralymphoid organs (10). CXCL13 and its receptor CXCR5 are responsible for the influx of B-lymphocytes into lymph nodes, and lymph nodes are therefore much smaller in CXCR5 knockout mice (11). However, also CCRs are involved in tissue homeostasis. An example is the role of CCR7 in the influx of lymphocytes into lymph nodes (12).

3. CHEMOKINE RECEPTORS IN CANCER

It has been known for more than a decade that tumor cells produce chemokines. Examples are CCL2 (13) and CCL5 (14) that attract macrophages and other leukocytes. In addition to chemokines, other factors such as CSF-1 (colony stimulating factor-1) also contribute to macrophage influx in certain tumors (15). The macrophages promote tumor growth, e.g. by producing metalloproteases (16) that release angiogenic and growth factors from the extracellular matrix. The relevance for therapy was recently shown by blocking CCL5 activity in CCL5-producing carcinomas. This led to reduced macrophage infiltration and impaired tumor growth (14). Another chemokine of major importance, at least in human cancers, is CXCL8 (interleukin-8). Production of CXL8 is correlated with malignancy in melanoma and ovarian, pancreatic and prostate carcinoma (17), and antibodies against CXCL8 inhibit growth and metastasis of human melanoma (18). CXCL8 may act as an autocrine growth factor for melanoma (19), but its major role is probably in angiogenesis (18). In addition, it may aid in attracting leukocytes. Indeed, it has been proposed to transfect the CXCL8 receptor CXCR2 into tumor-specific cytotoxic T-lymphocytes to promote their migration into tumors (20).

These results are in line with the notion that tumors are comparable to wounds, as described above, and that chemokines are required for infiltration of leukocytes, known to express the receptors. Chemokine receptors are also present on malignant hematopoietic cells, similarly as on their non-transformed counterparts. These receptors probably play a role in the dissemination of these cells to lymphoid tissues. In addition, we have shown that CXCR4 is required for dissemination of T-lymphoma cells to extralymphoid tissues such as liver and kidneys, as will be described below.

Chemokine receptors might be expected to be only expressed by leukocytes, given their predominant role in leukocyte trafficking. Surprisingly, however, several receptors were also found on other cells, such as colon epithelial cells (21), and in particular on many non-hematopoietic

tumor cell types (22). CXCR4 was most often detected, on a wide variety of carcinoma and sarcoma cells (10, 22-27). In the remainder of this chapter we will focus on the role of CXCR4 in cancer. Here, we will briefly mention observations on a few other receptors. CXCR2 is expressed by human melanomas and has been proposed to play a role in invasion (28). Furthermore, its ligand CXCL8 can stimulate growth of melanoma and pancreatic carcinoma cells in an autocrine fashion (29). This proliferation-enhancing effect of a chemokine is not unique for CXCL8, as we will emphasize below. However, as mentioned above, CXCL8 is also an important angiogenic factor (30). Which of the many functions of CXCL8 is most important for tumor progression remains to be demonstrated. The CXCL8 receptors CXCR1 and CXCR2 are expressed in gastric, colon and pancreatic carcinomas (31-33). CCR7 is sometimes expressed in melanoma, and lung, esophageal, gastric and breast carcinoma. In line with its role in the influx of lymphocytes into lymph nodes, CCR7 appears to promote lymph node metastasis of these tumors (23, 34-36). Finally, CCR10 is expressed in melanoma and may be involved in invasion of the skin (23).

4. CXCR4: ROLE IN DEVELOPMENT

Before describing the role of CXCR4 in cancer, it is appropriate to briefly summarize what is known about the normal function of this remarkably versatile receptor. CXCR4 was identified as the monocyte orphan receptor LESTR (37) and later as fusin, a co-receptor for certain strains of HIV virus (38,39). CXCL12 (SDF-1 = stromal cell-derived factor 1), which is its only ligand (40,41), was cloned from bone marrow stromal cells (8) and found to support the proliferation of bone marrow B-cell progenitors in the presence of interleukin-7 (42). It is noteworthy that this chemokine was originally identified as a growth factor, a property that is relevant for its role in cancer, as shown below. CXCL12 is an extremely potent lymphocyte and monocyte chemoattractant which, unlike other chemokines, is constitutively expressed in many non-inflamed tissues (8, 10, 40, 42). CXCR4 is unusually widely expressed, namely on T cells, B cells, monocytes, macrophages, dendritic cells, several neuronal cell types, endothelial cells, hematopoietic progenitors, platelets and even on some epithelial cells (21, 43-51).

In contrast to other chemokines and chemokine receptors, CXCL12 or CXCR4 gene disruption is embryonically lethal (43, 52-54). The embryos exhibit dysplasia of the ventricular septum of the heart. In the cerebellum, the migration of granule cells is affected. CXCR4 is also involved in vascular development in the gastrointestinal tract, particularly in the remodeling of the capillary network (54). This has, however, no consequences for the development of the gastrointestinal organs.

CXCR4 and CXCL12 have been implicated in the homing of hematopoietic precursors to the bone marrow, and CXCR4- and CXCL12-deficient mice have defects in development of B cells and myeloid cells but not T cells. B-cell development is blocked at the pro-B-cell stage. Cells of the myeloid lineage develop normally in the fetal liver, but fail to colonize the bone marrow. In the adult, CXCR4 is necessary for human stem cell engraftment (55), and inhibition of CXCR4 function leads to release of hematopoietic stem cells into the circulation. Finally, the colonization of the gonads by primordial germ cells is impaired in CXCL12 knockout mice (56, 57). Also in zebrafish, CXCL12 is essential for guiding the migration of primordial germ cells (58, 59).

5. EFFECTS OF CXCR4 ON PROLIFERATION

Guidance of cell migration is apparently the main role of CXCR4 in development. In addition, however, it promotes proliferation of certain cells. In knockout mice, B-cell development is not impaired by a defect in homing, but to a block in proliferation, in line with the original identification of CXCL12 as a B-cell growth factor. In the cerebellum, CXCR4 is probably not only required for proper migration but also for proliferation of granule cells, because CXCL12, together with Sonic Hedgehog, promotes the proliferation of the granule precursor cells (60). Furthermore, CXCL12 enhances the proliferation of $CD34^+$ hematopoietic stem cells and myeloid

progenitors (61), T-cell precursors and ovarian and lung cancer cells (62, 63). CXCL12 stimulates proliferation probably via the Erk and Akt pathways, as shown for glioblastoma cells (64).

6. ROLE OF CXCR4 IN T LYMPHOMA METASTASIS

We studied the dissemination mechanisms of lymphomas and myeloid leukemias, with emphasis on T-cell hybridomas, a model for T lymphoma. Using hepatocyte cultures as a model for liver invasion, we had observed that activated normal T lymphocytes are similarly invasive as highly metastatic lymphomas. This suggested that the lymphomas used similar invasion mechanisms as normal T cells. Indeed, when fused with non-invasive lymphoma cells, the T cells gave rise to highly invasive T-cell hybridomas that disseminated to multiple tissues but were especially quite invasive in the liver (65). The T-cell hybridomas rapidly invade fibroblast cultures using invasion mechanisms that are remarkably similar to those in vivo, as we showed by downregulation of multiple components of the required signal pathways (10, 66, 67). It is to be noted that details of these mechanisms are different for other hematopoietic malignancies, as we have shown for ESb lymphoma cells (68) and MDAY-D2 acute myeloid leukemia cells (69).

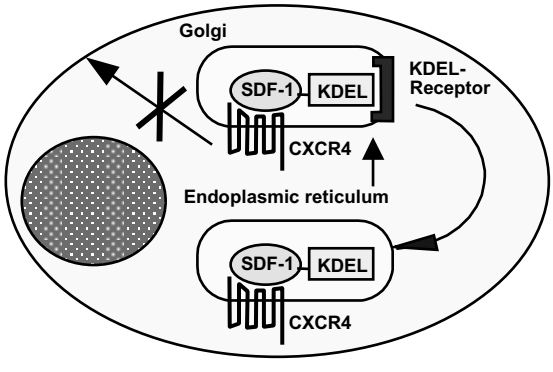


Figure 1. Method used to generate CXCR4-deficient cells: a cDNA encoding the ligand CXCL12 (SDF-1), extended with a KDEL sequence, is transfected. This protein binds newly synthesized CXCR4 as well as the KDEL-receptor, which normally binds resident ER proteins and transports them back to the ER. Thus, SDF-KDEL with its associated CXCR4 is retained in the ER and CXCR4 is prevented from reaching the cell surface.

Invasiveness of the T-cell hybridomas was inhibited by pertussis toxin, an inhibitor of Gi protein signaling. Furthermore, metastasis was blocked in cells in which we expressed the catalytic subunit of pertussis toxin (66), showing that Gi-protein-coupled receptors are involved, most likely chemokine receptors. CXCR4 and CCR8 are expressed by the T-cell hybridoma cells, and CXCR4 was the major candidate because of the constitutive expression of its ligand CXCL12 in virtually all tissues. To study its role, we made use of an approach proposed for gene therapy of AIDS (70). The aim was to remove the HIV co-receptor from the cell surface. CXCL12 with a C-terminal extension of four amino acids, KDEL, was expressed. This CXCL12-KDEL binds to the KDEL-receptor which retains proteins in the endoplasmic reticulum (ER). The CXCL12-KDEL binds newly synthesized CXCR4 which is therefore also retained in the ER and does not reach the surface (see fig. 1). The complete loss of surface CXCR4, thus achieved, blocked *in vitro* invasion as well as metastasis to all tissues (10), showing that CXCR4 was essential and the only chemokine receptor involved. Given the correlation between invasion into fibroblast cultures and metastasis, and the essential role of CXCR4 in both, the most likely role of CXCR4 is in the invasion of tissues by blood-borne T-cell hybridoma cells. However, an additional role in the proliferation of these tumor cells within the tissues, as we will describe below for carcinoma cells, can not be excluded.

7. CXCR4 IN CARCINOMA METASTASIS

Müller et al. (23) showed that CXCR4 plays an important role in breast carcinoma metastasis, using antibodies against CXCR4 that reduced metastasis formation of MDA-MB-231 cells in the lungs by ~ 70%. Subsequently, we showed that metastasis formation of mouse CT-26 colon carcinoma cells, in both liver and lungs, was almost completed

prevented when CXCR4 function was blocked (9). For this we used the same approach as described above for T-cell hybridoma cells (see fig. 1), by expressing a CXCL12-KDEL protein that prevents CXCR4 from reaching the cell surface (10, 70). Liver metastases were generated by injecting cells into the spleen. Strikingly, we observed no effect of the CXCR4 deficiency on the formation of tumors in the spleen. In contrast to the numerous liver metastases generated by the control cells, however, no metastases were formed at all by the CXCR4-deficient cells (9). Lung metastases were generated by injection of cells into a tail vein. Again, almost no metastases were formed and the few that did arise were derived from cells that were not completely devoid of CXCR4. These results demonstrated the important role of CXCR4 in metastasis formation of these colon carcinoma cells. Subsequently, Kang et al. (71) found that CXCR4 was one of a set of proteins, expressed in variants of the MDA-MB-231 breast carcinoma cell line with enhanced metastasis to the bone. The cDNAs encoding CXCR4 and two of the other proteins were then transfected into the regular MDA-MB-231 cells that did not express them. This enabled these transfectant cells to form bone metastases. However, also a different set of these proteins, but not including CXCR4, promoted bone metastasis formation. Finally, transfection of CXCR4 into B16 melanoma cells was found to enhance lung metastasis (72). Together, these results indicate a prominent role for CXCR4 in metastasis of different tumor types. Although not yet demonstrated, it seems likely that this is also true for the wide variety of other tumors that express CXCR4.

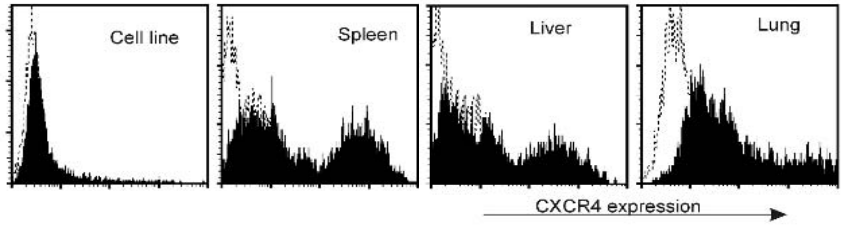


Figure 2. Although CXCR4 mRNA can be detected by RT-PCR in CT-26 colon carcinoma cells in vitro, very little is present on the cell surface, as shown in the left panel. In vivo, however, CXCR4 is strongly upregulated and expressed on a large percentage of the cells. Open histograms: control cells, only treated with secondary antibody (reprinted with permission from Zeelenberg et al., Cancer Res. 63:3833-3839, 2003).

8. UPREGULATION OF CXCR4 IN VIVO

Müller et al. (23) proposed that the role of CXCR4 in lung metastasis was to promote invasion of blood-borne carcinoma cells into the lungs, triggered by the CXCL12 that is constitutively expressed in that tissue. It is remarkable, however, that these authors continued treatment with the anti-CXCR4 antibodies after i.v. injection during the whole assay period, until the mice were sacrificed and the extent of metastasis assessed. It is well-established that more than 99% of i.v. injected non-lymphoid tumor cells die within 24 h (73, 74), so that invasion of the lung tissue should occur within this first day. Antibody treatment beyond the first few days should therefore not be necessary if CXCR4 is only involved in invasion. Thus, the antibodies may have blocked additional functions of CXCR4 at later stages of metastasis formation.

In the CT-26 colon carcinoma cells, we had detected CXCR4 expression by RT-PCR. Subsequent FACS analysis, however, revealed very little CXCR4 on the surface of CT-26 cells *in vitro* (see fig. 2). The cells that we injected into a tail vein were therefore essentially CXCR4-negative. In the liver and lung metastases, as well as in the spleen tumor, CXCR4 was greatly upregulated (fig. 2). To measure CXCR4 expression shortly after lung colonization, we injected a hundredfold larger cell

dose (5 x 10^6). FACS analysis of cells isolated from the lungs showed that CXCR4 was upregulated *in vivo* (see fig. 3), and that maximal expression was reached after 5-7 days (9). As emphasized above, CXCR4-deficiency led to an almost complete block of metastasis. Clearly, the essential function of CXCR4 must be exerted after the blood-borne cells had invaded the lung parenchyma.

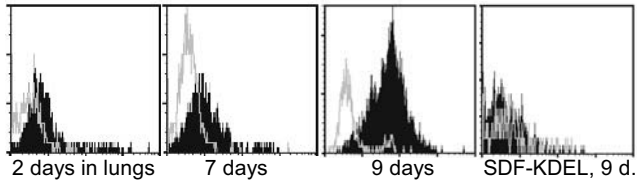


Figure 3. CXCR4 surface levels on CT26 cells, isolated from the lungs 2, 7 or 9 days after tail vein injection, showing that CXCR4 is upregulated in vivo, and maximally after ~9 days. Right panel: SDF-KDEL-expressing cells after 9 days, which are CXCR4-negative, showing that the SDF-KDEL does prevent the CXCR4 from reaching the surface. Note that the number of cells that could be analyzed was very small, since the SDF-KDEL cells grew very slowly in the lungs (reprinted with permission from Zeelenberg et al., Cancer Res. 63:3833-3839, 2003).

Upregulation was not due to selection of a sn number of cells with constitutively high CXC levels that may have been present in the injected c population, since CXCR4 expression was lost up *ex vivo* culture within 1-2 days. Therefore, CXC induction must be due to signals from microenvironment. CXCR4 expression was recen shown to be induced by HIF-1α (75,76), transcription factor stimulated by hypoxia. T "signal" could therefore simply be the lack oxygen in the center of a growing metastasis. Thi very unlikely, however, since we obser upregulation of CXCR4 after 2-7 days, wh metastases are still very small, if they have grown all. Especially in the lungs, the initial upregulat can therefore not be due to hypoxia, although it n play a role later. A second possibility is interact with surrounding cells. It is not known whether influx of tumor stromal cells such as activated leukocytes and fibroblasts has already occurred at these early stages. Unfortunately, it is virtually impossible to find the few isolated metastatic cells in tissue sections, and thus to observe the associated cells. If such stromal cells are not yet present, only interactions with normal lung cells can be responsible. A third option is that CXCR4 is actually suppressed *in vitro*, e.g. by serum factors, but so far we have not been able to induce CXCR4 *in vitro* by changing the culture conditions.

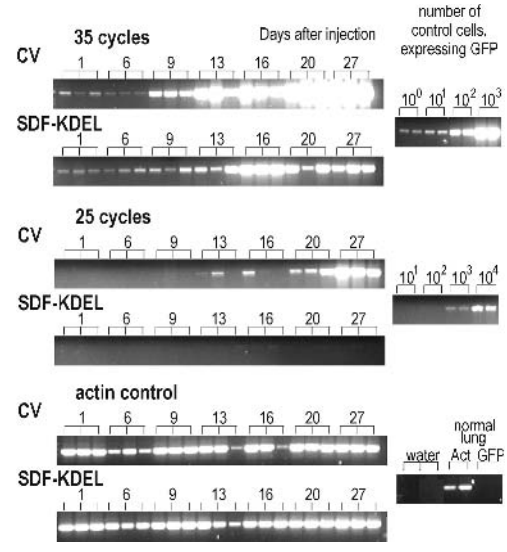


Figure 4. Detection of CT-26 cells in the lungs by GFP-specific RT-PCR. After 35 cycles one cell can be detected in the sample (2% of lungs), i.e. ~50 cells in the complete lungs. The results show that similar numbers of control vector- (CV) transduced and SDF-KDEL-expressing (CXCR4-deficient) cells get established in the lungs, but that control cells expand more rapidly. The slow proliferation of the SDF-KDEL cells stops after 16 days. After 25 cycles, ~50,000 cells can be detected. After 13-20 days control cells are detected but even after 4 weeks no CXCR4-deficient cells. Bottom: actin controls. Bottom right: water controls and actin and (lack of) GFP in lungs of non-injected mice. (Reprinted with permission from Zeelenberg et al., 2003, Cancer Res. 63: 3833-3839).

9. CXCR4 IS REQUIRED FOR GROWTH OF METASTASES

To follow the outgrowth of lung metastases, we used GFP-expressing cells and RT-PCR, to detect as little as 50 cells (9). We found that CXCR4-deficient cells colonized the lungs similarly as control cells (fig. 4). This explains why pertussis toxin had no effect. Similarly as described above for lymphoma cells, we expressed the pertussis toxin catalytic subunit in the CT-26 cells and this blocked Gi protein activity completely. In contrast to the lymphoma cells (10), this did not affect metastasis of the CT-26 cells (9). Since Gi is required for CXCR4-induced migration, this also argues against a role of CXCR4 in invasion. The RT-PCR results revealed that the CXCR4-deficient cells remained present for a prolonged period, but proliferated much more slowly. Furthermore, the slow expansion stopped after 16 days, after a 1000-fold increase, and cell numbers appear to decline thereafter (fig. 4). Growth therefore stops at ~ 1000 cells per metastasis. At this stage, hypoxia may become a problem, and CXCR4 signals may be required to deal with this, as suggested by the hypoxia-induced CXCR4 expression described above. Such signals may trigger the synthesis of angiogenesis factors such as VEGF (77).

The notion that CXCR4 signals promote proliferation is in line with effects on other cells, as described above. In most cases where it was investigated, CXCR4 proliferation signals such as Erk phosphorylation were blocked by pertussis toxin (78), which would argue against involvement since metastasis formation was not susceptible to pertussis toxin. In some cells, however, CXCR4-induced proliferation signals were not inhibited by the toxin, in particular signals activating the p38 MAPK (79). In the carcinoma cells, such Gi-independent pathways must be involved.

CXCR4 is also upregulated in the spleen tumors, formed after injection of cells into the spleen. Yet, the CXCR4-deficient cells form such tumors as readily as the control cells. The spleen tumors were formed from a large number of cells (10^5) and proliferation may have been stimulated by interactions between these tumor cells or by the production of large amounts of other factors, in addition to CXCL12, by the tumor cells. Proliferation may also be enhanced by wound factors in the wound made by the injection needle. This suggests that CXCR4 is required in particular when isolated cells have to initiate tumor formation, such as cells that invade into lung or liver tissue.

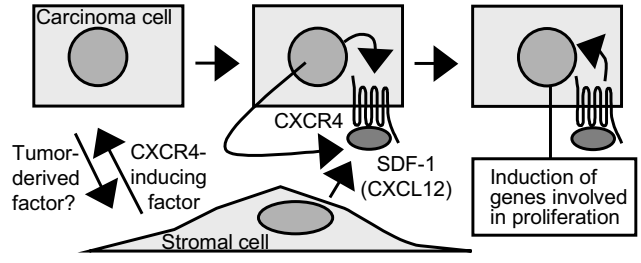


Figure 5. Model for the role of CXCR4 in carcinoma metastasis: In vivo CXCR4 is induced, possibly by a factor produced by stromal cells. CXCL12 (SDF-1), produced by stromal cells and/or by the tumor cells themselves, binds to this CXCR4. This results in signals that lead to induction of genes involved in proliferation in vivo. In later stages, hypoxia may further increase CXCR4 levels. The induced genes may include angiogenesis factors such as VEGF.

10. CXCL12/SDF-1: PARACRINE OR AUTOCRINE?

Once CXCR4 is expressed by the metastatic cells, they can be stimulated by CXCL12, which is present in all tissues tested so far, including the lungs and liver. CXCL12 is also abundantly produced by tumor cells, including the CT-26 cells. CXCL12 (SDF-1) is one of the "wound" factors produced by fibroblasts in response to serum (1), and one of the "wound" proteins that are produced by tumor cells, as discussed in the first paragraph of this chapter. This autocrine CXCL12 probably contributes to stimulation of the CXCR4-expressing cells and can be regarded as an autocrine growth factor. However, since CXCR4 is important for outgrowth when metastases are still small and therefore will not produce much CXCL12, a contribution of environmental CXCL12 seems likely. The model shown in fig. 5 illustrates the different aspects of the proposed roles of CXCR4 and CXCL12 in metastasis formation.

11. CXCR5 IN CARCINOMAS

RT-PCR of chemokine receptors in CT-26 cells revealed expression of CXCR5. This was surprising since it was so far only detected on lymphocytes, particularly B-cells and a T-cell subset. Again, CXCR5 was hardly present on the cell surface *in vitro*, but strongly upregulated *in vivo*. The possible role of CXCR5 in metastasis remains to be demonstrated. Remarkably, we found that the CXCR5 ligand CXCL13 (BCA-1) enhanced growth of CT-26 cells that had been transfected with CXCR5 cDNA and expressed it *in vitro*. Even in 10% fetal calf serum, but especially in serum-free keratinocyte medium, CXCL13 enhanced proliferation. Moreover, we found CXCL13 in the metastases, so that it might stimulate growth *in vivo*. The possible contribution of CXCR5 to metastasis formation is being investigated.

12. CONCLUSION

Chemokines and their receptors play important roles in tumor development, because they attract and activate cells that contribute to malignancy. CXCR4 is the most widely expressed chemokine receptor in a variety of tumor cell types. It plays a major, if not essential, role in the formation of breast and colon carcinoma metastases. For a colon carcinoma, we have shown that CXCR4 signals promote proliferation of the tumor cells in early phases of metastasis formation, after upregulation of CXCR4 expression by as yet unknown signals from the microenvironment. CXCR4 is a co-receptor for certain strains of HIV virus (38, 39) and therefore drugs have been developed that block this interaction with HIV (80, 81). These drugs also interfere with CXCL12 binding and inhibit CXCR4 function. Moreover, they have already been tested in clinical trials for AIDS treatment (82, 83), so their use for cancer therapy can be initiated more rapidly than for other newly developed anti-cancer reagents. CXCR4 antagonists may be most effective in the suppression of outgrowth of micrometastases.

ACKNOWLEDGEMENTS

Our experimental work, described in this chapter, was supported by grant NKI 98-1679 from the Dutch Cancer Society. We thank Joost Meijer for some of the data on CXCR5, and Lisette Ruuls-Van Stalle and Ton Schrauwers for expert technical assistance.

REFERENCES

1. Iyer, V.R., Eisen, M.B., Ross, D.T., Schuler, G., Moore, T., Lee, J.C., Trent, J.M., Staudt, L.M., Hudson, J., Boguski, M.S., Lashkari, D., Shalon, D., Botstein, D., and Brown, P.O., 1999, The transcriptional program in the response of human fibroblasts to serum. Science, 283:83-87.
2. Dvorak, H.F., 1986, Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N Engl J Med, 315:1650-1659.

3. Moser, B., and Loetscher, P., 2001, Lymphocyte traffic control by chemokines. Nat Immunol, 2:123-128.
4. Schall, T.J., 1994, The Chemokines. In The Cytokine Handbook, 419-460. Thomson, A., ed, Academic Press, New York, USA.
5. Murphy, P.M., Baggiolini, M., Charo, I.F., Hebert, C.A., Horuk, R., Matsushima, K., Miller, L.H., Oppenheim, J.J. and Power, C.A., 2000, International union of pharmacology. XXII. Nomenclature for chemokine receptors. Pharmacol Rev, 52:145-176.
6. Zlotnik, A. and Yoshie, O., 2000, Chemokines: a new classification system and their role in immunity. Immunity, 12:121-127.
7. Murdoch, C. and Finn, A., 2000, Chemokine receptors and their role in inflammation and infectious diseases. Blood, 95:3032-3043.
8. Tashiro, K., Tada, H., Heilker, R., Shirozu, M., Nakano, T., and Honjo, T., 1993, Signal sequence trap: a cloning strategy for secreted proteins and type I membrane proteins. Science, 261: 600-603.
9. Zeelenberg, I.S., Ruuls-Van Stalle, L., Roos, E., 2003, The chemokine receptor CXCR4 is required for outgrowth of colon carcinoma micrometastases. Cancer Res, 63: 3833-3839.
10. Zeelenberg, I.S., Ruuls-Van Stalle, L., and Roos, E., 2001, Retention of CXCR4 in the endoplasmic reticulum blocks dissemination of a T cell hybridoma. J Clin Invest, 108: 269-277.
11. Förster, R., Mattis, A.E., Kremmer, E., Wolf, E., Brem, G. and Lipp, M., 1996, A putative chemokine peceptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. Cell, 87:1037-1047.
12. Förster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Müller, I., Wolf, E., and Lipp, M., 1999, CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell, 99:23-33.
13. Negus, R.P., Stamp, G.W., Relf , M.G., Burke, F., Malik, S.T., Bernasconi, S., Allavena, P., Sozzani, S., Mantovani, A., and Balkwill, F.R., 1995, The detection and localization of monocyte chemoattractant protein-1 (MCP-1) in human ovarian cancer. J Clin Invest, 95:2391-2396.
14. Robinson, S.C., Scott, K.A,, Wilson, J.L., Thompson, R.G., Proudfoot, A.E., and Balkwill, F.R., 2003, A chemokine receptor antagonist inhibits experimental breast tumor growth. Cancer Res, 63:8360-8365.
15. Lin, E.Y., Nguyen, A.V., Russell, R.G., and Pollard, J.W., 2001, Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med, 193:727-740.
16. Coussens, L.M., Tinkle, C.L., Hanahan, D., and Werb, Z., 2000, MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. Cell, 103:481-490.
17. Mian, B.M., Dinney, C.P., Bermejo, C.E., Sweeney, P., Tellez, C., Yang, X.D., Gudas, J.M., McConkey, D.J., and Bar-Eli, M., 2003, Fully human anti-interleukin 8 antibody inhibits tumor growth in orthotopic bladder cancer xenografts via down-regulation of matrix metalloproteases and nuclear factor-kappaB. Clin Cancer Res, 9:3167-3175.
18. Huang, S., Mills, L., Mian, B., Tellez, C., McCarty, M., Yang, X.D., Gudas, J.M., and Bar-Eli, M., 2002, Fully humanized neutralizing antibodies to interleukin-8 (ABX-IL8) inhibit angiogenesis, tumor growth, and metastasis of human melanoma. Am J Pathol, 161:125-134.
19. Schadendorf, D., Moller, A., Algermissen, B., Worm, M., Sticherling, M., and Czarnetzki, B.M., 1993, IL-8 produced by human malignant melanoma cells in vitro is an essential autocrine growth factor. J Immunol, 151:2667-2675.
20. Kershaw, M.H., Wang, G., Westwood, J.A., Pachynski, R.K., Tiffany, H.L., Marincola, F.M., Wang, E., Young, H.A., Murphy, P.M., and Hwu, P., 2002, Redirecting migration of T cells to chemokine secreted from tumors by genetic modification with CXCR2. Hum Gene Ther, 13:1971-1980.
21. Jordan, N.J., Kolios, G., Abbot, S.E., Sinai, M.A., Thompson, D.A., Petraki, K., and Westwick, J., 1999, Expression of functional CXCR4 chemokine receptors on human colonic epithelial cells. J Clin Invest, 104:1061-1069.
22. Balkwill, F., 2003, Chemokine biology in cancer. Semin Immunol, 15:49-55.
23. Müller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M.E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S.N., Barrera, J.L., Mohar, A., Verastegui, E., and Zlotnik, A., 2001, Involvement of chemokine receptors in breast cancer metastasis. Nature, 410:50-56.
24. Taichman, R.S., Cooper, C., Keller, E.T., Pienta, K.J., Taichman, N.S., and McCauley, L.K., 2002, Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. Cancer Res, 62:1832-1837.
25. Scotton, C.J., Wilson, J.L., Milliken, D., Stamp, G., and Balkwill, F.R., 2001, Epithelial cancer cell migration: a role for chemokine receptors?. Cancer Res, 61:4961-4965.
26. Geminder, H., Sagi-Assif, O., Goldberg, L., Meshel, T., Rechavi, G.,Witz, I.P., and Ben Baruch, A., 2001, A possible role for CXCR4 and its ligand, the CXC chemokine stromal cell-derived factor-1, in the development of bone marrow metastases in neuroblastoma. J Immunol, 167:4747-4757.
27. Libura, J., Drukala, J., Majkam M,, Tomescu, O., Navenot, J.M., Kucia, M., Marquez, L., Peiper, S.C.,

Barr, F.G., Janowska-Wieczorek, A., and Ratajczak, M.Z., 2002, CXCR4-SDF-1 signaling is active in rhabdomyosarcoma cells and regulates locomotion, chemotaxis, and adhesion. Blood, 100:2597-2606.

28. Luca, M., Huang, S., Gershenwald, J.E., Singh, R.K., Reich, R., Bar-Eli, M., 1997, Expression of interleukin-8 by human melanoma cells up-regulates MMP-2 activity and increases tumor growth and metastasis. Am J Pathol, 151:1105-1113.
29. Payne, A.S., and Cornelius, L.A., 2002, The role of chemokines in melanoma tumor growth and metastasis. J Invest Dermatol, 118:915-922.
30. Li, A., Dubey, S., Varney, M.L., Dave, B.J., and Singh, R.K., 2003, IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. J Immunol, 170:3369-3376.
31. Kitadai,Y., Haruma, K., Mukaida, N., Ohmoto, Y., Matsutani, N., Yasui, W., Yamamoto, S., Sumii, K., Kajiyama, G., Fidler, I.J., and Tahara, E., 2000, Regulation of disease-progression genes in human gastric carcinoma cells by interleukin 8. Clin Cancer Res, 6:2735-2740.
32. Li, A., Varney, M.L., and Singh, R.K., 2001, Expression of interleukin 8 and its receptors in human colon carcinoma cells with different metastatic potentials. Clin Cancer Res 7:3298-3304.
33. Kuwada, Y., Sasaki, T., Morinaka, K., Kitadai, Y., Mukaida, N., and Chayama, K., 2003, Potential involvement of IL-8 and its receptors in the invasiveness of pancreatic cancer cells. Int J Oncol, 22:765-771.
34. Mashino, K., Sadanaga, N., Yamaguchi, H., Tanaka, F., Ohta, M., Shibuta, K., Inoue, H., and Mori, M., 2002, Expression of chemokine receptor CCR7 is associated with lymph node metastasis of gastric carcinoma. Cancer Res, 62:2937-2941.
35. Ding,Y., Shimada, Y., Maeda, M., Kawabe, A., Kaganoi, J., Komoto, I., Hashimoto, Y., Miyake, M., Hashida, H., and Imamura, M., 2003, Association of CC chemokine receptor 7 with lymph node metastasis of esophageal squamous cell carcinoma. Clin Cancer Res, 9:3406-3412.
36. Takanami, I., 2003, Overexpression of CCR7 mRNA in non-small cell lung cancer: correlation with lymph node metastasis. Int J Cancer, 105:186-189.
37. Loetscher, M., Geiser, T., O'Reilly, T., Zwahlen, R., Baggiolini, M., and Moser, B., 1994, Cloning of a human seven-transmembrane domain receptor, LESTR, that is highly expressed in leukocytes. J Biol Chem, 269:232-237.
38. Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R.E., Hill, C.M., Davis, C.B., Peiper, S.C., Schall, T.J., Littman, D.R., and Landau, N.R., 1996, Identification of a major co-receptor for primary isolates of HIV-1. Nature, 381:661-666.
39. Feng,Y., Broder, C.C., Kennedy, P.E., and Berger, E.A., 1996, HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science, 272:872-877.
40. Bleul, C.C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., and Springer, T.A., 1996, The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. Nature, 382:829-833.
41. Oberlin, E., Amara, A., Bachelerie, F., Bessia, C., Virelizier, J.L., Arenzana-Seisdedos, F., Schwartz, O., Heard, J.M., Clark-Lewis, I., Legler, D.F., Loetscher, M., Baggiolini, M., and Moser, B., 1996, The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. Nature, 382:833-835.
42. Nagasawa,T., Kikutani, H., and Kishimoto, T., 1994, Molecular cloning and structure of a pre-B-cell growth-stimulating factor. Proc Natl Acad Sci U.S.A, 91:2305-2309.
43. Zou, Y.R., Kottmann, A.H.,Kuroda, M., Taniuchi, I., and Littman, D.R., 1998, Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature, 393:595-599.
44. Sozzani, S., Luini, W., Borsatti, A., Polentarutti, N., Zhou, D., Piemonti, L., D'Amico, G., Power, C.A., Wells, T.N., Gobbi, M., Allavena, P., and Mantovani, A., 1997, Receptor expression and responsiveness of human dendritic cells to a defined set of CC and CXC chemokines. J Immunol, 159:1993-2000.
45. Bleul, C.C., Wu, L., Hoxie, J.A., Springer, T.A., and Mackay, C.R., 1997, The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. Proc Natl Acad Sci U.S.A., 94:1925-1930.
46. Murdoch, C., Monk, P.N., and Finn, A., 1999, Functional expression of chemokine receptor CXCR4 on human epithelial cells. Immunology, 98:36-41.
47. Deichmann, M., Kronenwett, R., and Haas, R., 1997, Expression of the human immunodeficiency virus type-1 coreceptors CXCR-4 (fusin, LESTR) and CKR-5 in CD34+ hematopoietic progenitor cells. Blood, 89:3522-3528.
48. Hesselgesser, J., Halks-Miller, M., DelVecchio, V., Peiper, S.C., Hoxie, J., Kolson, D.L., Taub, D., and Horuk, R., 1997, CD4-independent association between HIV-1 gp120 and CXCR4: functional chemokine receptors are expressed in human neurons. Curr Biol, 7:112-121.
49. Wang, J.F., Liu, Z.Y., and Groopman, J.E., 1998, The alpha-chemokine receptor CXCR4 is expressed on the megakaryocytic lineage from progenitor to

platelets and modulates migration and adhesion. Blood, 92:756-764.

50. Zaitseva, M., Blauvelt, A., Lee, S., Lapham, C.K., Klaus-Kovtun, V., Mostowski, H., Manischewitz, J., and Golding, H., 1997, Expression and function of CCR5 a nd CXCR4 on human Langerhans cells and macrophages: implications for HIV primary infection. Nat Med, 3:1369-1375.
51. Gupta, S.K., Lysko, P.G., Pillarisetti, K., Ohlstein, E., and Stadel, J.M., 1998, Chemokine receptors in human endothelial cells. Functional expression of CXCR4 and its transcriptional regulation by inflammatory cytokines. J Biol Chem, 273:4282-4287.
52. Ma, Q., Jones, D., Borghesani, P.R., Segal, R.A., Nagasawa, T., Kishimoto, , Bronson, R.T., and Springer, T.A., 1998, Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4 and SDF-1-deficient mice. Proc Natl Acad Sci U.S.A., 95:9448-9453.
53. Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T., 1996, Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature, 382:635-638.
54. Tachibana, K., Hirota, S., Iizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., Kitamura, Y., Matsushima, K., Yoshida, N., Nishikawa, S., Kishimoto, T., and Nagasawa, T., 1998, The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature, 393:591-594.
55. Peled, A., Petit, I., Kollet, O., Magid, M., Ponomaryov, T., Byk, T., Nagler, A., Ben Hur, H., Many, A., Shultz, L., Lider, O., Alon, R., Zipori, D., and Lapidot, T., 1999, Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science, 283:845-848.
56. Molyneaux, K.A., Zinszner, H., Kunwar, P.S., Schaible, K., Stebler, J., Sunshine, M.J., O'Brien, W., Raz, E., Littman, D., Wylie, C., and Lehmann, R., 2003, The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. Development, 130:4279-4286.
57. Ara, T., Nakamura, Y., Egawa, T., Sugiyama, T., Abe, K., Kishimoto, T., Matsui, Y., and Nagasawa. T., 2003, Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1). Proc Natl Acad Sci U.S.A., 100:5319-5323.
58. Doitsidou, M., Reichman-Fried, M., Stebler, J., Koprunner, M., Dorries, J., Meyer, D., Esguerra, C.V., Leung, T., and Raz, E., 2002, Guidance of primordial germ cell migration by the chemokine SDF-1. Cell, 111:647-659.
59. Knaut, H., Werz, C., Geisler, R., and Nusslein-Volhard, C., 2003, A zebrafish homologue of the chemokine receptor Cxcr4 is a germ-cell guidance receptor. Nature, 421:279-282.
60. Klein, R.S., Rubin, J.B., Gibson, H.D., De Haan, E.N., Alvarez-Hernandez, X., Segal, R.A., and Luster, A.D., 2001, SDF-1 alpha induces chemotaxis and enhances Sonic hedgehog-induced proliferation of cerebellar granule cells. Development, 128:1971-1981.
61. Lataillade, J.J., Clay, D., Dupuy, C., Rigal, S., Jasmin, C., Bourin, P., and Bousse-Kerdiles, M.C., 2000, Chemokine SDF-1 enhances circulating CD34(+) cell proliferation in synergy with cytokines: possible role in progenitor survival. Blood, 95:756-768.
62. Kijima, T., Maulik, G., Ma, P.C., Tibaldi, E.V., Turner, R.E., Rollins, B., Sattler, M., Johnson, B.E., and Salgia, R., 2002, Regulation of cellular proliferation, cytoskeletal function, and signal transduction through CXCR4 and c-Kit in small cell lung cancer cells. Cancer Res, 62:6304-6311.
63. Scotton, C.J., Wilson, J.L., Scott, K., Stamp, G., Wilbanks, G.D., Fricker, S., Bridger, G., and Balkwill, F.R., 2002, Multiple actions of the chemokine CXCL12 on epithelial tumor cells in human ovarian cancer. Cancer Res, 62:5930-5938.
64. Barbero, S., Bonavia, R., Bajetto, A., Porcile, C., Pirani, P., Ravetti, J.L., Zona, G.L., Spaziante, Florio, R.T., and Schettini, G., 2003, Stromal cell-derived factor 1alpha stimulates human glioblastoma cell growth through the activation of both extracellular signal-regulated kinases 1/2 and Akt. Cancer Res, 63:1969-1974.
65. La Rivière, G., Schipper, C., Collard, J.G., and Roos, E., 1988, Invasiveness in hepatocyte and fibroblast monolayers and metastatic potential of T-cell hybridomas in mice. Cancer Res. 48:3405-3410
66. Soede, R.D.M., Wijnands, Y.M., Van Kouteren-Cobzaru, I., and Roos, E., 1998, ZAP-70 tyrosine kinase is required for LFA-1-dependent T-cell migration and invasion. J Cell Biol, 142:1371-1379.
67. Soede, R.D.M., Zeelenberg, I.S., Wijnands, Y.M., Kamp, M., and Roos, E., 2001, SDF-1-induced LFA-1 activation during T-lymphoma dissemination requires Gq/11, RhoA and myosin, as well as Gi and Cdc42. J Immunol, 166:4293-4301.
68. Stroeken, P.J.M., Van Rijthoven, E.A., Van der Valk, M.A., and Roos, E., 1998, Targeted disruption of the beta-1 integrin gene in a lymphoma cell line greatly reduces metastatic capacity. Cancer Res, 58: 1569-1577.
69. Soede, R.D.M., Wijnands, Y.M., Kamp, M., Van der Valk, M.A., and Roos, E., 2000, Gi and Gq/11 proteins are involved in dissemination of myeloid leukemia cells to liver and spleen, whereas bone

marrow colonization involves Gq/11 but not Gi. Blood, 96: 691-698.

70. Chen, J.D., Bai, X., Yang A.G., Cong, Y., and Chen, S.Y., 1997, Inactivation of HIV-1 chemokine co-receptor CXCR-4 by a novel intrakine strategy. Nature Med, 3: 1110-1116.
71. Kang, Y., Siegel, P.M., Shu, W., Drobnjak, M., Kakonen, S.M/, Cordon-Cardo, C., Guise, T.A., and Massagué, J. 2003, A multigenic program mediating breast cancer metastasis to bone. Cancer Cell, 3:537-549.
72. Cardones, A.R., Murakami, T., and Hwang, S.T., 2003, CXCR4 enhances adhesion of B16 tumor cells to endothelial cells in vitro and in vivo via beta-1 integrin. Cancer Res, 63:6751-6757.
73. Fidler, I.J., 1970, Metastasis: quantitative analysis of distribution and fate of tumor emboli labeled with 125 I-5-iodo-2'-deoxyuridine. J Natl Cancer Inst, 45:773-782.
74. Liotta, L.A., Vembu, D., Saini, R.K., and Boone, C., 1978, In vivo monitoring of the death rate of artificial murine pulmonary micrometastases. Cancer Res, 38:1231-1236.
75. Staller, P., Sulitkova, J., Lisztwan, J., Moch, H., Oakeley, E.J., and Krek, W., 2003, Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. Nature, 425:307-311.
76. Schioppa, T., Uranchimeg, B., Saccani, A., Biswas, S.K., Doni, A., Rapisarda, A., Bernasconi, S., Saccani, S., Nebuloni, M., Vago, L., Mantovani, A., Melillo, G., and Sica, A., 2003, Regulation of the chemokine receptor CXCR4 by hypoxia. J Exp Med, 198:1391-1402.
77. Kijowski, J., Baj-Krzyworzeka, M., Majka, M., Reca, R., Marquez, L.A., Christofidou-Solomidou, M., Janowska-Wieczorek, A., and Ratajczak, M.Z., 2001, The SDF-1-CXCR4 axis stimulates VEGF secretion and activates integrins but does not affect proliferation and survival in lymphohematopoietic cells. Stem Cells, 19:453-466.
78. Tilton, B., Ho, L., Oberlin, E., Loetscher, P., Baleux, F., Clark-Lewis, I., and Thelen, M., 2000, Signal transduction by CXC chemokine receptor 4. Stromal cell-derived factor 1 stimulates prolonged protein kinase B and extracellular signal-regulated kinase 2 activation in T lymphocytes. J Exp Med, 92:313-324.
79. Del Corno, M., Liu, Q.H., Schols, D., De Clerq, E., Gessani, S., Freedman, B.D. and Collman, R.G., 2001, HIV-1 gp120 and chemokine activation of Pyk2 and mitogen-activated protein kinases in primary macrophages mediated by calcium-dependent, pertussis toxin-insensitive chemokine receptor signaling. Blood, 98:2909-2916.
80. Doranz, B.J., Grovit-Ferbas, K., Sharron, M.P., Mao, S.H., Goetz, M.B., Daar, E.S., Doms, R.W., and O'Brien, W.A., 1997, A small-molecule inhibitor directed against the chemokine receptor CXCR4 prevents its use as an HIV-1 coreceptor. J Exp Med 186:1395-1400.
81. Donzella, G.A., Schols, D., Lin, S.W., Este, J.A., Nagashima, K.A., Maddon, P.J., Allaway, G.P., Sakmar, T.P., Henson, G., De Clercq, E., and Moore, J.P., 1998, AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. Nature Med, 4:72-77.
82. Hendrix, C.W., Flexner, C., MacFarland, R.T., Giandomenico, C., Fuchs, E.J., Redpath, E., Bridger, G., and Henson, G.W., 2000, Pharmacokinetics and safety of AMD-3100, a novel antagonist of the CXCR4 chemokine receptor, in human volunteers. Antimicrob Agents Chemother, 44:1667-1673.
83. Doranz, B.J., Filion, L.G., Diaz-Mitoma, F., Sitar, D.S. Sahai, J., Baribaud, F., Orsini, M.J., Benovic, J.L., Cameron, W., Doms, R.W., 2001, Safe use of the CXCR4 inhibitor ALX40-4C in humans. AIDS Res. Hum. Retroviruses, 17: 475-486.

Chapter 14

Energy Homeostasis and the Tumor/Host Interaction: The role of the Brain

Michael F. Wiater
Program in Neuroscience, College of Veterinary Medicine, Washington State University, Pullman, WA 99164-6520, USA

Abstract: The defensive regulation of energy homeostasis by neural and endocrine systems is examined to evaluate the role of the brain in macroenvironmental metabolic control systems that help counterattack the aggressive tumor. Brain homeostatic mechanisms (neural and hormonal) discussed are those linked to metabolic rhythms, food intake and adiposity. Homeostasis is discussed in terms of rheostasis, the low probability of dysregulation, and the potential risks for the defense. The perspective of this review is that many of the metabolic alterations observed in tumor progression may be due to appropriate central homeostatic regulation. Clear deficits in homeostatic regulation during tumor growth have not been unequivocally demonstrated. Yet the brain of the host is clearly under duress due to the tumor. Clarification of homeostatic macroenvironmental regulatory responses may be useful in developing strategies that collaborate with these brain mechanisms. Further analysis of these regulatory systems may identify key changes that ultimately subserve the lethal failure of all host responses. Strategies that protect the brain from the pathological consequences of tumor growth (glucoprivation, oxidative stress, ketogenic diets) and thereby enable a stronger defense are discussed.

Key words: Anorexia, cachexia, circadian rhythms, glucoprivation, ketogenic diet, leptin, melatonin, neuropeptide Y, oxidative stress, suprachiasmatic nucleus

1. ENERGY HOMEOSTASIS

Considerable attention has been focused in recent years on the microenvironmental interaction between the host and tumor, yet relatively little attention has been focused on the macroenvironmental interaction between the host and tumor, particularly with the role of the brain. This is curious because the brain is the site of potent metabolic homeostatic defense systems. Much of what occurs locally is monitored and adjusted centrally. Indeed, disparity in outcomes between *in vitro* and *in vivo* analyses is often due to interactive contributions by the brain.

Homeostasis is regulated through dynamic control mechanisms that defend the host from perturbations that threaten the miliu intérieur (1-4). These energy-related phenotypes are central to the evolved organization of the higher organism that may host a tumorous neoplasm. The macroenvironmental management of homeostasis occurs in the brain where there are regulatory mechanisms for the complex control of food intake (5-11), adiposity (6-12), glucose (13-14), and metabolic rhythms (14-22) that rely in part on peripheral information derived from hormonal and neural input and in part on the local environment within the brain (e.g., glucose sensing). During the progression of aggressive tumor growth these defended parameters in the host are often altered to manifest a metabolic syndrome that features unusual rhythms (23-26), glucose intolerance (27-28), insulin

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 245-258.

resistance (27-28), increased gluconeogenesis (29), increased free fatty acid turnover, oxidation and clearance (30), cachexia (31-32) and anorexia (31-32).

The perspective of this review is that many of the metabolic alterations observed in tumor progression may be due to central homeostatic regulation that is often appropriate, sometimes disturbed, but seldom dysregulated. Whether the response is appropriate or disturbed has been difficult to establish. For example, food restriction inhibits tumor growth and the host response to the tumor is to inhibit food intake (anorexia/cachexia) (33-36). If food restriction is additionally reduced by fasting the tumor grows faster[37]. Thus the brain seems to have achieved just the right balance of food intake and food restriction to minimize the growth potential of the tumor. Yet there are risks for this strategy due to the dire long-term consequences of anorexia/cachexia. Similarly, the brain and the tumor compete for glucose and the brain can enhance gluconeogenesis to meet its glucose needs.

Clarification of homeostatic macroenvironmental regulatory responses may be useful in developing strategies that collaborate with these brain mechanisms. Moreover, further analysis of these regulatory systems may identify the key events that ultimately subserve the lethal failure of all host responses. For example, homeostatic systems may be misled by or blind to the re-activated embryogenic systems often observed in malignant tumors (e.g., proteolysis-inducing factor (31)). Thus the etiology of lethality in cancer pathology may plausibly be due to tumor factors that disturb, foil, or overwhelm homeostatic regulation, even when that regulation is working appropriately.

Progress has been made in the last decade that furthers our understanding of some key neural circuits utilized for energy homeostasis, in particular the integrated hypothalamic and brainstem circuits for metabolic rhythms, feeding, fuel, adiposity. Key hormonal metabolic signals to the brain (e.g., leptin) have been identified and extensively examined. Cross-talk between the research fields of energy homeostasis and cancer may be fruitful for both disciplines.

Evidence on the role of macroenvironmental regulatory mechanisms residing in the brain for energy homeostasis as an adaptive and/or maladaptive response by the host to tumor progression will be briefly reviewed in this chapter.

2. HOMEOSTASIS, RHEOSTASIS AND DYSREGULATION

Much of medicine is assisted homeostasis. But parsing malignant metabolism and parceling out the contribution of the host and the tumor can be difficult. The tumor perturbs, the host responds. Most of the metabolic characteristics of malignant metabolism probably reflect the interaction of host and tumor.

The metabolic syndrome that occurs during cancer malignancy has some features of starvation, infection and late term pregnancy[33] as well as some features of another metabolic syndrome, diabetes. In each analogy however there are significant differences and in the final analysis cancer appears to induce its own metabolic syndrome[38]. As with diabetes the contribution of host homeostatic mechanisms to each of these metabolic alterations has been difficult to assess. And certainly there are significant differences in etiology for each metabolic syndrome. But just as certainly there are common features and a limited set of control system weapons to work with in the defense of energy homeostasis. To understand that set of control systems is one aim of those who study energy homeostasis.

Altered homeostatic parameters alone do not constitute dysregulatory pathology. Indeed it may be more appropriate to reconsider homeostasis as rheostasis where the defended level changes, but the defense of the level remains intact. For example, as Mrosovsky (4) has eloquently articulated, hibernation, starvation, infection, and pregnancy are physiological conditions where appropriate regulatory changes occur. The organism might run a fever, not eat, conserve energy and/or reduce activity. Each of these adaptations may become pathological but each represents a risk that favors survival. Obesity, for example, derived as a phenotype from adaptive selection that favored survival during sustained food deprivation (39). The fat survived. Hypertension may be required to adequately provide circulation especially when there

is about a mile of additional capillary length for each pound of fat. Hyperglycemia may assure glucose delivery during insulin insufficiency (40). Fever fights infection but may itself become pathological. These examples suggest that pathology does not require dysregulation.

Indeed true dysregulation is probably rare and would best be characterized by no defense at all. The host seldom misinterprets or is blind to the homeostatic insult, especially in chronic conditions. One important example may help clarify this point. Hypoglycemia is an acute risk in insulin treated diabetes and can be fatal. The phenomenon is called hypoglycaemia-associated autonomic failure (HAAF) and is often referred to as hypoglycemia unawareness (41). In HAAF the regulatory responses transduced in the medullary brainstem (e.g., glucoprivic feeding, stimulation of epinephrine, glucocorticoid and glucagon secretion, enhanced gluconeogenesis) do not occur (42). When studied in rats HAAF was produced by an acute prior induction of hypoglycemia during which the normal regulatory responses occurred, followed in a few hours by a second bout of hypoglycemia. During the second bout the normal regulatory responses did not occur. Hypoglycemia unawareness in these rats, however, did not occur when glucocorticoids were removed by prior adrenalectomy (42). Thus we see that hypoglycemia unawareness may reflect either 1. homeostatic unawareness; 2. a regulated, yet adverse, reaction to a competition between homeostatic responses (acquisition of glucose versus stress induced glucocorticoids); or 3. a regulated, yet adverse, reaction to components of the same homeostatic response (acquisition of glucose) in which glucocorticoids may play a direct role.

The lesson then is that dysregulation can not be assumed to occur. A demonstration of a changed response, or even an inappropriate non-response, is not necessarily a demonstration of dysregulation. In addition, integrated responses to complex challenges can create competition between regulatory subsystems, which may result in a lack of metabolic stability[4]. When not extreme such a competition is usually resolved with metabolic stability. For example, rats will leave a warm environment to acquire food in a cold environment (-15º C) but they will eat that food as quickly as possible (43). The suggestion from this perspective on homeostasis during cancer is that the identification, and experimental manipulation, of environmental impediments (e.g., glucoprivation, cyclic AMP deficits, and oxidative stress) on function is teleologically necessary in order to understand the brain-derived defense of the energy realm. In addition, experiments on the direct impact of tumor-derived molecules (e.g., proteolysis-inducing factor and Zn-alpha (2)-glycoprotein (31) on brain outcomes are needed, as are further analysis of brain energy systems under the influence of a malignant tumor.

Clearly, without further study, the complex perturbations caused by cancer that render the host inadequately defended will continue to a lethal outcome. Assisted homeostasis through a better understanding of the role of the brain in cancer may be critical in tipping the counterbalance of this defense in favor of the host.

3. ENERGY HOMEOSTASIS CIRCUITS IN THE BRAIN

Neural and neuroendocrine mechanisms for the defense of energy homeostasis have been extensively elaborated on during the last decade (5-12, 14-22, 44-48). These reviews have identified the regulatory signal to the brain from adipose tissue as the cytokine leptin, a hormone derived primarily from adipose tissue. Leptin levels in the circulation reflect the level of adiposity. The administration of exogenous leptin to rodents potently reduces body weight (49-55). This reduction in body weight is almost entirely due to loss of stored lipid fuels and is not due to loss of protein (56-58). This pattern differs from starvation, during which both lipid and protein are lost (59-60).

Leptin's central actions are critical for its effects on feeding and body weight. First, hypophagia can be produced by central administration of leptin at doses several fold lower than equally-effective systemic doses, and this effect is achieved without any increase in the circulating levels of leptin (56, 61), either by efflux from the cerebral spinal fluid into venous blood or by enhancing peripheral leptin

secretion. Second, central administration of leptin is sufficient to reverse the obesity of ob/ob ($Lepr^{ob}$/$Lepr^{ob}$) mice lacking the gene for leptin (50). Although peripheral administration of leptin also causes anorexia and weight loss, these actions of leptin may be mediated centrally since leptin is able to gain access to the brain from the circulation. Finally, selective deletion of neuronal leptin receptors abolished leptin's anorexic effect and resulted in obesity in mice (62); and epigenetic expression of leptin receptors only in the brain reversed the obese phenotype of the db/db mouse ($Lepr^{db}$/$Lepr^{db}$), which is without leptin receptors due to an autosomal recessive mutation (63).

The related cytokines ciliary neurotrophic factor (CNTF), interleukin-6 (IL-6) and leukemia inhibitory factor (LIF), which signal through the highly related glycoprotein-130 (gp-130) JAK/STAT receptor circuitry, also dramatically decrease fat pads to a similar degree when chronically given centrally into the ventricles (64-68). These members of the interleukin (IL)-6-receptor family of cytokines appear to redundantly activate a brain system that controls fat mass to the extent that rodent models live in good health without adipose tissue for most of their adult life (68). Redundancy in signalling is a common feature of this system (69-71).

Yet these cytokines that employ the gp-130 signal transducing subunit also interact with specificity (69). Oncostatin M (OSM) regulated IL-6 expression in glia, but LIF did not (72). OSM did not produce anorexia, but LIF and interleukin-11 (IL-11) did produce anorexia in short-term feeding tests (68).

Numerous neuropeptide effectors in the brain that are implicated in the control of feeding occur downstream of leptin and have been extensively studied (5-12, 14-22, 45-48, 73, 74). Orexigenic neuropeptides inhibited by leptin include neuropeptide Y (NPY) (75-76), galanin (77), melanin concentrating hormone (MCH) (75) and agouti-related peptide (AGRP) (75-76). Anorexigenic neuropeptides stimulated by leptin include corticotropin releasing hormone (77-78), glucagon-like peptide 1 (GLP-1)79, alpha-melanocyte stimulating hormone (80), neurotensin (75, 81) and cocaine- and amphetamine-regulated transcript (CART) (82). Neurotrophins such as brain-derived neurotropin factor (BDNF) (83, 84), CNTF (85-88) and the neurotropin regulated VGF (90-91) have emerged as important in this matrix of neural signals necessary for energy homeostasis, as has leukemia inhibitory factor (LIF) (85, 89). The specific role of each signalling agent is beginning to be clarified (83). Thus there is an extensive brain circuitry that subserves the control of food intake (e.g., anorexia, hypogeusia, hyposmia, nausea, satiation, aversion, and appetite), metabolism (e.g., gluconeogenesis, free fatty acid release, compartmentalization of fuels), adiposity (fat cachexia) and perhaps muscle mass (muscle cachexia).

4. WHY IS BRAIN CIRCUITRY FOR ENERGY HOMEOSTASIS OF INTEREST IN CANCER?

In the sections that follow six particular topics will be briefly considered with each section providing only a partial, and necessarily incomplete, suggested answer to this question. More complex brain functions that affect the host/tumor interaction but involve external events and behaviors that transcend energy homeostasis (e.g., biopsychosocial oncology (93) or psychoneuroimmunology (94), such as social stress (95), social defeat (96) or social dominance (97) will not be discussed because these topics are beyond the scope of this review.

4.1 Regulatory feeding responses appear intact during tumor progression

Although anorexia, cachexia and numerous metabolic disturbances are manifest in many cancer models, the normal controls of feeding are still substantially intact (33-34). That is, the anorexia can be returned to normophagia under some experimental conditions. In a classic review Seoras Morrison asked his version of the central question of cachexia/anorexia: “Why the host does not respond to change in need with change in intake?” (33). Why doesn’t the energy-depleted host simply invoke hyperphagia? For example, cold exposure (5º C vs.

24º C) for two days normalized food intake in rats with tumor-induced anorexia (98). And the hyperphagia induced by insulin-induced glucoprivation was intact in Walker 256 carcinoma-bearing Sprague-Dawley rats (34). Interestingly, obese mice maintained on a food for which they had developed a conditioned taste aversion lived longer with B16 melanoma than those maintained on a food that was not aversive (99). These examples indicate that the metabolic changes observed during tumor progression may be due to a regulatory response to the tumor and are not due to incapacity of the regulatory system.

Moreover cachexia and anorexia do not require the presence of the tumor (34). As with parabiosis (100), direct transfer of blood from a cancer-bearing host to a non-cancer-bearing host results in cachexia/anorexia without a tumor (101). And, indeed, removal of the tumor, except in the late stages of cachexia, reverses both the anorexia and cachexia (34, 102). Thus the cachectic/anorectic response interaction by the host to the tumor does not require the tumor and does not require structural alterations to the host that can not be rapidly adjusted.

The answer to Seoras Morrison's central question, therefore, may be that the host has already responded appropriately to the tumor with just the right amount of food restriction.

4.2 There is an altered energy environment within the brain during tumor progression

Alteration in homeostatic function may occur because of alterations in the metabolic environment within the brain, as a distant function of tumor growth. Tumor growth involves vascular leakiness within the tumor induced by a unique tumor angiogenesis that produces a necrotic condition characterized by hypoxia, elevated glycolysis even under aerobic conditions, high glucose turnover, glucose deprivation, adverse acidity and increased interstitial fluid pressure (103-104). These elements of tumor growth alter the brain environment by reducing glucose availability and increasing oxidative stress.

The high glucose turnover of the aggressive tumor reduced glucose utilization by the brain and increased utilization of lactate and 3-hydroxybutyrate (ketones) (105). Indeed it is probable that the proximal cause of death with rapidly growing non-metastasizing tumors is often acute hypoglycemia (106). Survival for sarcoma-bearing hypoglycemic mice was not enhanced by administration of glucagon, but drinking of glucose by food-deprived mice did enhance survival. And adrenalectomy shortened survival time (106).

The homeostatic responses to glucoprivation are transduced in the ventrolateral medulla of the brainstem (107-108) and include hyperphagia, gluconeogenesis and thus the sympathoadrenal stimulation of epinephrine, glucagon and glucocorticoids. The need to provide nutrients to the brain quickly is paramount during acute hypoglycemia.

One way to reduce the brain's need for glucose, and the adverse consequences of oxidative stress, is to substitute ketones for glucose as fuel for the brain (109). Several reports (110-116) have examined the mostly positive effects of a ketogenic diet based on medium-chain triglycerides on non-brain tumor cachexia and tumor growth but not on survival rates or brain function. Glycerol supplementation may also be helpful (117). And recently a direct role for glucose supplementation has been beneficial (118). Additional studies with diets that may support brain function during cancer are warranted. From the perspective of this review the support of brain function should be considered a different objective than ameliorating anorexia/cachexia.

Oxidative stress was increased within the brain, with highest levels of increased stress observed in the hypothalamus, during the growth of the Walker-256 tumor, due to the elevated rate of oxygen consumption, the high level of endogenous polyunsaturated fats (119) and iron (120). TNFα has been implicated in this increased stress (122). Specific sites within the hypothalamus are rich in TNF-α receptors, especially the paraventricular nucleus, the supraoptic nucleus and the arcuate nucleus (123). Central catecholamines are particularly susceptible to oxidative stress (123) and critical participants in energy homeostasis and especially in glucose regulation (107-108).

Oxidative injury to catecholamines has been reported in the locus coeruleus (124) and ventrolateral medulla (VLM) (125). The VLM is a critical site for transduction of numerous homeostatic responses, including those stimulated by hypoxia and glucoprivation. Melatonin functions as a neuroprotectant during oxidative stress to catecholamines in part due to upregulation of glial derived neurotrophic factor (GDNF) (124). Oxidative stress induced by the tumor stimulated glycose-6-phosphate dehydrogenase activity, pentose phosphate pathway activity and elevated flux of substrates, while brain mitochondrial activity was inhibited (119).

Thus in these two ways, and probably others (e.g., the aberrant presence in the brain of tumor-derived molecules), the brain environment is altered by the presence of the tumor.

4.3 Altered expression of neuropeptides and monoamines in brain circuitry for energy homeostasis during tumor malignancy

The hypothalamus is an important site for integrating metabolic information and regulating energy homeostasis and metabolism (5-9, 12, 14-15, 20, 73-78, 80, 82-83). Alterations in signalling within the hypothalamus during tumor progression have usually been reviewed in the context of cancer anorexia (31, 126-137). For example, there are reportedly decreased NPY fibers in anorectic tumor-bearing rats in the parvocellular region of the paraventricular nucleus, as well as in the supraoptic, suprachiasmatic and arcuate nuclei (138). Most of the NPY fibers in the hypothalamus are located in these nuclei. This decrease in NPY fibers tends to support these authors' hypothesis that the anorexia induced by the tumor alters the structure of the neuronal signalling pathway for food intake, of which NPY is one important part. Indeed, when NPY was acutely delivered directly into the perifornical hypothalamus in MCA sarcoma-bearing rats before the expression of anorexia hyperphagia in a four-hour food intake test was at first induced comparable to non-tumor bearing controls (139). While elevated food intake in tumor-bearing rats who received acute hypothalamic injections of NPY continued to occur during the ongoing experiment over the next two weeks and five injections, the elevation was greatly attenuated in tumor bearing rats compared to controls without the MCA sarcoma that received hypothalamic injections of NPY. A similar refractory response to perifornical NPY was observed with chronic minipump infusion (139). This refractory feeding response induced by perifornical NPY in tumor-bearing rats may reflect refractory adenylate cyclase AMP formation (131).

The effect of a tumor on NPY expression and signalling in the hypothalamus is far from clear, which may reflect differences between tumors, in the brain site examined, experimental designs, rodent strains, multiple roles for NPY and/or other factors. Some studies report decreased expression of NPY (137, 141-143), other studies report increased expression (144-148) and yet other studies report normal expression (149-150). A more complete critical review of the NPY feeding system and its alteration by a malignant tumor is overdue but beyond the scope of this review. However, further exploration of the NPY feeding system, and related systems within the prevue of brain regulated energy homeostasis, seems warranted. Indeed, the direct examination in tumor bearing rodents of the effects of infusion into the brain of either energy related molecules such as this orexigenic neuropeptide NPY, or of tumor-related molecules into non-tumor bearing hosts, seems to be a neglected area of cancer research with only a few examples (139, 151-152), even when experiments with proinflammatory cytokines (66, 67, 126, 131) are included. Perhaps to refocus research on brain pathology engendered by a malignant tumor and on brain responses to that tumor would reenergize research in this neglected area.

In addition to NPY, there are expression changes that occur for other neuropeptides and monoamines critical to neural communication in the circuitry for energy homeostasis during the progression of malignant tumors. Alterations in brain serotonin (141, 143), dopamine (141, 143), MCH (145), orexin (145) and interleukin-1 beta (150) have been reported.

Which subset of homeostatic controls these changes reflect (anorexia, cachexia, the regulation of

adiposity or muscle, metabolites) is unclear. But clearly the brain is reacting to the presence of the tumor with alterations in this critical neural network that serves energy homeostasis.

4.4 Destruction of the medial basal hypothalamus with gold thioglucose, or agouti blockade of the melanocortin-4 receptor, accelerates lipid wasting and demise in mice

In 1971 Liebelt and co-workers induced hyperphagia and obesity in two strains of mice by destruction of the medial basal hypothalamus with gold thioglucose (GTG) (153). They predicted that the hyperphagia induced by this treatment would override the hypophagia induced by either of two strains of tumor, the CBA 2663 stomach tumor or the C57Bl sarcoma. To their surprise a profound anorexia and cachexia were accelerated in these obese mice, who died by day twenty of tumor growth. The lean mice lived until the end of the experiment, at day thirty of tumor growth, and never expressed anorexia or cachexia. Tumor growth was not accelerated in the obese mice and the tumors remained small but lethal. Carcass weight loss was primarily lipid in the GTG tumor-bearing mice. A parallel experiment utilized the agouti yellow (Ay/a) obese mice, which have a dominant mutation of the agouti gene so that there is a blockade of the melanocortin-4 receptor and insensitivity to αMSH (154), produced the same pattern to tumor bearing.

There are several factors in common to these two mouse models of obesity. The ability of central leptin to reduce body weight, adiposity and food intake is attenuated in both the GTG mouse and the agouti yellow obese mouse (155-156). In both models there is insensitivity to POMC and αMSH157. And both obesity models require the neurotropin-induced polypeptide VGF (158), which is also important in energy homeostasis. Thus the circuitry of energy homeostasis in the hypothalamus is blocked with catastrophic consequence in tumor bearing mice. Further work in cancer with these models is warranted, but clearly both the circuitry and the site of action are important in the host defense against tumor lethality.

4.5 Suprachiasmatic nucleus-induced rhythms inhibit tumor growth

Time-keeping is important for energy homeostasis. Metabolism is processed with rhythm (14-22). Food is anticipated with activity (22). Insulin is secreted in anticipation of food (20). There are circadian rhythms for metabolites such as glucose (14), and metabolic hormones such as ghrelin and leptin (159). Food (21), or glucose, but not lipid, reset the biological clock (160). Disrupted sleep also disrupts these rhythms (161).

The suprachiasmatic nucleus (SCN) in the hypothalamus serves as a site for photic transduction of the light/dark cycle; as an entrainer for its endogenous clock; and, as a site for rhythm synchronization with non-photic stimuli (14, 17-20, 162, 163). Ablation of the SCN in rodents removes photic entrained rhythms and accelerates tumor growth (164, 165). In a recent experiment, Filipski and co-workers demonstrated that a simple advance of the light/dark cycle by eight hours every two days accelerated Glasgow osteosarcoma tumor growth (166).

Circadian rhythms are often disrupted in cancer and chronotherapies are currently under investigation for improved management of cancer (24, 26). Sleep disorders are a common feature of cancer (23). Brain derived rhythms inhibit tumor growth and sustain normal sleep. And the tumor may disrupt those rhythms. Thus there is a need for further investigation of the role of circadian rhythms in tumor growth and malignancy.

4.6 The pineal gland and melatonin

The pineal gland is located within the brain and is the source of the hormone melatonin (167-168). Both the pineal gland and melatonin are involved in energy homeostasis (169-170). And both melatonin and the pineal can interact with tumor growth, which has stimulated an interest in the antitumor properties of this hormonal system (171-176).

In one study the removal of the pineal gland stimulated the growth of melanoma (177). And in another study administration of melatonin shortened the survival rate of mice with the Ehrlich ascites

tumor (178). And overexpression of the melatonin MT1 receptor suppressed mammary tumor formation (179).

These examples, supported by correlational studies (171-176), have generated a recent surge of interest in the role of the pineal axis in the treatment of malignant tumors.

5. SUMMARY

The brain is the ultimate defender of energy homeostasis and cancer is the ultimate aggressor. The metabolic syndrome expressed in this terrible struggle is due in part to both a stout defense and a violent aggression. Disentangling this interaction requires further experiment but may prove to be important in the management of malignancy. Fundamental components of the defense are apparent and suggest that the brain is responding appropriately, with roles for particular sites (e.g., the medial basal hypothalamus, the suprachiasmatic nucleus, the pineal gland), that mediate the control of rhythms, food intake, and adiposity. The environment within the brain is altered by the tumor, which may compromise the defense. Analysis of the altered environment (e.g., glucoprivation, oxidative stress, tumor-derived molecules, alterations in neuropeptides and monomines) may lead to an improved defense. And, finally, analysis of the antitumor strategies of this brain-derived defense may lead to assisted homeostasis: interventions that serve to tip the counterbalance in favour of the host.

REFERENCES

1. Bernard, C., 1878, Leçons sur les phénomènes de la vie communs aux animaux et aux végétaux. J-B Baillière et fils, Paris, France.
2. Cannon, W.B., 1929, Organization for physiological homeostasis. Physiol Rev, 9:399-431.
3. Cannon, W.B., 1939, The wisdom of the body. W.W. Norton & Co. Inc., New York, NY, USA.
4. Mrosovsky, N., 1990, Rheostasis: the physiology of change. : Oxford University Press. New York
5. Saper, C.B., Chou, T.C., and Elmquist, J.K. 2002 The need to feed: homeostatic and hedonic control of eating. Neuron 36: 199-211.
6. Berthoud, H.R. 2002 Multiple neural systems controlling food intake and body weight. Neurosci Biobehav Rev 26: 393-428.
7. Blevins, J.E., Schwartz, M.W., and Baskin, D.G. 2002 Peptide signals regulating food intake and energy homeostasis. Can J Physiol Pharmacol 80: 396-406.
8. Horvath, T.L., Diano, S., and Tschop, M. 2004 Brain circuits regulating energy homeostasis. Neuroscientist 10: 235-46.
9. Flier, J., and Maratos-Flier, E. 2000 Energy homeostasis and body weight. Curr Biol 10: R215-7.
10. Havel, P.J. 2001 Peripheral signals conveying metabolic information to the brain: short-term and long-term regulation of food intake and energy homeostasis. Exp Biol Med (Maywood) 226: 963-77.
11. Meier, U., and Gressner, A.M. 2004 Endocrine regulation of energy metabolism: review of pathobiochemical and clinical chemical aspects of leptin, ghrelin, adiponectin, and resistin. Clin Chem 50: 1511-25.
12. Cowley, M.A., Pronchuk, N., Fan, W., Dinulescu, D.M., Colmers, W.F., and Cone, R.D. 1999 Integration of NPY, AGRP, and melanocortin signals in the hypothalamic paraventricular nucleus: evidence of a cellular basis for the adipostat. Neuron 24: 155-63.
13. Epstein, A.N., Nicolaïdis, S. and Miselis, R. 1975. The glucoprivic control of food intake and the glucostatic theory of feeding behaviour. Pp 146-168 in Neural integration of physiological mechanisms and behavior, Mogenson G.J. and Calaresu, F.R, eds. Toronto: University of Toronto Press.
14. La Fleur, S.E. 2003 Daily rhythms in glucose metabolism: suprachiasmatic nucleus output to peripheral tissue. J Neuroendocrinol 15: 315-22.
15. Kreier, F., Kalsbeek, A., Ruiter, M., Yilmaz, A., Romijn, J.A., Sauerwein, H.P., Fliers, E., and Buijs, R.M. 2003 Central nervous determination of food storage--a daily switch from conservation to expenditure: implications for the metabolic syndrome. Eur J Pharmacol 480: 51-65.
16. Schibler, U., Ripperger, J., and Brown, S.A. 2003 Peripheral circadian oscillators in mammals: time and food. J Biol Rhythms 18: 250-60.
17. Challet, E., Caldelas, I., Graff, C., and Pevet, P. 2003 Synchronization of the molecular clockwork by light- and food-related cues in mammals. Biol Chem 384: 711-9.
18. Rutter, J., Reick, M., and McKnight, S.L. 2002 Metabolism and the control of circadian rhythms. Annu Rev Biochem 71: 307-31.
19. Nagai, K., Nagai, N., Sugahara, K., Niijima, A., and Nakagawa, H. 1994 Circadian rhythms and energy metabolism with special reference to the

suprachiasmatic nucleus. Neurosci Biobehav Rev 18: 579-84.
20. Strubbe, J.H., and van Dijk, G. 2002 The temporal organization of ingestive behaviour and its interaction with regulation of energy balance. Neurosci Biobehav Rev 26: 485-98.
21. Stephan, F.K. 2002 The "other" circadian system: food as a Zeitgeber. J Biol Rhythms 17: 284-92.
22. Mistlberger, R.E. 1994 Circadian food-anticipatory activity: formal models and physiological mechanisms. Neurosci Biobehav Rev 18: 171-95.
23. Lee, K., Cho, M., Miaskowski, C., and Dodd, M. 2004 Impaired sleep and rhythms in persons with cancer. Sleep Med Rev 8: 199-212.
24. Sephton, S., and Spiegel, D. 2003 Circadian disruption in cancer: a neuroendocrine-immune pathway from stress to disease? Brain Behav Immun 17: 321-8.
25. Fu, L., and Lee, C.C. 2003 The circadian clock: pacemaker and tumour suppressor. Nat Rev Cancer 3: 350-61.
26. Mormont, M.C., and Levi, F. 2003 Cancer chronotherapy: principles, applications, and perspectives. Cancer 97: 155-69.
27. Argiles, J.M., Almendro, V., Busquets, S., and Lopez-Soriano, F.J. 2004 The pharmacological treatment of cachexia. Curr Drug Targets 5: 265-77.
28. Tayek, J.A. 1992 A review of cancer cachexia and abnormal glucose metabolism in humans with cancer. J Am Coll Nutr 11: 445-56.
29. Thompson, M.G, and Palmer, R.M. 1998 Signalling pathways regulating protein turnover in skeletal muscle. Cell Signal 10: 1-11.
30. Baron, A., Migita, T., Tang, D., and Loda, M. 2004 Fatty acid synthase: a metabolic oncogene in prostate cancer? J Cell Biochem 91: 47-53.
31. Tisdale, M.J. 2004 Tumor-host interactions. J Cell Biochem 93: 871-7.
32. van Halteren, H.K., Bongaerts, G.P., and Wagener, D.J. 2003 Cancer cachexia: what is known about its etiology and what should be the current treatment approach? Anticancer Res 23: 5111-5.
33. Morrison, S.D. 1976 Control of food intake in cancer cachexia: a challenge and a tool. Physiol Behav 17: 705-714.
34. Morrison, S.D. 1981 Control of food intake in experimental tumor growth. Cancer Treat Rep 65(Suppl 5): 9-14.
35. Molotkov, A., Satoh, M., and Tohyama, C. 1998 Tumor growth and food intake in interleukin-6 gene knock-out mice. Cancer Lett 132: 187-92.
36. Thompson, C.I., Kreider, J.W., and Margules, D.L. 1984 Food intake during tumor growth: anorexia in genetically obese ob/ob mice and hyperphagia in lean mice. Physiol Behav 32: 935-9.
37. Rofe, A.M., Porter, S.J., Bais, R., and Coyers, R.A.J. 1985 The metabolic response of tumour-bearing mice to fasting. Br J Cancer 52: 619-623.
38. Devereux, D.F., Redgrave, T.G., Loda, M.F., Clowes, G.H. Jr, and Deckers, P.J. 1985 Tumor-associated metabolism in the rat is a unique physiologic entity. J Surg Res 38: 149-53.
39. Coleman, D.L. 1979 Obesity genes: beneficial effects in heterozygous mice. Science 203: 663-5.
40. Cabanac, M. and Russek, M. 1982 Régulation et contrôle en biologie. Les Presses de l'Université Laval, Québec.
41. Cryer, P.E. 2002 Hypoglycaemia: the limiting factor in the glycaemic management of Type I and Type II diabetes. Diabetologia 45: 937-48.
42. Sanders, N.M., and Ritter, S. 2001 Acute 2DG-induced glucoprivation or dexamethasone abolishes 2DG-induced glucoregulatory responses to subsequent glucoprivation. Diabetes 50: 2831-6.
43. Johnson, K.G., and Cabanac, M. 1982 Homeostatic competition between food intake and temperature regulation in rats. Physiol Behav 28: 675-9.
44. Mrosovsky, N. 1986 Body fat: what is regulated? Physiol Behav 38: 407-14.
45. Cancello, R., Tounian, A., Poitou, Ch., and Clement, K. 2004 Adiposity signals, genetic and body weight regulation in humans. Diabetes Metab 30: 215-27.
46. Wynne, K., Stanley, S., and Bloom, S. 2004 The gut and regulation of body weight. J Clin Endocrinol Metab 89: 2576-82.
47. Tremblay, A. 2004 Dietary fat and body weight set point. Nutr Rev 62: S75-7.
48. Cone, R.D., Cowley, M.A., Butler, A.A., Fan, W., Marks, D.L., and Low, M.J. 2001 The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. Int J Obes Relat Metab Disord 25 Suppl 5: S63-7.
49. Pelleymounter, M.A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T., and Collins, F. 1995 Effects of the obese gene product on body weight regulation in ob/ob mice. Science 269: 540-543
50. Campfield, L.A., Smith, F.J., Guisez, Y., Devos, R., and Burn, P. 1995 Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. Science 269: 546-549.
51. Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D., Lallone, R.L., Burley, S.K., and Friedman, J.M. 1995 Weight regulation effects of the plasma protein encoding the obese gene. Science 269: 543-6.
52. Matson, C.A., Wiater, M.F., and Weigle, D.S. 1996 Leptin and the regulation of body adiposity: A critical review. Diabetes Rev 4: 488-508.
53. Baile, C.A., Della-Fera, M.A., and Martin, R.J. 2000 Regulation of metabolism and body fat mass by leptin. Annu Rev Nutr 20: 105-27.

54. Spiegelman, B.M., and Flier, J.S. 2001 Obesity and the regulation of energy balance. Cell 104: 531-543.
55. Friedman, J.M., and Halaas, J.L. 1998 Leptin and the regulation of body weight in mammals. Nature 395: 763-70.
56. Azain, M.J., Wang, T., Hulsey, M.G., Qian, H., Hartzell, D.L., and Baile, C.A. 1999 Effects of intracerebroventricularly administered leptin on protein selection in the rat. Physiol Behav 66: 537-541.
57. Kaibara, A., Moshyedi, A., Auffenberg, T., Abouhamze, A., Copeland, E.M., III, Kalra, S., and Moldawer, L.L. 1998 Leptin produces anorexia and weight loss without inducing an acute phase response or protein wasting. Amer J Physiol 274: R1518-R1525.
58. Chen, Y., and Heiman, M.L. 2000. Chronic leptin administration promotes lipid utilization until fat mass is greatly reduced and preserves lean mass of normal female rats. Reg Peptides 92: 113-119.
59. Dallman, M.F., Akana, S.K., Bhatnagar, S., Bell, M.E., Choi, S., Chu, A., Horsley, C., Levin, N., Meijer, O., Soriano, L.R., Strack A.M., and Viau, V. 1999 Starvation: early signals, sensors, and sequelae. Endocrinology 140: 4015-4023.
60. Goodman, M.N., and Ruderman, N.B. 1980 Starvation in the rat. I. Effect of age and obesity on organ weights, RNA, DNA, and protein. Am J Physiol 239: E269-E276.
61. van Dijk, G., Seeley, R.J., Thiele, T.E., Friedman, M.I., Ji, H., Wilkinson, C.W., Burn, P., Campfield, L.A., Tenenbaum, R., Baskin, D.G., Woods, S.C., and Schwartz, M.W. 1999 Metabolic, gastrointestinal, and CNS neuropeptide effects of brain leptin administration in the rat. Am J Physiol 276: R1425-33.
62. Cohen, P., Zhao, C., Cai, X., Montez, J.M., Rohani, S.C. Feinstein, P., Mombaerts, P., and Friedman, J.M. 2001 Selective deletion of leptin receptor in neurons leads to obesity. J Clin Invest 108: 1113-21.
63. Kowalski, T.J., Liu, S.M., Leibel, R.L., and Chua, S.C. Jr. 2001 Transgenic complementation of leptin-receptor deficiency. I. Rescue of the obesity/diabetes phenotype of LEPR-null mice expressing a LEPR-B transgene. Diabetes 50: 425-35.
64. Duff, E., Li, C.L., Hartzell, D.L., Choi, Y.H., Della-Fera, M.A., and Baile, C.A. 2004 Ciliary neurotrophic factor injected icv induces adipose tissue apoptosis in rats. Apoptosis 9: 629-34.
65. Beretta, E., Dhillon, H., Kalra, P.S., and Kalra, S.P. 2002 Central LIF gene therapy suppresses food intake, body weight, serum leptin and insulin for extended periods. Peptides 23: 975-84.
66. Jansson, J.O., Wallenius, K., Wernstedt, I., Ohlsson, C., Dickson, S.L., and Wallenius, V. 2003 On the site and mechanism of action of the anti-obesity effects of interleukin-6. Growth Horm IGF Res 13 Suppl A: S28-32.
67. Wallenius, K., Wallenius, V., Sunter, D., Dickson, S.L., and Jansson, J.O. 2002 Intracerebroventricular interleukin-6 treatment decreases body fat in rats. Biochem Biophys Res Commun 293: 560-5.
68. Plata-Salaman, CR. 1996 Anorexia induced by activators of the signal transducer gp 130. Neuroreport 7: 841-4.
69. Ishihara, K., and Hirano, T. 2002 Molecular basis of the cell specificity of cytokine action. Biochim Biophys Acta 1592: 281-96.
70. Taga, T., and Kishimoto, T. 1997 gp130 and the interleukin-6 family of cytokines. Annu. Rev. Immunol. 15: 797–819.
71. Heinrich, P.C., Behrmann, I., Haan, S., Hermanns, H.M., Muller-Newen, G., and Schaper, F. 2003 Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J 374: 1-20.
72. Van Wagoner, N.J., Choi, C., Repovic, P., and Benveniste, E.N. 2000 Oncostatin M regulation of interleukin-6 expression in astrocytes: biphasic regulation involving the mitogen-activated protein kinases ERK1/2 and p38. J Neurochem 75: 563-75.
73. Hakansson, M.L., Brown, H., Ghilardi, N., Skoda, R.C., and Meister, B. 1998. Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus. J Neurosci 18: 559-72.
74. Schwartz, M.W., Seeley, R.J., Campfield, L.A., Burn, P., and Baskin, D.G. 1996 Identification of targets of leptin action in rat hypothalamus. J Clin Invest 98: 1101-6.
75. Sahu, A. 1998 Evidence suggesting that galanin (GAL), melanin-concentrating hormone (MCH), neurotensin (NT), proopiomelanocortin (POMC) and neuropeptide Y (NPY) are targets of leptin signaling in the hypothalamus. Endocrinology 139: 795-8.
76. Korner, J., Savontaus, E., Chua, S.C. Jr, Leibel, R.L., and Wardlaw, S.L. 2001 Leptin regulation of Agrp and Npy mRNA in the rat hypothalamus. J Neuroendocrinol 13: 959-66.
77. Costa, A., Poma, A., Martignoni, E., Nazppi, G., Ur, E., and Grossman, A. 1997 Stimulation of corticotrophin-releasing hormone release by the obese (ob) gene product, leptin, from hypothalamic explants. NeuroReport 8: 1131-34.
78. Uehara, Y., Shimizu, H., Ohtani, K., Sato, N., and Mori, M. 1998 Hypothalamic corticotropin-releasing hormone is a mediator of the anorexigenic effect of leptin. Diabetes 47: 890-93.
79. Mercer, J.G., Moar, K.M., Findlay, P.A., Hoggard, N., and Adam, C.L. 1998 Association of leptin receptor (OB-Rb), NPY, and GLP-1 gene expression in the ovine and murine brainstem. Regul Pept 75-76: 271-278.

80. Forbes, S., Bui, S., Robinson, B.R., Hochgeschwender, U., and Brennan, M.B. 2001 Integrated control of appetite and fat metabolism by the leptin-prooopiomelanocortin pathway. Proc Natl Acad Sci USA 98: 4233-4237.
81. Sahu, A., Carraway, R.E., and Wang, Y.P. 2001 Evidence that neurotensin mediates the central effect of leptin on food intake in rat. Brain Res 888: 343-347.
82. Kristensen, P., Judge, M.E., Thim, L., Ribel, U., Christjansen, K.N., Wulff, B.S., Clausen, J.T., Jensen, P.B., Madsen, O.D., Vrang, N., Larsen, P.J., and Hastrup, S. 1998 Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature 393: 72-76.
83. Pelleymounter, M.A., Cullen, M.J., and Wellman, C.L. 1995 Characteristics of BDNF-induced weight loss. Exp Neurol 131: 229-38.
84. Kernie, S.G., Liebl, D.J., and Parada, L.F. 2000 BDNF regulates eating behavior and locomotor activity in mice. EMBO J 19: 1290-300.
85. Prima, V., Tennant, M., Gorbatyuk, O.S., Muzyczka, N., Scarpace, P.J., and Zolotukhin, S. 2004 Differential modulation of energy balance by leptin, ciliary neurotrophic factor, and leukemia inhibitory factor gene delivery: microarray deoxyribonucleic acid-chip analysis of gene expression. Endocrinology 145: 2035-45.
86. Mattson, M.P. 2001 Lose weight STAT: CNTF tops leptin. Trends Neurosci 24: 313-4.
87. Duff, E., Li, C.L., Hartzell, D.L., Choi, Y.H., Della-Fera, M.A., and Baile, C.A. 2004 Ciliary neurotrophic factor injected icv induces adipose tissue apoptosis in rats. Apoptosis 9: 629-34.
88. Anderson, K.D., Lambert, P.D., Corcoran, T.L., Murray, J.D., Thabet, K.E., Yancopoulos, G.D., and Wiegand, S.J. 2003 Activation of the hypothalamic arcuate nucleus predicts the anorectic actions of ciliary neurotrophic factor and leptin in intact and gold thioglucose-lesioned mice. J Neuroendocrinol 15: 649-60.
89. Beretta, E., Dhillon, H., Kalra, P.S., and Kalra, S.P. 2002 Central LIF gene therapy suppresses food intake, body weight, serum leptin and insulin for extended periods. Peptides 23: 975-84.
90. Levi, A., Ferri, G.L., Watson, E., Possenti, R., and Salton, S.R. 2004 Processing, distribution, and function of VGF, a neuronal and endocrine peptide precursor. Cell Mol Neurobiol 24: 517-33.
91. Salton, S.R. 2003 Neurotrophins, growth-factor-regulated genes and the control of energy balance. Mt Sinai J Med 70: 93-100.
92. Leibowitz, S.F., and Wortley, K.E. 2004 Hypothalamic control of energy balance: different peptides, different functions. Peptides 25: 473-504.
93. Temoshok, L.R., and Wald, R.L. 2002 Change is complex: rethinking research on psychosocial interventions and cancer. Integr Cancer Ther 1: 135-45.
94. Ben-Eliyahu, S. 2003 The promotion of tumor metastasis by surgery and stress: immunological basis and implications for psychoneuroimmunology. Brain Behav Immun 17 Suppl 1: S27-36.
95. Strange, K.S., Kerr, L.R., Andrews, H.N., Emerman, J.T., and Weinberg, J. 2000 Psychosocial stressors and mammary tumor growth: an animal model. Neurotoxicol Teratol. 2000 Jan-Feb;22(1):89-102.
96. Stefanski, V., and Ben-Eliyahu, S. 1996 Social confrontation and tumor metastasis in rats: Defeat and ß–adrenergic mechanisms. Physiol Behav 60: 277-282.
97. Temoshock, L., Peeke, H.V.S., and Mehard, C.W 1988 Individual behaviour differences related to induced tumor growth in the female syrian hamster: two studies. Int. J. Neurosci 38: 199-209.
98. Stevenson, J.A., Box, B.M., and Wright, R.B. 1963 The effect of a cold environment on malignant anorexia. Can J Biochem Physiol 41: 531-2.
99. Thompson, C.I., Margules, D.L., Kreider, J.W., Boha, S.P., Rejer, R.E. Jr, Quirey, R.A., and Reitz, J.A. 1993 Propensity to form conditioned taste aversions augments anorexia in obese (ob/ob) mice with B16 melanoma. Behav Neurosci 107: 786-98.
100. Norton, J.A., Moley, J.F., Green, M.V., Carson, R.E., and Morrison, S.D. 1985 Parabiotic transfer of cancer anorexia/cachexia in male rats. Cancer Res 45: 5547-52.
101. Illig, K.A., Maronian, N., and Peacock, J.L. 1992 Cancer cachexia is transmisible in plasma J Surg Res 52: 353-358.
102. Donovan, H. 1954 Malignant cachexia. Proc R Soc Med 47: 27-31.
103. McDonald, D.M., and Choyke, P.L. 2003 Imaging of angiogenesis: from microscope to clinic. Nat Med 9: 713-25.
104. Måseide, K., Kalliomäki, T., and Hill, R.P. Microenvironmental Effects on Tumour Progression and Metastasis. Chapter in this book.
105. Mulligan, H.D., and Tisdale, M.J. 1991 Metabolic substrate utilization by tumour and host tissues in cancer cachexia. Biochem J 277: 321-6.
106. Svaninger, G., Gelin, J., and Lundholm, K. 1989 The cause of death in non-metastasizing sarcoma-bearing mice. A study with relevance for tumor treatment experiments in mice. Eur J Cancer Clin Oncol 25: 1295-302.
107. Ritter, S., Dinh, T.T., and Zhang, Y. 2000 Localization of hindbrain glucoreceptive sites controlling food intake and blood glucose. Brain Res 856: 37-47.
108. Ritter, S., Bugarith, K., and Dinh, T.T. 2001 Immunotoxic destruction of distinct catecholamine subgroups produces selective impairment of

glucoregulatory responses and neuronal activation. J Comp Neurol 432: 197-216.

100. Veech, R.L. 2004 The therapeutic implications of ketone bodies: the effects of ketone bodies in pathological conditions: ketosis, ketogenic diet, redox states, insulin resistance, and mitochondrial metabolism. Prostaglandins Leukot Essent Fatty Acids. 2004 70: 309-19.
110. Beck, S.A., and Tisdale, M.J. 1989 Nitrogen excretion in cancer cachexia and its modification by a high fat diet in mice. Cancer Res 49: 3800-4.
111. Tisdale, M.J., Brennan, R.A., and Fearon, K.C. 1987 Reduction of weight loss and tumour size in a cachexia model by a high fat diet. Br J Cancer 56: 39-43.
112. Fearon, K.C., Borland, W., Preston, T., Tisdale, M.J., Shenkin, A., and Calman, K.C. 1988 Cancer cachexia: influence of systemic ketosis on substrate levels and nitrogen metabolism. Am J Clin Nutr 47: 42-8.
113. Fearon, K.C. 1988 Nutritional pharmacology in the treatment of neoplastic disease. Baillieres Clin Gastroenterol 2: 941-9.
114. Tisdale, M.J., and Brennan, R.A. 1988 A comparison of long-chain triglycerides and medium-chain triglycerides on weight loss and tumour size in a cachexia model. Br J Cancer 58: 580-3.
115. Beck, S.A., and Tisdale, M.J. 1989 Effect of insulin on weight loss and tumour growth in a cachexia model. Br J Cancer 59: 677-81.
116. Nebeling, L.C., and Lerner, E. 1995 Implementing a ketogenic diet based on medium-chain triglyceride oil in pediatric patients with cancer. J Am Diet Assoc 95: 693-7.
117. Wang, H.Y., Hochwald, S., Port, J., Harrison, L.E., Ng, B., and Burt, M. 1995 Hypoglycemia with glycerol salvage: a role in anti-neoplastic therapy? Anticancer Res 15: 1343-8.
118. Bozzetti, F., Gavazzi, C., Mariani, L., and Crippa, F. 2004 Glucose-based total parenteral nutrition does not stimulate glucose uptake by humans tumours. Clin Nutr 23: 417-21.
119. Freitas, J.J., Pompeia, C., Miyasaka, C.K., and Curi, R. 2001 Walker-256 tumor growth causes oxidative stress in rat brain. J Neurochem 77: 655-63.
120. Zaleska, M.M., and Floyd, R.A. 1985 Regional lipid peroxidation in rat brain in vitro: possible role of endogenous iron. Neurochem Res 10: 397-410.
121. Theologides, A., Ingersoll-Stroubos, A.M., and Apple, F.S. 1994 TNF-alpha effect on oxygen free radical scavenging and generating enzymes in rat liver. Biochem Mol Biol Int 33: 205-10.
122. Nadeau, S., and Rivest, S. 1999 Effects of circulating tumor necrosis factor on the neuronal activity and expression of the genes encoding the tumor necrosis factor receptors (p55 and p75) in the rat brain: a view from the blood-brain barrier. Neuroscience 93: 1449-64.
123. Smythies, J., and Galzigna, L. 1998 The oxidative metabolism of catecholamines in the brain: a review. Biochim Biophys Acta 1380: 159-62.
124. Chen, K.B., Lin, A.M., and Chiu, T.H. 2003 Oxidative injury to the locus coeruleus of rat brain: neuroprotection by melatonin. J Pineal Res 35: 109-17.
125. Kishi, T., Hirooka, Y., Kimura, Y., Ito, K., Shimokawa, H., and Takeshita, A. 2004 Increased reactive oxygen species in rostral ventrolateral medulla contribute to neural mechanisms of hypertension in stroke-prone spontaneously hypertensive rats. Circulation 109: 2357-62.
126. Ramos, E.J., Suzuki, S., Marks, D., Inui, A., Asakawa, A., and Meguid, M.M. 2004 Cancer anorexia-cachexia syndrome: cytokines and neuropeptides. Curr Opin Clin Nutr Metab Care 7: 427-34.
127. Davis, M.P., Dreicer, R., Walsh, D., Lagman, R., and LeGrand, S.B. 2004 Appetite and cancer-associated anorexia: a review. J Clin Oncol 22: 1510-7.
128. Laviano, A., Russo, M., Freda, F., and Rossi-Fanelli, F. 2002 Neurochemical mechanisms for cancer anorexia. Nutrition 18: 100-5.
129. Inui, A., and Meguid, M.M. 2003 Cachexia and obesity: two sides of one coin? Curr Opin Clin Nutr Metab Care 6: 395-9.
130. Fanelli, F.R. and Laviano, A. Cancer anorexia: a model for the understanding and treatment of secondary anorexia. Int J Cardiology 85: 67-72.
131. Turrin, N.P., Ilyin, S.E., Gayle, D.A., Plata-Salaman, C.R., Ramos, E.J., Laviano, A., Das, U.N., Inui, A., and Meguid, M.M. 2004 Interleukin-1beta system in anorectic catabolic tumor-bearing rats. Curr Opin Clin Nutr Metab Care 7: 419-26.
132. Inui, A. 1999 Cancer anorexia-cachexia syndrome: are neuropeptides the key? Cancer Res 59: 4493-501.
133. Inui, A. 1999 Neuropeptide Y: a key molecule in anorexia and cachexia in wasting disorders? Mol Med Today 5: 79-85.
134. Wisse, BE.., Schwartz M.W., and Cummings, D.E. 2003 Melanocortin signaling and anorexia in chronic disease states. Ann N Y Acad Sci 994: 275-81.
135. Chance, W.T., Balasubramaniam, A., and Fischer, J.E. 1995 Neuropeptide Y and the development of cancer anorexia. Ann Surg 221: 579-87.
136. Norton, J.A., Peacock, J.L., and Morrison, S.D. 1987 Cancer cachexia. Crit Rev Oncol Hematol 7: 289-327.
137. Chance, W. T., Balasubramaniam, A., Sheriff, S., and Fischer, J. E. 1994 Possible role of neuropeptide Y in experimental cancer anorexia. Pp 185-201 in:

Jacobs, M., ed. Diet and cancer: Markers, prevention and treatment. New York: Plenum Press.

138. Makarenko, I.G., Meguid, M.M., Gatto, L., Chen, C., and Ugrumov, M.V. 2003 Decreased NPY innervation of the hypothalamic nuclei in rats with cancer anorexia. Brain Res 961: 100-8.
139. Chance, W.T., Balasubramaniam, A., Thompson, H., Mohapatra, B., Ramo, J., and Fischer, J.E. 1996 Assessment of feeding response of tumor-bearing rats to hypothalamic injection and infusion of neuropeptide Y. Peptides 17: 797-801.
140. Chance, W.T., Balasubramaniam, A., Borchers, M., and Fischer, J.E. 1995 Refractory hypothalamic adenylate cyclase in anorectic tumor-bearing rats: implications for NPY-induced feeding. Brain Res 691: 180-4.
141. Meguid, M.M., Ramos, E.J., Laviano, A., Varma, M., Sato, T., Chen, C., Qi, Y., and Das, U.N. 2004 Tumor anorexia: effects on neuropeptide Y and monoamines in paraventricular nucleus. Peptides 25: 261-6.
142. Chance, W.T., Balasubramaniam, A., Dayal, R., Brown, J., and Fischer, J.E. 1994 Hypothalamic concentration and release of neuropeptide Y into microdialysates is reduced in anorectic tumor-bearing rats. Life Sci 54: 1869-74.
143. Ramos, E.J., Suzuki, S., Meguid, M.M., Laviano, A., Sato, T., Chen, C., and Das, U. 2004 Changes in hypothalamic neuropeptide Y and monoaminergic system in tumor-bearing rats: pre- and post-tumor resection and at death. Surgery 136: 270-6.
144. McCarthy, H.D., McKibbin, P.E., Perkins, A.V., Linton, E.A., and Williams, G. 1993 Alterations in hypothalamic NPY and CRF in anorexic tumor-bearing rats. Am J Physiol 264: E638-43.
145. Nara-ashizawa, N., Tsukada, T., Maruyama, K., Akiyama, Y., Kajimura, N., and Yamaguchi, K. 2001 Response of hypothalamic NPY mRNAs to a negative energy balance is less sensitive in cachectic mice bearing human tumor cells. Nutr Cancer 41: 111-8.
146. Nara-ashizawa., N., Tsukada, T., Maruyama, K., Akiyama, Y., Kajimura, N., Nagasaki, K., Iwanaga, T., and Yamaguchi, K. 2001 Hypothalamic appetite-regulating neuropeptide mRNA levels in cachectic nude mice bearing human tumor cells. Metabolism 50: 1213-9.
147. Chance, W.T., Sheriff, S., Kasckow, J.W., Regmi, A,, and Balasubramaniam, A. 1998 NPY messenger RNA is increased in medial hypothalamus of anorectic tumor-bearing rats. Regul Pept 75-76: 347-53.
148. Jensen, P.B., Blume, N., Mikkelsen, J.D., Larsen, P.J., Jensen, H.I., Holst, J.J., and Madsen, O.D. 1998 Transplantable rat glucagonomas cause acute onset of severe anorexia and adipsia despite highly elevated NPY mRNA levels in the hypothalamic arcuate nucleus. J Clin Invest 101: 503-10.
149. Bing, C., Taylor, S., Tisdale, M.J., and Williams, G. 2001 Cachexia in MAC16 adenocarcinoma: suppression of hunger despite normal regulation of leptin, insulin and hypothalamic neuropeptide Y. J Neurochem 79: 1004-12.
150. Plata-Salaman, C.R., Ilyin, S.E., and Gayle, D. 1998 Brain cytokine mRNAs in anorectic rats bearing prostate adenocarcinoma tumor cells. Am J Physiol 275: R566-73.
151. Wisse, B.E., Frayo, R.S., Schwartz, M.W., and Cummings, D.E. 2001 Reversal of cancer anorexia by blockade of central melanocortin receptors in rats. Endocrinology 142: 3292-301.
152. Chance, W. T., van Lammeren, F. M., and Fischer, J. E. 1988 Feeding elicited by cholinergic and adrenergic hypothalamic stimulation of anorectic tumor-bearing rats. Pharmacol. Biochem. Behav 31: 209–213.
153. Liebelt, R.A., Liebelt, A.G., Johnston, H.M. 1971 Lipid mobilization and food intake in experimentally obese mice bearing transplanted tumors. Proc Soc Exp Biol Med 138: 482-90.
154. Boston, B.A. 2001 Pro-opiomelanocortin and weight regulation: from mice to men. J Pediatr Endocrinol Metab 14 Suppl 6: 1409-16.
155. Rahmouni, K., Haynes, W.G., Morgan, D.A, and Mark A.L. 2002 Selective resistance to central neural administration of leptin in agouti obese mice. Hypertension 39: 486-90.
156. Fei, H., Okano, H.J., Li, C., Lee, G.H., Zhao, C., Darnell, R., and Friedman, J.M. 1997 Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues. Proc Natl Acad Sci U S A 94: 7001-5.
157. Anderson, K.D., Lambert, P.D., Corcoran, T.L., Murray, J.D., Thabet, K.E., Yancopoulos, G.D., and Wiegand, S.J. 2003 Activation of the hypothalamic arcuate nucleus predicts the anorectic actions of ciliary neurotrophic factor and leptin in intact and gold thioglucose-lesioned mice. J Neuroendocrinol 15: 649-60.
158. Hahm, S., Fekete, C., Mizuno, T.M., Windsor, J., Yan, H., Boozer, C.N., Lee, C., Elmquist, J.K., Lechan, R.M., Mobbs, C.V., and Salton, S.R. 2002 VGF is required for obesity induced by diet, gold thioglucose treatment, and agouti and is differentially regulated in pro-opiomelanocortin- and neuropeptide Y-containing arcuate neurons in response to fasting. J Neurosci 22: 6929-38.
159. Kalra, S.P., Bagnasco, M., Otukonyong, E.E., Dube, M.G., and Kalra, P.S. 2003 Rhythmic, reciprocal ghrelin and leptin signaling: new insight in the development of obesity. Regul Pept 111: 1-11.

160. Stephan, F.K., and Davidson, A.J. 1998 Glucose, but not fat, phase shifts the feeding-entrained circadian clock. Physiol Behav 65: 277-88.
161. Everson, C.A., and Wehr, T.A. 1993. Nutritional and metabolic adaptations to prolonged sleep deprivation in the rat. Am J Physiol 264: R376-87.
162. Hastings, M.H., and Herzog, E.D. 2004 Clock genes, oscillators, and cellular networks in the suprachiasmatic nuclei. J Biol Rhythms 19: 400-13.
163. Buijs, R.M., van Eden, C.G., Goncharuk, V.D., and Kalsbeek, A. 2003 The biological clock tunes the organs of the body: timing by hormones and the autonomic nervous system. J Endocrinol 177: 17-26.
164. Filipski, E., King, V.M., Li, X., Granda, T.G., Mormont, M.C., Claustrat, B., Hastings, M.H., and Levi, F. 2003 Disruption of circadian coordination accelerates malignant growth in mice. Pathol Biol (Paris) 51: 216-9.
165. Filipski, E., King, V.M., Li, X., Granda, T.G., Mormont, M.C., Liu, X., Claustrat, B., Hastings, M.H., and Levi, F. 2002 Host circadian clock as a control point in tumor progression. J Natl Cancer Inst 94: 690-7.
166. Filipski, E., Delaunay, F., King, V.M., Wu, M.W., Claustrat, B., Grechez-Cassiau, A., Guettier, C., Hastings, M.H., and Francis, L.2004 Effects of chronic jet lag on tumor progression in mice. Cancer Res 64: 7879-85.
167. Csernus, V., and Mess, B. 2003 Biorhythms and pineal gland. Neuro Endocrinol Lett 24: 404-11.
168. Simonneaux, V., and Ribelayga, C. 2003 Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. Pharmacol Rev 55: 325-95.
169. Morgan, P.J., Ross, A.W., Mercer, J.G., and Barrett, P. 2003 Photoperiodic programming of body weight through the neuroendocrine hypothalamus. J Endocrinol 177: 27-34.
170. Barrenetxe, J., Delagrange, P., and Martinez, J.A. 2004 Physiological and metabolic functions of melatonin. J Physiol Biochem 60: 61-72.
171. Sanchez-Barcelo, E.J., Cos, S., Fernandez, R., and Mediavilla, M.D. 2003 Melatonin and mammary cancer: a short review. Endocr Relat Cancer 10: 153-9.
172. Blask, D.E., Dauchy, R.T., Sauer, L.A., Krause, J.A., and Brainard, G.C. 2002 Light during darkness, melatonin suppression and cancer progression. Neuro Endocrinol Lett 23 Suppl 2: 52-6.
173. Lissoni, P. 2002 Is there a role for melatonin in supportive care? Support Care Cancer 10: 110-6.
174. Karasek, M., and Pawlikowski, M. 1999 Pineal gland, melatonin and cancer. NEL Review. Neuro Endocrinol Lett 20: 139-144.
175. Sauer, L.A., Dauchy, R.T., and Blask, D.E. 2001 Polyunsaturated fatty acids, melatonin, and cancer prevention. Biochem Pharmacol 61: 1455-62.
176. Blask, D.E., Sauer, L.A., and Dauchy, R.T. 2002 Melatonin as a chronobiotic/anticancer agent: cellular, biochemical, and molecular mechanisms of action and their implications for circadian-based cancer therapy. Curr Top Med Chem 2: 113-32.
177. El-Domeiri, A.A., and Das Gupta, T.K. 1976 The influence of pineal ablation and administration of melatonin on growth and spread of hamster melanoma. J Surg Oncol 8: 197-205.
178. Catrina, S.B., Curca, E., Catrina, A.I., Radu, C., and Coculescu, M. 2002 Melatonin shortens the survival rate of Ehrlich ascites-inoculated mice. Neuro Endocrinol Lett 22: 432-4.
179. Collins, A., Yuan, L., Kiefer, T.L., Cheng, Q., Lai, L., and Hill, S.M. 2003 Overexpression of the MT1 melatonin receptor in MCF-7 human breast cancer cells inhibits mammary tumor formation in nude mice. Cancer Lett 189: 49-57.

Chapter 15

Anti-Angiogenic and Pro-Apoptotic Effects of Dietary Restriction in Experimental Brain Cancer: Role of Glucose and Ketone Bodies

T.N. Seyfried and P. Mukherjee
Biology Department, Boston College, Chestnut Hill, MA 02467, USA

Abstract: Angiogenesis involves neovascularization or the formation of new capillaries from existing blood vessels and is associated with the processes of tissue inflammation, wound healing, and tumorigenesis. Recent studies from this laboratory show that moderate restriction of dietary energy intake (dietary restriction, DR) has powerful anti-angiogenic and pro-apoptotic effects against the CT-2A experimental mouse astrocytoma. DR reduces blood glucose levels while elevating ketone bodies. As most brain tumour cells are dependent on glycolysis for energy due to mitochondrial defects, they are unable to switch from glucose to ketone bodies for energy. An energy source shift from glucose to ketone bodies will enhance the bioenergetic potential of normal brain cells while reducing tumour cell growth and tissue inflammation. It is suggested that cancer therapeutics that reduce tumour growth, while also reducing food intake and body weight, may operate in large part through the anti-angiogenic and pro-apoptotic effects of DR.

Key words: Astrocytoma, caloric restriction, cell death, IGF-1, inflammation, vascularity, angiogenesis, apoptosis, metabolic control theory, brain cancer, CT-2A management, anti-angiogenic therapies, ketogenic diet

1. INTRODUCTION

The long-term prognosis remains poor for most patients with malignant brain tumours despite advances in the molecular genetics of cancer and in brain imaging techniques (1, 2). Surgical resection followed by radiation is the standard therapy today as it has been for over five decades. Chemotherapy also has had little positive benefit on malignant glioma management and is often associated with adverse effects that diminish quality of life (1, 3). It is also unlikely that therapeutic targeting of tumour-associated mutations will be effective in brain tumour management, as most tumour mutations arise as epiphenomena of tissue disorganization and their involvement with tumour initiation, promotion, or progression has not been conclusively established (4-7). Clearly, alternative therapies are needed that can better manage brain tumours while permitting a decent quality of life.

2. BRAIN TUMOUR ANGIOGENESIS

Angiogenesis involves neovascularization or the formation of new capillaries from existing blood vessels and is associated with the processes of tissue inflammation, wound healing, and tumorigenesis (8-10). A significant literature suggests that vascularity is rate limiting for the formation of solid tumours including brain tumours (10-15). The malignancy and invasiveness of tumours are also correlated with the degree of their vascularity since prognosis is generally better for tumours that are less vascular than for those that are more vascular (15-18). The inhibition of vascularity is therefore considered an

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 259-270.

important therapeutic strategy for controlling tumour growth (14, 19-26).

Factors that influence the migration and proliferation of endothelial cells may underlie the mechanisms of angiogenesis. Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen that is a reliable biomarker of angiogenesis in human brain tumours (16, 20, 27-30). Besides VEGF, several of other growth factors and cytokines act as angiogenesis inducers (8, 31, 32). Elevated IGF-1 levels are associated with enhanced brain tumour progression and angiogenesis (33-35). Indeed, reduced IGF-1 levels are associated with reduced angiogenesis and increased apoptosis (36, 37). In general, the degree of tumour vascularity reflects a relative balance between angiogenesis inducers and inhibitors (24, 38).

Tumour progression is thought to involve a change in the balance of angiogenesis inducers over inhibitors (24). The inducers and inhibitors of angiogenesis may originate from both the tumour cells and from tumour-infiltrating host cells, e.g., endothelial cells and macrophages (8, 9, 39-41). Anti-angiogenic therapies may therefore influence brain tumour growth through effects on these cells. We recently suggested that dietary energy restriction may shift the microenvironment of brain tumours from a pro-angiogenic to an anti-angiogenic state through multiple effects on the tumour cells and on the tumour associated host cells (42).

3. DIETARY RESTRICTION AND CANCER

Dietary restriction (DR) is produced from a total restriction of dietary nutrients and differs from starvation in that DR reduces total caloric energy intake without causing anorexia or malnutrition (36, 43-47). In 1914, Rous first suggested that underfeeding might inhibit mouse tumour growth by delaying tumour vascularity (angiogenesis) from the host (48). Later studies showed that the anti-tumour effects of DR resulted from caloric restriction *per se* and not from the restriction of any specific dietary component such as proteins, vitamins, minerals, fats, or carbohydrates (36, 43, 44, 49). In addition to tumour growth inhibition, DR also produces a marked increase in general health consistent with the notion that *ad libitum* feeding of sedentary rodents or humans is overfeeding (44, 45, 50). Although the restriction of dietary energy intake (caloric restriction) underlies the anti-tumour effects and health benefits of DR, the molecular mechanisms for these phenomena have not been clearly described.

4. ANTI-ANGIOGENIC AND PRO-APOPTOTIC EFFECTS OF DR IN AN EXPERIMENTAL ASTROCYTOMA

4.1 Brain Tumour Model

In our initial study, we investigated the effects of DR on the orthotopic growth and angiogenic properties of the experimental mouse astrocytoma, CT-2A (42). This syngeneic brain tumour was generated in our laboratory after implantation of 20-methylcholanthrene into the cerebral cortex of a C57BL/6J mouse according to the procedure of Zimmerman (51, 52). Histologically, the CT-2A brain tumour is broadly classified as a poorly differentiated highly malignant anaplastic astrocytoma (51). The tumour grows orthotopically as a soft, noncohesive, and highly vascularized mass.

4.2 Intracerebral tumour implantation

We implanted the CT-2A tumour into the cerebral cortex of C57BL/6 mice using a trocar as previously described (53, 54). Small CT-2A tumour pieces (about 1 mm^3) from a C57BL/6J donor mouse were used for the cerebral implants. We prefer the initiation of brain tumours from intact tumour pieces rather than from cultured cells since the pieces already contain an established microenvironment that facilitates tumour growth. Moreover, tumours initiated from tissue pieces more closely match the natural tumour environment and do not require adaptation from an unnatural cell culture environment.

4.3 Dietary restriction

Prior to the initiation of our experiments, we separated and randomly assigned mice to either a control group that was fed AL (*ad libitum*) or to an experimental group that was fed a total DR of 30% (70% of the control group) (42). Each mouse was housed singly and was given a cotton-nesting pad for warmth. In our initial experiments, DR was initiated 7 days prior to tumour implantation and was continued for either 11 or 14 days after implantation (42). Total DR maintains a constant ratio of nutrients to energy, i.e., the average daily food intake (grams) for the AL fed mice was determined every other day and the DR-fed mice were given 70% of that quantity on a daily basis (36). All mice received PROLAB RMH 3000 chow (Purina, LabDiet, Richmond, IN), which contained a balance of mouse nutritional ingredients and, according to the manufacture's specification, delivered about 4.4 Kcal/g gross metabolizable energy. Body weights of all mice were recorded every other day.

4.4 Tumour growth

We analyzed intracerebral tumour growth directly by measuring total tumour dry weight. Tumours were dissected from normal appearing brain tissue, were frozen, and then lyophilized to remove water. From our experience, total tumour dry weight is a more accurate measure of CT-2A tumour growth than total wet weight because individual tumours can vary in the degree of hemorrhage and edema.

4.5 Influence or DR on CT-2A Growth, Angiogenesis, and Apoptosis

We found that CT-2A brain tumour growth was about 80% less under moderate DR than under AL feeding (Figure 1). This reduction in tumour growth greatly exceeded the 12% reduction in body weight during the 22-day experiment. Several previous studies showed that moderate DR could reduce the growth of histologically diverse non-neural tumours (36, 43, 46, 48). Our studies are the first to document this phenomenon in a brain tumour model and suggest that brain tumours are especially vulnerable to the growth-inhibitory effects of DR. We have since documented this phenomenon in other mouse and human brain tumour models (109).

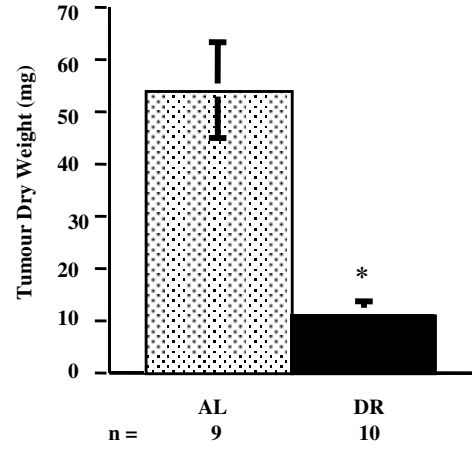


Figure 1. Influence of DR on the intracerebral growth of the CT-2A brain tumour. DR was initiated 7 days before tumour implantation and was continued for 14 days after implantation. Values are expressed as means + SEM and n = the number of tumour-bearing mice examined in each group. The asterisk indicates that the dry weight of the treated tumours was significantly lower than that of the control tumours ($P < 0.001$, two tailed t-test). (with permission from BJC).

The DR-induced reduction in CT-2A growth was also associated with significant reduction in the number and size of blood vessels and with significant elevation in TUNEL positive cells (apoptosis) (Table 1 and Figure 2). In other words, we found that DR is both anti-angiogenic and pro-apoptotic in this brain tumour model. These effects also occurred without causing significant reductions in CT-2A cell proliferation as assessed using the proliferating cell nuclear antigen (PCNA) assay (42). Other investigators have also reported that anti-angiogenic growth factors and cytokines can reduce tumour microvessel density, increase apoptosis, but have little effect on cell proliferation (55-58). Our

results therefore support previous findings that DR produces a pattern of biomarker changes similar to the changes seen following anti-angiogenic therapies (36, 59).

Table 1. Effects of dietary restriction on microvessel density, apoptosis, and proliferation index in the CT-2A brain tumour.

Treatment [a]	Microvessel density [b] vessels/high-power field	Apoptotic index % [c]	Proliferation index % [d]
AL	27.3 ± 3.9	3.8 ± 0.9	71.5 ± 5.8
DR	13.0 ± 2.0 *	9.9 ± 0.6 **	69.7 ± 4.9

[a] Animals were fed either *ad libitum* (AL) or under dietary restriction (DR) as described in Text. Three independent tumours chosen at random were analyzed in each group and all values are expressed as means ± SEM. The asterisks indicate that the values from the DR group differed from AL group at $P < 0.05$ *, and $P < 0.01$ ** as determined by the two tailed t-test.

[b] Factor VIII- positive microvessels were averaged in three hotspot areas of each tumour section at 200 x.

[c] Apoptotic index, determined from the TUNEL assay, 400x.

[d] Proliferation index, determined from the PCNA assay, 400x. (with permission from BJC)

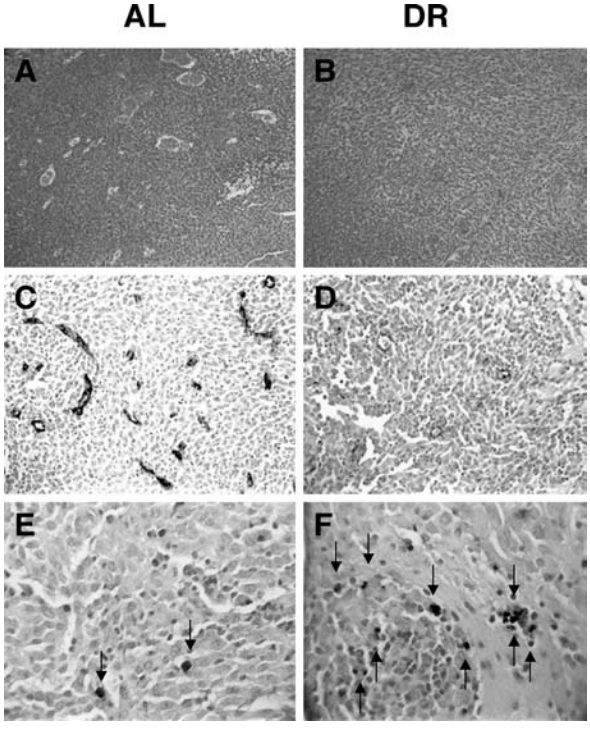


Figure 2. Influence of DR on microvessel density and apoptosis in the CT-2A brain tumour. DR was initiated 7 days before intracerebral tumour implantation and was continued for 11 days. H & E stained tumour sections in an AL mouse (A) and in a DR mouse (B) (100 x). Factor VIII immunostaining from the tumour grown in an AL mouse (C) and in a DR mouse (D) (200 x). TUNEL positive apoptotic cells (arrows) from the tumour grown in an AL mouse (E) and in a DR mouse (F) (400 x). Each stained section was representative of the entire tumour. All images were produced from digital photography. (with permission from BJC)

4.6 Implications of DR for Anti-Angiogenic Therapeutics

Our findings with the CT-2A brain tumour are relevant to those *in vivo* studies where food intake and body weight are reduced in conjunction with anti-angiogenic or anti-cancer therapies. For example, if a new anti-angiogenic drug reduces both body weight and tumour growth in experimental test subjects, it is necessary for the investigators to demonstrate the extent to which the angiogenic effect is due specifically to the drug and not to DR. Tannenbaum and Mukherjee previously mentioned that tumour therapies, which secondarily restrict food intake or assimilation, may produce changes in tumour growth that could be mistaken for a primary effect (44, 59). Recent studies also indicate that many anti-tumour drugs may also have 'accidental' anti-angiogenic effects (60). We suggest that cancer therapeutics that reduce tumour growth, while also reducing food intake and body weight, may operate in large part through the anti-angiogenic and pro-apoptotic effects of DR.

The inclusion of both pair-fed controls and active body weight controls in the analysis of new experimental drugs could help distinguish the anti-angiogenic and pro-apoptotic effects of the drug from that of DR. We recently found that complete starvation of mice for two days was necessary in order for an active body weight control group to match the weight loss in mice injected i.p. with temozolomide (100 mg/kg) (Mukherjee and Seyfried, unpublished observation). As some drugs may reduce food assimilation, active body weight controls must be evaluated together with pair-fed controls. Unfortunately, many scientific reports of new anti-angiogenic drugs fail to include all of the necessary control groups needed to distinguish

specific from nonspecific effects. It is our contention that few if any systemic anti-angiogenic brain tumour therapies will be as effective as DR in reducing angiogenesis.

5. INFLUENCE OF GLUCOSE AND KETONE BODIES ON CT-2A TUMOUR GROWTH

5.1 Brain Tumours Lack Metabolic Versatility

Reductions in plasma levels of glucose and elevations in ketone bodies (acetoacetate and β-hydroxybutyrate) are key biomarker changes associated with DR (49, 61). In contrast to normal brain neurons and glia, that can metabolize ketone bodies for energy when blood glucose levels decrease as occurs during fasting or caloric restriction (47, 61-63), gliomas and most tumour cells lack this metabolic versatility and are largely dependent on glycolytic energy (64-68). Defects in ketone body metabolism, the mitochondrial TCA cycle, and electron transport chain systems are thought to underlie the dependence of tumour cells on glycolytic energy (69-73). Hence, therapies that exploit the genetic and metabolic weakness of brain tumour cells should be effective in controlling brain cancer.

5.2 Ketogenic Diet Management of Pediatric Astrocytoma

Support for the concept that brain tumours are vulnerable to metabolic stress came in 1995, when Nebeling and coworkers reported that a ketogenic diet (KD) could manage advanced stage malignant astrocytoma in two female pediatric patients (74). The KD is a high fat, low protein, low carbohydrate diet that has been used for decades to treat patients with refractory epilepsies (47, 75, 76). It was not clear, however, whether the KD controlled pediatric astrocytoma through effects on plasma glucose or ketone bodies since the diet was administered under restricted conditions where blood glucose levels were also reduced (74). Although the findings with pediatric astrocytoma generated considerable interest in the brain tumour field (77), no further studies were conducted in humans to evaluate the anti-tumour effects of the KD. The reason for this is not clear since one of the patients is still alive and well at the time of this writing (Nebeling, personal communication). Clearly, further studies are warranted on the use of the KD and other diet therapies for brain cancer management.

5.3 Influence of Diet on CT-2A Tumour Growth and on Circulating Levels of Glucose and Ketone bodies

As a follow-up to the Nebeling study, we evaluated the efficacy of the KD in the CT-2A mouse astrocytoma. To determine if the content or composition of dietary calories was responsible for tumour growth inhibition, we compared the effects of the low carbohydrate, high fat KD with a high carbohydrate, low fat standard (SD) diet under both restricted and unrestricted feeding conditions (49). The nutritional composition of the two diets is shown in Table 2. After tumour implantation as before, we randomly assigned the mice to one of four diet groups that received either: 1) the standard diet fed *ad libitum* or unrestricted (SD-UR), 2) the KD fed *ad libitum* or unrestricted (KD-UR), 3) the SD restricted to 40% (SD-R), and 4) the KD restricted to 40% of the control standard diet (KD-R). The average daily food intake (grams) for the UR fed mice was determined every other day and the R-fed mice were given 60% of the SD-UR group amount on a daily basis. This ensured that the mice in both R mouse groups received a similar number of total calories throughout the study. The dietary treatments were initiated 24 hours following tumour implantation and were continued for 13 days. The study was terminated at this time to avoid the stress of tumour burden. We also recorded body weights of all mice every other day.

Table 2. Composition (%) of the standard diet (SD) and the ketogenic diet (KD)

Components	SD [a]	KD [b]
Fat (F)	6	75
Protein (P)	27	14
Carbohydrates (C)	62	0
Fiber	5	12
Kcal/g	4.4	7.8
F/P + C	0.07	5.4

[a] Standard diet was obtained from Purina, LabDiet, Richmond, IN.

[b] Ketogenic diet was obtained from Zeigler Bros. Inc., Gardners, PA.

The CT-2A tumour grew rapidly and to a similar large size in both groups of UR-fed mice (Figure 3). Restricted feeding, of either the SD or the KD, significantly reduced tumour growth. The R-fed mice shown in Figure 3A were representative of those mice with the largest tumours in their respective groups. The UR-fed mice, however, were not representative of those with the largest tumours. Tumour dry weights were approximately 74 % less in both R-fed groups than in their respective UR-fed control groups (Figure 3B). The reduction in tumour growth exceeded the 12-15% reduction in final body weight in the R-fed groups (49). All implanted tumours grew in both the UR-fed and R-fed groups suggesting that restricted feeding of either the SD or the KD did not prevent tumour "take" or establishment, but significantly reduced the intracerebral growth of the malignant CT-2A brain

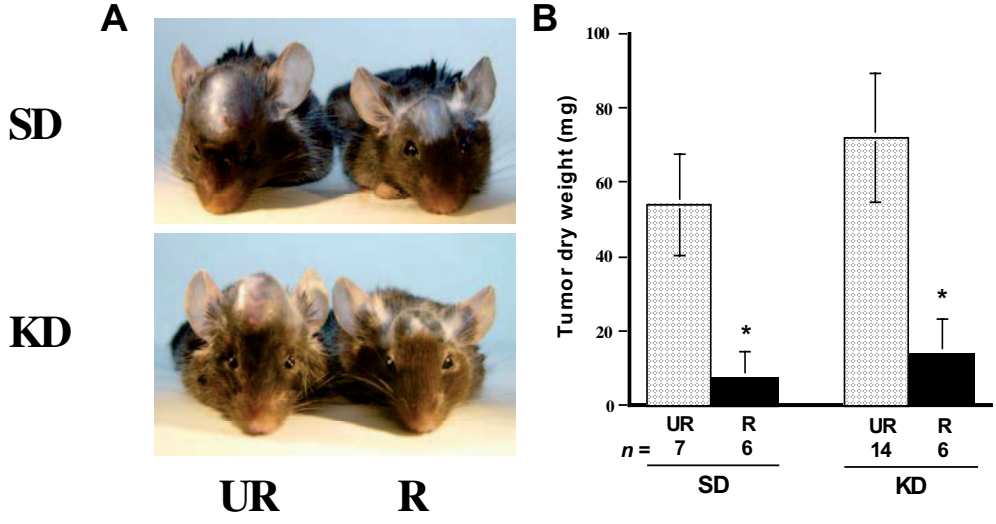


Figure 3. Influence of diet on the intracerebral growth of the CT-2A brain tumour. Dietary treatment was initiated 1 day after tumour implantation and was continued for 13 days. The visual representation (A) and quantitative assessment (B) of tumour growth in C57BL/6J mice receiving either the standard diet (SD) or ketogenic diet (KD) under either unrestricted (UR) or restricted (R) feeding. Values in B are expressed as means with 95% confidence intervals, and n = the number of mice examined in each group. The dry weights of the tumours in R groups were significantly lower than those in the UR groups at $P < 0.01$.

CT-2A growth reduction was associated with reduced blood glucose levels (Table 3). We used linear regression analysis to show that blood glucose levels could predict CT-2A growth (Figure 4) (49). Although blood ketone levels were elevated under restriction of either diet, elevated ketone levels alone could not account for reduced tumour growth because tumour growth was rapid in the UR-KD group despite the presence of high ketone levels (Table 3). These findings support the previous observations of Fearon and co-workers who showed that the failure of a KD to restrict growth of the

Walker 256 rat tumour resulted from the failure of ketosis to reduce glucose availability (78). This is also consistent with our previous findings that blood glucose levels remain high in epileptic mice that consume the KD ad libitum and do not lose body weight (75). We suggest that reduced blood glucose may have contributed to the management of advanced stage malignant astrocytoma with the KD used in the Nebeling study (74, 77). Hence, reduced glucose, associated with reduced caloric intake, is a key factor in the metabolic control of the mouse CT-2A tumour and also possibly for the human pediatric astrocytomas in the Nebeling study.

Table 3. Influence of diet on plasma glucose, ß-OHB, and IGF-1 levels in mice bearing the CT-2A intracerebral brain tumour[a]

Diet[b]	Groups[c]	Glucose (mmol/L)	ß -OHB (mmol /L)	IGF-1 (ng/ml)
SD	UR	9.1 ± 0.9 (7) [d]	0.6 ± 0.1 (7)	208 ± 25 (6)
	R	5.2 ± 1.1* (6)	1.4 ± 0.2* (6)	117 ± 36* (6)
KD	UR	11.4 ± 1.4 (14)	1.0 ± 0.3 (14)	294 ± 30 (5)
	R	5.7 ± 1.5* (6)	1.3 ± 0.6 (6)	193 ± 57* (6)

[a] Values are expressed as means + 95% confidence intervals.
[b] Animals were fed either a standard chow diet (SD) or a ketogenic diet (KD).
[c] UR (unrestricted feeding) and R (restricted to 60% of the SD-UR group as described in Text).
[d] Numbers in parentheses indicate the number of independent tumor-bearing mice examined in each group. The asterisks indicate that the values of the R groups differed from those of their respective UR groups at $P < 0.01$ (analyzed by ANOVA, one way) (with permission from BJC)

In contrast to the situation with prostate cancer and other non-neural cancers (36, 43, 79, 80), little is known about the influence of diet on the progression of brain cancer. We found that orthotopic growth of the CT-2A brain tumour was similarly rapid during the unrestricted feeding of either a high carbohydrate, low fat SD or a high-fat, low carbohydrate KD. On the other hand, CT-2A growth was significantly reduced when either diet was restricted to 60% of the control diet. These findings indicate that orthotopic CT-2A brain tumour growth, like prostate tumour growth, is influenced more by the amount of dietary calories than by the origin or source of the calories (36, 43). Hence, diet and lifestyle may influence the progression of brain cancer.

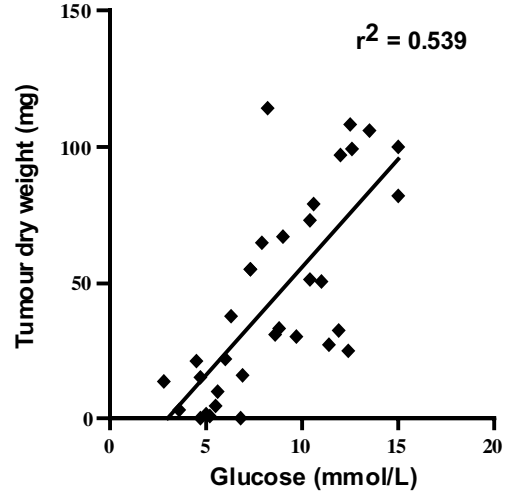


Figure 4. Linear regression analysis of plasma glucose and CT-2A-tumour growth in mice from both the SD and KD dietary groups combined (n = 34). These analyses included the values for plasma glucose and tumour growth of individual mice from both the UR and R-fed groups. The linear regression was highly significant at $P < 0.001$. (with permission from BJC)

5.4 Influence of Restricted Diets on Plasma IGF-1 levels in CT-2A Tumour Mice

As with glucose, we found that circulating IGF-1 levels were significantly lower in each R-fed mouse group than in the respective UR-fed group (Table 3). Linear regression analysis also showed that plasma glucose is predictive of plasma IGF-1 levels (49). These observations agree with previous findings that glucose regulates IGF-1 expression (81, 82). Since reduced IGF-1 levels are associated with reduced angiogenesis and increased apoptosis (36, 37), our findings provide further evidence that DR is anti-angiogenic and pro-apoptotic and that either blood glucose or IGF-1 levels may be useful biomarkers for predicting the effects of DR on brain tumour growth and angiogenesis (49).

6. METABOLIC CONTROL THEORY AND BRAIN CANCER MANAGEMENT

Metabolic control theory applies principles of bioenergetics for the control or management of complex diseases (47, 83, 84). Since metabolism is a universal process underlying all phenotypes, modification of metabolism can potentially modify phenotype. The theory is based on the idea that compensatory genetic and biochemical pathways regulate the bioenergetic potential of glycolysis, the tricarboxylic acid (TCA) cycle, and the electron transport chain. This produces a flexible and versatile metabolic system that is capable of restoring an orderly adaptive behavior to widely disordered conditions involving complex gene-environmental interactions (47, 83, 85). As biological chaos underlies the progression of brain tumours (4), principles of metabolic control theory may be effective in managing brain cancer.

We suggest that accumulated tumour mutations restrict the metabolic versatility of CT-2A tumour cells. As DR inhibits glycolysis, DR would force the CT-2A cells to switch from glucose to alternative non-carbohydrate energy metabolites, e.g., ketone bodies (47, 49). While this switch occurs readily in normal cells, the switch should be more difficult for the tumour cells due to their genetic defects (49). Regardless of whether the genetic defects arise as a cause or consequence of tumour growth, they will to some degree restrict metabolic flexibility. DR would therefore produce catastrophic energy failure and apoptosis in those CT-2A cells that lack metabolic flexibility and are solely dependent on glycolysis. Recent findings also showed that the glycolysis inhibitor 2-deoxy-D-glucose (2DG) or glucose deprivation enhances apoptosis through caspase-3 activation and PARP cleavage in human breast and lung cancer cells (86-88). Since DR reduces blood glucose levels, it is possible that the DR-induced apoptosis in the CT-2A tumour occurs through similar glucose-dependent caspase-3 apoptotic pathways. Our most recent findigs support this hypothesis (109).

We found that DR alone is incapable of killing all CT-2A tumour cells since the tumours continue to grow, though slowly, despite persistently reduced glucose and elevated ketone body levels (Mukherjee and Seyfried, unpublished). The survival of some tumour cells under the metabolic stress of DR may result in part from DR-enhanced gluconeogenesis (89). The liver and kidney are largely involved in gluconeogenesis during calorie restriction. It is also likely that the major glucose transporter, GLUT-1, is up-regulated in CT-2A cells following reductions in circulating glucose levels. Glycerol, released through hydrolysis of triacylglycerol and converted to glucose, will also contribute to circulating glucose levels that can be used by the tumour cells. These physiological adaptations to DR could provide just enough energy to maintain the survival and growth of CT-2A tumour cells. Nevertheless, the CT-2A tumour cells under DR are weakened and likely susceptible to additional forms of metabolic stress.

6.1 Role of Ketone Bodies in CT-2A Management

If the anti-tumour effects of restricted caloric intake are associated with reduced glucose levels and glycolytic energy, a question arises as to what role elevated ketone levels might have in CT-2A management. We suggest that ketone body metabolism, while providing normal brain cells with an alternative high-energy substrate, also reduces the inflammatory activities of tumour-associated host cells (stromal cells) (49). Ketone body metabolism reduces oxygen free radicals, enhances tolerance to hypoxia, and may prevent organ dysfunction from inflammatory processes (84, 90-93). Indeed, Dong et al reported that moderate calorie restriction could reduce the proinflammatory properties of macrophages while enhancing their phagocytic function (94). This is important since activated macrophages contribute to tumour angiogenesis (39, 95). Macrophages secrete numerous pro-angiogenic factors including VEGF, and the degree of tumour angiogenesis is generally associated with the number of macrophages (8, 9, 11, 17, 38-40, 94, 96-99). Uncoupling the detrimental inflammatory activities of macrophages from their potentially beneficial phagocytic activities is considered important for the eventual management of brain cancer (4). Hence, a shift in energy metabolism from glucose to ketone bodies will enhance the bioenergetic potential of

normal brain cells on the one hand while reducing tumour cell growth and tumour inflammatory properties on the other hand.

The key to controlling brain cancer will depend to a large extent on the combined effects of lowering glucose availability while increasing ketone availability. Our findings show, however, that ketone elevation alone is incapable of reducing brain tumour growth unless glucose is also reduced. This indicates that the brain tumour cells will continue to metabolize glucose for energy despite the presence of elevated ketones. Although brain tumour cells may take up ketone bodies, ketone bodies cannot be metabolized for energy if the mitochondria are defective. Roeder and coworkers showed that cultured brain tumour cells use glucose for energy and ketones for lipid synthesis (100). It is our contention that experimental brain tumour management may be achieved if glucose levels can be lowered to maximally stress the glycolytic-dependent tumour cells while providing enough ketone bodies to satisfy the energy needs of normal brain cells.

7. IS DR A PRACTICAL ANTI-ANGIOGENIC AND PRO-APOPTOTIC THERAPY FOR BRAIN CANCER IN THE CLINIC?

The pioneering studies of Nebeling and co-workers using the ketogenic diet to treat pediatric astrocytoma suggests that pediatric brain cancer can be managed with diet therapies that reduce glucose and elevate ketone bodies (74, 77). It is yet unclear if DR can produce anti-angiogenic and pro-apoptotic effects in human brain tumours similar to those we found in the CT-2A mouse astrocytoma. We think this may not be the case since basal metabolic rate is significantly less in humans than in mice (101). The anti-angiogenic and pro-apoptotic effects of moderate DR may therefore be less in human brain tumours than in mouse brain tumours.

In contrast to mice, however, adult humans are capable of complete fasting for prolonged periods with minimal adverse effects (47). We, therefore, speculate that a total food fast in adult humans will produce the physiological conditions of moderate DR in mice. This comes from recent findings that a total food fast lowers blood glucose and IGF-1 levels while elevating blood ketone body levels in healthy non-obese humans (102). Moreover, periodic fasting is known to improve general health to include reduction of tumour growth (103). Fasting will also elevate circulating glucocorticoid levels that will further reduce tumour angiogenesis and edema (104, 105). Glucocorticoids restrict glucose availability to tumour cells and thus will enhance tumour cell apoptosis and reduce angiogenesis (106). Although the synthetic glucocorticoid, dexamethasone, is often administered to brain tumour patients, severe adverse effects are associated with the long-term use of this compound (107). A total food fast in adult humans with brain tumours may, therefore, produce anti-angiogenic and pro-apoptotic effects similar to those that we found in DR mice bearing the CT-2A astrocytoma. For chilhood brain tumors, on the other hand, the pediatric dietary protocol of Nebeling may be most effective for tumour management (77).

A frequent criticism of the use of fasting or DR as a therapy for brain cancer comes from the misconception that voluntary food restriction (anorexia) may exacerbate patient weight loss from tumour-associated cachexia. In other words, how can fasting be justified as an anti-angiogenic/pro-apoptotic brain tumour therapy if the patient is already loosing weight from the tumour? Weight loss associated with cancer cachexia, however, differs from weight loss associated with anorexia since cachexia can occur without anorexia and is produced from factors actively released by the tumour (108). We suggest that fasting or DR may antagonize cachexia by reducing tumour size and thereby reducing the levels of pro-cachexic factors. Although appearing counterintuitive, fasting may facilitate patient weight gain once the fast is broken. The timing of the fast is another critical variable for use as a potential brain tumour therapy. We suggest that the therapeutic benefit of fasting will be best when initiated soon after brain tumour diagnosis or surgical resection, i.e., at a time when normal brain cells can easily switch from glucose to ketone body metabolism. This energy switch may be more difficult following radiation or chemotherapies that

reduce the physiological health of normal brain cells and may actually contribute to brain tumour progression (4).

In summary, we suggest that identification of the anti-angiogenic and pro-apoptotic mechanisms of DR in experimental mouse and human brain tumour models will facilitate translation of this diet therapy to the clinic. It is interesting that an enormous effort is presently underway in the pharmaceutical industry to identify new cancer drugs with anti-angiogenic and pro-apoptotic effects. Since DR already produces these effects, in addition to improving general physiological health, it is surprising that a greater research emphasis is not devoted to this area. We suggest that deciphering the molecular and biochemical mechanisms by which DR reduces angiogenesis and enhances apoptosis may produce new brain tumor drugs that are more effective and biologically friendly than those currently available.

ACKNOWLEDGEMENTS

This work was supported in part from NIH grant (HD39722), the Boston College Research Expense Fund, and a grant from the American Institute of Cancer Research (AICR). We thank Julie Kasperzyk, Richard McGowan, Mohga El-Abbadi, Todd Sanderson, Laura Abate, and Grant Balkema for technical assistance.

REFERENCES

1. Shapiro, W. R., 1999, Arch Neurol, 56:429-32.
2. McLendon, R. E, and Halperin, E. C., 2003, Cancer, 98:1745-8.
3. Yung, W. K., Albright, R. E., Olson, J., Fredericks, R., Fink, K., Prados, M. D., Brada, M., Spence, A., Hohl, R. J., Shapiro, W., Glantz, M., Greenberg, H., Selker, R. G., Vick, N. A., Rampling, R., Friedman, H., Phillips, P., Bruner, J., Yue, N., Osoba, D., Zaknoen, S., and Levin, V. A., 2000, Br J Cancer, 83:588-593.
4. Seyfried, T. N, 2001, Perspect Biol Med, 44:263-82.
5. Sonnenschein, C., and Soto, A. M., 1999, The Society of Cells: Cancer and the Control of Cell Proliferation. Springer-Verlag, New York.
6. Sonnenschein, C., and Soto, A.M., 2000, Mol Carcinog, 29:205-11.
7. Bergstein, I., 2003, Mol Carcinog, 36:1-5.
8. Sunderkotter, C., Steinbrink, K., Goebeler, M., Bhardwaj, R., and Sorg, C., 1994, J Leukoc Biol, 55:410-422.
9. Folkman, J., 1992, Semin Cancer Biol, 3:65-71.
10. Jendraschak, E., and Sage, E.H., 1996, Semin Cancer Biol, 7:139-146.
11. Nishie, A., Ono, M., Shono, T., Fukushi, J., Otsubo, M., Onoue, H., Ito, Y., Inamura, T., Ikezaki, K., Fukui, M., Iwaki, T., and Kuwano, M., 1999, Clin Cancer Res, 5:1107-13.
12. Assimakopoulou, M., Sotiropoulou Bonikou, G., Maraziotis, T., Papadakis, N., and Varakis, I., 1997, Anticancer Res, 17:4747-53.
13. Wesseling, P., Ruiter, D. J., and Burger, P. C., 1997, J Neurooncol, 32:253-265.
14. Kim, K.J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., and Ferrara, N., 1993, Nature, 362:841-844.
15. Leon, S. P., Folkerth, R. D., and Black, P. M., 1996, Cancer, 77:362-372.
16. Takano, S., Yoshii, Y., Kondo, S., Suzuki, H., Maruno, T., Shirai, S., and Nose, T., 1996, Cancer Res, 56:2185-2190.
17. Leek, R. D., Lewis, C. E., Whitehouse, R., Greenall, M., Clarke, J., and Harris, A. L., 1996, Cancer Res, 56:4625-9.
18. Izycka-Swieszewska, E., Rzepko, R., Borowska-Lehman, J., Stempniewicz, M., and Sidorowicz, M., 2003, Folia Neuropathol, 41:15-21.
19. Bergers, G., Javaherian, K., Lo, K.-M., Folkman, J., and Hanahan, D., 1999, Science, 284:808-812.
20. Cheng, S. Y., Huang, H. J., Nagane, M., Ji, X. D., Wang, D., Shih, C. C., Arap, W., Huang, C. M., and Cavenee, W. K., 1996, Proc. Natl Acad Sci USA, 93:8502-8507.
21. Hsu, S. C., Volpert, O. V., Steck, P. A., Mikkelsen, T., Polverini, P. J., Rao, S., Chou, P., and Bouck, N. P., 1996, Cancer Res, 56:5684-5691.
22. Brooks, P. C., Stromblad, S., Klemke, R., Visscher, D., Sarkar, F. H., and Cheresh, D. A., 1995, J. Clin. Invest, 96:1815-1822.
23. Boehm, T., Folkman, J., Browder, T., and O'Reilly, M. S., 1997, Nature , 390:404-407.
24. Hanahan, D., and Folkman, J., 1996, Cell, 86:353-64.
25. Arap, W., Pasqualini, R., and Ruoslahti, E., 1998, Science, 279:377-380.
26. Friedlander, M., Brooks, P. C., Shaffer, R. W., Kincaid, C. M., Varner, J. A., and Cheresh, D. A., 1995, Science, 270:1500-2.
27. Berkman, R. A., Merrill, M. J., Reinhold, W. C., Monacci, W. T., Saxena, A., Clark, W. C., Robertson, J. T., Ali, I. U., and Oldfield, E. H., 1993, J Clin Invest, 91:153-159.

28. Samoto, K., Ikezaki, K., Ono, M., Shono, T., Kohno, K., Kuwano, M., and Fukui, M., 1995, Cancer Res, 55:1189-1193.
29. Obermair, A., Bancher Todesca, D., Bilgi, S., Kaider, A., Kohlberger, P., Mullauer Ertl, S., Leodolter, S., and Gitsch, G., 1997, J Natl Cancer Inst, 89:1212-1217.
30. Chaudhry, I. H., O'Donovan, D. G., Brenchley, P. E., Reid, H., and Roberts, I. S., 2001, Histopathology, 39:409-15.
31. Pintucci, G., Bikfalvi, A., Klein, S., and Rifkin, D. B., 1996, Semin Thromb Hemost, 22:517-524.
32. Jensen, R. L., 1998, Surg Neurol, 49:189-95, discussion 196.
33. Hirano, H., Lopes, M. B., Laws, E. R., Jr., Asakura, T., Goto, M., Carpenter, J. E., Karns, L. R., and VandenBerg, S. R., 1999, Neuro-oncol, 1:109-19.
34. Yu, H., and Rohan, T., 2000, J Natl Cancer Inst, 92:1472-89.
35. Trojan, L. A., Kopinski, P., Wei, M. X., Ly, A., Glogowska, A., Czarny, J., Shevelev, A., Przewlocki, R., Henin, D., and Trojan, J., 2002, Acta Biochim Pol, 49:979-90.
36. Mukherjee, P., Sotnikov, A. V., Mangian, H. J., Zhou, J. R., Visek, W. J. and Clinton, S. K., 1999, J Natl Cancer Inst, 91:512-523.
37. Kari, F. W., Dunn, S. E., French, J. E., and Barrett, J. C., 1999, J Nutr Health Aging, 3:92-101.
38. Lund, E. L., Spang Thomsen, M., Skovgaard Poulsen, H., and Kristjansen, P. E., 1998, Acta Neurol Scand, 97:52-62.
39. Bingle, L., Brown, N. J., and Lewis, C. E., 2002, J Pathol, 196:254-65.
40. Berse, B., Brown, L. F., Van de Water, L., Dvorak, H. F., and Senger, D. R., 1992, Mol Biol Cell, 3: 211-220.
41. Senger, D. R., Van de Water, L., Brown, L. F., Nagy, J. A., Yeo, K. T., Yeo, T. K., Berse, B., Jackman, R. W., Dvorak, A. M., and Dvorak, H. F., 1993, Cancer Metastasis Rev, 12:303-324.
42. Mukherjee, P., El-Abbadi, M.M., Kasperzyk, J. L., Ranes, M. K., and Seyfried, T. N., 2002, Br J Cancer, 86:1615-21.
43. Tannenbaum, A., 1959, In Physiopathology of Cancer,. Homburger, F., ed, 517-562. Paul B. Hober, NY.
44. Tannenbaum, A., 1942, Can Res, 2:460-467.
45. Weindruch, R., and Walford, R. L., 1988, The retardation of aging and disease by dietary restriction. Thomas, Springfield, IL.
46. Kritchevsky, D., 1999, Toxicol Sci, 52:13-6.
47. Greene, A. E., Todorova, M. T., and Seyfried, T. N., 2003, J Neurochem, 86:529-37.
48. Rous, P., 1914, J Exp Med 20:433-451.
49. Seyfried, T. N., Sanderson, T. M., El-Abbadi, M. M., McGowan, R., and Mukherjee, P., 2003, Br J Cancer, 89:1375-82.
50. Keenan, K. P., Ballam, G. C., Soper, K. A., Laroque, P., Coleman, J. B., and Dixit, R., 1999, Toxicol Sci 52:24-34.
51. Seyfried, T. N., El-Abbadi, M., and Roy, M. L., 1992, Mol Chem Neuropathol, 17:147-167.
52. Zimmerman, H. M., and Arnold, H., 1941, Cancer Res, 1:919-938.
53. Seyfried, T. N., Yu, R. K., Saito, M., and Albert, M., 1987, Cancer Res, 47:3538-3542.
54. Ranes, M. K., El-Abbadi, M., Manfredi, M. G., Mukherjee, P., Platt, F. M., and Seyfried, T. N., 2001, Br J Cancer, 84:1107-14.
55. Beecken, W. D., Fernandez, A., Joussen, A. M., Achilles, E. G., Flynn, E., Lo, K. M., Gillies, S. D., Javaherian, K., Folkman, J., and Shing, Y., 2001, J Natl Cancer Inst, 93:382-7.
56. Holmgren, L., O'Reilly, M. S., and Folkman, J., 1995, Nat Med, 1:149-153.
57. O'Reilly, M. S., Holmgren, L., Chen, C., and Folkman, J., 1996, Nat Med, 2:689-92.
58. Tanaka, T., Manome, Y., Wen, P., Kufe, D. W., and Fine, H. A., 1997, Nat Med, 3:437-42.
59. Mukherjee, P., Zhau, J.-R., Sotnikov, A. V., and Clinton, S. K., 1999, In Antiangiogenic Agents in Cancer Therapy, Teicher, B. A., ed, 237-261. Humana Press, Totowa, NJ.
60. Kerbel, R. S., Viloria-Petit, A., Klement, G., and Rak, J., 2000, Eur J Cancer, 36:1248-57.
61. Greene, A. E., Todorova, M. T., McGowan, R., and Seyfried, T. N., 2001, Epilepsia, 42:1371-8.
62. Clarke, D. D., and Sokoloff, L., 1999, In Basic Neurochemistry, Siegel, G. J., Agranoff, B. W., Albers, R. W., Fisher, S. K., and Uhler, M. D., eds, 637-669. Lippincott-Raven, New York.
63. Owen, O. E., Morgan, A. P., Kemp, H. G., Sullivan, J. M., Herrera, M. G., and Cahill, G. F., Jr., 1967, J Clin Invest, 46:1589-95.
64. Oudard, S., Boitier, E., Miccoli, L., Rousset, S., Dutrillaux, B., and Poupon, M. F., 1997, Anticancer Res, 17:1903-11.
65. Mies, G., Paschen, W., Ebhardt, G., and Hossmann, K. A., 1990, J Neurooncol, 9:17-28.
66. Aronen, H. J., Pardo, F. S., Kennedy, D. N., Belliveau, J. W., Packard, S. D., Hsu, D. W., Hochberg, F. H., Fischman, A. J., and Rosen, B. R., 2000, Clin Cancer Res, 6:2189-200.
67. Fearon, K. C., Borland, W., Preston, T., Tisdale, M. J., Shenkin, A., and Calman, K. C. 1988, Am J Clin Nutr, 47:42-8.
68. Roslin, M., Henriksson, R., Bergstrom, P., Ungerstedt, U., and Bergenheim, A. T., 2003, J Neurooncol, 61:151-60.
69. Lichtor, T., and Dohrmann, G. J., 1986, Neurosurgery, 19:896-9.
70. John, A. P., 2001, Med Hypotheses, 57:429-31.
71. Warberg, O., 1956, Science, 123:309-314.

72. Tisdale, M. J., and Brennan, R. A., 1983, Br J Cancer, 47:293-7.
73. Fredericks, M., and Ramsey, R. B., 1978, J Neurochem, 31:1529-31.
74. Nebeling, L. C., Miraldi, F., Shurin, S. B., and Lerner, E., 1995, J Am Coll Nutr, 14:202-8.
75. Todorova, M. T., Tandon, P., Madore, R. A., Stafstrom, C. E., and Seyfried, T. N., 2000, Epilepsia, 41:933-40.
76. Freeman, J. M., Freeman, J. B., and Kelly, M. T., 2000, The Ketogenic Diet: A Treatment for Epilepsy Demos, New York.
77. Nebeling, L. C., and Lerner, E., 1995, J Am Diet Assoc, 95:693-7.
78. Fearon, K. C., Tisdale, M. J., Preston, T., Plumb, J. A., and Calman, K. C., 1985, Br J Cancer, 52:87-92.
79. Bosland, M. C., Oakley-Girvan, I., and Whittemore, A. S., 1999, J Natl Cancer Inst, 91:489-91.
80. Kritchevsky, D., 1999, In Nutritional Oncology, Heber, D., Blackburn, G. L., and Go, V. L. W., eds. 5-10, Academic Press, Boston.
81. Wang, L., Yang, H., and Adamo, M. L., 2000, Biochem Biophys Res Commun, 269:336-46.
82. Straus, D. S., and Burke, E. J., 1995, Endocrinology, 136:365-8.
83. Strohman, R., 2002, Science, 296:701-3.
84. Veech, R. L., Chance, B., Kashiwaya, Y., Lardy, H. A., and Cahill, G. F., Jr., 2001, IUBMB Life, 51:241-7.
85. Greenspan, R. J., 2001, Nat Rev Genet, 2:383-7.
86. Aft, R. L., Zhang, F. W., and Gius, D., 2002, Br J Cancer, 87:805-12.
87. Lee, Y. J., Galoforo, S. S., Berns, C. M., Tong, W. P., Kim, H. R., and Corry, P. M., 1997, J Cell Sci, 110:Pt 5,681-6.
88. Shim, H., Chun, Y. S., Lewis, B. C., and Dang, C. V., 1998, Proc Natl Acad Sci USA, 95:1511-6.
89. Spindler, S. R., 2001, Ann N Y Acad Sci, 928:296-304.
90. Sharman, M. J., Kraemer, W. J., Love, D. M., Avery, N. G., Gomez, A. L., Scheett, T. P., and Volek, J. S., 2002, J Nutr, 132:1879-85.
91. Marcondes, S., Turko, I. V., and Murad, F., 2001, Proc Natl Acad Sci USA, 98:7146-51.
92. Kirsch, J. R., and D'Alecy, L. G., 1984, Stroke, 15:319-23.
93. Yu, B. P., Lee, D. W., Marler, C. G., and Choi, J. H., 1990, Proc Soc Exp Biol Med, 193:13-5.
94. Dong, W., Selgrade, M. K., Gilmour, I. M., Lange, R. W., Park, P., Luster, M. I., and Kari, F. W., 1998, Am J Respir Cell Mol Biol, 19:462-9.
95. Polverini, P. J., 1999, In Antiangiogenic Agents in Cancer Therapy, Teicher, B. A., ed, 65-75. Humana Press, Totowa, New Jersey.
96. Hirano, H., Tanioka, K., Yokoyama, S., Akiyama, S., and Kuratsu, J., 2001, J Neurosurg, 95:89-95.
97. Mitchell, R. A., and Bucala, R., 2000, Semin Cancer Biol, 10:359-66.
98. Badie, B., and Schartner, J. M., 2000, Neurosurgery, 46:957-61.
99. Sharkey, A. M., Charnock Jones, D. S., Boocock, C. A., Brown, K. D., and Smith, S. K., 1993, J Reprod Fertil, 99:609-6015.
100. Roeder, L. M., Poduslo, S. E., and Tildon, J. T., 1982, J Neurosci Res, 8:671-82.
101. Kleiber, M., 1975, The Fire of Life: An Introduction to Animal Energetics. Robert E. Krieger Publishing Co., Malabar, Florida.
102. Savendahl, L., and Underwood, L. E., 1999, J Nutr, 129:2005-8.
103. Shelton, H. M., 1974, Fasting for Renewal of Life. Amer Nat Hygene Soc, Inc., Tampa, FL.
104. Birt, D. F., Yaktine, A., and Duysen, E., 1999, J Nutr, 129:571S-574S.
105. Kadekaro, M., Ito, M., and Gross, P. M., 1988, Neuroendocrinology, 47:329-34.
106. Badruddoja, M. A., Krouwer, H. G., Rand, S. D., Rebro, K. J., Pathak, A. P., and Schmainda, K. M., 2003, Neuro-oncol, 5:235-43.
107. Goodman, L. S., and Gilman, A., 1975, The Pharmacological Basis of Therapeutics. Macmillan, Inc., New York.
108. Tisdale, M. J., 2001, Nutrition, 17:438-42.
109. Mukherjee, P., Abate, L. E., and Seyfried T. N., 2004, Clin Can Res, 10(16): 5322-29.

Chapter 16

Dietary Restriction of Specific Amino Acids Modulates Tumor and Host Interactions

G. G. Meadows and Y.-M. Fu

Cancer Prevention & Research Center, Department of Pharmaceutical Sciences, The Pharmacology & Toxicology Graduate Program, and the College of Pharmacy, Box 646510, Washington State University, Pullman, WA 99164-6510, USA

Abstract: Nutrients modulate host metabolism that lead to changes in biological and molecular responses. Thus, it is not surprising that tumor cells also are affected by changes in host nutrition. Because tumors have altered or mutated genes, they often respond differently to metabolic changes than the host. Many tumors have altered or specific requirements for amino acids, and restriction often can curtail growth/cell death, invasion, and/or metastasis of tumor cells without detrimental effects on the host. This chapter reviews the effects that various specific amino acid restriction has on tumor cells themselves and the effects on interaction between the host stromal components. The cell signalling pathways that modulate attachment, motility and invasion are discussed. Also included are *in vivo* studies encompasing the interactions between the host immune response and B16BL6 melanoma during dietary restriction of tyrosine and phenylalanine. Lastly, the potential for amino acid restriction as one element of adjuvant therapy to control cancer progression is discussed.

Key words: Amino acids, metastasis, signaling, stroma, immune response, cell cycle, growth

1. INTRODUCTION

Cancer is a group of more than 100 different diseases and it arises from accumulation of mutations in various genes (1). Many genes are differentially expressed in cancer cells as compared to their original tissues (2-9). Gene mutations and their altered expression in cancer are believed to be responsible for the altered behavior in cancer cells such as un-controlled growth, evasion of apoptosis, and invasive and metastatic ability (1, 10).

Plants, some of which we consume in our diet, contain factors that prevent cancer, inhibit growth and alter the malignant behavior of cancer cells to control their progression (9, 11-13). In addition, during the last thirty years, scientists found that many cancer cells exhibit relative specific amino acid dependency. For example, PC3 prostate cancer, Yoshida sarcoma, and melanoma are methionine (Met)-dependent (14-17). Melanomas as well as many other cancers also are tyrosine/phenylalanine (Tyr/Phe)-dependent (15, 16, 18-23).

As outlined in Figure 1, specific amino acid dependency is the metabolic signature of cancer cells that arises from gene alterations and is linked to alterations in cell signaling pathways and malignant behaviors. The restriction of specific amino acids in different tumor cells not only inhibits growth, invasion, and metastasis, but also differentially regulates signaling pathways and gene expression (2, 3, 18-28). Thus, the relative specific amino acid dependency of cancer cells can be regarded as a

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 271-283.

molecular target for cancer therapy. The most notable clinically available therapy that targets the L-asparagine dependency of human leukemias is L-asparaginase. L-asparaginine is a nonessential amino acid, which cannot be synthesized by many leukemic cells. Thus, it is not toxic to normal cells.

To make the use of specific amino acid restriction practical for treatment of human cancer patients, the amino acid specificity of particular cancers must be known. Because of the metabolic importance of amino acids, it is also essential to investigate the potential adverse effects of specific amino acid restriction on host cells, since this could have an impact not only on toxicity but also could have a positive or negative impact on the tumor-host interaction and cancer growth and progression. In this chapter, we focus on the various mechanisms whereby specific amino acid restriction modulates tumor and host cells. At the end we discuss the pre-clinical studies with Met and with Tyr/Phe restriction that support their use as adjunctive therapies for cancer.

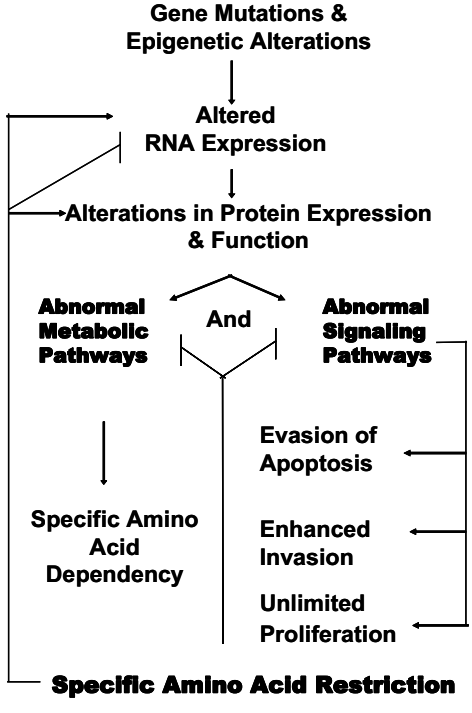


Figure 1. A scheme connecting gene mutations and alterations to abnormal metabolic/cell signaling pathways and the malignant behaviors of cancer cells. ↑, stimulation; ⊥, inhibition.

2. SPECIFIC AMINO ACID RESTRICTION SELECTIVELY AFFECTS TUMOR CELL GROWTH

Unlimited proliferation and protection from programmed cell death (apoptosis) are two major characteristics of solid tumor cells. Specific amino acid restriction inhibits cancer cell proliferation in a cell cycle specific manner. We have primarily studied the effects of Met, Tyr/Phe, and glutamine (Gln) on melanoma and prostate cancer cells. We found that Tyr/Phe restriction arrests different tumor cells in the G0/G1 phase of the cell cycle, including murine B16, B16BL6 melanoma *in vitro and in vivo,* and humanA375 melanoma, human PC3 and DU145 prostate cancer cells *in vitro* (22, 23, 28). These effects correlate with decreased [^{3}H]thymidine uptake, further indicating that limitation of these amino acids decreases DNA synthesis (23).

The cell cycle regulators of melanoma cells are selectively modulated by Tyr/Phe restriction. Restriction of these amino acids selectively inhibits cyclin D1 expression in B16BL6 melanoma without affecting cyclin-dependent kinase or cyclin-dependent kinase inhibitor expression (23). Moreover, Tyr/Phe restriction also inhibits cyclin-dependent kinase 4 of A375 human melanoma cells. Met restriction and Gln restriction also arrest proliferation through selective blockade of the cell cycle in both melanoma and prostate cancer cells. Met restriction primarily blocks melanoma cells in S phase with only a modest 10% increase in the G0/G1 phase (23). Interestingly, Met restriction has a different effect on cyclin D expression and on cyclin-dependent kinase expression by inhibiting cyclin D3 and cyclin-dependent kinase 2, respectively (23). In PC3 and DU145 prostate cancer cells, Met and Gln restriction uniformly blocks the G0/G1 phase of the cell cycle (28). Restriction of Tyr/Phe and Met also inhibits proliferating cell nuclear antigen (PCNA) in the melanoma cells (23). These findings indicate that there are similarities as well as differences in tumor cells in response to amino acid restriction. This is further underscored by the effects of specific amino acid restriction on protein synthesis.

Interestingly, in DU145 prostate cancer cells, Tyr/Phe, Met, and Gln restriction initially stimulates

uptake of [^{3}H]alanine, an indicator of protein synthesis, between 2-3 fold during the first two days after depriving these cells of the specific amino acids (28). [^{3}H]alanine uptake decreases progressively in Gln-restricted cells to about of 40% of amino acid unrestricted cells (control) by day 4 and in Met-restricted cells to about 10% of control. However, a [^{3}H] alanine uptake remains elevated in the cells restricted in Tyr/Phe and is about 160% of control after 4 days. These results further emphasize the specific and differential effect that cells exhibit to selective amino acid restriction and stress the fact that one cannot assume that protein synthesis is always inhibited under these conditions.

We evaluated the effects of Tyr/Phe restriction on growth of primary B16 and B16BL6 melanoma cells *in vivo* (23, 29-32). In all cases, Tyr/Phe restriction reduces tumor growth. In B16BL6 melanoma, this is associated with a selective block in the G0/G1 phase in the tumors and also in the expression of PCNA and cyclin D1. Although *in vitro* restriction of specific amino acids also inhibits proliferation of fibroblasts, there is no observed effect on adjacent connective tissue with dietary restriction of Tyr/Phe in this mouse melanoma model. This could be due to the fact that fibroblasts *in vivo* are not in an active state of proliferation. Thus, the effect of specific amino acid restriction *in vivo* on these cells is likely to be minimal.

3. AMINO ACID RESTRICTION INDUCES APOPTOSIS IN SOME TUMOR CELLS, BUT NOT IN OTHERS, AND EACH TUMOR CELL LINES BEHAVES DIFFERENTLY

Evasion of apoptosis (programmed cell death) is an important feature of solid tumor cells. The control of cell death is tightly linked to the cell cycle. Specific amino acid restriction of Tyr/Phe, Met, and Gln induces apoptosis of several cancer cell lines such as B16BL6 melanoma, A375 melanoma, PC3 and DU145 prostate cancer as assessed by DNA fragmentation, Tunel staining, annexin V positivity, and electron microscopy (22, 23, 28, 33). Figure 2 shows an electron micrograph documenting the progressive process of apoptosis in B16BL6 melanoma cells restricted for Tyr/Phe.

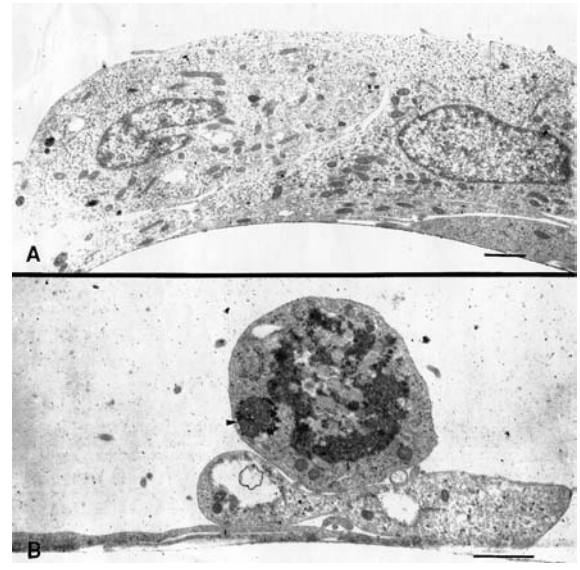


Figure 2. Electron micrograph of B16BL6 melanoma cells grown in Tyr/Phe limited MEM medium containing 4 μg/ml of Tyr and 4 μmg/ml Phe for 3 days (transverse sections). **A.** Early apoptosis of a melanoma cells (left side). Note the shrunken nucleus, the migration of chromatin to form dense masses that abut the nuclear membrane, vacuoles in the cytoplasm, and morphologically looking normal mitochondria. **B.** Late phase of apoptosis showing a melanoma cell with condensed chromatin-apoptotic body (arrow), nuclear fragmentation, and swollen mitochondria. Bars = 1 μm.

Apoptosis induced by specific amino acid restriction is a relatively slow process in contrast to cell death receptor-mediated apoptosis (22, 28, 34). In fact, amino acid restriction does not alter cell surface death receptors (33). It is important to point out that various tumor cell lines respond differently to specific amino acid restriction. For example, Tyr/Phe but not Gln deprivation induces apoptosis in A375 melanoma cells (22). PC3 cells are resistant to induction of apoptosis by Tyr/Phe restriction, but sensitive to Met and Gln restriction; while DU145 cells undergo apoptosis in response to all of the amino acid restrictions (28). Thus, it is not possible to assume that all cancer cells even within the same classification exhibit the same amino acid dependency.

Interestingly, specific amino acid restriction does not induce apoptosis in human infant foreskin fibroblasts, human prostate epithelial cells, and neonatal mouse epidermal cells (22, 28). It is not known why normal cells are resistant to the induction of apoptosis by specific amino acid restriction. However, we do know that normal cells and tumor cells sense amino acids in different ways. For example, the insulin-producing pancreatic β cell, which secretes insulin, senses plasma lysine and arginine levels; however, there is no known "sensor" for specific amino acids on the surface of cancer cells. Amino acid-transporters and glutathione on the membrane transfer amino acid from outside to inside the tumor cell. It is known that these transporters in human hepatoma and melanoma cells are not damaged by specific amino acids restriction (27, 35). Therefore, the proximal event controlling the cancer cell during amino acid restriction is the reduction in intracellular amino acid levels (27).

It is likely that the reduced level of intracellular specific amino acids acts as an inside signal to inhibit the cell survival pathways since the intracellular concentrations of crucial amino acids in tumor cells are about 2- to 3-fold higher than in normal cells (27). The trigger-on points for inducing apoptosis of tumor cells differs from that of normal cells. The tumor cells appear to be more sensitive to intracellular amino acid concentration, and when the levels decrease below a certain threshold, this induces apoptosis. This could partially explain the differences in amino acid dependency between cancer and normal cells.

Some of the signals that induce apoptosis of tumor cells during amino acid restriction are known; however, the roles(s) of many down-stream signaling pathway(s) still require identification. In A375 melanoma, Tyr/Phe restriction induces apoptosis within 48 hr and this is accompanied by proteolytic cleavage of poly ADP-ribose polymerase (19). The first step in this process is activation of caspase 8, and this leads to the cleavage of Bid to a truncated form, tBid. The tBid translocates to the mitochondria and recruits cytoplasmic Bax. This results in the release of cytochrome c from the mitochondria into the cytosol where it acts together with Apaf-1, ATP and caspase 9 to initiate the activation of caspase 3, caspase 7 and ultimately to execute cell death (19). Whether this is a general pathway or is specific to each individual amino acid restriction or tumor cell line is currently not known.

4. SPECIFIC AMINO ACID RESTRICTION MODULATES INTERACTION OF CANCER CELL WITH HOST STROMAL COMPONENTS

Metastasis involves extensive interactions between invasive tumor cells and the host stromal components at the primary tumor and at secondary metastatic sites. Invasion of cancer cells through extracellular matrix (ECM) involves a three-step process. At the primary tumor site, the tumor cell must attach to constituents of the ECM. Then, proteolytic enzymes are secreted and activated. They degrade adjacent ECM constituents, or activate pro-enzymes in the ECM to active enzymes that degrade ECM components. This allows for tumor cell migration into the area where the ECM is altered by the degradative enzymes.

The fact that dietary restriction of specific amino acids, particularly Tyr/Phe, inhibits metastasis of melanoma and other tumors suggests that there is modulation of interaction between the melanoma cell and the host stromal ECM. We found *in vitro* that specific amino acid restriction does not alter attachment of melanoma cells to endothelial cells (36), but does inhibit attachment of melanoma cells to components of ECM including laminin, fibronectin, and heparin sulfate proteoglycans, and to fibroblasts (20, 21, 37). Specific amino acid restriction does not reduce the total amount of cellular fibronectin, laminin, or heparin sulfate proteolycans in melanoma cells [(20) and unpublished data]. Integrins are cell surface receptors that bind to ECM components during cell attachment. Specific amino acid restriction selectively reduces cell surface integrins in melanoma and prostate cancer cells (37). This is consistent with the inhibitory effects of amino acid restriction on cell attachment and also indicates that integrin-mediated cell signaling and cell migration are modulated (22, 28).

The proteolytic enzymes that degrade ECM are produced by both cancer and host stromal cells. Metalloproteinases (MMPs) play an important role in tumor invasion because of their ability to degrade a variety of ECM and basement membrane components. *In vitro* restriction of specific amino acids affects the secretion, activity, and expression of MMPs in murine and human melanoma cells. As shown in Figure 3A, A375 melanoma cells express MMP-2 and MMP-9. Tyr/Phe deprivation almost totally abrogates secretion of MMP-9 and greatly reduces the secretion of MMP-2 into the conditioned medium. Additionally, the remaining MMP-2 is present in an inactive form as is demonstrated by the absence of the <72kDa band. On the other hand, Met deprivation does not affect MMP-2 secretion and actually increases MMP-9 secretion. In keeping with the lack of effect of amino acid restriction on apoptosis of normal cells, Figure 3B shows that neither Met, Phe, Tyr, nor Tyr/Phe affects MMP-2 secretion and activity in the human infant foreskin fibroblasts (Figure 3B) or neonatal mouse epidermal cells (not shown). Also, MMP-9 is not secreted in any significant amount in these cells. These data further support the relative resistance of normal cells to amino acid restriction.

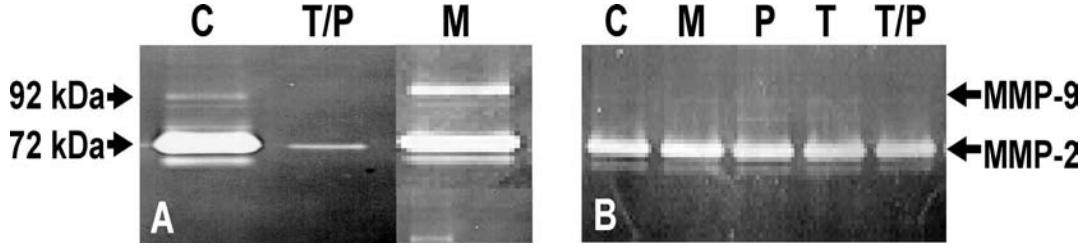


Figure 3. Differential effect of amino acid restriction on secretion of matrix metalloproteinases between human A375 melanoma and infant foreskin fibroblasts. Conditioned medium was obtained and analyzed by zymography as previously described (21) in cells cultured for 3 days in complete MEM containing 10% fetal bovine serum or in MEM containing 10% fetal bovine serum lacking Met (M), Phe (P), Tyr (T) or Tyr/Phe (T/P). The enzyme activities of the MMPs were determined by zymography. A. Zymogram of A375 melanoma. B. Zymogram of human infant foreskin fibroblasts.

The urokinase-type (uPA) and tissue-type (tPA) plasminogen activators and their inhibitors also especially important to in melanoma invasion and tumor progression (38, 39). uPA and tPA convert plasminogen, which is abundant in ECM, into plasmin. Plasmin is a protease that degrades several components of ECM, such as fibronectin, laminin, and type IV collagen. Specific amino acid restriction reduces uPA and tPA secretion of melanoma cells while increasing secretion of their inhibitors (20, 21). By the contrast, specific amino acid restriction does not inhibit secretion of these proteins in normal cells (Figure 4). Therefore, the inhibition of uPA and tPA in cancer cell by specific amino acid restriction plays a major role in modulation of interactions between cancer cells with host stromal cells.

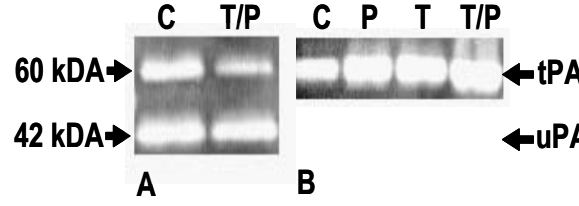


Figure 4. Relative lack of effect of Tyr and Phe on secretion of tPA and uPA in normal cells. A. Neonatal mouse epidermal cells and B. human infant foreskin fibroblasts were cultured in complete, C; Phe-free, P; Tyr-free, or Tyr/Phe-free MEM for 3 days and then conditioned media were collected. The conditioned media were analyzed for enzyme assay via zymography as previously described (20, 21).

5. EFFECTS OF AMINO ACID RESTRICTION ON MOTILITY AND INVASION OF TUMOR CELLS

Specific Tyr/Phe restriction inhibits the invasion and chemoinvasion of a variety of murine and human melanoma cell lines including moderately pigmented murine B16BL6 (20), nonpigmented A375 (21), highly pigmented SK-MEL-28 (27), and moderately pigmented MeWo (unpublished data). Thus, there is no association with pigmentation characteristics and the sensitivity of melanoma cells to Tyr/Phe deprivation. B16BL6, SK-MEL-28, and A375 cells were also examined to determine if other amino acids also decrease invasion. Met deprivation decreases invasion of all three cell lines; however, the inhibition of invasion is less pronounced for B16BL6 and A375 melanoma as compared to Tyr/Phe deprivation. Gln deprivation has no effect on invasion of B16BL6 melanoma or A375 melanoma (20, 27) and leucine deprivation increases invasion of B16BL6 melanoma (20). Although Phe deprivation decreases invasion of B16Bl6 and A375 melanoma, Tyr deprivation exhibits a greater effect and the degree of inhibition is similar to combined Tyr and Phe deprivation.

Tyr/Phe deprivation also inhibits invasion of human MDA-MB-231 breast cancer cells (27) and human PC3 and DU145 prostate cancer cells (28). Met deprivation similarly decreases invasion of DU145 but the anti-invasive activity of PC3 is greater during Met deprivation than during Tyr/Phe deprivation (27). Another difference is that PC3 is insensitive to Gln deprivation, however, Gln inhibits invasion of DU145 in a fashion similar to Tyr/Phe deprivation (27).

Neonatal murine epidermal cells are invasive through growth-factor reduced Matrigel and their invasive ability is insensitive to Tyr/Phe deprivation (20). Similarly, invasion of human infant foreskin fibroblasts also are non responsive to deprivation of these amino acids (21).

Taken together, these data indicate that tumor cells, even within the same tumor type exhibit a unique signature with regard to their dependence and response to amino acids. This signature is evident not only in the susceptibility of tumor cells to apoptosis induced by amino acid restriction, but also with regard to secretion of proteases and invasive characteristics. While tumor cells exhibit such changes, we do not find any significant effects on normal cells. Moreover, a unifying characteristic of all tumor cells studied to date is that Tyr/Phe restriction exhibits a major inhibitory effect on invasion and metastasis.

6. AMINO ACID RESTRICTION MODULATES CELL SIGNALING PATHWAYS THAT MODULATE INVASION

Amino acid restriction modulates a number of important cell signaling molecules. Unlike the insulin-producing pancreatic β cell in which insulin secretion is sensitive to plasma lysine and arginine levels, there is no such “sensor” for specific amino acids found on the surface of cancer cells. Amino acid-transporters and glutathione on the membrane transfer amino acid from outside to inside the tumor cell. These transporters in human hepatoma and melanoma cells are not damaged by specific restriction of amino acids (27, 35). Moreover, glutathione is not reduced during amino acid restriction (unpublished results). The proximal event for melanoma cells during amino acid restriction is the reduction in intracellular amino acid levels (27). At the cellular level, metabolic regulation consists of intracellular compartmentalization of substrates and enzymes and the activity and amount of these enzymes. Previous studies on abnormalities in carbohydrate, lipid, protein, vitamin and energy metabolism in cancer cells do not explain the specific amino acid dependency of solid tumor cells including melanoma (11, 12, 40-46).

Reduced cytoplasmic amino acid levels trigger a series of alterations in metabolic and signaling pathways to inhibit invasion and, in some cases, induce apoptosis. Specific amino acid restriction most likely targets the metabolic abnormalities of cancer that are created as a result of genetic mutation or alterations that leads to altered cellular protein expression (Figure 1). However, the current knowledge of metabolism does not suggest that specific metabolic reactions are linked directly to

invasion. Since some signaling molecules are also enzymes, changes in amount, cellular distribution, and phosphorylation of these enzymes are part of the metabolic regulatory process that influences invasion.

Cells respond to amino acid restriction *in vitro* by altering their size and shape (28). Change in cell shape and size requires restructuring of the cytoskeleton, which can alter integrin-mediated cell signaling. Focal adhesion kinase (FAK) is a major mediator of integrin signaling and a key regulator of focal adhesion dynamics and cell movement (47-49,50). Also, FAK is constitutively active in human malignant melanoma and is related to its aggressive behavior (51). We found in A375 melanoma and in DU145 prostate cancer cells that Tyr/Phe restriction *in vitro* decreases FAK protein expression and FAK phosphorylation (22,28). Tyr/Phe restriction in PC3 cells does not alter FAK expression or phosphorylation (28). Met restriction, although it does not inhibit FAK protein expression, does decrease FAK phosphorylation in PC3 cells. FAK expression and phosphorylation of non tumorigenic human infant foreskin fibroblasts is not affected by Tyr/Phe or Met restriction. Invasion of all these tumor cell lines is inhibited by Tyr/Phe. Thus, it appears that Tyr/Phe restriction evokes FAK-dependent and FAK-independent pathways that control invasion.

The mitogen activated protein kinase (MAPK) pathway is another cell signaling pathway that is associated with melanoma and prostate cancer cell invasion (52-55) and this pathway controls the expression of invasive proteases (54, 55). The expression and phosphorylation of extracellular-regulated kinase (ERK), one of the cell signaling molecules in the MAPK pathway, are decreased in DU145 cells during Tyr/Phe restriction (28); however, Tyr/Phe restriction does not affect ERK in PC3 cells. Met restriction does not affect expression of ERK protein, but does decrease the phosphorylation to non detectable levels. Since Tyr/Phe deprivation decreases invasion of PC3 cells, these results indicate that invasion of cancer cells also is controlled by both ERK-dependent and ERK-independent signaling pathways.

From these limited studies, it is apparent that there is no one single cell signaling pathway through which amino acid deprivation regulates invasion of tumor cells. More studies are required to determine if there are common elements of multiple pathways by which amino acids regulate tumor cell migration and invasion.

7. INTERACTION BETWEEN HOST IMMUNE RESPONSE AND B16BL6 MELANOMA IN VIVO DURING DIETARY RESTRICTION OF TYR/PHE

It is well known that the immune response can impact the establishment as well as the progression of tumors. Few studies have examined the effect of dietary restriction of Tyr/Phe on the immune system. We found that Tyr/Phe restriction reduces splenic natural killer (NK) cell cytolytic activity in $B6D2F_1$ mice when examined *ex-vivo* (56). The cytolytic activity of splenic NK cells, however, is enhanced *in vitro* by interleukin 2 and *in vivo* by poly I:C (56) indicating that the cells from mice fed the Tyr/Phe restricted diet are still responsive (23). In healthy human beings, dietary restriction of Tyr/Phe for six weeks significantly increases the numbers of NK, CD4+ and CD8+ T lymphocytes in peripheral blood without compromising NK or IL2-stimulated NK cytolytic activity (57). Another finding is that platelet aggregative responses to platelet aggregating factor and adenosine diphosphate are appreciably decreased in subjects that consumed the Tyr/Phe diet (57). It is well known that platelet aggregation contributes to tumor metastasis; therefore, the inhibition of platelet aggregation in the host by Tyr/Phe restriction could be another factor that further inhibits cancer progression.

Additional studies in mice indicate that dietary restriction of The Tyr/Phe does not impair the ability of host lymphocytes to infiltrate primary tumors (Figure 5) and that growth of B16BL6 melanoma is significantly inhibited in $B6D2F_1$ mice (23). Although Figure 5 indicates that there is considerable lymphocytic infiltrate into the tumor, we do not know the phenotype of the infiltrating cells or their ability to attack the tumor cells.

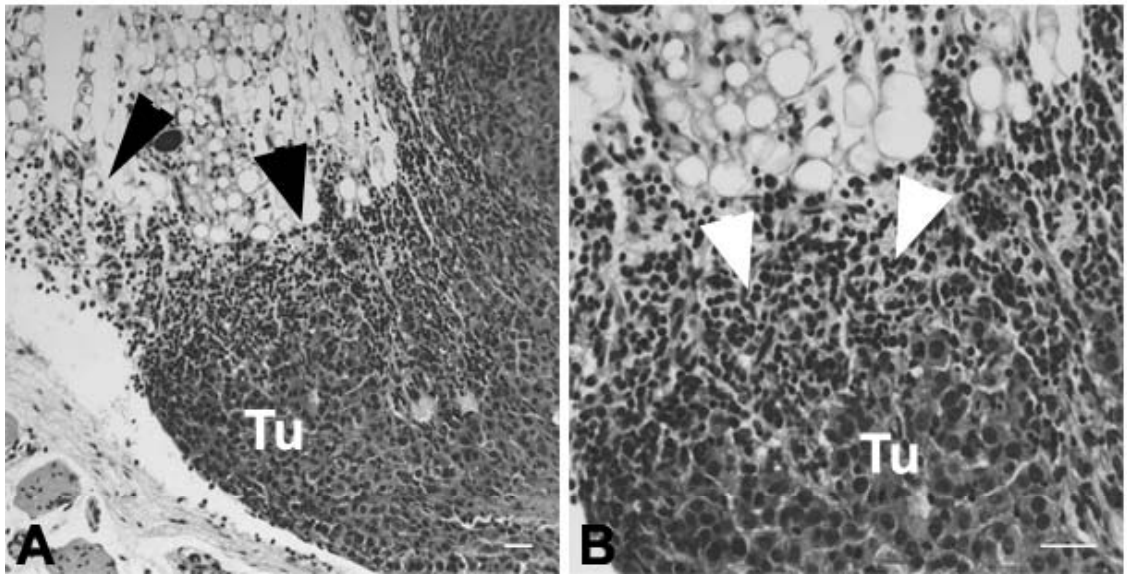


Figure 5. Dietary Tyr/Phe restriction does not impair infiltration of lymphocytes. Mice were fed a Tyr/Phe restricted diet for 14 days and then inoculated with B16BL6 melanoma cells into the dorsal hip of female B6D2F1 mice (23). This figure is a hematoxylin-eosin stained primary tumor and surrounding host tissue section from a mouse consuming the Tyr/Phe restricted diet for 12 days after tumor implantation. A. Low power view. B. High power view. Tu, tumor. Black and white arrows indicate tumor infiltrating lymphocytes. Bars equal to 10μm.

Another outcome from this study is that there are no differences in the number of blood vessels in tumors from mice fed a normal diet and mice fed a Tyr/Phe restricted diet. Thus, it is unlikely that the dietary restriction alters angiogenesis. Also, characteristic of the tumors from mice fed the Tyr/Phe diet is decreased PCNA and cyclin D1 protein expression coupled with an increase of about 91% in the G0/G1 phase of the cell cycle (23). While these data indicate that the decrease in growth is cell cycle related, they do not exclude a role for apoptosis (19, 22) or immune factors in control of local tumor growth. Moreover, it is remarkable that the cell cycle block is evident in the tumors since plasma Tyr levels are only reduced from 72±2 (S.E.) to 46±1 (S.E.) nmol/ml and Phe levels are reduced from 53±1 (S.E.) to 37±1 (S.E.) nmol/ml in mice with 7-day old melanoma tumors (29). These data underscore the extraordinary sensitivity of the melanoma tumor to modest decreases in Tyr/Phe levels. While the exact mechanism underlying the effect of dietary Tyr/Phe restriction on tumor growth and metastasis is not known, it is clear that a variety of factors are involved.

8. AMINO ACID RESTRICTION AS A POTENTIAL ADJUVANT TO CONTROL CANCER GROWTH AND PROGRESSION

In this chapter, we reviewed and discussed a number of aspects by which specific amino acid restriction modulates tumor-host interactions. Based on our previous studies, dietary restriction of Tyr/Phe not only inhibits the growth and metastasis of solid tumors, but also enhances survival of primary and metastatic tumor-bearing mice (58-61). In addition to enhancement of apoptosis (in some tumors), decreased proliferation, decreased invasion, and altered expression of cell signaling molecules associated with these processes, amino acid restriction also modulates the expression of a number of genes (26, 27). The significance of these

changes are being evaluated and not fully understood.

The control of gene transcription by amino acid deprivation is an emerging area of research (24, 26, 62). In fact normal cells, like tumor cells, sense low levels of amino acids by up regulating genes and altering metabolism (63-65). We found that tumor cells in contrast to normal cells are quite sensitive to reduced levels of amino acids most likely because of the genetic mutations or alterations that induce altered metabolic and regulatory pathways. This, in itself, could be a key factor in using selective amino acid deprivation as one treatment for cancer, since unlike standard chemotherapy that is less selective and is also toxic to host cells, amino acid restriction induces few, if any, adverse side effects (57).

Current cancer chemotherapy relies heavily on cytotoxic drugs and patients often derive only marginal benefit from this type of therapy due to host toxicity. Thus, new approaches to enhance traditional chemotherapy or to find broader targets to inhibit growth and metastasis of cancer are greatly needed. Furthermore, it has become increasingly clear that diet and dietary factors can impact not only the incidence of cancer, but also inhibits metastasis. It also is practical to use amino acid restriction in treating cancer. Amino acids that are absorbed from the intestinal epithelia are transported into the portal vein, and about 80% are metabolized in the liver leaving only 20% to enter the systemic circulation. The mean plasma half-life of amino acids is 15 minutes. Thus, amino acid metabolism is targetable.

There are a number of reports in the literature that amino acid restriction can act synergistically with other agents used to treat cancer. Met restriction, for example, is known to increase efficacy of other treatments and also reduce their toxicity toward host cells and these studies were recently reviewed (66). Different approaches to reduce plasma Met levels such as dietary Met restriction, Met-deprived total parenteral nutrition, enzyme administration, and gene therapy also were recently reviewed (67). Of primary clinical significance is the preliminary phase I study by Lu and Epner (17) in which they show impressive data regarding the ability of dietary Met restriction to substantially lower plasma Met levels in patients with advanced cancer.

We have carefully documented that dietary and enzyme restriction of Tyr/Phe alone and in combination with chemotherapy can control melanoma and other cancers including leukemia, lung, liver, breast, and prostate (27, 29, 32, 58, 61, 68, 69). Furthermore these effects are not due to loss in body weight, decreased food intake, or general starvation. The antitumor and antimetastatic effects are likely due to the combined effects on the host and tumor. Melanoma tumors themselves are altered as a result of exposure to low levels of Tyr/Phe *in vitro* as well as *in vivo*. For example, melanoma cells isolated from mice fed a low Tyr/Phe diet and then cultured *in vitro* in complete medium are less able to establish metastases when re-inoculated into mice fed a normal diet (18, 61, 70). They also exhibit reduced invasion through Matrigel and Growth Factor Reduced Matrigel (71). This indicates that the alteration in metastatic phenotype is relatively stable (70). A reduced metastatic phenotype is also generated after culturing melanoma cells in Tyr/Phe restricted media *in vitro* (72). Importantly, the induced change in metastatic phenotype is not produced in cells cultured in media low in Met, Gln, Leu, or arginine (72). We also found that Tyr/Phe restriction *in vitro* enhances the therapeutic response of adriamycin sensitive and multi drug resistant leukemic cells to adriamycin (68). Thus, there is sufficient rationale to pursue clinical trials utilizing Tyr/Phe restriction. Moreover, patients with phenylketonuria and tyrosinemia have been treated for many years with Tyr and/or Phe restriction without major adverse effects. Thus, using Tyr/Phe restriction as a therapeutic approach to control cancer is safe.

On factor that limits dietary restriction as a method to reduce Tyr and/or Phe levels is patient compliance. We found in our initial human study that compliance was not difficult because of the availability of a number of low Phe (and Tyr) foods and protein supplements (57). Other novel methods are also being developed that might also be clinically useful. One such methods relies on the use of polyhemaglogin-tyrosinase to degrade tyrosine, and this enzymes decreases growth of B16F10 melanoma in mice (73, 74).

Tumor growth, invasion, and metastasis involve many interrelated and dysregulated steps. Because of genetic instability, tumor cells are continuously subject to altered environmental and host forces. The selective pressures placed on the host and the tumor regulates how tumor cells interact with host cells. It is clear that the host environment and in addition to the specific characteristics of tumor cells, is a vital component in determining whether the tumor will remain dormant, invade locally, or metastasize. Invasion and metastasis are multicomponent processes and thus difficult to inhibit by one type of targeted therapy. Unfortunately, the toxic effects of traditional chemotherapy on the host are often extensive and limit the usefulness and effectiveness of this therapeutic approach. New therapies are drastically needed to control cancer and one advantage of using selective amino acid restriction is that its effects are multifaceted, beneficially affecting both the host and the tumor at many levels. This approach can enhance chemotherapy, prevent host toxicity, and block invasion and metastasis. Understanding the specific amino acid dependency of tumors, and then capitalizing on this dependency could become an essential strategy for controlling cancer, especially metastatic disease that currently is virtually incurable.

ACKNOWLEDGMENTS

The authors acknowledge the following specific grants that have supported our current research and described in this chapter. They are R01CA77604 from NIH, DAMD17-00-1-0107 from the Department of Defence Prostate Cancer Research Program, and 01A22 from the American institute for Cancer Research.

REFERENCES

1. Weber, B. L., 2002, Cancer genomics. Cancer Cell, 1:37-45.
2. Persad, S., S. Attwell, V. Gray, M. Delcommenne, A. Troussard, J. Sanghera, and Dedhar, S., 2000, Inhibition of integrin-linked kinase (ILK) suppresses activation of protein kinase B/Akt and induces cell cycle arrest and apoptosis of PTEN-mutant prostate cancer cells. Proc Natl Acad Sci, 97:3207-3212.
3. Ramaswamy, S., Nakamura, N., Vazquez, F., Batt, D. B., Perera, S. Roberts, T. M., and Sellers, W. R., 1999, Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. Proc Natl Acad Sci, 96:2110-2115.
4. Peters, M. A. and Ostrander, E. A., 2001, Prostate cancer: simplicity to complexity. Nature Genetics, 27:134-135.
5. Konishi, N., Nakamura, M., Kishi, Nishimine, M., Ishida, E., and. Shimada, K., 2002, Heterogeneous methylation and deletion patterns of the NIK4a/ARF locus within prostate carcinomas. Am J Pathol, 160:1207-1214.
6. Porkka, K. P. and Visakorpi, T., 2001, Detection of differentially expressed genes in prostate cancer by combining suppression subtractive hybridization and cDNA library array. J Pathol, 193:73-79.
7. Waghray, A., F. Schober, F. Feroze, F. Yao, J. Virgin, and Chen, Y. Q., 2001, Identification of differenctially expressed genes by serial analysis of gene expression in human prostate cancer. Cancer Res, 61:4283-4286.
8. Cristofano, A. D. and Pandolfi, P. P., 2000, The multiple roles of PTEN in tumor suppression. Cell, 100:387-390.
9. Gronberg, H., 2003, Prostate cancer epidemiology. Lancet, 361:859-864.
10. Hanahan, D. and Weinberg, R., 2000, The hallmark of cancer. Cell, 100:57-70.
11. Zhang, Y., Ni, J., Messing, E. M., Chang, E., Yang, C. R., and Yeh, S., 2002, Vitamin E succinate inhibits the function of androgen receptor and the expression of prostate-specific antigen in prostate cancer cells. Proc Natl Acad Sci U S A, 99:7408-7413.
12. Zhang, X. K., 2002, Vitamin A and apoptosis in prostate cancer. Endocr Relat Cancer, 9: 87-102.
13. Wargovich, M. J., 1997, Experimental evidence for cancer preventive elements in foods. Cancer Lett, 114:11-17.
14. Connolly, J. M., Coleman, M. and Rose, D. P., 1997, Effects of dietary fatty acids on DU145 human prostate cancer cell growth in athymic nude mice. Nutr Cancer, 29:114-119.
15. Guo, H., Lishko, V. K., Herrera, H., Groce, A., Kubota, T., and Hoffman, R. M. 1993, Therapeutic tumor-specific cell cycle block induced by methionine starvation in vivo. Cancer Res, 53:5676-5679.
16. Poirson-Bichat, F., Gonfalone, G., Bras-Gonçalves, R. A., Dutrillaux, B., and Poupon, M. F., 1997, Growth of methionine-dependent human prostate cancer (PC-3) is inhibited by ethionine combined with methionine starvation. Br J Cancer, 75:1605-1612.

17. Lu, S. and Epner, D. E., 2000, Molecular mechanisms of cell cycle block by methionine restriction in human prostate cancer cells. Nutr Cancer, 38:123-130.
18. Elstad, C. A., Meadows, G. G., and Abdallah, R. M., 1990, Specificity of the suppression of metastatic phenotype by tyrosine and phenylalanine restriction. Clin Exp Met, 8:393-416.
19. Ge, X., Fu, Y.-M., Li, Y.-Q., and Meadows, G. G., 2000, Induction of apoptosis by tyrosine and phenylalanine deprivation requires activation of caspases in A375 human melanoma cells. Proc Am Assoc Cancer Res, 41:509.
20. Pelayo, B. A., Fu, Y.-M., and Meadows, G. G., 1999, Inhibition of B16BL6 melanoma invasion by tyrosine and phenylalanine deprivation is associated with decreased secretion of plasminogen activators and increased plasminogen inhibitors. Clin Expl Met, 17:841-848.
21. Pelayo, B. A., Fu, Y.-M., and Meadows, G. G., 2001, Decreased tissue plasminogen activator and increased plasminogen activator inhibitors are associated with inhibition of invasion in human A375 melanoma deprived of tyrosine and phenylalanine. Int J Oncol, 18:877-883
22. Fu, Y.-M., Yu, Z.-X., Pelayo, B. A., Ferrans, V. J., and Meadows, G. G., 1999, Focal adhesion kinase-dependent apoptosis of melanoma induced by tyrosine and phenylalanine deficiency. Cancer Res, 59:758-765.
23. Fu, Y.-M., Yu, Z.-X., Ferrans, V. J., and Meadows, G. G., 1997, Tyrosine and phenylalanine restriction induces G0/G1 cell cycle arrest in murine melanoma in vitro and in vivo, Nutr Cancer, 29:104-113.
24. Marten, N. W., Burke, E. J., Hayden, J. M., and Straus, D. S. 1994, Effect of amino acid limitation on the expression of 19 genes in rat hepatoma cells. FASEB J, 8:538-54.
25. Tong, D. Li, G., L., Chopra, D. P., and. Poter, A. T., 1998, Extended survivability of prostate cancer cells in the absence of trophic factors: increased proliferation, evasion of apoptosis, and the role of apoptosis proteins. Cancer Res, 58:3466-3479.
26. Meadows, G. G., Zhang, H. and Ge, X. 2001, Specific amino acid deficiency alters the expression of genes in human melanoma and other tumor cell lines. J Nutr, 131:3047S-3050S.
27. Meadows, G. G., Ge, X., Zhang, X., Oros, D. E., and Fu, Y.-M., 2002, Inhibition of invasion and metastasis during specific amino acid restriction associated with metastasis suppressor and other gene changes. In Cancer Metastasis Related Genes, 3: 2191-208, Kluwer, The Netherlands.
28. Fu, Y.-M., Yu, Z. X., Li, Y.-Q., Ge, X., Sanchez, P. J., Fu, X., and Meadows, G. G., 2003, Specific amino acid dependency regulates invasiveness and viability of androgen-independent prostate cancer cells. Nutr Cancer, 45:60-73.
29. Meadows, G. G., Pierson, H. F. Abdallah, R. M. and Desai, P. R., 1982, Dietary influence of tyrosine and phenylalanine on the response of B16 melanoma to carbidopa-levodopa methyl ester chemotherapy. Cancer Res, 42:3056-3063.
30. Meadows, G. G. and Oeser, D. E., 1983, Response of B16 melanoma-bearing mice to varying dietary levels of phenylalanine and tyrosine. Nutr Rep Int, 28:1073-1082.
31. Meadows, G. G., DiGiovanni, J., Minor, L. and Elmer, G. W., 1976, Some biological properties and an in vivo evaluation of tyrosine phenol-lyase on growth of B-16 melanoma. Cancer Res, 36:167-171.
32. Elmer, G. W., Minor, L., Meadows, G. G., Spackman, D. H., and Riley, V., 1978, Increased tyrosine phenol-lyase activity in mice following pyridoxal phosphate administration. Cancer Res, 38:3663-3667.
33. Ge, X., Fu, Y.-M., Li, Y.-Q., and Meadows, G. G., 2002, Activation of caspases and cleavage of Bid are required for tyrosine and phenylalanine deficiency-induced apoptosis of human A375 melanoma cells. Arch Biochem Biophys, 403:50-58.
34. Ballif, A. B. and J. Blenis, 2001, Molecular mechanisms mediating mammalian mitogen-activate protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. Cell Growth & Differentiation, 12:397-408.
35. Wasa, M., Bode, B. P., and Souba, W. W., 1996, Adaptive regulation of amino acid transport in nutrient-deprived human hepatomas. Am J Surg, 171:163-9.
36. Robertson, N. P., Starkey, J. R., Hamner, S., and Meadows, G. G., 1989, Tumor cell invasion of three-dimensional matrices of defined composition: evidence for a specific role for heparan sulfate in rodent cell lines. Cancer Res, 49:1816-1823.
37. Fu. Y.-M., Li, Y.-Q., Zhang, H., Ge, X., Fu, X., and Meadows, G. G., 2002, Specific amino acid dependency modulates expression of integrins and invasiveness in solid tumor cells. Proc Am Assoc Cancer Res, 43:541.
38. Meissauer, A., Kramer, M. D. Hofmann, M., Erkell, J., Jacob, E., Schirrmacher, V.,and Brunner, G., 1991, Urokinase-type and tissue-type plasminogen activators are essential for in vitro invasion of human melanoma cells. Exp Cell Res, 192:453-459.
39. de Vries, T. J., Quax, P. H., Denijn, M., Verrijp, K. N. , Verheijen, J. H., Verspaget, H. W., Weidle, U. H., Ruiter, D. J., and van Muijen, G. N., 1994, Plasminogen activators, their inhibitors, and urokinase receptor emerge in late stages of melanocytic tumor progression. Am J Pathol, 144:70-81.

40. Guo, X., Knudsen, B. S., Peehl, D. M., Ruiz, A., Bok, D., Rando, R. R., Rhim, J. S., Nanus, D. M., and Gudas, L. J., 2002, Retinol metabolism and lecithin:retinol acyltransferase levels are reduced in cultured human prostate cancer cells and tissue specimens. Cancer Res, 62:1654-1661.
41. Gugliucci, A., Ranzato, L., Scorrano, L., Colonna, R., Petronilli, V., Cusan, C., Prato, M., Mancini, M., Pagano, F., and Bernardi, P., 2002, Mitochondria are direct targets of the lipoxygenase inhibitor MK886. A strategy for cell killing by combined treatment with MK886 and cyclooxygenase inhibitors. J Biol Chem, 277:31789-31795.
42. Joshi, B., Li, L., Taffe, B. G., Zhu, Z., Wahl, S., Tian, H., Ben-Josef, E., Taylor, J. D., Porter, A. T., and Tang, D. G., 1999, Apoptosis induction by a novel anti-prostate cancer compound, BMD188 (a fatty acid-containing hydroxamic acid), requires the mitochondrial respiratory chain. Cancer Res, 59:4343-4355.
43. Tang, D. G., La, E., Kern, J., and Kehrer, J. P., 2002, Fatty acid oxidation and signaling in apoptosis. Biol Chem, 383:425-442.
44. Jiang, W., Zhu, Z., and Thompson, H. J., 2003, Effect of energy restriction on cell cycle machinery in 1-methyl-1- nitrosourea-induced mammary carcinomas in rats. Cancer Res, 63:1228-1234.
45. Rieber, M. S. and Rieber, M., 1993, Specific tyrosinases associated with melanoma replicative senescence and melanogenesis. Cancer Res, 53:2469-2471.
46. Zhuang, L., Lin, J., Lu, M. L., Solomon, K. R., and Freeman, M. R., 2002, Cholesterol-rich lipid rafts mediate Akt-regulated survival in prostate cancer cells. Cancer Res, 62: 2227-2231.
47. Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B., and Parsons, J. T., 1992, pp125FAK, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Proc Natl Acad Sci, 89:5192-5196.
48. Lipfert, L., Haimovich, B., Schaller, M. D., Cobb, B. S., Parsons, J. T., and Brugge, J. S., 1992, Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125FAK in platelets. J Cell Biol, 119:905-912.
49. Juliano, R. L. and Haskill, S., 1993, Signal transduction from the extracellular matrix. J Cell Biol, 120:577-585.
50. Parsons, J. T., Martin, K. H., Slack, J. K., Taylor, J. M., and Weed, S. A., 2000, Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. Oncogene, 19:5606-5613.
51. Kahana, O., Micksche, M., Witz, I. P., and Yron, I., 2002, The focal adhesion kinase (P125FAK) is constitutively active in human malignant melanoma. Oncogene, 21:3969-3977.
52. Gioeli, D., Mandell, J. W., Petroni, G. R., Frierson Jr., H. F., and Weber, M. J., 1999, Activation of mitogen-activated protein kinase associated with prostate cancer progression. Cancer Res, 59:279-284.
53. Putz, T., Culig, Z., Eder, I. E., Nessler-Menardi, C., Bartsch, G., Grunicke, H.,and Überall, F. 1999, Epidermal growth factor (EGF) receptor bloackade inhibits the action of EGF, insulin-like growth factor I, and a protein kinase A activator on the mitogen-activated protein kinase pathways in prostate cancer cell lines. Cancer Res, 59:227-233.
54. Smalley, K. S., 2003, A pivotal role for ERK in the oncogenic behaviour of malignant melanoma?. Int J Cancer, 104:527-532.
55. Ge, X., Fu, Y., and Meadows, G. G., 2002, U0126, a mitogen-activated protein kinase kinase inhibitor, inhibits the invasion of human A375 melanoma cells. Cancer Lett, 179:133-140.
56. Abdallah, R. M., Starkey, J. R.,and Meadows, G. G., 1983, Alcohol and related dietary effects on mouse natural killer-cell activity. Immunology, 50:131-137.
57. Norris, J. R., Meadows, G. G., Massey, L. K., Starkey, J. R., Sylvester, D. M., and Liu, S.-Y., 1990, Tyrosine- and phenylalanine-restricted formula diet augments immunocompetence in healthy humans. Am J Clin Nutr, 51:188-196.
58. Abdallah, R. M., Starkey, J. R., and Meadows, G. G., 1987, Dietary restriction of tyrosine and phenylalanine: inhibition of metastasis of three rodent tumors. J Natl Cancer Inst, 78:759-766.
59. Pierson, H. F. and Meadows, G. G. 1983, Sodium ascorbate enhancement of carbidopa-levodopa methyl ester antitumor activity against pigmented B16 melanoma. Cancer Res, 43:2047-2051.
60. Pierson, H. F. and Meadows, G. G., 1985, Influence of supplemental ascorbate on the antitumor activity of 5-hydroxydopa, a purported cytotoxic metabolite. Cancer Lett, 29:157-168.
61. Elstad, C. A., Meadows, G. G., Aslakson, C. J., and Starkey, J. R., 1994, Evidence for nutrient modulation of tumor phenotype: impact of tyrosine and phenylalanine restriction. In Diet and Cancer: Markers, Prevention, and Treatments. M. M. Jacobs, ed, 171-183. Plenum Press, New York, USA.
62. Sica, A., Dorman, L., Viggiano, V., Cippitelli, M., Ghosh, P., Rice, N., and Young, H. A., 1997, Interaction of NF-κB and NFAT with the interferon-γ promoter. J Biol Chem, 272:30412-30420.
63. Jousse, C., Averous, J., Bruhat, A., Carraro, V., Mordier, S., and Fafournoux, P., 2004, Amino acids as regulators of gene expression: molecular mechanisms. Biochem Biophys Res Commun, 313:447-452.
64. Palii, S. S., Chen, H., Kilberg, M. S., 2004, Transcriptional control of the human sodium-coupled neutral amino acid transporter system A gene by

amino acid availability is mediated by an intronic element. J Biol Chem, 279:3463-3471.

65. Zhong, C., Chen, C., and Kilberg, M. S., 2003, Characterization of the nutrient-sensing response unit in the human asparagine synthetase promoter. Biochem J, 372:603-609.
66. Epner, D. E., 2001, Can dietary methionine restriction increase the effectiveness of chemotherapy in treatment of advanced cancer?. J Am Coll Nutr, 5:443S-475S.
67. Cellarier, E., Durando, X., Vasson, M. P., Farges, M. C., Demiden, A., Maurizis, J. C., Madelmont, J. C., and Chollet, P., 2003, Methionine dependency and cancer treatment. Cancer Treat Rev, 29:489-499.
68. Elstad, C. A., B. D. Thrall, G. Raha, and G. G. Meadows, 1996, Tyrosine and phenylalanine restriction sensitizes adriamycin-resistant P388 leukemia cells to adriamycin. Nutr Cancer, 25:47-60.
69. Meadows, G. G., Abdallah, R. M., and Starkey, J. R., 1986, Interaction between specific dietary factors and experimental chemotherapy of metastatic melanoma. Cancer Chemother Pharmacol, 16:229-236.
70. Elstad, C. A. and Meadows, G. G., 1990, Phenotypic stability of B16-BL6 melanoma exposed to low levels of tyrosine and phenylalanine. Anticancer Res, 10:1313-1318.
71. Uhlenkott, C. E., Huijzer, J. C., Cardeiro, D. J., Elstad, C. A., and Meadows, G. G., 1996, Attachment, invasion, chemotaxis, and proteinase expression of B16-BL6 melanoma cells exhibiting a low metastatic phenotype after exposure to dietary restriction of tyrosine and phenylalanine. Clin Expl Met, 14:125-137.
72. Elstad, C. A., and Meadows, G. G., 1993, Modulation of B16-BL6 murine melanoma metastatic phenotype by tyrosine and phenylalanine restriction in the absence of host selection pressures. Anticancer Res, 13:523-528.
73. Yu, G. and Chang, T. M. S., 2004, Effects of long-term oral administration of polymeric microcapsules containing tyrosinase on maintaining decreased systemic tyrosine levels in rats. J Pharm Sci, 93:831-837.
74. Chang, T. M. S., 2003, Future generations of red blood cell substitutes. J Intern Med, 253:527-535.

Chapter 17

Role of Tumour Microenvironment in Chemoresistance

Yong Wei and Jessie L.-S. Au
College of Pharmacy, Ohio State University, Columbus, OH 43210, USA

Abstract: Preclinical and clinical findings indicate multiple tumour micro-environmental factors, including growth factors, cytokines, cell-cell and cell-matrix adhesion molecules and hypoxia, protect solid tumours from therapeutic interventions. Experimental evidence have defined some of the resistance mechanisms, which have led to the development of innovative approaches aiming at specific targets. While some of these newer approaches have yielded therapeutic benefits in selected tumour types, considerable challenges remain in the management of the majority of patients with solid tumours. This chapter reviews the various tumour microenvironmental factors that contribute to drug resistance. These factors exert their effects through direct promoting resistance effectors and/or indirectly modulating other environmental factors. Furthermore, cooperative regulation, cross-talk and redundancy at different levels of signaling cascades affect the tumour progression and drug resistance, and can diminish the effectiveness of the single target therapeutic approach. A better understanding of the intersecting resistance pathways has the potential of leading to new therapeutic paradigms aiming at multiple targets, in order to overcome the microenvironment-conferred survival advantage to tumour cells.

Key words: Drug resistance, clinical drug resistance, solid tumour, microenvironment, soluble factor, epidermal growth factor, fibroblast growth factor, insulin-like growth factor, hepatocyte growth factor, scatter factor, transforming growth factor-β, interleukin 4, interleukin 6, interleukin 10, cell adhesion molecule, integrin, E-cadherin, hypoxia, HIF1, redundancy, cross-talk, therapeutic implications

1. INTRODUCTION

Since the first demonstration of antitumour activity of aminopterin (4-aminopteroyl-glutamic acid) in childhood acute leukaemia patients by Farber and colleagues in 1948 (1), considerable efforts have been spent on developing effective cancer chemotherapeutic agents. Curative or survival benefits have been achieved in a few selected tumour types (2). However, clinical drug resistance remains a major obstacle in most cancers, especially adult solid tumours (2). Studies using monolayer-cultured cells have defined several genetic mechanisms of drug resistance. Examples include (a) activation and/or overexpression of cell membrane drug efflux transporters (e.g., P-glycoprotein and other ATP-binding cassette transporters such as multi-drug resistance-associated proteins), breast cancer resistance protein, and lung resistance-related protein (3-8), (b) altered expression or activation of detoxifying enzymes such as glutathione S-transferase (9, 10), quantitative or qualitative alterations of drug targets (11-15), and (c) defects in apoptosis regulatory proteins (16-18). In spite of the promising preclinical data indicating therapeutic advantages by reversing these genetic resistance mechanisms, the clinical results of these experimental approaches have been largely disappointing (19). On the other hand, there is growing evidence suggesting that

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 285-321.

epigenetic factors or proteins present in the tumour microenvironment play important roles in clinical drug resistance.

Teicher and colleagues (20) demonstrated that repeated administration of alkylating agents to mice bearing syngeneic mammary tumours yielded subclones which, when reimplanted in other recipient mice, showed cross-resistance to alkylating agents. They further showed that this acquired resistance was exhibited only *in vivo* but not in monolayer cultures of the disaggregated tumour cells. Hoffman and colleagues developed a surgical orthotropic implantation technique (OIT) (21), where patient or animal tumour fragments are implanted into the tumour-originating organs. These orthotropic implants maintain clinically relevant tumour properties including progression, metastasis and chemosensitivity. For example, the orthotropic human small cell lung cancer in mice showed a clinically relevant chemosensitivity profile (i.e., sensitive to cisplatin and resistant to mitomycin C), whereas the same tumour implanted in subcutaneous sites shows the opposite profile (22). Similar results were obtained for colorectal cancer (23, 24), fibrosarcoma (24) and renal cancer (25). Our laboratory similarly found that lung and lymph node metastases lost their chemoresistance when reimplanted in subcutaneous sites (26).

Organ-specific chemosensitivity is also observed in patients. Table 1 outlines the various tumour types displaying different sensitivity to chemotherapy. For example, breast, colorectal, testicular and ovarian cancers usually are responsive to chemotherapy initially (2). In contrast, patients with renal, pancreatic and oesophageal cancers show very low initial response rate (2), even though the tumour cell lines derived from these cancers are equally sensitivity to chemotherapy as cell lines derived from the other more chemo-responsive tumour types (27).

These earlier studies suggest a critical role of tumour microenvironment on preclinical and clinical chemosensitivity or chemoresistance. In solid tumours, cancer cells are surrounded by vasculature and stromal tissues. The tumour-stromal interaction results in tumour-specific expression of soluble factors and extracellular matrix components, some of which promote tumour growth and invasiveness. Dysregulated tumour progression promotes active but abnormal angiogenesis and higher hypoxia level in tumours, which also affect tumour progression and chemosensitivity.

Table 1. Organ specific responses to chemotherapy. adapted from (2).

Curable by chemotherapy
Acute leukaemia
High grade non-Hodgkin's lymphoma
Hodgkin's disease
Choriocarcinoma
Germ cell tumours
Wilms' tumour
Ewing's sarcoma
Osteosarcoma
Neuroblastoma
Chemotherapy improves survival
Breast cancer
Ovarian cancer
Small cell lung cancer
Bladder cancer
Colorectal cancer
Gastric cancer
Modest survival improvement: Tumour symptomatic response only
Non-small cell lung cancer
Metastatic cancer of unknown primary origin
Endometrial cancer
Soft tissue sarcoma
Carcinoids
Head and neck cancer
Pancreatic cancer
Brain tumours
Mesothelioma
Esophageal cancer
Poorly sensitive to chemotherapy
Prostate cancer
Adrenocortical cancer
Melanoma
Renal cancer
Thyroid cancer

This chapter focuses on the effects of tumour environmental factors on sensitivity to chemotherapy and/or radiotherapy, with special emphasis on growth factors, cytokines, cell adhesion molecules and hypoxia. The information on each factor is discussed in the following order, (a) general information, (b) distribution and/or expression status

in cancer patients, (c) association with disease progression and/or resistance in preclinical models and cancer patients, (d) resistance mechanisms, and (e) current status as a therapeutic target and development of modulators.

2. CHEMORESISTANCE INDUCED BY SOLUBLE FACTORS

Multiple growth factors and cytokines cause resistance to anti-cancer drugs in cell culture and animal tumour models. In keeping with the focus of clinically relevant resistance, we will discuss the soluble factors that satisfy the following criteria, (a) inducible by chemotherapy, (b) associated with chemotherapy outcome or patient prognosis, (c) affecting the efficacy of chemotherapy *in vitro* and/or *in vivo*, and/or (d) useful targets for achieving chemosensitization. Table 2 shows the growth factors and cytokines that satisfy these criteria. The receptors for these growth factors and cytokines, which are integral components of the corresponding intracellular signalling pathways, are also discussed. Note that some of these factors may cause either chemoresistance or chemosensitization depending on the experimental systems.

2.1 Growth factors

2.1.1 Epidermal growth factors/ Epidermal growth factor receptors

Aberrant activation of epidermal growth factor receptor (EGFR) or human EGFR family members, e.g., EGFR and human EGF receptor 2 (HER2), either through overexpression of receptors and/or elevation of cognate ligands, e.g., EGF and transforming growth factor-α, promotes tumour cell proliferation, survival, invasion, metastasis, and angiogenesis, resulting in enhanced tumourigenesis and progression (28-30). Other mechanisms independent of EGFR/HER2 expression, e.g., constitutively active mutation of these receptors, transactivation by other receptors including G-protein coupled receptors, interleukin receptors, estrogen receptors and cell adhesion molecules, can also cause aberrant activation of EGFRs (28-31).

In patients, higher expression of EGFR family proteins and/or cognate ligands is associated with worse prognosis, shorter survival, and resistance to radiotherapy and chemotherapy in multiple solid tumour types (28, 29, 32-39).

In vitro and *in vivo* preclinical studies have shown that activation of EGFR and HER2 leads to activation of the downstream Ras/Raf/ MAPK, STAT3/7 and PI3K/AKT pathways, resulting in modulations of apoptosis regulatory proteins and thereby protecting tumour cells from cell death and causing resistance to several classes of antitumour drugs (28, 29, 40, 41). The protective effect mediated by EGFR activation is more pronounced in anoikis, or apoptosis due to loss of cell attachment, suggesting a link between EGFR-mediated survival pathways and adhesion molecules (40, 42).

Paradoxically, studies in several experimental tumour models have demonstrated that activation of EGFR and HER2 (a) reduces cell adhesion and thereby enhances apoptosis (43, 44), (b) inhibits DNA topisomerase II and thereby promotes DNA damage (45), and (c) accelerates the cell proliferation rate and thereby increases the sensitivity of tumour cells to chemotherapeutics (46-49). In patients, several studies on node-negative and node-positive breast cancer patients show that the efficacy of doxorubicin-containing adjuvant therapy is dependent on HER2 status, with higher response rate and longer survival in patients with higher HER2 expression (50-54). Similarly, in patients with advanced urothelial carcinoma, patients with HER2-positive tumours are more likely to respond to paclitaxel and show lower death rate (55, 56).

Additional preclinical studies have demonstrated that EGFR-targeting approaches, by using either monoclonal antibody or small molecule tyrosine kinase inhibitors (TKIs), enhance the antitumour activity of chemotherapy and radiotherapy *in vitro* and *in vivo* (28, 57). These encouraging preclinical results have led to significant efforts to develop and evaluate HER2 and EGFR modulators in patients, as monotherapy or in combination with standard radiotherapy or chemotherapy (28, 58-60).

Table 2. Clinical relevance of soluble chemoresistance factors. Abbreviations are: BC, breast cancer; BLC, bladder cancer; CC, cervical cancer; CRC, colerectal cancer; GBM, glioblastoma; GC, gastric cancer, GLM, gliomas; HC, hepatocellular carcinoma; Mel, melanoma; MM, multiple myeloma; NSCLC, non-small cell lung cancer; OC, ovarian cancer; PaC, pancreatic cancer; PC, prostate cancer; RCC, renal cell carcinoma; SCCHN squamous cell head and neck cancer; SCCOP, squamous cell cancer of the oropharynx; SCLC, small cell lung cancer; TC, thyroid cancer; UC urothelial carcinoma. If the tumour type is not specifically mentioned, the association is found in most of the common solid tumour types. ND, not studied or not reported.

Factors/Receptors	Disease prognosis with elevated expressions/levels		Usefulness as therapeutic targets	
	Tumour progression	Treatment outcome	Preclinical models	Patients
EGFs/EGFRs	Worse: carcinogenesis, advanced stages, increased metastasis, angiogenesis, shorter survival	Resistance: radiatherapy in SCCHN and GLM Chemotherapy in many solid tumours *Favourable: HER2 favourable for adjuvant chemotherapy in BC and taxane based therapy in UC*	EGFR tyrosine kinase inhibitors and EGFR antibody improve effectiveness of chemotherapy and radiatherapy in multiple solid tumours	Same agents have activity as single agent and/or improve effectiveness of chemotherapy in advanced NSCLC, SCCHN, CRC as 2nd or 3rd line treatments
FGF2/FGFRs	Worse: advanced stages, increased metastasis, angiogenesis, shorter survival *Favourable: in primary BC, OC, advanced GLM*	Resistance: chemotherapy in SCLC	Improve effectiveness of chemotherapy in PC, BC, RCC, PaC, BLC, CRC by a non-specific inhibitor suramin	In phase II evaluation, suramin improve effectiveness of chemotherapy in advanced NSCLC
IGFs/IGFR1	Worse: carcinogenesis, increased metastasis, shorter survival	Resistance: chemotherapy and radiatherapy in BC	Improve effectiveness of chemotherapy in multiple solid tumours	ND
HGF/SF/c-Met	Worse: carcinogenesis, advanced stages, increased metastasis, angiogenesis, shorter survival	Resistance: radiatherapy in SCCOP, chemotherapy in MM	ND	ND
TGFβ	Worse: advanced stages, increased metastasis, angiogenesis, shorter survival *Favourable: Suppressive in early stage of solid* tumours	Resistance: chemotherapy and radiatherapy in CC	Improve effectiveness of chemotherapy in BC, GC, PC, HC	ND
IL4	Worse: shorter survival in RCC	Resistance: chemotherapy in TC	ND	ND
IL6	Worse: advanced stages, increased metastasis, shorter survival	Resistance: chemotherapy in PC, BC, RCC, GBM	Improve effectiveness of chemotherapy in PC, RCC, MM	ND
IL10	Worse: advanced stages, increased metastasis, recurrence, shorter survival *Favourable: stage 1 NSCLC*	Resistance: chemotherapy in TC, GC, CRC, NSCLC, Mel	Improve effectiveness of chemotherapy in GBM	In Phase II evaluation, AS101 Improves effectiveness of chemotherapy in NSCLC

In pivotal clinical trials, trastuzumab (Herceptin), a monoclonal anti-HER2 antibody, shows activity in HER2-positive metastatic breast cancer as single agent (61, 62) and in combination therapy with multiple standard chemotherapy regimens, e.g., anthyracycline plus cyclophosphomide, paclitaxel (63). The activity of trastuzumab is associated with the HER2 expression status. Trastuzumab is currently being evaluated as adjuvant therapy in patients with primary breast cancer (64-66). These studies have established the therapeutic value of trastuzumab and indirectly the HER2-targeting approach in breast cancer. The situation in other cancer types is less promising. In spite of strong preclinical data (67, 68), trastuzumab fails to show activity either as monotherapy or in combination with standard chemotherapy such as cisplatin plus gemcitabine or docetaxel in patients with HER2-positive advanced non-small cell lung cancer or prostate cancer (69-71). This failure is presumably, at least partly, due to compensation by coexpression of EGFR1 (72, 73).

EGFR modulators, including tyeosine kinase inhibitors (TKIs) (i.e., gefitnib or Iressa, erlotinib or Tarceva) and monoclonal antibody (cetuximab or Erbitux), are well tolerated in patients. These agents show activity in patients with advanced chemotherapy-refractory squamous cell head and neck cancer, non-small cell lung cancer, pancreatic cancer and colorectal cancer, either as monotherapy (e.g., gefitinib as third line treatment of non-small cell lung cancer patients) or in combination with standard chemotherapy (e.g., cetuximab in combination with irinotecan in irinothecan-refractory colorectal cancer patients) (28, 74-80). However, in large randomized phase III trials, all three modulators failed to show superior response rate and survival in chemotherapy-naïve, advanced non-small cell lung cancer (gefitnib and erlotinib) or colorectal cancer (cetuximab) compared to standard chemotherapy (28, 74-76, 79, 81, 82).

Unlike the association between HER2 expression and responsiveness to trastuzumab in breast cancer, patient response to EGFR modulators are not correlated with the EGFR expression. Two recent studies have identified mutations in the tyrosine kinase domain of EGFR in a subset of non-small cell lung cancer patients (less than 10% in American patients and ~30% in Japanese patients) as potential prognostic indicator of patient response. These mutations result in enhanced intensity and duration of EGFR activation by EGF and the corresponding survival signals as well as enhanced sensitivity to EGFR inhibition by gefitnib (83-85).

There are several interesting aspects to the profiles of clinical activities of the various HER2 and EGFR modulators. First, the finding that the EGFG/HER2-targeting approach results in therapeutic benefits in several major tumour types suggests an common role of EGFR in chemosensitivity/chemoresistance of solid tumours. Second, the success in chemotherapy-refractory patients together with the failure in chemotherapy-naïve patients suggests an role of EGFR in the clinically acquired resistance to platinum-, irinotecan-, and taxane-based therapy. The selective benefits of EGFR modulators in the second/third-line setting are also consistent with a scenario of selection of subclones carrying mutated EGFR receptors. Third, the failure of EGFR modulators as first-line treatment in lung and colorectal cancer, together with the opposite effects of trastuzumab in breast and lung cancer patients, suggest the presence of redundant, compensatory survival signalling from other HER2/EGFR family members or other growth factors.

2.1.2 Fibroblast growth factors and their receptors

FGFs constitute a large family of 22 growth factors with molecular weights ranging from 17 to 34 kDa. FGFs are expressed in most, if not all, tissues. FGF1 and/or FGF2 (also called acidic and/or basic FGFs) are involved in the development and function of numerous organ systems, induce cell proliferation, migration, survival, and angiogenesis, and stimulate wound healing and repair, under *in vivo* and *in vivo* conditions (86-90).

FGF2 has been extensively studied. The FGF2 gene encodes several different isoforms. The low molecular weight (18kD) isoform is present in extracellular compartment and the high molecular weight (22 and 24 KD) isoforms are localized in intracellular compartment. The following discussion focuses on the extracellular FGF2. Multiple studies

have implicated FGF2 in chemoresistance, whereas the role of FGF1 was demonstrated recently by our laboratory (26).

The binding of FGFs to FGF receptors (FGFRs) in the presence of heparan sulfate proteoglycans results in FGFR dimerization. The FGFR family includes four members and, through various possibilities of alternative splicing, potentially consists of up to 100 isoforms (91). FGFR1, FGFR2, and FGFR3 are widely expressed in adult human tissues whereas the distribution of FGFR4 is more limited. The 7 major FGFR isoforms have different ligand-binding specificity, which is determined by the alternative splicing in Ig domain III (87, 92, 93). Activation of FGFRs results in activation of different signalling pathways leading to gene transcription and diverse responses (88, 94, 95). The signal transduction pathways of FGF2, including Ras-Raf-MEK-MAP kinase, PLC-DAG-PKC, and PLC-PI3K-Akt pathways; are implicated in cell survival (96-107).

Depending on cell types and growth conditions, FGF2 can cause mitogenesis or inhibit cell growth (108-110) and can either induce resistance or sensitization to cytotoxic insults (111) under *in vitro* conditions. On one hand, addition of exogenous FGF2 or over-expression of FGF2 confers resistance to chemotherapy (etoposide, cisplatin, fludarabine, doxorubicin, methotrexate, hydroxyurea, 5-fluorouracil, paclitaxel, N-(phosphonacetyl)-L-aspartic acid) in solid tumour cells (i.e., small lung cancer, prostate, bladder), chronic lymphocytic leukaemia cells, and fibroblasts (111-113). On the other hand, exogenous FGF2 or FGF2 overexpression enhances the sensitivity of breast, prostate, ovarian and pancreatic tumour cells and fibroblasts to chemotherapeutic agents (i.e., cisplatin, etoposide, 5-fluorouracil, doxorubicin, carboplatin, and docetaxel), and to oxidative stress (111).

Consistent with the dual roles of FGF2 in chemoresistance and chemosensitization, FGF2 also shows opposite effects in prognosis of cancer patients. Some studies reported association between high FGF2 expression and higher tumour apoptotic indices or improved overall and disease free survival and association between lower FGF2 levels and increased tumour size or higher tumour stage in breast cancer (114-118), ovarian cancer (119) and pediatric high-grade gliomas (120). Conversely, other studies reported associations between increased local FGF2 expression and shorter survival in nodal-negative breast cancer (121), and between elevated systemic and/or local tissue FGF2 levels and worse prognosis and shorter survival in leukaemia and lymphoma (122, 123), in solid tumours including non-small and small cell lung cancer (124-127), colorectal cancer (128), renal cell carcinoma (129), advanced carcinoma of head and neck (130), gastric cancer (131, 132), non-Hodgkin's lymphoma (133, 134), oesophageal carcinomas (135), thyroid carcinomas (136), malignant solitary fibrous tumour (137), mesothelioma (138), and Wilms' tumour (139). In pancreatic cancer, there is no relationship between FGF2 level and postoperative recurrence and survival, but increased FGF receptor expression is associated with shorter survival (140). A similar observation in non-small cell lung cancer patients has been reported (141). Mutation in the transmembrane domain of FGFR4 is associated with shorter disease-free survival in breast cancer (121, 142), colorectal cancer (142) and high-grade soft tissue sarcoma (143). Constitutively active FGFR3 mutation has been found in bladder and cervix carcinomas (144). Furthermore, elevated serum FGF2 levels are associated with poor response to chemotherapy in small cell lung cancer (145), suggesting a direct contribution of FGF2 to resistance.

The mechanisms of FGF2-conferred survival appear to be context-dependent, differ in different cells and differ in response to different stress signals. The FGF2-induced chemoresistance in small cell lung cancer cells is mediated through activation of the MAP kinase pathway resulting in upregulation of anti-apoptotic proteins Bcl-2, Bcl-XL and IAPs (146, 147), and the resistance in fibroblasts is mediated through MDM2 induction and the subsequent inhibition of p53 pathways (148). The mechanism of chemosensitization in breast tumour cells is presumably due to Bcl-2 down-regulation (111, 149, 150).

In addition to inducing resistance in tumour cells, FGF2 also protects endothelial cell against radiation- or chemotherapy-induced cell death, which in turn results in chemoresistance.

Alternatively, FGF2 may regulate the expression and signalling of other environmental survival factors (151-153). For example, in multiple myeloma, FGF2 secreted by tumour cells stimulates IL6 secretion from stromal cells, and IL6 in turn stimulates tumour cells to secrete more FGF2 (151). As discussed below, IL6, similar to FGF2, also protects tumour cells from cytotoxicity conferred by chemotherapy.

The reasons of the dual effects of FGF2 on chemosensitivity or chemoresistance are not clear. As FGF2 functions are regulated by multiple environmental factors, e.g. heparan sulfate proteoglycans (154-157), cell-matrix adhesion (158), cell-cell interaction (159-161), it is tempting to postulate that the switch between induction of resistance or sensitization is governed by intersecting microenvironmental factors..

The earlier studies on FGF2-induced chemoresistance used exogenous FGF2 concentrations that far exceed the concentrations in patient plasma and urine samples (10-50 vs <1 ng/ml) (26, 111) , thus raising questions on the clinical relevance of this mechanism. Our laboratory recently demonstrated that a second FGF, i.e., FGF1, amplified the FGF2 effect such that combinations of FGF1 and FGF2, at clinically relevant concentrations, induce up to 10-fold resistance to several anticancer drugs (26). We further showed that monoclonal FGF antibodies and/or a non-specific inhibitor of FGF1 and FGF2, suramin, reversed the FGF-induced resistance and significantly improved the sensitivity of human xenograft tumours to multiple chemotherapeutic agents, i.e., paclitaxel, 5-fluorouracil, doxorubicin and irinotecan (26, 162, 163), under *in vitro* and *in vivo* conditions. The suramin chemosensitization was broad spectrum and applied to colorectal, renal cell, breast, pancreatic, and bladder cancer (164-167), and was attained at dosing regimens yielding low, FGF-inhibitory but non-toxic suramin concentrations. These encouraging preclinical results have motivated several phase I/II clinical trials using non-toxic suramin regimens in lung, breast and kidney cancers. The first phase II trial in non-small cell lung cancer has been completed and the results suggest therapeutic efficacy using FGF-inhibitory suramin regimens (168).

2.1.3 Insulin-like growth factors and their receptors.

The insulin-like growth factor (IGF) family consists of two extracellular ligands, IGF-1 and IGF-2. The two membrane IGF receptors are IGF1R and IGF2R. Binding of IGF1 and IGF2 to IGF1R initiates the signalling cascades. Six circulating IGF binding proteins (IGFBP1-6) compete with IGF1R for binding with IGFs. IGF2R is responsible for the hydrolysis of the IGF/IGFBP1-6 complex, thereby making IGFs available for binding to IGF1R (169, 170).

The IGF signalling system regulates cell proliferation, apoptosis and differentiation and thereby plays critical roles in the development and physiological growth control of most if not all tissues. Aberrant activation of the IGF system contributes to carcinogenesis, tumour progression and metastasis in experimental tumour models (169-172). In patients, elevated activation of the IGF system, resulting from either increased serum IGF level or decreased IGFBP level, is associated with increased risk of breast, colon, prostate and lung cancers (169, 173-178). Similarly, the overall IGF system activity, represented either by increased expression of IGF and/or IGF1R or decreased levels of IGFBPs and/or IGF2R, is associated with poor prognosis and/or shorter disease- free interval or overall survival time in patients with ovarian cancer (179, 180), colorectal cancer (181, 182), head and neck cancer (183), non-small cell lung cancer (184), multiple myeloma (185), breast cancer (172, 186-190) and pulmonary adenocarcinoma (191).

Interestingly, an inverse association between the activation level of the IGF system and the prognosis or survival of breast cancer patients was not observed in patients undergoing surgical intervention (188, 189), but was observed in patients receiving chemotherapy or radiotherapy (187, 190). This suggests that the poor prognosis associated with the high IGF system activity is not due to enhanced tumour progression but rather due to resistance to chemotherapy and/or radiotherapy.

In tumour cells, activation of IGF1R stimulates cell proliferation and, through activation of MAPKs and PI3K/AKT pathways, also inhibits apoptosis induced by stress conditions such as treatments with

cytotoxic drugs or radiation, or deprivation of growth factors and/or nutrients (192-196). These two effects result in tumour cell resistance to multiple anticancer drugs, including doxorubicin, cisplatin, 5-fluorouracil, camptothecin, mitomycin C, actinomycin D, lovastatin, Cox-2 inhibitors, and to cytokines, e.g., tumour necrosis factor and interferon-γ (192, 193, 197-208).

Approaches to target the IGF signaling system have been evaluated in *in vitro* and *in vivo* preclinical tumour models (209, 210). IGF1R shows a high degree of similarity to insulin receptor (up to 70% homology). This, together with the wide distribution and broad physiological functions in normal tissues of these receptors, raise the concern of host toxicity. Hence, approaches targeting the IGF1R-specific gene sequences, including antisense RNA, ribozymes, triplex and small interfering RNA, are favored over the more conventional approaches using small molecule tyrosine kinase inhibitors and monoclonal antibodies. Antisenses against IGF1R, either by vector-expressed or chemical synthesized oligonucleotides, effectively (a) downregulate IGF1R and consequently inhibit survival in cultured cells, (b) through inhibition of tumourigenicity and metastasis, exert *in vivo* antitumour activity in multiple tumour types, and (c) enhance the cytotoxicity of several drugs in cultured Ewing's sarcoma, bladder cancer and prostate cancer cells (209, 210). These studies further yielded the unexpected finding that IGF1R reduction stimulates the host immune response, which in turn enhances the antitumour efficacy of the IGF system targeting approach. This finding has resulted in a pilot clinical trial in patients with malignant astrocytomas, where autologous glioma cells are collected, treated ex vivo with IGF1R antisense oligodeoxynucleotide, and then placed in small diffusion chambers that are reimplanted in patients (210-212).

2.1.4 Hepatocyte growth factor/scatter factor and receptor.

Hepatocyte growth factor/scatter factor, HGF/SF, and its specific receptor c-Met are involved in tumourigenesis and tumour progression (213-217). HGF/SF are predominantly expressed by mesenchymal cells. Although the HGF/c-Met system in tumours is primarily activated through endocrine or paracrine mechanisms (217, 218), autocrine activation has also been reported in *in vitro* and *in vivo* tumour models (219-222). For example, HGF/SF is predominantly expressed in tumour but not stromal cells present in non-small cell lung tumours (222).

Elevated serum and tissue HGF levels and aberrant *c-met* expression (constitutively active mutation and overexpression) are found in multiple tumours including the most common and most malignant types such as breast cancer, non-small cell lung cancer, multiple myeloma and pancreatic cancer (223-229).

In nearly all tumour types, enhanced activation of the HGF/c-Met system in patients is associated with resistance to radiotherapy and chemotherapy, and with worse prognosis and shorter survival (228-236). It is noted, however, that HGF exhibits cytotoxicity and enhances apoptosis induced by paclitaxel and cisplatin in ovarian carcinoma cell lines (237).

In experimental models, exogenous HGF protects human cancer cells (i.e., breast, leiomyosarcoma, gastric, prostate, glioblastoma and rhabdomyosarcoma) as well as endothelial cells from cell death induced by ion radiation or cytoxtoxic drugs including doxorubicin, cisplatin, etoposide, camptothecin, paclitaxel and tumour necrosis factor (238-248). The protective effects are derived from its anti-apoptotic (238-240, 242, 243 ,245 ,247) and/or enhanced DNA repair function (246, 248).

Several specific small molecular inhibitors of the HGF/SF/c-Met system or the biological agonist NK4 (HGF N-terminal four Kringle domain variant), which is a proteolytic cleavage product of HGF that competitively inhibits the binding of HGF to its receptor (249), have shown antitumour activity as single agents, in *in vitro* and *in vivo* preclinical models. Whether these agents enhance the activity of the conventional cytotoxic agents is not known.

2.2 Cytokines

Cytokines, a large family of immune modulator proteins, have been used to activate the immune system in cancer biotherapy or immunotherapy. A

recent review discusses the roles of cytokines in tumour pathogenesis and immunotherapy (250). Among the cytokines, transforming growth factor-β (TGFβ) and Th2 interleukins, secreted by T helper cells and tumour cells, contribute to tumour progression and protect tumour cells from cytotoxic chemotherapy, as follows.

2.2.1 Transforming growth factor-β

TGFβ is a pleiotropic growth factor and regulates multiple cellular functions including proliferation, adhesion, migration, and differentiation (251-257). TGFβ also induces the expression of matrix metalloproteinases, matrix components and adhesion molecules. These various effects together enable the remodelling of the microenvironment to provide for the appropriate physiological functions (251-257).

There are two types of TGFβ receptors, Type 1 and Type II. These receptors are widely distributed and expressed in normal and tumour cells. Members of the TGFβ family of proteins (TGFβ1 through TGFβ3 in mammalian species) bind to specific TGFβ receptors, followed by heterodimerization of the lignad-bound Type I and Type II receptors, and activation of the corresponding serine/threonine kinases (258). The activated receptors initiate multiple intracellular signaling cascades; the best characterized of which is the Smads-mediated signaling and transcriptional regulation pathway (258-260). TGFβ also activates TAK1 (TGFβ-activated kinase 1) (261) and small G-proteins (i.e., Ras, RhoA and RhoB), resulting in the activation of different MAPKs pathways, including ERK, p38 and JNK (262-268). In addition, TGFβ inhibits the phosphatase 2A-mediated activation of p70S6K, which is a ribosomal protein that regulates protein synthesis (269, 270). TGFβ also activates the PI3k/AKT survival pathways after a lag time; the delayed nature of this effect suggests the involvement of other mediating factors (271, 272).

TGFβ exhibits both tumour suppressing and tumour promoting functions (257, 273-275). TGFβ suppresses early stage carcinogenesis by inhibiting the growth of neoplastic cells of epithelial lineage. The tumour promoting function is largely through its suppression of host immune responses, stimulation of angiogenesis and promotion of tumour cell invasion and metastasis.

TGFβ overproduction is observed in most common tumour types, including prostate (276-285), breast (286-291), lung (292-300), hepatic (301-303), colorectal (304), gastric (305, 306) and brain cancer (307), and is associated with increased pathological stages, metastasis and/or poor prognosis. In most cases, TGFβ overproduction is associated with loss of responsiveness of tumour cells to TGFβ mediated growth inhibition, through alterations in various steps of the TGFβ signalling cascade, i.e., downregulation of TGFβ receptors, mutation of Smad, and upregulation of c-myc (282, 283, 285, 294, 296, 298, 308-314).

Pretreatment serum TGFβ level is a predictor of the outcome of radiation therapy in cervical cancer; higher levels are associated with worse locoregional control and shorter survival (315). However, pretreatment serum TGFβ level is not associated with acute radiation morbidity (315). These data indicate the selective effect of TGFβ on the radiosensitivity of tumour cells and not normal tissues.

Teicher and colleagues have conducted a series of elegant studies demonstrating the role of TGFβ in chemoresistance (20, 316-328). These investigators established an *in vivo* acquired drug resistance mouse mammary tumour model by repeated administration of alkylating agents to tumour-bearing mice (20). The key findings are as follows. First, the resistance phenotype was lost in monolayer cultures of the disaggregated tumour cells, indicating the involvement of environmental factors (20). Second, the implantation of the resistant tumour on one side of a mouse reduced the sensitivity of bone marrow cells and of the sensitive parent tumour implanted in the opposite side of the same host, indicating the present of circulating soluble resistance factors (20, 324, 326-328). Third, tumour morphological studies demonstrated the more fibrous nature, increased blood vessel density and increased metastatic potential of the resistant tumours, as compared to the parent, chemosensitive tumours (20, 324, 326-328). Based on these morphological changes that are typical of TGFβ functions, the investigators evaluated and established the role of TGFβ in chemoresistance.

First, mice bearing the resistant subclone showed higher pretreatment serum and intratumoural TGFβ levels compared to mice bearing the chemosensitive parent tumour (319, 321, 323). Second, blocking TGFβ by neutralizing antibodies (323) or the natural inhibitor decorin (319, 321) sensitizes the resistant tumours to chemotherapy. Third, over-expressing TGFβ by transfection in the parent chemosensitive cells resulted in chemoresistant tumours *in vivo* (319). This chemoresistance development was accompanied by several other changes observed in the acquired resistance tumour model developed by repeated challenges with chemotherapy, including elevation of serum TGFβ level and resistance of bone marrow cells. Furthermore, the chemoresistance due to TGFβ transfection was reversed by decorin (319). Finally, serum and intratumoural TGFβ levels are enhanced by chemotherapy, suggesting TGFβ as a mediator of chemoresistance acquired after therapy (316, 318). Similar findings have been reported in other tumour models including prostate, liver and gastric cancers, thus indicating the broad-spectrum nature of the TGFβ induced drug resistance (318, 320, 322).

In spite of the abundant evidence suggesting an important role of TGFβ in the chemoresistance observed in tumour-bearing animals, exogenous TGFβ does not induce resistance in monolayer cultures. The differences of TGFβ effects under *in vitro* and *in vivo* conditions suggest the involvement of additional factors present in tumour microenvironment. For example, in hepatocellular carcinoma cells, TGFβ promotes survival pathways including PI3K/AKT and FAK, an effect that is dependent on integrin-mediated adhesion and is most likely due to activation of integrin-linked kinase (329). In addition, TGFβ, together with growth factor signalling (IGF, EGF), through activation of receptor tyrosine kinases and Ras, stimulate epithelial-to-mesenchymal transition (i.e., squamous carcinoma to spindle carcinoma)(257). Furthermore, both direct and indirect effects of TGFβ, including host immune suppression, increased vascular endothelial growth factor (VEGF) production, remodelling of extracellular matrix and modulation of cell-cell adhesion molecule expression and signalling, contribute to angiogenesis in tumours. These various findings suggest the *in vivo* TGFβ-mediated chemoresistance as a result of the effects of complex networking between TGFβ and other environmental factors on the different compartments in a solid tumour, i.e., tumour cells, stroma, and blood vessel, as well as the host immune system.

Due to the critical roles of TGFβ in tumour progression and resistance to chemo- and radiotherapy, TGFβ and the associated signalling pathways are attractive cancer therapy targets. However, the fact that TGFβ also suppresses early stage tumour development and promotes carcinogenesis introduces the uncertainty that inhibition of TGFβ may lead to undesirable outcome. A better understanding and differentiation of the molecular mechanisms of these various TGFβ effects may provide more specific targeting approaches to blocking its tumour promoting and chemoresistance functions while retaining its tumour suppressive function. Furthermore, inhibitors of TGFβ signaling may have promise as enhancers of chemotherapy or radiotherapy, as suggested by tumor model studies where inhibition of TGFβ by neutrilization antibodies or the natural inhibitor decorin enhanced the efficacy of chemotherapy.

2.2.2 Interleukins

Various cytokines including interleukins (IL) are secreted by two types of T helper cells, i.e., types 1 and 2 or Th1 and Th2. Th1 cells express IL2, interferons and tumour necrosis factor β and mediate cellular immune response. Th2 cells express IL4, 5, 6, 10, 13 and mediate humoral immune response (330). In tumour-bearing animals, TGFβ shifts the balance between Th1 and Th2 responses toward Th2 response by inducing the overproduction of IL10, which initiates Th2 functions and inhibits Th1 functions.

Overproduction of Th2 cytokines and/or aberrant activation of the signalling pathways of Th2 cytokines have been found in patients with multiple types of advanced cancer, and in most cases are indicators for poor prognosis or short survival. For example, elevated serum IL6 level is observed in advanced metastatic prostate cancer, hormone-refractory metastatic breast cancer, glioblastoma multiforme, and renal cell carcinoma, and is

predictive of poor prognosis and shorter survival in these patients following chemotherapy and/or immunotherapy (331-338). Elevated serum IL10 levels, in some cases accompanied by elevated IL6 and/or IL8 levels, have been found in patients with gastric and colon cancer, aggressive Hodgkin's lymphoma, metastatic melanoma, advanced non-small cell lung cancer, hepatocellular carcinoma, and are associated with recurrence and/or shorter survival following therapy (339-354). On the other hand, a lack of IL10 expression in the tumour tissue of stage 1 non-small cell lung cancer patients is associated with a poor prognosis and shorter survival, suggesting a tumour suppressive function of IL10 in early stage disease (355). These biphasic effects of IL10 mirror the biphasic effects of TGFβ on tumour progression, and raise the interesting question whether the TGFβ effects are mediated through Th2 cytokines.

IL4 is overexpressed in thyroid cancers and high IL4 levels are associated with the resistance of thyroid cancer to chemotherapy (356). However, IL4 level is not associated with cancer progression, response to chemotherapy or immunotherapy, or prognosis in other tumour types. In fact, IL4 shows antitumour activity in breast and renal cell carcinoma. In the Japanese population, IL4 levels are no effects whereas genetic polymorphisms of IL4 receptor α gene result in heightened IL4 signalling and Th2 immunity and are associated with higher incidence and poor prognosis in renal cell carcinoma (330).

Since Th1-meidated immunity is the major antitumour immunity mechanism under *in vivo* conditions, Th2-mediated immunity, by suppressing Th1 immunity, results in tumour promotion and/or resistance. However, multiple lines of evidence support the notion that Th2 cytokines, including IL4, IL6, and IL10, confer survival advantage to tumour cells directly. Primary cultures of disaggregated thyroid cancer cells from patients produce IL4 and IL10 and cause the over-expression of anti-apoptotic proteins Bcl-2 and Bcl-XL and thereby confer resistance to cytotoxic chemotherapy (356). These findings are in agreement with the clinical observation that high level of Bcl-2 and Bcl-XL in thyroid tumours is associated with high resistance to chemotherapy (356). Likewise, murine B16 melanoma cells and primary cultures of human stomach adenocarcinoma and glioblastoma multiforme produce high levels of IL10, which protects tumour cells from cytotoxic chemotherapy (357). The IL10-induced chemoresistance was observed under *in vitro* and *in vivo* conditions, and is mediated by STAT3-dependent upregulation of anti-apoptotic Bcl-2 family proteins (357). Similarly, autocrine or paracrine activation of IL6 signalling induces multidrug resistance in breast, prostate, pancreatic, cervical and oesophageal carcinoma cells. IL-6 induced resistance is mediated by activation of STAT3, MAPK and/or PI3K/AKT pathways, through upregulation of anti-apoptotic Mcl1 and Bcl-2 family proteins, mdr1 drug transporter and/or detoxification enzyme glutathione transferase.

IL6 and IL10 have been investigated as potential therapeutic targets. Blocking IL10 actions using an inhibitor AS101, an immunomodulator, inhibits STAT3 activation, downregulates anti-apoptotic Bcl-2 family protein and sensitizes aggressive human glioblastoma multiforme to paclitaxel treatment under *in vitro* and *in vivo* conditions in preclinical models. The chemosensitization effect of AS101 was achieved at nontoxic drug levels (357). A subsequent phase II trial using AS101 in combination with chemotherapy in unresectable or metastatic non-small cell lung cancer patients shows higher response rate and lower toxicity, partially validating the concept of using IL10 inhibition as a chemosensitizer in patients (358).

Inhibition of IL6 or IL6 receptor using blocking antibodies sensitizes renal carcinoma and prostate cancer cells to anti-tumour drugs, e.g., etoposide, cisplatin and mitomycin C (359, 360). Sant7, a modified high affinity analog of IL6 that binds to IL6R without initiating downstream signaling, inhibits multiple myeloma and prostate cancer cell growth and sensitizes tumour cell to cytotoxic drugs (361, 362).

3. CELL ADHESION MOLECULES

Extensive studies have demonstrated the critical roles of cell adhesion to extracellular matrix and tumour/stromal cell interaction in tumourigenesis

and tumour progression (160, 363-374). The two major categories of adhesion/interaction molecules are (a) integrins which are the major mediators of cell-matrix adhesion, and (b) cadherins, selectins and members of the immunoglobulin superfamily cell adhesion molecules (CAM-Ig), which mainly mediate cell-cell interaction. Multiple lines of evidence support important roles for these compounds in mediating chemoresistance of solid tumours, as follows.

First, the expression levels of adhesion molecules, including ICAM1(375-383), CD44 (384-386), NCAM (387-390), LFA-3 (383), E-cadherin (391, 392), P-cadherin (393), integrin β1(394, 395), are correlated with poor prognosis, resistance to chemotherapy and radiotherapy, and shorter survival in multiple types of solid tumours and leukaemia, suggesting a potential role of cell adhesion mediated clinical drug resistance.

Second, conventional cytotoxic chemotherapy upregulates the expression of adhesion molecules in solid tumours, suggesting alteration in cell adhesion as a response to chemotherapy. A comparison of the gene expression profiles in three lung cancer patients using the cDNA array technique shows significant increases in adhesion molecules, including matrix metalloproteinases, integrins, endonexin, collagens and FGFR3, in post-chemotherapy lung cancer tissues compared to normal lung tissues from other donors (396). Similarly, patients with Barrett's-associated adenocarcinoma showed significantly elevated E-cadherin expression following chemotherapy or radiotherapy compared to patients who did not receive therapy (397). Higher E-cadherin levels are also associated with a shorter survival in patients receiving chemotherapy or radiotherapy, but this association was not observed in patients that did not receive therapy (397), demonstrating direct contribution of this responsive induction of E-cadherin to chemoresistance. The role of adhesion in chemoresistance was further demonstrated in series of studies on small cell lung xenograft tumours, the adhesion-dependent chemoresistance mimic the *in vivo* resistance in patients and involves altered extracellular matrix and cell adhesion molecules expression, constitutive activation of MAPK and AKT pathways and modulation of apoptosis molecules (398, 399).

Third, preclinical studies have demonstrated that specific cell adhesion to either extracellular matrix or neighbouring stromal/cancer cell causes drug resistance in different experimental models. Altered expression of extracellular matrix components e.g., collagen IV and membrane integrins, is associated with acquired resistance in tumour cells398, 400, 401. Adhesion to protein or non-protein extracellular matrix components, e.g., fibronectin, collagens, tenacin, laminin and hyaluronan, protects multiple types of tumour cells against apoptosis induced by cytotoxic drugs or radiation (399, 402-412). The protective action of the extracellular matrix (ECM) is mediated by integrin activation and signalling; several recent reviews summarize the integrins promoting drug resistance (e.g., integrin α4β1, α5β1) and the corresponding experimental systems (408-411). Activation of these integrins leads to activation of the downstream PI3K/AKT, MAPK and PLCγ pathways, resistance by inhibiting cell death through regulation of apoptosis regulatory proteins (e.g., Bcl-XL and Bad), decreased cell proliferation through upregulation of the CDK inhibitor p27 protein, and decreased DNA damage by downregulation of DNA topisomerase II (408).

Fourth, tumour-stromal contact and cell-cell contact (e.g., when tumour cells are cultured as multi-cellular spheroids) alter tumour cell sensitivity to cytotoxic treatment (410-414). For the former, the contact between myeloma cells and bone marrow stroma *in vitro* resulted in protection of myeloma cells from the cytotoxicity of a topoisomerase II inhibitor mitoxantrone, as well as induction of yet-unknown soluble factors that mediated partial inhibition of apoptosis and accelerated tumour cell proliferation (413). E-cadherin has been identified as an important player in the cell-cell contact dependent resistance, and its inhibition by a blocking antibody reversed the drug resistance in cultured colon cancer spheroids (415). The mechanisms for the resistance mediated by cell-cell contact are not known, but could be due to direct or indirect mechanisms.

4. HYPOXIA

Dysregulated tumour growth and progression cause imbalance between oxygen supply and consumption. These, together with structural and functional dysfunction of intratumoural vasculature, induce higher level of hypoxia in solid tumours. The phenomenon and the mechanisms of hypoxia-induced tumour cell resistance to radiation were discovered more than 70 years ago (416, 417). Since then, the availability and application of quantitative polarographic oxygen electrode technique (pO2 histograph) and other techniques using antibodies for detecting hypoxic markers have enabled detailed studies on the characteristics, development, and clinical relevance of hypoxia in human tumours (418, 419).

Hypoxia is observed in almost all types of human solid tumours, with substantial inter- and intra-tumour heterogeneity (420). Hypoxia contributes to tumour progression and invasion, and affects the prognosis in patients with various types of solid tumours (419, 420). The extensive studies on uterine cervix and head and neck tumours have shown that hypoxia in patient tumours is independent of tumour size, stages/grade and pathological types (419, 420). However, a high level of hypoxia is correlated with the tumour grade in other tumours, e.g., brain tumour(421). Hypoxia is associated with worse prognosis in non-small cell lung (422-424), brain (421, 425-427), and head and neck cancer (419), presumably due to enhanced malignancy and resistance to radiotherapy or chemotherapy.

Hypoxia directly or indirectly affects tumour sensitivity to radiation or chemotherapy drugs through chemical, biological and/or micro-evolutional mechanisms (418-420, 428), as follows.

Chemically, oxygen is required for enhancing the radiation-induced DNA damage and thereby enhancing cell kill. Hence, hypoxia causes resistance to radiotherapy. Typically, a 2.5-3 folds higher radiation is required to kill cells under fully hypoxia condition compared to aerobic conditions (428).

Hypoxia induces multiple biological responses simultaneously, through transcriptional and post-transcriptional mechanisms. Hypoxia-induced factor-1 (HIF1), mainly acts as a transcription factor, is the key element mediating the downstream transcriptional response in mammalian cells (418, 429-433). HIF1 is a heterodimer of the oxygen-regulated HIF1a and the constitutively expressed HIF1b. In the presence of oxygen and iron cofactors, proline hydroxylase hydroxylates HIF1a, resulting in its ubiqutin-mediated proteosome degradation initiated by the binding to VHL (von Hippel-Lindau tumour suppressor). This process, which serves to control the HIF1 level, is inhibited by hypoxia. Other oxygen sensing system may also be involved in HIF1 induction. Increases in HIF1 levels enable binding of HIF1 to hypoxia-response-elements in target genes and thereby regulates the transcription of these genes. Hypoxia also activates common stress-responsive transcription factors, e.g., p53, NFkb (434), AP-1 (Jun and c-fos heterodimer)(435, 436). Furthermore, APE-1/Ref1, a widely expressed dual-function protein, is activated under hypoxia and, through post-translational modifications, regulates transcriptional factors, leading to proteomic changes and subsequent biological responses to hypoxia and reoxygenation (435, 437-440).

The oxygenation status affects tumour cell sensitivity, under *in vitro* and *in vivo* conditions, to DNA-active agents. The mechanisms include decreasing the free radical generation (e.g., belomycin, etoposide), by causing acidosis which decreases the activity of the weakly basic drugs (e.g., vinblastin, doxorubicin, bleomycin), by causing elevated levels of glutathione which competes for alkylation of DNA or proteins, e.g., melphalan, cyclophosphamide, 1-nitrosourea (BCNU), or indirectly by complex biological consequences (see below). Besides inducing these drug-specific resistance mechanisms, hypoxia also causes resistance through broad-based mechanisms, as follows.

First, hypoxia induces G0/G1 phase cell cycle arrest through HIF-1 dependent upregulation of cyclin dependent kinase inhibitors p27/Kip1 and p21/Cip1 in tumour cells and fibroblasts (441-446). Downregulation of cyclin D, cyclin E and upregulation of 15/ink4a may also be involved in hypoxia-induced G0/G1 arrest. Because most chemotherapeutic drugs preferentially kill active dividing cells and/or target tumour cells at specific

cell cycle stages, slow down of cell proliferation by hypoxia protects tumour cells from drug toxicity. Furthermore, p27/kip1 protects tumour cells from hypoxia, nutrition depletion-induced cell death, and confers survival benefits in the presence of cyototoxic drugs.

Second, hypoxia modulates the expression and the balance of pro- and anti-apoptotic proteins. Chronic/severe hypoxia causes cell death mainly via mitochondria permeation-mediated apoptotic and necrotic pathways. Hypoxia induces the expression of the pro-apoptotic protein NIP3 and its homologue NIX, in a wide range of cell lines, an effect that requires HIF1 (447, 448). The expression of NIP3 is found in the perinecrotic region in human tumours (447, 449); its induction causes cells to undergo caspase-independent necrosis-like cell death while its inhibition by antisense RNA abolishes hypoxia-induced cell death (450-452). This data suggest that NIP3, and possibly NIX as well, mediate hypoxia-induced necrosis. However, hypoxia, through both HIF1-dependent and HIF1-independent pathways, also transcriptionally and/or post-transcriptionally upregulates the anti-apoptotic proteins Bcl-2, Bcl-XL and IAP family members (453), and downregulates the pro-apoptotic proteins Bid, Bad and Bax (454), and thereby protects tumour cells from hypoxia-induced cell death.

Third, the expression of ATP-binding cassette drug efflux protein P-glycoprotein (455, 456) is upregulated in human tumour and endothelia cells under hypoxic conditions, probably as a part of adaptative reactions to hypoxia (see also below). The induction requires prolonged chronic hypoxia, is dependent on HIF1, and is rapidly reversed by reoxygenation (456-458). Similarly, breast cancer resistance protein (BCRP or ABCG2) is upregulated by hypoxia *via* an HIF-dependent mechanism, and thereby protects tumour cells from hypoxia-induced cell death (459). These drug efflux transporters, by decreasing the intracellular drug accumulation, confer drug resistance (4-6, 460-463).

Fourth, hypoxia reorganizes and modulates the intra-tumour microenvironment, by upregulating vascular endothelial growth factor (464-468) and its receptors (469-471), FGF1 and/or FGF2 (468, 472), HGF/c-Met system (473, 474), IGFII (475, 476), IGFBP1 (477, 478), TGFβ1 and 3 (479), TGFα /EGFR system (480), IL1(481-483), IL6 (484-486) and IL8 (487-490). Many of these signalling pathways confer survival advantage to tumour cells as discussed in earlier sections.

Fifth, tumour cells, unlike normal cells, can survive and even benefit from hypoxic conditions through genetic and epigenetic adaptive changes. As a persistent stress, hypoxia selects for cells more adaptive to adverse conditions, and causes cross-resistance to therapy. Hypoxia and the associated acidosis, as well as nutrient deprivation, diminish DNA repair and cause genetic instability, accelerating the long-term micro-evolutionary process. The frequency of mutation and chromosome alteration increases 5-folds in tumour cells grown as solid tumours in mice or under hypoxic culture conditions, as compared to the same cells grown as monolayer cultures under aerobic conditions (491). Hypoxia induces genetic instability by downregulating the expression of the Mlh1 gene, a key component in DNA mismatch repair system (MMR), and thereby causes a deficiency in the MMR functionality (492), which in turn (a) increases genomic mutations and facilitates the selection of more aggressive and resistant tumours cells, and (b) activates adaptive responses to low oxygen level and/or nutrient depletion, including altered oxygen transport, iron metabolism, glycolysis and pH regulation and promoting angiogenesis. These changes affect the activity or delivery of chemotherapeutics and initiate micro-environmental remodeling by modulating the expression of growth factors, cytokines, matrix metalloproteinases, adhesion molecules and extracellular matrix components, resulting in enhanced invasiveness, metastasis and drug resistance. Hypoxia also accelerates the selection of transformed epithelial cells that are apoptosis-deficient (493, 494).

Therapeutic approaches targeting hypoxia, either through HIF1 blocking or use hypoxia activated prodrug, have been developed (418, 428). Among them, tirapazamine, a prodrug preferentially activated under hypoxic condition, has been evaluated clinically; its ability to improve the activity chemotherapy in advanced non-small cell lung and breast cancer patients has been demonstrated in randomized trials (495, 496).

5. INTERACTION BETWEEN TUMOUR- AND MICROENVIRONMENT-DERIVED FACTORS

Interactions between tumour- and microenvironment-derived factors affect chemosensitivity or chemoresistance in two ways. First, these factors can modulate each other and act cooperatively on several levels, e.g., regulation of expression of factors to induce environmental remodelling, cooperative activation between adhesion molecules and receptor tyrosine kinases, and cross-talk between downstream signalling pathways. In addition to the examples discussed in the above sections, growth factors or cytokines can activate changes in adhesion molecules, and cell-cell or cell-matrix adhesion can promote expression of survival-conferring soluble factors. Interactions between adhesion molecules (e.g., integrins, cadherins and adhesion molecules) and receptor tyrosine kinases on the cell membrane regulate the downstream signalling pathways and cell survival in multiple experimental models. In addition, N-cadherin, which is upregulated to replace E-cadherin during tumour progression in solid tumours, a phenomenon called cadherin switch, is able to activate or augment the signalling of the FGF system. Simultaneous upregulation of adhesion molecules and FGFR3 in tumour tissues obtained from advanced lung cancer patients after chemotherapy further suggests a common response of tumour- and microenvironment-derived factors to cytotoxic insults. Individual factors may also have direct and indirect effects on multiple levels. For example, in addition to triggering the protective mechanisms on hypoxic cells, hypoxia initiates environment change by regulating the expression of certain growth factors, matrix components and adhesion molecules, and thereby protects hypoxic tumour cells as well as the neighbouring non-hypoxic cells from stress. These various interactions often confer survival advantages to tumour cells.

Second, there is a high degree of redundancy between the intracellular signalling pathways activated by receptors and adhesion molecules. Cross-talk between these pathways regulates the intensity and duration of the activation and plays critical role in signalling differentiation. An example is the redundant intracellular signalling pathways of integrins and growth factors. The effects of redundant signalling are two fold. On one hand, the activation of one factor can compensate for the blocking of the activation of the second factor, e.g., the EGF-mediated protection is attenuated when cells are adherent to extracellular matrix components. Furthermore, the redundant provides for a more reliable protection, and, hence, it is more difficult to overcome the survival advantage by blocking only a single target.

6. CONCLUSIONS AND PERSPECTIVES

The recent advances of cancer biology and genetics provide unprecedented opportunities for innovative therapeutic paradigms. Abundant preclinical and clinical evidence indicates tumour resistance to therapy, either intrinsic or acquired, is determined by three major groups of tumour microenvironmental factors, i.e., soluble factors, adhesion molecules and hypoxia. The implications of the complex interplay between these factors and their redundant signalling pathways are two-fold. First, they highlight the importance of using appropriate experimental models. For example, the tumour-stromal interaction is not addressed by the monolayer culture system that is widely used in the experimental therapeutics field. Future successes in therapy development depend on establishing experimental models that can capture the various components of the tumour microenvironment that contribute to the protection of tumour cells against chemotherapy or radiotherapy. The availability of such models is also critical to the elucidation of the survival mechanisms conferred by environmental factors. Experimental systems and techniques, such as, 3-dimensional cultures, tumour-stromal cell co-cultures, and orthotropic tumours, especially the surgical orthotropic implantation of tumour cells from individual patients, include microenvironment compartments, and are more likely to yield clinically relevant information. Second, it is reasonable to postulate that approaches aiming at a single target

are not likely to yield significant and durable therapeutic successes. A logical approach is to aim at multiple targets, simultaneously eliminating the survival benefits conferred by multiple factors, present in either tumour and/or stromal compartments. Additional challenges include the chemotherapy-induced microenvironment remodelling, the kinetics of signalling initiated by tumour- and environment-derived factors and the interaction of these signalling pathways resulting in chemoresistance.

ACKNOWLEDGEMENT

The authors thank Dr. M.G. Wientjes for his critical review and input. This work was supported in part by research grants R37CA49816, R01CA77091, R01CA78577, R21CA91547 and R01CA93871 from the National Cancer Institute, NIH, DHHS.

REFERENCES

1. Farber, S., Diamond, L.K., Mercer, R.D., Sylvester, R.F., and Wolff, R.D., 1948, Temporary remissions in acute leukemia in children produced by folic acid antagonist, 4-aminopteroyl-glutamic acid (aminopterin). N Engl J, 238:787-793.
2. Nygren, P., 2001, What is cancer chemotherapy? Acta Oncol, 40:166-174.
3. Borges-Walmsley, M. I., McKeegan, K. S., and Walmsley, A. R., 2003, Structure and function of efflux pumps that confer resistance to drugs. Biochem J, 376:313-338.
4. Leonessa, F., and Clarke, R., 2003, ATP binding cassette transporters and drug resistance in breast cancer. Endocr Relat Cancer, 10:43-73.
5. Varadi, A., Szakacs, G., Bakos, E., and Sarkadi, B., 2002, P glycoprotein and the mechanism of multidrug resistance. Novartis Found Symp, 243:54-65.
6. Gottesman, M.M., Fojo, T., and Bates, S.E., 2002, Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer, 2:48-58.
7. Litman, T., Druley, T.E., Stein, W. D., and Bates, S.E., 2001, From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance. Cell Mol Life Sci, 58:931-959.
8. Persidis, A., 1999, Cancer multidrug resistance. Nat Biotechnol, 17:94-95.
9. Townsend, D.M., and Tew, K.D., 2003, The role of glutathione-S-transferase in anti-cancer drug resistance. Oncogene, 22:7369-7375.
10. Hayes, J.D., and Pulford, D.J., 1995, The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit Rev Biochem Mol Biol, 30:445-600.
11. Rasheed, Z.A., and Rubin, E.H., 2003, Mechanisms of resistance to topoisomerase I-targeting drugs. Oncogene, 22:7296-7304.
12. Dingemans, A.M., Pinedo, H.M., and Giaccone, G., 1998, Clinical resistance to topoisomerase-targeted drugs. Biochim Biophys Acta, 1400:275-288.
13. Orr, G. A., Verdier-Pinard, P., McDaid, H., and Horwitz, S.B., 2003, Mechanisms of Taxol resistance related to microtubules. Oncogene, 22:7280-7295.
14. Drukman, S., and Kavallaris, M., 2002, Microtubule alterations and resistance to tubulin-binding agents (review). Int J Oncol, 21:621-628.
15. Sangrajrang, S., and Fellous, A., 2000, Taxol resistance. Chemotherapy, 46:327-334.
16. Hersey, P., and Zhang, X. D., 2003, Overcoming resistance of cancer cells to apoptosis. J Cell Physiol, 196:9-18.
17. Tolomeo, M., and Simoni, D., 2002, Drug resistance and apoptosis in cancer treatment: development of new apoptosis-inducing agents active in drug resistant malignancies. Curr Med Chem Anti -Canc Agents, 2:387-401.
18. Reed, J.C., 1995, Bcl-2: prevention of apoptosis as a mechanism of drug resistance. Hematol Oncol Clin North Am , 9:451-473.
19. Nygren, P., and Larsson, R., 2003, Overview of the clinical efficacy of investigational anticancer drugs. J Intern Med, 253:46-75.
20. Teicher, B.A., Herman, T.S., Holden, S.A., Wang, Y.Y., Pfeffer, M.R., Crawford, J.W., and Frei, III, E., 1990, Tumor resistance to alkylating agents conferred by mechanisms operative only in vivo. Science, 247:1457-1461.
21. Hoffman, R.M., 1999, Orthotopic metastatic mouse models for anticancer drug discovery and evaluation: a bridge to the clinic. Invest New Drugs, 17:343-359.
22. Kuo, T.H., Kubota, T., Watanabe, M., Furukawa, T., Kase, S., Tanino, H., Saikawa, Y., Ishibiki, K., Kitajima, M., and Hoffman, R. M., 1993, Site-specific chemosensitivity of human small-cell lung carcinoma growing orthotopically compared to subcutaneously in SCID mice: the importance of orthotopic models to obtain relevant drug evaluation data. Anticancer Res, 13:627-630.
23. Wilmanns, C., Fan, D., O'Brian, C.A., Bucana, C.D., and Fidler, I.J., 1992, Orthotopic and ectopic organ environments differentially influence the sensitivity

of murine colon carcinoma cells to doxorubicin and 5-fluorouracil. Int J Cancer, 52:98-104.

24. Fidler, I.J., Wilmanns, C., Staroselsky, A., Radinsky, R., Dong, Z., and Fan, D., 1994, Modulation of tumor cell response to chemotherapy by the organ environment. Cancer Metastasis Rev, 13:209-222.
25. Ahn, K.S., Jung, Y.S., Kim, J., Lee, H., and Yoon, S.S., 2001, Behavior of murine renal carcinoma cells grown in ectopic or orthotopic sites in syngeneic mice. Tumour Biol, 22:146-153.
26. Song, S., Wientjes, M.G., Gan, Y., and Au, J.L., 2000, Fibroblast growth factors: an epigenetic mechanism of broad spectrum resistance to anticancer drugs. Proc Natl Acad Sci USA, 97:8658-8663.
27. Paull, K.D., Shoemaker, R.H., Hodes, L., Monks, A., Scudiero, D.A., Rubinstein, L., Plowman, J., and Boyd, M.R., 1989, Display and analysis of patterns of differential activity of drugs against human tumor cell lines: development of mean graph and COMPARE algorithm. J Natl Cancer Inst, 81:1088-1092.
28. Laskin, J.J., and Sandler, A.B., 2004, Epidermal growth factor receptor: a promising target in solid tumours. Cancer Treat Rev, 30:1-17.
29. Ciardiello, F. and Tortora, G., 2003, Epidermal growth factor receptor (EGFR) as a target in cancer therapy: understanding the role of receptor expression and other molecular determinants that could influence the response to anti-EGFR drugs. Eur J Cancer, 39:1348-1354.
30. Holbro, T., Civenni, G., and Hynes, N.E., 2003, The ErbB receptors and their role in cancer progression. Exp Cell Res, 284:99-110,
31. Lichtner, R.B., 2003, Estrogen/EGF receptor interactions in breast cancer: rationale for new therapeutic combination strategies. Biomed Pharmacother, 57:447-451.
32. Nicholson, R.I., Gee, J.M., and Harper, M.E., 2001, EGFR and cancer prognosis. Eur J Cancer, 37Suppl 4:S9-15.
33. Lorenzo, G.D., Bianco, R., Tortora, G., and Ciardiello, F., 2003, Involvement of growth factor receptors of the epidermal growth factor receptor family in prostate cancer development and progression to androgen independence. Clin Prostate Cancer, 2:50-57.
34. Sartor, C.I., 2003, Epidermal growth factor family receptors and inhibitors: radiation response modulators. Semin.Radiat.Oncol., 13: 22-30.
35. Ang, K.K., Andratschke, N.H., and Milas, L., 2004, Epidermal growth factor receptor and response of head-and-neck carcinoma to therapy. Int J Radiat Oncol Biol Phys, 58:959-965.
36. Meden, H., Marx, D., Roegglen, T., Schauer, A., and Kuhn, W., 1998, Overexpression of the oncogene c-erbB-2 (HER2/neu) and response to chemotherapy in patients with ovarian cancer. Int J Gynecol Pathol, 17:61-65.
37. Felip, E., Del Campo, J.M., Rubio, D., Vidal, M.T., Colomer, R., and Bermejo, B., 1995, Overexpression of c-erbB-2 in epithelial ovarian cancer. Prognostic value and relationship with response to chemotherapy. Cancer, 75:2147-2152.
38. Niibe, Y., Nakano, T., Ohno, T., Suzuki, Y., Oka, K., and Tsujii, H., 2003, Prognostic significance of c-erbB-2/HER2 expression in advanced uterine cervical carcinoma with para-aortic lymph node metastasis treated with radiation therapy. Int J Gynecol Cancer, 13: 849-855.
39. Chakravarti, A., Dicker, A., and Mehta, M., 2004, The contribution of epidermal growth factor receptor (EGFR) signaling pathway to radioresistance in human gliomas: a review of preclinical and correlative clinical data. Int J Radiat Oncol Biol Phys, 58:927-931.
40. Kari, C., Chan, T. O., Rocha, d. Q., and Rodeck, U. 2003, Targeting the epidermal growth factor receptor in cancer: apoptosis takes center stage. Cancer Res, 63:1-5.
41. Kumar, R., Mandal, M., Lipton, A., Harvey, H., and Thompson, C.B., 1996, Overexpression of HER2 modulates bcl-2, bcl-XL, and tamoxifen-induced apoptosis in human MCF-7 breast cancer cells. Clin Cancer Res, 2:1215-1219.
42. Golubovskaya, V., Beviglia, L., Xu, L. H., Earp, III, H. S., Craven, R., and Cance, W., 2002, Dual inhibition of focal adhesion kinase and epidermal growth factor receptor pathways cooperatively induces death receptor-mediated apoptosis in human breast cancer cells. J Biol Chem, 277:38978-38987.
43. Cao, L., Yao, Y., Lee, V., Kiani, C., Spaner, D., Lin, Z., Zhang, Y., Adams, M.E., and Yang, B.B., 2000, Epidermal growth factor induces cell cycle arrest and apoptosis of squamous carcinoma cells through reduction of cell adhesion. J Cell Biochem, 77:569-583.
44. Kottke, T.J., Blajeski, A.L., Martins, L.M., Mesner, Jr., P.W., Davidson, N.E., Earnshaw, W.C., Armstrong, D.K., and Kaufmann, S.H., 1999, Comparison of paclitaxel-, 5-fluoro-2'-deoxyuridine-, and epidermal growth factor (EGF)-induced apoptosis. Evidence for EGF-induced anoikis. J Biol Chem, 274:15927-15936.
45. Allen, G. C., Lubas, S., Wax, M. K., and Devore, R. F., III 1996.Epidermal growth factor regulates topoisomerase II activity and drug sensitivity in human KB cells. Otolaryngol.Head Neck Surg., 114:785-792.
46. Boudny, V., Murakami, Y., Nakano, S., and Niho, Y., 1999, Expression of activated c-erbB-2 oncogene induces sensitivity to cisplatin in human gallbladder adenocarcinoma cells. Anticancer Res, 19:5203-5206.

47. Kroning, R., Jones, J.A., Hom, D.K., Chuang, C. C., Sanga, R., Los, G., Howell, S. B., and Christen, R. D., 1995, Enhancement of drug sensitivity of human malignancies by epidermal growth factor. Br J Cancer, 72:615-619.
48. Amagase, H., Tamura, K., Hashimoto, K., Fuwa, T., Murakami, T., and Yata, N., 1990, Response of A431 experimental human solid xenograft to mitomycin C in combination with human epidermal growth factor in mice. J Pharmacobiodyn, 13:263-268.
49. Ling, Y.H., Donato, N.J., and Perez-Soler, R., 2001, Sensitivity to topoisomerase I inhibitors and cisplatin is associated with epidermal growth factor receptor expression in human cervical squamous carcinoma ME180 sublines. Cancer Chemother Pharmacol, 47:473-480.
50. Thor, A.D., Berry, D.A., Budman, D.R., Muss, H.B., Kute, T., Henderson, I.C., Barcos, M., Cirrincione, C., Edgerton, S., Allred, C., Norton, L., and Liu, E.T., 1998, erbB-2, p53, and efficacy of adjuvant therapy in lymph node-positive breast cancer. J Natl Cancer Inst, 90:1346-1360.
51. Campiglio, M., Somenzi, G., Olgiati, C., Beretta, G., Balsari, A., Zaffaroni, N., Valagussa, P., and Menard, S., 2003, Role of proliferation in HER2 status predicted response to doxorubicin. Int J Cancer, 105:568-573.
52. Paik, S., Bryant, J., Park, C., Fisher, B., Tan-Chiu, E., Hyams, D., Fisher, E.R., Lippman, M.E., Wickerham, D.L., and Wolmark, N., 1998, erbB-2 and response to doxorubicin in patients with axillary lymph node-positive, hormone receptor-negative breast cancer. J Natl Cancer Inst, 90:1361-1370.
53. Clark, G.M., 1998, Should selection of adjuvant chemotherapy for patients with breast cancer be based on erbB-2 status?. J Natl Cancer Inst, 90:1320-1321.
54. Muss, H.B., Thor, A.D., Berry, D.A., Kute, T., Liu, E.T., Koerner, F., Cirrincione, C.T., Budman, D.R., Wood, W.C., and Barcos, M., 1994, c-erbB-2 expression and response to adjuvant therapy in women with node-positive early breast cancer. N Engl J Med, 330:1260-1266.
55. Gandour-Edwards, R., Lara, Jr., P.N., Folkins, A.K., LaSalle, J.M., Beckett, L., Li, Y., Meyers, F.J., and DeVere-White, R., 2002, Does HER2/neu expression provide prognostic information in patients with advanced urothelial carcinoma?. Cancer, 95: 1009-1015.
56. Baselga, J., Seidman, A.D., Rosen, P.P., and Norton, L., 1997, HER2 overexpression and paclitaxel sensitivity in breast cancer: therapeutic implications. Oncology (Huntingt), 11:43-48.
57. Koizumi, F., Kanzawa, F., Ueda, Y., Koh, Y., Tsukiyama, S., Taguchi, F., Tamura, T., Saijo, N., and Nishio, K., 2004, Synergistic interaction between the EGFR tyrosine kinase inhibitor gefitinib ("Iressa") and the DNA topoisomerase I inhibitor CPT-11 (irinotecan) in human colorectal cancer cells. Int J Cancer, 108:464-472.
58. Montemurro, F., Valabrega, G., and Aglietta, M., 2004, Trastuzumab-based combination therapy for breast cancer. Expert Opin Pharmacother, 5:81-96.
59. Ligibel, J.A. and Winer, E.P., 2002, Trastuzumab/chemotherapy combinations in metastatic breast cancer. Semin Oncol, 29:38-43.
60. Vogel, C.L., Cobleigh, M.A., Tripathy, D., Gutheil, J.C., Harris, L.N., Fehrenbacher, L., Slamon, D.J., Murphy, M., Novotny, W.F., Burchmore, M., Shak, S., and Stewart, S.J., 2001, First-line Herceptin monotherapy in metastatic breast cancer. Oncology, 61Suppl2: 37-42.
61. Cobleigh, M.A., Vogel, C.L., Tripathy, D., Robert, N.J., Scholl, S., Fehrenbacher, L., Wolter, J.M., Paton, V., Shak, S., Lieberman, G., and Slamon, D.J., 1999, Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol, 17:2639-2648.
62. Baselga, J., Tripathy, D., Mendelsohn, J., Baughman, S., Benz, C.C., Dantis, L., Sklarin, N.T., Seidman, A.D., Hudis, C.A., Moore, J., Rosen, P.P., Twaddell, T., Henderson, I.C., and Norton, L., 1996, Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. J Clin Oncol, 14:737-744.
63. Slamon, D.J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J., and Norton, L., 2001, Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Eng J Med, 344:783-792.
64. Tan, A.R. and Swain, S.M., 2003, Ongoing adjuvant trials with trastuzumab in breast cancer. Semin Oncol, 30:54-64.
65. Piccart-Gebhart, M.J., 2001, Herceptin: the future in adjuvant breast cancer therapy. Anticancer Drugs, 12 Suppl 4:S27-S33.
66. Slamon, D. and Pegram, M., 2001, Rationale for trastuzumab (Herceptin) in adjuvant breast cancer trials. Semin Oncol, 28:13-19.
67. Bunn, Jr., P.A., Helfrich, B., Soriano, A.F., Franklin, W.A., Varella-Garcia, M., Hirsch, F. R., Baron, A., Zeng, C., and Chan, D.C., 2001, Expression of Her-2/neu in human lung cancer cell lines by immunohistochemistry and fluorescence in situ hybridization and its relationship to in vitro cytotoxicity by trastuzumab and chemotherapeutic agents. Clin Cancer Res, 7:3239-3250.

68. Hirsch, F.R., Helfrich, B., Franklin, W.A., Varella-Garcia, M., Chan, D.C., and Bunn, Jr., P.A., 2002, Preclinical studies of gemcitabine and trastuzumab in breast and lung cancer cell lines. Clin Breast Cancer, 3Suppl 1:12-16,
69. Gatzemeier, U., Groth, G., Butts, C., Van Zandwijk, N., Shepherd, F., Ardizzoni, A., Barton, C., Ghahramani, P., and Hirsh, V., 2004, Randomized phase II trial of gemcitabine-cisplatin with or without trastuzumab in HER2-positive non-small-cell lung cancer. Ann Oncol, 15:19-27,
70. Lara, Jr., P.N., Chee, K.G., Longmate, J., Ruel, C., Meyers, F.J., Gray, C.R., Edwards, R.G., Gumerlock, P.H., Twardowski, P., Doroshow, J.H., and Gandara, D.R., 2004, Trastuzumab plus docetaxel in HER-2/neu-positive prostate carcinoma: final results from the California Cancer Consortium Screening and Phase II Trial. Cancer, 100:2125-2131,
71. Ziada, A., Barqawi, A., Glode, L.M., Varella-Garcia, M., Crighton, F., Majeski, S., Rosenblum, M., Kane, M., Chen, L., and Crawford, E.D., 2004, The use of trastuzumab in the treatment of hormone refractory prostate cancer; phase II trial. Prostate, 60:332-337,
72. Andre, F., Le Chevalier, T., and Soria, J.C., 2004, Her2-neu: a target in lung cancer? Ann Oncol, 15 3-4.
73. Fernandes, A., Hamburger, A.W., and Gerwin, B.I., 1999, ErbB-2 kinase is required for constitutive stat 3 activation in malignant human lung epithelial cells. Int J Cancer, 83:564-570.
74. Thomas, M., 2003, Epidermal growth factor receptor tyrosine kinase inhibitors: application in non-small cell lung cancer. Cancer Nurs, 26:21S-25S.
75. Giaccone, G., 2004, The role of gefitinib in lung cancer treatment. Clin Cancer Res, 10:4233s-4237s.
76. Perez-Soler, R., 2004, The role of erlotinib (Tarceva, OSI 774) in the treatment of non-small cell lung cancer. Clin Cancer Res, 10:4238s-4240s.
77. Xiong, H.Q., Rosenberg, A., LoBuglio, A., Schmidt, W., Wolff, R.A., Deutsch, J., Needle, M., and Abbruzzese, J.L., 2004, Cetuximab, a monoclonal antibody targeting the epidermal growth factor receptor, in combination with gemcitabine for advanced pancreatic cancer: a multicenter phase II Trial. J Clin Oncol, 22:2610-2616.
78. Saltz, L.B., Meropol, N.J., Loehrer, Sr., P.J., Needle, M.N., Kopit, J., and Mayer, R.J., 2004, Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. J Clin Oncol, 22:1201-1208.
79. Reynolds, N.A. and Wagstaff, A.J., 2004, Cetuximab: in the treatment of metastatic colorectal cancer. Drugs, 64:109-118.
80. Herbst, R.S. and Hong, W.K., 2002, IMC-C225, an anti-epidermal growth factor receptor monoclonal antibody for treatment of head and neck cancer. Semin Oncol, 29:18-30.
81. Herbst R.S., Prager D., Hermann R., Miller V., Fehrenbacher L., Hoffman P., Johnson B., Sandler A.B., Mass R., and Johnson D.H., 2004, TRIBUTE - A phase III trial of erlotinib HCl (OSI-774) combined with carboplatin and paclitaxel (CP) chemotherapy in advanced non-small cell lung cancer (NSCLC). [Abstract]. Journal of Clinical Oncology, ASCO Annual Meeting Proceedings (Post-Meeting Edition), 22:#7011.
82. Gatzemeier U., Pluzanska A., Szczensna A., Kaukel E., Roubec J., Brennscheidt U., De Rosa F., Muller B., and Von Pawel J., 2004, Results of a phase III trial of erlotinib (OSI-774) combined with cisplatin and gemcitabine (GC) chemotherapy in advanced non-small cell lung cancer (NSCLC). [Abstract]. Journal of Clinical Oncology, ASCO Annual Meeting Proceedings (Post-Meeting Edition), 22:#7010.
83. Paez, J.G., Janne, P.A., Lee, J.C., Tracy, S., Greulich, H., Gabriel, S., Herman, P., Kaye, F.J., Lindeman, N., Boggon, T.J., Naoki, K., Sasaki, H., Fujii, Y., Eck, M.J., Sellers, W.R., Johnson, B.E., and Meyerson, M., 2004, EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science, 304:1497-1500.
84. Lynch, T.J., Bell, D.W., Sordella, R., Gurubhagavatula, S., Okimoto, R.A., Brannigan, B.W., Harris, P.L., Haserlat, S.M., Supko, J.G., Haluska, F.G., Louis, D.N., Christiani, D.C., Settleman, J., and Haber, D.A., 2004, Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Eng J Med, 350:2129-2139.
85. Sordella, R., Bell, D.W., Haber, D.A., and Settleman, J., 2004, Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science, 305:1163-1167.
86. Bikfalvi, A., Klein, S., Pintucci, G., and Rifkin, D.B., 1997, Biological roles of fibroblast growth factor-2. Endocr Rev, 18: 26-45.
87. Ornitz, D.M. and Itoh, N., 2001, Fibroblast growth factors. Genome Biol, 2:REVIEWS3005.
88. Burgess, W.H. and Maciag, T., 1989, The heparin-binding (fibroblast) growth factor family of proteins. Annu Rev Biochem, 58:575-606.
89. Fuks, Z., Persaud, R.S., Alfieri, A., McLoughlin, M., Ehleiter, D., Schwartz, J.L., Seddon, A.P., Cordon-Cardo, C., and Haimovitz-Friedman, A., 1994, Basic fibroblast growth factor protects endothelial cells against radiation-induced programmed cell death in vitro and in vivo. Cancer Res, 54:2582-2590,
90. Yayon, A. and Klagsbrun, M., 1990, Autocrine regulation of cell growth and transformation by basic fibroblast growth factor. Cancer Metastasis Rev, 9:191-202.

91. Johnson, D.E. and Williams, L.T., 1993, Structural and functional diversity in the FGF receptor multigene family. Adv Cancer Res, 60:1-41.
92. Ornitz, D.M., Xu, J., Colvin, J.S., McEwen, D.G., MacArthur, C.A., Coulier, F., Gao, G., and Goldfarb, M., 1996, Receptor specificity of the fibroblast growth factor family. J Biol Chem, 271:15292-15297.
93. Valve, E.M., Nevalainen, M.T., Nurmi, M.J., Laato, M.K., Martikainen, P.M., and Harkonen, P.L., 2001, Increased expression of FGF-8 isoforms and FGF receptors in human premalignant prostatic intraepithelial neoplasia lesions and prostate cancer. Lab Invest, 81:815-826.
94. Dell'Era, P., Mohammadi, M., and Presta, M., 1999, Different tyrosine autophosphorylation requirements in fibroblast growth factor receptor-1 mediate urokinase-type plasminogen activator induction and mitogenesis. Mol Biol Cell, 10:23-33.
95. Spivak-Kroizman, T., Lemmon, M.A., Dikic, I., Ladbury, J.E., Pinchasi, D., Huang, J., Jaye, M., Crumley, G., Schlessinger, J., and Lax, I., 1994, Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. Cell, 79:1015-1024.
96. Friesel, R.E. and Maciag, T., 1995, Molecular mechanisms of angiogenesis: fibroblast growth factor signal transduction. FASEB J, 9:919-925.
97. Xing, J., Ginty, D.D., and Greenberg, M.E., 1996, Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. Science, 273:959-963.
98. Tan, Y., Ruan, H., Demeter, M.R., and Comb, M.J., 1999, p90(RSK) blocks bad-mediated cell death via a protein kinase C-dependent pathway. J Biol Chem, 274:34859-34867.
99. Shimamura, A., Ballif, B.A., Richards, S.A., and Blenis, J., 2000, Rsk1 mediates a MEK-MAP kinase cell survival signal. Curr Biol, 10:127-135.
100. Zong, W.X., Edelstein, L.C., Chen, C., Bash, J., and Gelinas, C., 1999, The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-kappaB that blocks TNFalpha-induced apoptosis. Genes Dev., 13:382-387.
101. Ghoda, L., Lin, X., and Greene, W.C., 1997, The 90-kDa ribosomal S6 kinase (pp90rsk) phosphorylates the N-terminal regulatory domain of IkappaBalpha and stimulates its degradation in vitro. J Biol Chem, 272 21281-21288.
102. Bertolotto, C., Maulon, L., Filippa, N., Baier, G., and Auberger, P., 2000, Protein kinase C theta and epsilon promote T-cell survival by a rsk-dependent phosphorylation and inactivation of BAD. J Biol Chem, 275:37246-37250.
103. Delehedde, M., Seve, M., Sergeant, N., Wartelle, I., Lyon, M., Rudland, P.S., and Fernig, D.G., 2000, Fibroblast growth factor-2 stimulation of p42/44MAPK phosphorylation and IkappaB degradation is regulated by heparan sulfate/heparin in rat mammary fibroblasts. J Biol Chem, 275:33905-33910.
104. Du, K., and Montminy, M., 1998, CREB is a regulatory target for the protein kinase Akt/PKB. J Biol Chem, 273:32377-32379.
105. Chen, C., Edelstein, L.C., and Gelinas, C., 2000, The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L). Mol Cell Biol, 20:2687-2695.
106. Bonni, A., Brunet, A., West, A.E., Datta, S.R., Takasu, M.A., and Greenberg, M.E., 1999, Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science, 286:1358-1362.
107. Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S., and Reed, J.C., 1998, Regulation of cell death protease caspase-9 by phosphorylation. Science, 282:1318-1321.
108. Fenig, E., Wieder, R., Paglin, S., Wang, H., Persaud, R., Haimovitz-Friedman, A., Fuks, Z., and Yahalom, J., 1997, Basic fibroblast growth factor confers growth inhibition and mitogen-activated protein kinase activation in human breast cancer cells. Clin Cancer Res, 3:135-142.
109. Wang, H., Rubin, M., Fenig, E., DeBlasio, A., Mendelsohn, J., Yahalom, J., and Wieder, R., 1997, Basic fibroblast growth factor causes growth arrest in MCF-7 human breast cancer cells while inducing both mitogenic and inhibitory G1 events. Cancer Res, 57:1750-1757.
110. Schweigerer, L., Neufeld, G., and Gospodarowicz, D., 1987, Basic fibroblast growth factor as a growth inhibitor for cultured human tumor cells. J Clin Ivest, 80:1516-1520.
111. Coleman, A.B., 2003, Positive and negative regulation of cellular sensitivity to anti-cancer drugs by FGF-2. Drug Resist Updat, 6:85-94.
112. Huang, A., Jin, H., and Wright, J.A., 1994, Aberrant expression of basic fibroblast growth factor in NIH-3T3 cells alters drug resistance and gene amplification potential. Exp Cell Res, 213:335-339.
113. Huang, A. and Wright, J.A., 1994, Fibroblast growth factor mediated alterations in drug resistance, and evidence of gene amplification. Oncogene, 9:491-499.
114. Linderholm, B.K., Lindh, B., Beckman, L., Erlanson, M., Edin, K., Travelin, B., Bergh, J., Grankvist, K., and Henriksson, R., 2003, Prognostic correlation of basic fibroblast growth factor and vascular endothelial growth factor in 1307 primary breast cancers. Clin Breast Cancer, 4:340-347.
115. Smith, K., Fox, S.B., Whitehouse, R., Taylor, M., Greenall, M., Clarke, J., and Harris, A.L., 1999,

Upregulation of basic fibroblast growth factor in breast carcinoma and its relationship to vascular density, oestrogen receptor, epidermal growth factor receptor and survival. Ann Oncol, 10:707-713.

116. Blanckaert, V.D., Hebbar, M., Louchez, M.M., Vilain, M.O., Schelling, M.E., and Peyrat, J.P., 1998, Basic fibroblast growth factor receptors and their prognostic value in human breast cancer. Clin Cancer Res, 4:2939-2947.
117. Colomer, R., Aparicio, J., Montero, S., Guzman, C., Larrodera, L., and Cortes-Funes, H., 1997, Low levels of basic fibroblast growth factor (bFGF) are associated with a poor prognosis in human breast carcinoma. Br J Cancer, 76:1215-1220.
118. Yiangou, C., Gomm, J.J., Coope, R.C., Law, M., Luqmani, Y.A., Shousha, S., Coombes, R. C., and Johnston, C.L., 1997, Fibroblast growth factor 2 in breast cancer: occurrence and prognostic significance. Br J Cancer, 75:28-33.
119. Obermair, A., Speiser, P., Reisenberger, K., Ullrich, R., Czerwenka, K., Kaider, A., Zeillinger, R., and Miksche, M., 1998, Influence of intratumoral basic fibroblast growth factor concentration on survival in ovarian cancer patients. Cancer Lett, 130:69-76.
120. Bredel, M., Pollack, I.F., Campbell, J.W., and Hamilton, R.L., 1997, Basic fibroblast growth factor expression as a predictor of prognosis in pediatric high-grade gliomas. Clin Cancer Res, 3:2157-2164.
121. Faridi, A., Rudlowski, C., Biesterfeld, S., Schuh, S., Rath, W., and Schroder, W., 2002, Long-term follow-up and prognostic significance of angiogenic basic fibroblast growth factor (bFGF) expression in patients with breast cancer. Pathol Res Pract, 198:1-5.
122. Tabone, M.D., Landman-Parker, J., Arcil, B., Coudert, M.C., Gerota, I., Benbunan, M., Leverger, G., and Dosquet, C., 2001, Are basic fibroblast growth factor and vascular endothelial growth factor prognostic indicators in pediatric patients with malignant solid tumors?. Clin Cancer Res, 7:538-543.
123. Nguyen, M., Watanabe, H., Budson, A.E., Richie, J.P., Hayes, D.F., and Folkman, J., 1994, Elevated levels of an angiogenic peptide, basic fibroblast growth factor, in the urine of patients with a wide spectrum of cancers. J Natl Cancer Inst, 86:356-361.
124. Iwasaki, A., Kuwahara, M., Yoshinaga, Y., and Shirakusa, T., 2004, Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) levels, as prognostic indicators in NSCLC. Eur J Cardiothorac Surg, 25:443-448.
125. Ruotsalainen, T., Joensuu, H., Mattson, K., and Salven, P., 2002, High pretreatment serum concentration of basic fibroblast growth factor is a predictor of poor prognosis in small cell lung cancer. Cancer Edpdemiol Biomarkers Prev, 11:1492-1495.
126. Joensuu, H., Anttonen, A., Eriksson, M., Makitaro, R., Alfthan, H., Kinnula, V., and Leppa, S., 2002, Soluble syndecan-1 and serum basic fibroblast growth factor are new prognostic factors in lung cancer. Cancer Res, 62:5210-5217.
127. Ito, H., Oshita, F., Kameda, Y., Suzuki, R., Ikehara, M., Arai, H., Mitsuda, A., Saito, H., Yamada, K., Noda, K., and Nakayama, H., 2002, Expression of vascular endothelial growth factor and basic fibroblast growth factor in small adenocarcinomas. Oncol Rep, 9:119-123.
128. Kos, M. and Dabrowski, A., 2002, Tumour's angiogenesis--the function of VEGF and bFGF in colorectal cancer. Ann Univ Mariae Curie Sklodowska [Med], 57:556-561.
129. Rasmuson, T., Grankvist, K., Jacobsen, J., and Ljungberg, B., 2001, Impact of serum basic fibroblast growth factor on prognosis in human renal cell carcinoma. Eur J Cancer, 37:2199-2203.
130. Dietz, A., Rudat, V., Conradt, C., Weidauer, H., Ho, A., and Moehler, T., 2000, Prognostic relevance of serum levels of the angiogenic peptide bFGF in advanced carcinoma of the head and neck treated by primary radiochemotherapy. Head Neck, 22:666-673.
131. Anzai, H., Kitadai, Y., Bucana, C.D., Sanchez, R., Omoto, R., and Fidler, I.J., 1998, Expression of metastasis-related genes in surgical specimens of human gastric cancer can predict disease recurrence. Eur J Cancer, 34:558-565.
132. Noda, M., Hattori, T., Kimura, T., Naitoh, H., Kodama, T., Kashima, K., and Pignatelli, M. 1997, Expression of fibroblast growth factor 2 mRNA in early and advanced gastric cancer. Acta Oncol, 36:695-700.
133. Pazgal, I., Zimra, Y., Tzabar, C., Okon, E., Rabizadeh, E., Shaklai, M., and Bairey, O., 2002, Expression of basic fibroblast growth factor is associated with poor outcome in non-Hodgkin's lymphoma. Br J Cancer, 86:1770-1775.
134. Salven, P., Orpana, A., Teerenhovi, L., and Joensuu, H., 2000, Simultaneous elevation in the serum concentrations of the angiogenic growth factors VEGF and bFGF is an independent predictor of poor prognosis in non-Hodgkin lymphoma: a single-institution study of 200 patients. Blood, 96:3712-3718.
135. Tanaka, S., Ueo, H., Mafune, K., Mori, M., Wands, J.R., and Sugimachi, K., 2001, A novel isoform of human fibroblast growth factor 8 is induced by androgens and associated with progression of esophageal carcinoma. Dig Dis Sci, 46:1016-1021.
136. Boelaert, K., McCabe, C.J., Tannahill, L.A., Gittoes, N.J., Holder, R.L., Watkinson, J.C., Bradwell, A.R., Sheppard, M.C., and Franklyn, J.A., 2003, Pituitary tumor transforming gene and fibroblast growth factor-2 expression: potential prognostic indicators

in differentiated thyroid cancer. J Clin Endocrinol Metab, 88:2341-2347.
137. Sun, Y., Naito, Z., Ishiwata, T., Maeda, S., Sugisaki, Y., and Asano, G., 2003, Basic FGF and Ki-67 proteins useful for immunohistological diagnostic evaluations in malignant solitary fibrous tumor. Pathol Int, 53:284-290.
138. Strizzi, L., Vianale, G., Catalano, A., Muraro, R., Mutti, L., and Procopio, A., 2001, Basic fibroblast growth factor in mesothelioma pleural effusions: correlation with patient survival and angiogenesis. Int J Oncol, 18:1093-1098.
139. Lin, R.Y., Argenta, P.A., Sullivan, K.M., and Adzick, N.S., 1995, Diagnostic and prognostic role of basic fibroblast growth factor in Wilms' tumor patients. Clin Cancer Res, 1:327-331.
140. Ohta, T., Yamamoto, M., Numata, M., Iseki, S., Tsukioka, Y., Miyashita, T., Kayahara, M., Nagakawa, T., Miyazaki, I., Nishikawa, K., and Yoshitake, Y., 1995, Expression of basic fibroblast growth factor and its receptor in human pancreatic carcinomas. Br J Cancer, 72:824-831.
141. Volm, M., Koomagi, R., Mattern, J., and Stammler, G., 1997, Prognostic value of basic fibroblast growth factor and its receptor (FGFR-1) in patients with non-small cell lung carcinomas. Eur J Cancer, 33:691-693.
142. Bange, J., Prechtl, D., Cheburkin, Y., Specht, K., Harbeck, N., Schmitt, M., Knyazeva, T., Muller, S., Gartner, S., Sures, I., Wang, H., Imyanitov, E., Haring, H.U., Knayzev, P., Iacobelli, S., Hofler, H., and Ullrich, A., 2002, Cancer progression and tumor cell motility are associated with the FGFR4 Arg(388) allele. Cancer Res, 62:840-847.
143. Morimoto, Y., Ozaki, T., Ouchida, M., Umehara, N., Ohata, N., Yoshida, A., Shimizu, K., and Inoue, H., 2003, Single nucleotide polymorphism in fibroblast growth factor receptor 4 at codon 388 is associated with prognosis in high-grade soft tissue sarcoma. Cancer, 98:2245-2250.
144. Cappellen, D., De Oliveira, C., Ricol, D., de Medina, S., Bourdin, J., Sastre-Garau, X., Chopin, D., Thiery, J.P., and Radvanyi, F., 1999, Frequent activating mutations of FGFR3 in human bladder and cervix carcinomas. Nat Genet, 23:18-20.
145. Ueno, K., Inoue, Y., Kawaguchi, T., Hosoe, S., and Kawahara, M., 2001, Increased serum levels of basic fibroblast growth factor in lung cancer patients: relevance to response of therapy and prognosis. Lung Cancer, 31:213-219.
146. Pardo, O.E., Arcaro, A., Salerno, G., Raguz, S., Downward, J., and Seckl, M.J., 2002, Fibroblast growth factor-2 induces translational regulation of Bcl-XL and Bcl-2 via a MEK-dependent pathway: correlation with resistance to etoposide-induced apoptosis. J Biol Chem, 277:12040-12046.
147. Pardo, O.E., Lesay, A., Arcaro, A., Lopes, R., Ng, B.L., Warne, P.H., McNeish, I.A., Tetley, T.D., Lemoine, N.R., Mehmet, H., Seckl, M.J., and Downward, J., 2003, Fibroblast growth factor 2-mediated translational control of IAPs blocks mitochondrial release of Smac/DIABLO and apoptosis in small cell lung cancer cells. Mol Cell Biol, 23:7600-7610.
148. Shaulian, E., Resnitzky, D., Shifman, O., Blandino, G., Amsterdam, A., Yayon, A., and Oren, M., 1997, Induction of Mdm2 and enhancement of cell survival by bFGF. Oncogene, 15:2717-2725.
149. Maloof, P., Wang, Q., Wang, H., Stein, D., Denny, T.N., Yahalom, J., Fenig, E., and Wieder, R., 1999, Overexpression of basic fibroblast growth factor (FGF-2) downregulates Bcl-2 and promotes apoptosis in MCF-7 human breast cancer cells. Breast Cancer Res Treat, 56:153-167.
150. Wang, Q., Maloof, P., Wang, H., Fenig, E., Stein, D., Nichols, G., Denny, T.N., Yahalom, J., and Wieder, R., 1998, Basic fibroblast growth factor downregulates Bcl-2 and promotes apoptosis in MCF-7 human breast cancer cells. Exp Cell Res, 238:177-187.
151. Bisping, G., Leo, R., Wenning, D., Dankbar, B., Padro, T., Kropff, M., Scheffold, C., Kroger, M., Mesters, R.M., Berdel, W.E., and Kienast, J., 2003, Paracrine interactions of basic fibroblast growth factor and interleukin-6 in multiple myeloma. Blood, 101:2775-2783.
152. El Hariry, I., Pignatelli, M., and Lemoine, N., 2001, R. FGF-1 and FGF-2 regulate the expression of E-cadherin and catenins in pancreatic adenocarcinoma. Int J Cancer, 94:652-661.
153. El Hariry, I., Pignatelli, M., and Lemoine, N. R., 2001, FGF-1 and FGF-2 modulate the E-cadherin/catenin system in pancreatic adenocarcinoma cell lines. Br J Cancer, 84:1656-1663.
154. Qiao, D., Meyer, K., Mundhenke, C., Drew, S. A., and Friedl, A., 2003, Heparan sulfate proteoglycans as regulators of fibroblast growth factor-2 signaling in brain endothelial cells. Specific role for glypican-1 in glioma angiogenesis. J Biol Chem, 278:16045-16053.
155. Mundhenke, C., Meyer, K., Drew, S., and Friedl, A., 2002, Heparan sulfate proteoglycans as regulators of fibroblast growth factor-2 receptor binding in breast carcinomas. Am J Pathol, 160:185-194.
156. Lambrecht, V., Le, B., X, Toillon, R.A., Boilly, B., and Hondermarck, H., 1998, Alterations in both heparan sulfate proteoglycans and mitogenic activity of fibroblast growth factor-2 are triggered by inhibitors of proliferation in normal and breast cancer epithelial cells. Exp Cell Res, 245:239-244.
157. Delehedde, M., Deudon, E., Boilly, B., and Hondermarck, H., 1996, Heparan sulfate

proteoglycans play a dual role in regulating fibroblast growth factor-2 mitogenic activity in human breast cancer cells. Exp Cell Res, 229:398-406.

158. Genersch, E., Ferletta, M., Virtanen, I., Haller, H., and Ekblom, P., 2003, Integrin alphavbeta3 binding to human alpha5-laminins facilitates FGF-2- and VEGF-induced proliferation of human ECV304 carcinoma cells. Eur J Cell Biol, 82:105-117.
159. De Medina, S.G., Popov, Z., Chopin, D.K., Southgate, J., Tucker, G.C., Delouvee, A., Thiery, J.P., and Radvanyi, F., 1999, Relationship between E-cadherin and fibroblast growth factor receptor 2b expression in bladder carcinomas. Oncogene, 18:5722-5726.
160. Cavallaro, U., Niedermeyer, J., Fuxa, M., and Christofori, G., 2001, N-CAM modulates tumour-cell adhesion to matrix by inducing FGF-receptor signalling. Nat Cell Biol, 3:650-657.
161. Boyer, B., Dufour, S., and Thiery, J.P., 1992, E-cadherin expression during the acidic FGF-induced dispersion of a rat bladder carcinoma cell line. Exp Cell Res, 201:347-357.
162. Zhang, Y., Song, S., Yang, F., Au, J.L., and Wientjes, M.G., 2001, Nontoxic doses of suramin enhance activity of doxorubicin in prostate tumors. J Pharmacol Exp Ther, 299:426-433.
163. Song, S., Wientjes, M.G., Walsh, C., and Au, J.L., 2001, Nontoxic doses of suramin enhance activity of paclitaxel against lung metastases. Cancer Res, 61:6145-6150.
164. Xin Y., Chen D., Song S., Lyness G., Wientjes G., and Au J., 2004, Low-dose suramin enhances antitumor activity of mitomycin C in bladder tumors.[Abstract]. Proceedings of the AACR, 45:#2002.
165. Ogden A., Song S., Wientjes G., and Au J., 2004, Nontoxic doses of suramin enhance the activity of gemcitabine and paclitaxel in pancreatic tumors.[Abstract]. Proceedings of the AACR, 45:#2148.
166. Yu B., Song S., Wientjes G., and Au J., 2003, Suramin enhances activity of CPT-11 in human colorectal xenograft tumors [Abstract]. Proceedings of the AACR, 44:#763.
167. Lyness G., Jang S., Gan Y., Zhang Y., Wientjes G., and Au J., 2003, Fibroblast growth factors and chemoresistance in renal cell carcinomas [Abstract]. Proceedings of the AACR, 44:#4718.
168. Villalona-Calero M.A., Otterson G.A., Wientjes G., Kobayashi K., Jensen R., Young D., Yeh T., Song S., Grever M., and Au J., 2004, Phase II study of low dose suramin as a sensitizer of paclitaxel/carboplatin (P/C) in non small cell lung cancer (NSCLC). [Abstract]. Journal of Clinical Oncology, 2004 ASCO Annual Meeting Proceedings (Post-Meeting Edition), 22:#7108.
169. Yu, H., and Rohan, T., 2000, Role of the insulin-like growth factor family in cancer development and progression. J Natl Cancer Inst, 92:1472-1489.
170. LeRoith, D. and Roberts, Jr., C.T., 2003, The insulin-like growth factor system and cancer. Cancer Lett, 195:127-137.
171. Samani, A.A., and Brodt, P., 2001, The receptor for the type I insulin-like growth factor and its ligands regulate multiple cellular functions that impact on metastasis. Surg Oncol Clin N Am, 10:289-312,viii.
172. Lee, A.V., Hilsenbeck, S.G., and Yee, D., 1998, IGF system components as prognostic markers in breast cancer. Breast Cancer Res Treat, 47:295-302.
173. Kaaks, R. 2002.Nutrition, energy balance and colon cancer risk: the role of insulin and insulin-like growth factor-I. IARC Sci Publ, 156:289-293.
174. Chan, J.M., Stampfer, M.J., Giovannucci, E., Ma, J., and Pollak, M., 2000, Insulin-like growth factor I (IGF-I), IGF-binding protein-3 and prostate cancer risk: epidemiological studies. Growth Horm IGF Res, 10Suppl A:S32-S33.
175. Giovannucci, E., 1999, Insulin-like growth factor-I and binding protein-3 and risk of cancer. Horm Res, 51Suppl3:34-41.
176. Spitz, M.R., Barnett, M.J., Goodman, G.E., Thornquist, M.D., Wu, X., and Pollak, M., 2002, Serum insulin-like growth factor (IGF) and IGF-binding protein levels and risk of lung cancer: a case-control study nested in the beta-Carotene and Retinol Efficacy Trial Cohort. Cancer Edpdemiol Biomarkers Prev, 11:1413-1418.
177. Schernhammer, E.S., 2002, In-utero exposures and breast cancer risk: joint effect of estrogens and insulin-like growth factor? Cancer Causes Control, 13:505-508.
178. Pollak, M., 2000, Insulin-like growth factor physiology and cancer risk. Eur J Cancer, 36:1224-1228.
179. Baron-Hay, S., Boyle, F., Ferrier, A., and Scott, C., 2004, Elevated serum insulin-like growth factor binding protein-2 as a prognostic marker in patients with ovarian cancer. Clin Cancer Res, 10:1796-1806.
180. Gu, L., Shigemasa, K., and Ohama, K., 2004, Increased expression of IGF II mRNA-binding protein 1 mRNA is associated with an advanced clinical stage and poor prognosis in patients with ovarian cancer. Int J Oncol, 24:671-678.
181. Peters, G., Gongoll, S., Langner, C., Mengel, M., Piso, P., Klempnauer, J., Ruschoff, J., Kreipe, H., and von Wasielewski, R., 2003, IGF-1R, IGF-1 and IGF-2 expression as potential prognostic and predictive markers in colorectal-cancer. Virchows Arch, 443:139-145.
182. Kawamoto, K., Onodera, H., Kondo, S., Kan, S., Ikeuchi, D., Maetani, S., and Imamura, M., 1998, Expression of insulin-like growth factor-2 can

predict the prognosis of human colorectal cancer patients: correlation with tumor progression, proliferative activity and survival. Oncology, 55:242-248.

183. Jamieson, T.A., Brizel, D.M., Killian, J.K., Oka, Y., Jang, H.S., Fu, X., Clough, R.W., Vollmer, R.T., Anscher, M.S., and Jirtle, R.L., 2003, M6P/IGF2R loss of heterozygosity in head and neck cancer associated with poor patient prognosis. BMC Cancer, 3:4.
184. Chang, Y.S., Wang, L., Liu, D., Mao, L., Hong, W.K., Khuri, F.R., and Lee, H.Y., 2002, Correlation between insulin-like growth factor-binding protein-3 promoter methylation and prognosis of patients with stage I non-small cell lung cancer. Clin Cancer Res, 8:3669-3675.
185. Standal, T., Borset, M., Lenhoff, S., Wisloff, F., Stordal, B., Sundan, A., Waage, A., and Seidel, C., 2002, Serum insulinlike growth factor is not elevated in patients with multiple myeloma but is still a prognostic factor. Blood, 100:3925-3929.
186. Rocha, R.L., Hilsenbeck, S.G., Jackson, J.G., Lee, A.V., Figueroa, J.A., and Yee, D., 1996, Correlation of insulin-like growth factor-binding protein-3 messenger RNA with protein expression in primary breast cancer tissues: detection of higher levels in tumors with poor prognostic features. J Natl Cancer Inst, 88:601-606.
187. Holdaway, I.M., Mason, B.H., Lethaby, A.E., Singh, V., Harvey, V.J., Thompson, P.I., and Evans, B.D., 2003, Serum insulin-like growth factor-I and insulin-like growth factor binding protein-3 following chemotherapy for advanced breast cancer. ANZ J Surg, 73:905-908.
188. Railo, M.J., von Smitten, K., and Pekonen, F., 1994, The prognostic value of insulin-like growth factor-I in breast cancer patients. Results of a follow-up study on 126 patients. Eur J Cancer, 30A:307-311.
189. Bonneterre, J., Peyrat, J.P., Beuscart, R., and Demaille, A., 1990, Prognostic significance of insulin-like growth factor 1 receptors in human breast cancer. Cancer Res, 50:6931-6935.
190. Turner, B.C., Haffty, B.G., Narayanan, L., Yuan, J., Havre, P.A., Gumbs, A.A., Kaplan, L., Burgaud, J.L., Carter, D., Baserga, R., and Glazer, P.M., 1997, Insulin-like growth factor-I receptor overexpression mediates cellular radioresistance and local breast cancer recurrence after lumpectomy and radiation. Cancer Res, 57:3079-3083.
191. Takanami, I., Imamuma, T., Hashizume, T., Kikuchi, K., Yamamoto, Y., Yamamoto, T., and Kodaira, S., 1996, Insulin-like growth factor-II as a prognostic factor in pulmonary adenocarcinoma. J Surg Oncol, 61:205-208.
192. Grothey, A., Voigt, W., Schober, C., Muller, T., Dempke, W., and Schmoll, H.J., 1999, The role of insulin-like growth factor I and its receptor in cell growth, transformation, apoptosis, and chemoresistance in solid tumors. J Cancer Res Clin Oncol, 125:166-173.
193. Hadsell, D.L. and Abdel-Fattah, G., 2001, Regulation of cell apoptosis by insulin-like growth factor I. Adv Exp Med Biol, 501:79-85.
194. Vincent, A.M. and Feldman, E.L. 2002.Control of cell survival by IGF signaling pathways. Growth Horm IGF Res, 12:193-197.
195. Le Roith, D., 2000, Regulation of proliferation and apoptosis by the insulin-like growth factor I receptor. Growth Horm IGF Res, 10SupplA:S12-S13.
196. Isgaard, J., and Tivesten, A., 1999, The role of growth hormone and insulin-like growth factor I in the regulation of apoptosis. Growth Horm IGF Res, 9SupplA:125-128.
197. Garrouste, F., Remacle-Bonnet, M., Fauriat, C., Marvaldi, J., Luis, J., and Pommier, G., 2002, Prevention of cytokine-induced apoptosis by insulin-like growth factor-I is independent of cell adhesion molecules in HT29-D4 colon carcinoma cells-evidence for a NF-kappaB-dependent survival mechanism. Cell Death Differ, 9:768-779.
198. Gil-Ad, I., Shtaif, B., Luria, D., Karp, L., Fridman, Y., and Weizman, A., 1999, Insulin-like-growth-factor-I (IGF-I) antagonizes apoptosis induced by serum deficiency and doxorubicin in neuronal cell culture. Growth Horm IGF Res, 9:458-464.
199. Geier, A., Hemi, R., Haimsohn, M., Beery, R., Malik, Z., and Karasik, A., 1994, Epidermal growth factor and insulin-like growth factor-1 protect MDA-231 cells from death induced by actinomycin D: the involvement of growth factors in drug resistance. In Vitro Cell Dev Biol Anim, 30A:336-343.
200. Gooch, J.L., Van Den Berg, C.L., and Yee, D., 1999, Insulin-like growth factor (IGF)-I rescues breast cancer cells from chemotherapy-induced cell death--proliferative and anti-apoptotic effects. Breast Cancer Res Treat, 56:1-10.
201. Guo, Y.S., Jin, G.F., Houston, C.W., Thompson, J.C., and Townsend, Jr., C.M., 1998, Insulin-like growth factor-I promotes multidrug resistance in MCLM colon cancer cells. J Cell Physiol, 175:141-148.
202. Sekharam, M., Zhao, H., Sun, M., Fang, Q., Zhang, Q., Yuan, Z., Dan, H.C., Boulware, D., Cheng, J.Q., and Coppola, D., 2003, Insulin-like growth factor 1 receptor enhances invasion and induces resistance to apoptosis of colon cancer cells through the Akt/Bcl-x(L) pathway. Cancer Res, 63:7708-7716.
203. Levitt, R.J. and Pollak, M., 2002, Insulin-like growth factor-I antagonizes the antiproliferative effects of cyclooxygenase-2 inhibitors on BxPC-3 pancreatic cancer cells. Cancer Res, 62:7372-7376.

204. Liu, Y.C., Leu, C.M., Wong, F.H., Fong, W.S., Chen, S.C., Chang, C., and Hu, C.P., 2002, Autocrine stimulation by insulin-like growth factor I is involved in the growth, tumorigenicity and chemoresistance of human esophageal carcinoma cells. J Biomed Sci, 9:665-674.
205. Wan, X. and Helman, L.J., 2002, Effect of insulin-like growth factor II on protecting myoblast cells against cisplatin-induced apoptosis through p70 S6 kinase pathway. Neoplasia, 4:400-408.
206. Sun, H.Z., Wu, S.F., and Tu, Z.H., 2001, Blockage of IGF-1R signaling sensitizes urinary bladder cancer cells to mitomycin-mediated cytotoxicity. Cell Res, 11:107-115.
207. Butt, A.J., Firth, S.M., and Baxter, R.C., 1999, The IGF axis and programmed cell death. Immunol Cell Biol, 77:256-262.
208. Werner, H. and Le Roith, D., 1997, The insulin-like growth factor-I receptor signaling pathways are important for tumorigenesis and inhibition of apoptosis. Crit Rev Oncog, 8:71-92.
209. Bohula, E.A., Playford, M.P., and Macaulay, V.M., 2003, Targeting the type 1 insulin-like growth factor receptor as anti-cancer treatment. Anticancer Drugs, 14:669-682.
210. Salisbury, A.J. and Macaulay, V.M., 2003, Development of molecular agents for IGF receptor targeting. Horm Metab Res, 35:843-849.
211. Andrews, D.W., Resnicoff, M., Flanders, A.E., Kenyon, L., Curtis, M., Merli, G., Baserga, R., Iliakis, G., and Aiken, R.D., 2001, Results of a pilot study involving the use of an antisense oligodeoxynucleotide directed against the insulin-like growth factor type I receptor in malignant astrocytomas. J Clin Oncol, 19:2189-2200.
212. Liu, X., Turbyville, T., Fritz, A., and Whitesell, L., 1998, Inhibition of insulin-like growth factor I receptor expression in neuroblastoma cells induces the regression of established tumors in mice. Cancer Res, 58:5432-5438.
213. Birchmeier, C., Birchmeier, W., Gherardi, E., and Vande Woude, G.F., 2003, Met, metastasis, motility and more. Nat.Rev.Mol Cell Biol, 4:915-925.
214. Zhang, Y.W. and Vande Woude, G.F., 2003, HGF/SF-met signaling in the control of branching morphogenesis and invasion. J Cell Biochem, 88:408-417.
215. Danilkovitch-Miagkova, A. and Zbar, B., 2002, Dysregulation of Met receptor tyrosine kinase activity in invasive tumors. J Clin Invest, 109:863-867.
216. Haddad, R., Lipson, K.E., and Webb, C.P., 2001, Hepatocyte growth factor expression in human cancer and therapy with specific inhibitors. AntiCancer Res, 21:4243-4252.
217. Trusolino, L. and Comoglio, P.M., 2002, Scatter-factor and semaphorin receptors: cell signalling for invasive growth. Nat Rev Cancer, 2:289-300.
218. Birchmeier, C. and Gherardi, E., 1998, Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. Trends Cell Biol, 8:404-410.
219. Vadnais, J., Nault, G., Daher, Z., Amraei, M., Dodier, Y., Nabi, I. R., and Noel, J., 2002, Autocrine activation of the hepatocyte growth factor receptor/met tyrosine kinase induces tumor cell motility by regulating pseudopodial protrusion. J Biol Chem, 277:48342-48350.
220. Yi, S. and Tsao, M.S., 2000, Activation of hepatocyte growth factor-met autocrine loop enhances tumorigenicity in a human lung adenocarcinoma cell line. Neoplasia, 2:226-234.
221. Jeffers, M., Rong, S., Anver, M., and Vande Woude, G.F., 1996, Autocrine hepatocyte growth factor/scatter factor-Met signaling induces transformation and the invasive/metastastic phenotype in C127 cells. Oncogene, 13:853-856.
222. Tsao, M.S., Yang, Y., Marcus, A., Liu, N., and Mou, L., 2001, Hepatocyte growth factor is predominantly expressed by the carcinoma cells in non-small-cell lung cancer. Hum Pathol, 32:57-65.
223. Chen, Y.S., Wang, J.T., Chang, Y.F., Liu, B.Y., Wang, Y.P., Sun, A., and Chiang, C.P., 2004, Expression of hepatocyte growth factor and c-met protein is significantly associated with the progression of oral squamous cell carcinoma in Taiwan. J Oral Pathol Med, 33:209-217.
224. Parr, C., Watkins, G., Mansel, R.E., and Jiang, W.G., 2004, The hepatocyte growth factor regulatory factors in human breast cancer. Clin Cancer Res, 10:202-211.
225. Nardone, H.C., Ziober, A.F., LiVolsi, V.A., Mandel, S.J., Baloch, Z.W., Weber, R.S., Mick, R., and Ziober, B.L., 2003, c-Met expression in tall cell variant papillary carcinoma of the thyroid. Cancer, 98:1386-1393.
226. Takeuchi, H., Bilchik, A., Saha, S., Turner, R., Wiese, D., Tanaka, M., Kuo, C., Wang, H.J., and Hoon, D.S., 2003, c-MET expression level in primary colon cancer: a predictor of tumor invasion and lymph node metastases. Clin Cancer Res, 9:1480-1488.
227. Tolgay, O., I, Dolled-Filhart, M., D'Aquila, T.G., Camp, R.L., and Rimm, D.L., 2003, Tissue microarray-based studies of patients with lymph node negative breast carcinoma show that met expression is associated with worse outcome but is not correlated with epidermal growth factor family receptors. Cancer, 97:1841-1848.
228. Maulik, G., Shrikhande, A., Kijima, T., Ma, P. C., Morrison, P.T., and Salgia, R., 2002, Role of the hepatocyte growth factor receptor, c-Met, in

oncogenesis and potential for therapeutic inhibition. Cytokine Growth Factor Rev, 13:41-59.

229. Furukawa, T., Duguid, W.P., Kobari, M., Matsuno, S., and Tsao, M.S., 1995, Hepatocyte growth factor and Met receptor expression in human pancreatic carcinogenesis. Am J Pathol, 147:889-895.
230. Baykal, C., Ayhan, A., Al, A., Yuce, K., and Ayhan, A., 2003, Overexpression of the c-Met/HGF receptor and its prognostic significance in uterine cervix carcinomas. Gynecol Oncol, 88:123-129.
231. Gohji, K., Nomi, M., Niitani, Y., Kitazawa, S., Fujii, A., Katsuoka, Y., and Nakajima, M., 2000, Independent prognostic value of serum hepatocyte growth factor in bladder cancer. J Clin Oncol, 18:2963-2971.
232. Tsarfaty, I., Alvord, W.G., Resau, J.H., Altstock, R.T., Lidereau, R., Bieche, I., Bertrand, F., Horev, J., Klabansky, R.L., Keydar, I., and Vande Woude, G.F., 1999, Alteration of Met protooncogene product expression and prognosis in breast carcinomas. Anal.Quant.Cytol.Histol., 21:397-408.
233. Nakajima, M., Sawada, H., Yamada, Y., Watanabe, A., Tatsumi, M., Yamashita, J., Matsuda, M., Sakaguchi, T., Hirao, T., and Nakano, H., 1999, The prognostic significance of amplification and overexpression of c-met and c-erb B-2 in human gastric carcinomas. Cancer, 85:1894-1902.
234. Porte, H., Triboulet, J.P., Kotelevets, L., Carrat, F., Prevot, S., Nordlinger, B., DiGioia, Y., Wurtz, A., Comoglio, P., Gespach, C., and Chastre, E., 1998, Overexpression of stromelysin-3, BM-40/SPARC, and MET genes in human esophageal carcinoma: implications for prognosis. Clin Cancer Res, 4:1375-1382.
235. Tokunou, M., Niki, T., Eguchi, K., Iba, S., Tsuda, H., Yamada, T., Matsuno, Y., Kondo, H., Saitoh, Y., Imamura, H., and Hirohashi, S., 2001, c-MET expression in myofibroblasts: role in autocrine activation and prognostic significance in lung adenocarcinoma. Am J Pathol, 158:1451-1463.
236. Cheng, H.L., Trink, B., Tzai, T.S., Liu, H.S., Chan, S.H., Ho, C.L., Sidransky, D., and Chow, N.H., 2002, Overexpression of c-met as a prognostic indicator for transitional cell carcinoma of the urinary bladder: a comparison with p53 nuclear accumulation. J Clin Oncol, 20:1544-1550.
237. Rasola, A., Anguissola, S., Ferrero, N., Gramaglia, D., Maffe, A., Maggiora, P., Comoglio, P.M., and Di Renzo, M.F., 2004, Hepatocyte growth factor sensitizes human ovarian carcinoma cell lines to paclitaxel and cisplatin. Cancer Res, 64:1744-1750.
238. Bowers, D.C., Fan, S., Walter, K.A., Abounader, R., Williams, J.A., Rosen, E.M., and Laterra, J., 2000, Scatter factor/hepatocyte growth factor protects against cytotoxic death in human glioblastoma via phosphatidylinositol 3-kinase- and AKT-dependent pathways. Cancer Res, 60:4277-4283.
239. Aebersold, D.M., Kollar, A., Beer, K.T., Laissue, J., Greiner, R.H., and Djonov, V., 2001, Involvement of the hepatocyte growth factor/scatter factor receptor c-met and of Bcl-xL in the resistance of oropharyngeal cancer to ionizing radiation. Int J Cancer, 96:41-54.
240. Jankowski, K., Kucia, M., Wysoczynski, M., Reca, R., Zhao, D., Trzyna, E., Trent, J., Peiper, S., Zembala, M., Ratajczak, J., Houghton, P., Janowska-Wieczorek, A., and Ratajczak, M.Z., 2003, Both hepatocyte growth factor (HGF) and stromal-derived factor-1 regulate the metastatic behavior of human rhabdomyosarcoma cells, but only HGF enhances their resistance to radiochemotherapy. Cancer Res, 63:7926-7935.
241. Wang, X., Zhou, Y., Kim, H.P., Song, R., Zarnegar, R., Ryter, S.W., and Choi, A.M., 2004, Hepatocyte growth factor protects against hypoxia/reoxygenation-induced apoptosis in endothelial cells. J Biol Chem, 279:5237-5243.
242. Derksen, P.W., de Gorter, D.J., Meijer, H.P., Bende, R.J., van Dijk, M., Lokhorst, H.M., Bloem, A.C., Spaargaren, M., and Pals, S.T., 2003, The hepatocyte growth factor/Met pathway controls proliferation and apoptosis in multiple myeloma. Leukemia, 17:764-774.
243. Nakagami, H., Morishita, R., Yamamoto, K., Taniyama, Y., Aoki, M., Yamasaki, K., Matsumoto, K., Nakamura, T., Kaneda, Y., and Ogihara, T., 2002, Hepatocyte growth factor prevents endothelial cell death through inhibition of bax translocation from cytosol to mitochondrial membrane. Diabetes, 51:2604-2611.
244. Ma, H., Calderon, T.M., Fallon, J.T., and Berman, J.W., 2002, Hepatocyte growth factor is a survival factor for endothelial cells and is expressed in human atherosclerotic plaques. Atherosclerosis, 164:79-87.
245. Gao, M., Fan, S., Goldberg, I.D., Laterra, J., Kitsis, R.N., and Rosen, E.M., 2001, Hepatocyte growth factor/scatter factor blocks the mitochondrial pathway of apoptosis signaling in breast cancer cells. J Biol Chem, 276:47257-47265.
246. Fan, S., Ma, Y.X., Gao, M., Yuan, R.Q., Meng, Q., Goldberg, I.D., and Rosen, E.M., 2001, The multisubstrate adapter Gab1 regulates hepatocyte growth factor (scatter factor)-c-Met signaling for cell survival and DNA repair. Mol Cell Biol, 21:4968-4984.
247. Aebersold, D.M., Kollar, A., Beer, K.T., Laissue, J., Greiner, R.H., and Djonov, V., 2001, Involvement of the hepatocyte growth factor/scatter factor receptor c-met and of Bcl-xL in the resistance of oropharyngeal cancer to ionizing radiation. Int J Cancer, 96:41-54.
248. Fan, S., Ma, Y.X., Wang, J.A., Yuan, R.Q., Meng, Q., Cao, Y., Laterra, J.J., Goldberg, I.D., and Rosen,

E.M., 2000, The cytokine hepatocyte growth factor/scatter factor inhibits apoptosis and enhances DNA repair by a common mechanism involving signaling through phosphatidyl inositol 3' kinase. Oncogene, 19:2212-2223.

249. Date, K., Matsumoto, K., Shimura, H., Tanaka, M., and Nakamura, T., 1997, HGF/NK4 is a specific antagonist for pleiotrophic actions of hepatocyte growth factor. FEBS Lett, 420: 1-6,
250. Dranoff, G., 2004, Cytokines in cancer pathogenesis and cancer therapy. Nat Rev Cancer, 4:11-22.
251. Cohen, Jr., M.M., 2003, TGF beta/Smad signaling system and its pathologic correlates. Am.J Med.Genet., 116A:1-10.
252. Cordeiro, M.F., 2002, Beyond Mitomycin: TGF-beta and wound healing. Prog Retin Eye Res, 21:75-89.
253. Moustakas, A., Pardali, K., Gaal, A., and Heldin, C.H., 2002, Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. Immunol Lett, 82:85-91.
254. Siegel, P.M. and Massague, J., 2003, Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. Nat Rev Cancer, 3:807-821.
255. Verrecchia, F. and Mauviel, A., 2002, Transforming growth factor-beta signaling through the Smad pathway: role in extracellular matrix gene expression and regulation. J Invest Dermatol, 118:211-215.
256. Massague, J., Blain, S.W., and Lo, R.S., 2000, TGFbeta signaling in growth control, cancer, and heritable disorders. Cell, 103:295-309.
257. Derynck, R., Akhurst, R.J., and Balmain, A., 2001, TGF-beta signaling in tumor suppression and cancer progression. Nat Genet, 29:117-129.
258. Derynck, R. and Zhang, Y.E., 2003, Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature, 425:577-584.
259. Massague, J., 2000, How cells read TGF-beta signals. Nat Rev Mol Cell Biol, 1:169-178.
260. Massague, J., 1998, TGF-beta signal transduction. Annu Rev Biochem, 67:753-791.
261. Shibuya, H., Yamaguchi, K., Shirakabe, K., Tonegawa, A., Gotoh, Y., Ueno, N., Irie, K., Nishida, E., and Matsumoto, K., 1996, TAB1: an activator of the TAK1 MAPKKK in TGF-beta signal transduction. Science, 272:1179-1182.
262. Yue, J., and Mulder, K.M., 2000, Requirement of Ras/MAPK pathway activation by transforming growth factor beta for transforming growth factor beta 1 production in a smad-dependent pathway. J Biol Chem, 275:35656.
263. Park, B.J., Park, J.I., Byun, D.S., Park, J.H., and Chi, S.G., 2000, Mitogenic conversion of transforming growth factor-beta1 effect by oncogenic Ha-Ras-induced activation of the mitogen-activated protein kinase signaling pathway in human prostate cancer. Cancer Res, 60:3031-3038.
264. Mulder, K.M., 2000, Role of Ras and Mapks in TGFbeta signaling. Cytokine Growth Factor Rev, 11:23-35.
265. Kivinen, L., and Laiho, M., 1999, Ras- and mitogen-activated protein kinase kinase-dependent and -independent pathways in p21Cip1/Waf1 induction by fibroblast growth factor-2, platelet-derived growth factor, and transforming growth factor-beta1. Cell Growth Differ, 10:621-628.
266. Yamamoto, H., Atsuchi, N., Tanaka, H., Ogawa, W., Abe, M., Takeshita, A., and Ueno, H., 1999, Separate roles for H-Ras and Rac in signaling by transforming growth factor (TGF)-beta. H-Ras is essential for activation of MAP kinase, partially required for transcriptional activation by TGF-beta, but not required for signaling of growth suppression by TGF-beta. Eur.J Biochem., 264:110-119.
267. Reimann, T., Hempel, U., Krautwald, S., Axmann, A., Scheibe, R., Seidel, D., and Wenzel, K.W., 1997, Transforming growth factor-beta1 induces activation of Ras, Raf-1, MEK and MAPK in rat hepatic stellate cells. FEBS Lett, 403:57-60.
268. Atfi, A., Djelloul, S., Chastre, E., Davis, R., and Gespach, C., 1997, Evidence for a role of Rho-like GTPases and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in transforming growth factor beta-mediated signaling. J Biol Chem, 272:1429-1432.
269. Petritsch, C., Beug, H., Balmain, A., and Oft, M., 2000, TGF-beta inhibits p70 S6 kinase via protein phosphatase 2A to induce G(1) arrest. Genes Dev, 14:3093-3101.
270. Griswold-Prenner, I., Kamibayashi, C., Maruoka, E.M., Mumby, M.C., and Derynck, R., 1998, Physical and functional interactions between type I transforming growth factor beta receptors and Balpha, a WD-40 repeat subunit of phosphatase 2A. Mol Cell Biol, 18:6595-6604.
271. Horowitz, J.C., Lee, D.Y., Waghray, M., Keshamouni, V.G., Thomas, P.E., Zhang, H., Cui, Z., and Thannickal, V.J., 2004, Activation of the pro-survival phosphatidylinositol 3-kinase/AKT pathway by transforming growth factor-beta1 in mesenchymal cells is mediated by p38 MAPK-dependent induction of an autocrine growth factor. J Biol Chem, 279:1359-1367.
272. Shin, I., Bakin, A.V., Rodeck, U., Brunet, A., and Arteaga, C.L., 2001, Transforming growth factor beta enhances epithelial cell survival via Akt-dependent regulation of FKHRL1. Mol Biol Cell, 12:3328-3339.
273. Wakefield, L.M. and Roberts, A.B., 2002, TGF-beta signaling: positive and negative effects on tumorigenesis. Curr Opin Genet Dev, 12:22-29.
274. Sun, L., 2004, Tumor-suppressive and promoting function of transforming growth factor beta. Front Biosci, 9:1925-1935.

275. Roberts, A.B. and Wakefield, L.M., 2003, The two faces of transforming growth factor beta in carcinogenesis. Proc Natl Acad Sci USA, 100:8621-8623.
276. Wikstrom, P., Stattin, P., Franck-Lissbrant, I., Damber, J.E., and Bergh, A. 1998, Transforming growth factor beta1 is associated with angiogenesis, metastasis, and poor clinical outcome in prostate cancer. Prostate, 37:19-29.
277. Eastham, J.A., Truong, L.D., Rogers, E., Kattan, M., Flanders, K.C., Scardino, P.T., and Thompson, T.C., 1995, Transforming growth factor-beta 1: comparative immunohistochemical localization in human primary and metastatic prostate cancer. Lab Invest, 73:628-635.
278. Steiner, M.S., Zhou, Z.Z., Tonb, D.C., and Barrack, E.R., 1994, Expression of transforming growth factor-beta 1 in prostate cancer. Endocrinology, 135:2240-2247.
279. Muir, G.H., Butta, A., Shearer, R.J., Fisher, C., Dearnaley, D.P., Flanders, K.C., Sporn, M.B., and Colletta, A.A., 1994, Induction of transforming growth factor beta in hormonally treated human prostate cancer. Br J, Cancer, 69:130-134.
280. Knabbe, C., Klein, H., Zugmaier, G., and Voigt, K.D., 1993, Hormonal regulation of transforming growth factor beta-2 expression in human prostate cancer. J Steroid Biochem Mol Biol, 47:137-142.
281. Truong, L.D., Kadmon, D., McCune, B.K., Flanders, K.C., Scardino, P.T., and Thompson, T.C., 1993, Association of transforming growth factor-beta 1 with prostate cancer: an immunohistochemical study. Hum Pathol, 24:4-9.
282. Wikstrom, P., Damber, J., and Bergh, A., 2001, Role of transforming growth factor-beta1 in prostate cancer. Microsc Res Tech, 52:411-419.
283. Kim, I.Y., Ahn, H.J., Lang, S., Oefelein, M.G., Oyasu, R., Kozlowski, J.M., and Lee, C., 1998, Loss of expression of transforming growth factor-beta receptors is associated with poor prognosis in prostate cancer patients. Clin Cancer Res, 4:1625-1630.
284. Shariat, S.F., Menesses-Diaz, A., Kim, I.Y., Muramoto, M., Wheeler, T.M., and Slawin, K.M., 2004, Tissue expression of transforming growth factor-beta1 and its receptors: correlation with pathologic features and biochemical progression in patients undergoing radical prostatectomy. Urology, 63:1191-1197.
285. Wikstrom, P., Bergh, A., and Damber, J.E., 2000, Transforming growth factor-beta1 and prostate cancer. Scand J Urol Nephrol, 34:85-94.
286. MacCallum, J., Bartlett, J.M., Thompson, A.M., Keen, J.C., Dixon, J.M., and Miller, W.R., 1994, Expression of transforming growth factor beta mRNA isoforms in human breast cancer. Br J Cancer, 69:1006-1009.
287. Auvinen, P., Lipponen, P., Johansson, R., and Syrjanen, K., 1995, Prognostic significance of TGF-beta 1 and TGF-beta 2 expressions in female breast cancer. AntiCancer Res, 15:2627-2631.
288. Kong, F.M., Anscher, M.S., Murase, T., Abbott, B.D., Iglehart, J.D., and Jirtle, R.L., 1995, Elevated plasma transforming growth factor-beta 1 levels in breast cancer patients decrease after surgical removal of the tumor. Ann Surg, 222:155-162.
289. Auvinen, P., Lipponen, P., Johansson, R., and Syrjanen, K., 1995, Prognostic significance of TGF-beta 2 expression in female breast cancer. Eur J Cancer, 31A: 851.
290. Thompson, A.M., Kerr, D.J., and Steel, C.M., 1991, Transforming growth factor beta 1 is implicated in the failure of tamoxifen therapy in human breast cancer. Br J Cancer, 63:609-614.
291. Ghellal, A., Li, C., Hayes, M., Byrne, G., Bundred, N., and Kumar, S., 2000, Prognostic significance of TGF beta 1 and TGF beta 3 in human breast carcinoma. AntiCancer Res, 20:4413-4418.
292. Hasegawa, Y., Takanashi, S., Kanehira, Y., Tsushima, T., Imai, T., and Okumura, K., 2001, Transforming growth factor-beta1 level correlates with angiogenesis, tumor progression, and prognosis in patients with nonsmall cell lung carcinoma. Cancer, 91:964-971.
293. Saji, H., Nakamura, H., Awut, I., Kawasaki, N., Hagiwara, M., Ogata, A., Hosaka, M., Saijo, T., Kato, Y., and Kato, H., 2003, Significance of expression of TGF-beta in pulmonary metastasis in non-small cell lung cancer tissues. Ann Thorac Cardiovasc Surg, 9:295-300.
294. Gold, L.I., 1999, The role for transforming growth factor-beta (TGF-beta) in human cancer. Crit Rev Oncog, 10:303-360.
295. Bennett, W.P., el Deiry, W.S., Rush, W.L., Guinee, Jr., D.G., Freedman, A.N., Caporaso, N.E., Welsh, J.A., Jones, R.T., Borkowski, A., Travis, W.D., Fleming, M.V., Trastek, V., Pairolero, P.C., Tazelaar, H.D., Midthun, D., Jett, J.R., Liotta, L.A., and Harris, C.C., 1998, p21waf1/cip1 and transforming growth factor beta 1 protein expression correlate with survival in non-small cell lung cancer. Clin Cancer Res, 4:1499-1506.
296. Takanami, I., Tanaka, F., Hashizume, T., and Kodaira, S., 1997, Roles of the transforming growth factor beta 1 and its type I and II receptors in the development of a pulmonary adenocarcinoma: results of an immunohistochemical study. J Surg Oncol, 64:262-267.
297. Takanami, I., Tanaka, F., Hashizume, T., Kikuchi, K., Yamamoto, Y., Yamamoto, T., and Kodaira, S., 1997, Transforming growth factor-beta isoforms expressions in pulmonary adenocarcinomas as prognostic markers: an immunohistological study of

one hundred and twenty patients. Oncology, 54:122-128.

298. de Jonge, R.R., Garrigue-Antar, L., Vellucci, V.F., and Reiss, M., 1997, Frequent inactivation of the transforming growth factor beta type II receptor in small-cell lung carcinoma cells. Oncol Res, 9:89-98.
299. Takanami, I., Imamuma, T., Hashizume, T., Kikuchi, K., Yamamoto, Y., Yamamoto, T., and Kodaira, S., 1996, Expression of PDGF, IGF-II, bFGF and TGF-beta 1 in pulmonary adenocarcinoma. Pathol Res Pract, 192:1113-1120.
300. Takanami, I., Imamura, T., Hashizume, T., Kikuchi, K., Yamamoto, Y., and Kodaira, S., 1994, Transforming growth factor beta 1 as a prognostic factor in pulmonary adenocarcinoma. J Clin Pathol, 47:1098-1100.
301. Okumoto, K., Hattori, E., Tamura, K., Kiso, S., Watanabe, H., Saito, K., Saito, T., Togashi, H., and Kawata, S., 2004, Possible contribution of circulating transforming growth factor-beta1 to immunity and prognosis in unresectable hepatocellular carcinoma. Liver Int, 24:21-28.
302. Tsai, J.F., Chuang, L.Y., Jeng, J.E., Yang, M.L., Chang, W.Y., Hsieh, M.Y., Lin, Z.Y., and Tsai, J.H., 1997, Clinical relevance of transforming growth factor-beta 1 in the urine of patients with hepatocellular carcinoma. Medicine (Baltimore), 76:213-226.
303. Tsai, J.F., Jeng, J.E., Chuang, L.Y., Yang, M.L., Ho, M.S., Chang, W.Y., Hsieh, M.Y., Lin, Z.Y., and Tsai, J.H., 1997, Elevated urinary transforming growth factor-beta1 level as a tumour marker and predictor of poor survival in cirrhotic hepatocellular carcinoma. Br J Cancer, 76:244-250.
304. Robson, H., Anderson, E., James, R.D., and Schofield, P.F., 1996, Transforming growth factor beta 1 expression in human colorectal tumours: an independent prognostic marker in a subgroup of poor prognosis patients. Br J Cancer, 74:753-758.
305. Saito, H., Tsujitani, S., Oka, S., Kondo, A., Ikeguchi, M., Maeta, M., and Kaibara, N., 1999, The expression of transforming growth factor-beta1 is significantly correlated with the expression of vascular endothelial growth factor and poor prognosis of patients with advanced gastric carcinoma. Cancer, 86:1455-1462.
306. Nakamura, M., Katano, M., Kuwahara, A., Fujimoto, K., Miyazaki, K., Morisaki, T., and Mori, M., 1998, Transforming growth factor beta1 (TGF-beta1) is a preoperative prognostic indicator in advanced gastric carcinoma. Br J Cancer, 78:1373-1378.
307. Hulshof, M.C., Sminia, P., Barten-Van Rijbroek, A.D., and Gonzalez, G.D., 2001, Prognostic value of plasma transforming growth factor-beta in patients with glioblastoma multiforme. Oncol Rep, 8:1107-1110.
308. Buck, M.B., Fritz, P., Dippon, J., Zugmaier, G., and Knabbe, C., 2004, Prognostic significance of transforming growth factor beta receptor II in estrogen receptor-negative breast cancer patients. Clin Cancer Res, 10:491-498.
309. Fukai, Y., Fukuchi, M., Masuda, N., Osawa, H., Kato, H., Nakajima, T., and Kuwano, H., 2003, Reduced expression of transforming growth factor-beta receptors is an unfavorable prognostic factor in human esophageal squamous cell carcinoma. Int J Cancer, 104:161-166.
310. Xie, W., Bharathy, S., Kim, D., Haffty, B.G., Rimm, D.L., and Reiss, M., 2003, Frequent alterations of Smad signaling in human head and neck squamous cell carcinomas: a tissue microarray analysis. Oncol Res, 14:61-73.
311. Xie, W., Rimm, D.L., Lin, Y., Shih, W.J., and Reiss, M., 2003, Loss of Smad signaling in human colorectal cancer is associated with advanced disease and poor prognosis. Cancer J, 9:302-312.
312. Fukuchi, M., Fukai, Y., Masuda, N., Miyazaki, T., Nakajima, M., Sohda, M., Manda, R., Tsukada, K., Kato, H., and Kuwano, H., 2002, High-level expression of the Smad ubiquitin ligase Smurf2 correlates with poor prognosis in patients with esophageal squamous cell carcinoma. Cancer Res, 62:7162-7165.
313. Xie, W., Mertens, J.C., Reiss, D.J., Rimm, D.L., Camp, R.L., Haffty, B.G., and Reiss, M., 2002, Alterations of Smad signaling in human breast carcinoma are associated with poor outcome: a tissue microarray study. Cancer Res, 62:497-505.
314. Boulay, J.L., Mild, G., Lowy, A., Reuter, J., Lagrange, M., Terracciano, L., Laffer, U., Herrmann, R., and Rochlitz, C., 2002, SMAD4 is a predictive marker for 5-fluorouracil-based chemotherapy in patients with colorectal cancer. Br J, Cancer, 87:630-634.
315. Dickson, J., Davidson, S.E., Hunter, R.D., and West, C.M., 2000, Pretreatment plasma TGF beta 1 levels are prognostic for survival but not morbidity following radiation therapy of carcinoma of the cervix. Int J Radiat Oncol Biol Phys, 48:991-995.
316. Keyes, K., Cox, K., Treadway, P., Mann, L., Shih, C., Faul, M.M., and Teicher, B.A., 2002, An in vitro tumor model: analysis of angiogenic factor expression after chemotherapy. Cancer Res, 62:5597-5602.
317. Teicher, B.A., 2001, Malignant cells, directors of the malignant process: role of transforming growth factor-beta. Cancer Metastasis Rev, 20:133-143.
318. Liu, P., Menon, K., Alvarez, E., Lu, K., and Teicher, B.A., 2000, Transforming growth factor-beta and response to anticancer therapies in human liver and gastric tumors in vitro and in vivo. Int J Oncol, 16:599-610.

319. Teicher, B.A., Ikebe, M., Ara, G., Keyes, S.R., and Herbst, R.S., 1997, Transforming growth factor-beta 1 overexpression produces drug resistance in vivo: reversal by decorin. In Vivo, 11:463-472.
320. Teicher, B.A., Kakeji, Y., Ara, G., Herbst, R. S., and Northey, D., 1997, Prostate carcinoma response to cytotoxic therapy: in vivo resistance. In Vivo, 11:453-461.
321. Teicher, B.A., Maehara, Y., Kakeji, Y., Ara, G., Keyes, S.R., Wong, J., and Herbst, R., 1997, Reversal of in vivo drug resistance by the transforming growth factor-beta inhibitor decorin. Int J Cancer, 71:49-58.
322. Kakeji, Y., Maehara, Y., Ikebe, M., and Teicher, B.A. 1997Dynamics of tumor oxygenation, CD31 staining and transforming growth factor-beta levels after treatment with radiation or cyclophosphamide in the rat 13762 mammary carcinoma. Int.J Radiat.Oncol.BiolPhys., 37:1115-1123.
323. Teicher, B.A., Holden, S.A., Ara, G., and Chen, G. 1996.Transforming growth factor-beta in in vivo resistance. Cancer Chemother Pharmacol, 37:601-609.
324. Chen, G., Teicher, B.A., and Frei, III, E., 1998, Biochemical characterization of in vivo alkylating agent resistance of a murine EMT-6 mammary carcinoma. Implication for systemic involvement in the resistance phenotype. Cancer Biochem Biophys, 16:139-155.
325. Teicher, B.A., Ara, G., Keyes, S.R., Herbst, R. S., and Frei, E., III 1998.Acute in vivo resistance in high-dose therapy. Clin Cancer Res, 4:483-491.
326. Chatterjee, D., Liu, C. J., Northey, D., and Teicher, B.A. 1995.Molecular characterization of the in vivo alkylating agent resistant murine EMT-6 mammary carcinoma tumors. Cancer Chemother Pharmacol, 35:423-431.
327. Kobayashi, H., Man, S., Graham, C.H. Kapitain, S. J., Teicher, B.A., and Kerbek, R.S., 1993, Acquired multicellular-mediated resistance to alkylating agents in cancer. Proc Natl Acad Sci, USA, 90:3294-3298.
328. Teicher, B.A., Chatterjee, D., Liu, J.T., Holden, S.A., and Ara, G., 1993, Protection of bone-marrow granulocyte-macrophage colony-forming units in mice bearing in vivo alkylating-agent-resistant EMT-6 tumors. Cancer Chemother Pharmacol, 32:315-319.
329. Xu, Z., Ma, D. Z., Wang, L.Y., Su, J.M., and Zha, X.L., 2003, Transforming growth factor-beta1 stimulated protein kinase B serine-473 and focal adhesion kinase tyrosine phosphorylation dependent on cell adhesion in human hepatocellular carcinoma SMMC-7721 cells. Biochem Biophys Res Commun, 312:388-396.
330. Nakamura, E., Megumi, Y., Kobayashi, T., Kamoto, T., Ishitoya, S., Terachi, T., Tachibana, M., Matsushiro, H., Habuchi, T., Kakehi, Y., and Ogawa, O., 2002, Genetic polymorphisms of the interleukin-4 receptor alpha gene are associated with an increasing risk and a poor prognosis of sporadic renal cell carcinoma in a Japanese population. Clin Cancer Res, 8:2620-2625.
331. De Vita, F., Orditura, M., Auriemma, A., Infusino, S., Roscigno, A., and Catalano, G., 1998, Serum levels of interleukin-6 as a prognostic factor in advanced non-small cell lung cancer. Oncol Rep, 5:649-652.
332. De Vita, F., Romano, C., Orditura, M., Galizia, G., Martinelli, E., Lieto, E., and Catalano, G., 2001, Interleukin-6 serum level correlates with survival in advanced gastrointestinal cancer patients but is not an independent prognostic indicator. J Interferon Cytokine Res, 21:45-52.
333. DeMichele, A., Martin, A.M., Mick, R., Gor, P., Wray, L., Klein-Cabral, M., Athanasiadis, G., Colligan, T., Stadtmauer, E., and Weber, B., 2003, Interleukin-6 -174G-->C polymorphism is associated with improved outcome in high-risk breast cancer. Cancer Res, 63:8051-8056.
334. Drachenberg, D.E., Elgamal, A.A., Rowbotham, R., Peterson, M., and Murphy, G.P., 1999, Circulating levels of interleukin-6 in patients with hormone refractory prostate cancer. Prostate, 41:127-133.
335. Gado, K., Domjan, G., Hegyesi, H., and Falus, A., 2000, Role of INTERLEUKIN-6 in the pathogenesis of multiple myeloma. Cell BiolInt, 24:195-209.
336. Plante, M., Rubin, S.C., Wong, G.Y., Federici, M.G., Finstad, C.L., and Gastl, G.A., 1994, Interleukin-6 level in serum and ascites as a prognostic factor in patients with epithelial ovarian cancer. Cancer, 73:1882-1888.
337. Thiounn, N., Pages, F., Flam, T., Tartour, E., Mosseri, V., Zerbib, M., Beuzeboc, P., Deneux, L., Fridman, W.H., and Debre, B., 1997, IL-6 is a survival prognostic factor in renal cell carcinoma. Immunol Lett, 58:121-124.
338. Yokoe, T., Iino, Y., and Morishita, Y., 2000,Trends of IL-6 and IL-8 levels in patients with recurrent breast cancer: preliminary report. Breast Cancer, 7:187-190.
339. Kozlowski, L., Zakrzewska, I., Tokajuk, P., and Wojtukiewicz, M.Z., 2003, Concentration of interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-10 (IL-10) in blood serum of breast cancer patients. Rocz Akad Med Bialymst, 48:82-84.
340. Lech-Maranda, E., Baseggio, L., Bienvenu, J., Charlot, C., Berger, F., Rigal, D., Warzocha, K., Coiffier, B., and Salles, G., 2003, The interleukin-10 gene promoter polymorphisms influence the clinical outcome of diffuse large B-cell lymphoma, Blood.
341. Hattori, E., Okumoto, K., Adachi, T., Takeda, T., Ito, J., Sugahara, K., Watanabe, H., Saito, K., Saito, T., Togashi, H., and Kawata, S., 2003, Possible

contribution of circulating interleukin-10 (IL-10) to anti-tumor immunity and prognosis in patients with unresectable hepatocellular carcinoma. Hepatol Res, 27:309-314.

342. Salgami, E.V., Efstathiou, S.P., Vlachakis, V., Sekara, E. V., Syrigos, K.N., and Roussou, P.P., 2002, High pretreatment interleukin-10 is an independent predictor of poor failure-free survival in patients with Hodgkin's lymphoma. Haematologia (Budap), 32:377-387.
343. Aydin, F., Yilmaz, M., Ozdemir, F., Kavgaci, H., Yavuz, M.N., and Yavuz, A.A., 2002, Correlation of serum IL-2, IL-6 and IL-10 levels with International Prognostic Index in patients with aggressive non-Hodgkin's lymphoma. Am J Clin Oncol, 25:570-572.
344. Martinez-Escribano, J.A., Moya-Quiles, M.R., Muro, M., Montes-Ares, O., Hernandez-Caselles, T., Frias, J.F., and Alvarez-Lopez, M.R., 2002, Interleukin-10, interleukin-6 and interferon-gamma gene polymorphisms in melanoma patients. Melanoma Res, 12:465-469.
345. Chandler, S.W., Rassekh, C.H., Rodman, S.M., and Ducatman, B.S., 2002, Immunohistochemical localization of interleukin-10 in human oral and pharyngeal carcinomas. Laryngoscope, 112:808-815.
346. Galizia, G., Lieto, E., De Vita, F., Romano, C., Orditura, M., Castellano, P., Imperatore, V., Infusino, S., Catalano, G., and Pignatelli, C., 2002, Circulating levels of interleukin-10 and interleukin-6 in gastric and colon cancer patients before and after surgery: relationship with radicality and outcome. J Interferon Cytokine Res, 22:473-482.
347. Galizia, G., Orditura, M., Romano, C., Lieto, E., Castellano, P., Pelosio, L., Imperatore, V., Catalano, G., Pignatelli, C., and De Vita, F., 2002, Prognostic significance of circulating IL-10 and IL-6 serum levels in colon cancer patients undergoing surgery. Clin Immunol, 102:169-178.
348. Vassilakopoulos, T.P., Nadali, G., Angelopoulou, M.K., Siakantaris, M.P., Dimopoulou, M.N., Kontopidou, F.N., Rassidakis, G.Z., Doussis-Anagnostopoulou, I.A., Hatzioannou, M., Vaiopoulos, G., Kittas, C., Sarris, A.H., Pizzolo, G., and Pangalis, G.A., 2001, Serum interleukin-10 levels are an independent prognostic factor for patients with Hodgkin's lymphoma. Haematologica, 86:274-281.
349. Hatanaka, H., Abe, Y., Kamiya, T., Morino, F., Nagata, J., Tokunaga, T., Oshika, Y., Suemizu, H., Kijima, H., Tsuchida, T., Yamazaki, H., Inoue, H., Nakamura, M., and Ueyama, Y., 2000, Clinical implications of interleukin (IL)-10 induced by non-small-cell lung cancer. Ann Oncol, 11:815-819.
350. De Vita, F., Orditura, M., Galizia, G., Romano, C., Lieto, E., Iodice, P., Tuccillo, C., and Catalano, G., 2000, Serum interleukin-10 is an independent prognostic factor in advanced solid tumors. Oncol Rep, 7:357-361.
351. De Vita, F., Orditura, M., Galizia, G., Romano, C., Roscigno, A., Lieto, E., and Catalano, G., 2000, Serum interleukin-10 levels as a prognostic factor in advanced non-small cell lung cancer patients. Chest, 117:365-373.
352. Sarris, A.H., Kliche, K.O., Pethambaram, P., Preti, A., Tucker, S., Jackow, C., Messina, O., Pugh, W., Hagemeister, F.B., McLaughlin, P., Rodriguez, M.A., Romaguera, J., Fritsche, H., Witzig, T., Duvic, M., Andreeff, M., and Cabanillas, F., 1999, Interleukin-10 levels are often elevated in serum of adults with Hodgkin's disease and are associated with inferior failure-free survival. Ann Oncol, 10:433-440.
353. Cortes, J., and Kurzrock, R., 1997, Interleukin-10 in non-Hodgkin's lymphoma. Leuk.Lymphoma, 26:251-259.
354. Blay, J.Y., Burdin, N., Rousset, F., Lenoir, G., Biron, P., Philip, T., Banchereau, J., and Favrot, M.C., 1993, Serum interleukin-10 in non-Hodgkin's lymphoma: a prognostic factor. Blood, 82:2169-2174.
355. Soria, J.C., Moon, C., Kemp, B.L., Liu, D.D., Feng, L., Tang, X., Chang, Y.S., Mao, L., and Khuri, F.R., 2003, Lack of interleukin-10 expression could predict poor outcome in patients with stage I non-small cell lung cancer. Clin Cancer Res, 9:1785-1791.
356. Stassi, G., Todaro, M., Zerilli, M., Ricci-Vitiani, L., Di Liberto, D., Patti, M., Florena, A., Di Gaudio, F., Di Gesu, G., and De Maria, R., 2003, Thyroid cancer resistance to chemotherapeutic drugs via autocrine production of interleukin-4 and interleukin-10. Cancer Res, 63:6784-6790.
357. Sredni, B., Weil, M., Khomenok, G., Lebenthal, I., Teitz, S., Mardor, Y., Ram, Z., Orenstein, A., Kershenovich, A., Michowiz, S., Cohen, Y.I., Rappaport, Z.H., Freidkin, I., Albeck, M., Longo, D.L., and Kalechman, Y., 2004, Ammonium trichloro(dioxoethylene-o,o')tellurate (AS101) sensitizes tumors to chemotherapy by inhibiting the tumor interleukin 10 autocrine loop. Cancer Res, 64:1843-1852.
358. Sredni, B., Albeck, M., Tichler, T., Shani, A., Shapira, J., Bruderman, I., Catane, R., Kaufman, B., and Kalechman, Y., 1995, Bone marrow-sparing and prevention of alopecia by AS101 in non-small-cell lung cancer patients treated with carboplatin and etoposide. J Clin Oncol, 13:2342-2353.
359. Mizutani, Y., Bonavida, B., Koishihara, Y., Akamatsu, K., Ohsugi, Y., and Yoshida, O., 1995, Sensitization of human renal cell carcinoma cells to cis-diamminedichloroplatinum(II) by anti-interleukin 6 monoclonal antibody or anti-

interleukin 6 receptor monoclonal antibody. Cancer Res, 55:590-596.
360. Borsellino, N., Belldegrun, A., and Bonavida, B., 1995, Endogenous interleukin 6 is a resistance factor for cis-diamminedichloroplatinum and etoposide-mediated cytotoxicity of human prostate carcinoma cell lines. Cancer Res, 55:4633-4639.
361. Honemann, D., Chatterjee, M., Savino, R., Bommert, K., Burger, R., Gramatzki, M., Dorken, B., and Bargou, R.C., 2001, The IL-6 receptor antagonist SANT-7 overcomes bone marrow stromal cell-mediated drug resistance of multiple myeloma cells. Int J Cancer, 93:674-680.
362. Borsellino, N., Bonavida, B., Ciliberto, G., Toniatti, C., Travali, S., and D'Alessandro, N., 1999, Blocking signaling through the Gp130 receptor chain by interleukin-6 and oncostatin M inhibits PC-3 cell growth and sensitizes the tumor cells to etoposide and cisplatin-mediated cytotoxicity. Cancer, 85:134-144.
363. Cavallaro, U. and Christofori, G., 2004, Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. Nat Rev Cancer, 4:118-132.
364. Persad, S. and Dedhar, S., 2003, The role of integrin-linked kinase (ILK) in cancer progression. Cancer Metastasis Rev, 22:375-384.
365. Felding-Habermann, B., 2003, Integrin adhesion receptors in tumor metastasis. Clin Exp Metastasis, 20:203-213.
366. Lozano, E., Betson, M., and Braga, V.M., 2003, Tumor progression: Small GTPases and loss of cell-cell adhesion. Bioessays, 25:452-463.
367. Pupa, S.M., Menard, S., Forti, S., and Tagliabue, E., 2002, New insights into the role of extracellular matrix during tumor onset and progression. J Cell Physiol, 192:259-267.
368. Hirohashi, S. and Kanai, Y., 2003, Cell adhesion system and human cancer morphogenesis. Cancer Sci., 94:575-581.
369. Christofori, G., 2003, Changing neighbours, changing behaviour: cell adhesion molecule-mediated signalling during tumour progression. EMBO J, 22:2318-2323.
370. Hecker, T.P. and Gladson, C.L., 2003, Focal adhesion kinase in cancer. Front Biosci, 8:s705-s714.
371. Conacci-Sorrell, M., Zhurinsky, J., and Ben Ze'ev, A., 2002, The cadherin-catenin adhesion system in signaling and cancer. J Clin Ivest, 109:987-991.
372. Stracke, M.L., Murata, J., Aznavoorian, S., and Liotta, L.A., 1994, The role of the extracellular matrix in tumor cell metastasis. In Vivo, 8:49-58.
373. Mason, M.D., Davies, G., and Jiang, W.G., 2002, Cell adhesion molecules and adhesion abnormalities in prostate cancer. Crit Rev Oncol Hematol, 41:11-28.
374. Cavallaro, U. and Christofori, G., 2001, Cell adhesion in tumor invasion and metastasis: loss of the glue is not enough. Biochim Biophys Acta, 1552:39-45.
375. Maeda, K., Kang, S.M., Sawada, T., Nishiguchi, Y., Yashiro, M., Ogawa, Y., Ohira, M., Ishikawa, T., and Hirakawa-YS, C.K., 2002, Expression of intercellular adhesion molecule-1 and prognosis in colorectal cancer. Oncol Rep, 9:511-514.
376. Hoffmann, R., Franzke, A., Buer, J., Sel, S., Oevermann, K., Duensing, A., Probst, M., Duensing, S., Kirchner, H., Ganser, A., and Atzpodien, J., 1999, Prognostic impact of in vivo soluble cell adhesion molecules in metastatic renal cell carcinoma. Br J, Cancer, 79:1742-1745.
377. Tacyildiz, N., Yavuz, G., Gozdasoglu, S., Unal, E., Ertem, U., Duru, F., Ikinciogullari, A., Babacan, E., Ensari, A., and Okcuoglu-Cavdar, A., 1999, Serum levels and differential expression of intercellular adhesion molecule-1 in childhood leukemia and malignant lymphoma: prognostic importance and relationship with survival. Pediatr Hematol Oncol, 16:149-158.
378. Zhang, G.J. and Adachi, I., 1999, Serum levels of soluble intercellular adhesion molecule-1 and E-selectin in metastatic breast carcinoma: correlations with clinicopathological features and prognosis. Int J Oncol, 14:71-77.
379. Dosquet, C., Coudert, M.C., Lepage, E., Cabane, J., and Richard, F., 1997, Are angiogenic factors, cytokines, and soluble adhesion molecules prognostic factors in patients with renal cell carcinoma? Clin Cancer Res, 3:2451-2458.
380. Terol, M.J., Lopez-Guillermo, A., Bosch, F., Villamor, N., Cid, M.C., Rozman, C., Campo, E., and Montserrat, E., 1998, Expression of the adhesion molecule ICAM-1 in non-Hodgkin's lymphoma: relationship with tumor dissemination and prognostic importance. J Clin Oncol, 16:35-40.
381. Shimizu, Y., Minemura, M., Tsukishiro, T., Kashii, Y., Miyamoto, M., Nishimori, H., Higuchi, K., and Watanabe, A., 1995, Serum concentration of intercellular adhesion molecule-1 in patients with hepatocellular carcinoma is a marker of the disease progression and prognosis. Hepatology, 22:525-531.
382. Ogawa, Y., Hirakawa, K., Nakata, B., Fujihara, T., Sawada, T., Kato, Y., Yoshikawa, K., and Sowa, M., 1998, Expression of intercellular adhesion molecule-1 in invasive breast cancer reflects low growth potential, negative lymph node involvement, and good prognosis. Clin Cancer Res, 4:31-36.
383. Archimbaud, E., Thomas, X., Campos, L., Magaud, J.P., Dore, J.F., and Fiere, D., 1992, Expression of surface adhesion molecules CD54 (ICAM-1) and CD58 (LFA-3) in adult acute leukemia: relationship with initial characteristics and prognosis. Leukemia, 6:265-271.

384. Gonzalez-Moles, M.A., Bravo, M., Ruiz-Avila, I., Esteban, F., Rodriguez-Archilla, A., Gonzalez-Moles, S., and Arias, B., 2003, Adhesion molecule CD44 as a prognostic factor in tongue cancer. AntiCancer Res, 23:5197-5202.
385. Vis, A.N., Noordzij, M.A., Fitoz, K., Wildhagen, M.F., Schroder, F.H., and van der Kwast, T.H., 2000, Prognostic value of cell cycle proteins p27(kip1) and MIB-1, and the cell adhesion protein CD44s in surgically treated patients with prostate cancer. J Urol, 164:2156-2161.
386. Horst, E., Meijer, C.J., Radaszkiewicz, T., Ossekoppele, G.J., Van Krieken, J.H., and Pals, S.T., 1990, Adhesion molecules in the prognosis of diffuse large-cell lymphoma: expression of a lymphocyte homing receptor (CD44), LFA-1 (CD11a/18), and ICAM-1 (CD54). Leukemia, 4:595-599.
387. Hage, R., Elbers, H.R., Brutel, d.l.R., and van den Bosch, J.M., 1998, Neural cell adhesion molecule expression: prognosis in 889 patients with resected non-small cell lung cancer. Chest, 114:1316-1320.
388. Poley, S., Stieber, P., Nussler, V., Pahl, H., and Fateh-Moghadam, A., 1997, Evaluation of serum neural cell adhesion molecule as a prognostic marker in multiple myeloma. AntiCancer Res, 17:3021-3024.
389. Pujol, J.L., Simony, J., Demoly, P., Charpentier, R., Laurent, J.C., Daures, J.P., Lehmann, M., Guyot, V., Godard, P., and Michel, F.B., 1993, Neural cell adhesion molecule and prognosis of surgically resected lung cancer. Am Rev Respir Dis, 148:1071-1075.
390. Kibbelaar, R.E., Moolenaar, K.E., Michalides, R.J., Van Bodegom, P.C., Vanderschueren, R.G., Wagenaar, S.S., Dingemans, K.P., Bitter-Suermann, D., Dalesio, O., and Van Zandwijk, N., 1991, Neural cell adhesion molecule expression, neuroendocrine differentiation and prognosis in lung carcinoma. Eur J Cancer, 27:431-435.
391. Bremnes, R.M., Veve, R., Hirsch, F.R., and Franklin, W.A., 2002, The E-cadherin cell-cell adhesion complex and lung cancer invasion, metastasis, and prognosis. Lung Cancer, 36:115-124.
392. Kuniyasu, H., Ellis, L.M., Evans, D.B., Abbruzzese, J.L., Fenoglio, C.J., Bucana, C.D., Cleary, K.R., Tahara, E., and Fidler, I.J., 1999, Relative expression of E-cadherin and type IV collagenase genes predicts disease outcome in patients with resectable pancreatic carcinoma. Clin Cancer Res, 5:25-33.
393. Stefansson, I.M., Salvesen, H.B., and Akslen, L.A., 2004, Prognostic impact of alterations in P-cadherin expression and related cell adhesion markers in endometrial cancer. J Clin Oncol, 22:1242-1252.
394. Oshita, F., Kameda, Y., Ikehara, M., Tanaka, G., Yamada, K., Nomura, I., Noda, K., Shotsu, A., Fujita, A., Arai, H., Ito, H., Nakayama, H., and Mitsuda, A., 2002, Increased expression of integrin beta1 is a poor prognostic factor in small-cell lung cancer. AntiCancer Res, 22:1065-1070.
395. Vihinen, P., Nikkola, J., Vlaykova, T., Hahka-Kemppinen, M., Talve, L., Heino, J., and Pyrhonen, S., 2000, Prognostic value of beta1 integrin expression in metastatic melanoma. Melanoma Res, 10:243-251.
396. Ohira, T., Akutagawa, S., Usuda, J., Nakamura, T., Hirano, T., Tsuboi, M., Nishio, K., Taguchi, F., Ikeda, N., Nakamura, H., Konaka, C., Saijo, N., and Kato, H., 2002, Up-regulated gene expression of angiogenesis factors in post-chemotherapeutic lung cancer tissues determined by cDNA macroarray. Oncol Rep, 9:723-728.
397. Turner, J.R., Torres, C.M., Wang, H.H., Shahsafaei, A., Richards, W.G., Sugarbaker, D., and Odze, R.D., 2000, Preoperative chemoradiotherapy alters the expression and prognostic significance of adhesion molecules in Barrett's-associated adenocarcinoma. Hum Pathol, 31:347-353.
398. Kraus, A.C., Ferber, I., Bachmann, S.O., Specht, H., Wimmel, A., Gross, M.W., Schlegel, J., Suske, G., and Schuermann, M., 2002, In vitro chemo- and radio-resistance in small cell lung cancer correlates with cell adhesion and constitutive activation of AKT and MAP kinase pathways. Oncogene, 21:8683-8695.
399. Rintoul, R.C. and Sethi, T., 2002, Extracellular matrix regulation of drug resistance in small-cell lung cancer. Clin.Sci (Lond), 102:417-424.
400. Liang, Y., Meleady, P., Cleary, I., McDonnell, S., Connolly, L., and Clynes, M., 2001, Selection with melphalan or paclitaxel (Taxol) yields variants with different patterns of multidrug resistance, integrin expression and in vitro invasiveness. Eur J Cancer, 37:1041-1052.
401. Sherman-Baust, C.A., Weeraratna, A.T., Rangel, L.B., Pizer, E.S., Cho, K.R., Schwartz, D.R., Shock, T., and Morin, P.J., 2003, Remodeling of the extracellular matrix through overexpression of collagen VI contributes to cisplatin resistance in ovarian cancer cells. Cancer Cell, 3:377-386.
402. Miyamoto, H., Murakami, T., Tsuchida, K., Sugino, H., Miyake, H., and Tashiro, S., 2004, Tumor-stroma interaction of human pancreatic cancer: acquired resistance to anticancer drugs and proliferation regulation is dependent on extracellular matrix proteins. Pancreas, 28:38-44.
403. Cordes, N., Blaese, M.A., Plasswilm, L., Rodemann, H.P., and Van Beuningen, D., 2003, Fibronectin and laminin increase resistance to ionizing radiation and the cytotoxic drug Ukrain in human tumour and normal cells in vitro. Int J Radiat Biol, 79:709-720.
404. Vincent, T., Molina, L., Espert, L., and Mechti, N., 2003, Hyaluronan, a major non-protein

glycosaminoglycan component of the extracellular matrix in human bone marrow, mediates dexamethasone resistance in multiple myeloma. Br J, Haematol, 121:259-269.
405. de la Fuente, M.T., Casanova, B., Moyano, J. V., Garcia-Gila, M., Sanz, L., Garcia-Marco, J., Silva, A., and Garcia-Pardo, A., 2002, Engagement of alpha4beta1 integrin by fibronectin induces in vitro resistance of B chronic lymphocytic leukemia cells to fludarabine. J Leukoc Biol, 71:495-502.
406. Damiano, J.S., Hazlehurst, L.A., and Dalton, W.S., 2001, Cell adhesion-mediated drug resistance (CAM-DR) protects the K562 chronic myelogenous leukemia cell line from apoptosis induced by BCR/ABL inhibition, cytotoxic drugs, and gamma-irradiation. Leukemia, 15:1232-1239.
407. Elliott, T. and Sethi, T., 2002, Integrins and extracellular matrix: a novel mechanism of multidrug resistance. Expert Rev Anticancer Ther, 2:449-459.
408. Shain, K.H., and Dalton, W.S., 2001, Cell adhesion is a key determinant in de novo multidrug resistance (MDR): new targets for the prevention of acquired MDR. Mol Cancer Ther, 1:69-78.
409. Hazlehurst, L.A., and Dalton, W.S., 2001, Mechanisms associated with cell adhesion mediated drug resistance (CAM-DR) in hematopoietic malignancies. Cancer Metastasis Rev, 20:43-50.
410. Shain, K.H., Landowski, T.H., and Dalton, W.S., 2000, The tumor microenvironment as a determinant of cancer cell survival: a possible mechanism for de novo drug resistance. Curr Opin Oncol, 12:557-563.
411. Hazlehurst, L.A., Landowski, T.H., and Dalton, W.S., 2003, Role of the tumor microenvironment in mediating de novo resistance to drugs and physiological mediators of cell death. Oncogene, 22:7396-7402.
412. Dalton, W.S., 1999, The tumor microenvironment as a determinant of drug response and resistance. Drug Resist Updat, 2:285-288.
413. Nefedova, Y., Landowski, T.H., and Dalton, W.S., 2003, Bone marrow stromal-derived soluble factors and direct cell contact contribute to de novo drug resistance of myeloma cells by distinct mechanisms. Leukemia, 17:1175-1182.
414. Morin, P.J., 2003, Drug resistance and the microenvironment: nature and nurture. Drug Resist Updat, 6:169-172.
415. Green, S.K., Francia, G., Isidoro, C., and Kerbek, R.S., 2004, Antiadhesive antibodies targeting E-cadherin sensitize multicellular tumor spheroids to chemotherapy in vitro. Mol Cancer Ther, 3:149-159.
416. Gray, L.H., Conger, A.D., Ebert, M., Hornsey, S., and Scott, O.C., 1953, The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. Br J, Radiol, 26:638-648.
417. Crabtree, H.G. and Cramer, W., 1933, The action of radium on cancer cells I. II. Some factors determining the susceptibility of cancer cells to radium. Proc R Soc Ser B, 113:238-250.
418. Harris, A. L., 2002, Hypoxia--a key regulatory factor in tumour growth. Nat Rev Cancer, 2:38-47.
419. Hockel, M., and Vaupel, P., 2001, Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst, 93:266-276.
420. Vaupel, P., Thews, O., and Hoeckel, M., 2001, Treatment resistance of solid tumors: role of hypoxia and anemia. Med.Oncol, 18:243-259.
421. Evans, S.M., Judy, K.D., Dunphy, I., Jenkins, W.T., Nelson, P.T., Collins, R., Wileyto, E.P., Jenkins, K., Hahn, S.M., Stevens, C.W., Judkins, A.R., Phillips, P., Geoerger, B., and Koch, C.J., 2004, Comparative measurements of hypoxia in human brain tumors using needle electrodes and EF5 binding. Cancer Res, 64:1886-1892.
422. Choi, N., Baumann, M., Flentjie, M., Kellokumpu-Lehtinen, P., Senan, S., Zamboglou, N., and Kosmidis, P., 2001, Predictive factors in radiotherapy for non-small cell lung cancer: present status. Lung Cancer, 31:43-56.
423. Saunders, M.I., 1994, Predictive testing of radiosensitivity in non-small cell carcinoma of the lung. Lung Cancer, 10Suppl 1:S83-S90.
424. Swinson, D.E., Jones, J.L., Richardson, D., Cox, G., Edwards, J.G., and O'Byrne, K.J., 2002, Tumour necrosis is an independent prognostic marker in non-small cell lung cancer: correlation with biological variables. Lung Cancer, 37:235-240.
425. Cruickshank, G.S. and Rampling, R., 1994, Does tumour related oedema contribute to the hypoxic fraction of human brain tumours? Acta Neurochir.Suppl (Wien.), 60:378-380.
426. Raza, S.M., Lang, F.F., Aggarwal, B.B., Fuller, G.N., Wildrick, D.M., and Sawaya, R., 2002, Necrosis and glioblastoma: a friend or a foe? A review and a hypothesis. Neurosurgery, 51:2-12.
427. Spence, A.M., Muzi, M., and Krohn, K.A., 2002, Molecular imaging of regional brain tumor biology. J Cell Biochem.Suppl, 39:25-35.
428. Brown, J.M., 1999, The hypoxic cell: a target for selective cancer therapy--eighteenth Bruce F. Cain Memorial Award lecture. Cancer Res, 59:5863-5870.
429. Pugh, C.W., and Ratcliffe, P.J., 2003, Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med, 9:677-684.
430. Pugh, C.W. and Ratcliffe, P.J., 2003, The von Hippel-Lindau tumor suppressor, hypoxia-inducible factor-1 (HIF-1) degradation, and cancer pathogenesis. Semin Cancer Biol, 13:83-89.
431. Semenza, G.L., Agani, F., Feldser, D., Iyer, N., Kotch, L., Laughner, E., and Yu, A. 2000.Hypoxia,

HIF-1, and the pathophysiology of common human diseases. Adv Exp Med Biol, 475:123-130.
432. Semenza, G.L., 2000, HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol, 88:1474-1480.
433. Maxwell, P. H., Pugh, C.W., and Ratcliffe, P.J., 2001, Activation of the HIF pathway in cancer. Curr Opin Genet Dev, 11:293-299.
434. Royds, J.A., Dower, S.K., Qwarnstrom, E.E., and Lewis, C.E., 1998, Response of tumour cells to hypoxia: role of p53 and NFkB. Mol Pathol, 51:55-61.
435. Kao, K.S., Xanthoudakis, S., Curran, T., and O'Dwyer, P.J., 1994, Activation of AP-1 and of a nuclear redox factor, Ref-1, in the response of HT29 colon cancer cells to hypoxia. Mol Cell Biol, 14:5997-6003.
436. Minet, E., Michel, G., Mottet, D., Piret, J.P., Barbieux, A., Raes, M., and Michiels, C., 2001, c-JUN gene induction and AP-1 activity is regulated by a JNK-dependent pathway in hypoxic HepG2 cells. Exp Cell Res, 265:114-124.
437. Flaherty, D.M., Monick, M.M., and Hunninghake, G.W., 2001, AP endonucleases and the many functions of Ref-1. Am J Respir Cell Mol Biol, 25:664-667.
438. Hall, J.L., Wang, X., Van, A., Zhao, Y., and Gibbons, G.H., 2001, Overexpression of Ref-1 inhibits hypoxia and tumor necrosis factor-induced endothelial cell apoptosis through nuclear factor-kappab-independent and -dependent pathways. Circ Res, 88:1247-1253.
439. Wang, N. and Stemerman, M.B., 2001, Ref-1 and transcriptional control of endothelial apoptosis. Circ Res, 88:1223-1225.
440. Kelley, M.R., Cheng, L., Foster, R., Tritt, R., Jiang, J., Broshears, J., and Koch, M., 2001, Elevated and altered expression of the multifunctional DNA base excision repair and redox enzyme Ape1/ref-1 in prostate cancer. Clin Cancer Res, 7:824-830.
441. Wang, G., Reisdorph, R., Clark, Jr., R.E., Miskimins, R., Lindahl, R., and Miskimins, W.K., 2003, Cyclin dependent kinase inhibitor p27(Kip1) is upregulated by hypoxia via an ARNT dependent pathway. J Cell Biochem, 90:548-560.
442. Goda, N., Ryan, H.E., Khadivi, B., McNulty, W., Rickert, R.C., and Johnson, R.S., 2003, Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia. Mol Cell Biol, 23:359-369.
443. Fujii, T., Otsuki, T., Moriya, T., Sakaguchi, H., Kurebayashi, J., Yata, K., Uno, M., Kobayashi, T., Kimura, T., Jo, Y., Kinugawa, K., Furukawa, Y., Morioka, M., Ueki, A., and Tanaka, H., 2002, Effect of hypoxia on human seminoma cells. Int J Oncol, 20:955-962.
444. Krtolica, A., Krucher, N.A., and Ludlow, J.W., 1999, Molecular analysis of selected cell cycle regulatory proteins during aerobic and hypoxic maintenance of human ovarian carcinoma cells. Br J, Cancer, 80:1875-1883.
445. Krtolica, A., Krucher, N.A., and Ludlow, J.W., 1998, Hypoxia-induced pRB hypophosphorylation results from downregulation of CDK and upregulation of PP1 activities. Oncogene, 17:2295-2304.
446. Carmeliet, P., Dor, Y., Herbert, J.M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., Koch, C.J., Ratcliffe, P., Moons, L., Jain, R.K., Collen, D., Keshert, E., and Keshet, E., 1998, Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature, 394:485-490.
447. Sowter, H.M., Ratcliffe, P.J., Watson, P., Greenberg, A.H., and Harris, A.L., 2001, HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. Cancer Res, 61:6669-6673.
448. Bruick, R.K., 2000, Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. Proc Natl Acad Sci, USA, 97:9082-9087.
449. Sowter, H.M., Ferguson, M., Pym, C., Watson, P., Fox, S.B., Han, C., and Harris, A.L., 2003, Expression of the cell death genes BNip3 and NIX in ductal carcinoma in situ of the breast; correlation of BNip3 levels with necrosis and grade. J Pathol, 201:573-580.
450. Kothari, S., Cizeau, J., McMillan-Ward, E., Israels, S.J., Bailes, M., Ens, K., Kirshenbaum, L.A., and Gibson, S.B., 2003, BNIP3 plays a role in hypoxic cell death in human epithelial cells that is inhibited by growth factors EGF and IGF. Oncogene, 22:4734-4744.
451. Regula, K.M., Ens, K., and Kirshenbaum, L.A., 2002, Inducible expression of BNIP3 provokes mitochondrial defects and hypoxia-mediated cell death of ventricular myocytes. Circ Res, 91:226-231.
452. Guo, K., Searfoss, G., Krolikowski, D., Pagnoni, M., Franks, C., Clark, K., Yu, K.T., Jaye, M., and Ivashchenko, Y., 2001, Hypoxia induces the expression of the pro-apoptotic gene BNIP3. Cell Death Differ, 8:367-376.
453. Park, S.Y., Billiar, T.R., and Seol, D.W., 2002, Hypoxia inhibition of apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Biochem Biophys Res Commun, 291:150-153.
454. Erler, J.T., Cawthorne, C.J., Williams, K.J., Koritzinsky, M., Wouters, B.G., Wilson, C., Miller, C., Demonacos, C., Stratford, I.J., and Dive, C., 2004, Hypoxia-mediated down-regulation of Bid and Bax in tumors occurs via hypoxia-inducible factor 1-dependent and -independent mechanisms

and contributes to drug resistance. Mol Cell Biol, 24:2875-2889.

455. Wartenberg, M., Ling, F.C., Muschen, M., Klein, F., Acker, H., Gassmann, M., Petrat, K., Putz, V., Hescheler, J., and Sauer, H., 2003, Regulation of the multidrug resistance transporter P-glycoprotein in multicellular tumor spheroids by hypoxia-inducible factor (HIF-1) and reactive oxygen species. FASEB J, 17:503-505.
456. Comerford, K.M., Wallace, T.J., Karhausen, J., Louis, N.A., Montalto, M.C., and Colgan, S.P., 2002, Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. Cancer Res, 62:3387-3394.
457. Liang, B.C., 1996, Effects of hypoxia on drug resistance phenotype and genotype in human glioma cell lines. J Neurooncol, 29:149-155.
458. Sakata, K., Kwok, T.T., Murphy, B.J., Laderoute, K.R., Gordon, G.R., and Sutherland, R.M., 1991, Hypoxia-induced drug resistance: comparison to P-glycoprotein-associated drug resistance. Br J, Cancer, 64:809-814.
459. Krishnamurthy, P., Ross, D.D., Nakanishi, T., Bailey-Dell, K., Zhou, S., Mercer, K.E., Sarkadi, B., Sorrentino, B.P., and Schuetz, J.D., 2004, The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. J Biol Chem, 279:24218-24225.
460. Brinkmann, U., 2002, Functional polymorphisms of the human multidrug resistance (MDR1) gene: correlation with P glycoprotein expression and activity in vivo. Novartis Found Symp, 243:207-210.
461. Doyle, L.A. and Ross, D.D., 2003, Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). Oncogene, 22:7340-7358.
462. Germann, U.A., 1996, P-glycoprotein--a mediator of multidrug resistance in tumour cells. Eur J Cancer, 32A:927-944.
463. Gottesman, M.M., Pastan, I., and Ambudkar, S.V., 1996, P-glycoprotein and multidrug resistance. Curr Opin Genet Dev, 6:610-617.
464. Mazure, N.M., Brahimi-Horn, M.C., and Pouyssegur, J., 2003, Protein kinases and the hypoxia-inducible factor-1, two switches in angiogenesis. Curr Pharm Des, 9:531-541.
465. Choi, K.S., Bae, M.K., Jeong, J.W., Moon, H.E., and Kim, K.W., 2003, Hypoxia-induced angiogenesis during carcinogenesis. J Biochem Mol Biol, 36:120-127.
466. Berra, E., Pages, G., and Pouyssegur, J., 2000, MAP kinases and hypoxia in the control of VEGF expression. Cancer Metastasis Rev, 19:139-145.
467. Levy, A.P., Levy, N.S., Iliopoulos, O., Jiang, C., Kaplin, Jr., W.G., and Goldberg, M.A., 1997, Regulation of vascular endothelial growth factor by hypoxia and its modulation by the von Hippel-Lindau tumor suppressor gene. Kidney Int, 51:575-578.
468. Ishibashi, H., Shiratuchi, T., Nakagawa, K., Onimaru, M., Sugiura, T., Sueishi, K., and Shirasuna, K., 2001, Hypoxia-induced angiogenesis of cultured human salivary gland carcinoma cells enhances vascular endothelial growth factor production and basic fibroblast growth factor release. Oral Oncol, 37:77-83.
469. Baek, J.H., Jang, J.E., Kang, C.M., Chung, H.Y., Kim, N.D., and Kim, K.W., 2000, Hypoxia-induced VEGF enhances tumor survivability via suppression of serum deprivation-induced apoptosis. Oncogene, 19:4621-4631.
470. Brogi, E., Schatteman, G., Wu, T., Kim, E.A., Varticovski, L., Keyt, B., and Isner, J.M., 1996, Hypoxia-induced paracrine regulation of vascular endothelial growth factor receptor expression. J Clin Ivest, 97:469-476.
471. Suzuki, H., Seto, K., Shinoda, Y., Mori, M., Ishimura, Y., Suematsu, M., and Ishii, H, 1999, Paracrine upregulation of VEGF receptor mRNA in endothelial cells by hypoxia-exposed hep G2 cells. Am.J Physiol, 276:G92-G97.
472. Le, Y.J. and Corry, P.M., 1999, Hypoxia-induced bFGF gene expression is mediated through the JNK signal transduction pathway. Mol Cell Biochem, 202:1-8.
473. Scarpino, S., Cancellario, d.F., Di Napoli, A., Pasquini, A., Marzullo, A., and Ruco, L.P., 2004, Increased expression of Met protein is associated with up-regulation of hypoxia inducible factor-1 (HIF-1) in tumour cells in papillary carcinoma of the thyroid. J Pathol, 202:352-358.
474. Pennacchietti, S., Michieli, P., Galluzzo, M., Mazzone, M., Giordano, S., and Comoglio, P.M., 2003, Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell, 3:347-361.
475. Bae, S.K., Bae, M.H., Ahn, M.Y., Son, M.J., Lee, Y.M., Bae, M.K., Lee, O.H., Park, B.C., and Kim, K.W., 1999, Egr-1 mediates transcriptional activation of IGF-II gene in response to hypoxia. Cancer Res, 59:5989-5994.
476. Kim, K.W., Bae, S.K., Lee, O.H., Bae, M.H., Lee, M.J., and Park, B.C., 1998, Insulin-like growth factor II induced by hypoxia may contribute to angiogenesis of human hepatocellular carcinoma. Cancer Res, 58:348-351.
477. Sugawara, J., Tazuke, S.I., Suen, L.F., Powell, D.R., Kaper, F., Giaccia, A.J., and Giudice, L.C., 2000, Regulation of insulin-like growth factor-binding protein 1 by hypoxia and 3',5'-cyclic adenosine monophosphate is additive in HepG2 cells. J Clin Endocrinol Metab, 85:3821-3827.
478. Sugawara, J., Suh, D.S., Faessen, G.H., Suen, L.F., Shibata, T., Kaper, F., Giaccia, A.J., and Giudice,

L.C., 2000, Regulation of insulin-like growth factor-binding protein-1 by nitric oxide under hypoxic conditions. J Clin Endocrinol Metab, 85:2714-2721.

479. Patel, B., Khaliq, A., Jarvis-Evans, J., McLeod, D., Mackness, M., and Boulton, M., 1994, Oxygen regulation of TGF-beta 1 mRNA in human hepatoma (Hep G2) cells. Biochem Mol BiolInt, 34:639-644.
480. Gunaratnam, L., Morley, M., Franovic, A., de Paulsen, N., Mekhail, K., Parolin, D.A., Nakamura, E., Lorimer, I.A., and Lee, S., 2003, Hypoxia inducible factor activates the transforming growth factor-alpha/epidermal growth factor receptor growth stimulatory pathway in VHL(-/-) renal cell carcinoma cells. J Biol Chem, 278:44966-44974.
481. Cooper, A.L. and Beasley, D., 1999, Hypoxia stimulates proliferation and interleukin-1alpha production in human vascular smooth muscle cells. Am J Physiol, 277:H1326-H1337.
482. Hagberg, H., Gilland, E., Bona, E., Hanson, L.A., Hahin-Zoric, M., Blennow, M., Holst, M., McRae, A., and Soder, O., 1996, Enhanced expression of interleukin (IL)-1 and IL-6 messenger RNA and bioactive protein after hypoxia-ischemia in neonatal rats. Pediatr Res, 40:603-609.
483. Liu, M.T., Huang, H.M., Jeng, K.C., Ou, S.C., and Kuo, J.S., 2000, Induction of cytokine genes and IL-1alpha by chemical hypoxia in PC12 cells. Life Sci, 67:2147-2157.
484. Maeda, Y., Matsumoto, M., Hori, O., Kuwabara, K., Ogawa, S., Yan, S.D., Ohtsuki, T., Kinoshita, T., Kamada, T., and Stern, D.M., 1994, Hypoxia/reoxygenation-mediated induction of astrocyte interleukin 6: a paracrine mechanism potentially enhancing neuron survival. J Exp Med, 180:2297-2308.
485. Matsui, H., Ihara, Y., Fujio, Y., Kunisada, K., Akira, S., Kishimoto, T., and Yamauchi-Takihara, K., 1999, Induction of interleukin (IL)-6 by hypoxia is mediated by nuclear factor (NF)-kappa B and NF-IL6 in cardiac myocytes. Cardiovasc Res, 42:104-112.
486. Muraoka, K., Shimizu, K., Sun, X., Zhang, Y.K., Tani, T., Hashimoto, T., Yagi, M., Miyazaki, I., and Yamamoto, K., 1997, Hypoxia, but not reoxygenation, induces interleukin 6 gene expression through NF-kappa B activation. Transplantation, 63:466-470.
487. Grutkoski, P.S., Graeber, C.T., D'Amico, R., Keeping, H., and Simms, H.H., 1999, Regulation of IL-8RA (CXCR1) expression in polymorphonuclear leukocytes by hypoxia/reoxygenation. J Leukoc Biol, 65:171-178.
488. Shi, Q., Abbruzzese, J.L., Huang, S., Fidler, I.J., Xiong, Q., and Xie, K., 1999, Constitutive and inducible interleukin 8 expression by hypoxia and acidosis renders human pancreatic cancer cells more tumorigenic and metastatic. Clin Cancer Res, 5:3711-3721.
489. Xu, L., Xie, K., Mukaida, N., Matsushima, K., and Fidler, I.J., 1999, Hypoxia-induced elevation in interleukin-8 expression by human ovarian carcinoma cells. Cancer Res, 59:5822-5829.
490. Yoshino, O., Osuga, Y., Koga, K., Hirota, Y., Yano, T., Tsutsumi, O., Fujimoto, A., Kugu, K., Momoeda, M., Fujiwara, T., and Taketani, Y., 2003, Upregulation of interleukin-8 by hypoxia in human ovaries. Am J Reprod Immunol, 50:286-290.
491. Reynolds, T.Y., Rockwell, S., and Glazer, P.M., 1996, Genetic instability induced by the tumor microenvironment. Cancer Res, 56:5754-5757.
492. Mihaylova, V.T., Bindra, R.S., Yuan, J., Campisi, D., Narayanan, L., Jensen, R., Giordano, F., Johnson, R.S., Rockwell, S., and Glazer, P.M., 2003, Decreased expression of the DNA mismatch repair gene Mlh1 under hypoxic stress in mammalian cells. Mol Cell Biol, 23:3265-3273.
493. Kim, C.Y., Tsai, M.H., Osmanian, C., Graeber, T.G., Lee, J.E., Giffard, R.G., DiPaolo, J.A., Peehl, D.M., and Giaccia, A.J., 1997, Selection of human cervical epithelial cells that possess reduced apoptotic potential to low-oxygen conditions. Cancer Res, 57:4200-4204.
494. Graeber, T.G., Osmanian, C., Jacks, T., Housman, D.E., Koch, C.J., Lowe, S.W., and Giaccia, A.J., 1996, Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature, 379:88-91.
495. von Pawel, J., von Roemeling, R., Gatzemeier, U., Boyer, M., Elisson, L.O., Clark, P., Talbot, D., Rey, A., Butler, T.W., Hirsh, V., Olver, I., Bergman, B., Ayoub, J., Richardson, G., Dunlop, D., Arcenas, A., Vescio, R., Viallet, J., and Treat, J., 2000, Tirapazamine plus cisplatin versus cisplatin in advanced non-small-cell lung cancer: A report of the international CATAPULT I study group. Cisplatin and Tirapazamine in Subjects with Advanced Previously Untreated Non-Small-Cell Lung Tumors. J Clin Oncol, 18:1351-1359.
496. Peters, K.B., and Brown, J.M., 2002, Tirapazamine: a hypoxia-activated topoisomerase II poison. Cancer Res, 62:5248-5253.

Chapter 18

Integrin-Mediated Resistance to Chemotherapy-Induced Apoptosis in Cancer Cells

R.C. Rintoul and T. Sethi
Department of Respiratory Medicine, Royal Infirmary of Edinburgh, Edinburgh, UK

Abstract: Tumour recurrence following chemotherapy remains a major obstacle to successful cancer treatment limiting both survival times and cure rates. The problem of residual disease has given rise to the hypothesis that some chemotherapy-naïve tumour cells are able to evade the initial pro-apoptotic effects of chemotherapy. The identification of drug resistance mechanisms offers a potential strategy for improving the efficacy of cancer chemotherapy. Until recently investigation of drug resistance has predominantly focussed on mechanisms selected for by chronic drug exposure. However, despite the identification of several mechanisms, no unifying hypothesis has been described to explain how these mechanisms interact to allow a tumour cell to evade death induced by early exposure to chemotherapeutic agents. Recently, several groups have described an integrin-mediated mechanism of drug resistance to chemotherapy. In this chapter we review the work that has been undertaken to elucidate the mechanisms underlying this phenomenon. The identification of signalling pathways underlying integrin-mediated drug resistance offers the possibility of designing novel therapeutic agents that could be administered in conjunction with conventional chemotherapy in order to augment chemosensitivity.

Key words: Cancer, chemotherapy, drug resistance, integrins, extracellular matrix, signal transduction, tumors/turmours, myltiple myelona, glioma, leukaemia,/leukemia

1. INTRODUCTION

National Cancer Institute statistics indicate that there will be over 1.25 million new cases of cancer in the US this year and an estimated 550,000 Americans will die from cancer (1). Despite reductions in age-adjusted rates of cancer death, the total number of recorded cancer deaths in the US continues to rise due to an aging and expanding population.

For patients with haematological or metastatic tumours the mainstay of treatment is chemotherapy and radiotherapy. Despite improvements to chemotherapy and radiotherapy regimens the problem of residual disease and tumour relapse following treatment remains a major obstacle to successful therapy. A prime example of this occurs in small cell lung cancer, a particularly aggressive form of lung cancer characterised by the development of early and widespread metastases. Despite initial response rates of 80-100% with combination chemotherapy in limited disease, relapse often occurs after only 6-8 months (2). Following disease recurrence, median survival is about 4 months. Survival beyond five years occurs in only 3 to 8% of patients. Unfortunately, despite multiple trials examining new chemotherapy and radiotherapy regimens this figure has remained virtually unchanged since the advent of chemotherapy for this disease in 1969 (3).

The recrudescence of disease in patients with small cell lung cancer as well as those with haematological malignancies, germ cell tumours, ovarian and breast cancers, has given rise to the hypothesis that some chemotherapy-naïve tumour cells are able to evade the initial pro-apoptotic effects of chemotherapy. It is generally considered that drug resistant cells arise from genetic changes

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 323-333.

that occur either spontaneously because of inherent genetic instability or as a result of chemotherapy-induced genetic damage. Over the last quarter of a century much effort has been directed at identifying and targeting drug-resistance mechanisms as a strategy for improving the efficacy of cancer chemotherapy.

In this chapter we will review some of the recent work that has been done on integrin-mediated drug resistance, a form of drug resistance that may explain the ability of tumour cells to evade the effects of cytotoxic agents.

2. DRUG RESISTANCE MECHANISMS

Historically, much effort has focussed on identifying mechanisms of acquired drug resistance. Classically, investigations of drug resistance have focussed on the single cell by selecting for drug resistant cells following exposure to cytotoxic agents. Such studies have identified mechanisms that:

a) Reduce intracellular drug concentration as a result of increased efflux through drug pumps e.g. P-glycoprotein (PgP/MDR-1) (4), multidrug resistance associated proteins (MRP 2-7) (5, 6), breast cancer resistance protein (7) and the lung cancer resistance protein drug pumps (8).
b) Promote up regulation of drug detoxification enzymes such as the glutathione-S-transferases (9, 10).
c) Alter the intracellular drug target (alterations in topoisomerase II) (11).

Although these mechanisms have been shown to have profound effects on tumour cell survival *in vitro*, their clinical importance is still unclear (12, 13). To date, it has not been possible to translate the *in vitro* results into any significant clinical benefit. Furthermore, no unifying hypothesis has been described to explain how these mechanisms interact to allow a tumour cell to evade death induced by early exposure to chemotherapeutic agents. It is significant that acquired drug resistance mechanisms are often absent in the unselected tumour cell (14). Factors that allow cell survival following acute cytotoxic drug exposure may differ from mechanisms selected for by chronic drug exposure. Moreover, the unicellular model, which has often been used to identify acquired drug resistance mechanisms, does not take into consideration host-tumour interactions that may modulate the development of the *de novo* drug-resistant phenotype.

3. THE TUMOUR MICROENVIRONMENT

Two aspects of the tumour microenvironment which are perceived as being particularly important in modulating the host-tumour cell relationship are a) stromal factors and b) cell-cell interactions.

a) Stromal factors

Teicher et al., (1990) observed that by treating mice bearing mammary tumours with alkylating agents over a six month period, a drug resistant tumour phenotype was selected (15). However, it was not possible to replicate this model using a unicellular tissue culture system. This suggested that there was some factor(s) in the *in vivo* microenvironment which was important in mediating the expression of the drug resistant phenotype. Subsequent work has identified a number of soluble factors in the extracellular milieu that can have marked effects on tumour chemoresistance. Haemopoietic cytokines such as granulocyte macrophage colony stimulating factor, granulocyte colony stimulating factor and interleukin-6 (16), vascular endothelial growth factor (17), nitric oxide (18) and transforming growth factor beta (19) have all been implicated in negatively regulating drug response.

b) Cell-cell interactions

In vivo, cells exist within a three-dimensional conformation unlike the two-dimensional in vitro monolayer culture system often used for unicellular studies. In an attempt to replicate the in vivo situation, the spheroid culture model was developed. Spheroid growth can be achieved by culturing cells within a continually rotating spinner flask or by coating a standard tissue culture flask with agar or polyHEMA (20). This model is thought to more closely mimic the architecture of a solid tumour with the formation of cell-cell junctions and intracellular signalling cascades activated by a variety of adhesion molecules including integrins, cadherins, selectins, and members of the immunoglobulin superfamily. Paradoxically, however, this degree of complexity limits the utility of such a model. Although a number of studies have shown that cells within this environment are conferred a survival advantage against cytotoxic insult (21, 22), it is difficult to dissect the signal transduction pathways

contributing to drug resistance without the ability to investigate the role of specific adhesion receptors. Therefore, several groups developed models examining the interaction between cancer cells and individual components of the extracellular environment.

4. INTEGRIN MEDIATED DRUG RESISTANCE (TABLE 1)

Our group developed a model to examine the interaction between SCLC cells and extracellular matrix (ECM) proteins (23). This was based on the observation that SCLC cells, *in vivo*, are surrounded by an extensive stroma of extracellular matrix at both primary and metastatic sites, which contains large amounts of fibronectin, laminin and collagen IV (Figure 1). Adhesion studies revealed that the main cell surface receptors regulating cell adhesion to ECM proteins are members of the integrin family. Integrin receptors are heterodimers composed of one α and one β subunit. To date, 18 α and 8 β subunits have been identified and comprise 22 distinct receptors (24). While the extracellular binding specificity of an integrin is generated jointly by both subunits, they display varying degrees of specificity for both ligand and cell type. For instance, fibronectin mediates cell adhesion through integrins α3β1, α5β1 and αvβ1. Collagen and laminin bind predominantly via α2β1, α3β1 and α6β1 integrins. The main β integrin expressed by SCLC cells is β1 and the principle α integrins expressed are α2, α3, α6 and αv (25, 26).

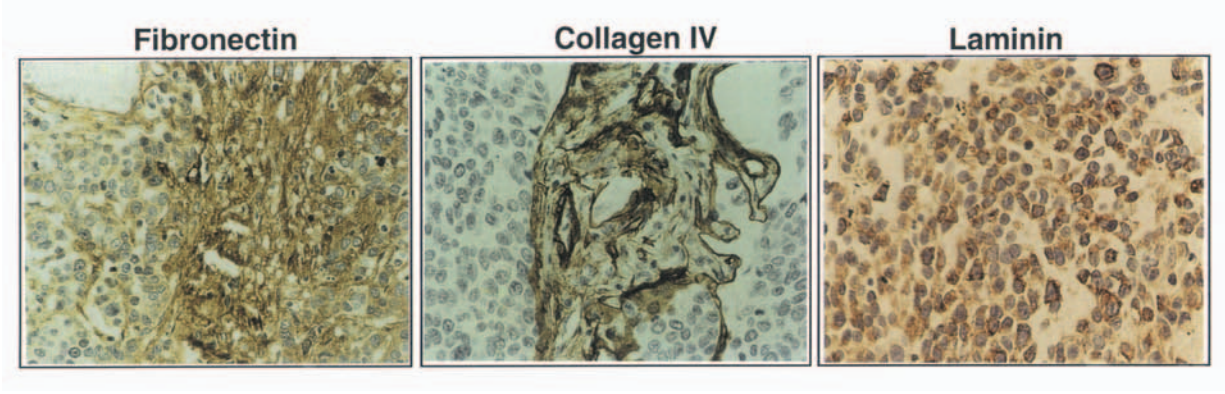


Figure 1. Immunohistochemical staining of ECM proteins around SCLC cells in vivo. Fibronectin (A) and collagen IV (B) immunoreactivity in SCLC. C, intra/pericellular immunoreactivity for laminin. Magnification X400.

In order to test the hypothesis that ECM proteins might protect SCLC cells from chemotherapy-induced apoptosis, we treated several SCLC cell lines with a variety of chemotherapeutic agents including etoposide, doxorubicin, cisplatin and cyclophosphamide in the presence and absence of various ECM proteins. When cells were adhered to fibronectin, laminin or collagen IV, they were markedly protected from chemotherapy-induced apoptosis compared with those non-specifically adhered to poly-L-lysine. Critically, co-incubation with a function-blocking β1 integrin antibody, but not with isotype-matched control antibodies, abolished the ECM-mediated protection. Collectively, these data showed that β1 integrin-mediated adhesion to ECM proteins was protecting SCLC cells from chemotherapy-induced apoptosis.

Dalton's group reported a similar phenomenon in multiple myeloma (27). Myeloma, a disease characterised by the clonal development of plasma cells, has a predilection for the bone marrow which is rich in extracellular matrix proteins. They showed that adhesion of myeloma cell lines to fibronectin via α4β1 and α5β1 integrins conferred resistance to the chemotherapeutic agents doxorubicin and melphalan. Furthermore, they observed that cells selected for drug resistance with either doxorubicin or melphalan over-expressed integrin α4β1. When removed from chemotherapy selection pressure, the

drug resistant cell lines reverted to a drug sensitive, low α4-expressing phenotype.

Subsequent work by the same group has shown cell adhesion mediated drug resistance in both chronic myeloid leukaemia (CML) and acute myeloid leukaemia (AML) (28, 29). Of particular interest was the observation that CML cells displayed significant inhibition of apoptosis in response to a range of structurally and functionally diverse agents including the alkylating agent melphalan, an anti-metabolite (Ara-C), a topoisomerase II inhibitor (mitoxantrone), and the BCR/ABL kinase inhibitors STI-571 and tyrphostin AG957 (28). Similarly, among solid tumours, the breast cancer cell lines MDA-MB-231 and MDA-MB-435 were shown to be protected from vincristine and paclitaxel via α2β1 and α5β1 integrins (30). Furthermore, Uhm et al., (1999) showed that glioma cells are protected from the topoisomerase inhibitor topotecan when adhered to vitronectin through αvβ3 and αvβ5 (31). The latter report is the only description to date that does not involve integrin β1.

5. INTRACELLULAR SIGNALLING MECHANISMS REGULATING INTEGRIN-MEDIATED DRUG RESISTANCE (TABLE 1)

It is now firmly established that ECM-integrin signalling profoundly influences the major cellular programmes of growth, differentiation and apoptosis and multiple cellular functions have been shown to be dependent upon integrin signalling. The identification of integrin-mediated drug resistance and the potential promise of circumventing this form of drug resistance has driven work to dissect the underlying regulatory signalling mechanisms.

Despite the vast amount of work that has been done on integrin signalling, no specific integrin mediators have been identified. Instead, the majority of signalling molecules implicated in ECM-integrin interactions are the rather ubiquitous mediators of signal transduction. Upon binding of ligands to integrin receptors, integrin cross-linking and clustering occurs (32). This promotes the formation of focal adhesions at the cell membrane. A large number of structural and signalling proteins have been shown to be concentrated at these sites. Early work demonstrated that tyrosine kinase inhibitors could block the formation of focal adhesions (33). This led to the identification of focal adhesion kinase (FAK), a 125 kDa non-receptor tyrosine kinase. FAK undergoes rapid tyrosine kinase phosphorylation following integrin ligation and clustering (34, 35). The mechanism by which integrins activate FAK is incompletely understood. Although FAK is unable to phosphorylate other substrates directly, integrin dependent autophosphorylation allows it to interact with docking or adapter proteins including paxillin, tensin and Grb-2 (36-38). These in turn are able to activate downstream signalling mediators including the Ras/Raf/mitogen activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI 3-kinase)/protein kinase B (PKB) pathway (39).

Following the initial observation that ECM adhesion to integrins prevented chemotherapy induced apoptosis, it was hypothesised that matrix-ligand interactions may result in up-regulation of survival pathways and down-regulation of pro-apoptotic pathways. These observations had parallels with those of Frisch and Francis (1994) who had previously noted that when endothelial or epithelial cells become detached from their matrix they undergo apoptosis or 'anoikis' (40). Khwaja et al., (1997) subsequently showed that this integrin-mediated mechanism was PI 3-kinase and PKB dependent (41).

Initial work in small cell lung cancer showed that β1 integrin-mediated cell adhesion to ECM proteins inhibited chemotherapy-induced apoptosis by blocking caspase-3 activation and subsequent apoptosis (23). This mechanism was shown to be protein tyrosine kinase dependent, since it was reversed by the tyrosine phosphorylation inhibitor, tyrphostin-25. Subsequent work showed that inhibition of PI 3-kinase also abolished fibronectin-mediated protection (author's unpublished observations). More recently Krystal et al., (2002) have reported that activation of PKB using a constitutively active mutant protects SCLC cells from etoposide-mediated cytotoxicity and that inhibition of PI 3-kinase/PKB using either a dominant negative PKB or the chemical PI 3-kinase inhibitor, LY294002 enhanced etoposide-induced apoptosis (44). Similarly, activated PKB promotes survival and resistance to both chemotherapy and radiotherapy in non-small cell lung cancer (45). Further support for the involvement of PI 3-kinase and its downstream mediator PKB in integrin-mediated drug resistance has come from work in breast cancer cell lines. Aoudjit et al., (2001) observed that matrix-mediated resistance to paclitaxel and vincristine correlated with an increase in activation of PKB (30). Pre-treatment of cells with the PI 3-kinase inhibitor LY294002 or

Table 1. Characteristics of integrin-mediated drug resistance in cancer cells.

Tumour cell type	Matrix protein	Integrin receptor	Cytotoxic agent	Mechanism of resistance
Small cell lung cancer (H69, H345,H510)[23]	Fn, Ln, Collagen IV	α2β1, α3β1, α6β1, αvβ1	Etoposide, Doxorubicin Cisplatin, Cyclophosphamide	β1 integrin mediated tyrosine phosphorylation mediates caspase-3 inhibition
Breast Cancer (MDA-MB-231/435)[30]	Fn, Collagen 1	α2β1, α5β1	Vincristine, Paclitaxel	β1 integrin signalling inhibits cytochrome c release in a PI 3-kinase/PKB dependent mechanism.
Glioma[31]	Vitronectin	αvβ3, αvβ5	Topotecan	Increased Bcl-2 and Bcl-X_L
Multiple Myeloma (8226)[29]	Fn	α4β1, α5β1	Melphalan, Doxorubicin	Matrix adhesion promotes increased p27kip1 levels
Chronic Myeloid Leukaemia (K562)[28]	Fn	α5β1	Melphalan, Mitoxantrone, Ara-C, AG957, STI-571	Mechanism unknown
Acute Myeloid Leukaemia (U937, HL60)[42]	Fn	α4β1	Daunorubicin, Ara-C	Induction of PI 3-K/PKB/Bcl-2 pathway
Multiple myeloma (8226)[27]	Fn	α4β1, α5β1	Doxorubicin, melphalan	Exact mechanism unclear but not due to reduced drug accumulation or up regulation of anti-apoptotic Bcl-2 family members
Histiocytic lymphoma (U937)[43]	Fn	α5β1	Mitoxantrone, Doxorubicin, Etoposide	Altered topoisomerase IIβ activity/localisation

transfection of a dominant negative PI 3-kinase abrogated protection against chemotherapy-induced apoptosis. Similarly, farnesylated PKB protects breast cancer cell lines from anoikis and suppresses chemotherapy-induced apoptosis (46, 47). Most recently, PI 3-kinase/PKB has been reported to mediate chemoresistance in acute myeloid leukaemia cell lines (42).

Although, most of the work done on integrin-mediated drug resistance points towards β1 integrin as being the key integrin involved, it is likely that there may be more than one downstream signalling pathway involved. Despite the evidence above indicating that the PI 3-kinase/PKB pathway plays a central role in mediating drug resistance, it is likely that there is cell-type specificity. In his recent review on the subject, Damiano (2002) states that he has been unable to find any evidence for this pathway's activation in fibronectin-adhered myeloma cells (48). It is known that integrin engagement activates the MAP kinase pathway, which contributes to adhesion-mediated survival signalling in some cell types (49, 50). This raises the possibility that in some cases the MAP kinase pathway rather than the PI 3-kinase/PKB pathway may mediate drug resistance. In support of this, inhibition of the MAP kinase pathway has been shown to enhance paclitaxel-induced apoptosis in several cancer cell types (51). Furthermore, over-expression of β1 integrin confers resistance to chemotherapy-induced apoptosis in hepatoma cells via a MAP kinase dependent pathway but not a PI 3-kinase/PKB one (52). However, Aoudjit and Vuori (2001) noted in breast cancer cells that while treatment with the MEK inhibitor PD098059 enhanced paclitaxel-induced apoptosis, it did not affect the ability of fibronectin or collagen 1 to protect the cells against apoptosis, i.e. it seems that activation of the MAP kinase pathway is not necessary for integrin-mediated inhibition of paclitaxel-induced apoptosis [30]. Inhibition of the MAP kinase pathway may also affect growth factor signalling which recent reports suggest may also contribute to drug resistance mechanisms. Fibroblast growth factor 2 (FGF-2) has been shown to be capable of protecting small cell lung cancer cells from etoposide-induced apoptosis via a MEK-dependent pathway (53, 54). This latter finding raises the possibility that in some cases integrin-mediated drug resistance signalling may converge or involve cross-talk with cytokine and growth factor receptors.

The downstream signal transduction pathway(s) by which PKB protects cells from apoptosis has received intensive study and has been the subject of recent reviews (55, 56). It is thought the Bcl-2 family of proteins are involved. Bcl-2 belongs to a family of related and interacting members, some of which are anti-apoptotic and some of which are pro-apoptotic. In breast cancer cell lines it has been shown that the release of cytochrome-c into the cytosol is a critical event in the activation of the apoptotic signalling cascade (30). Cytosolic release of cytochrome-c results in activation of caspase-9

which results in subsequent activation of effector caspases such as caspase-3. These caspases promote cleavage of apoptosis regulators and house-keeping proteins resulting in DNA fragmentation and apoptosis. PI 3-kinase and PKB-mediated integrin signalling, by blocking the release of cytochrome-c, inhibited the activation of caspase-9 and subsequent cell death in paclitaxel treated breast cancer cells. Although the mechanisms that control the release of cytochrome-c are poorly understood it is thought that its release from the mitochondrion is controlled by a balance of pro and anti-apoptotic members of the Bcl-2 family. It has been shown previously that growth factors acting via PKB can phosphorylate the pro-apoptotic Bcl-2 family protein Bad thereby preventing it from antagonising Bcl-2 and Bcl-X_L and consequently inhibiting apoptosis through inhibition of caspase-3 (57, 58). In the same way, matrix adhesion of breast cancer and acute myeloid leukaemia cells blocked chemotherapy-induced inhibition of Bcl-2 thereby promoting survival (30, 42). This inhibition was shown to be PI 3-kinase dependent. Similarly, ligand engagement of $\alpha 4\beta 1$ confers resistance of B-cell chronic lymphatic leukaemia cells to the pro-apoptotic drug fludarabine as a result of up-regulation of Bcl-X_L (59). Furthermore, in glioma cells, vitronectin-mediated protection from chemotherapy-induced apoptosis correlated with increased ratios of Bcl-2:Bax and Bcl-X_L:Bax (31).

Despite these findings it is likely that there is more than one mechanism regulating integrin-mediated inhibition of apoptosis. In small cell lung cancer cell lines (23) and myeloma cell lines (27) adhesion to matrix did not alter the expression of Bcl-2 or related proteins. Kouniavsky et al., (2002) observed that although expression of Bcl-2 and Bcl-X_L was increased in colon cancer cells cultured on matrix, there was no correlation between protection by matrix and expression of these proteins (60). Recent work suggests that integrin signalling can also modify drug-induced apoptosis by regulating the cell cycle.

5.1 ECM effect on cell cycle effectors

There is now a large body of work reporting the multiple effects of integrin signalling on the cell cycle (61). Cell cycle progression is driven by the sequential activation of cyclin dependent kinases which form complexes with specific activating cyclins. It is well recognised that cell cycle progression is dependent upon transition through the G1/S and G2/M checkpoints. Chemotherapeutic agents predominantly bring about cell death by inducing apoptosis in response to DNA damage-induced cell cycle arrest in G1/S and G2/M (62). Prolonged cell cycle arrest, in the absence of DNA repair, normally leads to caspase-3 activation and the induction of apoptosis. To date, limited work has been performed examining the effect of matrix adhesion on cell cycle kinetics. Much of this has focussed on the cyclin dependent kinase inhibitor p27kip1. Adhesion of a myeloma cell line to fibronectin which resulted in increased resistance to chemotherapy was correlated with G1 arrest, increased p27kip1 levels and inhibition of cyclin A and E-associated kinase activity. Detachment from matrix led to progression into S phase associated with a fall in p27kip1 levels and reversion to a drug sensitive phenotype (29). Furthermore, p27kip1 anti-sense successfully reduced protein levels in adhered cells and restored drug sensitivity whilst not affecting cell adhesion. These findings supported previous work reporting that over-expression of p27kip1 protected cells from chemotherapy-induced apoptosis. Furthermore, St Croix et al., (1996) previously demonstrated that when cells are grown in spheroids, p27kip1 levels were increased compared with monolayer cultures (63). The increase in p27kip1 was correlated with G1 arrest and increased resistance to alkylating agents. However, the *in vivo* situation may be far more complicated than these data suggest due to constantly fluctuating levels of p27kip1 during cell cycle progression. Furthermore, there may exist a degree of cell type specificity as in MDA-MB-231 breast cancer cells for instance, over-expression of p27kip1 is associated with the induction of apoptosis rather than protection from apoptosis (64).

5.2 Effects of matrix adhesion on chemotherapy- induced DNA damage

Many chemotherapeutic agents bring about apoptosis by promoting DNA damage. When matrix-mediated protection from chemotherapy was initially identified, several groups hypothesised that extracellular matrix may confer drug resistance by limiting the amount of DNA damage induced by chemotherapy. Dalton et al., identified a 40-60% reduction in etoposide-induced DNA double strand breaks when U937 lymphoma cells were adhered to fibronectin (65). This was due to a reduction in topoisomerase II activity secondary to alterations in the nuclear distribution of the enzyme. Similarly, activation of $\beta 1$ integrins in tumour-derived endothelial cells or lung endothelial cells resulted in

a decrease in etoposide and bleomycin-induced DNA damage as measured by DNA sedimentation and in situ nick translation (66, 67). However, the effect of adhesion on topoisomerase II mediated DNA damage may be cell-type specific. In small cell lung cancer topoisomerase II activity in response to etoposide was not altered regardless of whether cells were adhered to fibronectin or plastic (23).

6. CLINICAL EVIDENCE TO SUPPORT INTEGRIN-MEDIATED DRUG RESISTANCE

Regardless of the compelling evidence that is accumulating to show that integrin-mediated matrix adhesion protects cancer cells from chemotherapy-induced apoptosis, it must not be forgotten that the majority of this work has been performed *in vitro*. It is well recognised that due to the complex interactions of multiple stimuli *in vivo*, effects seen in vitro are not always reproducible *in vivo*. Even with advances in the development of *in vitro* models for cancer it is not yet possible to fully recreate the complex microenvironment in which cancer cells exist *in vivo*.

Despite the paucity of models examining integrin-mediated drug resistance *in vivo*, there is increasing clinical evidence accruing to support the hypothesis. In small cell lung cancer an association between the expression of β1 integrin and response to chemotherapy and survival has been identified. Patients whose tumours had high levels of β1 integrin expression were significantly more resistant to combination chemotherapy than those whose tumours had low expression of β1 (68). Using multivariate regression analysis the authors concluded that clinical stage and β1 integrin were independent risk factors for survival following chemotherapy: high expression of β1 in tumour cells is a poor prognostic factor in patients with SCLC. Similarly, we reported that in small cell lung cancer patients who had received chemotherapy, those with extensive matrix around their tumours had a significantly shorter survival time from diagnosis than did patients with focal or no matrix (23). Recently, a study on patients with acute myeloid leukaemia examined the relationship between α4β1 expression and response to chemotherapy (42). The complete remission rate for the α4β1 positive patients was significantly lower than that of the α4β1 negative patients. By contrast the relapse rate was higher in the α4β1 positive patients than in the α4β1 negative patients.

7. CONCLUSIONS AND POTENTIAL THERAPEUTIC INTERVENTIONS

Despite the recent identification of a number of mechanisms of acquired drug resistance, none have explained how tumour cells are able to evade the initial pro-apoptotic effects of chemotherapy. The identification of integrin-mediated drug resistance offers a mechanism as to how this may occur. It is thought that even if 0.1% of cells survive, this is probably sufficient to allow tumour recurrence. Surviving, genetically damaged cells are then selected out and continue to proliferate leading to persistence of, and recurrence of, disease. These cells may eventually display the markers of acquired drug resistance discussed previously. The work presented above demonstrates that cell adhesion to matrix proteins has the potential to suppress apoptosis induced by a wide variety of chemotherapeutic agents. Although it is likely that several different intracellular signalling mechanisms are responsible for this phenomenon depending on cell type, the constant observation is that the process is integrin-mediated and predominantly through β1 integrins.

The identification of the mechanisms underlying integrin-mediated drug resistance offers the possibility of designing novel therapeutic agents that could be administered in conjunction with conventional chemotherapy in order to augment chemosensitivity. Several inhibitors of integrins are currently being investigated as potential cancer therapies. However, all of the current trials which are utilising either anti-integrin antibodies or cyclic peptide inhibitors of integrins, are evaluating whether these agents have significant anti-angiogenesis or anti-metastatic roles (69-71). There are no on-going clinical studies examining whether integrin inhibition will improve response to chemotherapy. However, the results of these studies are eagerly awaited and may lead to the development of agents with clinical utility as chemosensitisers.

Downstream of the integrin receptor, obvious targets are components of the signal transduction pathways that regulate the integrin mediated survival pathway. Our work, together with that of others indicates that both tyrosine kinase inhibitors and PI 3-kinase inhibitors may be potential chemosensitisers. Although work to date has used

broad spectrum tyrosine kinase inhibitors which may have undesirable side-effects *in vivo*, specific inhibitors for a number of tyrosine kinases have been described (72). The central role of PI 3-kinase in tumourigenesis and chemoresistance makes it an attractive target for drug development. Recently, two groups have reported the use of either inositol phosphates or phosphatidylinositol analogues to inhibit the growth of various cancer cell lines (73, 74). In another approach the PI 3-kinase inhibitor LY294002 was used to treat athymic mice that had been inoculated intraperitoneally with an ovarian cancer cell line (75). In the LY294002 treated group, mean tumour burden and ascites production was markedly reduced compared with controls. This is the first description of an animal model to test the efficacy of LY294002 *in vivo*. Although LY294002 is a 'broad-spectrum' PI 3-kinase inhibitor there were apparently no systemic side effects in the control group. Isoform-specific inhibitors of PI 3-kinase are now under development and may prove to play an adjuvant role to conventional chemotherapy.

Notwithstanding the large amount of work that has been undertaken attempting to refine and improve chemotherapy regimens in order to boost response and cure rates, the survival figures for a number of tumours have remained relatively static for many years. This has led many investigators to believe that novel therapeutic strategies will only come through a better understanding of the basic molecular processes governing proliferation and apoptosis. In time it is hoped that modulation of the integrin-mediated survival pathway either at the level of the receptor or the downstream signalling pathway may augment the cytotoxicity of chemotherapeutic agents and allow for improved clinical results at lower doses of drugs with decreased side-effects.

REFERENCES

1. Greenlee, R. T., Hill-Harmon, M. B., Murray, T., and Thun, M., 2001, Cancer statistics. CA Cancer J Clin, 51:15-36.
2. Simon, G., Ginsberg, R. J., and Ruckdeschel, J. C., 2001, Small-cell lung cancer. Chest Surg Clin N Am, 11:165-188, ix.
3. Green, R. A., Humphrey, E., Close, H., and Patno, M. E., 1969, Alkylating agents in bronchogenic carcinoma. Am J Med, 46:516-525.
4. Kartner, N., Riordan, J. R., and Ling, V., 1983, Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. Science, 221:1285-1288.
5. Roninson, I. B., Chin, J. E., Choi, K. G., Gros, P., Housman, D. E., Fojo, A., Shen, D. W., Gottesman, M. M., and Pastan, I., 1986, Isolation of human mdr DNA sequences amplified in multidrug-resistant KB carcinoma cells. Proc Natl Acad Sci USA, 83:4538-4542.
6. Cole, S. P., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M., and Deeley, R. G., 1992, Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science, 258:1650-1654.
7. Doyle, L. A., Yang, W., Abruzzo, L. V., Krogmann, T., Gao, Y., Rishi, A. K., and Ross, D. D., 1998, A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci USA, 95:15665-15670.
8. Scheffer, G. L., Wijngaard, P. L., Flens, M. J., Izquierdo, M. A., Slovak, M. L., Pinedo, H. M., Meijer, C. J., Clevers, H. C., and Scheper, R. J., 1995, The drug resistance-related protein LRP is the human major vault protein. Nat Med, 1:578-582.
9. Hall, A. G., 1999, Glutathione and the regulation of cell death. Adv Exp Med Biol, 457:199-203.
10. Salinas, A. E. and Wong, M. G., 1999, Glutathione S-transferases--a review. Curr Med Chem, 6:279-309.
11. Kaufmann, S. H., 1989, Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. Cancer Res, 49:5870-5878.
12. Narasaki, F., Matsuo, I., Ikuno, N., Fukuda, M., Soda, H., and Oka, M., 1996, Multidrug resistance-associated protein (MRP) gene expression in human lung cancer. Anticancer Res, 16:2079-2082.
13. Nooter, K., Bosman, F. T., Burger, H., van Wingerden, K. E., Flens, M. J., Scheper, R. J., Oostrum, R. G., Boersma, A. W., van der, G. A., and Stoter, G., 1996, Expression of the multidrug resistance-associated protein (MRP) gene in primary non-small-cell lung cancer. Ann Oncol, 7:75-81.
14. Kreisholt, J., Sorensen, M., Jensen, P. B., Nielsen, B. S., Andersen, C. B., and Sehested, M., 1998, Immunohistochemical detection of DNA topoisomerase IIalpha, P-glycoprotein and multidrug resistance protein (MRP) in small-cell and non-small-cell lung cancer. Br J Cancer, 77:1469-1473.
15. Teicher, B. A., Herman, T. S., Holden, S. A., Wang, Y. Y., Pfeffer, M. R., Crawford, J. W., and Frei, E., III, 1990, Tumor resistance to alkylating agents conferred by mechanisms operative only in vivo. Science, 247:1457-1461.
16. Lotem, J. and Sachs, L., 1992, Hematopoietic cytokines inhibit apoptosis induced by transforming

growth factor beta 1 and cancer chemotherapy compounds in myeloid leukemic cells. Blood, 80: 1750-1757.

17. Dias, S., Choy, M., Alitalo, K., and Rafii, S., 2002, Vascular endothelial growth factor (VEGF)-C signaling through FLT-4 (VEGFR-3) mediates leukemic cell proliferation, survival, and resistance to chemotherapy. Blood, 99:2179-2184.
18. Xu, W., Liu, L., Smith, G. C., and Charles, G., 2000, Nitric oxide upregulates expression of DNA-PKcs to protect cells from DNA-damaging anti-tumour agents. Nat Cell Biol, 2:339-345.
19. Teicher, B. A., Ikebe, M., Ara, G., Keyes, S. R., and Herbst, R. S., 1997, Transforming growth factor-beta 1 overexpression produces drug resistance in vivo: reversal by decorin. In Vivo, 11:463-472.
20. Desoize, B., Gimonet, D., and Jardiller, J. C., 1998, Cell culture as spheroids: an approach to multicellular resistance. Anticancer Res, 18:4147-4158.
21. Durand, R. E. and Sutherland, R. M., 1972, Effects of intercellular contact on repair of radiation damage. Exp Cell Res, 71:75-80.
22. Desoize, B. and Jardillier, J., 2000 Multicellular resistance: a paradigm for clinical resistance? Crit Rev Oncol Hematol, 36:193-207.
23. Sethi, T., Rintoul, R. C., Moore, S. M., MacKinnon, A. C., Salter, D., Choo, C., Chilvers, E. R., Dransfield, I., Donnelly, S. C., Strieter, R., and Haslett, C., 1999, Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: a mechanism for small cell lung cancer growth and drug resistance in vivo. Nat Med, 5:662-668.
24. Jin, H. and Varner, J., 2004, Integrins: roles in cancer development and as treatment targets. Br J Cancer, 90:561-565.
25. Hirasawa, M., Shijubo, N., Uede, T., and Abe, S., 1994, Integrin expression and ability to adhere to extracellular matrix proteins and endothelial cells in human lung cancer lines. Br J Cancer, 70:466-473.
26. Falcioni, R., Cimino, L., Gentileschi, M. P., D'Agnano, I., Zupi, G., Kennel, S. J., and Sacchi, A., 1994, Expression of beta 1, beta 3, beta 4, and beta 5 integrins by human lung carcinoma cells of different histotypes. Exp Cell Res, 210:113-122.
27. Damiano, J. S., Cress, A. E., Hazlehurst, L. A., Shtil, A. A., and Dalton, W. S., 1999, Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. Blood, 93:1658-1667.
28. Damiano, J. S., Hazlehurst, L. A., and Dalton, W. S., 2001, Cell adhesion-mediated drug resistance (CAM-DR) protects the K562 chronic myelogenous leukemia cell line from apoptosis induced by BCR/ABL inhibition, cytotoxic drugs, and gamma-irradiation. Leukemia, 15:1232-1239.
29. Hazlehurst, L. A., Damiano, J. S., Buyuksal, I., Pledger, W. J., and Dalton, W. S., 2000, Adhesion to fibronectin via beta1 integrins regulates p27kip1 levels and contributes to cell adhesion mediated drug resistance (CAM-DR). Oncogene, 19:4319-4327.
30. Aoudjit, F. and Vuori, K., 2001, Integrin signaling inhibits paclitaxel-induced apoptosis in breast cancer cells. Oncogene, 20:4995-5004.
31. Uhm, J. H., Dooley, N. P., Kyritsis, A. P., Rao, J. S., and Gladson, C. L., 1999, Vitronectin, a glioma-derived extracellular matrix protein, protects tumor cells from apoptotic death. Clin Cancer Res, 5:1587-1594.
32. Kornberg, L. J., Earp, H. S., Turner, C. E., Prockop, C., and Juliano, R. L., 1991, Signal transduction by integrins: increased protein tyrosine phosphorylation caused by clustering of beta 1 integrins. Proc Natl Acad Sci USA, 88:8392-8396.
33. Burridge, K., Turner, C. E., and Romer, L. H., 1992, Tyrosine phosphorylation of paxillin and pp125FAK accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. J Cell Biol, 119:893-903.
34. Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B., and Parsons, J. T., 1992, pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Proc Natl Acad Sci USA, 89:5192-5196.
35. Hanks, S. K., Calalb, M. B., Harper, M. C., and Patel, S. K., 1992, Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. Proc Natl Acad Sci USA, 89:8487-8491.
36. Vuori, K., Hirai, H., Aizawa, S., and Ruoslahti, E., 1996, Introduction of p130cas signaling complex formation upon integrin-mediated cell adhesion: a role for Src family kinases. Mol Cell Biol, 16:2606-2613.
37. Abassi, Y. A., Rehn, M., Ekman, N., Alitalo, K., and Vuori, K., 2003, p130Cas Couples the tyrosine kinase Bmx/Etk with regulation of the actin cytoskeleton and cell migration. J Biol Chem, 278:35636-35643.
38. Schlaepfer, D. D. and Hunter, T., 1997, Focal adhesion kinase overexpression enhances ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src. J Biol Chem, 272:13189-13195.
39. Chen, H. C., Appeddu, P. A., Isoda, H., and Guan, J. L., 1996, Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. J Biol Chem, 271:26329-26334.
40. Frisch, S. M. and Francis, H., 1994, Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol, 124:619-626.

41. Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P. H., and Downward, J., 1997, Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. EMBO J, 16:2783-2793.
42. Matsunaga, T., Takemoto, N., Sato, T., Takimoto, R., Tanaka, I., Fujimi, A., Akiyama, T., Kuroda, H., Kawano, Y., Kobune, M., Kato, J., Hirayama, Y., Sakamaki, S., Kohda, K., Miyake, K., and Niitsu, Y., 2003, Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. Nat Med, 9:1158-1165.
43. Hazlehurst, L. A. and Dalton, W. S., 2001, Mechanisms associated with cell adhesion mediated drug resistance (CAM-DR) in hematopoietic malignancies. Cancer Metastasis Rev, 20:43-50.
44. Krystal, G. W., Sulanke, G., and Litz, J., 2002, Inhibition of phosphatidylinositol 3-kinase-Akt signaling blocks growth, promotes apoptosis, and enhances sensitivity of small cell lung cancer cells to chemotherapy. Mol Cancer Ther, 1:913-922.
45. Brognard, J., Clark, A. S., Ni, Y., and Dennis, P. A., 2001, Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. Cancer Res, 61:3986-3997.
46. Schmidt, M., Hovelmann, S., and Beckers, T. L., 2002, A novel form of constitutively active farnesylated Akt1 prevents mammary epithelial cells from anoikis and suppresses chemotherapy-induced apoptosis. Br J Cancer, 87:924-932.
47. Knuefermann, C., Lu, Y., Liu, B., Jin, W., Liang, K., Wu, L., Schmidt, M., Mills, G. B., Mendelsohn, J., and Fan, Z., 2003. HER2/PI-3K/Akt activation leads to a multidrug resistance in human breast adenocarcinoma cells. Oncogene, 22:3205-3212.
48. Damiano, J. S., 2002, Integrins as novel drug targets for overcoming innate drug resistance. Curr Cancer Drug Targets, 2:37-43.
49. Aoudjit, F. and Vuori, K., 2001, Matrix attachment regulates Fas-induced apoptosis in endothelial cells: a role for c-flip and implications for anoikis. J Cell Biol, 152:633-643.
50. Le Gall, M., Chambard, J. C., Breittmayer, J. P., Grall, D., Pouyssegur, J., and Obberghen-Schilling, E., 2000, The p42/p44 MAP kinase pathway prevents apoptosis induced by anchorage and serum removal. Mol Biol Cell, 11:1103-1112.
51. MacKeigan, J. P., Collins, T. S., and Ting, J. P., 2000, MEK inhibition enhances paclitaxel-induced tumor apoptosis. J Biol Chem, 275:38953-38956.
52. Zhang, H., Ozaki, I., Mizuta, T., Matsuhashi, S., Yoshimura, T., Hisatomi, A., Tadano, J., Sakai, T., and Yamamoto, K., 2002, Beta 1-integrin protects hepatoma cells from chemotherapy induced apoptosis via a mitogen-activated protein kinase dependent pathway. Cancer, 95:896-906.
53. Pardo, O. E., Lesay, A., Arcaro, A., Lopes, R., Ng, B. L., Warne, P. H., McNeish, I. A., Tetley, T. D., Lemoine, N. R., Mehmet, H., Seckl, M. J., and Downward, J., 2003, Fibroblast growth factor 2-mediated translational control of IAPs blocks mitochondrial release of Smac/DIABLO and apoptosis in small cell lung cancer cells. Mol Cell Biol, 23:7600-7610.
54. Pardo, O. E., Arcaro, A., Salerno, G., Raguz, S., Downward, J., and Seckl, M. J., 2002, Fibroblast growth factor-2 induces translational regulation of Bcl-XL and Bcl-2 via a MEK-dependent pathway: correlation with resistance to etoposide-induced apoptosis. J Biol Chem, 277:12040-12046.
55. Downward, J., 1998, Mechanisms and consequences of activation of protein kinase B/Akt. Curr Opin Cell Biol, 10:262-267.
56. Brazil, D. P., Park, J., and Hemmings, B. A., 2002, PKB binding proteins. Getting in on the Akt. Cell, 111:293-303.
57. del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G., 1997, Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt Science, 278:687-689.
58. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E., 1997, Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell, 91:231-241.
59. de la Fuente, M. T., Casanova, B., Cantero, E., Hernandez, d. C., Garcia-Marco, J., Silva, A., and Garcia-Pardo, A., 2003, Involvement of p53 in alpha4beta1 integrin-mediated resistance of B-CLL cells to fludarabine. Biochem Biophys Res Commun, 311:708-712.
60. Kouniavsky, G., Khaikin, M., Zvibel, I., Zippel, D., Brill, S., Halpern, Z., and Papa, M., 2002, Stromal extracellular matrix reduces chemotherapy-induced apoptosis in colon cancer cell lines. Clin Exp Metastasis, 19:55-60.
61. Schwartz, M. A. and Assoian, R. K., 2001, Integrins and cell proliferation: regulation of cyclin-dependent kinases via cytoplasmic signaling pathways. J Cell Sci, 114:2553-2560.
62. Hannun, Y. A., 1997, Apoptosis and the dilemma of cancer chemotherapy. Blood, 89:1845-1853.
63. St Croix, B., Florenes, V. A., Rak, J. W., Flanagan, M., Bhattacharya, N., Slingerland, J. M., and Kerbel, R. S., 1996, Impact of the cyclin-dependent kinase inhibitor p27Kip1 on resistance of tumor cells to anticancer agents. Nat Med, 2:1204-1210.
64. Katayose, Y., Kim, M., Rakkar, A. N., Li, Z., Cowan, K. H., and Seth, P., 1997, Promoting apoptosis: a novel activity associated with the cyclin-dependent kinase inhibitor p27. Cancer Res, 57:5441-5445.

65. Hazlehurst, L. A., Valkov, N., Wisner, L., Storey, J. A., Boulware, D., Sullivan, D. M., and Dalton, W. S., 2001, Reduction in drug-induced DNA double-strand breaks associated with beta1 integrin-mediated adhesion correlates with drug resistance in U937 cells. Blood, 98:1897-1903.
66. Hoyt, D. G., Rusnak, J. M., Mannix, R. J., Modzelewski, R. A., Johnson, C. S., and Lazo, J. S., 1996, Integrin activation suppresses etoposide-induced DNA strand breakage in cultured murine tumor-derived endothelial cells. Cancer Res, 56:4146-4149.
67. Jones, C. B., McIntosh, J., Huang, H., Graytock, A., and Hoyt, D. G., 2001, Regulation of bleomycin-induced DNA breakage and chromatin structure in lung endothelial cells by integrins and poly(ADP-ribose) polymerase. Mol Pharmacol, 59:69-75.
68. Oshita, F., Kameda, Y., Ikehara, M., Tanaka, G., Yamada, K., Nomura, I., Noda, K., Shotsu, A., Fujita, A., Arai, H., Ito, H., Nakayama, H., and Mitsuda, A., 2002, Increased expression of integrin beta1 is a poor prognostic factor in small-cell lung cancer. Anticancer Res, 22:1065-1070.
69. Kerr, J. S., Slee, A. M., and Mousa, S. A., 2002, The alpha v integrin antagonists as novel anticancer agents: an update. Expert Opin Investig Drugs, 11:1765-1774.
70. Burke, P. A., DeNardo, S. J., Miers, L. A., Lamborn, K. R., Matzku, S., and DeNardo, G. L., 2002, Cilengitide targeting of alpha(v)beta(3) integrin receptor synergizes with radioimmunotherapy to increase efficacy and apoptosis in breast cancer xenografts. Cancer Res, 62:4263-4272.
71. Eskens, F. A., Dumez, H., Hoekstra, R., Perschl, A., Brindley, C., Bottcher, S., Wynendaele, W., Drevs, J., Verweij, J., and van Oosterom, A. T., 2003, Phase I and pharmacokinetic study of continuous twice weekly intravenous administration of Cilengitide (EMD 121974), a novel inhibitor of the integrins alphavbeta3 and alphavbeta5 in patients with advanced solid tumours. Eur J Cancer, 39:917-926.
72. Levitzki, A. and Gazit, A., 1995, Tyrosine kinase inhibition: an approach to drug development. Science, 267:1782-1788.
73. Razzini, G., Berrie, C. P., Vignati, S., Broggini, M., Mascetta, G., Brancaccio, A., and Falasca, M., 2000, Novel functional PI 3-kinase antagonists inhibit cell growth and tumorigenicity in human cancer cell lines. FASEB J, 14:1179-1187.
74. Hu, Y., Qiao, L., Wang, S., Rong, S. B., Meuillet, E. J., Berggren, M., Gallegos, A., Powis, G., and Kozikowski, A. P., 2000, 3-(Hydroxymethyl)-bearing phosphatidylinositol ether lipid analogues and carbonate surrogates block PI3-K, Akt, and cancer cell growth. J Med Chem, 43:3045-3051.
75. Hu, L., Zaloudek, C., Mills, G. B., Gray, J., and Jaffe, R. B., 2000, In vivo and in vitro ovarian carcinoma growth inhibition by a phosphatidylinositol 3-kinase inhibitor (LY294002). Clin Cancer Res, 6:880-886.

Chapter 19

Bone Metastasis Microenvironment Participates in the Development of Androgen Ablation Refractoriness and Chemotherapy Resistance of Prostate Cancer Cells Residing in the Skeleton: Clinical Implications

M. Koutsilieris, R. Tenta, D. Tiblalexi, N. Pitulis, E. Sotiriou, P. Lembessis, J. Bogdanos, C. Milathianakis and A. Sourla

Department of Experimental Physiology, Medical School, University of Athens, Goudi-Athens, 115 27, Greece

Abstract: The development of resistance to anti-cancer therapies is a major hurdle preventing long-lasting clinical responses to conventional or investigational therapies in hormone refractory prostate cancer. Herein, we analyze the molecular evidence which show that bone metastasis microenvironment survival factors, mainly paracrine, growth hormone (GH)-independent, urokinase-type plasminogen activator (uPA)-mediated production of insulin-like growth factor 1 (IGF-1) and endocrine, GH-dependent production of IGF-1 (mainly liver-derived IGF-1), produce an epigenetic form of cancer cells resistance to pro-apoptotic therapies. In addition, we review the conceptual framework of a novel hormone manipulation for hormone refractory metastatic prostate cancer (combination of dexamethasone and somatostatin analog (SM-A)), which yielded durable objective responses and major improvement of bone pain and performance status in stage D3 prostate cancer patients.

Key words: Osteoblastic metastasis, survival factors, metastasis microenvironment, androgen ablation refractoriness, uPA, growth hormone, prostate cancer, ET-1, insulin-like growth factor 1, Liver, epigenetic, pro-apoptotic, somatostatin, bone, TGFβ, osteoblasts, blood-brain barrier (BBB), brain vasculature, brain ECM, integrins, selectins, tetraspanins, FAK, MMPs, uPA/uPAR, HSPGs, HIF-1, VEGF, chemokines

1. CLINICAL SIGNIFICANCE OF BONE METASTASIS IN PROSTATE CANCER

In prostate cancer, the bones represent the most frequent site for metastasis (1). The typical clinical presentation of the disease spread to the bones includes pain, spinal cord compression, pathologic fractures and anaemia (2, 3). Pain is usually the first symptom of the disease bone involvement that it is produced by the mechanical and chemical stimulation of pain receptors in the periosteum/endosteum (4). Spinal cord compression results by the extradural expansion of the metastatic tumor while spinal angulation is secondary to vertebral collapses or dislocations produced by pathologic fractures. Back pain, motor weakness, sensory loss and autonomic dysfunction are the common symptoms of spinal cord compression (5, 6). Pathologic fractures occur mostly as a result of the tumor mass weakening of bone architecture, whereas anaemia is secondary to the infiltration of bone marrow by metastatic tumor cells (7, 8). Therefore, the development of bone metastases is

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 335-344.

undoubtedly the major cause for morbidity and mortality in prostate cancer patients.

Unfortunately, there exists no therapy capable of improving the median survival of 2.5 years assigned to prostate cancer patients with bone involvement (stage D2), even though most of them respond objectively to androgen ablation manipulations (9). In addition, as of yet no salvage chemotherapy can improve, so far, the median survival of 12 months assigned to patients progressing to androgen ablation refractory stage (stage D3) of the disease (10). However, the selective spread of prostate cancer in the skeleton (osteotropism), along with its consistent ability to incite locally the practically unique, in its nature for solid tumor, blastic reaction of host tissue (osteoblastic metastasis), strongly suggests that there exist specific autocrine, paracrine, intracrine, and endocrine regulatory mechanisms, which are activated at the interface of metastatic prostate cancer cell growth and bone (11). Notably, the sites of the disease spread to the bones coincide almost always with the same sites of disease progression to its terminal androgen ablation refractory stage (stage D3). Therefore, it is conceivable that bone metastasis microenvironment that induces the osteoblastic reaction can also alter tumor cell biology by "turning on" the molecular pathways of tumor cell survival, locally.This can explain the development of tumor cell refractoriness to anticancer therapies, particularly observed at disease metastatic sites in skeleton (12).

2. BONE MICROENVIRONMENT AND PROSTATE CANCER METASTASIS IN SKELETON

The development of bone metastasis, includes specific host tissue recognition of circulating prostate cancer cells, which is followed by tumor cell migration/invasion into the bone matrix, and finally by establishment of local cell interactions with bone matrix-residing cells, leading to the osteoblastic metastasis (13-17). Analysis of the pathophysiological mechanisms implicated at the initial stages of bone metastasis, such as the osteotropism of metastatic prostate cancer cells, is beyond the scope of this review article.

Theoretically, at this initial stage of prostate cancer cells' arrival in bones, tumor cells should be more or less equally distributed into the metabolically active "red" bone marrow-containing bones (18). Obviously, a critical number of tumor cells per ml of peripheral blood should be necessary to allow efficient bone colonization. Indeed, based on animal model data, the development of a single metastatic lesion requires the presence of more than 10,000 tumor cells circulating in the blood stream (19, 20). Consequently, bones of particular high content of "red" bone marrow are expected to be the most frequent targets of initial implantation of circulating tumor cells. Indeed, clinical studies have confirmed the increased susceptibility for metastasis of bones rich in metabolically active "red" bone marrow (21, 22).

However, at this initial stage of bone invasion (micrometastasis stage), tumor cell survival is challenged by the intense immunological surveillance of bone marrow. Consequently, tumor cells, in order to survive, need to migrate into bone matrix (Figure 1). In the bone matrix, bone matrix-residing cells provide a plethora of growth/survival factors, such as insulin like growth factor 1 (IGFs), transforming growth factor βs (TGFβs), bone morphogenetic proteins (BMPs), basic fibroblast-derived growth factor (bFGF), which all have a supportive role for tumor cell growth and survival. This crucial migration of tumor cells into bone matrix employs the activation of a tumor cell-orchestrated, but nevertheless, osteoclast-mediated bone resorption, locally (11, 18, 23-25). So far, there exist no data to support a direct tumor cell-mediated bone resorption in the absence of osteoclasts. Indeed, at quiescence (resting phase) of bones, osteoclasts are not part of bone matrix-residing cells, therefore, it is fair to conclude that prostate cancer cells should stimulate bone resorption by attracting pre-osteoclasts (chemotaxis) and by stimulating osteoclast differentiation (fusion and formation of mature osteoclasts; Figure 1).

Indeed, prostate cancer cells express humoral factors, such as the macrophage colony-stimulating factor (MCSF), transforming growth factor beta family (TGFβς), parathyroid hormone related

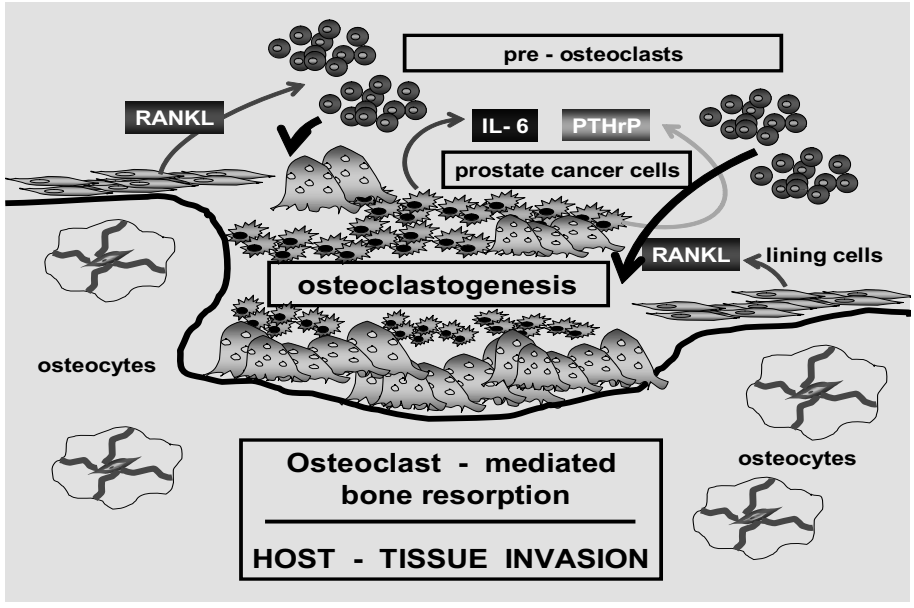


Figure 1. Tumor cell-orchestrated induction of osteoclastogenesis. Note the role of tumor cell-produced IL-6, PTHrP and bone cell-produced RANKL for the activation of osteoclastogenesis, which results in the activation of osteoclast-mediated bone resorption at the sites of bone metastasis.

protein (PTHrP), interleukin-1 (IL-1) and IL-6, regulating osteoclastogenesis, locally (25-30). It is, herefore, fair to conclude that the local cell-cell interactions between tumor cells and bone cells, which are established after tumor cell migration into the bone matrix would either favor the predominance of bone formation or bone resorption, thereby producing blastic, lytic or mixed type of bone lesions

In this context, a large number of bone metastasis microenvironment humoral factors, such as proteases (prostatic specific antigen (PSA), urokinase-type plasminogen activator (uPA), metalloproteinase (MMPs)), bone morphogenetic proteins (BMPs), osteoprotegerin (OPG), endothelin-1 (ET-1), IGFs, platelet-derived growth factor (PDGF), TGFβs, IL-6, and bFGF have been shown to contribute to the unique ability of metastatic prostate cancer cells of producing osteoblastic lesions (11, 16, 31-49).

Among bone metastasis microenvironment-related humoral factors ET-1, a potent vasoconstrictor peptide, has been recently implicated in the pathophysiology of osteoblastic bone metastases. ET-1 levels were found increased in plasma of prostate cancer patients with bone metastasis. In addition, in vitro studies demonstrated that ET-1 increases osteoblast proliferation and osteoblast-specific gene expression. Indeed, prostate cancer cells produce ET-1 while the expression of the ET-1 clearance receptor B is lost during prostate cancer progression to androgen ablation refractory phenotype. Furthermore, the overexpression of ET-1 in murine bone was shown to increase of bone formation, a result that was effectively blocked by the administration of ET-1 antagonist. ET-1 stimulates the proliferation of a variety of cell types, however, it was recently shown that ET-1 is more potent as mitogenic cofactor of several growth factors, including IGFs. In addition, ET-1 exerts survival factor actions, inhibiting apoptosis of prostate cancer cells, in vitro.

In addition, IGFs are also abundant in human bones (bones are second to liver for IGFs content), and have chemotactic, proliferative and antiapoptotic effects on a wide variety of cells, including prostate cancer cells and pre-osteoblasts. IGF-1 exerts its action through the IGF-1.R, a

tyrosine kinase receptor, while its interactions to IGF-1.R are modulated by six IGFBPs. All IGFBPs contain higher affinity for IGFs that this of IGF-1.R. Notably, more than 90% of the circulating IGFs are bound to IGFBP-3, suggesting that IGFBP-3 is a major modulator of IGFs activity in biological fluids. Moreover, IGFBP-3 exerts IGF-independent growth actions and pro-apoptotic effects on several cancer cell types (11, 43, 50). Our group, among others, has shown that prostate cancer cells regulate IGFs levels at metastatic sites by selective uPA-mediated IGFBP-3 proteolysis (32, 34, 37, 40, 51-54), mediating the uPA mitogenic activity for osteoblasts (25, 31, 32, 40, 50-53, 55).

Moreover, the tumor cell-activated growth substances, such as IGFs and TGFbs stimulate the production of osteogenic BMP-7, PDGF-BB, bFGF by the bone matrix-residing cells (lining cells and osteocytes), which in their turn, stimulate recruitment, proliferation, and final differentiation (Runx2 expression) of osteoprogenitor cells to mature osteoblasts (49, 56). Therefore, bone metastasis microenvironment-related growth factors via OPG taper down osteoclastogenesis, thereby reducing osteoclast-mediated bone resorption, while uPA, TGFβ1, BMP-7, IL-6, bFGF, PTHrP, IGFs, Runx2 and ET-1 favour recruitment – proliferation – differentiation of osteoprogenitor cells to mature osteoblasts, thereby producing the osteoblastic reaction, locally (Figure 2).

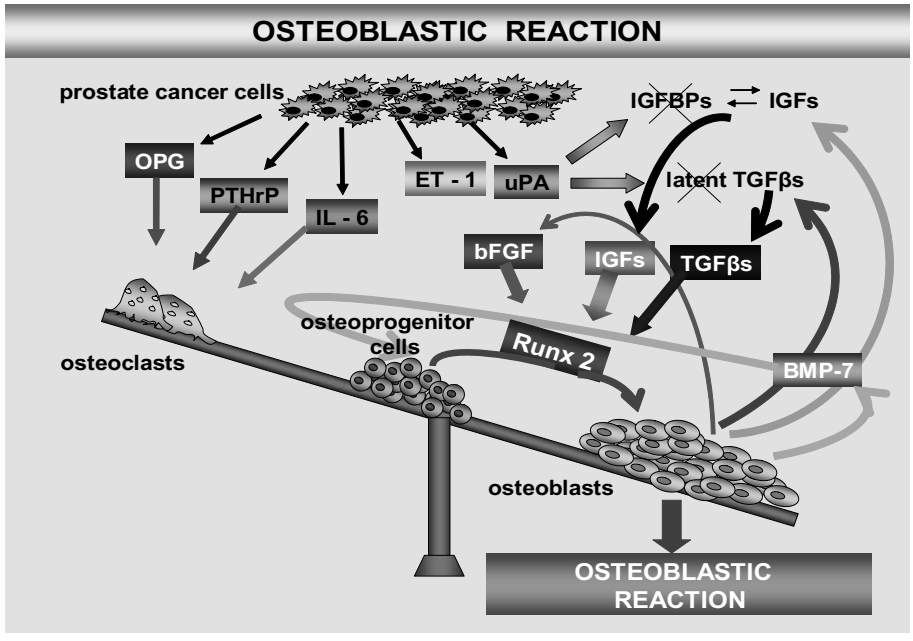


Figure 2. Prostate cancer-produced and bone matrix cell-derived growth substances in the pathophysiology of osteoblastic metastasis in prostate cancer. Note the importance of the prostate cancer cell-derived osteoprotegerin (OPG), which can inhibit osteoclastogenesis (antagonizing RANKL) the same time that bone metastasis microenvironment-related growth factors favour the recruitment of the osteoprogenitor cells, which then proliferate and differentiate(Runx2 expression) to mature osteoblasts, locally.

3. BONE METASTASIS MICROENVIRONMENT AND DEVELOPMENT OF ANDROGEN ABLATION REFRACTORINESS AND CHEMOTHERAPY RESISTANCE OF PROSTATE CANCER CELLS IN BONES

Although the androgen regulatory axis has been widely studied in the context of prostate cancer progression and metastases, it is becoming apparent that many other factors are important in the biology of prostate carcinoma growing into bones. These include growth factors, cytokines, and hormones (8, 11, 12, 18, 29, 46, 47, 50, 54, 57-61). In the prostate cancer field, refractoriness to androgen ablation and chemotherapy was originally determined at the genetic level by cell clones possessing mutations/chromosomal abnormalities. Consequently, it was initially suggested that bone-associated growth factors, such as EGF, bFGF, tumor necrosis factor α (TNFα), IGF-1, PDGF, TGFβ1, IL-6 and IL-1β act differentially on androgen-insensitive (PC-3 and DU145) vs androgen sensitive (LNCaP, VCaP, MDA PCa 2a, PCa 2b, and DUCaP) prostate cancer cells. However, recent data suggested that androgen-independent prostate cancer cells do not respond preferentially to mitogens of bone metastasis microenvironment (62). Therefore, the development of refractoriness is not the result of selective mitogenic action of bone microenvironment-related growth factors on genetically altered tumor cells.

It is, therefore, conceivable that the bone microenvironment-related growth factors by inhibiting androgen ablation-induced and chemotherapy-induced apoptosis of tumor cells result in androgen ablation- and chemotherapy resistant-growth in skeleton (Figure 3).

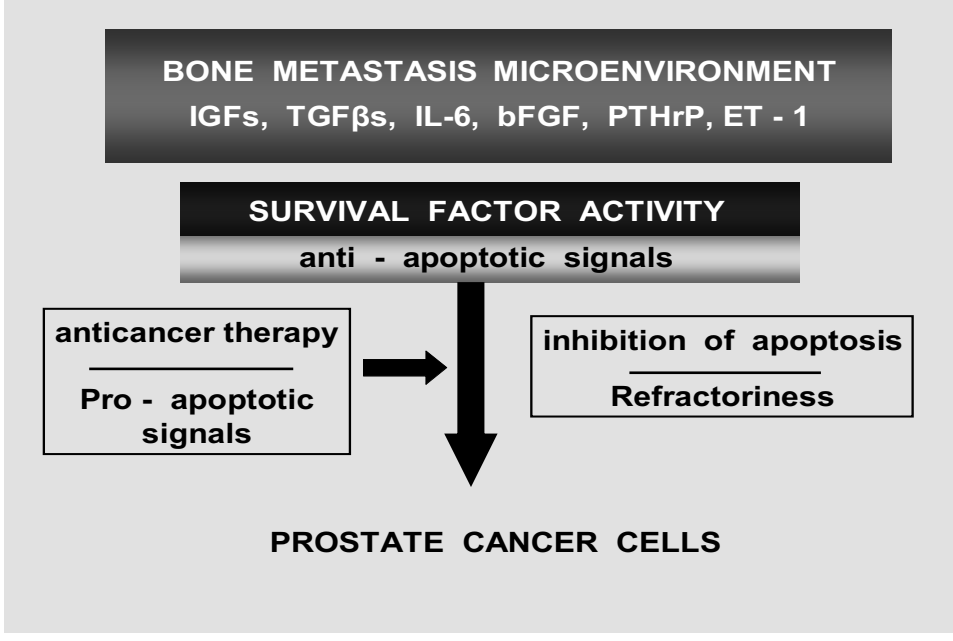


Figure 3. Schematic representation of the major role of bone metastasis microenvironment-related survival factors in the development of prostate cancer cell refractoriness to anticancer therapies in bone metastasis.

Indeed, bone metastasis microenvironment growth factors rescued metastatic prostate cancer cells from anticancer drug-induced apoptosis (11, 50, 51, 54, 63-65). Also, IGF-1 and other bone-related growth factors, such as TGFβ1, IL-6, bFGF were shown remarkable ability to neutralize the cytotoxic actions of doxorubicin on PC-3 cells, in vitro. Although, IL-6 was more potent than IGF-1 in

neutralizing the doxorubicin-induced apoptosis of PC-3 cells, the interpretation of these in vitro data should take into account other biological parameters, such as the inhibition of proliferation by TGFβ1 and the minimal effect of IL-6 and bFGF on the proliferation of PC-3 cells. Thus, the role of TGFβ1, IL-6 and bFGF as survival factors is perhaps less important than that of IGF-1, which has been shown to rescue prostate cancer cells from doxorubicin-induced apoptosis and to stimulate the growth of surviving prostate cancer cells. Furthermore, anti-apoptotic activity of these survival substances has been tested in vitro at final concentrations ranging from 1 ng/ml up to 25 ng/ml, which are super-physiological for IL-6, bFGF and TGFβ1 with respect to those detected in biological fluids (peripheral blood: normal range of IGF-1 = 100-280 ng/ml versus normal range for IL-6; bFGF and TGFβ1 < 1 ng/ml). Therefore, IGF-1 was rightfully named as the major survival factor for prostate cancer cells in bone metastasis microenvironment (66). In addition, it is important to note that bone metastasis microenvironment-growth factors, such as IL-6, IGF-1, TGFβ1, bFGF can increase the expression of PTHrP mRNA of prostate cancer cells and PTHrP overexpression is directly linked with a significant survival factor activity in prostate cancer cell lines (26, 27, 67).

Therefore, whereas most chemotherapy approaches to the treatment of prostate carcinoma rely on the responsiveness of this tissue to the androgen-estrogen axis, a novel approach for therapy may be needed in patients with far advanced prostate cancer, an approach based on the manipulation of these local growth/survival regulators of tumor cell survival, such as IGFs, TGFβ1, IL-6, ET-1 and PTHrP (50, 61, 63, 64, 68-71). Indeed, clinical trials aiming the suppression of IGF-1 activity in bone metastasis have employed a novel combination therapy consisting of somatostatin analogs (SM-A), which can reduce the GH-dependent IGF-1 bioavailability, and dexamethasone, which can reduce the GH-independent/uPA-mediated local increase of IGF-1 bioavailability (uPA-orchestrated hydrolysis of IGFBPs in bone metastasis). This novel combination therapy showed clinical efficiency and efficacy to re-introduce objective and sustainable objective responses to androgen ablation therapy in androgen refractory prostate cancer patients (64, 68).

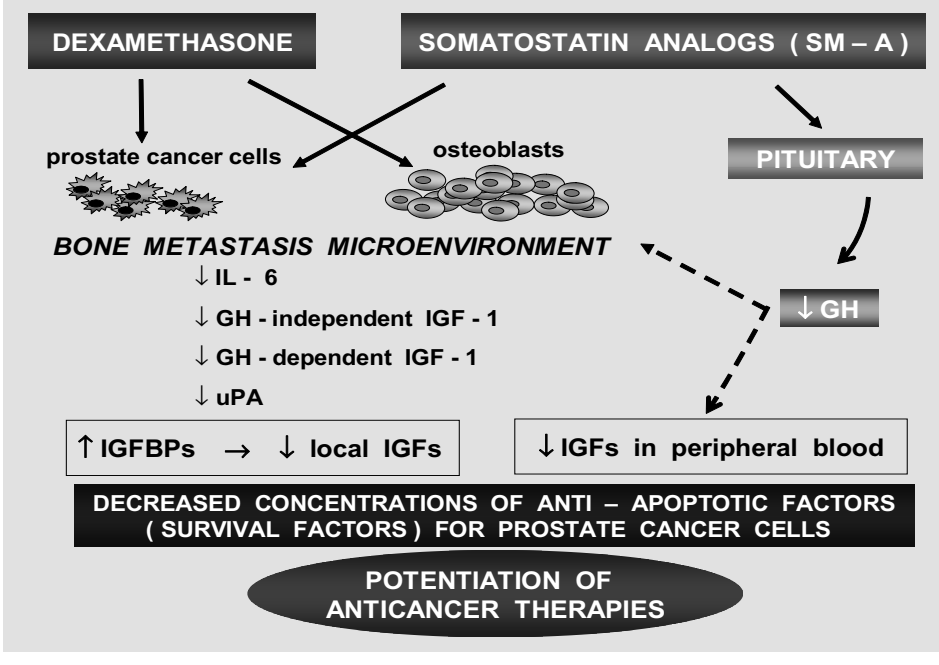


Figure 4. Schematic representation of a bone-targeted hormone manipulation consisted of somatostatic analog and dexamethasone, which produces clinical responses in patients with androgen ablation refractory (stage D3) prostate cancer.

Also, recent studies have demonstrated that dexamethasone alone can reduce IL-6 levels in peripheral blood of patients with androgen ablation refractory prostate cancer and this IL-6 reduction conforms with objective clinical responses in such patients with far advanced prostate cancer (72). Therefore, hormone manipulations (bone-targeted therapy) that can reduce the circulating levels of bone metastasis microenvironment-related growth factors, such as IGFs and IL-6 produce objective clinical responses in stage D3 prostate cancer patients (Figure 4).

4. CONCLUSION

Analysis of the molecular mode of action of bone metastasis microenvironment-related anti-apoptotic factors on the survival of prostate cancer cells may enable a more complete understanding of the development of prostate cancer cell refractoriness to anti-cancer therapies in the stage D3 prostate cancer patients. The bone-targeted therapeutic approach of a combination therapy, using dexamethasone and SM-A illustrates a novel paradigm in cancer treatment: anti-tumor treatment strategies may not only aim at directly inducing cancer cell apoptosis, but can also target the tumor metastasis microenvironment, and neutralize the protection it confers on metastatic cancer cells. The low toxicity profile of such a novel therapeutic approach calls for its testing in a randomized controlled setting in metastatic prostate cancer and, conceivably, in other IGF-1-responsive and IL-6-responsive malignancies.

REFERENCES

1. Soloway, M.S., et al., 1988, Stratification of patients with metastatic prostate cancer based on extent of disease on initial bone scan. Cancer, 61(1): 195-202.
2. Mohla, S., et al., 2003, Third North American Symposium on Skeletal Complications of Malignancy: summary of the scientific sessions. Cancer, 97(3 Suppl):719-25.
3. McMurtry, C.T., and McMurtry, J.M., 2003, Metastatic prostate cancer: complications and treatment. J Am Geriatr Soc, 51(8):1136-42.
4. Pollen, J.J., and Schmidt, J.D., 1979, Bone pain in metastatic cancer of prostate. Urology, 13(2):129-34.
5. Ruff, R.L. and Lanska, D.J., 1989, Epidural metastases in prospectively evaluated veterans with cancer and back pain. Cancer, 63(11):2234-41.
6. Liskow, A., et al., 1986, Epidural cord compression in association with genitourinary neoplasms. Cancer, 58(4):949-54.
7. Benjamin, R., 2002, Neurologic complications of prostate cancer. Am Fam Physician, 65(9):1834-40.
8. De, S., et al., 2003, Molecular pathway for cancer metastasis to bone. J Biol Chem, 278(40):39044-50.
9. Eaton, C.L. and Coleman, R.E., 2003, Pathophysiology of bone metastases from prostate cancer and the role of bisphosphonates in treatment. Cancer Treat Rev, 29(3):189-98.
10. David, A.K., Khwaja R., and Hudes, G.R., 2003, Treatments for improving survival of patients with prostate cancer. Drugs Aging, 20(9):683-99.
11. Bogdanos, J., et al., 2003, Endocrine/paracrine/autocrine survival factor activity of bone microenvironment participates in the development of androgen ablation and chemotherapy refractoriness of prostate cancer metastasis in skeleton. Endocr Relat Cancer, 10(2):279-89.
12. Mitsiades, C.S. and Koutsilieris, M., 2001, Molecular biology and cellular physiology of refractoriness to androgen ablation therapy in advanced prostate cancer. Expert Opin Investig Drugs, 10(6):1099-115.
13. Reddi, A.H., et al., 2003, Mechanisms of tumor metastasis to the bone: challenges and opportunities. J Bone Miner Res, 18(2):190-4.
14. Kumar, C.C., 2003, Integrin alpha v beta 3 as a therapeutic target for blocking tumor-induced angiogenesis. Curr Drug Targets, 4(2):123-31.
15. Sweeney, P., et al., 2002, Anti-vascular endothelial growth factor receptor 2 antibody reduces tumorigenicity and metastasis in orthotopic prostate cancer xenografts via induction of endothelial cell apoptosis and reduction of endothelial cell matrix metalloproteinase type 9 production. Clin Cancer Res, 8(8):2714-24.
16. Nemeth, J.A., et al., 2002, Matrix metalloproteinase activity, bone matrix turnover, and tumor cell proliferation in prostate cancer bone metastasis. J Natl Cancer Inst, 94(1):17-25.
17. Scott, L.J., et al., 2001, Interactions of human prostatic epithelial cells with bone marrow endothelium: binding and invasion. Br J Cancer, 84(10):1417-23.
18. Karamanolakis, D., et al., 2002, Molecular evidence-based use of bone resorption-targeted therapy in prostate cancer patients at high risk for bone involvement. Mol Med, 8(11):667-75.

19. Fidler, I.J. 1990, Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Cloes memorial award lecture. Cancer Res, 50 (19):6130-8.
20. Liotta, L.A., Kleinerman, J., Saidel, G.M., 1974, Quantitative relationships of intravascular tumor cells, tumor vessels, and pulmnary metastases following tumor implantation. Cancer Res, 34 (5): 997-1004.
21. Smith, J.A., 2003, Bone histology at autopsy and matched bone scintigraphy findings in patients with hormone refractory prostate cancer: the effect of bisphosphonate therapy on bone scintigraphy results. Roudier MP, Vesselle H, True LD, Higano CS, Ott SM, King SH, Vessella RL, Department of Urology, University of Washington, Seattle, WA. Clin Exp Metastasis,20:171-180. Urol Oncol, 21(6):483.
22. Yu, K.K., and Hawkins, R.A., 2000, The prostate: diagnostic evaluation of metastatic disease. Radiol Clin North Am, 38(1):139-57, ix.
23. Yonou, H., et al., 2004, Intraosseous growth of human prostate cancer in implanted adult human bone: relationship of prostate cancer cells to osteoclasts in osteoblastic metastatic lesions. Prostate, 58(4):406-13.
24. Hsieh, C.L., et al., 2004, Cotargeting tumor and stroma in a novel chimeric tumor model involving the growth of both human prostate cancer and bone stromal cells. Cancer Gene Ther, 11(2):148-55.
25. Mundy, G.R., 1997, Mechanisms of bone metastasis. Cancer, 80(8 Suppl):1546-56.
26. Sugihara, A., et al., 1998, Expression of cytokines enhancing the osteoclast activity, and parathyroid hormone-related protein in prostatic cancers before and after endocrine therapy: an immunohistochemical study. Oncol Rep, 5(6):1389-94.
27. Blomme, E.A., et al., 1999, Skeletal metastasis of prostate adenocarcinoma in rats: morphometric analysis and role of parathyroid hormone-related protein. Prostate, 39(3):187-97.
28. Zhang, J., et al., 2001, Osteoprotegerin inhibits prostate cancer-induced osteoclastogenesis and prevents prostate tumor growth in the bone. J Clin Invest, 107(10):1235-44.
29. Shah, A.H., et al., 2002, Suppression of tumor metastasis by blockade of transforming growth factor beta signaling in bone marrow cells through a retroviral-mediated gene therapy in mice. Cancer Res, 62(24):7135-8.
30. Fizazi, K., et al., 2003, Prostate cancer cells-osteoblast interaction shifts expression of growth/survival-related genes in prostate cancer and reduces expression of osteoprotegerin in osteoblasts. Clin Cancer Res, 9(7):2587-97.
31. Koutsilieris, M., Rabbani, S.A., and Goltzman, D., 1986, Selective osteoblast mitogens can be extracted from prostatic tissue. Prostate, 9(2):109-15.
32. Koutsilieris, M., et al., 1987, Characteristics of prostate-derived growth factors for cells of the osteoblast phenotype. J Clin Invest, 80(4):941-6.
33. Killian, C.S., et al., 1993, Mitogenic response of osteoblast cells to prostate-specific antigen suggests an activation of latent TGF-beta and a proteolytic modulation of cell adhesion receptors. Biochem Biophys Res Commun, 192(2):940-7.
34. Koutsilieris, M., 1993, Osteoblastic metastasis in advanced prostate cancer. Anticancer Res, 13(2):443-9.
35. Ware, J.L., 1993, Growth factors and their receptors as determinants in the proliferation and metastasis of human prostate cancer. Cancer Metastasis Rev, 12(3-4):287-301.
36. Lang, S.H., Miller, W.R., and Habib, F.K., 1995, Stimulation of human prostate cancer cell lines by factors present in human osteoblast-like cells but not in bone marrow. Prostate, 27(5):287-93.
37. Burfeind, P., et al., 1996, Antisense RNA to the type I insulin-like growth factor receptor suppresses tumor growth and prevents invasion by rat prostate cancer cells in vivo. Proc Natl Acad Sci U S A, 93(14):7263-8.
38. Martinez, J., Silva, S., and Santibanez, J.F., 1996, Prostate-derived soluble factors block osteoblast differentiation in culture. J Cell Biochem, 61(1):18-25.
39. Ritchie, C.K., et al., 1997, The effects of growth factors associated with osteoblasts on prostate carcinoma proliferation and chemotaxis: implications for the development of metastatic disease. Endocrinology, 138(3):1145-50.
40. Festuccia, C., et al., 1999, Osteoblasts modulate secretion of urokinase-type plasminogen activator (uPA) and matrix metalloproteinase-9 (MMP-9) in human prostate cancer cells promoting migration and matrigel invasion. Oncol Res, 11(1):17-31.
41. Koeneman, K.S., Yeung, F., and Chung, L.W., 1999, Osteomimetic properties of prostate cancer cells: a hypothesis supporting the predilection of prostate cancer metastasis and growth in the bone environment. Prostate, 39(4):246-61.
42. Bissell, M.J., Le Beyec, J., and Anderson, R.L., 2002, Prostate cancer in bone: importance of context for inhibition of matrix metalloproteinases. J Natl Cancer Inst, 94(1):4-5.
43. Shariat, S.F., et al., 2002, Association of preoperative plasma levels of insulin-like growth factor I and insulin-like growth factor binding proteins-2 and -3 with prostate cancer invasion, progression, and metastasis. J Clin Oncol, 20(3):833-41.

44. Bratland, A., et al., 2003, The metalloproteinase inhibitor TIMP-2 is down-regulated by androgens in LNCaP prostate carcinoma cells. Clin Exp Metastasis, 20(6):541-7.
45. Mohammad, K.S. and Guise, T.A., 2003, Mechanisms of osteoblastic metastases: role of endothelin-1. Clin Orthop, (415 Suppl):S67-74.
46. Uehara, H., et al., 2003, Effects of blocking platelet-derived growth factor-receptor signaling in a mouse model of experimental prostate cancer bone metastases. J Natl Cancer Inst, 95(6):458-70.
47. Guise, T.A. and Mohammad, K.S., 2004, Endothelins in bone cancer metastases. Cancer Treat Res, 118:197-212.
48. LeRoy, B.E., Sellers, R.S., and Rosol, T.J., 2004, Canine prostate stimulates osteoblast function using the endothelin receptors. Prostate, 59(2):148-56.
49. Masuda, H., et al., 2004, Expression of bone morphogenetic protein-7 (BMP-7) in human prostate. Prostate, 59(1):101-6.
50. Koutsilieris, M., Mitsiades, C., and Sourla, A., 2000, Insulin-like growth factor I and urokinase-type plasminogen activator bioregulation system as a survival mechanism of prostate cancer cells in osteoblastic metastases: development of anti-survival factor therapy for hormone-refractory prostate cancer. Mol Med, 6(4):251-67.
51. Koutsilieris, M., et al., 1997, Growth factors mediate glucocorticoid receptor function and dexamethasone-induced regression of osteoblastic lesions in hormone refractory prostate cancer. Anticancer Res, 17(3A):1461-5.
52. Achbarou, A., et al., 1994, Urokinase overproduction results in increased skeletal metastasis by prostate cancer cells in vivo. Cancer Res, 54(9):2372-7.
53. Goltzman, D., 1997, Mechanisms of the development of osteoblastic metastases. Cancer, 80(8 Suppl):1581-7.
54. Mundy, G.R., 2002, Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer, 2(8):584-93.
55. Tso, C.L., et al., 2000, Androgen deprivation induces selective outgrowth of aggressive hormone-refractory prostate cancer clones expressing distinct cellular and molecular properties not present in parental androgen-dependent cancer cells. Cancer J, 6(4):220-33.
56. Brubaker, K.D., et al., 2003, Prostate cancer expression of runt-domain transcription factor Runx2, a key regulator of osteoblast differentiation and function. Prostate, 56(1):13-22.
57. Reese, D.M., et al., 2001, HER2 protein expression and gene amplification in androgen-independent prostate cancer. Am J Clin Pathol, 116(2):234-9.
58. Shariat, S.F., et al., 2001, Plasma levels of interleukin-6 and its soluble receptor are associated with prostate cancer progression and metastasis. Urology, 58(6):1008-15.
59. George, D.J., 2002, Receptor tyrosine kinases as rational targets for prostate cancer treatment: platelet-derived growth factor receptor and imatinib mesylate. Urology, 60(3 Suppl 1):115-21; discussion 122.
60. Kim, S.J., et al., 2003, Blockade of epidermal growth factor receptor signaling in tumor cells and tumor-associated endothelial cells for therapy of androgen-independent human prostate cancer growing in the bone of nude mice. Clin Cancer Res, 9(3):1200-10.
61. Zayzafoon, M., Abdulkadir, S.A., and McDonald, J.M., 2004, Notch signaling and ERK activation are important for the osteomimetic properties of prostate cancer bone metastatic cell lines. J Biol Chem, 279(5):3662-70.
62. Lee H-L, et al., 2002, The effect of growth factors and cytokines on the growth of androgen sensitive and insensitive human prostate cancer cells in vitro. Pro Am Asso Cancer Res , 43:187
63. Reyes-Moreno, C., et al., 1998, Osteoblast-derived survival factors protect PC-3 human prostate cancer cells from adriamycin apoptosis. Urology, 52(2):341-7.
64. Koutsilieris, M., Tzanela, M., and Dimopoulos, T., 1999, Novel concept of antisurvival factor (ASF) therapy produces an objective clinical response in four patients with hormone-refractory prostate cancer: case report. Prostate, 38(4):313-6.
65. Cheville, J.C., et al., 2002, Metastatic prostate carcinoma to bone: clinical and pathologic features associated with cancer-specific survival. Cancer, 95(5):1028-36.
66. Tenta, R., et al., 2004, Bone microenvironment-related growth factors modulate differentially the anticancer actions of zoledronic acid and doxorubicin on PC-3 prostate cancer cells. Prostate, 59(2):120-31.
67. Hullinger, T.G., et al., 1998, Effect of bone proteins on human prostate cancer cell lines in vitro. Prostate, 36(1):14-22.
68. Koutsilieris, M., et al., 2001, A combination therapy of dexamethasone and somatostatin analog reintroduces objective clinical responses to LHRH analog in androgen ablation-refractory prostate cancer patients. J Clin Endocrinol Metab, 86(12):5729-36.
69. Koutsilieris, M., et al., 2002, Combination of dexamethasone and a somatostatin analogue in the treatment of advanced prostate cancer. Expert Opin Investig Drugs, 11(2):283-93.
70. Hsieh, C.L., Kubo, H., and Chung, L.W., 2004, Gene therapy for prostate cancer bone metastasis. Gene therapy targeting bone metastasis. Cancer Treat Res, 118:231-90.

71. Dimopoulos, M.A., et al., 2004, Combination of LHRH analog with somatostatin analog and dexamethasone versus chemotherapy in hormone-refractory prostate cancer: a randomized phase II study. Urology, 63(1):120-5.

72. Akakura, K., et al., 2003, Possible mechanism of dexamethasone therapy for prostate cancer: suppression of circulating level of interleukin-6. Prostate, 56(2):106-9.

Chapter 20

Current and Future Therapeutic Targets of the Tumour-Host Microenvironment

Jareer Kassis, Emilyn Alejandro, Victoria Virador and Elise C. Kohn
Molecular Signalling Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892, USA

Abstract: The search for effective cancer therapies is one of the foremost priorities of modern-day research. In recent times, enormous strides have been made in the understanding of the molecular events that underlie cancer progression and in the development of promising therapies against relevant molecular targets. The molecular signalling pathways that modulate tumourigenesis, especially those involved in cell migration and adhesion are logical foci for molecular intervention. Angiogenesis is a validated target from which combination approaches are now being entertained, and agents against new and known targets are under development. Further advances are needed to use these agents to their best advantage and to demonstrate proof of concept of the target. To that end, new technologies are being developed and applied to patient trials. The local microenvironment and the tumour-host interface are dynamic areas that are understudied as therapeutic directions. Stromal therapy, focused to the paracrine interactions at the microenvironmental level is an important new direction.

Key words: Migration, adhesion, angiogenesis, molecular therapeutics, stem cells, microenvironment, tumour progression, membrane receptors, integrins, proteomic analysis, EMT, MET, VEGF, PDGF, fibroblast growth factor, growth factor receptors, extra cellular matrix

1. INTRODUCTION

Numerous abnormal events in a cell's microenvironment contribute to the threshold driving it to dysregulated growth. The sheer number of molecules that are involved in cell growth, and as such, abnormal growth, makes pinpointing a single therapeutic target not always feasible. The quest for a cure for cancer is unlikely to be satisfied by a single "killer drug". Very few if any cancers are truly monogenic phenotypes. Those that appear to be, such as chronic myelogenous leukemia, driven by the bcr-abl fusion gene, have taught us that cancer remains plastic and able to circumvent our best interventions. One method to overcome the plasticity of cancer is to recognize that cancer is not simply a disease of autonomous cells, but still a disease of local communication. The study of the tumour-host microenvironment is a leading area of investigation addressing this important biological and biochemical event and has already yielded many potential therapeutic targets.

Although abnormal tumour growth is the fundamental problem in cancer, it comes second in importance to tumour invasion and metastasis, the *sine qua non* of cancer. A tumour that does not invade or metastasize is generally non-malignant and more likely curable; it is when a tumour invades into the local environment and enters the vasculature or lymph nodes that patient survivability decreases

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 345-367.

dramatically. Hence, it becomes critical to address the cellular mechanisms of invasion and metastasis in the context of the tumour and its local milieu from which to identify and validate targets for therapeutic intervention. Invasion and metastasis can be broken into three main categories: Recognition of and association with the extracellular milieu, disruption and penetration of the surrounding milieu, and cell migration through the surrounding environment to the vascular or lymph vessels (1-3). The interaction between the tumour and its local micro-milieu is a key factor in invasion.

As such, current research has been centred heavily on elucidating factors that promote tumour cell invasion. There have been tremendous advances in dissecting the cellular signalling pathways and regulation of secreted factors, such as proteases. Many insights have been developed into the signalling and cellular machinery involved in migration through the extracellular matrix (ECM). Such signalling pathways are interconnected and interdependent. A series of individual disruptions in the proper function of key molecules can push a threshold that collectively shifts the cell's machinery towards dysregulated growth.

The microenvironment plays a major role in tumour growth and development prior to or concurrent with invasion by initiating and propagating cellular events. Tumour cells receive positive and negative stimuli from several sources. Cell-cell contact and cell-ECM contact are examples of outside-in signalling (4-6). In addition, soluble factors such as growth factors provide added input. These can result in inside-out signalling through secondary production and secretion of factors for autocrine and paracrine consumption and inside-out stimulation of cell-cell or cell-matrix interactions or release. Such mechanisms have similarities in their signalling. Growth factor receptors of the receptor tyrosine kinase class, such as the epidermal growth factor receptor (EGFR) or the platelet-derived growth factor receptor (PDGFR), activate a range of downstream molecules through direct or indirect phosphorylation and second messenger release (7, 8). Example molecules for which involvement in invasion, metastasis, and or malignant progression have been shown include phospholipase C-γ (PLCγ), extracellular-signal regulated kinase (ERK), and src. These molecules also may be stimulated by integrins, transmembrane receptors for ECM molecules. Thus, a significant number of cytoplasmic molecules serve as convergence points for different upstream sources. These convergence points are logical targets for molecular therapeutic intervention.

Tumours are not alone in their outside-in and inside-out signalling. Cellular components of the local microenvironment, inflammatory cells, endothelial cells, mesenchymal cells, are also dynamically interacting with the environment and responding to changes stimulated by the intrusion of the cancer. Hence, angiogenesis, the process of sustaining tumour microenvironment via sprouting of vasculature becomes essential. Angiogenesis is the endothelial cell response to the activation of its signalling pathways through exposure to paracrine growth factor and ECM changes. It requires the same events: interaction with the ECM, local proteolysis, migration and proliferation (1). The study of angiogenesis has taken priority among many investigators to discover methods of starving tumours through its disruption.

Cell signalling is a highly dynamic process, and select cell responses are linked to a cascade of pathways. A current focus is to pinpoint potential cellular and extracellular components that, when *collectively* inhibited, would produce successful blockade of the tumour and of its coupled support structures. The tumour-host interface provides one important area of exploration, especially as it is at this juncture where major mechanisms of tumour invasion occur.

2. THE CANCER MICROENVIRONMENT

A cell's normal function is characterized by its ability to maintain its balance through a myriad of internal and external stimuli to which it is exposed to during its life cycle (Figure 1). A combination of growth factors, extracellular and intracellular ion fluxes, extracellular matrix proteins, and other stimuli act in concert to maintain a cell's function. There are constant gradients of any particular ligand

or ion on either side of the cell membrane that act to maintain or reverse particular stimuli. Normal cells have a series of checks and balances to ensure that no stimulus is maintained beyond its physiologic requirement. It is failure of these controls that contributes to abnormal growth and invasive phenotypes. Therefore, an understanding of these checks and balances in the context of the local tumour microenvironment, and the cellular switches that control them, is a major goal for successful development of therapeutics. At least three caveats exist when trying to apply findings of bench research to actual therapies. First, much of the research is done in a two-dimensional *in vitro* environment in cell cultures that do not necessarily translate to a physiological three-dimensional environment. For example, cells surrounded by ECM in a three-dimensional setting do not form the same stress fibre and focal contact patterns as cells on two-dimensional settings (9). Second, the use of animal models is almost universal, although it is unknown how relevant these models are to human cancer. Third, artificial manipulation of pathways through mutation, xenograft carcinogen, and transgenic stimuli create models that are unlikely to recapitulate the human cancer molecularly. Thus, successful traversal of pathways in these models may be useful for proof of concept but may not provide accurate information related to human disease.

2.1 Membrane receptors interact with the microenvironment to modulate tumour progression

Growth factor receptors play a major role in relaying signals at the tumour-host interface. The extracellular domains of these transmembrane proteins bind a wide variety of ligands that are present in the extracellular milieu, secreted by adjoining or local cells (paracrine signalling) or the cell itself (autocrine signalling). Receptor tyrosine kinases contain cytoplasmic kinase domains that phosphorylate target tyrosine residues. These receptors also have cytosolic domain target tyrosine residues that can be autophosphorylated or transactivated by kinase domains of other receptors with which they are aggregated when stimulated (8, 10-12). Receptor tyrosine kinases also phosphorylate and activate downstream effector molecules tyrosine residues located on the recipient molecules' src homology 2 (SH2) or phosphotyrosine binding (PTB) domains (13). There are at least three potential ways that receptors can become dysregulated in their signalling: activating or blocking mutation, hyperactivity due to increased or prolonged stimulus, or overexpression of receptor quantity. Overexpression of receptors, due to genomic amplification, transcriptional activation and/or increased survival of message and/or protein is a common receptor event in transformed cells. Increased receptor availability means the potential for increased interaction with microenvironmental stimuli. Among the most studied of such overexpressed receptor tyrosine kinases is the prototypical epidermal growth factor receptor (EGFR), which has the advantage that it does not share its main ligands (such as EGF or TGFα) with any other receptor (8).

Studies of the EGFR have shed tremendous light on the plethora of signalling events that are triggered by growth factor receptors and continue to reveal significant aspects of cell function and growth. It is involved in almost all carcinomas studied to date in some fashion and is also a target in the microenvironment (14). EGFR is a major target for therapeutic intervention, most recently with FDA-approved drugs such as cetuximab (Erbitux) or gefinitib (Iressa). Both agents have been shown experimentally to regulate both the tumour and the local events, such as angiogenesis (15, 16). Proteins that lie parallel to or downstream of EGFR have provided valuable insight as to how signals associated with tumour-host interactions are propagated and act in concert to provide for cell signalling dysregulation. Cell migration, a rate limiting step in tumour invasion, is a prime example of such dysregulated signalling, and the role of EGFR in migration will be used for discussion in order to give a sense of the complexity of pinpointing proper therapeutic targets in the leading invasive edge.

2.2 Growth factor receptors sense environmental signals that stimulate cell migration

Growth factor-mediated migration involves a meshwork of cellular pathways that produce a series of events that cause a cell to translocate from one area to another (Figure 2). Prior to its actual movement, a cell becomes polarized and forms a leading edge at one end and a trailing edge on the other by creating a gradient of various ions across its membrane and cytoplasm (17, 18). In addition to extensive intracellular signalling, chemotactic stimuli in the form of ions, ECM proteins, or fragments from the microenvironment help mould the cell into a polarized state (19), and many studies illustrate the marked localization of individual cellular components to either the leading or trailing edge (20, 21). The next step in migration is extension, where the leading edge extends forward and attaches to a new scaffold position. Extension involves a series of signalling events coupled to actin cytoskeleton polymerization to extend the lamellipod forward (22). EGFR or other receptor tyrosine kinases and their downstream effectors stimulate members of the cytosolic Rac and Rho families that then regulate membrane ruffling and lamellipodial extension (23-25). Concurrent with extension is the process of forming a focal contact and recruitment of outside-in signalling partners to form a focal adhesion. The focal adhesion is the site of cell anchorage to the extracellular milieu and of its signalosome through which the information is propagated (26, 27).

The focal contact is the hallmark of an adherent cell, and it defines the structure and position of tumour cells in the microenvironment. It is rich in integrins, adhesion-related kinases, and other proteins or receptors that make up the signalosome. Many of the focal adhesion components are proteins that are directly or indirectly signalled by growth factor receptors, such as EGFR. Src is a non-receptor kinase that is activated by EGFR, integrins, and other transmembrane receptors and in turn binds to such molecules as focal adhesion kinase (FAK), a major component of the focal adhesion (28). Activated FAK is required for cell migration; FAK -/- cells have severely diminished migration which can then be restored by reintroducing FAK (29, 30). FAK is also activated by integrins, signalling through its partner proteins such as paxillin and talin (29, 31). These molecules are all localized to the focal adhesion and serve as building blocks of the complex.

Cell adhesion is an important potential target for therapeutic intervention because of its importance to migration and invasion of tumour and endothelial cells. Targeting adhesion, while not dealing with tumour growth, could restrict the tumour's ability to migrate and invade. However, such therapies may not be sufficient as tumour cells lose the apoptotic response to matrix-detachment, a process known as anoikis (32). This is an important differential between malignant cells and normal cells. Malignant invasive carcinoma cells are not attached to basement membrane as are normal epithelial cells, as they develop an acute survival mechanism. Interestingly, upregulation of survival pathways is associated with acquisition of the metastatic phenotype (33-35). Although it makes sense that a cell that does not adhere well is more agile and motile, a cell that has abnormally strong adhesion may be equally prone to higher invasion and especially metastasis, as it requires tumour cells attaching at distant locales. Such complexity in the mechanisms of adhesion only enhances the importance of further study and provides numerous potential sites for therapeutic intervention.

The next step of migration is cell body translocation, which involves a shift of the main bulk of the cell from the original point towards the new leading edge once focal adhesions have secured it. This step involves receptor signalling that cause the actin cytoskeleton to contract and pull the cell body forward. EGFR and other receptor tyrosine kinases are involved in this step. PLCγ, a downstream target of receptor tyrosine kinases, is activated by binding the autophosphorylated receptor tyrosine residues on the cytoplasmic tail through its SH2 domains. After it is phosphorylated by the activated receptor, PLCγ cleaves its substrate, phosphatidyl inositol bisphosphate (PIP_2), on the cell membrane. PIP_2 normally anchors a number of actin modifying proteins such as profilin, which is involved in actin polymerization, and gelsolin, involved in actin capping, that are released upon

PIP_2 cleavage (36-38). The cleavage products of PIP_2 are inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 is a potent activator of intracellular calcium release and secondary calcium influx that is needed to activate cytoskeletal reorganization (39, 40). DAG activates common and novel protein kinase C isozymes (PKC), functioning as calcium ion regulators as well as kinases to phosphorylate critical serine and threonine residues for further signal propagation to downstream effectors such as paxillin, ezrin, and akt (41-47).

The final step in migration is detachment of the trailing edge, which determines the rate of cell migration (48). It includes decomposition of the focal adhesions and release of the cell from its link to the local microenvironment. It is not completely clear what triggers a cell to dissolve focal adhesions. Cytoskeletal contractile forces pull the cell forward, with receptor signalling, ECM interaction, and cellular ion gradients playing a combined role in stimulating focal adhesion disassembly (26, 49). Integrins are shed as the cell detaches (50). This can result in further loss of signal and accelerate focal adhesion disassembly. Activation of src and ERK by receptor tyrosine kinases such as EGFR also activates calpain (51-53), a calcium-dependent protease which cleaves numerous focal adhesion proteins. A shift of balance from promotion of adhesion and motility to increased disassembly and would be a mechanism through which adhesion and migration could be shut down. Studies have shown that these principles of adhesion and migration apply in concept to three dimensional model environments, as would be the case *in vivo* (9).

2.3 Cells receive survival signals from the microenvironment

More recent studies have elucidated important links between seemingly independent pathways. For a long time, the proliferation, survival, and metastatic pathways were perceived as unrelated. The demonstration that apoptosis caused by loss of attachment to ECM, anoikis, is identical to that of growth factor starvation or other forms of programmed cell death, links the survival and invasion pathways. Proteins such as phosphatidylinositol 3' kinase (PI3K) that have been shown to be necessary and sufficient to protect many cell types, including endothelial cells, from anoikis and other forms of death are also activators of angiogenesis and metastasis (54-56). Lysophosphatidic acid (LPA) activates PI3K downstream of its G protein-coupled receptor (57). This results in upregulation of invasion and metastasis, inhibition of anoikis, and production of proangiogenic cytokines and proliferative stimuli.

Other links have been identified. PLCγ has also been shown to be phosphorylated by FAK (58). This implies an indirect recruitment of PLCγ by integrins, the major upstream activators of FAK and link to the outside microenvironment. In separate studies, FAK has been shown to interact with the tumour and metastasis suppressor PTEN (59), one function of which is to inhibit activity of the survival pathway protein Akt. Akt is regulated by PIP_3, the product of PI3K, which itself is activated downstream of the EGFR family and other receptor tyrosine kinases and is an activator of Akt (60-64). These findings reinforce that the cell is meshwork of interconnected signalling pathways responsive to outside-in receptor and ECM-mediated signalling. They also show that targeting the invasion and metastasis processes will also entail targeting tumour and endothelial cell survival. Confrontation at multiple signalling fronts would be expected to be at least additive and perhaps synergistic.

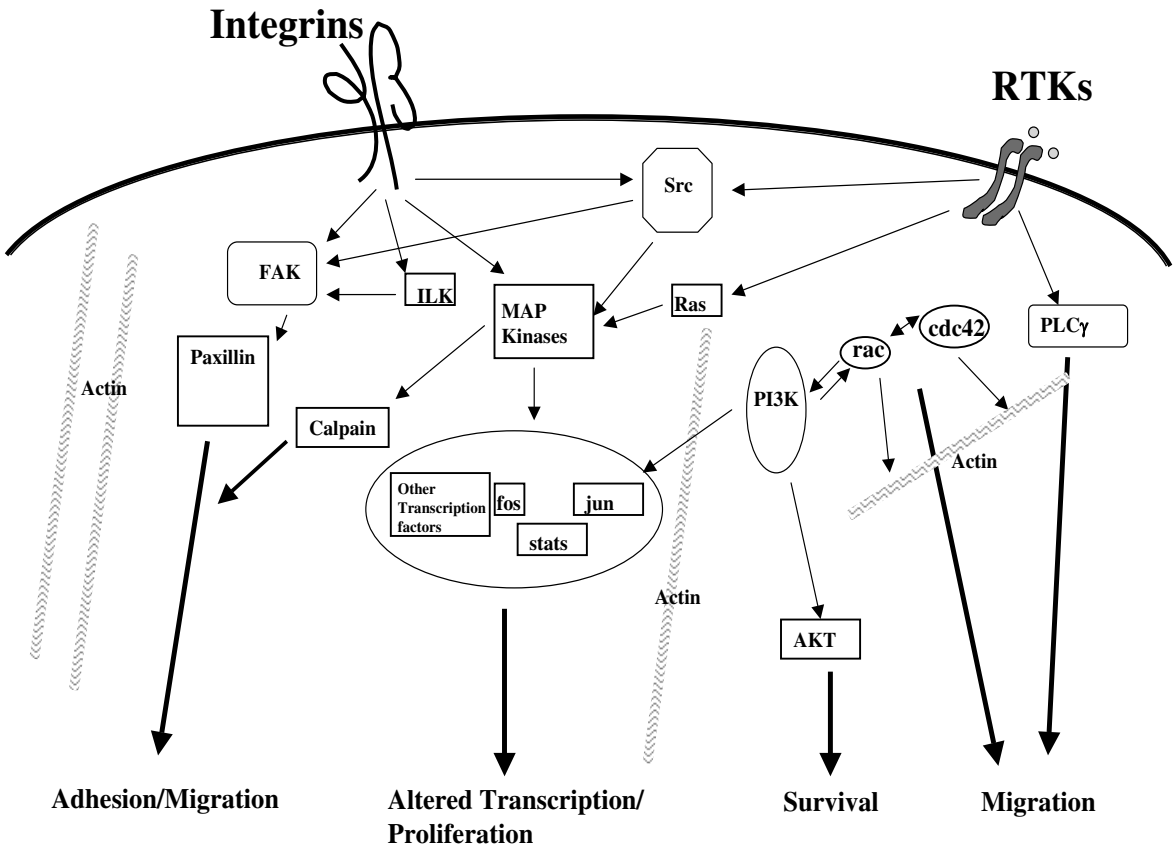


Figure 1. Some of the signalling pathways involved in tumour cells. Receptors at the membrane such as receptor tyrosine kinases (RTKs) or integrins propagate downstream effector molecules that, while stimulating phenotypic changes such as migration or proliferation, are also involved in cross-talk.

2.4 Non-receptor tyrosine kinase target proteins involved in microenvironment signalling

Integrins. Integrins are heterodimeric transmembrane receptors consisting of an α and β chain. Their function is to communicate between cells and their surrounding ECM through large extracellular domains, while transmitting signals to the intracellular milieu through the cortical actin cytoskeleton via short cytoplasmic domains (65, 66). Integrins crosstalk with proteins involved in cell survival, proliferation, and differentiation pathways showing their versatility (4). As integrins aggregate at the focal adhesion , they recruit their signalosome components that signal downstream to promote motility, survival, and/or proliferation. However, the redundancy in integrin ligands and signal targets has made understanding such recruitment and signalling difficult. This redundancy is in part traversed as integrins that bind multiple ECM ligands may propagate different intracellular messages. For example, it is thought that selective adapter proteins, such as caveolin (67), interact with some α subunits of integrin but not others to produce unique signalling. Integrin β1, a ligand for multiple ECM components, has been shown to be required for fibroblast and tumour cell migration across three-dimensional matrices (reviewed in (9)). Alternatively, other groups have shown that receptor affinity to their ligands can be switched from low to high via unidentified intracellular components (68).

Many studies have shown that blocking of integrins in turn inhibits tumour and endothelial cell migration. These events are dependent on the select integrin subunits to be blocked and the ligand substrate to which the integrin binds and the cell is bound to and/or across which the cell is migrating. Blockade of $\alpha5\beta1$ integrin inhibits migration of many cell types on vitronectin (69), but is not effective in blockade of binding to collagen type IV. Blocking of $\alpha6\beta1$ inhibits endothelial cell migration on laminin-8, but not on fibronectin (24). Integrins are also important in regulating angiogenesis, where they play a key role in outside-in signalling from the stromal and basement membrane ECMs to promote vascular proliferation and sprouting (70). Integrin $\alpha v\beta3$, a receptor for vitronectin, is a validated molecular target for intervention of angiogenesis. Inhibitors to $\alpha v\beta3$ (Vitaxin), $\alpha v\beta5$, or both (Cilengitide), as well as inhibitors of $\alpha5\beta1$ are currently in different phases of clinical trials (reviewed in (70)). Both peptidomimetics and blocking antibodies have been advanced from preclinical studies into clinical trials (71). This integrin is selectively partnered with vascular endothelial growth factor receptor-2, providing a mechanism for signal amplification and driving a logical combination of these agents with those that inhibit VEGFR2.

Among the downstream proteins activated by integrins are integrin-linked kinase (ILK), FAK, and src, all currently active targets for small molecule therapeutics. Combinatorial therapy of integrin inhibition with inhibitors of their downstream signalling and/or trans-signalling events may provide a less toxic but equally effective approach.

Matrix metalloproteinases. Several classes of proteases are under study and are targets for therapeutic intervention. The matrix metalloproteinases (MMPs) are the best studied to date. They are structurally related endopeptidases that dissolve and rearrange the extracellular matrix, are dependent on zinc, and are induced by growth factors or cytokines. MMPs are key interlocutors with the microenvironment in at least two ways. First, they degrade the ECM to carve a path through which cells migrate into the mesenchyme; and second, they release soluble factors from the extracellular matrix (72, 73). They are structurally divided into functional groups on the basis of their substrate (73-76). MMPs are also required for the invasive behaviour underlying angiogenesis (77). Increased MMP activity in tumours is often directly correlated with poorer prognosis, with many studies illustrating the role of various MMPs with different types of tumour invasion and metastasis (76, 78). There are at least four types of described tissue inhibitors of metalloproteinases (TIMPs). These interact with MMPs dynamically to balance ECM remodelling. MMPs are released or bound to membrane molecules as inactive zymogens that are activated proteolytically outside the cell membrane autocatalytically or by directly cleavage by extracellular proteinases (73). Attempts have been made to therapeutically target MMPs; therapeutics development can be and has been general or subclass specific and has been of limited success to date. Several first and second generation agents have had untoward toxicities (79, 80).

Rho-family GTPases. Rho family proteins have a vital role in changing cell morphology and organization of actin in adhesion, migration, and proliferation. They are molecular switches that are active when bound to GTP, from which they activate their downstream effector molecules, or inactive when bound to GDP (81). Three members of the Rho family stand out due to their role in membrane protrusion and ruffling: Rac, RhoG, and Cdc42. Rac stimulates membrane actin organization into lamellipodial extensions; RhoG, a newer member of the Rho family, may activate Rac (81), and Cdc42 induces actin polymerization into filopodial formation (81, 82). Effectors of these members of the Rho family also can function as upstream activators, creating circular loops to enhance their effects. These molecules also are key to cell polarization and are necessary for tumour and vascular remodelling. Cdc42 recruits the microtubule organizing centre towards the leading edge of the cell, leading to an overall increased presence of specific molecules involved in the protrusive machinery. PAK1, which itself can activate Cdc42 through stimulation by G protein-coupled receptors is also a target of Cdc42 (83). Cdc42 may also recruit PI3K, generating PIP_3 that stimulates Rac resulting in lamellipodial extension and inhibition of PTEN (83). Finally, through

positive feedback loops, Rac activates PI3K, leading to more Rac activation. Integrins which aggregate at the leading edge activate Rac, which leads to more integrin recruitment (66). On the trailing edge of the cell, RhoG, which both activates and is antagonistic to Rac, stabilizes the microtubules and facilitates focal adhesion disassembly, thus helping to release the rear end of the cell.

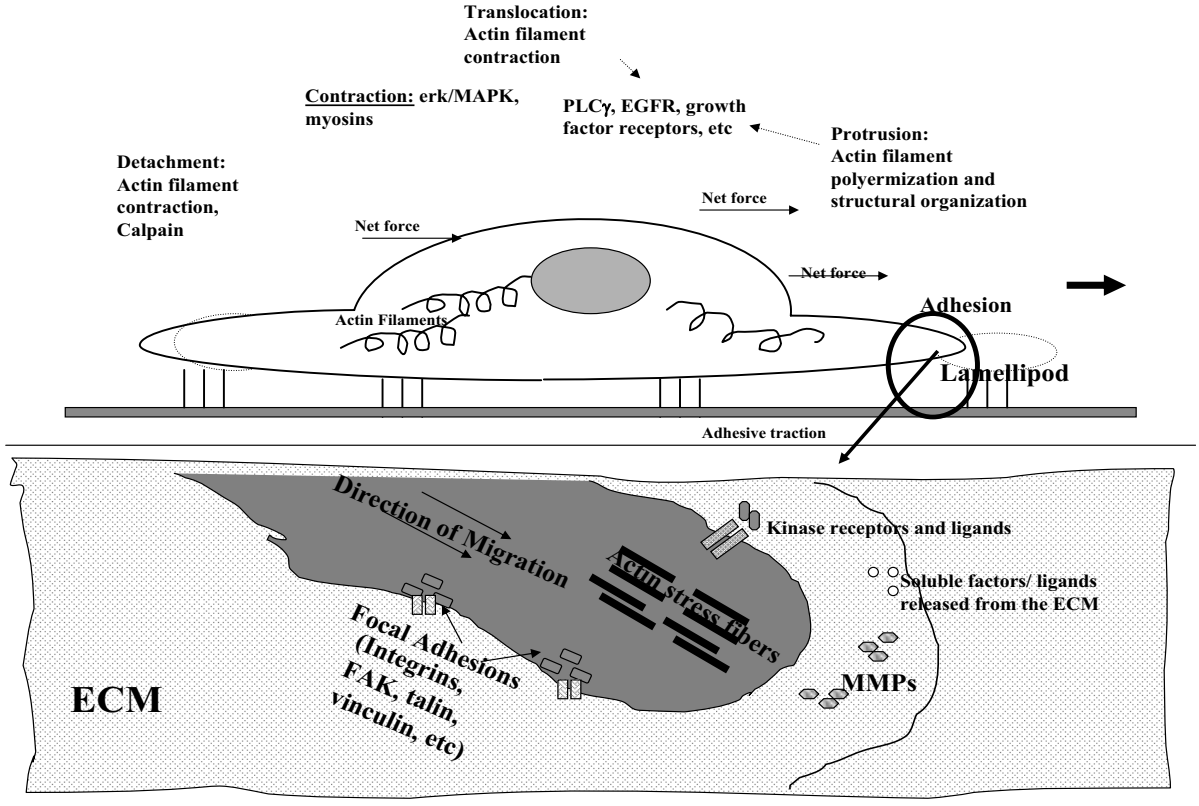


Figure 2. Cell migration and amplified molecular events at the tumour cell - ECM interface. A host of signalling pathways are involved in cell polarization, extension of the leading edge, cell body translocation, and detachment from the leading edge. In invasion, these events are dysregulated, and cells press through the ECM with abnormal focal adhesion signalosome and MMP secretion activity and also release soluble factors from the ECM to propagate the abnormal cellular signalling (Based on (26)).

2.5 Therapeutic targeting of signalling pathways governed by cancer cell – microenvironment interactions

Biological systems are redundant. This is necessary to allow the normal cell to maintain a dynamic stability in the face of a changing local environment (1). This is demonstrated in part by the ability of normal cells to tolerate some signal interruption whereas in some cases the tumour cell will die. Normal cells are less addicted to selected pathways and are able to use a redundant mechanism to protect themselves. Such a mechanism may be involved in either physiological or pathological situations. Angiogenesis is critical in wound healing, development, and reproduction, but is also seen in tumour dissemination. Thus, context is important for checks and balances to work. Activation of endothelial cells in corpus luteum development is under local microenvironment control allowing it to be turned off when necessary, whereas activation of endothelial cells in malignant

neovascularization and even in psoriasis is not responsive to stop messages if they exist. Cell migration through three-dimensional lattices uses proteolytic enzymes to carve a path for the cells. When proteolysis was impaired by the use of inhibitors, cells adapted to use a different method of migration, amoeboid migration. This was associated with a decrease in β1 integrin clustering (84).

2.5.1 Targeting the receptors

Many current therapeutic studies target the membrane receptors that are the most upstream of the dysregulated signalling pathways. To this end, targeting EGFR is one of the modern approaches to targeting tumours in patients. Gefitinib (Iressa) is one of several perceived promising therapeutic agents at this writing to target EGFR. It is a specific EGFR tyrosine kinase inhibitor that blocks autophosphorylation by binding to the ATP binding site of the kinase and results in inhibition of activation of downstream targets such as ERK or PAK-1 (85). This agent has been shown to increase survival in lung cancer patients when administered with chemotherapy in a phase II trial (86, 87). Preclinical data also has shown it to be useful in combination with other therapies such as radiation therapy and trastuzumab (Herceptin) (88). Other agents under investigation are not specific only to EGFR but can target two or more members of the EGFR/ErbB receptor family. GW572016 is a dual EGFR and ErbB2 tyrosine kinase inhibitor that has shown some degree of antitumour response (89). Erlotinib (OSI-774) is another EGFR/ErbB2 inhibitor that has show activity in patients with a number of cancers including ovarian and pancreatic tumours (90). These agents are of interest as it has been shown that overexpression of ErbB2 can blunt the response to EGFR-selective agents (91). The family of agents targeting ErbB family members may also be anti-angiogenic. It has been shown in experimental models that endothelial cells within the proximity of a EGF (or TGFα)-secreting tumour can upregulate their EGF receptors (92). This presents a plasticity of the local microenvironment that needs to be considered in signal transduction therapy.

An alternative approach to addressing paracrine microenvironment interactions is to look at the signal interrupting agents that have multiple targets, such as imatinib mesylate (Gleevec). This agent was originally developed for its ability to inhibit the abl kinase, within the bcr-abl fusion protein (93). Further studies have demonstrated that it also inhibits other members of the type III receptor tyrosine kinase family, c-kit and PDGFR. The c-kit target has come into attention due to the striking clinical responses of gastrointestinal stromal tumours (GIST), especially those with an activating mutation of c-kit (94). Also, recent preclinical modelling of STI-571 in combination with gemcitabine has shown promise in preclinical studies, especially in the pancreas (95).

3. ANGIOGENESIS: THE OTHER FRONTIER IN CANCER RESEARCH AND PREVENTION

Research over the past several decades has shown cancer tissues to require new blood vessel formation to proliferate, invade, and metastasize (1, 96-98). To obtain sufficient oxygen and nutrients, tumours need an increase in blood supply to grow. One way to achieve this is by stimulating angiogenesis, the process of new vessel formation from pre-existing vasculature in response to angiogenic stimuli released by tumour cells, stromal cells, and inflammatory cells recruited to the tumour site (99). The process of angiogenesis is a complex cascade that applies the same components of invasion as are used by malignant tumour cells coupled with final development of the capillary lumen (100). The newly formed normal microvasculature is then stabilized by deposition of basement membrane, recruitment and maturation of pericytes, and subsequent inhibition of endothelial cell growth (101). These maturation steps do not happen normally if at all in the microvasculature of malignancy.

Three decades ago, Judah Folkman proposed tumour neovascularization might be a novel target for cancer therapy (98, 102). Although inhibition of angiogenesis seems to be a promising cancer therapy, there are many caveats to consider. Treatments must selectively focus to the vasculature

network of tumours, and avoid neovascularization necessary for normal activities such as wound healing and reproductive function. Tumour vasculature is also characterized by endothelial cell proliferation, an event that is otherwise only seen in development, reproductive organ activity, and wound healing. Angiogenesis-associated endothelial cells proliferate at a higher rate than endothelial cells in normal tissue (103). Moreover, tumour-associated vessels are distinctly aberrant. They lack pericytes and are morphologically fragile, leaky, permeable to large molecules, and have reversible blood flow (99, 104). The complex process of angiogenesis is tightly regulated by pro- and anti-angiogenic growth factors. Clinical studies are ongoing both to stimulate angiogenesis such as in cardiovascular disease and diabetes wound healing, and to inhibit angiogenesis in cancer and proliferative processes.

3.1 Therapeutics in angiogenesis

VEGF and its Receptors. Endothelial cells interact with a variety of other cells within particular microenvironments. These include tumour cells, immune cells, fibroblasts, pericytes, and the ECM. The relationships and cross-talk among these cells and the microenvironment determine gene expression and whether endothelial cells survive, proliferate, or undergo apoptosis. The most important microenvironment interaction in tumour-associated angiogenesis is the tumour cell's ability to interact with endothelial cells. Compelling evidence suggests VEGFs and their receptors play critical roles in tumour-associated angiogenesis. VEGF is a family of potent pro-angiogenic growth factors that stimulate the proliferation, migration, differentiation, and survival of vascular and lymphatic endothelial cells. In mice, the loss of a single VEGF-A allele results in embryonic lethality due to impaired angiogenesis (105, 106), indicating that VEGF is critical for global development due to the requirement for healthy vasculature in all tissue microenvironments. It has also been shown that there is a correlation between the degree of vascularization of a tumour and VEGF expression (107-109). This is consistent with the laboratory-based findings that tumours produce VEGF and many other pro-angiogenic cytokines to which the local endothelial cells respond (110, 111).

VEGF-A, also known as vascular permeability factor, was purified from ovarian cancer xenograft ascites (112, 113). The angiogenic activity of VEGF is mediated through two VEGF receptors (114). VEGFR-1 or fms-like tyrosine kinase 1 (Flt-1) (115), and VEGFR-2, known as kinase domain receptor (KDR) in humans and fetal liver kinase (Flk-1) in mice (116). Although VEGFR-1 was the first to be identified as a VEGF receptor (116), its function is still unclear. However, VEGFR-2 has been identified for its critical role in developmental angiogenesis in Flk-1 null mice and is the VEGF receptor most linked to cancer as a potential biomarker and a therapeutic target (117).

VEGF expression is upregulated in most human carcinomas and neuroendocrine tumours (114, 118, 119). It has been shown that stromal support cells and endothelial cells, as well as tumour and inflammatory cells, can produce VEGF. VEGF is thus a paracrine pro-angiogenic factor in cancer, important in local regulation creating a permissive microenvironment. In a study of advanced epithelial ovarian carcinomas, patients with tumours expressing higher levels of VEGF had shorter survival compared to those who expressed lower levels (109, 119, 120).

Elevated VEGF levels have also been shown to predict recurrence and negative overall survival in patients with breast cancer (121, 122). VEGF overexpression is also correlated with inactivation of the von Hippel-Landau gene, which is thought to be a mechanism of angiogenesis in renal cell carcinoma (123, 124). These studies and others underscore the utility of anti-angiogenic approaches targeting VEGF. Several approaches to therapy are currently under investigation and listedin Table 1. Specific small molecule inhibitors and monoclonal antibodies to VEGFR-2/KDR have reached clinical trial with varied success (125, 126). Other promising reagents against VEGFR-2 and other receptor tyrosine kinases, such as BAY-43-9006 are now in trial (127). One of the most successful anti-angiogenic agents thus far is the recently FDA-approved bevacizumad (Avastin), a recombinant humanized version of the murine anti-human VEGF monoclonal antibody. This VEGF-neutralizing antibody

inhibited VEGF-induced signalling, resulting in reduced angiogenesis and tumour growth. Bevacizumad has rapid antivascular effects in human rectal cancer patients (106). Phase-I and -II studies have revealed bevacizumad is well tolerated as a single agent and in chemotherapy combination (128). A randomized clinical trial of bevacizumad in patients with renal cell cancer resulted in a statistically significant improvement in progression-free survival while reducing vascularity and tumour burden (129); a similar positive outcome was observed in colon cancer (128). Another neutralizing antibody specific to VEGF is HuMV833, a humanized form of monoclonal antibody MV833. This antibody inhibited a wide variety of solid tumour xenografts in nude mice (130). It was also shown to reduce the permeability of blood vessels, thus slowing cancer growth. Phase I data indicate that it is well tolerated and currently working its way through different phases of clinical development (131).

Platelet-Derived Growth Factor. PDGF is a family of glycopeptides that are important mitogens for many cell types (132). The A and B chains of PDGF combine to form AA, BB and AB dimers that bind to protein tyrosine kinase receptors α and β (133) Activation of PDGF has been shown to stimulate cell growth, migration, reorganization of actin, and inhibition of apoptosis (132, 134). Moreover, PDGF stimulates growth of pericytes that surround endothelial cells (135). Preclinical studies have shown that PDGF effects angiogenesis by increasing transcription and secretion of VEGF, demonstrating further its effects on local events (136). PDGF protected human umbilical endothelial cells from apoptosis caused by serum starvation through its induction of VEGF, indicating an indirect role of PDGF in angiogenesis (137). Direct evidence for a role of PDGF-B was demonstrated in mice deficient in PDGF-B (138), which produced ruptured capillary microaneurisms in late gestation due to lack of pericytes. Many studies have identified platelet-derived growth factor BB (PDGF-BB), the ligand for PDGFβ receptor, as a major growth factor involved in smooth muscle cell recruitment during angiogenesis (139, 140). In a rat model of vascular restenosis, inhibition of the PDGF signal transduction cascade pathway by a PDGF receptor inhibitor blocked smooth muscle cell proliferation and migration after ballooning injury *in vivo* (141). Many tumour types express PDGF and its receptors, which function in autocrine or paracrine fashions in some cancers and in angiogenesis. For example, PDGF is expressed in epithelial ovarian carcinomas in contrast to borderline ovarian tumours (142, 143).

PDGFβ receptor is one of the several molecular targets of imatinib mesylate (STI571/Gleevec), a potent inhibitor of PDGF receptor kinase and also of bcr-Abl and c-kit oncoprotein kinases (139, 144). Imatinib mesylate inhibits *in vitro* angiogenesis in fibrinogen-embedded mouse aorta (145). It has had remarkable effects against c-kit mutation positive gastrointestinal stromal tumours and moderate activity against those without mutation. Notably, it was reported that uptake of fluoro-deoxyglucose on positron emission tomography was remarkably reduced within 24 hr of administration of imatinib mesylate. However, more clinical studies are underway to further understand its anti-angiogenic activity (146-148).

SU11248 is a novel oral ozindole that has been identified to target the tyrosine kinase activities of VEGFR-2 and PDGF receptor. In preclinical models of human small cell lung cancer, SU11248 inhibited KIT and PDGFβ tyrosine kinase activity (149) Results of phase I studies of SU11248 have been shown to be safe and well tolerated, and phase II studies are currently underway (150). Another PDGF inhibitor under clinical investigation is a small organic molecule, N-[4-(trifluoromethyl)phenyl] 5-methylisoxazole-4-carboxamide (SU101, Leflunomide). It has been reported that administration of SU101 significantly reduced tumour weight and tumour volume in human colon carcinoma cells in a nude mouse model (151). SU101 is also noted to repress PDGF mediated tyrosine phosphorylation of PDGFβ in rat glioma and NIH3T3 cells that overexpressed human PDGFβ (152). A Phase II study of SU101 in patients with prostate cancer indicated that SU101 as a single agent can delay progression of metastatic cancer (153). These results are promising and further investigations are ongoing.

Fibroblast growth factor. Fibroblast growth factor-2 (FGF-2), also known as basic FGF (bFGF),

belongs to a large family of growth factors consisting of 14 to 16 kDa secreted proteins that are characterized by high-affinity binding to heparin within the ECM (154). Like VEGF, FGF-2 stimulates the proliferation, migration, differentiation, and survival of endothelial cells (96, 98). However, unlike VEGF and other growth factors which are homodimeric and are secreted by cells, FGF-2 is monomeric and lacks a signal sequence for secretion (155). FGF-2 null mice are viable, suggesting redundancy of nine distinct members of the FGF family (156). Although FGF-2 knockout mice have no apparent defects related to impaired angiogenesis, FGF-2 is clearly an angiogenic factor *in vivo*, where it has been shown that angiogenesis is modulated both by vascular endothelial cell-derived FGF-2 and VEGF in autocrine fashions (157). FGF-2 has also been shown to stimulate expression of $\alpha\beta$ integrins on developing blood vessels and is believed to play a role in migration and proliferation during angiogenesis (158).

The biological effects of FGF-2 are mediated through the FGF receptors, FGFR-1,-2,-3 and –4 (159). FGFRs are characterized by a split tyrosine kinase domain. Many tumour cell lines including HT-29 human colon and ovarian cancer cell lines can synthesize FGF-2 (160, 161). Overexpression of FGF-2 in low metastatic renal carcinoma cells have been shown to increase angiogenesis and metastatic potential *in vivo* (162). Furthermore, endogenous or exogenous *in vitro* synthesis of FGF-2 stimulates the proliferation and migration of endothelial cells (163), suggesting that FGF-2 plays an important role in the invasion and metastasis of renal and bladder cell carcinoma (162, 164). FGF-2 levels in serum of patients with breast cancer (165), renal cell carcinoma (166) and other cancers are inversely correlated with survival. FGF-2 is a major angiogenic factor in ovarian carcinoma; its mRNA expression levels in both tumour and stromal cells were comparable in tumours of long-term and short-term survivors (167). Clinical targeting of FGF receptors has not been successful overall, and this may be due to the redundancy in the large number of members of the family. Agents that target FGFR in combination with other receptor tyrosine kinases, such as SU101 described above, may circumvent this problem.

Table 1. Current angiogenesis inhibitors in clinical trials (Source: www.cancer.gov)

Drugs that block activators of angiogenesis	AE-941(Neovastat™; GW786034)
	Anti-VEGF antibodies (bevacizumab; Avastin™ and HuMV833)
	Interferon-alpha
	PTK787/ZK 222584
	SU5416, 6668,
	11248
	VEGF-Trap
	ZD6474
Drugs that inhibit endothelial cells directly	2-methoxyestradiol (2-ME)
	CC-5013 (thalidomide analogue)
	Combretastatin A4 phosphate
	LY317615 (protein kinase C ß inhibitor)
	Genestein
	Thalidomide
Drugs that inhibit endothelial-specific integrin/survival signalling	EMD 121974
	Anti-αvβ3 Integrin Antibody (Medi-522; Vitaxin™)
Fibroblast growth factor receptor inhibitors	SU101, SU6668
Drugs that inhibit PDGF receptor	Imatinib mesylate (Gleevec)
	SU6668
Drugs with non-specific mechanisms of action	Carboxyamidotriazole (CAI)
	Celecoxib (Celebrex®)
	Halofuginone Hydrobromide(Tempostatin™)
	Interleukin-12

Drugs that block activators of angiogenesis	AE-941(Neovastat™; GW786034) Anti-VEGF antibodies (bevacizumab; Avastin™ and HuMV833) Interferon-alpha PTK787/ZK 222584 SU5416, 6668, 11248 VEGF-Trap ZD6474 Rofecoxib (VIOXX®)

4. THERAPIES OF THE FUTURE: EPITHELIAL-MESENCHYMAL TRANSITIONS (EMT) OR MESENCHYMAL-EPITHELIAL TRANSITIONS (MET)

There is increasing evidence that many of the developmental processes involved in embryogenesis may be similarly used by the cancer cell in its growth and dissemination through the organism (reviewed in (168)). That guidance for embryonic development is derived from the mesenchyme points to a strong involvement of the mesenchyme in guiding cancer progression. Early embryonic cells need to convert from the epithelial-like phenotype; characterized as closely arranged, in proximity, and connected to its neighbours by gap junctions; to the mesenchymal-like, largely motile, and less differentiated cells with exploratory functions towards the environment. The process occurs again in the opposite direction, when cells need to form subsequent epithelial layers such as the conversion of metanephric mesenchyme to epithelial phenotype. It is important to understand the molecular basis of these transitions during embryogenesis, as they would give insight into potential MET transitions to be achieved in cancer treatments. These processes appear to be directed by signals received from the extracellular matrix for which an important regulator is TGF-β (169). One major change involved in EMT is the downregulation of E-cadherin. This is followed by the development of a cytoplasmic actin machinery that will facilitate migration. Therefore, intracellular pathways associated with integrins and E-cadherin, such as the wnt signalling pathway, must promote these transitions as a response to cytokine signalling from the environment.

Similarly, in tumourigenesis, it appears that at an early stage, even as the primary tumour is being formed, some cells may undergo an epithelial-mesenchymal transition associated with genetic instability (170) and with upregulation of vimentin, dispersion of cytokeratin, and loss of adhesion at intercellular borders. Signals such as increased hyaluronan (171) and tenascin (172) in the ECM promote this EMT. Some examples of other possible signals contributed by the stroma are transient or chemical or viral alterations of the stroma resulting in oncogenic signals such as an increase in IL-6 or possibility that reactive stroma can be caused by aberrant expression of stromelysin-1 (MMP-3) (reviewed in (173)).

Some of the same molecular pathways used in embryonic development have been found in EMT transitions in cancer (174). Genes that were specifically involved in EMT were identified by expression profiling of seven pairs of polarized mammary epithelial cells taken as cellular models of migration, local invasion and metastasis (175). Genes associated with PDGF signalling were upregulated in mesenchymal cells as were inflammatory genes known to respond to interferon. Vimentin, type-III and type-IV collagens and various other ECM proteins were induced. As expected E-cadherin and plakoglobin were downregulated in mesenchymal as compared to epithelial cells.

In contrast with the observed plasticity of embryonic and cancer cells, adult tissues are typically stable and cells are restricted in their proliferation by their neighbours. The elucidation of factors that govern this growth restriction or unleash the transitions in the adult cells opens up new avenues for future research aimed at therapeutic intervention.

MET/EMT transitions in adult tissues and their phenotypical consequences in cancer could be explained by the properties of adult stem cells. The plasticity of adult stem cells and their very existence remains a hotly debated issue (176, 177). Cancer cells can be plastic without definitely being stem cells and dedifferentiation during carcinogenesis and the changes associated with telomeric crisis may yield the same ultimate change. Nevertheless, a growing number of researchers are investigating whether cancer stem cells do exist. Adult stem cells have been described by their ability to differentiate into cells from different embryonic layers. (178-181). Whether reprogramming can occur at a single cell level or whether this observed transdifferentiation comes from cell fusion (182) or from the presence of uncharacterized precursors inside (183) does not change the fact that these observations suggest a new way of thinking about the cancer cell and its interactions with the environment (Figure 3). If indeed the cells undergoing EMT during cancer progression are true adult stem cells, they may undergo reversion of the process if they are allowed to upregulate E-cadherin and reform cell-cell junctions and cell-matrix adhesion (184).

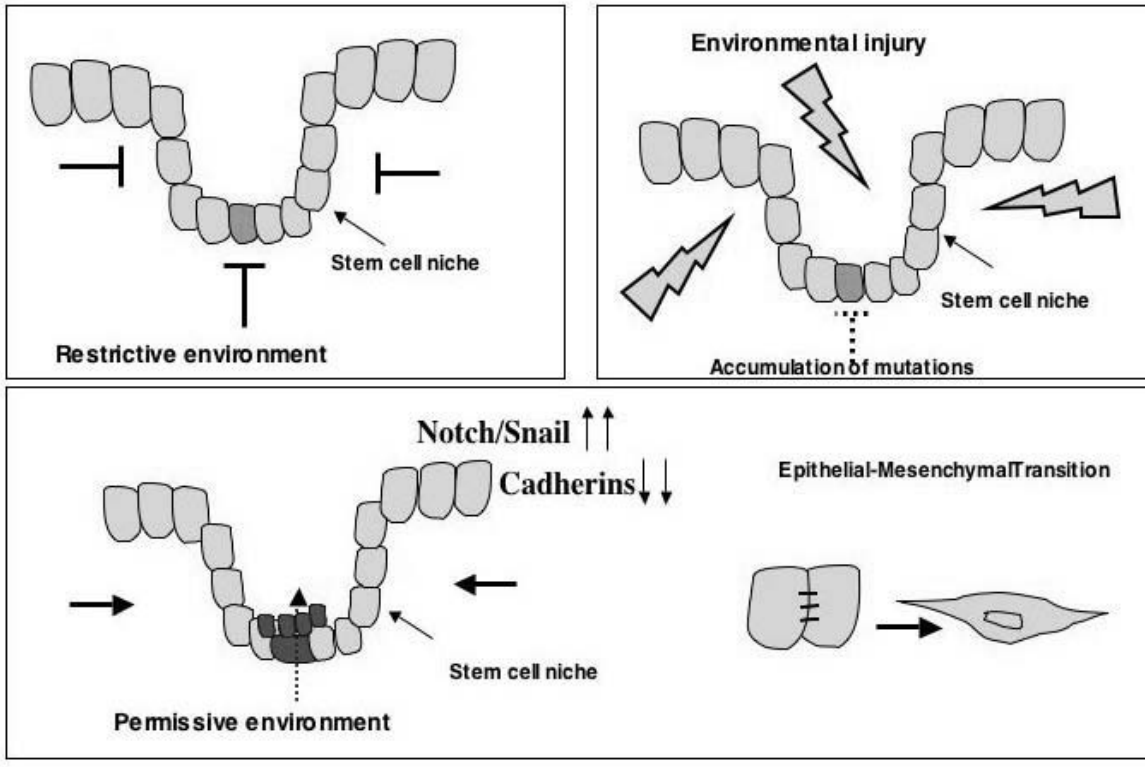


Figure 3. The traditional view of carcinogenesis, centered on single cells mutating to become invasive and then migrating through a defective (permissive) stroma, can be reconciled with the current view of the adult stem cells being potentially the cancer cells.

5. PROTEOMICS AND VALIDATION OF TARGETS

An emerging concept in the quest for successful therapies is the use of proteomic analysis (185). Identifying biomarkers that are clearly differentially expressed in invasive tumours would be of great benefit in early diagnosis of hard-to-detect diseases such as colon and ovarian cancer. To this end, there has been some marked progress in the development of novel screening approaches using cDNA microarrays, yielding new potential markers such as

villin for colon cancer and moesin for ovarian cancer (186). In separate studies, analysis of tissue samples from ovarian cancer patients obtained by laser capture microdissection revealed that FK506 binding protein, RhoGDI, and glyoxalase I were overexpressed in patients with advanced ovarian cancer compared to low-malignant potential patients (187). Tissue lysate microarrays, a mechanism to evaluate the phosphoproteome are being applied to clinical materials obtained from patients being treated with the newer molecular therapeutics. Other studies test serum from ovarian cancer patients to define signature patterns, which are hypothesized to contain significant protein biomarkers (188). Using a technique that screens proteins bound to albumin, a potent protein binder in the serum, many previously unsuspected proteins emerged as potential markers of ovarian cancer (189). If successful, such techniques can also be used to validate current therapeutic interventions by testing for biomarkers discovered by proteomics and verifying their return to more normal expression levels. Such avenues of research provide for exciting and more facile techniques in confronting cancer.

6. CONCLUSION

The benefits of advanced research in both basic molecular biology and molecular therapeutics are many. Identification of critical convergence points in signalling pathways used for cell migration and invasion with those of survival and proliferation, will lead to exploratory therapies and combinations of therapies targeted to those convergence points alone or in combination with agents focused at receptors or other signal nodes. Such explorations will lead to development of combinations of therapies and agents with multiple different unrelated targets (cocktail drugs and drug cocktails). This may be beneficial to reduce toxicity to bystander tissue and thus side effects for the patient while potentially providing improved intervention to the cancer. These approaches may also be demonstrated to be safe and biologically sound for use as primary chemoprevention agents. As more modern therapeutic approaches come into play, we will undoubtedly see a sprouting of such combination treatments that, when coupled with new technologies and understanding of the biology of neoplasia, will bring us significantly forward in the fight to eradicate cancer.

REFERENCES

1. Liotta, L. A., and Kohn, E. C., 2001, The microenvironment of the tumour-host interface. Nature, 411:375-379.
2. Liotta, L. A., Steeg, P. S., and Stetler-Stevenson, W. G., 1991, Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell, 64:327-336.
3. Stetler-Stevenson, W. G., Aznavoorian, S., and Liotta, L. A., 1993, Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol, 9:541-573.
4. Longhurst, C. M., and Jennings, L. K., 1998, Integrin-mediated signal transduction. Cell Mol Life Sci, 54:514-526.
5. Pece, S., and Gutkind, J. S., 2000, Signaling from E-cadherins to the MAPK pathway by the recruitment and activation of epidermal growth factor receptors upon cell-cell contact formation. J Biol Chem, 275:41227-41233.
6. Hubbard, A. K., and Rothlein, R., 2000, Intercellular adhesion molecule-1 (ICAM-1) expression and cell signaling cascades. Free Radic Biol Med, 28:1379-1386.
7. Anand-Apte, B., and Zetter, B., 1997, Signaling mechanisms in growth factor-stimulated cell motility. Stem Cells, 15:259-267.
8. Wells, A., 1999, EGF receptor. Int J Biochem Cell Biol, 31:637-643.
9. Friedl, P., and Brocker, E. B., 2000, The biology of cell locomotion within three-dimensional extracellular matrix. Cell Mol Life Sci, 57:41-64.
10. Petruzzelli, L., Herrera, R., and Rosen, O. M., 1984, Insulin receptor is an insulin-dependent tyrosine protein kinase: copurification of insulin-binding activity and protein kinase activity to homogeneity from human placenta. Proc Natl Acad Sci U S A, 81:3327-3331.
11. Downward, J., Waterfield, M. D., and Parker, P. J., 1985, Autophosphorylation and protein kinase C phosphorylation of the epidermal growth factor receptor. Effect on tyrosine kinase activity and ligand binding affinity. J Biol Chem, 260:14538-14546.
12. Claesson-Welsh, L., 1994, Signal transduction by the PDGF receptors. Prog Growth Factor Res, 5:37-54.

13. Pawson, T., 2002, Regulation and targets of receptor tyrosine kinases. Eur J Cancer, 38 Suppl 5: S3-10.
14. Mendelsohn, J. and Baselga, J., 2003, Status of epidermal growth factor receptor antagonists in the biology and treatment of cancer. J Clin Oncol, 21:2787-2799.
15. Baselga, J., 2002, Why the epidermal growth factor receptor? The rationale for cancer therapy. Oncologist, 7 Suppl 4:2-8.
16. Ciardiello, F., and Tortora, G., 2001, A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. Clin Cancer Res, 7:2958-2970.
17. Fay, F. S., Gilbert, S. H., and Brundage, R. A., 1995, Calcium signalling during chemotaxis. Ciba Found Symp, 188:121-135; discussion 136-140.
18. Huang, J. B., Kindzelskii, A. L., Clark, A. J., and Petty, H. R., 2004, Identification of channels promoting calcium spikes and waves in HT1080 tumor cells: their apparent roles in cell motility and invasion. Cancer Res, 64:2482-2489.
19. Lee, J., Ishihara, A., Oxford, G., Johnson, B., and Jacobson, K., 1999, Regulation of cell movement is mediated by stretch-activated calcium channels. Nature, 400:382-386.
20. Munevar, S., Wang, Y. L., and Dembo, M., 2001, Distinct roles of frontal and rear cell-substrate adhesions in fibroblast migration. Mol Biol Cell, 12:3947-3954.
21. Webb, D. J., Donais, K., Whitmore, L. A., Thomas, S. M., Turner, C. E., Parsons, J. T., and Horwitz, A. F., 2004, FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat Cell Biol, 6:154-161.
22. Chan, A. Y., Raft, S., Bailly, M., Wyckoff, J. B., Segall, J. E., and Condeelis, J. S., 1998, EGF stimulates an increase in actin nucleation and filament number at the leading edge of the lamellipod in mammary adenocarcinoma cells. J Cell Sci, 111(Pt 2):199-211.
23. Azuma, T., Witke, W., Stossel, T. P., Hartwig, J. H., and Kwiatkowski, D. J., 1998, Gelsolin is a downstream effector of rac for fibroblast motility. Embo J, 17:1362-1370.
24. Fujiwara, H., Gu, J., and Sekiguchi, K., 2004, Rac regulates integrin-mediated endothelial cell adhesion and migration on laminin-8. Exp Cell Res, 292:67-77.
25. Ridley, A. J., 2001, Rho GTPases and cell migration. J Cell Sci, 114:2713-2722.
26. Lauffenburger, D. A., and Horwitz, A. F., 1996, Cell migration: a physically integrated molecular process. Cell, 84:359-369.
27. Wehrle-Haller, B., and Imhof, B., 2002, The inner lives of focal adhesions. Trends Cell Biol, 12:382-389.
28. Schlaepfer, D. D., and Hunter, T., 1996, Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases. Mol Cell Biol, 16:5623-5633.
29. Schaller, M. D., 2001, Biochemical signals and biological responses elicited by the focal adhesion kinase. Biochim Biophys Acta, 1540:1-21.
30. Sieg, D. J., Hauck, C. R., Ilic, D., Klingbeil, C. K., Schaefer, E., Damsky, C. H., and Schlaepfer, D. D., 2000, FAK integrates growth-factor and integrin signals to promote cell migration. Nat Cell Biol, 2:249-256.
31. Sieg, D. J., Hauck, C. R., and Schlaepfer, D. D., 1999, Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. J Cell Sci, 112 (Pt 16):2677-2691.
32. Frisch, S. M., and Francis, H., 1994, Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol, 124:619-626.
33. Horiguchi, A., Oya, M., Uchida, A., Marumo, K., and Murai, M., 2003, Elevated Akt activation and its impact on clinicopathological features of renal cell carcinoma. J Urol, 169:710-713.
34. Itoh, N., Semba, S., Ito, M., Takeda, H., Kawata, S., and Yamakawa, M., 2002, Phosphorylation of Akt/PKB is required for suppression of cancer cell apoptosis and tumor progression in human colorectal carcinoma. Cancer, 94:3127-3134.
35. Shindoh, M., Adachi, M., Higashino, F., Yasuda, M., Hida, K., Nishioka, T., Ono, M., Takayama, S., Reed, J. C., Imai, K., Totsuka, Y., and Kohgo, T., 2000, BAG-1 expression correlates highly with the malignant potential in early lesions (T1 and T2) of oral squamous cell carcinoma. Oral Oncol, 36:444-449.
36. Sun, H. Q., Yamamoto, M., Mejillano, M., and Yin, H. L., 1999, Gelsolin, a multifunctional actin regulatory protein. J Biol Chem, 274:33179-33182.
37. Chou, J., Stolz, D. B., Burke, N. A., Watkins, S. C., and Wells, A., 2002, Distribution of gelsolin and phosphoinositol 4,5-bisphosphate in lamellipodia during EGF-induced motility. Int J Biochem Cell Biol, 34:776-790.
38. Sakisaka, T., Itoh, T., Miura, K., and Takenawa, T., 1997, Phosphatidylinositol 4,5-bisphosphate phosphatase regulates the rearrangement of actin filaments. Mol Cell Biol, 17:3841-3849.
39. Mooseker, M. S., Coleman, T. R., and Conzelman, K. A., 1986, Calcium and the regulation of cytoskeletal assembly, structure and contractility. Ciba Found Symp, 122:232-249.
40. Rodland, K. D., Wersto, R. P., Hobson, S., and Kohn, E. C., 1997, Thapsigargin-induced gene expression in nonexcitable cells is dependent on calcium influx. Mol Endocrinol, 11:281-291.

41. Gliki, G., Wheeler-Jones, C., and Zachary, I., 2002, Vascular endothelial growth factor induces protein kinase C (PKC)-dependent Akt/PKB activation and phosphatidylinositol 3'-kinase-mediates PKC delta phosphorylation: role of PKC in angiogenesis. Cell Biol Int, 26:751-759.
42. Amin, A. R., Ichigotani, Y., Oo, M. L., Biswas, M. H., Yuan, H., Huang, P., Mon, N. N., and Hamaguchi, M., 2003, The PLC-PKC cascade is required for IL-1beta-dependent Erk and Akt activation: their role in proliferation. Int J Oncol, 23:1727-1731.
43. Legg, J. W., Lewis, C. A., Parsons, M., Ng, T., and Isacke, C. M., 2002, A novel PKC-regulated mechanism controls CD44 ezrin association and directional cell motility. Nat Cell Biol, 4:399-407.
44. Ng, T., Parsons, M., Hughes, W. E., Monypenny, J., Zicha, D., Gautreau, A., Arpin, M., Gschmeissner, S., Verveer, P. J., Bastiaens, P. I., and Parker, P. J., 2001, Ezrin is a downstream effector of trafficking PKC-integrin complexes involved in the control of cell motility. Embo J, 20:2723-2741.
45. Zhang, M. I. and O'Neil, R. G., 2001, Kinetics of activation of a PKC-regulated epithelial calcium channel. Cell Calcium, 29:263-275.
46. Deucher, A., Efimova, T., and Eckert, R. L., 2002, Calcium-dependent involucrin expression is inversely regulated by protein kinase C (PKC) alpha and PKCdelta. J Biol Chem, 277:17032-17040.
47. Kamat, A., and Carpenter, G., 1997, Phospholipase C-gamma1: regulation of enzyme function and role in growth factor-dependent signal transduction. Cytokine Growth Factor Rev, 8:109-117.
48. Chen, W. T., 1981, Mechanism of retraction of the trailing edge during fibroblast movement. J Cell Biol, 90:187-200.
49. Giannone, G., Ronde, P., Gaire, M., Haiech, J., and Takeda, K., 2002, Calcium oscillations trigger focal adhesion disassembly in human U87 astrocytoma cells. J Biol Chem, 277:26364-26371.
50. Regen, C. M., and Horwitz, A. F., 1992, Dynamics of beta 1 integrin-mediated adhesive contacts in motile fibroblasts. J Cell Biol, 119:1347-1359.
51. Glading, A., Bodnar, R. J., Reynolds, I. J., Shiraha, H., Satish, L., Potter, D. A., Blair, H. C., and Wells, A., 2004, Epidermal growth factor activates m-calpain (calpain II), at least in part, by extracellular signal-regulated kinase-mediated phosphorylation. Mol Cell Biol, 24:2499-2512.
52. Carragher, N. O., Fincham, V. J., Riley, D., and Frame, M. C., 2001, Cleavage of focal adhesion kinase by different proteases during SRC-regulated transformation and apoptosis. Distinct roles for calpain and caspases. J Biol Chem, 276:4270-4275.
53. Xie, H., Pallero, M. A., Gupta, K., Chang, P., Ware, M. F., Witke, W., Kwiatkowski, D. J., Lauffenburger, D. A., Murphy-Ullrich, J. E., and Wells, A., 1998, EGF receptor regulation of cell motility: EGF induces disassembly of focal adhesions independently of the motility-associated PLCgamma signaling pathway. J Cell Sci, 111 (Pt 5):615-624.
54. Valentinis, B., Morrione, A., Peruzzi, F., Prisco, M., Reiss, K., and Baserga, R., 1999, Anti-apoptotic signaling of the IGF-I receptor in fibroblasts following loss of matrix adhesion. Oncogene, 18:1827-1836.
55. He, Z., Ismail, A., Kriazhev, L., Sadvakassova, G., and Bateman, A., 2002, Progranulin (PC-cell-derived growth factor/acrogranin) regulates invasion and cell survival. Cancer Res, 62:5590-5596.
56. Liu, X. W., Bernardo, M. M., Fridman, R., and Kim, H. R., 2003, Tissue inhibitor of metalloproteinase-1 protects human breast epithelial cells against intrinsic apoptotic cell death via the focal adhesion kinase/phosphatidylinositol 3-kinase and MAPK signaling pathway. J Biol Chem, 278:40364-40372.
57. Mills, G. B., and Moolenaar, W. H., 2003, The emerging role of lysophosphatidic acid in cancer. Nat Rev Cancer, 3:582-591.
58. Zhang, X., Chattopadhyay, A., Ji, Q. S., Owen, J. D., Ruest, P. J., Carpenter, G., and Hanks, S. K., 1999, Focal adhesion kinase promotes phospholipase C-gamma1 activity. Proc Natl Acad Sci USA, 96:9021-9026.
59. Tamura, M., Gu, J., Danen, E. H., Takino, T., Miyamoto, S., and Yamada, K. M., 1999, PTEN interactions with focal adhesion kinase and suppression of the extracellular matrix-dependent phosphatidylinositol 3-kinase/Akt cell survival pathway. J Biol Chem, 274:20693-20703.
60. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovanella, B. C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M. H., and Parsons, R., 1997, PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science, 275:1943-1947.
61. Tanaka, M., and Grossman, H. B., 2003, In vivo gene therapy of human bladder cancer with PTEN suppresses tumor growth, downregulates phosphorylated Akt, and increases sensitivity to doxorubicin. Gene Ther, 10:1636-1642.
62. Lu, Y., Lin, Y. Z., LaPushin, R., Cuevas, B., Fang, X., Yu, S. X., Davies, M. A., Khan, H., Furui, T., Mao, M., Zinner, R., Hung, M. C., Steck, P., Siminovitch, K., and Mills, G. B., 1999, The PTEN/MMAC1/TEP tumor suppressor gene decreases cell growth and induces apoptosis and anoikis in breast cancer cells. Oncogene, 18:7034-7045.
63. Krymskaya, V. P., Hoffman, R., Eszterhas, A., Kane, S., Ciocca, V., and Panettieri, R. A., Jr., 1999, EGF activates ErbB-2 and stimulates

phosphatidylinositol 3-kinase in human airway smooth muscle cells. Am J Physiol, 276:L246-255.
64. Marmor, M. D., Skaria, K. B., and Yarden, Y., 2004, Signal transduction and oncogenesis by ErbB/HER receptors. Int J Radiat Oncol Biol Phys, 58:903-913.
65. Ivaska, J., and Heino, J., 2000, Adhesion receptors and cell invasion: mechanisms of integrin-guided degradation of extracellular matrix. Cell Mol Life Sci, 57:16-24.
66. Ridley, A.J., Schwartz, M.A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T., and Horwitz, A. R., 2003, Cell migration: integrating signals from front to back. Science, 302:1704-1709.
67. Wary, K.K., Mainiero, F., Isakoff, S.J., Marcantonio, E.E., and Giancotti, F.G., 1996, The adaptor protein Shc couples a class of integrins to the control of cell cycle progression. Cell, 87:733-743.
68. Lollo, B.A., Chan, K.W., Hanson, E.M., Moy, V.T., and Brian, A.A., 1993, Direct evidence for two affinity states for lymphocyte function-associated antigen 1 on activated T cells. J Biol Chem, 268:21693-21700.
69. Kim, S., Bell, K., Mousa, S. A., and Varner, J. A., 2000, Regulation of angiogenesis in vivo by ligation of integrin alpha5beta1 with the central cell-binding domain of fibronectin. Am J Pathol, 156:1345-1362.
70. Jin, H., and Varner, J., 2004, Integrins: roles in cancer development and as treatment targets. Br J Cancer, 90: 61-565.
71. Sawyer, T.K., 2004, Cancer metastasis therapeutic targets and drug discovery: emerging small-molecule protein kinase inhibitors. Expert Opin Investig Drugs, 13:1-19.
72. Stetler-Stevenson, W.G., 1999, Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. J Clin Invest, 103:1237-1241.
73. Stetler-Stevenson, W.G. and Yu, A.E., 2001, Proteases in invasion: matrix metalloproteinases. Semin Cancer Biol, 11:143-152.
74. Johansson, N., Ahonen, M., and Kahari, V. M., 2000, Matrix metalloproteinases in tumor invasion. Cell Mol Life Sci, 57:5-15.
75. Pendas, A. M., Knauper, V., Puente, X. S., Llano, E., Mattei, M. G., Apte, S., Murphy, G., and Lopez-Otin, C., 1997, Identification and characterization of a novel human matrix metalloproteinase with unique structural characteristics, chromosomal location, and tissue distribution. J Biol Chem, 272:4281-4286.
76. Lynch, C. C. and Matrisian, L. M., 2002, Matrix metalloproteinases in tumor-host cell communication. Differentiation, 70:561-573.
77. Brown, M. R., Blanchette, J. O., and Kohn, E. C., 2000, Angiogenesis in ovarian cancer. Baillieres Best Pract Res Clin Obstet Gynaecol, 14:901-918.
78. Kerkela, E. and Saarialho-Kere, U., 2003, Matrix metalloproteinases in tumor progression: focus on basal and squamous cell skin cancer. Exp Dermatol, 12:109-125.
79. Fingleton, B., 2003, Matrix metalloproteinase inhibitors for cancer therapy:the current situation and future prospects. Expert Opin Ther Targets, 7:385-397.
80. Freije, J. M., Balbin, M., Pendas, A. M., Sanchez, L. M., Puente, X. S., and Lopez-Otin, C., 2003, Matrix metalloproteinases and tumor progression. Adv Exp Med Biol, 532:91-107.
81. Burridge, K. and Wennerberg, K., 2004, Rho and Rac take center stage. Cell, 116:167-179.
82. Karnoub, A. E., Symons, M., Campbell, S. L., and Der, C. J., 2004, Molecular Basis for Rho GTPase Signaling Specificity. Breast Cancer Res Treat, 84:61-71.
83. Li, Z., Hannigan, M., Mo, Z., Liu, B., Lu, W., Wu, Y., Smrcka, A. V., Wu, G., Li, L., Liu, M., Huang, C. K., and Wu, D., 2003, Directional sensing requires G beta gamma-mediated PAK1 and PIX alpha-dependent activation of Cdc42. Cell, 114:215-227.
84. Wolf, K., Mazo, I., Leung, H., Engelke, K., von Andrian, U. H., Deryugina, E. I., Strongin, A. Y., Brocker, E. B., and Friedl, P., 2003, Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis. J Cell Biol, 160:267-277.
85. Barnes, C. J., Bagheri-Yarmand, R., Mandal, M., Yang, Z., Clayman, G. L., Hong, W. K., and Kumar, R., 2003, Suppression of epidermal growth factor receptor, mitogen-activated protein kinase, and Pak1 pathways and invasiveness of human cutaneous squamous cancer cells by the tyrosine kinase inhibitor ZD1839 (Iressa). Mol Cancer Ther, 2:345-351.
86. Kelly, K., and Averbuch, S., 2004, Gefitinib: phase II and III results in advanced non-small cell lung cancer. Semin Oncol, 31:93-99.
87. Tiseo, M., Loprevite, M., and Ardizzoni, A., 2004, Epidermal growth factor receptor inhibitors: a new prospective in the treatment of lung cancer. Curr Med Chem Anti-Canc Agents, 4:139-148.
88. Warburton, C., Dragowska, W.H., Gelmon, K., Chia, S., Yan, H., Masin, D., Denyssevych, T., Wallis, A.E., and Bally, M.B., 2004, Treatment of HER-2/neu Overexpressing Breast Cancer Xenograft Models with Trastuzumab (Herceptin) and Gefitinib (ZD1839): Drug Combination Effects on Tumor Growth, HER-2/neu and Epidermal Growth Factor Receptor Expression, and Viable Hypoxic Cell Fraction. Clin Cancer Res, 10:2512-2524.
89. Zhou, H., Kim, Y. S., Peletier, A., McCall, W., Earp, H. S., and Sartor, C. I., 2004, Effects of the

EGFR/HER2 kinase inhibitor GW572016 on EGFR- and HER2-overexpressing breast cancer cell line proliferation, radiosensitization, and resistance. Int J Radiat Oncol Biol Phys, 58:344-352.

90. Herbst, R. S., 2003, Erlotinib (Tarceva): an update on the clinical trial program. Semin Oncol, 30:34-46.
91. Christensen, J. G., Schreck, R. E., Chan, E., Wang, X., Yang, C., Liu, L., Cui, J., Sun, L., Wei, J., Cherrington, J. M., and Mendel, D. B., 2001, High levels of HER-2 expression alter the ability of epidermal growth factor receptor (EGFR) family tyrosine kinase inhibitors to inhibit EGFR phosphorylation in vivo. Clin Cancer Res, 7:4230-4238.
92. Kim, S. J., Uehara, H., Karashima, T., Shepherd, D. L., Killion, J. J., and Fidler, I. J., 2003, Blockade of epidermal growth factor receptor signaling in tumor cells and tumor-associated endothelial cells for therapy of androgen-independent human prostate cancer growing in the bone of nude mice. Clin Cancer Res, 9:1200-1210.
93. La Rosee, P., O'Dwyer, M. E., and Druker, B. J., 2002, Insights from pre-clinical studies for new combination treatment regimens with the Bcr-Abl kinase inhibitor imatinib mesylate (Gleevec/Glivec) in chronic myelogenous leukemia: a translational perspective. Leukemia, 16:1213-1219.
94. DeMatteo, R. P., 2002, The GIST of targeted cancer therapy: a tumor (gastrointestinal stromal tumor), a mutated gene (c-kit), and a molecular inhibitor (STI571). Ann Surg Oncol, 9:831-839.
95. Hwang, R. F., Yokoi, K., Bucana, C. D., Tsan, R., Killion, J. J., Evans, D. B., and Fidler, I. J., 2003, Inhibition of platelet-derived growth factor receptor phosphorylation by STI571 (Gleevec) reduces growth and metastasis of human pancreatic carcinoma in an orthotopic nude mouse model. Clin Cancer Res, 9:6534-6544.
96. Folkman, J., 1995, Seminars in Medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis. N Engl J Med, 333:1757-1763.
97. Liotta, L. A., Kleinerman, J., and Saidel, G. M., 1974, Quantitative relationships of intravascular tumor cells, tumor vessels, and pulmonary metastases following tumor implantation. Cancer Res, 34:997-1004.
98. Folkman, J., 1971, Tumor angiogenesis: therapeutic implications. N Engl J Med, 285:1182-1186.
99. Sivridis, E., Giatromanolaki, A., and Koukourakis, M. I., 2003, The vascular network of tumours--what is it not for? J Pathol, 201:173-180.
100. Sieczkiewicz, G. J., Hussain, M., and Kohn, E. C., 2002, Angiogenesis and metastasis. Cancer Treat Res, 107:353-381.
101. Risau, W., 1997, Mechanisms of angiogenesis. Nature, 386:671-674.
102. Folkman, J., Merler, E., Abernathy, C., and Williams, G., 1971, Isolation of a tumor factor responsible or angiogenesis. J Exp Med, 133:275-288.
103. Goto, F., Goto, K., Weindel, K., and Folkman, J., 1993, Synergistic effects of vascular endothelial growth factor and basic fibroblast growth factor on the proliferation and cord formation of bovine capillary endothelial cells within collagen gels. Lab Invest, 69:508-517.
104. Carmeliet, P. and Jain, R. K., 2000, Angiogenesis in cancer and other diseases. Nature, 407:249-257.
105. Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell-Braxton, L., Hillan, K. J., and Moore, M. W., 1996, Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature, 380:439-442.
106. Willett, C. G., Boucher, Y., di Tomaso, E., Duda, D. G., Munn, L. L., Tong, R. T., Chung, D. C., Sahani, D. V., Kalva, S. P., Kozin, S. V., Mino, M., Cohen, K. S., Scadden, D. T., Hartford, A. C., Fischman, A. J., Clark, J. W., Ryan, D. P., Zhu, A. X., Blaszkowsky, L. S., Chen, H. X., Shellito, P. C., Lauwers, G. Y., and Jain, R. K., 2004, Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. Nat Med, 10:145-147.
107. Guidi, A. J., Abu-Jawdeh, G., Berse, B., Jackman, R. W., Tognazzi, K., Dvorak, H. F., and Brown, L. F., 1995, Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in cervical neoplasia. J Natl Cancer Inst, 87:1237-1245.
108. Guidi, A. J., Abu-Jawdeh, G., Tognazzi, K., Dvorak, H. F., and Brown, L. F., 1996, Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in endometrial carcinoma. Cancer, 78: 454-460.
109. Hartenbach, E. M., Olson, T. A., Goswitz, J. J., Mohanraj, D., Twiggs, L. B., Carson, L. F., and Ramakrishnan, S., 1997, Vascular endothelial growth factor (VEGF) expression and survival in human epithelial ovarian carcinomas. Cancer Lett, 121:169-175.
110. Price, J. T., Bonovich, M. T., and Kohn, E. C., 1997, The biochemistry of cancer dissemination. Crit Rev Biochem Mol Biol, 32:175-253.
111. Kumar, R., Kuniyasu, H., Bucana, C. D., Wilson, M. R., and Fidler, I. J., 1998, Spatial and temporal expression of angiogenic molecules during tumor growth and progression. Oncol Res, 10:301-311.
112. Ferrara, N., Gerber, H. P., and LeCouter, J., 2003, The biology of VEGF and its receptors. Nat Med, 9:669-676.

113. Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S., and Dvorak, H. F., 1983, Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science, 219:983-985.
114. Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z., 1999, Vascular endothelial growth factor (VEGF) and its receptors. Faseb J, 13:9-22.
115. de Vries, C., Escobedo, J. A., Ueno, H., Houck, K., Ferrara, N., and Williams, L. T., 1992, The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science, 255:989-991.
116. Terman, B. I., Dougher-Vermazen, M., Carrion, M. E., Dimitrov, D., Armellino, D. C., Gospodarowicz, D., and Bohlen, P., 1992, Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem Biophys Res Commun, 187:1579-1586.
117. Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L., and Schuh, A. C., 1995, Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature, 376:62-66.
118. Olson, T. A., Mohanraj, D., Carson, L. F., and Ramakrishnan, S., 1994, Vascular permeability factor gene expression in normal and neoplastic human ovaries. Cancer Res, 54: 276-280.
119. Paley, P. J., Staskus, K. A., Gebhard, K., Mohanraj, D., Twiggs, L. B., Carson, L. F., and Ramakrishnan, S., 1997, Vascular endothelial growth factor expression in early stage ovarian carcinoma. Cancer, 80:98-106.
120. Paley, P. J., Goff, B. A., Gown, A. M., Greer, B. E., and Sage, E. H., 2000, Alterations in SPARC and VEGF immunoreactivity in epithelial ovarian cancer. Gynecol Oncol, 78:336-341.
121. Eppenberger, U., Kueng, W., Schlaeppi, J. M., Roesel, J. L., Benz, C., Mueller, H., Matter, A., Zuber, M., Luescher, K., Litschgi, M., Schmitt, M., Foekens, J. A., and Eppenberger-Castori, S., 1998, Markers of tumor angiogenesis and proteolysis independently define high- and low-risk subsets of node-negative breast cancer patients. J Clin Oncol, 16:3129-3136.
122. Linderholm, B., Tavelin, B., Grankvist, K., and Henriksson, R., 1998, Vascular endothelial growth factor is of high prognostic value in node-negative breast carcinoma. J Clin Oncol, 16:3121-3128.
123. George, D. J., and Kaelin, W. G., Jr., 2003, The von Hippel-Lindau protein, vascular endothelial growth factor, and kidney cancer. N Engl J Med, 349:419-421.
124. Igarashi, H., Esumi, M., Ishida, H., and Okada, K., 2002, Vascular endothelial growth factor overexpression is correlated with von Hippel-Lindau tumor suppressor gene inactivation in patients with sporadic renal cell carcinoma. Cancer, 95:47-53.
125. Rosen, L. S., 2002, Inhibitors of the vascular endothelial growth factor receptor. Hematol Oncol Clin North Am, 16:1173-1187.
126. Patel, N., Sun, L., Moshinsky, D., Chen, H., Leahy, K. M., Le, P., Moss, K. G., Wang, X., Rice, A., Tam, D., Laird, A. D., Yu, X., Zhang, Q., Tang, C., McMahon, G., and Howlett, A., 2003, A selective and oral small molecule inhibitor of vascular epithelial growth factor receptor (VEGFR)-2 and VEGFR-1 inhibits neovascularization and vascular permeability. J Pharmacol Exp Ther, 306 838-845.
127. Hotte, S. J., and Hirte, H. W., 2002, BAY 43-9006: early clinical data in patients with advanced solid malignancies. Curr Pharm Des, 8:2249-2253.
128. Giantonio, B. J., Levy, D., O'Dwyer, N. J., Catalano, P. J., and Benson, A. B., 2003, Bevacizumab (anti-VEGF) plus IFL (irinotecan, fluorouracil, leucovorin) as front-line therapy for advanced colorectal cancer (advCRC): Results from the Eastern Cooperative Oncology Group (ECOG) Study E2200. In: Proc Am Soc Clin Oncol, No. 1024, Chicago, IL.
129. Yang, J. C., Haworth, L., Sherry, R. M., Hwu, P., Schwartzentruber, D. J., Topalian, S. L., Steinberg, S. M., Chen, H. X., and Rosenberg, S. A., 2003, A randomized trail of Bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. N Engl J Med, 349:427-434.
130. Asano, M., Yukita, A., and Suzuki, H., 1999, Wide spectrum of antitumor activity of a neutralizing monoclonal antibody to human vascular endothelial growth factor. Jpn J Cancer Res, 90:93-100.
131. Jayson, G., Mulater, C., Ranson, M., Zweit, J., Hasting, D., Julyan, P., Lawrance, J., McGown, A., Jackson, A., Haroon, H., Hakannson, L., Wagstaff, J., Groenewegen, G., Lehmann, F., Levitt, D., Tang, T., and Zweirzina, H., 2001, Anti-VEGF Antibody HuMV833: an EORTC Biological Treatment Development Group Phase I toxicity, pharmacokinetic, and pharmacodynamic study. In: Proc Am Soc Clin Oncol, No. 14, San Francisco, CA.
132. Heldin, C. H., and Westermark, B., 1999, Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev, 79:1283-1316.
133. Henriksen, R., Funa, K., Wilander, E., Backstrom, T., Ridderheim, M., and Oberg, K., 1993, Expression and prognostic significance of platelet-derived growth factor and its receptors in epithelial ovarian neoplasms. Cancer Res, 53:4550-4554.
134. Thommen, R., Humar, R., Misevic, G., Pepper, M. S., Hahn, A. W., John, M., and Battegay, E. J., 1997, PDGF-BB increases endothelial migration on cord movements during angiogenesis in vitro. J Cell Biochem, 64:403-413.
135. Sundberg, C., Ljungstrom, M., Lindmark, G., Gerdin, B., and Rubin, K., 1993, Microvascular

pericytes express platelet-derived growth factor-beta receptors in human healing wounds and colorectal adenocarcinoma. Am J Pathol, 143:1377-1388.

136. Wang, D., Huang, H. J., Kazlauskas, A., and Cavenee, W. K., 1999, Induction of vascular endothelial growth factor expression in endothelial cells by platelet-derived growth factor through the activation of phosphatidylinositol 3-kinase. Cancer Res, 59:1464-1472.
137. Reinmuth, N., Liu, W., Jung, Y. D., Ahmad, S. A., Shaheen, R. M., Fan, F., Bucana, C. D., McMahon, G., Gallick, G. E., and Ellis, L. M., 2001, Induction of VEGF in perivascular cells defines a potential paracrine mechanism for endothelial cell survival. Faseb J, 15:1239-1241.
138. Lindahl, P., Johansson, B. R., Leveen, P., and Betsholtz, C., 1997, Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science, 277:242-245.
139. Demetri, G. D., 2001, Targeting c-kit mutations in solid tumors: scientific rationale and novel therapeutic options. Semin Oncol, 28:19-26.
140. Greco, A., Roccato, E., Miranda, C., Cleris, L., Formelli, F., and Pierotti, M. A., 2001, Growth-inhibitory effect of STI571 on cells transformed by the COL1A1/PDGFB rearrangement. Int J Cancer, 92:354-360.
141. Myllarniemi, M., Calderon, L., Lemstrom, K., Buchdunger, E., and Hayry, P., 1997, Inhibition of platelet-derived growth factor receptor tyrosine kinase inhibits vascular smooth muscle cell migration and proliferation. Faseb J, 11:1119-1126.
142. Schmandt, R. E., Broaddus, R., Lu, K. H., Shvartsman, H., Thornton, A., Malpica, A., Sun, C., Bodurka, D. C., and Gershenson, D. M., 2003, Expression of c-ABL, c-KIT, and platelet-derived growth factor receptor-beta in ovarian serous carcinoma and normal ovarian surface epithelium. Cancer, 98:758-764.
143. Link, C.J., Jr., Kohn, E., and Reed, E., 1996, The relationship between borderline ovarian tumors and epithelial ovarian carcinoma: epidemiologic, pathologic, and molecular aspects. Gynecol Oncol, 60:347-354.
144. Capdeville, R., Buchdunger, E., Zimmermann, J., and Matter, A., 2002, Glivec (STI571, imatinib), a rationally developed, targeted anticancer drug. Nat Rev Drug Discov, 1:493-502.
145. Dudley, A., Gilbert, R. E., Thomas, D., Cox, A., Price, J. T., Best, J., and Jenkins, A., 2003, STI-571 inhibits in vitro angiogenesis. Biochem Biophys Res Commun, 310:135-142.
146. Atallah, E., Talpaz, M., O'Brien, S., Rios, M. B., Guo, J. Q., Arlinghaus, R., Fernandes-Reese, S., and Kantarjian, H., 2002, Chronic myelogenous leukemia in T cell lymphoid blastic phase achieving durable complete cytogenetic and molecular remission with imatinib mesylate (STI571; Gleevec) therapy. Cancer, 94:2996-2999.
147. Barbany, G., Hoglund, M., and Simonsson, B., 2002, Complete molecular remission in chronic myelogenous leukemia after imatinib therapy. N Engl J Med, 347:539-540.
148. Demetri, G. D., von Mehren, M., Blanke, C. D., Van den Abbeele, A. D., Eisenberg, B., Roberts, P. J., Heinrich, M. C., Tuveson, D. A., Singer, S., Janicek, M., Fletcher, J. A., Silverman, S. G., Silberman, S. L., Capdeville, R., Kiese, B., Peng, B., Dimitrijevic, S., Druker, B. J., Corless, C., Fletcher, C. D., and Joensuu, H., 2002, Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. N Engl J Med, 347:472-480.
149. Demetri, G. D., George, S., Heinrich, M. C., Fletcher, J. A., Fletcher, C. D. M., Desai, J., Cohen, D. P., Scigalla, P., Cherrington, J., and Van Den Abbeele, A. D., 2003, Clinical activity and tolerability of the multi-targeted tyrosine kinase inhibitor SU11248 in patients (pts) with metastatic gastrointestinal stromal tumor (GIST) refractory to imatinib mesylate. In: Proc Am Soc Clin Oncol No. 3273, Chicago, IL.
150. Raymond, E., Faivre, S., Vera, K., Delbaldo, C., Robert, C., Spatz, A., Bello, C., Brega, N., Scigalla, P., and Armand, J. P., 2003, Final results of a phase I and pharmacokinetic study of SU11248, a novel multi-target tyrosine kinase inhibitor, in patients with advanced cancers. In: Proc Am Soc Clin Oncol, No. 769, Chicago, IL.
151. Mall, J. W., Myers, J. A., Xu, X., Saclarides, T. J., Philipp, A. W., and Pollmann, C., 2002, [Leflunomide reduces the angiogenesis score and tumor growth of subcutaneously implanted colon carcinoma cells in the mouse model]. Chirurg, 73:716-720.
152. Shawver, L. K., Schwartz, D. P., Mann, E., Chen, H., Tsai, J., Chu, L., Taylorson, L., Longhi, M., Meredith, S., Germain, L., Jacobs, J. S., Tang, C., Ullrich, A., Berens, M. E., Hersh, E., McMahon, G., Hirth, K. P., and Powell, T. J., 1997, Inhibition of platelet-derived growth factor-mediated signal transduction and tumor growth by N-[4-(trifluoromethyl)-phenyl]5-methylisoxazole-4-carboxamide. Clin Cancer Res, 3:1167-1177.
153. Ko, Y. J., Chachou, A., Small, E., Reese, D., Kabbinavar, F., Taneja, S., DePaoli, A., Hannah, A., Balk, S., and Bubley, G., 1999, Phase II study of SU101 in patients with PSA-positive prostate cancer. In: Proc Am Soc Clin Oncol, No. 1220, Atlanta, GA.
154. Folkman, J., Klagsbrun, M., Sasse, J., Wadzinski, M., Ingber, D., and Vlodavsky, I., 1988, A heparin-binding angiogenic protein--basic fibroblast growth factor--is stored within basement membrane. Am J Pathol, 130:393-400.

155. Wiesmann, C., Muller, Y. A., and de Vos, A. M., 2000, Ligand-binding sites in Ig-like domains of receptor tyrosine kinases. J Mol Med, 78:247-260.
156. Dono, R., Texido, G., Dussel, R., Ehmke, H., and Zeller, R., 1998, Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. Embo J, 17:4213-4225.
157. Seghezzi, G., Patel, S., Ren, C. J., Gualandris, A., Pintucci, G., Robbins, E. S., Shapiro, R. L., Galloway, A. C., Rifkin, D. B., and Mignatti, P., 1998, Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. J Cell Biol, 141:1659-1673.
158. Brooks, P. C., Clark, R. A., and Cheresh, D. A., 1994, Requirement of vascular integrin alpha v beta 3 for angiogenesis. Science, 264:569-571.
159. Klagsbrun, M., and Baird, A., 1991, A dual receptor system is required for basic fibroblast growth factor activity. Cell, 67:229-231.
160. Netzer, P., Domek, M., Pai, R., Halter, F., and Tarnawski, A., 2001, Inhibition of human colon cancer cell growth by antisense oligodeoxynucleotides targeted at basic fibroblast growth factor. Aliment Pharmacol Ther, 15:1673-1679.
161. Crickard, K., Gross, J. L., Crickard, U., Yoonessi, M., Lele, S., Herblin, W. F., and Eidsvoog, K., 1994, Basic fibroblast growth factor and receptor expression in human ovarian cancer. Gynecol Oncol, 55:277-284.
162. Miyake, H., Hara, I., Yoshimura, K., Eto, H., Arakawa, S., Wada, S., Chihara, K., and Kamidono, S., 1996, Introduction of basic fibroblast growth factor gene into mouse renal cell carcinoma cell line enhances its metastatic potential. Cancer Res, 56:2440-2445.
163. Tsuboi, R., Sato, Y., and Rifkin, D. B., 1990, Correlation of cell migration, cell invasion, receptor number, proteinase production, and basic fibroblast growth factor levels in endothelial cells. J Cell Biol, 110:511-517.
164. Miyake, H., Yoshimura, K., Hara, I., Eto, H., Arakawa, S., and Kamidono, S., 1997, Basic fibroblast growth factor regulates matrix metalloproteinases production and in vitro invasiveness in human bladder cancer cell lines. J Urol, 157:2351-2355.
165. Sliutz, G., Tempfer, C., Obermair, A., Dadak, C., and Kainz, C., 1995, Serum evaluation of basic FGF in breast cancer patients. Anticancer Res, 15:2675-2677.
166. Dosquet, C., Coudert, M. C., Lepage, E., Cabane, J., and Richard, F., 1997, Are angiogenic factors, cytokines, and soluble adhesion molecules prognostic factors in patients with renal cell carcinoma? Clin Cancer Res, 3:2451-2458.
167. Davidson, B., Goldberg, I., Kopolovic, J., Gotlieb, W. H., Givant-Horwitz, V., Nesland, J. M., Berner, A., Ben-Baruch, G., Bryne, M., and Reich, R., 2000, Expression of angiogenesis-related genes in ovarian carcinoma--a clinicopathologic study. Clin Exp Metastasis, 18:501-507.
168. Prindull, G., and Zipori, D., 2004, Environmental guidance of normal and tumor cell plasticity: epithelial mesenchymal transitions as a paradigm. Blood, 103:2892-2899.
169. Bush, K. T., Sakurai, H., Steer, D. L., Leonard, M. O., Sampogna, R. V., Meyer, T. N., Schwesinger, C., Qiao, J., and Nigam, S. K., 2004, TGF-beta superfamily members modulate growth, branching, shaping, and patterning of the ureteric bud. Dev Biol, 266:285-298.
170. Klein, C. A., Blankenstein, T. J., Schmidt-Kittler, O., Petronio, M., Polzer, B., Stoecklein, N. H., and Riethmuller, G., 2002, Genetic heterogeneity of single disseminated tumour cells in minimal residual cancer. Lancet, 360:683-689.
171. Zoltan-Jones, A., Huang, L., Ghatak, S., and Toole, B. P., 2003, Elevated hyaluronan production induces mesenchymal and transformed properties in epithelial cells. J Biol Chem, 278:45801-45810.
172. Tanaka, K., Hiraiwa, N., Hashimoto, H., Yamazaki, Y., and Kusakabe, M., 2004, Tenascin-C regulates angiogenesis in tumor through the regulation of vascular endothelial growth factor expression. Int J Cancer, 108:31-40.
173. Tlsty, T. D., and Hein, P. W., 2001, Know thy neighbor: stromal cells can contribute oncogenic signals. Curr Opin Genet Dev, 11:54-59.
174. Piek, E., Moustakas, A., Kurisaki, A., Heldin, C. H., and ten Dijke, P., 1999, TGF-(beta) type I receptor/ALK-5 and Smad proteins mediate epithelial to mesenchymal transdifferentiation in NMuMG breast epithelial cells. J Cell Sci, 112 (Pt 24):4557-4568.
175. Jechlinger, M., Grunert, S., Tamir, I. H., Janda, E., Ludemann, S., Waerner, T., Seither, P., Weith, A., Beug, H., and Kraut, N., 2003, Expression profiling of epithelial plasticity in tumor progression. Oncogene, 22:7155-7169.
176. Jiang, Y., Henderson, D., Blackstad, M., Chen, A., Miller, R. F., and Verfaillie, C. M., 2003, Neuroectodermal differentiation from mouse multipotent adult progenitor cells. Proc Natl Acad Sci USA, 100 Suppl 1:11854-11860.
177. Wagers, A. J., Sherwood, R. I., Christensen, J. L., and Weissman, I. L., 2002, Little evidence for developmental plasticity of adult hematopoietic stem cells. Science, 297:2256-2259.
178. Toma, J. G., Akhavan, M., Fernandes, K. J., Barnabe-Heider, F., Sadikot, A., Kaplan, D. R., and

Miller, F. D., 2001, Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat Cell Biol, 3:778-784.

179. Krause, D. S., Theise, N. D., Collector, M. I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S., and Sharkis, S. J., 2001, Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell, 105:369-377.
180. Liang, L., and Bickenbach, J. R., 2002, Somatic epidermal stem cells can produce multiple cell lineages during development. Stem Cells, 20:21-31.
181. Cao, B., Zheng, B., Jankowski, R. J., Kimura, S., Ikezawa, M., Deasy, B., Cummins, J., Epperly, M., Qu-Petersen, Z., and Huard, J., 2003, Muscle stem cells differentiate into haematopoietic lineages but retain myogenic potential. Nat Cell Biol, 5:640-646.
182. Vassilopoulos, G., Wang, P. R., and Russell, D. W., 2003, Transplanted bone marrow regenerates liver by cell fusion. Nature, 422:901-904.
183. McKinney-Freeman, S. L., Jackson, K. A., Camargo, F. D., Ferrari, G., Mavilio, F., and Goodell, M. A., 2002, Muscle-derived hematopoietic stem cells are hematopoietic in origin. Proc Natl Acad Sci U S A, 99:1341-1346.
184. Gilchrist, A. J., Meuser, R., Turchinsky, J., Shaw, A. R., Pasdar, M., and Dixon, W. T., 2002, Cell adhesion-mediated transformation of a human SCLC cell line is associated with the development of a normal phenotype. Exp Cell Res, 276:63-78.
185. Liotta, L. A., Kohn, E. C., and Petricoin, E. F., 2001, Clinical proteomics: personalized molecular medicine. Jama, 286:2211-2214.
186. Nishizuka, S., Chen, S. T., Gwadry, F. G., Alexander, J., Major, S. M., Scherf, U., Reinhold, W. C., Waltham, M., Charboneau, L., Young, L., Bussey, K. J., Kim, S., Lababidi, S., Lee, J. K., Pittaluga, S., Scudiero, D. A., Sausville, E. A., Munson, P. J., Petricoin, E. F., 3rd, Liotta, L. A., Hewitt, S. M., Raffeld, M., and Weinstein, J. N., 2003, Diagnostic markers that distinguish colon and ovarian adenocarcinomas: identification by genomic, proteomic, and tissue array profiling. Cancer Res, 63:5243-5250.
187. Jones, M. B., Krutzsch, H., Shu, H., Zhao, Y., Liotta, L. A., Kohn, E. C., and Petricoin, E. F., 3rd, 2002, Proteomic analysis and identification of new biomarkers and therapeutic targets for invasive ovarian cancer. Proteomics, 2:76-84.
188. Stevens, E. V., Liotta, L. A., and Kohn, E. C., 2003, Proteomic analysis for early detection of ovarian cancer: a realistic approach? Int J Gynecol Cancer, 13 Suppl 2:133-139.
189. Mehta, A., Lowenthal, M., Johann, D., Rajapakse, V., Fusaro, V., Hoatson, S., Asmussen, H., Roberts, J., Butler, C., Pappas, R., Ross, S., Fishman, D., Petricoin III, E., and Liotta, L. A., 2004, Carrier albumin's binders in serum (CABs): Detecting ovarian cancer. In: American Association of Cancer Research, No. 4774, Orlando, FL.

Chapter 21

Bone Stromal Cells As Therapeutic Targets In Osseous Metastasis

R.A. Sikes[1], C.R. Cooper[2], G.L. Beck[3], F. Pruitt[4], M.L. Brown[5] and G. Balian[6]
[1]Laboratory for Cancer Ontogeny and Therapeutics, Department of Biological Sciences, University of Delaware, USA, [2]Cancer Biology Laboratory, Department of Biological Sciences, University of Delaware, USA, [3]Departments of Orthopedics & Biochemistry and Molecular Genetics, School of Medicine, University of Virginia Health System, USA, [4]Cancer Biology Laboratory, Department of Biological Sciences, University of Delaware, USA, [5]Department of Chemistry, University of Virginia, USA, [6]Departments of Orthopedics & Biochemistry and Molecular Genetics, School of Medicine, University of Virginia Health System, USA

Abstract: The dissemination of cancer from the primary site of growth to distant organs is an early event that leads to the random deposition of tumor cells throughout the organs of the body. Growth of these seeded cellular singularities into secondary, clinically manifest tumors is a notably non-random event. In addition to being the primary tumor site for multiple myeloma and several forms of bone cancer, the bone is the favored site for metastasis of breast, lung and prostate cancer. Once in the bone, these cancers interact with the bone microenvironment to become more aggressive and resistant to therapy. Therefore, the bone stroma is likely to play a major role in the support, growth and improved survival of the metastatic cancer cell. As such, therapeutic intervention targeting the bone stromal should enhance our ability to eliminate bone metastases.

Key words: Metastasis, neoplasia, bone, angiogenesis, small molecule inhibitor, phage display, biopanning, anti-angiogenic therapy, thalidomide, cell adhesion

1. INTRODUCTION

The sheer magnitude of cancer incidence in the United States has a huge impact from a public health perspective. In 2004 it has been estimated that there will be 1,368,030 new cases of cancer and 563,700 cancer related deaths (1). These numbers translate into almost one of every four deaths in the United States being cancer related. To further emphasize this point, cancer related deaths (22.9%) are second only to heart disease (29%) as the major killer of Americans. Of these 563,700 cancer related deaths, approximately two thirds will result from dissemination, or metastasis of the primary tumor to a secondary site(s) (2).

It long has been observed that cancer metastasis is a nonrandom event with certain cancers having a predilection for metastatic colonization of certain tissues with high frequency. Cancers of the breast, lung and prostate preferentially metastasize to marrow containing bone. Together these cancers account for about 80% of all metastases involving bone. For prostate cancer upwards of 85% of patients will have bone involvement at autopsy (3-5). For the patient, bone metastases have associated morbidities including pathological fracture and pain. Clinically, these bone metastases respond poorly to conventional therapies and generally result in poor survival. For example, in prostate cancer the median survival for patients with bone metastases and androgen dependent disease is 30-35 months while

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 369-386.

that of men with androgen independent disease is 4 months (2).

Once established in the bone all of these cancer types have one thing in common, they interact with the bone microenvironment. The result is a complex multi-directional paracrine interaction between the cancer cells and bone stromal cells that include bone marrow endothelial cells, bone marrow stromal cells, osteoblasts (OsB) and osteoclasts (OsC). The resultant outcome on bone architecture, either the overall deposition of new bone (osteoblastic) or degradation of bone (osteoclastic), is the sum effect of cancer cell: bone stromal cell interactions. A natural sequela to the complex interaction between cancer cells and bone stromal cells is a co-dependence of the cancer on the bone stromal cells for both cancer cell targeting to the bone marrow and the establishment of the metastasis in this environs. This dynamic mutualism proffers an opportunity for targeting the normal cells of the bone stroma as a means to eliminate the cancer invader by removing its primary means of support. In this chapter we will summarize some of the recent approaches and developments in this area with an emphasis on work in our laboratories and through collaborative efforts.

2. INTERVENTION WITH METASTATIC PROCESS: TARGETING BONE MARROW ENDOTHELIAL CELLS.

The adhesion of cancer cells to microvascular endothelial cells, particularly bone marrow endothelial cells, is a critical step in the metastatic process to bone. This interaction is described as the "Docking and Locking" hypothesis, whereby cancer cells transiently attach (dock) to the endothelium via selectins and then form stronger adhesive interactions via integrins (lock). In some cases, the preferential adhesion of certain cancer cells to particular organ microvessels determines their pattern of metastasis and correlates with cancer progression (6). That either of these two steps can be targeted for therapeutic intervention is the basic premise underlying many of the interventional strategies currently under development to treat and prevent bone metastases.

2.1 Phage Display

Random peptide phage display libraries have been used *in vivo* with nonrandom distribution of peptides isolated from phage bound to different organs (7, 8). This high degree of specificity of phage binding as imparted by the peptide epitope allowed investigators to determine that each organ's microvasculature had unique determinants (8, 9). Thus, the process of *in vivo* phage display has led to the identification of interacting receptors on cells (10). In addition to the cell surface targets that were found for phage display peptides, there have been reports of an intracellular fate for phage display vectors in mammalian cells (11, 12). These observations have raised the possibility that peptides identified by *in vivo* biopanning of a phage display library may be useful for targeted delivery of genes, therapeutics and perhaps even cells to specific organs. Furthermore, peptides that are directed to cell surface components, such as CD34 may prove useful for cell selection (13). Screening the phage library can lead to the identification of ligands to cell surface proteins in target cells and tissues (14-16). Since the construction of the peptide phage display library is random by design, many of the peptides that interact specifically with any given target tissue may not represent true protein epitopes but are likely to be protein mimotopes, peptides whose sequences fold to look like another protein but are unrelated at the amino acid level. These sequences from phage display libraries may be helpful as structural and functional mimics that serve as the basis for novel drug design for the interacting target.

Given this background on phage display it seemed reasonable that targeting peptides from a random phage display library could be found using *in vivo* biopanning that specifically adhered to bone or bone marrow stromal cells. Since prostate, breast and lung cancer preferentially metastasize to bone and previous data suggested that phage display-derived peptides may be targeting either cell surface receptors or their ligands in the extracellular matrix of the target tissue, then the logical targets for their interaction with bone reside either on the surface of

bone marrow endothelial cells, bone stromal cells, bone cells or bone matrix. These peptide sequences then may be investigated to determine their targeting specificity and potential therapeutic uses, such as the delivery of drugs to tumor or normal vascular tissue or possible intervention with tumor cell colonization at distant sites, any of which could help in the treatment of metastatic cancer.

2.1.1 Random Peptide Phage Display

Synthetic peptides may be a useful addition to the idea that gene targeting of tumor vasculature can be achieved with potentially beneficial consequences (15, 17-19). The specificity of protein binding molecules as a potential delivery system for drugs is clearly of interest to a variety of clinical areas as well as to the pharmaceutical industry in general (20, 21). Phage display and peptide discovery can lead to drug development. Aminopeptidase is a receptor for peptides that home to tumors, and the phenomenon could serve as an address system for targeting vasculature in normal tissues and in tumors (8, 17-19, 22). The inhibitory action of such peptides on tumor angiogenesis therefore has been proposed. Phage display peptides have not been restricted to targeting tumor vasculature however. A number of studies have shown that specific receptors on endothelial cells serve as ligands and, in addition, some peptides demonstrate cell-selectivity (22-24). Thus, a variety of cell surface proteins may prove to be the potential targets for a phage display peptide library.

In our most recent research using *in vivo* biopanning with a random 12-mer peptide phage display library and DNA sequencing, we isolated and identified 13 peptide sequences enriched selectively from their interaction with bone. The final 13 peptide sequences were detected repeatedly in phage that was isolated from bone and bone marrow. Our objectives were to establish the target cell population within bone (osteoblasts) and bone marrow (endothelial and mesenchymal) that bind to these peptides and confirm the tissue-specificity of peptide targeting. We also are pursuing the capacity of the selected peptides to modify cancer cell growth, viability, as well as their adhesion to and invasion through human bone marrow endothelial cells. Ultimately, we will identify the receptors for the peptides by affinity chromatography and two-dimensional peptide mapping of membrane proteins and extracellular matrix molecules.

We view the major clinical implications of our studies to be in the potential treatment of cancer cell metastases to bone, either with a selected peptide that specifically targets bone, or using the peptide to deliver a pharmacological agent. Identification of cell surface and matrix ligands will elucidate the mechanism of cancer cell metastasis to bone.

***In vivo* biopanning with a phage display peptide library.** Selection of a phage display 12 - mer peptide library was based on the likelihood that specific antibodies can be raised to the selected peptides, and that the combination of the peptides and their antibodies can provide a useful tool to confirm specificity of peptide-ligand binding. If a given peptide modulates cell adhesion, invasion or migration, then the antibodies are likely to serve as a tool to further examine the effects of peptides on cell attachment or cell invasion. Such properties are reminiscent of tumor cell attachment and invasion and they are taken into consideration in discussions of cancer cell metastases to bone. We further hypothesized that from a random phage display peptide library of 2.7×10^9 different peptide sequences that unique peptide sequences could be retrieved that interact specifically with cellular components of bone. If true, when these phage were reintroduced into mice, some, if not all, of the bone targeting peptides should be retrievable from bone. This is essentially biopanning *in vivo* (Figure 1). We found that this was indeed the case and that the peptides were obtained in bone and did not reappear in significant amounts, based on plaque forming units, in other tissues that were examined such as liver and kidney. Nevertheless, the targeting specificity is an important issue that will be examined further *in vivo* by selectively panning the peptides individually.

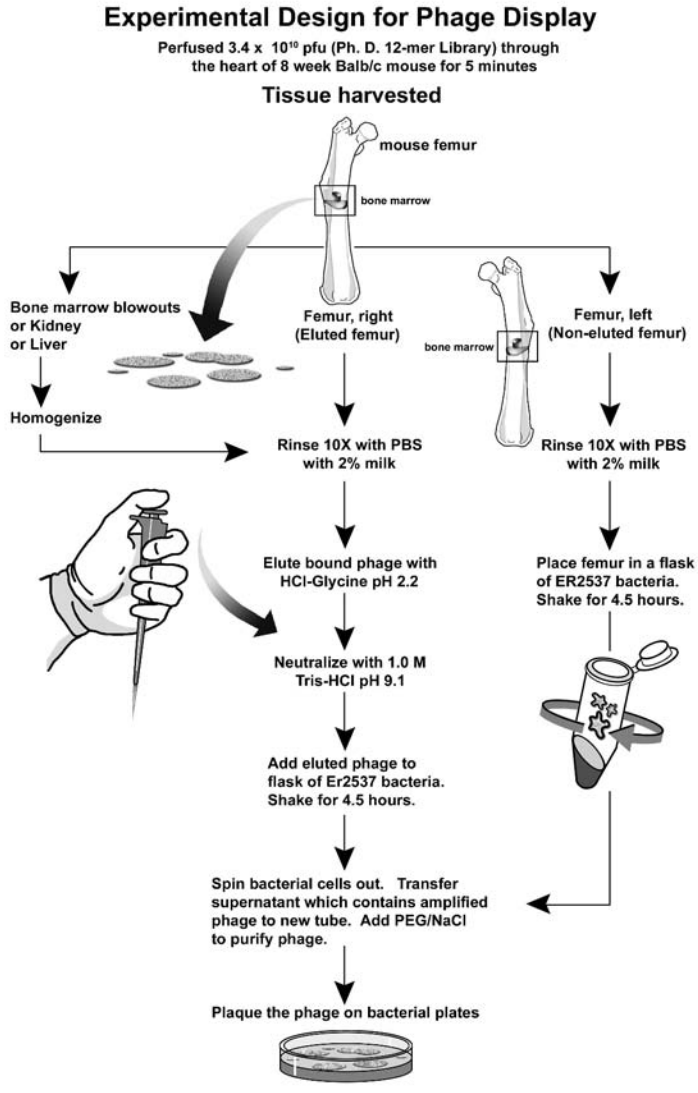


Figure 1. Schematic representation of *in vivo* biopanning methodology.

Cell binding by selected phage display peptides. The peptides were ranked based on the frequency with which they occurred in the plaques from which the DNA was prepared and sequenced (Table 1). Four of the sequences (L13, L19, R1, and R3) were synthesized, and the peptides were prepared either as biotinylated peptide or as the control non-biotinylated version of the sequence. Two of three peptides tested to date, L13 and R1, were able *in vitro* to bind cells from the skeleton. The cells used in these experiments were mesenchymal cells from bone marrow, D1 (Figure 2), or human bone marrow endothelial cells. An Avidin-FITC probe detected the biotinylated peptide in areas of the cells in culture representing either extrusions from the cells or at the end of cell processes; structures reminiscent of adhesion plaques or footprints (Figure 2, arrows). Furthermore, selected peptides were found to bind to tissue sections *in vitro*. Biotinylated L13 and R1 peptides and Avidin-FITC were used to detect the

interaction of the phage-derived peptides in thick sections of different tissues.

Table 1. Phage obtained from elution of murine femurs following third pass of biopanning in vivo. Phage are listed in the order of descending frequency of isolation.

NAME	PHAGE SEQUENCE FREQUENCY*	PERCENT FREQUENCY
L14	dec-30	40
R3	okt-29	34.5
L2	sep-28	32.1
L1	aug-28	28.6
R1	jul-29	24.1
R8	jul-29	24.1
L13	jul-30	23.3
L6	mei-28	17.9
L5	mei-28	17.9
R2	mei-29	17.2
L12	mei-30	16.7
L19	mei-30	16.7
L7	jan-28	3.6
L11	jan-30	3.3

This immunohistochemistry revealed significant binding to rib and long bone growth plate by both L13 and R1 (Figure 3). By contrast R3 peptide showed no staining. These experiments partially demonstrate the specificity of some of the peptides for bone and showed that the ability to bind to bone is not shared by all of the peptides isolated from the phage display library.

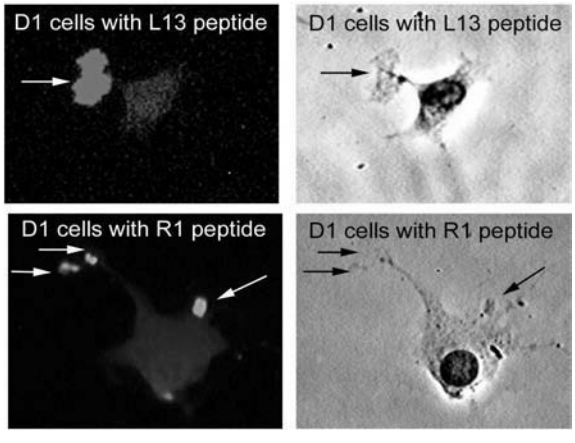
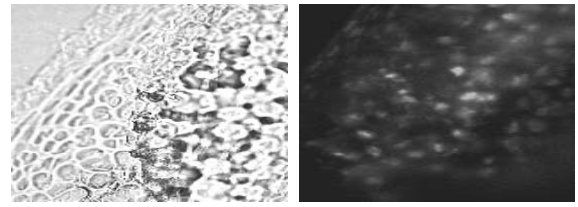


Figure 2. Bone-homing peptide binding to osteoblastic precursor, D1, with biotinylated phage-derived peptides, L13 and R1.



Peptide L13 binding to rib bone.

Figure 3. Immunocytochemistry of murine rib with biotinylated phage-derived peptide L13.

Prostate cancer cells adhere to bone marrow mesenchymal osteoprogenitors and to human bone marrow endothelial cells (hBMEC). The prostate literature is replete with references stating the dramatic tendency for prostate cancer metastases to establish within the marrow forming centers of bone (2, 4, 5, 25-30). We have established assays *in vitro* to test directly whether this proclivity is due to an inherent adhesive preference for bone marrow stromal or endothelial cells by more aggressive circulating prostate cancer cells (31, 32). The four phage-derived peptides isolated by biopanning were tested for their ability to alter the adhesion of prostate cancer cells to a human bone marrow endothelial cell line, hBMEC-1 (33)(Figure 4). In this assay, LNCaP cells were compared directly to the more metastatic, bone colonizing derivative, C4-2. These preliminary results show the possibility that the phage-derived peptides may have specific responses towards prostate cancer cell adhesion. In this assay, peptide R1 was relatively ineffective in its activity while R3 showed a dose-dependent inhibition of prostate cancer cell invasion for both cell lines. The peptide, L13, showed consistent suppression of prostate cancer cell invasion that was apparently saturated at the doses tested. L19, on the other hand, showed a remarkable difference between LNCaP and C4-2 cells. This peptide strongly inhibited the less aggressive LNCaP cell, while leaving the adhesion of the C4-2 cell line unaffected, demonstrating that these phage-derived peptides have cell type and dose-dependent activity. We are currently in the process of determining the effective concentration of peptide required to block prostate cancer cell adhesion to hBMEC cells as well as to determine the cognate binding partners of these

peptides. Future studies will examine the effect of these peptides on prostate cancer cell invasion and bone colonization *in vivo*. These blocking peptides thus may one day provide a therapeutic tool to slow prostate cancer colonization of the bone or provide the basis for developing bone endothelial targeted therapy.

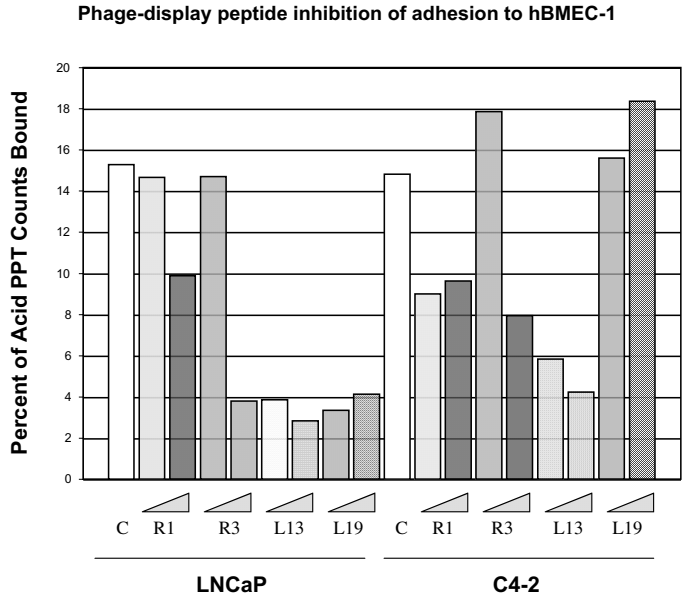


Figure 4. The ability of four phage-derived peptides to block prostate cancer cell adhesion to human bone marrow endothelial cells (hBMEC) was tested at either 50μM or 100μM. Cell line specific inhibition can be observed with L19 while L13 inhibits both cell lines from adhering to hBMEC. Dose-dependent decreases in cell adhesion are seen with R3 for both cell lines.

2.1.2 A2. Bone Marrow Derived Peptide Phage Display

Two studies demonstrated that the adhesion of prostate cancer cells to human bone marrow endothelium (HBME) does not correlate with metastatic preference and aggression (33). Lehr and Pienta (1998) (33) reported that although prostate cancer cells adhered preferentially to HBME cells when compared to human umbilical vein endothelial cells (HUVEC), there were no significant differences between the degree of cell adhesion to HBME between LNCaP (mildly aggressive and derived from a lymph node metastasis), DU145 (aggressive and derived from a brain metastasis) and PC-3 cells (aggressive and derived from a bone metastasis). It was anticipated that PC-3 cells would adhere to a greater extent to HBME cells when compared to the other prostate cancer cell lines because these other cell lines do not have a bone metastatic phenotype. We recently confirmed this observation using the sublines of the LNCaP human prostate progression model (31). This study reported that there was no difference between the adhesion of LNCaP cells and its more aggressive and spontaneously bone-homing derivative, C4-2, to a monolayer of HBME cells. Interestingly, the normal prostate cell line, P69, adhered better to HBME than both C4-2 and LNCaP, further suggesting that in the case of prostate cancer the adhesion of cancer cells to microvascular endothelial cells does not correlate with metastatic potential. One possibility is that a strong adhesive interaction may hinder the metastatic process by maintaining adhesion of cancer cells to the endothelium, thereby preventing transendothelial migration and invasion into the bone stroma. Yet, despite these new observations, the adhesion to HBME cells is an

important step in the metastatic cascade of prostate cancer to bone. Therefore, identifying surface proteins or cell adhesion molecules (CAMs) essential for this interaction would facilitate efforts to interrupt the metastatic process in prostate cancer.

Several studies, including our own, have determined that β1 integrins, type C surface lectin, and hyaluronan, are all expressed on PC-3 cells, mediate their interaction with HBME. These same studies excluded the utility of αvβ3, which is highly expressed by PC-3 cells when compared to other prostate cancer cell lines (30, 34). Surprisingly, β1 integrins expressed on HBME cells do not mediate their adhesion of PC-3 cell adhesion (35). These results were mostly obtained from adhesion blockade assays that used specific blocking antibodies for integrins and used hyaluronidase to strip the hyaluronan pericellular matrix from PC-3. Both procedures require knowledge of the existing proteins on the surface of both cell lines, which limits these procedures. Phage display is a novel approach involving the cloning of eukaryotic proteins into the capsid region of phage genome, theoretically allowing the phage to interact with the appropriate ligands on selected substrates, either extracellular matrix components or cells. This approach does not require such knowledge of existing surface proteins and, if done in a manner that significantly reduces background, can yield useful information.

Romanov et al. (36) reported phage 35, which contained the peptide sequence DPRATPGS, bound to the surface of LNCaP cells and their derivatives C4-2 and C4-2B. Functionally, this phage blocks the spreading of LNCaP, C4-2, and C4-2B on tissue culture plastic, but the cognate peptide 35 did not. The cognate peptide 35 however did increase the adhesion of C4-2 and C4-2B to HUVEC, increase invasion through Matrigel®, and increase the activation of matrix metalloproteinase-2 in these sublines. By using biopanning *in vitro* and comparing LNCaP to C4-2 cells using a circular peptide phage display library, we identified consistently the same peptide sequence (GWAGWGRPAE) on both LNCaP and C4-2 cells *in vitro*, suggesting that expression of proteins on the cell surface change minimally during the progression of LNCaP to the bone metastasizing C4-2. Another less appealing explanation is that the recognition of different surface epitopes by this type of library is not ideal (31). This result indicates that the adhesion of prostate cancer cells to HBME is not the sole determinant of prostate cancer's preference for bone, but supports the notion that distinct prostate cancer cell types may respond differently to the bone microenvironment depending on their overall phenotype and history. For example, the C4-2 subline may relocate to the bone better than LNCaP because the phenotype of C4-2 allows it to respond more favorably to stromal factors found in the bone marrow.

Surface proteins on HBME that mediate the adhesion of prostate cancer cells are not known and to this date, none have been reported. To determine the natural ligand on HBME cells for CAMs on PC-3 cells, Cooper and colleagues (37) used the T7 phage display system to generate a HBME phage library (Figure 5). Other studies used a phage library of synthetic peptides that may mimic the natural ligand, but the identity of the ligand has not been determined. By performing a subtractive biopanning step on a colon cancer cell, WiDr, which binds poorly to HBME cells, followed by three rounds of biopanning on PC-3 cells, a novel protein homologous to human reticulocalbin 1 that is preferentially expressed in HBME cells was isolated. Because of its tentative expression pattern, this protein is called Expressed in Human bone marrow endothelial Cells (EIHC) (37). Here, we report for the first time that EIHC is expressed equally in LNCaP, C4-2, and C4-2B sublines of the LNCaP progression model (Figure 6). Reticulocalbin and EIHC are putative endoplasmic reticulum proteins that contain six EF hands for binding calcium. The function of reticulocalbin in HBME and the LNCaP sublines is not known and is under investigation; however, it is believed that EIHC and reticulocalbin will have similar functions based on their similar amino acid sequence.

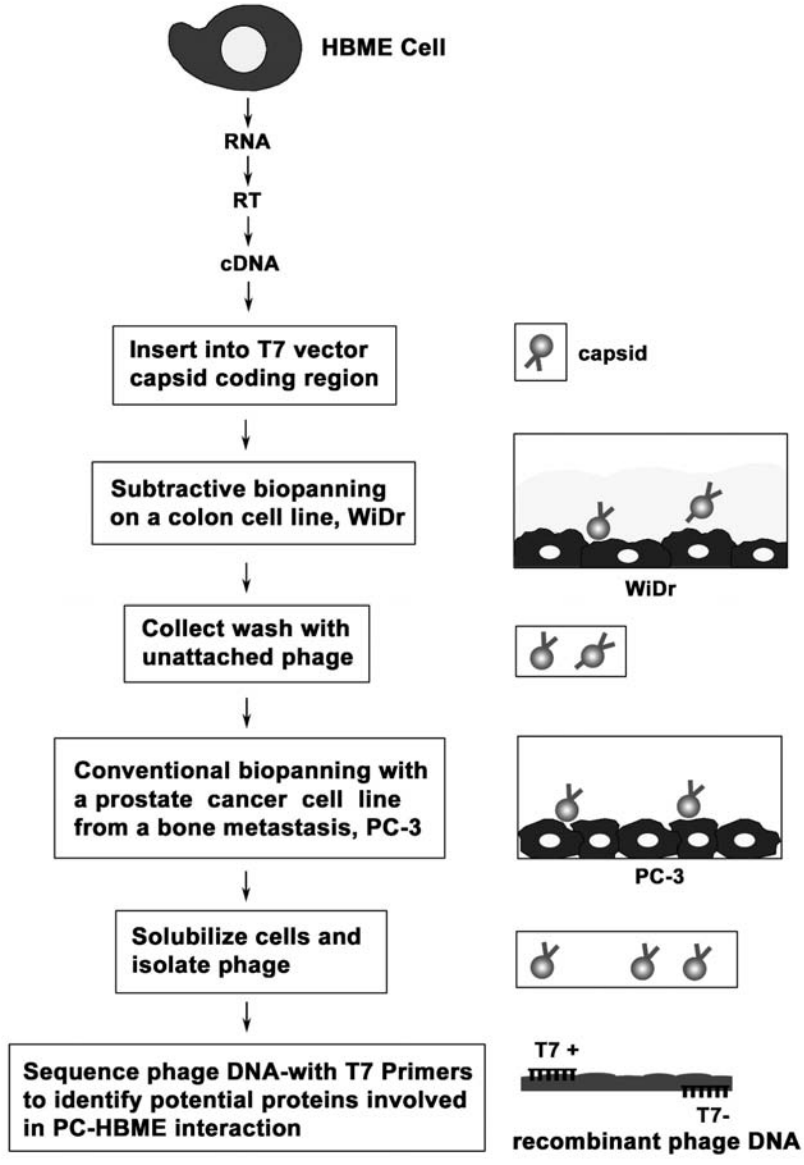


Figure 5. Schematic of the protocol generating the T7 phage library from human endothelial cell cultures.

Identifying the CAMs necessary for prostate cancer-endothelium interaction is essential in order to design a therapeutic intervention for the metastatic process specific for prostate cancer dissemination to the bone marrow. Using innovative and powerful approaches, such as phage display, will hopefully reveal these CAMs that can serve as targets for better drug development in the near future.

2.2 Anti-Angiogenic Therapy

It is well established that for a tumor or a tumor metastasis to grow beyond 0.5-1mm in diameter, the size beyond which the diffusion of oxygen is limited, neovascularization or angiogenesis must occur (38, 39). Angiogenesis is the process by which new vessels arise from pre-existing vessels is a

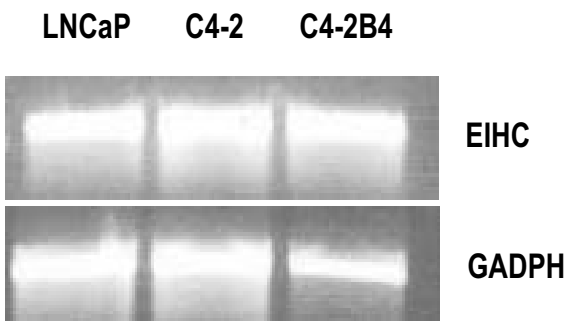


Figure 6. The expression of EIHC in the LNCaP progression cell model system

series influences to sprout from the existing vasculature, form tubules and elongate toward the angiogenic source, and finally complete the vascular network by connecting to other vessels (40). Tumor cells have been demonstrated to produce a number of angiogenic factors that include the two predominant endothelial cell growth factors, basic fibroblast growth factor (bFGF, FGF-2) and vascular endothelial growth factors (VEGF) (40). Finally, the degree of microvessel density (MVD) has been shown to correlate with the predisposition of a number of tumor types to metastasize and the patient's prognosis (41-65). For these reasons a number of investigators have pursued the development of anti-angiogenic compounds as cancer therapeutics. Several promising candidates have been discovered that are derivatives of larger proteins having completely disparate functions. Angiostatin was discovered in the serum and urine of mice specifically bearing primary tumors, and was reported to be responsible for the suppression of metastatic growth of Lewis lung carcinoma in these animals (66, 67). The naturally occurring human isoform of this protein is called angiostatin 4.5. Angiostatin is a fragment of plasminogen, which gets converted to the anti-angiogenic protein by membrane-associated beta-actin preferred to previously at a plasminogen receptor (68). This fragment inhibits angiogenesis by inducing apoptosis in endothelial cells via the caspase pathway (66). Endostatin, another endogenous angiogenic inhibitor, was isolated from hemangioendothelioma cells as a COOH-terminal segment of collagen XVIII. Like angiostatin, endostatin inhibits angiogenesis, by inhibiting the growth of endothelial cells (69). The mechanism for inhibitory effect of endostatin on endothelial cells is via a reduction in anti-apoptotic proteins Bcl-2 and Bcl-XL (69). Through binding integrin $\alpha 5\beta 1$, endostatin inhibits focal adhesion kinase (FAK) and its signaling to the p38 mitogen-activated protein kinase pathway (70). Finally, tumostatin, a noncollagenous (NC)1 fragment of collagen type IV, inhibits angiogenesis by specifically inhibiting endothelial cell-specific protein synthesis (71). The action of tumstatin is dependent on its binding to integrin $\alpha V\beta 3$ to inhibit FAK activation of the PKB/Akt and mTOR pathway (70). Therefore, both endostatin and tumstatin suppress angiogenesis by binding to specific integrin heterodimers on the endothelial cell surface that activate distinct signal pathways with the same outcome (i.e. endothelial cell apoptosis) (70). The fact that some patients may not tolerate these natural compounds or that they may be degraded rapidly has led to the development of small molecule inhibitors with anti-angiogenic efficacy, like thalidomide (See below) (72-76).

2.2.1 B1 Continuous low-dose Chemotherapy and Disease states

Morris and Scher (77), recently recommended the application of a "clinical states" model for designing trials tailored to the patient and the stage of the disease. The authors point out that there are an expanding number of treatment options available that are becoming increasingly tailored or perhaps better applied for particular points in the development/progression of a cancer. Therefore, care should be taken to match the therapy chosen with the object of treatment, namely the state at which the clinician finds the patient's disease. Using prostate cancer as an example, localized disease therapy should be optimized to minimize recurrence and rising prostate specific antigen (PSA) while patients with recurrent disease and rising PSA levels should be treated to minimize progression with similar concerns for prostate cancer at other steps in progression. Taking this model into account, it becomes evident that there are two targets for anti-angiogenic therapy. The first occurs when the primary tumor is small and neovascularization is just beginning and the second follows when the metastatic cell is beginning to grow at a distal site.

This philosophy reflects the emerging consensus that the most effective window for the use of anti-angiogenic compounds, such as thalidomide, is during the development of new blood vessels and not after the blood supply is established and the endothelium is no longer proliferative. The conditions that are prime for targeting neovascularization are most likely to occur when the tumor burden is considerably lower than that found in the population of patients currently recruited for phase I or II clinical trials.

The maximum tolerated dose (MTD) of a chemotherapeutic agent may not be the most effective method for administering these agents (78-80). Instead there is accumulating evidence that metronomic dosing, the continuous infusion of low-dose chemotherapy, is much better tolerated by patients and still delivers sufficiently toxic dosages of agents to kill the tumor supporting vasculature as well as some tumor cells in the population that may not have been cycling during a particular MTD exposure to a particular chemotherapeutic agent. This works since the tumor-induced neovascularization is the resultant outgrowth of normal endothelial cells in response to endothelial cell growth factors secreted by the tumor. The genomic instability of tumor cells allows them to become resistant to the MTD during the obligatory recovery periods required for patients during MTD therapeutic regimens. Since the endothelial cells are normal cells, they do not become resistant to the chemotherapy and are killed to a varying degree at each treatment interval. However, the recovery period between MTD administrations allows for neovascularization of the tumor before the next treatment. This explains, in part, some of the failures seen for anti-angiogenic therapy. Indeed, such chronically administered low-dosage or metronomic dosing strategies have shown regression of chemotherapy resistant tumors in animal models (81, 82) or partial to complete patient responses in patients with metastatic breast cancer (83). For molecules like thalidomide, that are known to have an anti-angiogenic mechanism, low dose administration of 100 mg/day (84) was just as effective as ≥200 mg/day (85, 86) and was tolerated better by the patients with fewer adverse incidents reported.

2.2.2 NOVEL THALIDOMIDE ANALOG DEVELOPMENT AND TESTING

The anti-angiogenic properties of thalidomide are not intrinsic to the parent molecule but rather are associated with a particular stereochemistry and/or with one or more of its metabolites (61, 73, 87-89). Stephens et al. (90) proposed that phthalimidoglutaramic acid and carboxybenzamidoglutarimide, the two initial metabolic products resulting from thalidomide's spontaneous hydrolysis, form the basis for thalidomide's action *in vivo*. The former has been implicated as the teratogenic and anti-angiogenic compound while the latter has demonstrated neither property. DNA intercalation of thalidomide derivatives has been proposed as a mechanism for its inherent teratogenic properties (91). The authors of this work proposed that this property can be avoided when derivatives are synthesized.

As thalidomide undergoes rapid hydrolysis *in vivo*, most, if not all, of the most valuable therapeutic effects likely result from the degradation products or will be derived from analogs based upon these structures or the thalidomide core structure. There have been hundreds of thalidomide analogues synthesized to date (72-76, 92) all based on rationale drug design. Furthermore, methods have been published for generating combinatorial libraries of analogs (76). The result is a considerable backlog in the testing of these molecules, in particular from the combinatorial libraries, for physiological efficacy. Indeed, Figg et al. (73) have demonstrated that most of these compounds will not have enhanced efficacy when compared to thalidomide or will not display the desired effects of being tumoricidal or anti-angiogenic with decreased side effects. For example, from 118 analogues synthesized by Figg et al. (93) that were tested specifically for anti-angiogenic properties, only 7 demonstrated potency in an anti-angiogenesis assay. Of the analogs synthesized by Xiao et al. (76) using combinatorial methods as described above, only 8 were tested (94). All 8 analogs had anti-angiogenic properties but none displayed anti-tumor properties. Preclinical evaluation to determine the suitability of most anti-angiogenic analogs for clinical trial is still in progress. The future holds great promise in

delivering several new small molecule thalidomide derivatives to the clinic.

To date there has been an enormous effort in the generation of new thalidomide derivatives with promising preclinical results (72-76, 92). Despite this effort, only one derivative CC-5013, has made it into clinical trial alongside the parental compound. The IMiD (Immunomodulatory Drug), CC-5013, has been demonstrated to be beneficial in the treatment of multiple myeloma, an osteolytic bone disease, and solid tumors (73, 75). CC-5013 is being evaluated in sixteen clinical trials overall, none of which are for prostate cancer. There is also recent evidence suggesting that SelCID-3 may have potent antitumor effects through the induction of G2-M cell cycle arrest and caspase 3-dependent apoptosis. The majority of these structural analogues were derived through modifications to the glutarimide moiety or by putting a phenyl ring in the phthalimide region. Recently investigators from the Brown group have attempted to tackle the challenge of expanding the phthalimide region of the molecule while preserving internal amides (72, 92). This has generated three functional classes of thalidomide analogs as evinced by testing *in vitro*; those that kill endothelial cells, those that kill prostate cancer cells and those that kill both with equal efficacy (72). Preliminary data suggests that one of these molecules is a novel microtubule depolymerizing agent. Given these developments in synthesis, testing and positive clinical trials results, the future of small molecule thalidomide analogs remains promising.

3. APPLICATIONS FOR MARROW-HOMING BONE STROMAL CELLS

Mesenchymal cell homing to bone and marrow. For many years, we have focused on the isolation and characterization of mesenchymal cells from bone marrow (95). The cells that were isolated are pluripotent, can differentiate into a variety of cell types *in vitro*, and are capable of targeting bone marrow upon intravenous administration of genetically labeled cells into host syngeneic mice (96). This bone marrow homing phenomenon was exploited for the purposes of gene delivery which can be useful for musculoskeletal gene therapy. By engineering cells to carry an IGF-1 cDNA, we showed homing to bone marrow and to fractures of bone (97). The genetically modified mesenchymal cells also were shown to target sites of injury in the musculoskeletal system such as defects that were created in cortical bone and femur fractures (98).

Bone marrow (mesenchymal) cells target bone metastases. Predilection of prostate cancer for bone metastasis suggests that cells of the bone marrow stroma provide a nurturing environment. The stromal-epithelial interaction is important for prostate cancer growth and survival (99, 100). In collaboration with Leland Chung's group we combined a bone stromal cell line expressing thymidine kinase with several prostate cancer cell lines *in vitro* and *in vivo* in an effort to explore and understand this relationship. A bone stromal cell line, D1, and a series of LNCaP-lineage related human prostate cancer cell lines were maintained in culture (95, 96, 101, 102). The D1 cell was transfected with lac Z (D1-bag) to allow for morphological identification. Retrovirus constructs containing the thymidine kinase (TK) or cytosine deaminase (CD) genes were used to transduce the β-galactosidase expressing D1-bag cells. D1-TK and D1-CD cells were co-cultured with prostate cancer cell lines *in vitro* and *in vivo*. The presence of toxic genes TK and CD in the bone stromal cells allows for cellular "suicide" by the administration of the pro-drug, acyclovir and 5-fluorocytosine, respectively. A co-culture of these genetically modified bone stromal cells with cells from the prostate cancer cell line LNCaP (androgen sensitive) and sublines C4-2 (androgen independent, tumorigenic and spontaneously metastatic to bone) and C4-2B (androgen-independent, bone metastatic, bone metastasis-derived) was performed to evaluate the interaction between the stromal cells and the various prostate cancer cell lines (99, 103, 104). The homing ability of the D1-bag cells to bone marrow was tested 2-4 weeks after intravenous injection by staining of the mouse femur with X-gal. A subcutaneous chimeric tumor model with 50% D1-TK and 50% C4-2 was established (99, 103, 104). The bystander cell kill was tested by administration of 40 mg/kg of acyclovir to the mice. Tumor size

was determined radiographically and PSA measured in serum.

In vitro co-culture of the bone marrow stromal cells with LNCaP and its subline C4-2 exhibited diffuse interaction at the cellular level. The bone metastatic subline C4-2B formed organized clusters that were completely surrounded by bone stromal cells. In this *in vitro* co-culture system, marked cellular killing occurred upon addition of acyclovir to the D1-TK cells compared to the unaltered bone stromal cells, D1. *In vivo* co-cultures of D1 cells containing thymidine kinase and C4-2 cells in the subcutaneous tissue stimulated an osseous metastasis in nude mice. Treatment with acyclovir attenuated tumor growth and decreased serum PSA levels. D1-bag cells delivered intravenously or by intramedullary injection home to the bone marrow and localize to sites of C4-2B metastasis in bone.

The morphological findings suggest intercellular communication between the stromal cells and the bone metastatic subline of LNCaP, C4-2B. This may explain the marked cellular killing which was observed in the co-culture assay of the bone marrow stromal cells which were transduced with TK and CD upon administration of acyclovir or 5-FC, respectively. The observation *in vivo* of decreased chimeric tumor volume and decreased serum PSA upon administration of acyclovir, combined with the ability of the stromal cells to home to the bone marrow after systemic injection, suggests that a viable treatment for osseous metastasis may be the destruction of the ability of bone stromal cells to nurture tumor growth.

There is now an accumulating body of evidence to indicate that bone marrow metastasis is an early event, occurring even prior to the detection of clinical symptoms (105). In studies with Chung et al. (99) and our collaboration with Hsieh et al (104), it has been shown that bone marrow mesenchymal cells can target bone metastases resulting in combined cell death in the tumor cell-D1-TK co-culture assay. This further demonstrates the potential efficacy of using bone-targeting bone stromal cells as therapeutic delivery vehicles. Further knowledge of the initial tumor cell-bone stroma interaction will facilitate the development of treatments that prevent osteoclastic and osteoblastic lesions with bone metastases. Such steps are already improving the targeting and efficiency of viral targeted gene expression with both tissue specific promoters (99, 106) and refinements to enhance those expression vectors using locally available steroid hormone response elements such as 1,25-dihydroxyvitamin D_3. (106) The identification of molecular interactions between tumor and bone cells that are important to the process of cancer cell dissemination to bone is a crucial step towards testing the physiological significance of these interactions *in vivo*.

4. CONCLUDING REMARKS REGARDING ADDITIONAL APPROACHES TARGETING BONE STROMAL CELLS

In this chapter we have summarized and highlighted many of our current and recent research approaches to the targeting of cancer metastasis involving bone by thinking about the unique interplay between bone metastasizing cancers and the bone microenvironment. The research highlighted above only touches the surface of some very exciting new approaches to this ongoing problem. Many investigators have made significant recent progress towards understanding the dynamic interplay between bone metastases and the changes that ensue in normal bone processes involved with bone remodeling.

As prostate cancer develops, it becomes more osteomimetic as described by Koeneman et al (107). Basically, this means that prostate cancer cells begin to express proteins commonly found in the bone microenvironment, both extracellular matrix molecules and soluble factors, long before they ever colonize that tissue (107-109). Taking advantage of this fact, Chung and colleagues have demonstrated the utility of targeting both tumor cells and bone stromal cells using the osteocalcin promoter to drive toxic gene therapy to kill both the invading tumor and the activating bone stromal cells as described above (99, 103, 104, 106, 109, 110).

Prostate cancer is not unique in this osteomimetic behavior as several bone metastasizing tumors, prostate, breast, multiple myeloma and lung

secrete parathyroid hormone-related protein (PTHrP) (111, 112), MIP-1α (113) or various interleukins like IL-6 and IL-8 (113-122), all of which are considered to be osteoclast activating factors in addition to contributing to the development of androgen independence in prostate cancer. All of these molecules are potential therapeutic targets.

As an indirect activator of osteoclasts, PTHrP is a potent and important molecule in the regulation of the resultant phenotype of the metastatic focus, whether or not it is osteoblastic or osteoclastic in nature. This is accomplished through the enhanced secretion of receptor activator of NFκB ligand (RANKL) by osteoblasts in response to PTHrP. RANKL then stimulates osteoclast activity directly. One of the consequences of this action of PTHrP is to decrease the natural RANKL receptor antagonist, osteoprotegerin (OPG). OPG normally acts as a decoy for RANKL preventing the formation of osteoclasts by RANKL binding to its receptor, RANK, on the precursors to osteoclasts. Since this pathway plays a central role in regulating bone turnover/remodeling, it is an attractive candidate for therapeutic intervention. Neutralizing monoclonal antibodies to PTHrP have been developed and found to be active in xenograft models (111, 123) and murine models of multiple myeloma (124). Guanine nucleotide analogs (125) and vitamin D_3 derivatives (126) were found to be effective at reducing PTHrP production by cancer cells. As the decoy receptor for RANKL, the administration of exogenous OPG has seen some success as has soluble RANK as determined by regulation of serum calcium levels and normalization of mineralized bone volume (112). In studies with human xenografts, OPG administration decreased the number of mature osteoclasts and osteolytic tumor lesions although care should be taken because this molecule can act as a tumor survival factor under appropriate circumstances (112).

Bisphosphonates are a class of small molecule inhibitors that accumulate in bone and act directly by decreasing osteoclastogenesis, decreasing resorption rates, and enhancing osteoclast apoptosis (127, 128). Overall, bisphosphonates inhibit the colonization of metastatic tumor cells in bone although the efficacy of this approach in osteoblastic cancers is unclear (2).

In conclusion, the understanding of the biology of bone development and turnover has led to the identification of several key regulatory molecules in these processes that have been commandeered by the invading cancer cells. As our level of understanding in this normal process of bone homeostasis increases, we will have new targets to assess for efficacy in the treatment of osseous metastases. It is our hope that these research efforts will provide well-tolerated, highly efficacious therapies to eradicate osseous metastases and the associated co-morbidity.

ACKNOWLEDGEMENTS

The funding for this research was provided by the following resources: University of Delaware Start-up funds (RAS, CRC), NIH K22 CA97117-01 (CRC), University of Delaware Research Foundation (RAS), Prostate Cancer Research Program DAMD 17-03-1-0043 (GB, RAS), The Jeffress Trust (MLB), the Developmental Therapeutics Program of the NCI, CA-105435-01 (MLB, RAS). Special thanks to Margie Barrett for graphic arts assistance and to Dr. M.C. Farach-Carson for critical reading of the manuscript.

REFERENCES

1. Jemal, A., Tiwari, R.C., Murray, T., et al., 2004, Cancer statistics, 2004. CA Cancer J Clin, 54(1):8-29.
2. Tu, S.M., and Lin, S.H., 2004, Clinical aspects of bone metastases in prostate cancer. Cancer Treat Res, 118:23-46.
3. Jacobs, S.C., 1983, Spread of prostatic cancer to bone. Urology, 21(4):337-344.
4. Koutsilieris, M., 1995, Skeletal metastases in advanced prostate cancer: cell biology and therapy. Crit Rev Oncol Hematol, 18(1):51-64.
5. Galasko, C.S., 1986, Skeletal metastases. Clin Orthop, 210:18-30.
6. Pauli, B.U., and Lee, C.L., 1988, Organ preference of metastasis. The role of organ-specifically modulated endothelial cells. Lab Invest, 58(4):-387.

7. Pasqualini, R., and Ruoslahti, E., 1996, Organ targeting in vivo using phage display peptide libraries. Nature, 380(6572):364-366.
8. Ruoslahti, E., and Rajotte, D., 2000, An address system in the vasculature of normal tissues and tumors. Annu Rev Immunol, 18:813-827.
9. Clement, G., Bisoffi, M., Finger, A.N., et al., 2003, Peptabodies as tools to test ligands isolated from phage-displayed peptide libraries. J Immunol Methods, 276(1-2):135-141.
10. Koivunen, E., Arap, W., Rajotte, D., et al., 1999, Identification of receptor ligands with phage display peptide libraries. J Nucl Med, 40(5):883-888.
11. Ivanenkov, V., Felici, F., and Menon, A.G., 1999, Uptake and intracellular fate of phage display vectors in mammalian cells. Biochim Biophys Acta, 1448(3):450-462.
12. Ivanenkov, V.V., Felici, F., and Menon A.G., 1999, Targeted delivery of multivalent phage display vectors into mammalian cells. Biochim Biophys Acta, 1448(3):463-472.
13. Tseng-Law, J., P. Szalay, R., Guillermo, et al., 1999, Identification of a peptide directed against the anti-CD34 antibody, 9C5, by phage display and its use in hematopoietic stem cell selection. Exp Hematol, 27(5):936-945.
14. Cwirla, S.E., Peters E.A., Barrett R.W., et al., 1990, Peptides on phage: a vast library of peptides for identifying ligands. Proc Natl Acad Sci U S A, 87(16):6378-6382.
15. Pasqualini, R., Koivunen, E., and Ruoslahti E., A peptide isolated from phage display libraries is a structural and functional mimic of an RGD-binding site on integrins. J Cell Biol, 130(5):1189-1196.
16. Sparks, A.B., Adey, N.B., Quilliam, L.A., et al., 1995, Screening phage-displayed random peptide libraries for SH3 ligands. Methods Enzymol, 255:498-509.
17. Arap, W., Pasqualini, R., and Ruoslahti, E., 1998, Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science, 279(5349):377-380.
18. Arap, W., Pasqualini, R., and Ruoslahti, E., 1998, Chemotherapy targeted to tumor vasculature. Curr Opin Oncol, 10(6):560-565.
19. Arap, W., Haedicke, W., Bernasconi, M., et al., 2002, Targeting the prostate for destruction through a vascular address. Proc Natl Acad Sci U S A, 99(3):1527-1531.
20. Devlin, J.J., Panganiban, L.C., and Devlin, P.E., 1990, Random peptide libraries: a source of specific protein binding molecules. Science, 249(4967):404-406.
21. Kay, B.K., Kasanov, J., Knight, S., et al., 2000, Convergent evolution with combinatorial peptides. FEBS Lett, 480(1):55-62.
22. Pasqualini, R., Koivunen, E., Kain, R., et al., 2000, Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. Cancer Res, 60(3):722-727.
23. Brown, K.C., 2000, New approaches for cell-specific targeting: identification of cell-selective peptides from combinatorial libraries. Curr Opin Chem Biol, 4(1):16-21.
24. White, S.J., Nicklin, S.A., Sawamura, T., et al., 2001, Identification of peptides that target the endothelial cell-specific LOX-1 receptor. Hypertension, 37(2 Part 2):449-455.
25. Rubens, R.D., and Mundy, G.R., 2000, Cancer and the skeleton. Martin Dunitz, Malden, MA, London, Blackwell Science, USA, viii:286.
26. Batson, O.V., 1967, The vertebral system of veins as a means for cancer dissemination. Prog Clin Cancer, 3:1-18.
27. Carlin, B.I., and Andriole, G.L., 2000, The natural history, skeletal complications, and management of bone metastases in patients with prostate carcinoma. Cancer, 88(12 Suppl):2989-2994.
28. Cher, M.L., 2001, Mechanisms governing bone metastasis in prostate cancer. Curr Opin Urol, 11(5):483-488.
29. Saitoh, H., Hida, M., Shimbo, T., et al., 1984, Metastatic patterns of prostatic cancer. Correlation between sites and number of organs involved. Cancer, 54(12):3078-3084.
30. Cooper, C.R., Chay, C.H., Gendernalik, J.D., et al., 2003, Stromal factors involved in prostate carcinoma metastasis to bone. Cancer, 97(3 Suppl):739-747.
31. Sikes, R.A., Nicholson, B.E., Koeneman, K.S., et al., 2004, Cellular interactions in the tropism of prostate cancer to bone. Int J Cancer, 110(4):497-503.
32. Cooper, C.R., McLean, L., Walsh, M., et al., 2000, Preferential adhesion of prostate cancer cells to bone is mediated by binding to bone marrow endothelial cells as compared to extracellular matrix components in vitro. Clin Cancer Res, 6(12):4839-4847.
33. Lehr, J.E., and Pienta, K.J., 1998, Preferential adhesion of prostate cancer cells to a human bone marrow endothelial cell line. J Natl Cancer Inst, 90(2):118-123.
34. Stewart, D.A., Cooper, C.R., and Sikes R.A., 2004, Changes in extracellular matrix (ECM) and ECM-associated proteins in the metastatic progression of prostate cancer. Reprod Biol Endocrinol, 2(1):2.
35. Cooper, C.R., McLean, L., Mucci, N.R., et al., 2000, Prostate cancer cell adhesion to quiescent endothelial cells is not mediated by beta-1 integrin subunit. Anticancer Res, 20(6B):4159-4162.
36. Romanov, V.I., Durand, D.B., and Petrenko V.A., 2001, Phage display selection of peptides that affect prostate carcinoma cells attachment and invasion. Prostate, 47(4):239-251.

37. Cooper, C.R., Chaib, H., Chay, C. et al., 2003, The identification of reticulocalbin's role in prostate cancer cell adhesion to human bone marrow endothelium by a novel phage display system. Oncology (Supplement), 17:42-43.
38. D'Amore, P.A., 1988, Antiangiogenesis as a strategy for antimetastasis. Semin Thromb Hemost, 14(1):73-78.
39. Fidler, I.J., 2003, The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat Rev Cancer, 3(6):453-458.
40. Bergers, G. and Benjamin, L.E., 2003, Tumorigenesis and the angiogenic switch. Nat Rev Cancer, 3(6):401-410.
41. Bono, A.V., Celato, N., Cova V., et al., 2002, Microvessel density in prostate carcinoma. Prostate Cancer Prostatic Dis, 5(2):123-127.
42. Bostwick, D., Wheeler, T., Blute, M., et al., 1996, Optimized microvessel density analysis improves prediction of cancer stage from prostate needle biopsies. Urology, 48:47-57.
43. Brawer, M.K., 1996, Quantitative microvessel density. A staging and prognostic marker for human prostatic carcinoma. Cancer, 78(2):345-349.
44. Lissbrant, I., Stattin, P., Damber, J., et al., 1997, Vascular density is a predictor of cancer-specific survival in prostatic carcinoma. Prostate, 33:38-45.
45. Matsushima, H., Goto, T., Hosaka, Y., et al., 1999, Correlation between proliferation, apoptosis, and angiogenesis in prostate carcinoma and their relation to androgen ablation. Cancer, 85(8):1822-1827.
46. Silberman, M., Partin, A., Veltri, R., et al., 1997, Tumor angiogenesis correlates with progression after radical prostatectomy but not with pathologic stage in Gleason sum 5 to 7 adenocarcinoma of the prostate. Cancer, 79:772-779.
47. Goddard, J.C., Sutton, C.D., Berry, D.P., et al., 2001, The use of microvessel density in assessing human urological tumours. BJU Int, 87(9):866-875.
48. Leek, R.D., 2001, The prognostic role of angiogenesis in breast cancer. Anticancer Res, 21(6B):4325-4331.
49. Sauer, G. and Deissler, H., 2003, Angiogenesis: prognostic and therapeutic implications in gynecologic and breast malignancies. Curr Opin Obstet Gynecol, 15(1):45-49.
50. Bamberger, E.S. and C.W. Perrett, Angiogenesis in epithelian ovarian cancer. Mol Pathol, 2002. 55(6):348-359.
51. Bamberger, E.S. and Perrett, C.W., 2002, Angiogenesis in benign, pre-malignant and malignant vulvar lesions. Anticancer Res, 22(6C):3853-3865.
52. Bristow, R.E., 1999, Endometrial cancer. Curr Opin Oncol, 11(5):388-393.
53. Abulafia, O., Triest, W.E., and Sherer, D.M., 1999, Angiogenesis in malignancies of the female genital tract. Gynecol Oncol, 72(2):220-231.
54. Goth, M.I., Hubina, E., Raptis, S., et al., 2003, Physiological and pathological angiogenesis in the endocrine system. Microsc Res Tech, 60(1):98-106.
55. Giatromanolaki, A., 2001, Prognostic role of angiogenesis in non-small cell lung cancer. Anticancer Res, 21(6B):4373-4382.
56. Moreto, M., Diagnosis of esophagogastric tumors. Endoscopy, 2001. 33(1):1-7.
57. Tarta, C., Teixeira, C.R., Tanaka, S., et al., 2002, Angiogenesis in advanced colorectal adenocarcinoma with special reference to tumoral invasion. Arq Gastroenterol, 39(1):32-38.
58. Kakeji, Y., Maehara, Y., Sumiyoshi, Y., et al., 2002, Angiogenesis as a target for gastric cancer. Surgery, 131(1 Suppl):S48-54.
59. Qin, L.X. and Tang, Z.Y., 2002, The prognostic molecular markers in hepatocellular carcinoma. World J Gastroenterol, 8(3):385-392.
60. Pluda, J.M. and Parkinson, D.R., 1996, Clinical implications of tumor-associated neovascularization and current antiangiogenic strategies for the treatment of malignancies of pancreas. Cancer 78(3 Suppl):680-687.
61. Rajkumar, S.V. and Witzig, T.E., 2000, A review of angiogenesis and antiangiogenic therapy with thalidomide in multiple myeloma. Cancer Treat Rev, 26(5):351-362.
62. Vacca, A., Ribatti, D., Roccaro, A.M., et al., 2001, Bone marrow angiogenesis in patients with active multiple myeloma. Semin Oncol, 28(6):543-550.
63. Molica, S., 2001, Angiogenesis in B-cell chronic lymphocytic leukemia: methods of study, clinical significance and prognostic implications. Leuk Lymphoma, 42(4):603-607.
64. Weidner, N., 1993, Tumor angiogenesis: review of current applications in tumor prognostication. Semin Diagn Pathol, 10(4):302-313.
65. Weidner, N., Semple, J.P., Welch, W.R., et al., 1991, Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. N Engl J Med, 324(1):1-8.
66. Hanford, H.A., Wong, C.A., Kassan, H., et al., 2003, Angiostatin(4.5)-mediated apoptosis of vascular endothelial cells. Cancer Res, 63(14):4275-4280.
67. O'Reilly, M.S., Holmgren, L., Shing, Y., et al., 1994, Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell, 79(2):315-328.
68. Wang, H., Schultz, R., Hong, J., et al., 2004, Cell surface-dependent generation of angiostatin4.5. Cancer Res, 64(1):162-168.
69. O'Reilly, M.S., Boehm, T., Shing, Y., et al., 1997, Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell, 88(2):277-285.

70. Sudhakar, A., Sugimoto, H., Yang, C., et al., 2003, Human tumstatin and human endostatin exhibit distinct antiangiogenic activities mediated by alpha v beta 3 and alpha 5 beta 1 integrins. Proc Natl Acad Sci U S A, 100(8):4766-4771.
71. Maeshima, Y., Sudhakar, A., Lively, J.C., et al., 2002, Tumstatin, an endothelial cell-specific inhibitor of protein synthesis. Science, 295(5552):140-143.
72. Capitosti, S.M., Hansen, T.P., and Brown, M.L., 2004, Thalidomide analogues demonstrate dual inhibition of both angiogenesis and prostate cancer. Bioorg Med Chem, 12(2):327-336.
73. Figg, W.D., Kruger, E.A., Price, D.K., et al., 2002, Inhibition of angiogenesis: treatment options for patients with metastatic prostate cancer. Invest New Drugs, 20(2):183-194.
74. Hashimoto, Y., 2002, Structural development of biological response modifiers based on thalidomide. Bioorg Med Chem, 10(3):461-479.
75. Marriott, J.B., Clarke, I.A., Czajka, A., et al., 2003, A novel subclass of thalidomide analogue with anti-solid tumor activity in which caspase-dependent apoptosis is associated with altered expression of bcl-2 family proteins. Cancer Res, 63(3):593-599.
76. Xiao, Z., Schaefer, K., Firestine, S., et al., 2002, Solid-phase synthesis of thalidomide and its analogues. J Comb Chem, 4(2):149-153.
77. Morris, M.J. and Scher, H.I., 2002, Novel therapies for the treatment of prostate cancer: current clinical trials and development strategies. Surg Oncol, 11(1-2):13-23.
78. Kerbel, R.S., Yu, J., Tran, J., et al., 2001, Possible mechanisms of acquired resistance to anti-angiogenic drugs: implications for the use of combination therapy approaches. Cancer Metastasis Rev, 20(1-2):79-86.
79. Kerbel, R.S., Klement, G., Pritchard, K.I., et al., 2002, Continuous low-dose anti-angiogenic/ metronomic chemotherapy: from the research laboratory into the oncology clinic. Ann Oncol, 13(1):12-15.
80. Browder, T., Butterfield, C.E. Kraling, B.M., et al., 2000, Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. Cancer Res, 60(7):1878-1886.
81. Klement, G., Baruchel, S., Rak, J., et al., 2000, Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J Clin Invest, 105(8):R15-24.
82. Klement, G., Huang, P., Mayer, B., et al., 2002, Differences in therapeutic indexes of combination metronomic chemotherapy and an anti-VEGFR-2 antibody in multidrug-resistant human breast cancer xenografts. Clin Cancer Res, 8(1):221-232.
83. Colleoni, M., Rocca, A., Sandri, M.T., et al., 2002, Low-dose oral methotrexate and cyclophosphamide in metastatic breast cancer: antitumor activity and correlation with vascular endothelial growth factor levels. Ann Oncol, 13(1):73-80.
84. Drake, M.J., Robson, W., Mehta, P., et al., 2003, An open-label phase II study of low-dose thalidomide in androgen-independent prostate cancer. Br J Cancer, 88(6):822-827.
85. Figg, W.D., Arlen, P., Gulley, J., et al., 2001, A randomized phase II trial of docetaxel (taxotere) plus thalidomide in androgen-independent prostate cancer. Semin Oncol, 28(4 Suppl 15):62-66.
86. Figg, W.D., Dahut, W., Duray, P., et al., 2001, A randomized phase II trial of thalidomide, an angiogenesis inhibitor, in patients with androgen-independent prostate cancer. Clin Cancer Res, 7(7):1888-1893.
87. Bauer, K.S., Dixon, S.C., and Figg, W.D., 1998, Inhibition of angiogenesis by thalidomide requires metabolic activation, which is species-dependent. Biochem Pharmacol, 55(11):1827-1834.
88. Richardson, P., Hideshima, T., and Anderson, K., 2002, Thalidomide: emerging role in cancer medicine. Annu Rev Med, 53:629-657.
89. Arlen, P.E.A., 2002, NCI Intramural Approach to Advanced Prostate Cancer. Clin Pros Canc, 1(3):153-162.
90. Stephens, T.D., Bunde, C.J., and Fillmore B.J, 2000, Mechanism of action in thalidomide teratogenesis. Biochem Pharmacol, 59(12):1489-1499.
91. Jonsson, N.A., 1972, Chemical structure and teratogenic properties. IV. An outline of a chemical hypothesis for the teratogenic action of thalidomide. Acta Pharm Suec, 9(6):543-562.
92. Capitosti, S.M., Hansen, T.P., and Brown, M.L., 2003, Facile synthesis of an azido-labeled thalidomide analogue. Org Lett, 5(16):2865-2867.
93. Ng, S.S., Gutschow, M., Weiss, M., et al., 2003, Antiangiogenic activity of N-substituted and tetrafluorinated thalidomide analogues. Cancer Res, 63(12):3189-3194.
94. Marks, M.G., Shi, J., Fry, M.O., et al., 2002, Effects of putative hydroxylated thalidomide metabolites on blood vessel density in the chorioallantoic membrane (CAM) assay and on tumor and endothelial cell proliferation. Biol Pharm Bull, 25(5):597-604.
95. Diduch, D.R., Coe, M.R., Joyner, C., et al., 1993, Two cell lines from bone marrow that differ in terms of collagen synthesis, osteogenic characteristics, and matrix mineralization. J Bone Joint Surg Am, 75(1):92-105.
96. Dahir, G.A., Cui, Q., Anderson, P., et al., 2000, Pluripotential mesenchymal cells repopulate bone marrow and retain osteogenic properties. Clin Orthop, 379 Suppl):S134-145.

97. Devine, M.J., Mierisch, C.M., Jang, E., et al., 2002, Transplanted bone marrow cells localize to fracture callus in a mouse model. J Orthop Res, 20(6):1232-1239.
98. Shen, F.H., Visger, J.M., Balian, G., et al., 2002, Systemically administered mesenchymal stromal cells transduced with insulin-like growth factor-I localize to a fracture site and potentiate healing. J Orthop Trauma, 16(9):651-659.
99. Chung, L.W., Hsieh, C.L., Law, A., et al., 2003, New targets for therapy in prostate cancer: modulation of stromal-epithelial interactions. Urology, 62(5 Suppl 1):44-54.
100. Sung, S.Y., and Chung, L.W., 2002, Prostate tumor-stroma interaction: molecular mechanisms and opportunities for therapeutic targeting. Differentiation, 70(9-10):506-521.
101. Thalmann, G.N., Sikes, R.A., Wu, T.T., et al., 2000, LNCaP progression model of human prostate cancer: androgen-independence and osseous metastasis. Prostate, 44(2):91-103, Jul 101;144(102).
102. Thalmann, G.N., Anezinis, P.E., Chang, S.M., et al., 1994, Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer. Cancer Res, 54(10):2577-2581.
103. Gardner, T.A., Ko, S.-C., Kao, C., et al., 1998, Exploiting stromal-epithelial interaction for model development and new strategies of gene therapy for prostate cancer and osteosarcoma metastases (review). Gene Ther. Mol. Biol., 2:41-58.
104. Hsieh, C.L., Gardner, T.A., Miao, L., et al., 2004, Cotargeting tumor and stroma in a novel chimeric tumor model involving the growth of both human prostate cancer and bone stromal cells. Cancer Gene Ther, 11(2):148-155.
105. Robinson, V.L., Kauffman, E.C., Sokoloff, M.H., et al., 2004, The basic biology of metastasis. Cancer Treat Res, 118:1-21.
106. Hsieh, C.L., Yang, L., Miao, L., et al., 2002, A novel targeting modality to enhance adenoviral replication by vitamin D(3) in androgen-independent human prostate cancer cells and tumors. Cancer Res, 62(11):3084-3092.
107. Koeneman, K.S., Yeung, F., and Chung, L.W., 1999, Osteomimetic properties of prostate cancer cells: a hypothesis supporting the predilection of prostate cancer metastasis and growth in the bone environment. Prostate, 39(4):246-261.
108. Thalmann, G.N., Sikes, R.A., Devoll, R.E., et al., 1999, Osteopontin: possible role in prostate cancer progression. Clin Cancer Res, 5(8):2271-2277.
109. Ko, S.C., Cheon, J., Kao, C., et al., 1996, Osteocalcin promoter-based toxic gene therapy for the treatment of osteosarcoma in experimental models. Cancer Res, 56(20):4614-4619.
110. Chung, L.W., 2003, Prostate carcinoma bone-stroma interaction and its biologic and therapeutic implications. Cancer, 97(3 Suppl): p. 772-778.
111. McCauley, L.K. and Schneider, A., 2004, PTHrP and skeletal metatasis. Cancer Treat Res, 118:125-147.
112. Brown, J.M., Zhang, J., and Keller, E.T., 2004, Opg, RANKl, and RANK in cancer metastasis: expression and regulation. Cancer Treat Res, 118:149-172.
113. Roodman, G.D. and Choi, S.J., 2004, MIP-1 alpha and myeloma bone disease. Cancer Treat Res, 118:83-100.
114. Lee, L.F., Louie, M.C., Desai, S.J., et al., 2004, Interleukin-8 confers androgen-independent growth and migration of LNCaP: differential effects of tyrosine kinases Src and FAK. Oncogene, 23(12):2197-2205.
115. Kim, O., Jiang, T., Xie, Y., et al., 2004, Synergism of cytoplasmic kinases in IL6-induced ligand-independent activation of androgen receptor in prostate cancer cells. Oncogene, 23(10):1838-1844.
116. Blaszczyk, N., Masri, B.A., Mawji, N.R., et al., 2004, Osteoblast-derived factors induce androgen-independent proliferation and expression of prostate-specific antigen in human prostate cancer cells. Clin Cancer Res, 10(5):1860-1869.
117. Xie, S., Lin, H.K., Ni, J., et al., 2004, Regulation of interleukin-6-mediated PI3K activation and neuroendocrine differentiation by androgen signaling in prostate cancer LNCaP cells. Prostate, 60(1):61-67.
118. Lee, S.O., Lou, W., Johnson, C.S., et al., 2004, Interleukin-6 protects LNCaP cells from apoptosis induced by androgen deprivation through the Stat3 pathway. Prostate, 60(3):178-186.
119. Pu, Y.S., Hour, T.C., Chuang, S.E., et al., 2004, Interleukin-6 is responsible for drug resistance and anti-apoptotic effects in prostatic cancer cells. Prostate, 60(2):120-129.
120. Royuela, M., Ricote, M., Parsons, M.S., et al., 2004, Immunohistochemical analysis of the IL-6 family of cytokines and their receptors in benign, hyperplasic, and malignant human prostate. J Pathol, 202(1):41-49.
121. Kim, J., Adam, R.M., Solomon, K.R., et al., 2004, Involvement of cholesterol-rich lipid rafts in interleukin-6-induced neuroendocrine differentiation of LNCaP prostate cancer cells. Endocrinology, 145(2):613-619.
122. Smith, P.C., Hobisch, A., Lin, D.L., et al., 2001, Interleukin-6 and prostate cancer progression. Cytokine Growth Factor Rev, 12(1):33-40.
123. Guise, T.A., Yin, J.J., Taylor, S.D., et al., 1996, Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. J Clin Invest, 98(7): 1544-1549.

124. Sordillo, E.M. and Pearse, R.N., 2003, RANK-Fc: a therapeutic antagonist for RANK-L in myeloma. Cancer, 97(3 Suppl): 802-812.
125. Gallwitz, W.E., Guise, T.A., and Mundy, G.R., 2002, Guanosine nucleotides inhibit different syndromes of PTHrP excess caused by human cancers in vivo. J Clin Invest, 110(10):1559-1572.
126. Tovar Sepulveda, V.A., and Falzon, M., 2002, Regulation of PTH-related protein gene expression by vitamin D in PC-3 prostate cancer cells. Mol Cell Endocrinol, 190(1-2):115-124.
127. Yoneda, T., Hashimoto, N., and Hiraga, T., 2004, Bisphosphonate actions on bone and visceral metastases. Cancer Treat Res, 118:213-229.
128. Yoneda, T., Hashimoto, N., and Hiraga, T., 2003, Bisphosphonate actions on cancer. Calcif Tissue Int, 73(4):315-318.

Chapter 22

Organotropism of Lung Cancer Metastasis and its Molecular Targeted Therapy

Seiji Yano, Soji Kakiuchi, Helong Zhang and Saburo Sone
Department of Internal Medicine and Molecular Therapeutics, University of Tokushima Graduate School, Tokushima, Japan

Abstract: The distribution of metastases is determined by an interaction between tumor cells ("seed") and the microenvironment of specific organs ("soil"). In fact, lung cancer produces metastasis to several particular organs, such as the liver, lung, lymph nodes, brain, and bone, suggesting organotropism on metastasis. But, the precise mechanisms determining organotropism remain unsolved. We established multiple-organ metastasis model by intravenous injection of human lung cancer cells into NK-cell depleted SCID mice. For the elucidation of the factors regulating organotropism of metastasis, we performed cDNA-microarray analyses (23,040 genes) of the metastatic foci of human lung cancer (SBC-5) cells developed in four different organs. Hierarchical clustering of 435 genes separated the four organ-specific groups of metastatic lesions very clearly. Of 435 genes, parathyroid hormone related-peptide (PTHrP) was highly expressed in bone metastasis, and inhibition of PTHrP resulting in specific inhibition of bone metastasis, suggesting usefulness of this approach to identify organ-specific therapeutic targets. Since no absolutely effective methods for curing metastatic tumors in different organs are available at present, combined use of the modalities which have anti-metastatic effect to single organ may be alternative approach to control multiple organ metastasis. Further examinations are warranted for developing novel molecular targeted therapy to control multiple-organ metastasis and improve the survival.

Key words: Organotropism, organ heterogeneity, lung cancer, metastasis, molecular target therapy, hormone metastasis model, cDNA microarray, bone metastasis, PTHrP, bisphosphonate, minodronate, combined therapy, parathyroid hormone related-peptide

1. INTRODUCTION

Lung cancer is a leading cause of malignancy-related death worldwide. It can be cured by surgical resection if found at early stage. More than 70% of lung cancer patients are, however, diagnosed at advanced stage, and the majority of these patients already developed metastasis. Despite of improvement of chemotherapy and radiotherapy, prognosis of advanced lung cancer patients is still poor (1). Lung cancer is known to produce metastasis to several particular organs, such as the liver, lung, lymph nodes, brain, and bone, suggesting organotropism on metastasis formation of this disease (2). No absolutely effective methods for curing metastatic tumors in different organs are available at present. Therefore, combined use of the modalities which have anti-metastatic effect to single organ may be alternative approach to control multiple organ metastasis.

It seems to be essential to understand molecular mechanisms of lung cancer metastasis for development of novel anti-metastatic modality. To produce metastasis, tumor cells must complete a series of sequential and selective steps (3) that

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 387-405.

include invasion of cancer cells from the primary site to blood vessels or the lymphatic system, survival in the circulation, intravascular transfer to distant organs, attachment to endothelial cells, extravasation into the parenchyma, and outgrowth into a secondary tumor with neovascularization. Throughout these multiple steps of metastasis, molecular interactions between cancer cells and their microenvironment(s) play important roles (4). Blood flow and other environmental factors influence the dissemination of cancer cells to specific organs (5). However, the organ specificity of metastasis (*i.e.*, some organs preferentially permit migration, invasion, and growth of specific cancer cells, but others do not) is a crucial determinant of metastatic outcome.

More than a century ago, Stephen Paget suggested that the distribution of metastases was not determined by chance, but rather that certain tumor cells ("seed") are likely to have an affinity for the microenvironment of specific organs ("soil") and that metastases occur only when the seed and soil are compatible (6). Various molecules such as adhesion molecules, cytokines, chemokines, hormones, and hormone receptors play important roles in preferential metastasis, but the precise mechanisms determining seed and soil compatibility remain unsolved.

2. ESTABLISHMENT OF MULTIPLE-ORGAN METASTASIS MODEL OF LUNG CANCER

2.1 Patient like patterns of metastasis in NK-cell depleted SCID mice.

To examine the cellular and molecular bases of organ tropism of lung cancer metastasis, we have established models of metastasis to multiple organs by intravenous injection of different human lung cancer cell lines to severe combined immune deficiency (SCID) mice depleted of natural killer (NK) cells (Figure 1) (7-12).

All the small-cell lung cancer (SCLC) cell lines (SBC-5, SBC-3, SBC-3/ADM, H69, H69/VP) formed metastatic nodules in multiple organs (liver, kidney, and lymph nodes).

Squamous cell carcinoma (RERF-LC-AI) cells metastasized mainly into the liver and kidneys, whereas adenocarcinoma (PC-14, A549) mainly produced colonies in the lungs. In addition, PC-14 and its highly metastatic variant PC14PE6 reproducibly produced malignant pleural effusions (12). The distribution of these tumor cells in the mice reproduced very well the distribution patterns of human metastatic lung cancers (in humans, *e.g.*, SCLC cells can form metastases in multiple organs, mainly systemic lymph nodes and liver, whereas lung adenocarcinomas produce lung metastasis and malignant pleural effusions).

2.2 Bone metastasis model using SBC-5 cells

Among these lung cancer cell lines, only SBC-5 cells reproducibly developed bone metastases (9). As assessed by X-ray photography, the osteolytic bone metastases produced by SBC-5 cells were detected as early as on day 28, and all recipient mice developed bone metastasis by day 35 (Table 1). The expression of PTHrP (parathyroid hormone related-peptide) in the cell lines was directly correlated with the formation of bone metastasis. Consistent with the formation of bone metastasis by SBC-5 cells, the levels of PTHrP and calcium in the mouse serum were increased in a time-dependent manner, suggesting that PTHrP produced by human lung cancer may play a crucial role in the formation of bone metastasis and hypercalcemia.

Histological type	Cell line	Metastasis formation				
		Lung	Liver	Kidney	LN	Bone
Small	H69	-	++	+	+++	-
	H69/VP	-	++	+	+++	-
	SBC-3	-	++	+	++	-
	SBC-5	+	++	+	+	++
Adeno	PC-14	+++	+	+	-	-
	PC14PE6	++++	+	+	-	-
	A549	++	-	-	-	-
Squamous	RERF-LC-AI	-	++	+	-	-
	H226	+++	-	-	-	-
Large	PC-13					

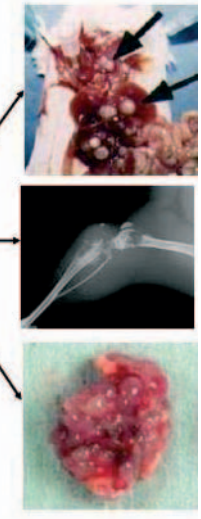


Figure 1. Patterns of metastasis by human lung cancer cell lines in NK-cell depleted SCID mice. Tumor cells (1x106) cells were intravenously inoculated into NK-cell depleted SCID mice on day 0. Metastasis formation were determined on days 28-42. (- None; + sometimes; ++ intermediate; +++ many, ++++ numerous).

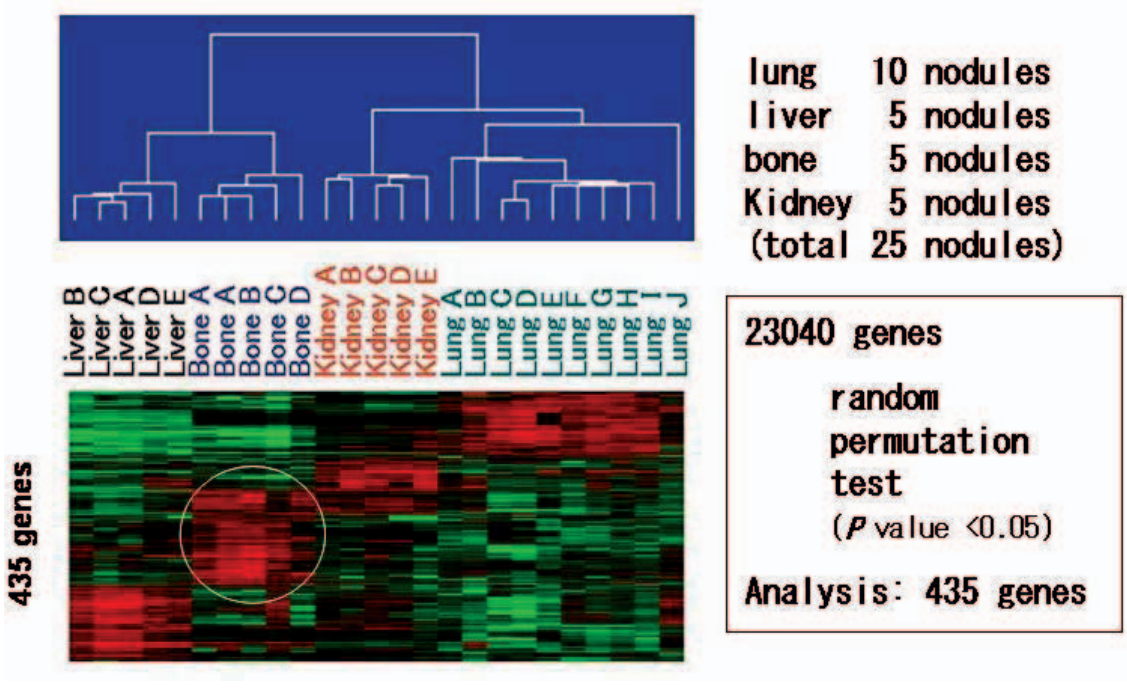


Figure 3. Cluster analysis of 25 metastatic lesions. 435 genes out of 23,040 genes were specifically expressed in each of the four metastasized organs.

Table 1. Genes predominantly expressed in metastasis in each of the four organs: **a**. lung, **b**. kidney, **c**. bone, **d**.liver
p value : p value of random permutation test; ratio : ratio of median value between 2 groups of random permutation test (see materials and methods).

a. Lung

Symbol	Description	p value	ratio
cell adhesion			
LGALS1	lectin, galactoside-binding, soluble, 1 (galectin 1)	<0.001	5.36
PCDHGC3	protocadherin gamma subfamily C, 3	<0.001	3.41
ITGB4	integrin, beta 4	<0.001	2.74
LGALS3BP	lectin, galactoside-binding, soluble, 3 binding protein	<0.01	2.28
SDC1	syndecan 1	<0.01	3.56
GJB2	gap junction protein, beta 2, 26kD (connexin 26)	<0.05	2.11
cytoskeleton/cell motility			
TUBB2	tubulin, beta, 2	<0.001	2.92
FLNA	filamin A, alpha (actin-binding protein-280)	<0.001	2.46
RHOC	ras homolog gene family, member C	<0.001	3.02
ACTC	actin, alpha, cardiac muscle	<0.001	4.84
ACTA1	actin, alpha 1, skeletal muscle	<0.001	5.40
ACTA2	actin, alpha 2, smooth muscle, aorta	<0.001	2.96
ACTB	actin, beta	<0.001	4.12
ACTG2	actin, gamma 2, smooth muscle, enteric	<0.001	5.49
ARPC4	actin related protein 2/3 complex, subunit 4 (20 kD)	<0.001	4.06
PTK9L	protein tyrosine kinase 9-like (A6-related protein)	<0.001	2.09
LMNA	lamin A/C	<0.001	3.97
RPS29	ribosomal protein S29	<0.001	2.02
PFN1	profilin 1	<0.01	2.16
ECM remodeling			
HTF9C	HpaII tiny fragments locus 9C	<0.001	2.87
FLJ11618	hypothetical protein FLJ11618	<0.01	2.11
cell-cell signaling (cytokine/chemokine)			
MIF	macrophage migration inhibitory factor	<0.001	2.01
TNFRSF1A	tumor necrosis factor receptor superfamily, member 1A	<0.001	2.22
SCYB13	small inducible cytokine B subfamily, member 13	<0.001	2.30
DDT	D-dopachrome tautomerase	<0.001	2.10
signal transduction			
FKBP8	FK506-binding protein 8 (38kD)	<0.001	2.48
PDAP1	PDGFA associated protein 1	<0.001	5.69
ITPK1	inositol 1,3,4-triphosphate 5/6 kinase	<0.001	2.21
TM4SF7	transmembrane 4 superfamily member 7	<0.001	3.75
IFITM1	interferon induced transmembrane protein 1 (9-27)	<0.01	5.92
ILK	integrin-linked kinase	<0.01	2.00
TRAF2	TNF receptor-associated factor 2	<0.05	2.10
immune response			
C3	complement component 3	<0.001	2.58
BF	B-factor, properdin	<0.001	2.13
HLA-A	major histocompatibility complex, class I, A	<0.001	2.86
HLA-B	major histocompatibility complex, class I, B	<0.001	2.77
HLA-C	major histocompatibility complex, class I, C	<0.001	2.16
HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	<0.001	19.77
HLA-DQB1	major histocompatibility complex, class II, DQ beta 1	<0.001	5.08
PSME2	proteasome (prosome, macropain) activator subunit 2	<0.001	2.64
IFITM2	interferon induced transmembrane protein 2 (1-8D)	<0.001	2.82

metabolism			
COX6B	cytochrome c oxidase subunit VIb	<0.001	2.64
COX8	cytochrome c oxidase subunit VIII	<0.001	2.84
COX7A2	cytochrome c oxidase subunit VIIa polypeptide 2 (liver)	<0.001	2.16
COX5B	cytochrome c oxidase subunit Vb	<0.001	2.18
GPX1	glutathione peroxidase 1	<0.001	4.99
GPX4	glutathione peroxidase 4 (phospholipid hydroperoxidase)	<0.001	2.67
MT2A	metallothionein 2A	<0.001	2.09
APOC1	apolipoprotein C-I	<0.001	6.44
FDXR	ferredoxin reductase	<0.001	2.58
HSD11B2	hydroxysteroid (11-beta) dehydrogenase 2	<0.001	2.63
ALAS1	aminolevulinate, delta-, synthase 1	<0.001	2.25
cell cycle/apoptosis/DNA repair			
PPP1CA	protein phosphatase 1, catalytic subunit, alpha isoform	<0.001	2.90
ERH	enhancer of rudimentary (Drosophila) homolog	<0.001	2.09
PCBP4	poly(rC)-binding protein 4	<0.01	2.46
CDC20	CDC20 (cell division cycle 20, S. cerevisiae, homolog)	<0.01	2.19
SFN	stratifin	<0.01	2.40
NOL3	nucleolar protein 3 (apoptosis repressor with CARD domain)	<0.01	2.64
transcription			
NFKBIA	NF-kappaB inhibitor	<0.001	2.15
DRAP1	DR1-associated protein 1 (negative cofactor 2 alpha)	<0.001	2.55
GATA2	GATA-binding protein 2	<0.001	2.92
MBD2	methyl-CpG binding domain protein 2	<0.001	3.25
protein synthesis/processing			
FAU	FBR-MuSV ubiquitously expressed (fox derived)	<0.001	2.74
RPS10	ribosomal protein S10	<0.001	2.12
RPLP2	ribosomal protein, large P2	<0.001	2.01
RPL18	ribosomal protein L18	<0.001	2.05
MRPL23	mitochondrial ribosomal protein L23	<0.001	3.15
PSMB8	proteasome (prosome, macropain) subunit, beta type, 8	<0.001	3.22
RPS26	ribosomal protein S26	<0.001	3.12
PMM2	phosphomannomutase 2	<0.001	2.06
FBXO2	F-box only protein 2	<0.001	2.22
EEF1D	eukaryotic translation elongation factor 1 delta	<0.001	2.24
unknown	27 genes		

b. Kidney

Symbol	Description	p value	ratio
cell adhesion			
LGALS9	lectin, galactoside-binding, soluble, 9 (galectin 9)	<0.001	2.50
ENTPD2	ectonucleoside triphosphate diphosphohydrolase 2	<0.01	2.79
CLDN17	claudin 17	<0.05	2.62
cytoskeleton/cell motility			
ACTR1A	ARP1 (actin-related protein 1, yeast) homolog A	<0.001	2.77
DKFZP586N1922	DKFZP586N1922 protein	<0.001	2.09
CYLN2	cytoplasmic linker 2	<0.001	2.07
EPLIN	epithelial protein lost in neoplasm beta	<0.001	3.43
CCT-7	HIV-1 Nef interacting protein	<0.01	2.35
CNN3	calponin 3, acidic	<0.05	3.84

ECM remodeling			
COL1A1	collagen, type I, alpha 1	<0.01	2.51
cell-cell signaling (cytokine/chemokine)			
BMP6	bone morphogenetic protein 6	<0.001	2.79
INHBA	inhibin, beta A (activin A, activin AB alpha polypeptide)	<0.001	2.35
signal transduction			
DUSP10	dual specificity phosphatase 10	<0.001	2.09
PRSS11	protease, serine, 11 (IGF binding)	<0.01	2.66
immune response			
HLA-DMA	major histocompatibility complex, class II, DM alpha	<0.01	2.48
TRB@	T cell receptor beta locus	<0.01	8.66
C1S	complement component 1, s subcomponent	<0.05	2.17
metabolism			
GCK	glucokinase (hexokinase 4, maturity onset diabetes of the young 2)	<0.001	2.52
GPX3	glutathione peroxidase 3 (plasma)	<0.001	11.30
HMGCL	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase	<0.01	3.45
cell cycle/apoptosis/DNA repair			
	Septin 6	<0.001	2.83
transcription			
P84	nuclear matrix protein p84	<0.001	2.27
NSAP1	NS1-associated protein 1	<0.001	2.17
ZNF258	Zinc finger protein 258	<0.001	2.12
GCN5L2	GCN5-like 2	<0.01	2.98
HSPC157	HSPC157 protein	<0.01	2.56
RNASE6PL	ribonuclease 6 precursor	<0.01	2.30
protein synthesis/processing			
HUGT1	UDP-glucose:glycoprotein glucosyltransferase 1	<0.001	2.10
UBE2N	ubiquitin-conjugating enzyme E2N	<0.001	2.24
SCAMP2	secretory carrier membrane protein 2	<0.01	2.35
Neurogenesis			
DCTN1	Dynactin 1 (p150, glued homolog, Drosophila)	<0.001	2.14
ITM2B	integral membrane protein 2B	<0.01	2.21
EFNB3	ephrin-B3	<0.01	2.49
the others			
ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide	<0.001	3.25
SRI	sorcin	<0.001	3.44
H1F2	H1 histone family, member 2	<0.001	2.15
SUT1	sulfate transporter 1	<0.05	2.15
unknown	32 genes		

c. Bone

Symbol	Description	p value	ratio
cell adhesion			
CELSR1	cadherin, EGF LAG seven-pass G-type receptor 1	<0.001	3.31
PLXNC1	plexin C1	<0.001	2.71
NEO1	neogenin (chicken) homolog 1	<0.001	2.06
PTPRM	protein tyrosine phosphatase, receptor type, M	<0.001	2.67
cytoskeleton/cell motility			
KIAA0855	golgin-67	<0.001	3.48

MESDC1	Mesoderm development candidate 1	<0.01	2.58
LIMK2	LIM domain kinase 2	<0.01	2.38
ECM remodeling			
KERA	keratocan	<0.001	2.05
COL3A1	collagen, type III, alpha 1	<0.01	3.29
cell-cell signaling (cytokine/chemokine)			
FST	follistatin	<0.001	3.97
MST1	macrophage stimulating 1 (hepatocyte growth factor-like)	<0.001	2.30
FAP	fibroblast activation protein, alpha	<0.001	2.01
FGFR1	fibroblast growth factor receptor 1	<0.001	2.07
signal transduction			
PRP4	serine/threonine-protein kinase PRP4 homolog	<0.001	2.13
PPP3CC	protein phosphatase 3 (formerly 2B)	<0.001	2.12
PTPN1	protein tyrosine phosphatase, non-receptor type 1	<0.001	2.12
AKT2	v-akt murine thymoma viral oncogene homolog 2	<0.001	2.14
CAV1	caveolin 1, caveolae protein, 22kD	<0.001	2.79
PRKAR1A	tissue-specific extinguisher 1	<0.05	2.01
immune response			
metabolism			
cell cycle/apoptosis/DNA repair			
TIA1	TIA1 cytotoxic granule-associated RNA-binding protein	<0.001	2.25
PRKDC	protein kinase, DNA-activated, catalytic polypeptide	<0.001	2.62
RAD51L3	RAD51 (S. cerevisiae)-like 3	<0.001	2.38
DDB1	damage-specific DNA binding protein 1 (127kD)	<0.001	2.16
BAK1	BCL2-antagonist/killer 1	<0.01	2.16
CDK3	Cyclin-dependent kinase 3	<0.01	2.00
transcription			
SFRS11	splicing factor, arginine/serine-rich 11	<0.001	2.02
DKFZP434P0721	similar to mouse Xrn1 / Dhm2 protein	<0.001	2.23
SIRT5	sir2-like 5	<0.001	2.23
TCEB1L	transcription elongation factor B (SIII), polypeptide 1-like	<0.001	2.46
EZH1	enhancer of zeste (Drosophila) homolog 1	<0.001	2.02
HSF2	heat shock transcription factor 2	<0.001	2.15
EGR4	early growth response 4	<0.001	2.22
HNRPU	heterogeneous nuclear ribonucleoprotein U	<0.01	3.33
GLI3	GLI-Kruppel family member GLI3	<0.01	2.27
EGR3	early growth response 3	<0.01	2.95
LZTR1	leucine-zipper-like transcriptional regulator, 1	<0.01	2.09
SMARCC1	SWI/SNF complex 155 kDa subunit	<0.05	2.36
protein synthesis/processing			
MTIF2	mitochondrial translational initiation factor 2	<0.001	2.08
MTHFD2	cyclohydrolase, NAD(+)-dependent	<0.01	2.27
RPL37A	ribosomal protein L37a	<0.01	2.04
SEC63L	SEC63, endoplasmic reticulum translocon component like	<0.01	2.36
LOC54516	similar to prokaryotic-type class I peptide chain release factors	<0.01	2.13
neurogenesis			
DPYSL2	dihydropyrimidinase-like 2	<0.001	3.51
GPM6B	glycoprotein M6B	<0.001	2.50
SLIT2	slit (Drosophila) homolog 2	<0.01	6.01

PRPS1	phosphoribosyl pyrophosphate synthetase 1	<0.01	2.10
the others			
ERF	Ets2 repressor factor	<0.001	2.24
DYT1	dystonia 1, torsion (autosomal dominant; torsin A)	<0.001	2.13
SLC11A2	solute carrier family 11, member 2	<0.001	2.37
MDM2	mouse double minute 2, human homolog of; p53-binding protein	<0.001	2.03
KTN1	kinectin 1 (kinesin receptor)	<0.001	2.45
POV1	prostate cancer overexpressed gene 1	<0.001	3.19
STK15	serine/threonine kinase 15	<0.001	2.36
INPPL1	inositol polyphosphate phosphatase-like 1	<0.001	2.02
GSK3B	glycogen synthase kinase 3 beta	<0.001	2.42
KDELR3	KDEL endoplasmic reticulum protein retention receptor 3	<0.001	2.39
TRF4-2	topoisomerase-related function protein 4-2	<0.001	2.37
RAB2	RAB2, member RAS oncogene family	<0.01	2.16
KIAA0102	KIAA0102 gene product	<0.01	2.24
SMT3H1	SMT3 (suppressor of mif two 3, yeast) homolog 1	<0.01	2.32
ENTPD5	ectonucleoside triphosphate diphosphohydrolase 5	<0.01	2.02
KIAA0939	KIAA0939 protein	<0.01	5.81
FUS1	lung cancer candidate	<0.01	2.18
LLGL2	lethal giant larvae (Drosophila) homolog 2	<0.01	2.11
MYB	v-myb avian myeloblastosis viral oncogene homolog	<0.01	2.03
unknown	79 genes		

d. Liver

Symbol	Description	p value	ratio
cell adhesion			
IGFBP7	insulin-like growth factor binding protein 7	<0.001	2.29
CDH2	cadherin 2, type 1, N-cadherin (neuronal)	<0.01	3.01
cytoskeleton/cell motility			
CBX1	chromobox homolog 1 (Drosophila HP1 beta)	<0.001	2.51
HECH	heterochromatin-like protein 1	<0.001	2.61
MYPT1	myosin phosphatase, target subunit 1	<0.001	3.00
SDCBP	syndecan binding protein (syntenin)	<0.001	2.26
CD2AP	CD2-associated protein	<0.01	2.21
ECM remodeling			
CTSL2	cathepsin L2	<0.01	2.06
P4HA1	prolyl 4-hydroxylase, alpha-1 subunit	<0.01	3.11
ADAM17	A disintegrin and metalloproteinase domain 17	<0.01	2.04
cell-cell signaling (cytokine/chemokine)			
LIF	leukemia inhibitory factor	<0.001	3.49
	IFN-gamma antagonist cytokine	<0.001	2.23
PBEF	pre-B-cell colony-enhancing factor	<0.01	2.52
signal transduction			
GNAS1	GNAS complex locus	<0.001	2.61
TIEG	TGFB inducible early growth response	<0.001	2.11
RHEB2	Ras homolog enriched in brain 2	<0.001	2.49
YWHAQ	14-3-3 protein tau	<0.01	2.22
PTPN12	protein tyrosine phosphatase, non-receptor type 12	<0.01	2.17
SSH3BP1	spectrin SH3 domain binding protein 1	<0.05	2.10
LOC56990	non-kinase Cdc42 effector protein SPEC2	<0.05	2.11

MTM1	myotubular myopathy 1	<0.05	2.14
immune response			
metabolism			
PGK1	phosphoglycerate kinase 1	<0.001	2.57
PDK1	pyruvate dehydrogenase kinase, isoenzyme 1	<0.001	6.21
LDHB	lactate dehydrogenase B	<0.001	3.37
ATQ1	antiquitin 1	<0.001	2.22
AGL	amylo,6-glucosidase, 4-alpha-glucanotransferase	<0.001	2.18
PHKB	phosphorylase kinase, beta	<0.001	2.71
ACLY	ATP citrate lyase	<0.05	2.40
cell cycle/apoptosis/DNA repair			
CCNG1	cyclin G1	<0.001	2.17
CAP-C	chromosome-associated polypeptide C	<0.001	2.53
BNIP3L	BCL2/adenovirus E1B 19kD-interacting protein 3-like	<0.01	2.15
REV3L	REV3 (yeast homolog)-like	<0.01	2.45
transcription			
TOP3	DNA topoisomerase III	<0.001	2.00
HNRPA2B1	heterogeneous nuclear ribonucleoprotein A2/B1	<0.001	2.73
DDX15	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 15	<0.001	3.26
HNRPA1	heterogeneous nuclear ribonucleoprotein A1	<0.001	2.45
RBMX	RNA binding motif protein, X chromosome	<0.001	2.21
HTATSF1	HIV TAT specific factor 1	<0.001	2.31
TAF172	TBP-associated factor 172	<0.001	2.18
TAF2B	TBP-associated factor 2	<0.001	2.64
LRRFIP1	leucine rich repeat (in FLII) interacting protein 1	<0.001	2.24
BTF3	basic transcription factor 3	<0.001	2.03
MYC	v-myc avian myelocytomatosis viral oncogene homolog	<0.001	2.50
BHLHB2	basic helix-loop-helix domain containing, class B, 2	<0.001	5.45
DBY	DEAD/H box polypeptide, Y chromosome	<0.001	2.33
MXI1	MAX-interacting protein 1	<0.01	2.86
ZNF9	zinc finger protein 9	<0.01	2.62
BAZ1A	bromodomain adjacent to zinc finger domain, 1A	<0.05	2.13
protein synthesis/processing			
RABGGTB	Rab geranylgeranyltransferase, beta subunit	<0.001	2.76
RPL9	ribosomal protein L9	<0.001	2.27
EIF3S6	eukaryotic translation initiation factor 3, subunit 6 (48kD)	<0.001	2.11
RPL6	ribosomal protein L6	<0.001	2.10
RPL7	ribosomal protein L7	<0.001	2.40
RPS3A	ribosomal protein S3A	<0.001	2.73
RPS4X	ribosomal protein S4, X-linked	<0.001	2.16
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	<0.001	2.57
LOC51280	golgi membrane protein GP73	<0.01	2.16
neurogenesis			
GPM6B	glycoprotein M6B	<0.001	3.87
STMN2	Stathmin-like 2	<0.001	3.78
GPI	glucose phosphate isomerase	<0.001	2.44
the others			
CSPG6	chondroitin sulfate proteoglycan 6 (bamacan)	<0.001	2.97
ST13	suppression of tumorigenicity 13(Hsp70-interacting protein)	<0.001	2.29
BET1	Golgi vesicular membrane trafficking protein p18	<0.001	2.15

SLC2A1	solute carrier family 2, member 1	<0.001	3.11
SLC16A1	solute carrier family 16, member 1	<0.001	2.35
RANBP2	RAN binding protein 2	<0.001	2.33
ATP5A1	mitochondrial ATP synthetase, oligomycin-resistant	<0.001	2.01
P115	vesicle docking protein p115	<0.01	2.54
SEC23B	Sec23 (S. cerevisiae) homolog B	<0.01	2.05
SCP2	sterol carrier protein 2	<0.01	2.56
UBA2	SUMO activating enzyme subunit 2	<0.05	2.50
SLC2A3	solute carrier family 2, member 3	<0.05	2.96
SSFA2	sperm specific antigen 2	<0.05	2.17
unknown	50 genes		

2.3 Usefulness of multiple-organ metastasis models for evaluation of organ heterogeneity of metastasis

A huge number of molecules has been reported to regulate metastasis. We demonstrated that overexpression of cytokines (IL-1β) (11) and adhesion molecules (CD82) (13) dramatically facilitated production of metastasis to various organs. On the other hand, metastasis formation is regulated by the interaction of tumor cells and host microenvironments. Since both tumor cells and host microenvironments are biologically heterogenous, modification of one particular molecule may not uniformly inhibit metastasis to multiple organs. In fact, we have previously shown that the antimetastatic effect of both M-CSF (14) and an inhibitor of MMP (15) are specific to particular organs, despite the fact that both macrophage colony-stimulating factor (M-CSF) and matrix metalloproteinase (MMP) inhibitors dramatically block the growth of subcutaneously inoculated tumor cells. For this reason, metastatic models should be used in preference to subcutaneous xenograft models for evaluating the therapeutic potential of new anticancer agents.

3. DETERMINATION OF ORGAN-SPECIFIC METASTASIS (ORGANOTROPISM)-RELATED GENES.

For the elucidation of a comprehensive survey of the factors regulating organotropism of metastasis and the cross-talk between cancer cells and microenvironment in each organ, we performed cDNA-microarray analyses (23,040 genes) of the metastatic foci of human SCLC (SBC-5) cells developed in four different murine organs (lung, liver, kidney, and bone) and compared gene-expression profiles among 25 of these lesions (10 in lung, 5 in liver, 5 in kidney, and 5 in bone) (Figure 2). To remove contamination of normal mouse mRNA and any experimental noises in the statistical analysis, we performed laser-capture microdissection of surrounding mouse normal tissues and then hybridized on the human cDNA microarrays (16). To identify genes that were specifically expressed in each of the four metastasized organs, we performed random permutation tests; this is an appropriate strategy for distinguishing two known subgroups. We used the following combinations: 10 lung metastases *versus* all 15 others; 5 liver metastases *versus* all 20 others; 5 kidney metastases *versus* all 20 others; and 5 bone metastases *versus* all 20 others. Hierarchical clustering of these 435 genes (Table 1) separated the four organ-specific groups of metastatic lesions very clearly (Figure 3). Figure 4 lists representative of the 435 genes, the median ratios of which between the two groups were >2 with P values <0.05, among the 23,040 genes examined on the microarray. These results suggest that metastatic cancer cells can grow in the different organs using with organ specific genes and their products, and that organotropism of SBC-5 cells can be determined by these factors.

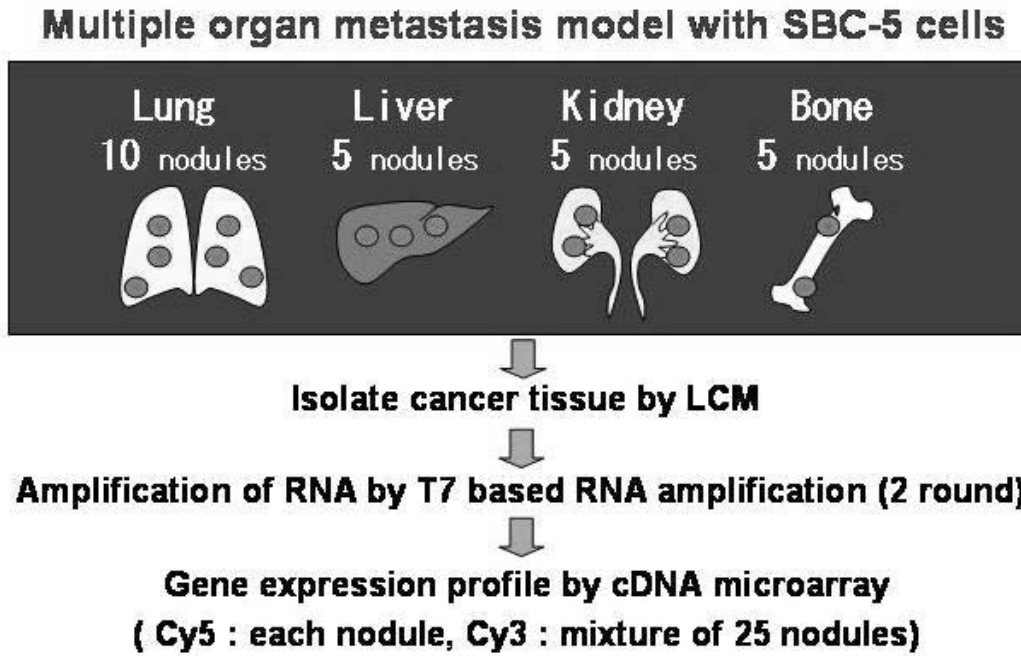


Figure 2. Strategy for detection of genes predominantly expressed in metastatic organs. Twenty five metastatic foci produced by SBC-5 cells (10 in lung, 5 in liver, 5 in kidney, and 5 in bone) were harvested. To remove contamination of normal mouse mRNA and any experimental noises in the statistical analysis, we then performed laser-capture microdissection (LCM) of surrounding mouse normal tissues and total RNA was harvested from the tumor cells. After amplification of RNA by T7 based RNA amplification (2 round), gene expression profile (23,040 genes) was examined by cDNA microarray analyses.

Lung	: *LGALS1, LGALS3BP, PCDHGC3, ILK, PDAP1 RHOC, ITBG4, SDC1, C3, MT2A, CALM et al*	**99 genes**
Liver	: *IGFBP7, CDH2, CTSL2, P4HA1, ADAM17 et al*	**123 genes**
Kidney	: *LGALS9, COL1A1, BMP6, INHBA, C1S et al*	**69 genes**
Bone	: *FGFR1, FST, PTHrP, COL3A1, MST1, FAP, POV1 et al*	**144 genes**

435 genes including unknown 186 genes

Figure 4. Genes with increased expression in metastatic nodules.

4. GENES DIFFERENTIALLY EXPRESSED BETWEEN "MICROMETASTASIS" AND "MACROMETASTASIS"

In vivo videomicroscopy studies have revealed that early phases of the metastatic process are completed quite efficiently through sequential steps, whereas growth phases of metastatic cells are very inefficient. Those observations suggest that regulators of tumor growth at secondary sites should be key targets for preventing metastasis. To further clarify the mechanism(s) operating later in the process of metastasis, we applied random permutation tests to compare lung-metastatic nodules classified according to the growth step from micrometastasis to macrometastasis. Nine metastatic lesions in the lung (five lesions were <1 mm and four were >2 mm) were analyzed and 105 differentially expressed genes were determined. Sixty-eight of the genes were predominantly expressed in the smaller lesions, and 37 were predominant in the larger lesions (Table 2). The 105 genes that were differentially expressed between the two groups were classified according to their function. A number of genes involved in the cell motility, cell adhesion, and extra cellular matrix (ECM) remodeling were predominantly expressed in micrometastasis. For example, *HSPB3*, *ACTB*, *ACTA2*, *TMSB10*, *MYH7*, *FLNA*, and *ARPC4*, the expressions of which were elevated in micrometastasis, coordinately form lamellipodia and new adhesion sites at the leading edge of the invading cells, and move the cell forward by contraction of actomyosin-based cytoskeletal filaments. *MMP1*, which encodes a secreted enzyme that breaks down interstitial collagens (types I, II, and III), was also up-regulated in the smaller lesions. On the other hand, none of the genes belonging to the categories documented above were highly expressed in the larger lesions. Enhanced expression of these genes in the smaller lesions might reflect active cellular movement and invasion of cancer cells in micrometastasis. Because the differential expression of 105 genes between the two groups might reflect differences in the biological features of these tumors, further investigations of nearly half of the genes of unknown functions listed here should provide important insights into the progression from micrometastasis to macrometastasis.

Table 2. Genes predominantly expressed in micrometastasis and macrometastasis
p value : p value of random permutation test, ratio : ratio of median value between 2 groups of random permutation test

a. Micrometastasis

Symbol	Description	p value	ratio
cell adhesion			
GP110	Adhesion regulating molecule 1	<0.001	2.00
SDC1	syndecan 1	<0.01	2.30
STEAP	six transmembrane epithelial antigen of the prostate	<0.01	2.03
CEACAM4	carcinoembryonic antigen-related cell adhesion molecule 4	<0.05	2.25
PCDHGC3	protocadherin gamma subfamily C, 3	<0.05	2.01
cytoskeleton/cell motility			
HSPB3	heat shock 27kD protein 3	<0.001	2.04
ARPC4	actin related protein 2/3 complex, subunit 4 (20 kD)	<0.001	2.03
ACTA2	actin, alpha 2, smooth muscle, aorta	<0.01	3.08
ACTB	actin, beta	<0.01	2.42
MYH7	myosin, heavy polypeptide 7, cardiac muscle, beta	<0.01	2.34
TMSB10	thymosin, beta 10	<0.05	2.04
ECM remodeling			
MMP1	matrix metalloproteinase 1 (interstitial collagenase)	<0.05	2.55
HTF9C	HpaII tiny fragments locus 9C (collagen type iii)	<0.05	2.43

cell-cell signaling (cytokine/chemokine)			
FGF19	fibroblast growth factor 19	<0.001	2.03
SCYB13	small inducible cytokine B subfamily, member 13	<0.05	2.05
EGFR	epidermal growth factor receptor	<0.05	5.79
signal transduction			
NR1I3	nuclear receptor subfamily 1, group I, member 3	<0.001	2.14
FSTL1	follistatin-like 1	<0.001	2.03
SHC1	SHC (Src homology 2 domain-containing) transforming protein 1	<0.01	2.04
IFITM1	interferon induced transmembrane protein 1 (9-27)	<0.05	2.76
immune response			
MD-2	MD-2 protein	<0.001	2.00
IFITM2	interferon induced transmembrane protein 2 (1-8D)	<0.01	2.56
IGKC	immunoglobulin kappa constant	<0.05	2.27
	DC classII histocompatibility antigen alpha-chain	<0.05	3.45
metabolism			
MAN1B1	mannosidase, alpha, class 1B, member 1	<0.001	2.23
FBP2	fructose,6-bisphosphatase 2	<0.001	2.29
NUCB1	nucleobindin 1	<0.01	2.39
PMM2	phosphomannomutase 2	<0.05	2.09
cell cycle/apoptosis/DNA repair			
transcription			
EEF1E1	eukaryotic translation elongation factor 1 epsilon 1	<0.001	2.00
NFX1	nuclear transcription factor, X-box binding 1	<0.05	2.07
protein synthesis/processing			
HUGT1	UDP-glucose:glycoprotein glucosyltransferase 1	<0.05	2.14
PPP1CA	protein phosphatase 1, catalytic subunit, alpha isoform	<0.05	2.45
neurogenesis			
THY1	Thy cell surface antigen	<0.001	2.84
the others			
RAB32	RAB32, member RAS oncogene family	<0.001	2.00
GBF1	golgi-specific brefeldin A resistance factor 1	<0.001	2.66
ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide	<0.01	4.31
FRZB	frizzled-related protein	<0.05	6.47
KIAA1011	synaptic nuclei expressed gene 2	<0.05	2.00
COPE	coatomer protein complex, subunit epsilon	<0.05	2.04
unknown	29 gene		

b. Macrometastasis

Symbol	Description	p value	ratio
cell adhesion			
cytoskeleton/cell motility			
ECM remodeling			
cell-cell signaling (cytokine/chemokine)			
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	<0.05	2.08
TGFBR2	transforming growth factor, beta receptor II (70-80kD)	<0.05	2.72
signal transduction			
RHEB2	Ras homolog enriched in brain 2	<0.001	2.01

RalGPS1A	Ral guanine nucleotide exchange factor RalGPS1A	<0.01	2.42
AKAP9	A kinase (PRKA) anchor protein (yotiao) 9	<0.05	2.20
RAB2L	RAB2, member RAS oncogene family-like	<0.05	2.02
GNAS1	G protein, alpha stimulating activity polypeptide 1	<0.05	2.22
CD47	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	<0.05	2.31
immune response			
metabolism			
LDHA	lactate dehydrogenase A	<0.01	2.51
cell cycle/apoptosis/DNA repair			
BNIP3L	BCL2/adenovirus E1B 19kD-interacting protein 3-like	<0.01	3.15
transcription			
BTF3	basic transcription factor 3	<0.01	2.22
ZNF277	zinc finger protein 277	<0.05	2.34
SMARCE1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily e, member 1	<0.05	2.13
protein synthesis/processing			
SMT3H1	SMT3 (suppressor of mif two 3, yeast) homolog 1	<0.001	2.19
HUGT1	UDP-glucose:glycoprotein glucosyltransferase 1	<0.05	2.41
neurogenesis			
SYNGR3	synaptogyrin 3	<0.001	2.01
the others			
NARF	nuclear prelamin A recognition factor	<0.01	2.08
GMPS	guanine monphosphate synthetase	<0.05	2.22
DNMT2	DNA (cytosine-5-)-methyltransferase 2	<0.05	2.83
unknown	18 genes		

5. MOLECULAR MECHANISMS OF BONE METASTASIS BY SBC-5 CELLS

Bone metastasis causes bone pain, hypercalcemia, nerve compression syndromes, and even fractures, resulting in decrease of quality of life of patients (17). Thus, the prevention and treatment of osteolytic bone metastasis based on understanding of its molecular mechanism are clinically important.

Resent reports indicate that bone destruction caused by bone metastasis is mediated by factors produced or induced by tumor cells that stimulate the formation and activation of osteoclasts, the normal bone-resorbing cells (18) (Figure 5). Several factors, including IL-1, IL-6, receptor activator of NF-kappa B (RANK) ligand, macrophage inflammatory protein-1-alpha (MIP-1α), and PTHrP, have been implicated as factors that enhance osteoclast formation and bone destruction in malignant diseases (19). We examined the cytokine production and metastatic potential of several human lung cancer cells in NK cell-depleted SCID mice, and found that only SBC-5 cells reproducibly developed bone metastasis and constitutively expressed a high level of PHTrP (9). Moreover, we found in microarray analyses that PTHrP was expressed by the tumor cells in all four metastatic sites (lung, liver, kidney, and bone) and that the expression levels of PTHrP in bone metastases tended to be higher than in the other organs. These findings strongly suggest that PTHrP may be responsible for bone metastasis of SBC-5 cells.

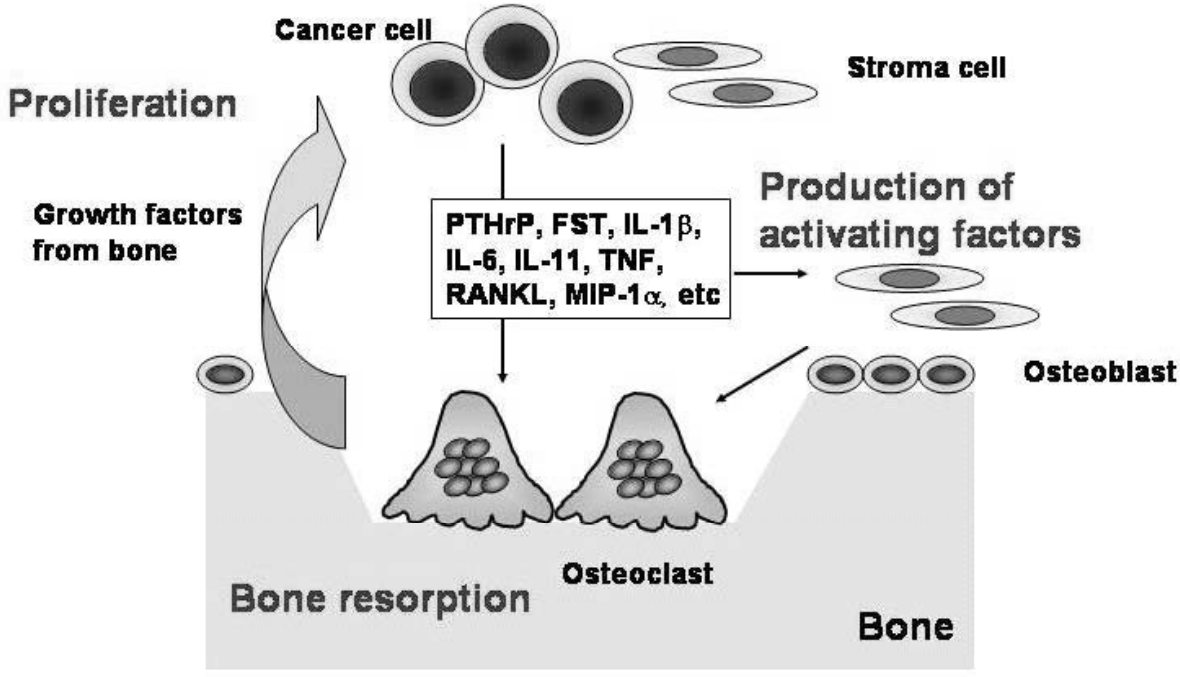


Figure 5. Molecular mechanism of osteolytic bone metastasis. Cytokines produced by tumor cells and/or host stromal cells directly, or indirectly (via activation of osteoblasts) activate osteoclasts. Activated osteoclasts cause boneresorption, providing the space for tumor cell growth and the release of growth factors from bone. Tumor cells proliferate with the stimulation by bone-derived growth factors.

In our microarray analyses, fibroblast growth factor receptor 1 (FGFR1) and follistatin (FST) were found to be overexpressed in cells metastasized in bone metastatic lesions (16) (Figure 4). FGFR1 is a receptor for fibroblast growth factors (FGFs) and its downstream signals influence mitogenesis and differentiation. Because FGFs are expressed abundantly in bone tissue (20), the microenvironment of bone is likely to be suitable for survival and proliferation of cancer cells that express FGFR1. Lung cancer commonly develops osteolytic bone metastasis. FST, an activin antagonist that can inhibit bone formation (21), might promote the bone absorption caused by metastatic cells and contribute to the release of the growth factors such as FGFs that are stored in bone tissue. This bidirectional interaction between tumor cells and the bone microenvironment seems to be important for developing bone metastasis.

6. MOLECULAR TARGETED THERAPY OF BONE METASTASIS

As described above, osteoclasts (the normal bone-resorbing cells) and their activating factors (such as PTHrP and FST) are ideal therapeutic targets of bone metastasis. We focused on PTHrP, which was overexpressed in the bone metastatic lesions, and examined therapeutic effect of anti-PTHrP neutralizing antibody in our bone-metastasis model with SBC-5 cells. Repeated treatment with anti-PTHrP neutralizing antibody to SBC-5-bearing SCID mice successfully inhibited the production of bone metastasis (Figure 6) (22). However the treatment was not effective to metastasis to other organs (liver, lung, lymph nodes), indicating bone-specitic antimetastatic effect of anti-PTHrP antibody.

Treatment	Number of metastatic colony (Median (Range))					Body weight (g)	Ca (mg/dl)
	Bone	Lung	Liver	Kidney	LN		
Control	5 (4-8)	14 (3-16)	87 (53-91)	1 (1-3)	6 (4-7)	17.8	12.1
Anti-PTHrP Ab	1 (1-5)*	7 (2-12)	66 (50-80)	1 (0-2)	4 (1-5)	19.9	9.5*

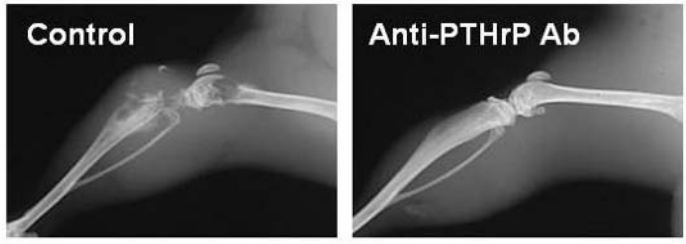


Figure 6. Anti-PTHrP antibody (Ab) inhibited bone metastasis by SBC-5 cells. SBC-5 (1x106) cells were intravenously inoculated into NK-cell depleted SCID mice on day 0. Anti-PTHrP Ab (200 μg/body) was intravenously administered on days -1, 7, 14, 21, and 28. Metastasis and a level of serum calcium were evaluated on day 35.

Hypercalcemia is another problem frequently observed in lung cancer patients. It can be caused by two different mechanisms, namely HHM (humoral hypercalcemia of malignancy) and LOH (local osteolytic hypercalcemia) (23). HHM is mediated via PTHrP produced by tumor cells. Since PTHrP indirectly stimulates bone resorption by osteoclasts and augments the reabsorption of calcium in the kidneys, the calcium level in the serum is elevated (9). In LOH, cytokines (IL-1, IL-6, TNF-α) locally produced by tumor cells promoted the proliferation and differentiation of osteoclast-lineage cells, induced bone resorption, then caused hypercalcemia (24). SBC-5 cells overexpressed PTHrP, whereas no IL-1, IL-6, or TNF-α was detected in the culture supernatants of SBC-5 cells. In addition, treatment with anti-PTHrP antibody improved the elevated serum calcium levels indicating that hypercalcemia observed in SBC-5 inoculated mice is due to PTHrP produced by SBC-5 cells (HHM mechanism). Collectively, anti-PTHrP antibody may be useful for control of bone metastasis and HHM of lung cancer patients.

Bisphosphonates are potent inhibitors of osteoclastic bone resorption, and have been widely used in the treatment of osteoporosis and hypercalcemia (25). Several bisphosphonate products, (clodronate, etidronate, alendronate, ibandronate, pamidronate), had been shown to prevent the production of bone metastasis, however, they could not improve the survival of advanced cancer patients with bone metastases (26). The third generation bisphosphonates (zoledronic acid and minodronate) are expected therapeutic effects against bone metastasis because of their high activity on osteoclast-mediated bone resorption. We have reported that minodronate (YM529), had a therapeutic effect against osteolytic bone metastasis (Figure 7) (27).

A

Treatment	Number of bone metastasis (Median (Range))		
	Bone	Liver	Lung
Control	14 (11-18)	72 (61-84)	27 (19-41)
YM529	3 (0-7) *	66 (58-88)	23 (12-31)
VP-16	7 (3-11)	41 (14-83)*	13 (8-21) *
YM529+VP-16	1 (0-3)*	43 (25-73)*	12 (9-23)*

B

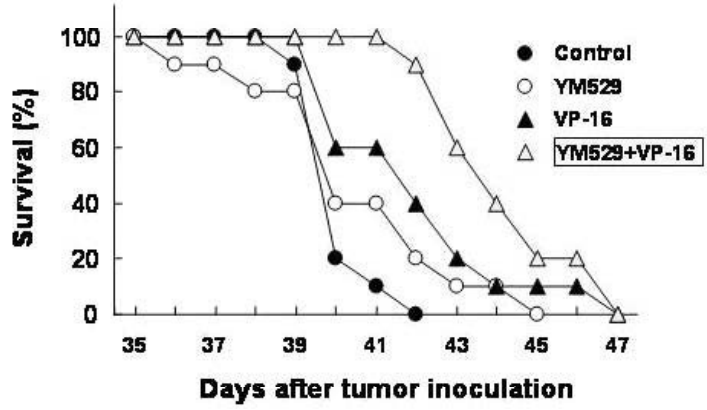


Figure 7. YM529 combined with VP-16 prolonged the survival of mice with multiple-organ metastasis by SBC-5 cells. SBC-5 (1x106) cells were intravenously inoculated into NK-cell depleted SCID mice on day 0. The mice were treated with intravenous administration of YM529 (0.2 μg) on day 7 and/or VP-16 (200 μg) on days 2, 3, 9, and 10. A: metastasis was evaluated on day 35. B: survival of the mice was evaluated.

Single treatment by YM529 after the establishment of micrometastasis inhibited bone metastasis, but not visceral metastasis, produced by SBC-5 cells, again indicating bone-sepecific anti-metastatic effects of YM529. However, treatment with YM529 alone could not prolong the survival significantly, although it remarkably inhibited bone metastasis (28). Because SBC-5 cells develop metastasis to various organs, selective inhibition of bone metastasis may be not sufficient for prolonging the survival. On the other hand, a chemotherapeutic agent, VP-16, suppressed metastasis to some visceral organs (the liver and lungs). Combined use of these two agents could produce a better effect in terms of survival and development of metastasis (28).

Collectively, the control of both bone metastasis and visceral metastases by multiple modalities may be necessary to prolong the survival of cancer patients with multiple organ metastases.

7. CONCLUSIONS

Metastasis to multiple organs is the major obstacle of treatment of lung cancer patients. There is organotropism on metastasis of lung cancer. Since no absolutely effective methods for curing metastatic tumors in different organs are available at present, combined use of the modalities which have antimetastatic effect to single organ may be alternative approach to control multiple organ metastasis. We established multiple-organ metastasis model of lung cancer in NK-cell depleted SCID mice. Using this model, we determined organotropism- related genes by means of cDNA microarray representing 23,040 genes and extracted

435 genes that seemed to reflect the organotropism of the metastatic cells and the cross-talk between cancer cells and microenvironment.

Of 435 genes, we demonstrated causal evidence of PTHrP on bone metastasis, suggesting usefulness of this approach to identify novel therapeutic targets. In addition, we showed that combined therapy with bisphosphonate (to control bone metastasis) and chemotherapy (to control visceral metastasis) is necessary for control multiple organ metastasis and prolongation of survival. Further examinations are warranted for developing novel molecular targeted therapy to control multiple organ metastasis and improve the survival of lung cancer patients.

REFERENCES

1. Mooi, W.J., 1996, Common lung cancers. In Spencer's Pathology of the Lung. Hazleton PS, ed, 1009-64, McGraw-Hill, New York.
2. Quint, L. E., Francis, I. R., Wahl, R.L., and Gross, B. H., 1996, Imaging of lung cancer. In Lung cancer: principles and practice. H.I. Pass, J.B. Mitchell, D.H. Johnson, and A.T. Turrisi, eds, 437–70, Lippincott-Raven. Philadelphia.
3. Fidler, I. J., and Ellis, L. M., 1994, The implications of angiogenesis for the biology and therapy of cancer metastasis. Cell, 79:185–8.
4. Fidler, I. J., Yano, S., Zhang, R. D., Fujimaki, T., and Bucana, C. D., 2002, The seed and soil hypothesis: vascularisation and brain metastases. Lancet Oncol, 3:53–7.
5. Weiss, L., 1992, Comments on hematogenous metastatic patterns in humans as revealed by autopsy. Clin & Exp Metastasis, 10:191–9.
6. Paget, S., 1889, The distribution of secondary growths in cancer of the breast. Lancet, 1:571-73.
7. Yano, S., Nishioka, Y., Izumi, K., Tsuruo, T., Tanaka, T., Miyasaka, M., and Sone, S., 1996, Novel metastasis model of human lung cancer in SCID mice depleted of NK cells. Int J Cancer, 67:211–7.
8. Hanibuchi, M., Yano, S., Nishioka, Y., Yanagawa, H., Kawano, T., and Sone, S., 1998, Therapeutic efficacy of mouse-human chimeric anti-ganglioside GM2 monoclonal antibody against multiple organ micrometastases of human lung cancer in NK cell-depleted SCID mice. Int . Cancer, 78:480-5.
9. Miki, T., Yano, S., Hanibuchi, M., and Sone, S., 2000, Bone metastasis model with multiorgan dissemination of human small-cell lung cancer (SBC-5) cells in natural killer cell-depleted SCID mice. Oncol Res, 12:209–17.
10. Yano, S., Nokihara, H., Yamamoto, A., Goto, H., Ogawa, H., Kanematsu, T., Miki, T. Uehara, H., Saijo, Y., Nukiwa, T., and Sone S., 2003, Multifunctional interleukin-1 β promotes metastasis of human lung cancer cells in SCID mice via enhanced expression of adhesion-, invasion- and angiogenesis-related molecules. Cancer Sci, 94:244-52.
11. Nokihara, H., Yanagawa, H., Nishioka, Y., Yano, S., Mukaida, N., Matsushima, K., and Sone, S., 2000, Natural killer cell-dependent suppression of systemic spread of human lung adenocarcinoma cells by monocyte chemoattractant protein-1 gene transfection in SCID mice. Cancer Res, 60:7002-7.
12. Yano, S., Shinohara, H., Herbst, R.S., Kuniyasu, H., Bucana, C.D., Ellis, L.M., and Fidler, I.J., 1893, Production of experimental malignant pleural effusions is dependent on invasion of the pleura and expression of vascular endothelial growth factor/vascular permeability factor by human lung cancer cells. Am J Pathol, 157:1893-903.
13. Shinohara T, Nishimura N, Hanibuchi M, Nokihara H, Miki T, Hamada H, and Sone S., 2001, Transduction of KAI1/CD82 cDNA promotes hematogenous spread of human lung-cancer cells in natural killer cell-depleted SCID mice. Int J Cancer, 94:16-23.
14. Yano, S., Nishioka, Y, Nokihara, N., and Sone, S., 1997, Macrophage colony-stimulating factor-gene transduction into human lung cancer cells differentially regulates metastasis formations in various organ microenvironments of NK-cell depleted SCID mice. Cancer Res, 57:784-9.
15. Shiraga, M., Yano, S., Yamamoto, A., Ogawa, H., Goto, H., Miki, T., Miki, K., Zhang, H., and Sone, S., 2002, Organ heterogeneity of host-derived matrix metalloproteinase expression and its involvement in multiple-organ metastasis by lung cancer cell lines. Cancer Res, 62:5967-73.
16. Kakiuchi, S., Daigo, Y., Tsunoda, T., Yano, S., Sone, S., and Nakamura, Y., 2003, Genome-wide analysis of organ-preferential metastasis of human small cell lung cancer in mice. Mol. Cancer Res, 1:485-99.
17. Sullivan F.J., 1996, Palliative radiotherapy for lung cancer: Lung cancer, principles and practice. 775-89, Lippincott-Raven, Philadelphia, PA.
18. Roodman, G.D., 2001, Biology of osteoclast activation in cancer. J Clin Oncol, 19:3562-71.
19. Orr F.W., Lee, J., Duivenvoorden W.C., and Singh, G., 2000, Pathophysiologic interactions in skeletal metastasis. Cancer, 88(12 Suppl):2912-8.
20. Mundy, G.R., 2002, Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer, 2:584–93.
21. Ebara, S., and Nakayama, K., 2002, Mechanism for the action of bone morphogenetic proteins and regulation of their activity. Spine, 27:S10–S15.

22. Miki, T., Yano, S., Hanibuchi, M., Kanematsu, T., Muguruma, H., and Sone, S., 2004, Parathyroid hormone-related protein (PTHrP) is responsible for production of bone metastasis, but not visceral metastasis, by human small cell lung cancer SBC-5 cells in natural killer cell-depleted SCID mice. Int J Cancer, 108:511-5.
23. Suva, L.J., Winslow, G.A., 1987, Wettenhall, R.E., Hammonds, R.G., Moseley, J.M., Diefenbach-Jagger, H., Rodda, C.P., Kemp, B.E., Rodriguez, H., Chen, E.Y., 1987, A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. Science, 237:893-6.
24. Jilka, R.L., Hangoc, G., Girasole, G., Passeri, G., Williams, D.C., Abrams, J.S., Boyce, B., Broxmeyer, H., and Manolagas, S.C., 1992, Increased osteoclast development after estrogen loss: mediation by interleukin-6. Science, 257:88-91.
25. Theriault, R.L., and Hortobagyi, G. N., 2001, The evolving role of bisphosphonates. Semin Oncol, 28:284-90.
26. Hillner, B. E., Ingle, J. N., Berenson, J. R., Janjan, N. A., Albain, K. S., Lipton, A., Yee, G., Biermann, J. S., Chlebowski, R. T., and Pfister, D. G., 2000, American Society of Clinical Oncology guideline on the role of bisphosphonates in breast cancer. American Society of Clinical Oncology Bisphosphonates Expert Panel. J Clin Oncol, 18:1378-91.
27. Zhang, H., Yano, S., Miki, T., Goto, H., Kanematsu, T., Muguruma, H., Uehara, H., and Sone, S., 2003, A novel bisophosphonate minodronate (YM529) specifically inhibites osteolytic bone metastasis produced by human small cell lung cancer cells in NK-cell depleted SCID mice. Clin Exp Metastasis, 20:153-9.
28. Yano, S., Zhang, H., Hanibuchi, M., Miki, T., Goto, H., Uehara, H., and Sone, S., 2003, Combined therapy by a new bisphosphonate, minodronate (YM529), with chemotherapy for multiple organ metastases of small cell lung cancer cells in severe combined immunodeficient mice. Clin Cancer Res, 9:5380-5.

Chapter 23

Maspin: A Novel Serine Protease Inhibitor

Shijie Sheng
Department of Pathology, Wayne State University School of Medicine, Protease Program of the Karmanos Cancer Insitute, Detroit, MI, USA

Abstract: Maspin is a novel serine protease inhibitor differentially expressed in several types of human cancers. Accumulated evidence indicates that maspin plays a tumor suppressive role at the steps of tumor growth, invasion, tumor-induced extracellular matrix remodeling and angiogenesis, and tumor metastasis. Several recent studies also suggest that maspin plays a role in restoring a more differentiated phenotype and enhances tumor cell sensitivity to drug-induced apoptosis. To date, the underlying molecular mechanisms of maspin remain elusive. This review is intended to summarize research progress made in several areas with a focus on whether maspin acts as a serine protease inhibitor. Towards the potential clinical application of maspin in anti-cancer therapies, this review also discusses the current challenges and future research directions.

Key words: Maspin, serpins, angiogenesis, serine protease inhibitor, apoptosis, cell suppression, tumor invasion, metastasis, prostate cancer, urokinase, urokinase receptor, extracellular matrix

1. INTRODUCTION

The maspin cDNA consists of 2584 nucleotides, encoding a 42 kDa protein (376 amino acids) with an overall sequence homology to serine protease inhibitors (serpins) (1). The protein sequence of maspin is highly conserved among human, mouse and rat (2, 3). The human maspin gene, along with several ovalbumin type serpins, has been mapped to chromosome 18q21.3-23 (1, 4, 5). Serpins in this gene cluster appear to be evolutionarily related (6) and may be regulated concertedly at epigenetic steps (7). Maspin produced by cultured human breast and prostate epithelial cells is a 42 kDa monomer which is present as a secreted, a cytoplasmic, as well as a cell surface-associated protein (1, 8-12).

Maspin has attracted a great deal of attention because it exhibits a tumor suppressive function both *in vitro* and in animal models (1, 13-15). Experiments using cell lines *in vitro* demonstrated an inhibitory role of maspin on tumor cell motility and invasion (1, 8, 16-20). In addition, maspin has been shown to inhibit angiogenesis in both *in vitro* and *in vivo* models (13, 21, 22). Recently, Cher et al. (23) showed that maspin overexpression in prostate carcinoma cells DU145 decreased tumor growth, tumor-induced osteolysis, and tumor angiogenesis (23), suggesting an exciting potential for maspin to block prostate cancer bone metastasis. Consistent with these experimental observations, clinical evidence demonstrates that maspin expression predicts a better prognosis for prostate, colon, thyroid, lung, and oral squamous cancers (reviewed in (24)), and maspin expression correlated with less tumor-induced angiogenesis (25, 26).

Emerging evidence further suggests that the tumor suppressive activity of maspin is not restricted to the steps of tumor invasion and metastasis. While

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 407-418.

the biological activities of maspin in inhibiting tumor cell motility and invasion is localized on the cell surface (8), the Sheng laboratory has shown that intracellular maspin plays an important role in up-regulating Bax expression and sensitizing tumor cells to cytokine- or drug-induced apoptosis (27, 28). These results help explain the earlier observations that maspin overexpression in transgenic mice is associated with increased apoptosis (13, 14, 21, 22, 29) and maspin-expressing tumor xenografts in mice are inhibited in tumor growth (1, 16, 23).

Despite the promising potential of maspin as a molecular marker for cancer diagnosis and prognosis, as a suppressor of tumor invasion and metastasis, and as a modifier of apoptosis-based cancer therapies, the underlying molecular mechanisms of maspin remain elusive. This review is intended to provide a critical review of the published data regarding the key issue of whether maspin acts as an inhibitory serpin.

2. THE BIOLOGICAL FUNCTIONS OF MASPIN

2.1 Extracellular Maspin and Tumor Invasion and Metastasis

In 1994 Zou and colleagues (1) first reported that re-expression of maspin by stable transfection of mammary carcinoma cells MDA-MB-435 significantly inhibited tumor cell invasion *in vitro* and metastasis in nude mice (1). This finding is supported by several *in vivo* experiments using genetically modified mouse models. For example, in WAP-TAg/WAP-maspin bitransgenic mice, maspin overexpression reduced angiogenesis and pulmonary metastasis (13). Using the syngeneic mammary tumor model, Shi et al. showed that maspin overexpression in TM40D mammary tumor cells blocked tumor local invasiveness and metastasis (14, 15). Recently, using a novel intraosseous SCID-Human (SCID-Hu) model that reproduces the organ- and species-specific prostate-bone metastasis (30), Cher et al. showed that maspin overexpression in prostate carcinoma cells DU145 decreased tumor growth, tumor-induced osteolysis, and tumor angiogenesis (23). The notion that maspin suppresses tumor-induced angiogenesis is consistent with earlier evidence that transgenic expression of maspin resulted in decreased angiogenesis and increased apoptosis in WAP-Tag/WAP-maspin bitransgenic mice (13, 22). Furthermore, these experimental observations are in line with a couple of correlative clinical studies. Hojo et al. showed a significant correlation of maspin expression with decreased microvessel staining in human breast cancer specimens (25). A similar finding was reported by Song et al. in human colon cancer (26).

Extensive *in vitro* experiments have been launched to define the maspin effect on tumor invasion. These *in vitro* experiments can be generally divided into two groups based on the form of maspin used. Endogenous maspin expression has been achieved by stable transfection or retrovirus infection in several tumor cell lines. In general, endogenous maspin expression significantly inhibits tumor cell invasion and motility (1, 8, 17, 18, 31). In the meantime, several forms of recombinant maspin protein have been produced and purified. Sheng et al. first described three forms of recombinant maspin proteins produced and purified from *E. coli*, yeast and baculo virus-infected insect cells (20). The recombinant maspins produced in yeast and baculo virus-infected insect cells were wild type full-length maspin, while the recombinant maspin produced in *E. coli* was fused N-terminal to glutathione-S-transferase. All three forms of recombinant maspin inhibited the invasion and motility of a series of cancer cell lines *in vitro* (8, 20, 32, 33). The effect of purified maspin on angiogenesis was further investigated by Zhang et al. (21) In their study published in 2000, maspin protein blocked endothelial cell migration toward basic fibroblast growth factor and vascular endothelial growth factor, and inhibited endothelial tube formation *in vitro*.

It is likely that maspin acts either on the cell surface or in the pericellular space to inhibit tumor invasion and metastasis since: (i) purified maspin added to the cell culture or administrated locally to tumor xenografts act similarly as endogenously expressed maspin to block tumor invasion, tumor-induced angiogenesis and tumor metastasis; and (ii)

endothelial cells and stromal cells do not express maspin, but are responsive to the inhibitory effect of purified maspin in *in vitro* motility and adhesion assays (32, 34). These data further suggests that maspin regulates common extracellular reactions involved in tumor invasion and motility.

A consensus that maspin depends on its reactive center loop (RCL) to inhibit tumor cell motility and invasion is derived from the evidence that the biological activity of both endogenous maspin and purified maspin can be reversed by a neutralizing antibody made against maspin RCL peptide (1, 8, 17, 19, 20). Further supporting this notion is the evidence that the N-terminal domain of maspin immediately upstream of its putative P_1P_1' site in the RCL region, resulting from limited cleavage by trypsin, has no effect on tumor cell invasion (20). In cell-based biological assays, Ngamkitidechakul et al. showed that maspin RCL peptide was sufficient to stimulate the adhesion of corneal stromal cells to type I collagen, fibronectin, and laminin, and stimulate the adhesion of breast cancer cells MDA-MB-231 to fibronectin (35). Chimeric maspin/ovalbumin, which has maspin RCL sequence replaced by that of ovalbumin, did not have any activity in parallel assays. In contrast, the substitution of the RCL of ovalbumin with that of maspin converted inactive ovalbumin into a fully active molecule (35). These data argue against the suggestion that maspin acts like noninhibitory serpin ovalbumin. However, it is worth noting that since only full-length maspin protein, but not maspin RCL peptide, has been detected in cells or biological samples so far, how the functionality of the maspin RCL can be totally independent of the general framework of maspin *in vivo* is not clear.

2.2 Intracellular Maspin and Cell Apoptosis

Since the discovery of the maspin gene, it has long been repeatedly noted that overexpression of maspin in cancer cells always results in growth inhibition in various *in vivo* tumor models (1, 13-16, 21, 23, 36). This phenomenon has not been reported in other tumor models where maspin-expressing tumor cells (either *via* stable transfection or viral infection) were implanted subcutaneously or orthotopically. In addition, neither recombinant maspin nor the endogenous re-expression of maspin directly inhibited tumor cell growth *in vitro* (8, 17, 20, 27). Thus, the difference between the *in vivo* and *in vitro* observations may reflect the differences in tumor microenvironments. Several recent studies suggest that the *in vivo* inhibitory effect of maspin on tumor growth is, at least in part, due to an increased apoptosis (13, 14, 22). A conceivable difference between the *in vitro* cell culture and *in vivo* tumor is the level of stress, such as oxidative stress. *In vitro* evidence shows that maspin expression may be induced by oxidative stress. Maspin may in turn further regulate cellular response to changes in the redox homeostasis. It has been reported that peroxisome proliferator-activated receptor-gamma (PPARγ)-induced maspin expression correlated with a more differentiated phenotype in both breast carcinoma cells (37) and colon cancer cells (38). Further evidence by Khalkhali-Ellis and colleagues suggested that maspin enhances nitric oxide-induced apoptosis of MCF-7 cells (39). Another conceivable difference between the *in vitro* cell culture and *in vivo* tumor may be cytotoxic cytokines that are mostly absent *in vitro* but are secreted by stromal or immune cells in the tumor microenvironment. The study by Jiang and colleagues showed that although maspin protein does not induce spontaneous cell death, endogenous maspin, but not exogenously added recombinant maspin or secreted maspin, significantly sensitized mammary carcinoma cells MDA-MB-435 to drug-induced apoptosis (27). A subsequent study by Liu et al. (28) revealed that maspin expression in DU145 cells led to increased Bax expression. Furthermore, the effect of maspin in sensitizing cells to drug-induced apoptosis depends on the Bax-mediated mitochondrial pathway. This finding is consistent with the evidence that maspin sensitizes the apoptotic response of breast and prostate carcinoma cells to various drugs ranging from death ligands to endoplasmic reticulum stress.

The evidence that maspin re-expression leads to tumor cell redifferentiation *in vivo* should encourage efforts to develop maspin-based differentiation therapies, while the link of maspin with the elevated Bax-mediated cellular sensitivity to apoptosis further suggests that maspin may be used as a modifier for

apoptosis-based cancer therapy. Maspin is the only proapoptotic serpin amongst all serpins so far implicated in apoptosis regulation.

3. COMPARING MASPIN WITH OTHER SERPINS

3.1 The Structural Considerations

It has been a long-standing issue whether maspin act as an inhibitory serpin to inhibit a serine protease that plays a key role in tumor invasion and metastasis. The essence of an inhibitory serpin is its ability to undergo dramatic conformational changes upon the initial interaction with the target serine protease, docking the target enzyme in a suicidal complex. This consensus is supported by structural analyses using either X-ray crystallographic and solution-based NMR analyses, and by enzyme kinetic data (reviewed in (40, 41)). Although the exact conformational changes and co-factor requirements vary, the inducibility of serpin conformational changes derives from several common structural features. The common core structure of serpins consists of three β-sheets (A, B and C), and 8-9 α-helices. The pseudo-substrate peptide bone P_1P_1' lies in an exposed loop sequence called reactive center loop (RCL). For a proteolytic inhibitory serpin, the amino acid residue at the P1 site is critical in determining the target specificity. Upon the formation the initial Michalis complex with the target enzyme, the P_1P_1' bond in serpin RCL is cleaved. The cleaved RCL is then inserted into the A β-sheets as the 4^{th} strand. The RCL insertion further stabilizes the serpin/enzyme complex and renders the release of the cleaved serpin a slow rate-limiting step.

The secondary and tertiary structures of all serpins are remarkably similar. In fact, the metastable nature of the serpin conformation is almost single-handedly determined by whether the RCL has the desired sequence compatibility with that of two adjacent A β-sheets (3A and 5A), and whether the RCL has an appropriate length in the hinge region N-terminal to the P_1P_1' bond (42). Maspin has an overall 30-35% sequence homology with other serpins (1), and based on a computer model has the overall serpin framework (43). However, it has been noted that maspin has a unique RCL sequence (Table 1). Based on two previous sequence alignments, maspin is characterized as a nonclassical and noninhibitory serpin. In the initial alignment (sequence in black box of Table 1), maspin RCL is 4 amino acids shorter as compared to other serpins. Furthermore, the hinge sequence, located 9-14 residues N-terminal to the P_1P_1' peptide bond of maspin, deviates significantly from the conserved sequence of inhibitory serpins (1). This sequence alignment does not perturb the overall sequence alignment of maspin with other serpins in other core domains. Using this sequence alignment, Fitzpatrick et al. generated a computer model for the tertiary structure of maspin using the crystal structure of noninhibitory serpin ovalbumin as a prototype. This theoretical structure of maspin suggests the absence of disulfide bonds and the presence of an unstable RCL that adopts a distorted helical structure (44). The second sequence alignment proposed by Dr. Gettins (sequence in red box of Table 1) suggests that a histidine is at the P_1 site of maspin RCL. Based on this alignment, maspin RCL has a length similar to that of other serpins. However, the hinge sequence of maspin remains deviant (reviewed in (41)). In addition, the sequence C-terminal to the RCK of maspin is 4 amino acids shorter than other serpins.

Since the protein structure of maspin has not been resolved, a third alignment can not be ruled out. As shown by the sequence in the blue box of Table 1, maspin is an arginine (Arg) serpin, with a full length RCL. According to this alignment, the maspin hinge sequence has a better homology to those of inhibitory serpins (serpin 1-15 in Table 1) than several known noninhibitory serpins (serpin 19-24 in Table 1). Among those amino acid residues in the maspin RCL that do not align well with their counterparts in inhibitory serpins, we noted that maspin has three glycine residues at P_3, P_{10} and P_{11} positions. Since glycine has only one hydrogen atom as a side chain, it can adopt a much wider range of conformations than the other residues (45). Thus, it is possible that a high proportion of glycine residues in the maspin RCL may play an important role to

allow a metastable conformation that will not be possible otherwise. On the other hand, since the hinge sequences of several inhibitory serpins such as bomapin, MEPI, α1-PI and A2, are also quite deviant from the most conserved sequences as seen in several other inhibitory serpins (PI8, SCCA1, SCCA2, antithrombin, α1-antichymotrypsin, PI6, kallistatin, PAI-2, headpin, and PI9), it remains a question what is the minimum degree of hinge sequence conservation that is sufficient to support a proteolytic inhibitory activity. It is noted that despite a closer sequence homology to noninhibitory ovalbumin, most serpins in the same gene cluster on chromosome 18q21, such as PAI-2, PI6, PI9, PI10, and headpin, are inhibitory against serine proteases (4, 5, 46-49). Interestingly, SCCA1 and SCCA2 that have a higher degree of hinge sequence conservation than several other inhibitory serpins have been shown to cross-inhibit cysteine proteases cathepsin S, K, L, and papain (50), raising the question whether a highly conserved hinge sequence necessarily dictates a more efficient inhibition against serine proteases.

Table 1. Reactive Center Loop Sequences for Selected Serpinsa.

No.	Serpin	P_{14}	P_1	P_{12}	P_1	P_{10}	P_9	P_8	P_7	P_6	P_5	P_4	P_3	P_2	P_1	P_1'	P_2'	P_3'	P_4'
1	B8; PI8	T	E	A	A	A	A	T	A	V	V	R	N	S	R	C	S	R	M
2	B3; SCCA1	A	E	A	A	A	A	T	A	V	V	G	F	G	S	S	P	A	S
3	B4; SCCA2	V	E	A	A	A	A	T	A	V	V	V	V	E	L	S	S	P	S
4	C1; antithrombin	S	E	A	A	A	S	T	A	V	V	I	A	G	R	S	L	N	P
5	A3; α1-ACT	T	E	A	S	A	A	T	A	V	K	I	T	L	L	S	A	L	V
6	B6; PI6	T	E	A	A	A	A	T	A	A	I	M	M	M	R	C	A	R	F
7	A4; kallistatin	T	E	A	A	A	A	T	T	F	A	I	K	F	F	S	A	Q	T
8	B2; PAI-2	T	E	A	A	A	G	T	G	G	V	M	T	G	R	T	G	H	G
9	B13; headpin	T	E	A	A	A	A	T	G	I	G	F	T	V	T	S	A	P	G
10	B9; PI9	T	E	A	A	A	A	S	S	C	F	V	V	A	E	C	C	M	E
11	B10; bomapin	R	E	A	A	A	G	S	G	S	E	I	D	I	R	I	R	V	P
12	I2; MEPI	S	E	A	A	T	S	T	G	I	H	I	P	V	I	M	S	L	A
13	A1; α1-PI	T	E	A	A	G	A	M	F	L	E	A	I	P	M	S	I	P	P
14	E1; PAI-1	T	V	A	S	S	S	T	A	V	I	V	S	A	R	M	A	P	E
15	A2;	T	E	A	T	G	A	P	H	L	E	E	K	A	W	S	K	Y	Q
16	B5; human maspin	T	E	D	G	G	D	S	I	E	V	P	G	A	R	I	L	Q	H
17	B5; human maspin	G	D	S	I	E	V	P	G	A	R	I	L	Q	H	K	D	E	L
18	B5, human maspin	G	D	S	I	E	V	P	G	A	-	-	-	-	R	I	L	Q	H
19	A6; cbg	V	D	T	A	G	S	T	G	V	T	L	N	L	T	S	K	P	I
20	A8; angiotensinogen	R	E	P	T	E	S	T	Q	Q	L	N	K	P	E	V	L	E	V
21	B1; ovalbumin	R	E	V	V	G	S	A	E	A	G	V	D	A	A	S	V	S	E
22	F1; PEDF	A	G	T	T	P	S	P	G	L	Q	P	A	H	L	T	F	P	L
23	H1; colligin 1	N	P	F	D	Q	D	I	Y	G	R	E	E	L	R	S	P	K	L
24	H2; colligin 1	N	P	F	D	Q	D	I	Y	G	R	E	E	L	R	S	P	K	L

3.2 Purified Maspin is Metastable

Because of the thermodynamically favored conformational liability, serpins tend to form polymers. The Z-variant of α1-PI is the best characterized serpin polymer, resulting either from a folding defect in which a folding intermediate has a substantially increased lifetime, or from a nonproductive RCL cleavage (reviewed in (41)). Structural analyses have confirmed that such polymerization is likely through a loop-sheet

insertion mechanism involving the RCL of one molecule and a β-sheet of another (51, 52). To date, there is no definitive experimental evidence to support whether maspin may allow efficient RCL insertion into the β-pleated sheets. However, it has been noted that purified recombinant maspin has poor stability (10, 43, 53). Data from transverse urea gradient gel electrophoresis and thermal denaturation experiments suggest that maspin has an exposed RCL and does not undergo the stressed-relaxed transition in solution which is typical for proteolytic inhibitory serpins (54). Limited digestion with trypsin or plasmin results in specific cleavage of maspin at the P_1P_1' bond (20). In addition, the dimerization of purified maspin is often associated with spontaneous specific cleavage of the P_1P_1' peptide bond. As compared to the intact monomeric maspin, both the dimerized and cleaved maspin exhibit a higher affinity for the heparin column (43), indicating major conformational changes not only in the RCL but also in the heparin-binding domain. Purified maspin also tends to undergo spontaneous three-state unfolding and polymerization under cell-free conditions (55). Using circular dichroism and intrinsic tryptophan fluorescence to monitor the conformational changes of maspin under urea denaturing conditions, Liu and colleagues showed that the unfolding and self-association of maspin involved three states: monomer form, unfolding intermediate, and dimer form (55).

Thus, despite its non-inhibitory activity in solution, maspin appears to be capable of undergoing major conformational changes such as RCL insertion. It is important to note that the instability of purified maspin is to be contrasted by endogenous maspin. In fact, endogenously expressed maspin, either in cell-associated fraction or secreted fraction, is found stable in an intact monomeric form. It is likely that the *in vivo* microenvironment of maspin is more complex, possibly involving other cognate factors that stabilize maspin.

3.3 The Novel Biochemical Behaviors of Maspin

In solution-based biochemical studies, recombinant maspin did not inhibit several purified proteases including tissue-type plasminogen activator (tPA), urokinase-plasminogen activator (uPA), trypsin, chymotrypsin, elastase, plasmin, and thrombin (53, 54). Instead, purified recombinant maspin is sensitive to limited proteolysis (20, 44), resulting in a single cleavage of the P_1P_1' bond (20). It is known that the inhibitory activity of several serpins can be significantly enhanced by co-factors such as low molecular weight heparin (56). Maspin binds to heparin affinity column with a low affinity (20). However, heparin and several other potential serpin co-factors failed to confer an inhibitory activity on purified maspin (54).

Interestingly, under certain circumstances, purified monomeric maspin has been shown to exert inhibitory effects on plasminogen activators. The study by Sheng et al. showed single-chain tissue plasminogen activator (sctPA) specifically interacts with the maspin RCL peptide and forms a stable complex with purified recombinant maspin (53). When incubated with free sctPA, maspin showed no proteolytic inhibitory effect. Rather, maspin activates sctPA in the presence of either an amidolytic substrate of tPA or a combination of plasminogen and plasmin substrate (21, 53). However, if sctPA was preactivated by fibrinogen/gelatin, maspin acts as a competitive inhibitor of sctPA at low concentrations (< 0.5 μM) and as a stimulator at higher concentrations (>0.5 μM). The 38-kDa C-terminal truncated maspin derived from specific P_1P_1' cleavage exclusively stimulates fibrinogen/gelatin-associated sctPA. Further analyses led to a novel kinetic model in which the N-terminal and C-terminal domains of maspin interact with the regulatory and catalytic domains of sctPA, respectively.

Although recombinant maspin does not inhibit uPA in solution, McGowen et al. showed that purified monomeric maspin produced in baculo virus-infected *Sf*9 insect cells binds specifically to the surface of prostate carcinoma DU145 cells, and inhibits the DU145 cell surface-mediated plasminogen activation. DU145 cells mediate plasminogen activation primarily by uPA (9). Consistently, the inhibitory effect of maspin on cell surface-bound uPA was similar to that of an uPA-neutralizing antibody and was reversed by a polyclonal antibody against the maspin RCL peptide. In parallel, cleaved or polymerized maspin

stimulated cell surface-mediated plasminogen activation. The Ki value for monomeric maspin in cell surface-mediated plasminogen activation was comparable to those for plasminogen activator inhibitor 1 (PAI-1) and plasminogen activator inhibitor 2 (PAI-2). These data demonstrate the potential of maspin to act as an inhibitory serpin, and an important role of cell surface in mediating the inhibitory interaction between maspin and uPA.

A report by Bass et al. showed that recombinant maspin produced in yeast did not inhibit tPA, uPAR-bound uPA and cell surface-associated uPA, albeit that the same maspin preparation inhibited the motility of tumor cells and vascular smooth muscle cells *in vitro* (33). While the issue of whether maspin inhibits tumor cell motility and invasion by inhibiting cell-associated uPA needs to be further clarified, it is important to raise a word of caution that experiments using purified recombinant maspin depend on both the quality and the quantity of maspin. As discussed above, purified maspin is metastable. Purified monomeric, but not cleaved or polymerized maspin has a proteolytic inhibitory effect against plasminogen activators. Furthermore, the effect of monomeric maspin on fibrinogen/gelatin-associated sctPA or cell surface-mediated plasminogen activation featured a bell-shaped dose-dependence, i.e. inhibitory at low concentrations and stimulatory at higher concentrations. This biphasic dose-dependence is similar to that found with the reaction between purified maspin and fibrinogen/gelatin-associated sctPA, and consistent with a bell-shaped dose-dependence in the maspin effect on tumor cell motility and invasiveness (20).

In contrast to the circumstantial proteolytic inhibitory effect of purified maspin, Biliran and colleagues showed that endogenously expressed maspin by DU145 cells led to a dramatic reduction in the release of active uPA into the conditioned culture medium. Consistently, the conditioned media of maspin transfectant clones had a significantly lower activity in converting plasminogen to plasmin (17). Of particular importance, maspin expression led to a significantly reduced level of cell surface-bound uPA and uPA receptor (uPAR) proteins. Treatment with receptor-associated protein (RAP), a specific inhibitor of low-density lipoprotein receptor-related protein (LRP), led to a significantly increased level of cell surface-associated uPA and uPAR in maspin transfectants but not in the mock control cells (17). Since uPAR/uPA complex interacts with LRP localized in cell surface caveolae lipid raft (57), and the binding of PAI-1 to the uPAR/uPA complex has been shown to trigger a rapid LRP-mediated internalization of the uPAR/uPA/PAI-1 complex, these data further support that maspin may act in a similar fashion as PAI-1 toward the cell surface-anchored uPAR/uPA complex.

The recent study of Cher et al. (23) provided the first evidence that maspin expression in stably transfected DU145 cells inhibits tumor-mediated ECM and collagen degradation. Cher and colleagues subsequently tested maspin transfected DU145 cells in a novel SCID-Hu model for prostate cancer bone metastasis and showed that expression of maspin correlated with decreased tumor growth, reduced osteolysis, and decreased angiogenesis. Furthermore, the maspin-expressing tumors are associated with a significantly reduced level of uPA and a dramatically increased fibrosis (23).

4. CURRENT ISSUES AND FUTURE DIRECTIONS

Regarding the underlying mechanisms of maspin for its multifaceted tumor suppressive activities, the deviant RCL sequence, the metastable biophysical properties and the novel biochemical behavior against plasminogen activators mandates a further clarification on whether maspin can still act as an inhibitory serpin. To date, neither of the following two possibilities can be definitively excluded: (i) Mapsin acts as an inhibitory serpin; and (ii) Maspin acts as a non-inhibitory serpin.

4.1 Maspin Acts as an Inhibitory Serpin

The evidence that the biological activities of maspin in inhibiting tumor cell motility and invasion requires its intact RCL, and that under biologically relevant conditions maspin inhibits both tPA and

uPA, support the hypothesis that maspin may act as a plasminogen activator inhibitor.

uPA is a particularly reasonable target for the tumor suppressive activities of maspin. A large body of literature demonstrates that the pericellular uPA-mediated proteolysis is facilitated by cell surface-anchored uPAR. Plasmin is a serine protease with a relatively broad spectrum of substrate specificity. The major biological function of plasmin is to dissolve fibrin clots in thrombolysis, to degrade several key ECM components, and to proteolytically activate ECM-associated growth factors or other protease zymogens. Thus, the cell surface-associated uPA/uPAR complex is responsible not only for initiating the powerful proteolytic cascade by converting plasminogen to plasmin, but also for regulating ECM and growth factor signaling pathways during wound healing, tumor invasion and angiogenesis.

The biological effect of maspin in cell motility and invasion has been localized on the cell surface. Among known endogenous uPA inhibitors, maspin inhibits cell surface-associated uPA with a Ki values similar to that for PAI-1 and PAI-2 (9). Furthermore, both maspin and PAI-1 have been shown to trigger rapid LRP-mediated uPA/uPAR internalization. In contrast to PAI-1 and PAI-2 that also free uPA, maspin did not inhibit uPA in the absence of cell surface. Additionally, PAI-1 is often up-regulated along with uPA and uPAR in tumor progression (32). In contrast, a loss of maspin and PAI-2 expression appears to correlate with invasion and metastasis in several types of cancer (reviewed in (24)).

The dependence of the proteolytic inhibitory activity of maspin on the coexistence of an allosteric tPA activating protein or the intact cell surface (for uPA) raises the possibility that maspin may undergo the conformational change as an inhibitory serpin when the target serine protease (tPA or uPA) adopts a transitional state conformation which is supported by the specific biological microenvironment. On the other hand, a serpin molecule such as PAI-1 may also be partitioned between its protease target (plasminogen activators) and extracellular matrix protein vitronectin. In fact, the balance between uPA-bound PAI-1 and vitronectin-bound PAI-1 may represent a biological switch between the anti-invasive and the pro-invasive activity of PAI-1 in tumor progression (58). The possibility remains that the proteolytic inhibitory effect of maspin requires a co-factor such as collagen I. Blacque et al. published the results of a yeast-two-hybrid screen using a C-terminal truncated form of maspin. In this study, α-2 chain of type I collagen was identified as a candidate maspin-associated molecule (59). While these results are yet to be independently confirmed, it is important to find out whether the potential interaction between maspin and collagen I further regulates the proteolytic inhibitory potential or the bio-availability of extracellular maspin.

The hypothesis that maspin inhibits the cell surface-associated uPA/uPAR system helps explain the suppressive effect of maspin at the step of tumor invasion and metastasis. It is also important to note that endogenous maspin re-expressed in both prostate and breast cancer cells inhibits tumor growth and induces tumor cell re-differentiation *in vivo*. Recent evidence by several groups showed that inhibition of the uPA/uPAR system leads to a decreased ratio of phosphorylated ERK1/2 to phosphorylated p38 MAPK, which in turn is associated with tumor dormancy. An inhibitory interaction between maspin and tumor cell surface-associated uPA/uPAR may lead to a similar decreased ratio of phosphorylated ERK1/2 to phosphorylated p38 MAPK and underlie the biological effect of maspin on tumor growth and re-differentiation.

4.2 Maspin Acts as a Non-inhibitory Serpin

Considering the novel sequence of the maspin RCL and the novel biochemical characteristics of maspin toward soluble serine proteases, it remains a possibility that maspin does not directly inhibit any active serine protease. In this case, the tumor suppressive activity of pericellular maspin and the apparent proteolytic inhibitory activity of maspin on fibrinogen-bound tPA or cell surface-associated uPA may be due to its interaction with the respective protease zymogens, which subsequently prevents the enzyme activation. As our earlier kinetic evidence suggests, purified recombinant maspin may use its N-terminal domain to interact with the regulatory

domain of tPA and use its RCL domain to interact with the catalytic domain of tPA. It is possible that such concerted multi-contact interaction prevents the allosteric activation of tPA by its physiological activators such as a fibrin clot. In the case of uPA, it has been shown that pericellular pro-uPA bound to uPAR is proteolytically activated. Both pro-uPA and active uPA can bind to uPAR. Furthermore, PAI-1 also binds to pro-uPA, although with a significantly lower affinity as compared to that with active uPA (60). It is important for the future studies to clarify whether the cell surface-dependent inhibition of uPA by maspin is because of maspin interaction with pro-uPA or with active uPA.

It is likely that maspin is protected by protein-protein interaction *in vivo* because purified maspin, but not endogenous maspin, is unstable in solution. Furthermore, since endogenous maspin is partitioned among several subcellular compartments (nucleus, cytoplasm, cell surface and secreted to the pericellular space), it is reasonable to hypothesize that maspin may exert multifaceted cellular and biochemical activities by interacting with different molecular targets in different microenvironments. Consistent with this notion, we have shown that intracellular maspin is associated with increased Bax expression and increased cellular sensitivity to drug-induced apoptosis (28).The apoptosis-sensitizing effect of maspin depends on both its N-terminal and C-terminal sequences. Meanwhile, a couple of recent correlative clinical studies revealed that maspin nuclear localization is associated with more differentiated phenotypes. It is worth noting that a non-inhibitory serpin Hsp47 is found to act as a stress-responsive chaperone molecule. It is yet to be explored whether maspin acts as a chaperone molecule either with or without a proteolytic inhibitory activity. In particular, since Hsp47 has been shown to chaperone collagen (61), it is important to find out whether maspin protects collagen I. Furthermore, as noted by Zhang et al. a maspin variant mutated at the RCL region was similarly effective as the full-length purified maspin in inhibiting angiogenesis. This result suggests yet another proteolysis-independent function of potential metabolites derived from maspin. Interestingly, an earlier report by O'Reilly and colleagues also showed that the N-terminal fragment of antithrombin was a potent inhibitor of tumor-induced angiogenesis *in vivo*. It is possible that maspin metabolites may be biologically active (62).

4.3 Concluding Remarks

The research progress made in the last decade has revealed several important anti-tumor activities of maspin. For future development of maspin-based anti-cancer therapies, it is critical to understand the molecular modes of maspin action. Although maspin sequence generally aligns with serpins, its biochemical characteristics appear to be between proteolytic inhibitory and non-inhibitory serpins. Furthermore, maspin is likely to be regulated differently in different subcellular compartments and biological microenvironments. It is particularly important to note that among all the serpins implicated in apoptosis regulation, maspin is the only pro-apoptotic serpin, thus may offer more desirable therapeutic benefits.

Towards the potential clinical application of maspin, it is important to raise a word of precaution. Since purified maspin is not as stable as endogenous maspin, and gives rise to a biphasic effect in cell biological and biochemical assays, future development of maspin-based therapeutic strategies may have to depend on the identification of a more defined maspin-mimic small molecular weight compound or a gene-therapy-like approach to deliver maspin expression to specific cell populations *in vivo*.

ACKNOWLEDGEMENTS

The author wishes to thank Mr. Jaron Lockett and Ms. Deborah Leicht for their skillful assistance in proofreading this manuscript.

REFERENCES

1. Zou, Z., Anisowicz, A., Hendrix, M. J., Thor, A., Neveu, M., Sheng, S., Rafidi, K., Seftor, E., and Sager, R., 1994, Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. Science, 263:526-529.

2. Zhang, M., Sheng, S., Maass, N., and Sager, R., 1997, mMaspin: the mouse homolog of a human tumor suppressor gene inhibits mammary tumor invasion and motility. Mol Med, 3:49-59.
3. Umekita, Y., Hiipakka, R. A., and Liao, S., 1997, Rat and human maspins: structures, metastatic suppressor activity and mutation in prostate cancer cells. Cancer Lett, 113: 87-93.
4. Bartuski, A. J., Kamachi, Y., Schick, C, Overhauser, J., and Silverman, G. A., 1997, Cytoplasmic antiproteinase 2 (PI8) and bomapin (PI10) map to the serpin cluster at 18q21.3. Genomics, 43:321-328.
5. Schneider, S. S., Schick, C., Fish, K. E., Miller, E., Pena, J. C., Treter, S. D., Hui, S. M., and Silverman, G. A., 1995, A serine proteinase inhibitor locus at 18q21.3 contains a tandem duplication of the human squamous cell carcinoma antigen gene. Proc Natl Acad Sci U S A, 92:3147-3151.
6. Scott, F. L., Eyre, H. J., Lioumi, M., Ragoussis, J., Irving, J. A., Sutherland, G. A., and Bird, P. I., 1999, Human ovalbumin serpin evolution: phylogenic analysis, gene organization, and identification of new PI8-related genes suggest that two interchromosomal and several intrachromosomal duplications generated the gene clusters at 18q21-q23 and 6p25. Genomics, 62:490-499.
7. Nakashima, T., Pak, S. C., Silverman, G. A., Spring, P. M., Frederick, M. J., and Clayman, G. L., 2000, Genomic cloning, mapping, structure and promoter analysis of HEADPIN, a serpin which is down-regulated in head and neck cancer cells. Biochim Biophys Acta, 1492: 441-446.
8. Sheng, S., Carey, J., Seftor, E. A., Dias, L., Hendrix, M. J., and Sager, R., 1996, Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells. Proc Natl Acad Sci U S A, 93:11669-11674.
9. McGowen, R., Biliran, H. Jr., Sager, R., and Sheng, S., 2000, The surface of prostate carcinoma DU145 cells mediates the inhibition of urokinase-type plasminogen activator by maspin. Cancer Res, 60: 4771-4778.
10. Pemberton, P. A., Tipton, A. R., Pavloff, N., Smith, J., Erickson, J. R., Mouchabeck, Z. M., and Kiefer, M. C. Maspin is an intracellular serpin that partitions into secretory vesicles and is present at the cell surface. J Histochem Cytochem, 45: 1697-1706, 1997.
11. Katz, A. B. and Taichman, L. B., 1999, A partial catalog of proteins secreted by epidermal keratinocytes in culture. J Invest Dermatol, 112: 818-821.
12. Shao, Z. M., Nguyen, M., Alpaugh, M. L., O'Connell, J. T., and Barsky, S. H., 1998, The human myoepithelial cell exerts antiproliferative effects on breast carcinoma cells characterized by p21WAF1/CIP1 induction, G2/M arrest, and apoptosis. Exp Cell Res, 24:394-403.
13. Zhang, M., Shi, Y., Magit, D., Furth, P. A., and Sager, R., 2000, Reduced mammary tumor progression in WAP-TAg/WAP-maspin bitransgenic mice. Oncogene, 19:6053-6058.
14. Shi, H. Y., Liang, R., Templeton, N. S., and Zhang, M., 2002, Inhibition of breast tumor progression by systemic delivery of the maspin gene in a syngeneic tumor model. Mol Ther, 5:755-761.
15. Shi, H. Y., Zhang, W., Liang, R., Abraham, S., Kittrell, F. S., Medina, D., and Zhang, M., 2001, Blocking tumor growth, invasion, and metastasis by maspin in a syngeneic breast cancer model. Cancer Res, 61:6945-6951.
16. Sager, R., Sheng, S., Anisowicz, A., Sotiropoulou, G., Zou, Z., Stenman, G., Swisshelm, K., Chen, Z., Hendrix, M. J., Pemberton, P., et al., 1994, RNA genetics of breast cancer: maspin as paradigm. Cold Spring Harb Symp Quant Biol, 59:537-546.
17. Biliran, H. J., and Sheng, S., 2001, Pleiotrophic inhibition of pericellular urokinase-type plasminogen activator system by endogenous tumor suppressive maspin. Cancer Res, 61:8676-8682.
18. Odero-Marah, V. A., Khalkhali-Ellis, Z., Chunthapong, J., Amir, S., Seftor, R. E., Seftor, E. A., and Hendrix, M. J., 2003, Maspin regulates different signaling pathways for motility and adhesion in aggressive breast cancer cells. Cancer Biol Ther, 2:398-403.
19. Seftor, R. E., Seftor, E. A., Sheng, S., Pemberton, P. A., Sager, R., and Hendrix, M. J., 1998, Maspin suppresses the invasive phenotype of human breast carcinoma. Cancer Res, 58:5681-5685.
20. Sheng, S., Pemberton, P. A., and Sager, R., 1994, Production, purification, and characterization of recombinant maspin proteins. J Biol Chem, 269: 30988-30993.
21. Zhang, M., Volpert, O., Shi, Y. H., and Bouck, N. Maspin is an angiogenesis inhibitor. Nat Med, 6: 196-199, 2000.
22. Shi, H. Y., Zhang, W., Liang, R., Kittrell, F., Templeton, N. S., Medina, D., and Zhang, M., 2003, Modeling human breast cancer metastasis in mice: maspin as a paradigm. Histol Histopathol, 18:201-206.
23. Cher, M. L., Biliran, H. R. Jr., Bhagat, S., Meng, Y., Che, M., Lockett, J., Abrams, J., Fridman, R., Zachareas, M., and Sheng, S., 2003, Maspin expression inhibits osteolysis, tumor growth, and angiogenesis in a model of prostate cancer bone metastasis. Proc Natl Acad Sci USA, 100:7847-7852.
24. Sheng, S., 2004, The promise and challenge toward the clinical application of maspin in cancer. Frontiers in Bioscience, 9:2733-2745.

25. Hojo, T., Akiyama, Y., Nagasaki, K., Maruyama, K., Kikuchi, K., Ikeda, T., Kitajima, M., and Yamaguchi, K., 2001, Association of maspin expression with the malignancy grade and tumor vascularization in breast cancer tissues. Cancer Lett, 171:103-110.
26. Song, S. Y., Lee, S. K., Kim, D. H., Son, H. J., Kim, H. J., Lim, Y. J., Lee, W. Y., Chun, H. K., and Rhee, J. C., 2002, Expression of maspin in colon cancers: its relationship with p53 expression and microvessel density. Dig Dis Sci, 47:1831-1835.
27. Jiang, N., Meng, Y., Zhang, S., Mensah-Osman, E., and Sheng, S., 2002, Maspin sensitizes breast carcinoma cells to induced apoptosis. Oncogene, 21:4089-4098.
28. Liu, J., Yin, S., Reddy, N., Spencer, C., and Sheng, S., (in press) 2004, Bax Mediates the apoptosis-sensitizing effect of maspin. Cancer Res.
29. Zhang, M., Magit, D., Botteri, F., Shi, H. Y., He, K., Li, M., Furth, P., and Sager, R., 1999, Maspin plays an important role in mammary gland development. Dev Biol, 215:278-287.
30. Nemeth, J. A., Harb, J. F., Barroso, U. Jr., He, Z., Grignon, D. J., and Cher, M. L., 1999, Severe combined immunodeficient-hu model of human prostate cancer metastasis to human bone. Cancer Res, 59:1987-1993.
31. Abraham, S., Zhang, W., Greenberg, N., and Zhang, M., 2003, Maspin functions as tumor suppressor by increasing cell adhesion to extracellular matrix in prostate tumor cells. J Urol, 169:1157-1161.
32. Ngamkitidechakul, C., Burke, J. M., O'Brien, W. J., and Twining, S. S., 2001, Maspin: synthesis by human cornea and regulation of in vitro stromal cell adhesion to extracellular matrix. Invest Ophthalmol Vis Sci, 42:3135-3141.
33. Bass, R., Fernandez, A. M., and Ellis, V., 2002, Maspin inhibits cell migration in the absence of protease inhibitory activity. J Biol Chem, 277:46845-46848.
34. Dokras, A., Gardner, L. M., Kirschmann, D. A., Seftor, E. A., and Hendrix, M. J., 2002, The tumour suppressor gene maspin is differentially regulated in cytotrophoblasts during human placental development. Placenta, 23:274-280.
35. Ngamkitidechakul, C., Warejcka, D. J., Burke, J. M., O'Brien, W. J., and Twining, S. S., 2003, Sufficiency of the reactive site loop of maspin for induction of cell-matrix adhesion and inhibition of cell . Conversion of ovalbumin to a maspin-like molecule. J Biol Chem, 278:31796-31806.
36. Lefter, L. P., Sunamura, M., Furukawa, T., Takeda, K., Kotobuki, N., Oshimura, M., Matsuno, S., and Horii, A., 2003, Inserting chromosome 18 into pancreatic cancer cells switches them to a dormant metastatic phenotype. Clin Cancer Res, 9:5044-5052.
37. Mueller, E., Sarraf, P., Tontonoz, P., Evans, R. M., Martin, K. J., Zhang, M., Fletcher, C., Singer, S., and Spiegelman, B. M., 1998, Terminal Differentiation of Human Breast Cancer through PPAR? Molecular Cell, 1:465-470.
38. Burgermeister, E., Tencer, L., and Liscovitch, M., 2003, Peroxisome proliferator-activated receptor-gamma upregulates caveolin-1 and caveolin-2 expression in human carcinoma cells. Oncogene, 22:3888-3900.
39. Khalkhali-Ellis, Z., and Hendrix, M. J., 2003 Nitric oxide regulation of maspin expression in normal mammary epithelial and breast cancer cells. Am J Pathol, 162:1411-1417.
40. Gettins, P. G., Backovic, M., and Peterson, F. C., 2004, Use of NMR to study serpin function. Methods, 32:120-129.
41. Gettins, P. G., 2002, Serpin structure, mechanism, and function. Chem Rev, 102:4751-4804.
42. Zhou, A., Carrell, R. W., and Huntington, J. A., 2001, The serpin inhibitory mechanism is critically dependent on the length of the reactive center loop. J Biol Chem, 276:27541-27547.
43. Sheng, S., Biliran, J. Jr., and McGowen, R., 2002, Maspin and Pericellular Plasminogen Activaiton in Cell-Matrix Interaction. In Maspin, ed. Hendrix, M. J.C., 57-67, Landes Bioscience, Georgetown, TX, USA.
44. Fitzpatrick, P. A., Wong, D. T., Barr, P. J., and Pemberton, P. A., 1996, Functional implications of the modeled structure of maspin. Protein Eng, 9:585-589.
45. Carl Branden, C., and Tooze, J., 1991, In Introduction to Protein Structure, Garland Publishing, Inc., New York, NY.
46. Sun, J., Rose, J. B., and Bird, P, 1995, Gene structure, chromosomal localization, and expression of the murine homologue of human proteinase inhibitor 6 (PI-6) suggests divergence of PI-6 from the ovalbumin serpins. J Biol Chem, 270:16089-16096.
47. Spring, P., Nakashima, T., Frederick, M., Henderson, Y., and Clayman, G., 1999, Identification and cDNA cloning of headpin, a novel differentially expressed serpin that maps to chromosome 18q. Biochem Biophys Res Commun, 264:299-304.
48. Askew, Y. S., Pak, S. C., Luke, C. J., Askew, D. J., Cataltepe, S., Mills, D. R., Kato, H., Lehoczky, J., Dewar, K., Birren, B., and Silverman, G. A., 2001, SERPINB12 is a novel member of the human ov-serpin family that is widely expressed and inhibits trypsin-like serine proteinases. J Biol Chem, 276:49320-49330.
49. Silverman, G. A., Bartuski, A. J., Cataltepe, S., Gornstein, E. R., Kamachi, Y., Schick, C., and Uemura, Y., 1998, SCCA1 and SCCA2 are

proteinase inhibitors that map to the serpin cluster at 18q21.3. Tumour Biol, 19:480-487.
50. Masumoto, K., Sakata, Y., Arima, K., Nakao, I., and Izuhara, K., 2003, Inhibitory mechanism of a cross-class serpin, the squamous cell carcinoma antigen 1. J Biol Chem, 278:45296-45304.
51. Carrell, R. W., Stein, P. E., Fermi, G., and Wardell, M. R., 1994, Biological implications of a 3 A structure of dimeric antithrombin. Structure, 2:257-270.
52. Stein, P. E., and Carrell, R. W., 1995, What do dysfunctional serpins tell us about molecular mobility and disease?. Nat Struct Biol, 2:96-113.
53. Sheng, S., Truong, B., Fredrickson, D., Wu, R., Pardee, A. B., and Sager, R., 1998, Tissue-type plasminogen activator is a target of the tumor suppressor gene maspin. Proc Natl Acad Sci USA, 95:499-504.
54. Pemberton, P. A., Wong, D. T., Gibson, H. L., Kiefer, M. C., Fitzpatrick, P. A., Sager, R., and Barr, P. J., 1995, The tumor suppressor maspin does not undergo the stressed to relaxed transition or inhibit trypsin-like serine proteases. Evidence that maspin is not a protease inhibitory serpin. J Biol Chem, 270:15832-15837.
55. Liu, T., Pemberton, P. A., and Robertson, A. D., 1999, Three-state unfolding and self-association of maspin, a tumor-suppressing serpin. J Biol Chem, 274:29628-29632.
56. Griffith, M. J., 1983, Heparin-catalyzed inhibitor/protease reactions: kinetic evidence for a common mechanism of action of heparin. Proc Natl Acad Sci USA, 80:5460-5464.
57. Stahl, A., and Mueller, B. M., 1995, The urokinase-type plasminogen activator receptor, a GPI-linked protein, is localized in caveolae. J Cell Biol, 129:335-344.
58. Schmitt, M., Harbeck, N., Thomssen, C., Wilhelm, O., Magdolen, V., Reuning, U., Ulm, K., Hofler, H., Janicke, F., and Graeff, H., 1997, Clinical impact of the plasminogen activation system in tumor invasion and metastasis: prognostic relevance and target for therapy. Thromb Haemost, 78:285-296.
59. Blacque, O. E., and Worrall, D. M., 2002, Evidence for a direct interaction between the tumor suppressor serpin, maspin, and types I and III collagen. J Biol Chem, 277:10783-10788.
60. Behrendt, N., List, K., Andreasen, P. A., and Dano, K., 2003, The pro-urokinase plasminogen-activation system in the presence of serpin-type inhibitors and the urokinase receptor: rescue of activity through reciprocal pro-enzyme activation. Biochem J, 371:277-287.
61. Kumei, Y., Morita, S., Shimokawa, H., Ohya, K., Akiyama, H., Hirano, M., Sams, C. F., and Whitson, P. A., 2003, Inhibition of HSP70 and a collagen-specific molecular chaperone (HSP47) expression in rat osteoblasts by microgravity. Ann N Y Acad Sci, 1010:476-480.
62. O'Reilly, M. S., Pirie-Shepherd, S., Lane, W. S., and Folkman, J., 1999, Antiangiogenic activity of the cleaved conformation of the serpin antithrombin. Science, 285:1926-1928.

Chapter 24

Metastasis Suppressor Genes: A Brief Review of an Expanding Field

Monica M. Richert and Danny R. Welch

Department of Pathology , Comprehensive Cancer Center, and the National Foundation for Cancer Research – Center for Metastasis Research, University of Alabama at Birmingham, Birmingham, AL, USA

Abstract: Metastasis of cancer cells from the primary tumor to form macroscopic lesions at the secondary site is the primary cause of morbidity and mortality in cancer patients. Thus, an understanding of the mechanisms controlling metastasis would allow more effective treatments resulting in better quality of life and higher survival rates. Metastasis suppressor genes prevent metastasis without affecting tumorigenesis and are therefore unique therapeutic targets. In this review, the thirteen defined metastasis suppressor genes are discussed and updated data on the mechanisms of action of these gene products are summarized. Furthermore, work ongoing on a number of potential metastasis suppressor genes for which functional data does not exist is summarized. Finally, important areas of ongoing research that will facilitate translation to the clinic are highlighted.

Key words: Metastasis, suppression, genes, cancer, tumorigenesis, KISS1, MKK4, Nm23, BRMS1, CRSP3, TXNIP, E-cadherin, SseCKS, KAI1, TIMPs, DRG-1, RKIP, RhoGDI2

1. INTRODUCTION

In 2003 the American Cancer Society estimated 1,334,100 new diagnoses of cancer in the United States. While overall 5 year survival rates are in the 60th percentile, an estimated 556,500 people died of cancer in 2003, approximately 1500 people per day. This translates into 1 in 4 deaths in the United States being cancer related. The majority of cancer related deaths are a result of metastasis from the primary tumor to secondary sites. Survival rates drop dramatically when a tumor spreads. For example, in breast cancer, the overall 5 year survival rate is approximately 97%. When the tumor cells have spread locally, survival drops to 78%, but with distant metastases, the rate plummets to 23%. Also, quality of life for those with metastatic disease is significantly worse than for those with tumors confined to the original site. Thus, better quality of life and higher survival rates depend on our ability to control metastatic progression.

In order to begin to clinically control metastatic progression we must obtain a comprehensive understanding of how a cell completes the metastatic cascade. Metastasis is the process through which tumor cells leave the primary tumor, travel to a distant site and grow to form a secondary mass (1, 2). The metastatic process is the end point of tumor development in which the cells within the tumor become more aggressive (3, 4). This movement of the tumor cells toward increased malignancy and metastasis is due to genetic instability and selection of sub-populations of cells (3). Eventually, some of the selected sub-populations acquire the ability to metastasize. The proportion of cells in a primary tumor that are able to metastasize depends on a number of factors. If cells acquire mutations allowing metastasis at an early stage of tumor development, then a higher percentage of the tumor

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 419-435.

cells will be capable of metastasis. Also, generally speaking, the larger a tumor is the more likely it is to spread, although size does not always correlate with a tumor's ability to metastasize (5, 6). Finally, signals from outside of the tumor cell can influence its ability to spread.

The ability of cells to metastasize is determined to a large extent by tumor-host interactions. The microenvironment can influence a tumor cell's ability to proliferate and the tumor cell can affect the microenvironment to elicit responses that either inhibit or promote growth of the tumor at the secondary site. These interactions occur through endocrine/paracrine and immunological mechanisms. The tumor cell can secrete cytokines and chemokines resulting in recruitment of neutrophils, macrophages, eosinophils and lymphocytes. These inflammatory cells can, in turn, secrete cytokines, proteases, cytotoxic mediators and membrane perforating agents (reviewed in (7)). Secretion of proteases can result in tissue remodeling, angiogenesis and the release of growth factors that can stimulate tumor cells to grow (7, 8). Therefore, the tumor cell and host environment can work together to allow growth of the metastasis. On the other hand, the microenvironment can respond with signals that kill or suppress the tumor cell. For example, activated macrophages that are recruited to the site can secrete IL-2, IL-12 and interferons that can kill the tumor cell (7). Why some tumor cells are allowed to grow into metastases while others are killed is an important question that remains unanswered, although theories addressing this question date back more than a century.

The seed and soil theory put forth by Sir Steven Paget is based on the tumor cell - host microenvironment interactions described above (9). This theory was originally used to explain why breast cancer predominantly spreads to bone. He proposed that the seed, or tumor cells, scatter in many directions through the vasculature, but will only grow when they encounter an appropriate organ microenvironment, or soil. Sites of metastasis are in part determined by the route taken through the body. Tumor cells can metastasize across body cavities, through the lymphatic system or through the vasculature. Most tumor cells that travel through the vasculature arrest in the first capillary bed they encounter (10, 11). Others selectively arrest in a specific tissue by using tissue specific endothelial cell surface molecules. These two methods of arrest may explain the organotropism of metastasis.

For metastasis to occur, a cell from the primary tumor must be able to complete every step of the metastatic cascade. It must detach from the primary tumor and enter into the lymphatic system or vasculature. The vasculature within the primary tumor is immature and, therefore, very permeable allowing the tumor cell ready access to the blood stream (12). When the tumor cell arrives in the vasculature it might remain as a single cell or join with other cells to form an embolus. This embolus can be made up of a single or multiple cell types. The single cell or embolus then travels through the vasculature where it must avoid being killed by shear forces. Eventually, the tumor cell or embolus arrests at a secondary site because of size restriction or through adherence to the vascular endothelium. The arrested cell then proliferates either within the vasculature and eventually breaks through, or extravasates into the neighboring tissue and then proliferates to form a secondary mass. Proliferation at the secondary site to form a mass is necessary for metastasis. Many tumor cells arrive at the secondary site and remain dormant - these are not metastases. This metastatic process is highly inefficient with $<0.001\%$ of the approximately 4×10^6 cells shed into the vasculature each day forming macroscopic tumors (13, 14). This inefficiency is due to cell death from a number of mechanisms including physical trauma, immune clearance and anoikis during the metastatic process (14, 15). Many of the cells extravasate into a tissue and then die at some later time (14).

It is obvious from the above description that the process of metastasis is not trivial. Each step of the metastatic cascade is regulated by a number of genes. In order for the metastatic cascade to be completed some of those genes must be mutated or mis-regulated. Therefore, identifying and manipulating metastasis regulating genes could allow decreased metastasis in the clinic. Metastasis suppressor genes are genes which interfere with at least one step of the metastatic cascade and, therefore, suppress metastasis (reviewed in (2, 16)). These genes prevent metastasis without affecting

tumorigenicity and are thus distinct from tumor suppressor genes. Tumor suppressor genes prevent both tumorigenesis and metastasis since tumor development is necessary for metastasis to take place. Therefore, tumor suppressors and metastasis suppressors are distinct therapeutic targets.

As mentioned above, some tumor cells arrive at the secondary site but do not initially proliferate. There are a number of theories as to how dormant cells exist within the secondary site (17-20). Some studies indicate that the cells survive but do not proliferate (21, 22), while others argue that it is a balance between proliferation and apoptosis (20). Another study indicates that the majority of cells that extravasate undergo apoptosis within 24 hours and that if apoptosis is inhibited metastatic potential increases (19). These studies complicate the understanding of metastasis, but it is likely that they are all correct. Tumors are inherently heterogeneous, and metastatic cells from different tumor types and at different secondary sites may act very differently. These dormant cells can remain at the secondary site for extended periods of time with no negative effect, but they have the potential to proliferate and form a mass at a later time.

Advances in technology in animal models have allowed detection of these single cells or small foci. This makes translation to the clinic more complex. How should a doctor treat a patient that has no metastatic disease but has dormant tumor cells at the secondary site? Should the patient go through the toxicity of chemotherapy if the chance of the dormant cells proliferating is very small? These are important questions that need to be addressed. Ideally, markers that can accurately predict the potential for forming macroscopic metastases will be used in the clinic to identify patients who need treatment for metastatic disease as well as those who can be spared that experience of chemotherapy. Many studies are currently underway to define patterns of gene expression that predict metastatic disease. Techniques such as microarray and proteomics are being employed to define profiles that indicate that a patient has the potential to develop metastases (23-32). These studies have been useful, but have also prompted controversy as to whether metastasis regulatory genes actually exist (6).

Studies using microarrays have shown that the expression patterns in primary tumors and metastases are similar, leading to the conclusion that there are no genes that specifically control metastasis, but rather, the same genes that control progression of tumor development control metastasis. It is not surprising that the primary tumors and metastases have similar profiles. Tumors are made up of very heterogeneous populations. Therefore, it is likely that some of the cells in the primary tumor would exhibit some of the mutations necessary for metastasis. Microarray studies do not allow examination of gene expression in single cells within the tumor. Therefore, there is currently no way to determine whether all of the mutations necessary for metastasis exist within the same cell. As already mentioned above, a number of genes regulate the process of metastasis. Thus multiple changes in gene expression are necessary before metastasis can occur. Within the heterogeneous primary tumor, cell A may have mutation X, cell B may have mutation Y and cell C may have mutation Z, but the chances of a single cell having all three mutations is improbable. However, all three mutations must be present in the same cell for metastasis to occur.

This was nicely demonstrated in studies by the Massagué and Guise laboratories (33). A sub-population of MDA MB 231 breast cancer cells that efficiently colonize bone (231 bone) were isolated and compared to parental cells by microarray. A number of genes were identified whose expression differed in the 231 bone cells. This profile was called the “bone metastasis signature”. In this profile MMP1, osteopontin, IL-11, a chemokine receptor (CXCR4) and connective tissue-derived growth factor were over-expressed. Transfection of each of these genes individually into the parental 231 cells increased metastasis only modestly, while co-transfection of combinations of these genes caused metastasis as efficiently as the 231 bone sub-population. This study demonstrates that defined gene combinations can control metastasis and underscores the complexity of the metastatic process. The results encourage the study of genes controlling metastasis as potential therapeutic targets.

Many studies have demonstrated alteration or genomic imbalances on a number of chromosomes that are associated with malignant progression and metastasis (24, 26, 34-36). This indicated that these regions of DNA may contain metastasis-associated genes. More specifically, when deletions of regions of DNA correlated with metastasis, metastasis-suppressor genes were likely to be found. To study this possibility a number of different techniques have been employed. Microcell mediated chromosomal transfer or MMCT was one of the first techniques used. This involves the introduction of an intact chromosome into a cell that has a chromosome with deletions. This technique was used to introduce chromosomes 2, 7, 8, 10, 11, 12, 13, 16, 17 and 20 into prostate cancer cells resulting in suppression of metastasis without affecting tumorigenicity (reviewed in (37)). This technique has also been used to introduce chromosome 6 into melanoma cells and chromosome 11 into breast cancer cells (38-40). Using this technique, a number of genes have been identified that suppress metastasis when expressed in metastatic cell lines. Other techniques such as differential display and subtractive hybridization comparing metastatic and non-metastatic cell lines have been used effectively to identify metastasis suppressor genes (41). More recently, microarry and proteomics have been added to the list of techniques used to discover genes involved in metastasis. The number of metastasis suppressor genes is growing rapidly. In the following section, each of the defined metastasis suppressor genes will be discussed.

2. METASTASIS SUPPRESSOR GENES

2.1 Nm23

Nm23 is located on chromosome 17q21 and was the first metastasis suppressor gene identified (42). It is a 17 kDa protein found by using differential display of metastatic and non-metastatic K1735 murine melanoma cell lines. Enforced expression prevented metastasis with no effect on tumorigenicity (reviewed in (43)). The human gene encodes a nucleoside diphosphate kinase (NDPK) `although the kinase activity is not responsible for metastatic suppression (44). NDPKs catalyze the transphosphorylation of the gamma phosphate of a deoxynucleoside diphosphate to a deoxynucleoside triphosphate with the formation of a phosphohistidine intermediate. Nm23 also is a histidine kinase and this activity is responsible for metastatic suppression (45). It forms a complex with kinase suppressor of ras (KSR) which is a scaffold protein for the mitogen activated protein kinases (MAPKs) (46). KSR phosphorylates Nm23 on serine 392 which is within a 14-3-3 binding site. MDA-MB-435 cells transfected with Nm23 demonstrate decreased MAPK phosphorylation, so Nm23 may signal through the ERK/MAPK pathway (47, 48). Nm23 H2 is a transcription promoting factor of the c-myc gene (49). Nm23 also interacts with granzyme A in the process of DNA damage induction in cytotoxic T cell apoptosis (50). This function relates to the NDPK activity and has not been reported in tumor cells.

Nm23 H1 and H2 are metastasis suppressors, while the other six Nm23 family members are not (44). Decreased expression of Nm23 correlates with metastatic potential in most tumor types, but interestingly expression in neuroblastomas correlates with increased aggressiveness indicating that the functions of this protein may be cell type specific (reviewed in (43)). Expression of Nm23 is decreased in late stage metastatic breast, endometrial, ovarian, melanoma and colon cancers (reviewed in (51-54)), while some studies have found no correlation of expression with metastasis. Cells transfected with Nm23 have decreased motility and invasion in vitro as well as decreased soft agar colonization in response to TGFβ. Expression may be controlled by epigenetic mechanisms as increased expression of Nm23 correlates with hypomethylation of the promoter (37, 47). A recent paper from the laboratory of Patricia Steeg demonstrates that Nm23 expression can be restored in metastatic breast cancer cells by treatment with dexamethasone and medroxyprogesterone acetate (55). Treatment with medroxyprogesterone acetate resulted in a 50% decrease in the colonization of metastatic breast cancer cells in soft agar. This study indicates the

possibility of modulating metastasis suppressor gene expression as a therapeutic intervention in the clinic.

2.2 KAI-1/CD82/C33

Kai1 is a member of the tetraspannin superfamily of transmembrane glycoproteins that are found on leukocytes and involved in cell adhesion (56). These proteins influence lymphocyte differentiation and function. The gene is found at 11p11.2, a region that is frequently implicated in breast cancer (57). Enforced expression of Kai1 suppresses metastasis from prostate and breast cancers as well as melanoma (58, 59). Decreased expression correlates inversely with aggressive behavior of breast cancer cell lines (60) and the metastatic potential of a variety of tumor types as determined by immunohistochemistry (37). Decreased expression directly correlates with survival (61) while expression levels are higher in normal breast and DCIS compared to infiltrating breast tumors (62).

Kai1 was identified as a metastasis suppressor by microcell mediated chromosomal transfer of chromosome 11 into AT3.1 and AT6.1 rat prostate cancer cell lines followed by subtractive hybridization of the suppressed cells versus parental cells (63). Increased expression of Kai1 results in an inhibition of invasion and motility of colon cancer cells. This protein acts as an adhesion molecule in leukocytes, but does not influence adhesion of tumor cells. It associates with EGFR to suppress lamellipodia and migration signaling (64). EGFR is down-regulated by ligand-induced receptor endocytosis. Kai1 may suppress metastasis by altering the balance between Kai1 and EGFR which could affect proliferative and migratory signals. Kai1 also associates with the cytoskeleton and promotes phosphorylation and association of vav (guanine exchange factor) with SLP76 (an adapter protein) resulting in de novo actin polymerization and another possible role in cell signaling (65).

Expression of Kai1 is decreased in urogenital, gynecological, prostate, breast, lung and pulmonary tumors and/or cancer cell lines (66). Loss of heterozygosity (LOH) of Kai1 is observed in many cancers. One possible means of down-regulation of Kai1 is promoter methylation, but studies give varying results (67). In bladder tumors, promoter methylation did not result in a change in gene expression (68). Mutations in the promoter region have been observed and could explain the down regulation of Kai1 in tumors. The promoter region of Kai1 interacts with and is activated by p53 and loss of Kai1 corresponds strongly to loss of p53, but in prostate cells, normal induction of p53 does not result in increased expression of Kai1 (69, 70). Therefore, over-expression of p53 may be required to affect Kai1 expression.

2.3 KISS1

KISS1 was identified using subtractive hybridization techniques following MMCT of chromosome 6 into human melanoma cells (71, 72). The gene maps to 1q32 indicating that it is a downstream effector of a gene on chromosome 6 (72). A deletion variant of chromosome 6 (neo6qdel16.3-q32) did not suppress metastasis when put into cells and those cells did not express KISS1 (72, 73). Loss of KISS1 in cancer cells correlates with LOH on the long arm of chromosome six corresponding to 6q16.3-q23 (74). Therefore, a regulator of KISS1 is located at 6q16.3-q23. Enforced expression of KISS1 suppressed metastasis of melanoma and breast cancer cells (75). In situ hybridization was used to demonstrate an 80% correlation between KISS1 loss and melanoma metastatic progression (74). In HT1080 cells, increased KISS1 resulted in decreased MMP-9 expression through decreased NFκB activation (76).

The short half life of this protein (<30 seconds) makes study difficult, but three groups have demonstrated the existence of a 54 amino acid amidated fragment of KISS1 termed metastin or kisspeptin (77, 78). Metastin binds to GPR54, a G - protein coupled receptor, to induce activation of phospholipase C, hydrolysis of PIP_2 as well as calcium and arachidonate release (77-81). This signaling cascade has been shown to affect focal adhesion kinases indicating a role for KISS1 in adhesion (78). Exogenous treatment with metastin of GPR54 transfected B16BL6 melanoma cells reduced metastasis and anchorage independent growth (78), but activity of the endogenous receptor has not been demonstrated in cancer cells. Recently, studies in

transgenic mice have implicated GPR54 activity in pubertal development and pregnancy, but this gives little definitive indication of the role of KISS1 in metastasis. KISS1 expression is high in early placenta and molar pregnancies and is low in choriocarcinoma cells indicating a role for KISS1 in the invasive and migratory properties of trophoblasts (82). KISS1 has recently been shown to form a stable complex with pro-MMPs. MMP-2,- 9, -14, -16 and -24 can cleave both KISS1 and metastin (83). Cleavage of metastin results in a decapeptide that can induce formation of focal adhesion and stress fibers in cells expressing GPR54 (83). Treatment of HT1080 cells with the metastin decapeptide and an MMP inhibitor (BB-94) resulted in a significant block in cell migration (83). This same decapeptide has been shown to inhibit trophoblast migration and proteolytic activity in vitro without affecting proliferation (84). These studies demonstrate the possibility that the cleavage products of KISS1 may be important for the suppression of metastasis however, the role has yet to be definitively established.

2.4 TXNIP/VDUP1

This protein functions upstream of KISS1 and was identified by comparing paired microarrays of metastatic and non-metastatic C8161 cells (32). The gene with the greatest differential expression was VDUP1 or vitamin D3 up-regulated protein 1, which was first found in HeLa cells treated with 1,25 dihydroxyvitamin D3. VDUP1 interacts with reduced thioredoxin (TRN) to inhibit both function and expression and was thus renamed TRN interacting protein or TXNIP (85, 86). Thioredoxin regulates MAPK signaling through suppression of ASK1 (apoptosis signal-regulating kinase) activation, and activates transcription factors to regulate stress-activated apoptosis (87, 88). This gene is encoded on chromosome 1. Increased TXNIP expression results in decreased thioredoxin and therefore cell growth arrest (89). A trend has been identified in which increased TRN correlates with increased tumor growth indicating that TXNIP may be a tumor suppressor gene.

2.5 CRSP3

CRSP3 is a cofactor necessary for SP1-mediated transcription. SP1 or specificity protein 1 binds to and acts through GC boxes (90, 91). The promoters of both KISS1 and TXNIP contain SP1 elements implicating CRSP3 as an upstream regulator of these proteins. CRSP3 maps to the distal end of the minimal region identified in the 6q16.3-q23 deletion variant as containing a metastasis suppressor. LOH of this region is common in melanoma.

Transfection into melanoma cells resulted in an increase in KISS1 and TXNIP and concomitant inhibition of metastasis without affect tumorigenicity. The expression of CRSP3 inversely correlates with the metastatic progression of melanoma. Therefore, the first metastatic pathway has been identified: CRSP3➔TXNIP➔KISS1 (32).

2.6 TIMPs

Tissue inhibitors of metalloproteinases or TIMPs are secreted proteins that selectively inhibit MMPs with a 1:1 stoichiometry (8, 92, 93). The TIMPs are critical for control of extravasation and tumor induced angiogenesis because each of these steps involves breakdown of the basement membrane. These proteins can inhibit tumorigenesis or metastasis depending on the cell type. In breast cancer, they are metastasis suppressors (94). The TIMPs are expressed in many tumor tissues and are found in the sera of cancer patients indicating that serum levels may be useful as a predictive tool (95-98). Their role in tumorigenicity and metastasis is hard to elucidate because they have many different effects. TIMP-1, -2 and -4 are anti-apoptotic, while TIMP-3 induces apoptosis. Also, TIMP-2 binds to MT1-MMP to bind and activate proMMP-2 (reviewed in (92)). It is believed that the suppression of metastasis by TIMPs is through inhibition of MMPs resulting in decreased invasion of tumor cells. Some mouse studies have indicated roles for TIMPs in other steps of the metastatic cascade (reviewed in (99)). Increased TIMP expression is associated with progression to metastasis in some studies. In one example, increased TIMP-1 correlated with the presence of lymph node metastases, while increased TIMP-2 correlated with

the occurrence of distant metastases. The balance between MMPs and TIMPs is critical and the imbalance may promote metastasis. It is also possible that the TIMPs have other functions, for example, TIMP-1 can stimulate growth in some cell lines (100). Finally, the increased expression of TIMP found in invasive tumors could be due to the increased expression of MMPs and there is no functional correlation between increased TIMP expression and tumor or metastatic progression.

2.7 Cadherins

Cadherins are calcium-dependent transmembrane glycoproteins found on the surface of epithelial cells. E-cadherin is made as a precursor protein of 135 kDa that is processed to a mature form of 120 kDa. It mediates calcium dependent homophillic cell-cell interactions through its extracellular domain. The cytoplasmic domain interacts with the actin cytoskeleton and α, β and γ catenins (101). Binding of E-cadherin to β-catenin blocks β-catenin nuclear translocation and therefore, transcription of c-myc and cyclin D1.

E-cadherin maps to 16q22.1 an area of the genome that often has LOH associated with late stage breast cancer (102). LOH of this gene correlates with shortened disease free survival, poor prognosis and metastasis (103). Methylation is one means of LOH (104). In one study, methylation of the promoter was found in 11/35 pre-invasive DCIS cases, 19/37 invasive lesions and 7/16 metastatic ductal carcinomas. Other means of LOH include redistribution within the cell, shedding of E-cadherin and competition from other proteins (reviewed in (105)). For example, EGFR stimulation with EGF or TGFβ results in dissociation of β-catenin and E-cadherin (106, 107). In most tumor types, mutation of the E-cadherin gene is rare, but breast and gastric cancer have a 50% mutation rate (102).

E-cadherin can be a tumor suppressor in some systems (108-111), but over-expression of E-cadherin decreases tumor cell motility, invasion and shedding from the primary tumor (110, 112). N-cadherin, which is high in osteoblasts, and cadherin 11 reduce metastasis to lung without changes in tumorigenicity (113). These two proteins are often over-expressed in metastatic breast and prostate cancer cells (114-116). Interestingly, over-expression results in induction of invasion and metastasis of breast cancer and melanoma cells (114, 117, 118).

2.8 MKK4/JNKK/SEK1

MKK4 was identified as a metastasis suppressor gene using chromosome 17 microcell hybrids. It is a member of the mitogen activated protein kinase pathway downstream of MEKK1 and upstream of activation of SAPK/JNK1 and $p38^{mapk}$ (119). This signaling pathway mediates proliferation, apoptosis and differentiation depending on the cellular context. Cancer cell lines that exhibit MEKK1 signaling defects were found to have alterations or deletions of portions of the MKK4 gene (120).

Enforced expression suppresses metastasis of prostate and ovarian cancer cells (121, 122). Expression of MKK4 is down-regulated in clinical ovarian cancer metastases and cell lines. MKK4 does not affect invasion or angiogenesis, and it has been shown that the suppressed cells complete every step of the metastatic cascade except growth at the secondary site (123, 124). There is an inverse correlation between Gleason Score in prostate cancer and MKK4 staining (125).

2.9 BRMS1

The Breast Metastasis Suppressor, BRMS1, was identified using differential display after MMCT of chromosome 11 into a breast cancer cell line (126). It is located at 11q13.1-q13.2, a region that is frequently altered in breast cancer metastasis. Enforced expression suppressed metastasis from breast cancer, bladder carcinoma and melanoma in vivo (126-132), but it does not suppress growth in vitro or in vivo, adhesion to laminin, fibronectin, collagens I and IV or matrigel, expression of gelatinases (MMP2 and 9) or heparanase, or invasion in vitro (128). Motility and growth in soft agar were moderately inhibited in BRMS1 transfectants (128).

Transfection of BRMS1 into MDA-MB-435 breast cancer cells results in the restoration of gap junctional intercellular communication with a concomitant increase in connexin 43 and decrease in

connexin 32 expression (127, 128, 130). This expression pattern of connexin 43 and 32 is equivalent to that of normal breast (133-138).

BRMS1 is found in the nucleus and has a glutamate rich region, an imperfect leucine zipper and 2 coiled-coil domains indicating that it has a role in a transcription complex. Yeast 2 hybrid and co-immunoprecipitation studies have demonstrated that it interacts with mSin3:HDAC complexes (139). Therefore, BRMS1 may control metastasis suppression by regulating gene expression.

2.10 SseCKS

Src-suppressed C kinase substrate or SseCKS is the mouse ortholog of human Gravin/KAP12. This protein functions as a cytoplasmic scaffold for protein kinase A and C (140). It is concentrated at the edge of cells and in podosomes. Expression levels of this gene are decreased in src and ras transformed rodent fibroblasts (141, 142). It is also low in metastatic prostate cancer cell lines. Enforced expression resulted in decreased lung metastasis in vivo and increased formation of filopodia-like projections and decreased anchorage independent growth in vitro (143). Over-expression suppressed v-src induced morphological transformation and tumorigenesis, possibly by reducing ERK activity (144).

2.11 RhoGDI2

Rho GDP dissociation inhibitors or RhoGDI work to stabilize and sequester in a cytoplasmic compartment the GDP bound form of Rho GTPases. Rho GTPases are guanine nucleotide binding proteins that cycle between an active GTP-bound and an inactive GDP-bound state. Increased RNA expression of RhoGDI2 correlates indirectly with decreased metastatic potential. Gene expression profiling in 105 bladder cancers demonstrated an inverse correlation between RhoDGI2 expression and invasive phenotype (145) and enforced expression in T24 human bladder carcinoma cells suppressed metastasis (146).

2.12 Drg-1/RTP/Cap43/rit43

Drg-1 is a differentiation associated gene in colon carcinomas. It was identified as a metastasis suppressor gene by differential display (41). When re-expressed in colorectal cancers Drg-1 suppressed in vitro invasion and liver metastasis in vivo (147). Metastases are also suppressed in prostate cancer cell lines transfected with Drg-1 and expression decreases with increasing Gleason Score in prostate cancer (148). In human bladder carcinoma it acts as a tumor suppressor (149).

While the function is unknown, Drg-1 may be acting downstream of MKK4 as it is induced similarly to JNK/SAPK (150). Supporting a role for this protein in cell signaling is data indicating that it is up-regulated by PTEN and p53 and is phosphorylated by protein kinase A (151).

2.13 RKIP

Raf kinase inhibitory protein or RKIP is a phosphatidylethanolamine binding protein that is a specific inhibitor of Raf binding to MEK. Binding of Raf to RKIP results in suppression of Raf1-induced transformation and AP-1 dependent transcription. RKIP can be phosphorylated by PKC resulting in release of RKIP from Raf1 (152). RKIP also modulates the activity of NFκB by antagonizing the activation of IκB kinase in response to TNFα and IL-1β (153).

Enforced expression of RKIP inhibits metastasis of prostate cancer cells without affecting tumorigenicity (154). There is an inverse correlation of expression of RKIP with stage of prostate cancer progression and Gleason score. Expression of RKIP also is associated with suppression of invasion and angiogenesis (154).

2.14 Potential Metastasis Suppressors

There is a growing list of genes that potentially can suppress metastasis. Most of the data supporting these genes as suppressors are correlative and, in some cases, the suppression of metastasis occurs along with decreased tumorigenicity. While all of the necessary studies needed to demonstrate that these genes meet the definition of metastasis

suppressors have not been completed, descriptions of these genes are included below.

Semaphorins are secreted and membrane bound protein involved in the collapse process of axons that have extended growth cones in several directions in response to environmental and growth cues (155, 156). **CRMP-1**, or collapsin response mediator protein 1, is a member of a family of proteins implicated in controlling cell movement (reviewed in (157)). CRMP-1 expression is inversely correlated with lung carcinoma progression and directly correlated to survival and time to relapse. CRMP-1 also has been shown to reduce the invasion of lung cancer cells in vitro (30). **Gelsolin** suppresses both tumorigenicity and metastasis of melanoma as well as bladder and lung cancer (158-160). It regulates motility by its involvement in actin assembly and disassembly. Gelsolin expression decreases soft agar colonization and chemotaxis to fibronectin in vitro. **Maspin** was identified by differential display RTPCR comparing normal mammary epithelial cells and mammary cancer cells. It is a serine protease inhibitor that sensitizes cells to apoptosis (161) and can inhibit angiogenesis (162). Similar to some metastasis suppressor genes, maspin expression is inversely correlated with methylation of its promoter (163). **HP1$^{HS\alpha}$** or heterochromatin-associated protein 1 is localized to the nucleus in non-metastatic cells, but its expression is decreased in highly invasive metastatic cells. Clinical correlations indicate that HP1$^{HS\alpha}$ may be a metastasis suppressor gene for breast cancer (164). **CD44** has been shown to suppress metastasis in AT3.1 prostate carcinoma cells without affecting tumorigenicity (165). The story becomes more complex because CD44 exists as multiple isoforms and, while the standard isoform (CD44-s) can reduce lung metastases by >60%, it is not clear which of the isoforms are relevant for cancer and metastasis. **SHP-2** is a cytoplasmic tyrosine phosphatase that is involved in signal transduction downstream of growth factor receptors. It has been shown that expression of SHP-2 results in decreased migration and spreading of fibroblasts on fibronectin in vitro (166). Expression of a mutant form of SHP-2 results in reduced focal adhesion kinase de-phosphorylation and decreased association with paxillin. **Csk** or C-terminal Src kinase is a cytoplasmic tyrosine kinase that phosphorylates and negatively regulates the Src family kinases. Over-expression of Csk results in decreased cell adhesion, migration and invasion in vitro through decreased focal contacts and increased cell-cell contacts (167). Over-expression of Csk also can suppress metastasis of colon cancer cells (168). **Claudin-4** is a transmembrane protein involved in the formation of tight junctions. Enforced expression in pancreatic cancer cells increases cell/cell adhesion and decreases colony formation and invasion in vitro. Expression of claudin-4 inversely correlates with malignant progression. In experimental metastasis assays, expression of claudin-4 decreases lung colonization, but the effect on primary tumor growth is unknown (169). Finally, there are a large number of genes that are likely metastasis suppressors, but are not yet published and therefore, are not included here. This indicates that the study of metastasis suppression is a rapidly growing field that will provide important insights into the progression of and, ultimately, the cure for cancer.

3. CONCLUSIONS

Metastasis is the critical clinical step in tumor progression. As patients are diagnosed earlier due to improved detection methods, survival rates increase. When diagnosis occurs before the establishment of metastases the prognosis is better and treatments are more effective. Unfortunately, it is not always possible to detect the spread of cancer at the time of diagnosis. In the case of breast cancer, approximately one quarter of patients diagnosed with node-negative cancer develop metastases. Therefore, it is important to develop a means to identify patients in whom the cancer is likely to spread. To do this, it is necessary to understand the genetic and biochemical mechanisms that underlie metastasis.

While those who study metastasis suppressor genes hope that they can be used to predict the spread of cancer and be targets for therapeutics, it is not yet known what role metastasis suppressors will play. It is clear, though, that understanding the role of metastasis suppressor genes in the development of secondary masses will provide insight in to the mechanisms that control cancer spread. Already,

studies demonstrating the mechanisms of action of some of the metastasis suppressor genes have raised questions about long held beliefs as to how metastasis occurs. The genes described in this review have been identified by several different laboratories using different model systems. They have been studied using a variety of experimental systems and the amount of information as to the mechanism of action varies with each, but pathways are beginning to emerge that connect previously independent metastasis suppressors and some common elements are becoming clearer.

First, metastasis suppressors are found in every cellular compartment. This is similar to genes that control cell cycle, apoptosis and differentiation. Many of the metastasis suppressors are involved in processes such as signal transduction and, as such, function to amplify signals. This is necessary for controlling complex, multigenic phenotypes such as metastasis. Pathways involving the metastasis suppressors are beginning to emerge and should help to guide the field toward a better understanding of the molecular functions of the metastasis suppressor genes. Second, many of the metastasis suppressors block metastasis in multiple cancer types indicating that there are common pathways that control metastasis in different tumor types. Third, the cellular context in which the gene is expressed is important. A growing number of the metastasis suppressors affect tumorigenicity in some cell types and metastasis in others. This demonstrates the complexity of the processes of tumorigenicity and metastasis. Fourth, many of the metastasis suppressors function by blocking growth at the secondary site (123, 124, 170). This also supports the idea that these genes act according to their cellular context or microenvironment. The tumor cells can grow at the site of the primary tumor, but not at the secondary site indicating that something outside of the tumor cell is involved in controlling its growth. This further complicates the study of metastasis because it suggests that genes involved in metastasis may control metastasis to certain sites but not others. Finally, despite the strict definition of metastasis suppression the number of metastasis suppressor genes is increasing. There is no way to know how many metastasis suppressor genes exist, but it is likely that the number is limited when examining the basic pathways. When the downstream signals are included the process becomes quite complex.

There are two areas of study that are particularly important for understanding metastasis and its potential focus as a clinical target. First, how does the microenvironment affect metastasis? And, second, what are the mechanisms that result in loss of expression of the metastasis suppressors?

Observations from many laboratories support the idea that microenvironment is important in the function of metastasis suppressor genes. For example, a number of the metastasis suppressor genes have been shown to have no effect on the early stages of the metastatic cascade, but rather to suppress proliferation at the secondary site (123, 124, 170). It has also been shown that single breast cancer cells removed from a secondary site can form a tumor when injected into a mammary fat pad, but when they metastasize again they do not form secondary masses. Further, preliminary data from our lab indicates that some metastasis suppressor genes can suppress metastasis to some organs, but not to others (J.F. Harms and D.R. Welch, unpublished). All of these data strongly support the idea that the cellular context is extremely important for control of metastasis.

Understanding the role of the microenvironment will help to define which patients can be expected to have metastatic disease and to what organs. If we can understand why tumor cells choose the particular secondary site that they do, what cues they are given to allow them to grow, and why some tumor cells remain dormant while others go on to form secondary masses, we can begin to target these steps of metastasis with therapeutic intervention.

Loss of expression of metastasis suppressor genes is not simply due to mutation. Many studies indicate that the metastasis suppressor genes are differentially expressed rather than mutated (reviewed in (171)). There are several possible explanations for how expression can be regulated to result in loss of expression in the metastatic cells including decreased protein translation (172, 173), methylation of the promoter (174, 175), acetylation of histones (174, 176-178) and decreased mRNA or protein stability (179, 180). In each of these cases, it may be possible to force re-expression of these

genes in the clinical setting. An example of this was described for Nm23 (181). Treatment of metastatic breast cancer cell lines with either dexamethasone or medroxyprogesterone acetate results in an enhancement of Nm23 expression. The same laboratory had demonstrated earlier that treatment with 5-azacytidine results in hypomethylation of the Nm23 promoter and a restoration of Nm23 expression (47). While similar studies have not been undertaken for other metastasis suppressor genes that are regulated by epigenetic changes, these data strongly suggest the possibility that metastasis suppressor genes could be regulated by pharmacological means resulting in inhibition of metastasis, decreased morbidity and increased survival rates.

ACKNOWLEDGMENTS

We would like to thank members of the laboratory for their assistance. For work described from our lab, we gratefully acknowledge the generous support of the U.S. Public Health Service, National Cancer Institute (CA87728, CA62168, CA89109, CA101955), the U.S. Army Medical Research and Materiel Command (DAMD17-02-1-0541, DAMD17-01-1-0362, DAMD-17-96-1-6152), and the National Foundation for Cancer Research.

REFERENCES

1. Lee, K., Jessop, H., Suswillo, R., Zaman, G., Lanyon, L., 2003, Endocrinology: bone adaptation requires oestrogen receptor-alpha. Nature, 424:389.
2. Steeg, P.S., 2003, Metastasis suppressors alter the signal transduction of cancer cells. Nature Rev Cancer, 3:55-63.
3. Welch, D.R., 1985, Tomasovic SP. Implications of tumor progression on clinical oncology. Clin Exptl Metastasis, 3:151-188.
4. Nowell, P., 1976, The clonal evolution of tumor cell populations. Science, 194:23-28.
5. Bernards, R., Weinberg, R.A., 2002, Metastasis genes: A progression puzzle. Nature, 418:823.
6. Hahn, W.C., and Weinberg, R.A., 2002, Rules for making human tumor cells. N Engl J Med, 347:1593-1603.
7. Coussens, L.M., and Werb, Z., 2002, Inflammation and cancer. Nature, 420:860-867.
8. Egeblad, M., and Werb, Z., 2002, New functions for the matrix metalloproteinases in cancer progression. Nature Rev Cancer, 2:161-174.
9. Paget, S., 1889, The distribution of secondary growths in cancer of the breast. Lancet, 1:571-573.
10. Chambers, A.F., Groom, A.C., and MacDonald, I.C., 2002, Metastasis: Dissemination and growth of cancer cells in metastatic sites. Nature Rev Cancer, 2:563-572.
11. Chambers, A. F., MacDonald, I.C., Schmidt, E. E. et al., 1995, Steps in tumor metastasis: New concepts from intravital videomicroscopy. Cancer Metastasis Rev, 14:279-301.
12. Jain, R.K., Munn, L.L., Fukumura, D., 2002, Dissecting tumour pathophysiology using intravital microscopy. Nature Rev Cancer, 2:266-276.
13. Weiss, L., 2000, Concepts of metastasis. Cancer Metastasis Rev, 19:219-234.
14. Weiss, L., 1990, Metastatic inefficiency. Adv Cancer Res, 54:159-211.
15. Fidler, I.J., 1973, Selection of successive tumor lines for metastasis. Nature New Biol, 242:148-149.
16. Shevde, L.A., Welch, D.R., 2003, Metastasis suppressor pathways - an evolving paradigm. Cancer Lett, 198:1-20.
17. Cameron, M.D., Schmid,t E.E., Kerkvliet, N., et al., 2000, Temporal progression of metastasis in lung: Cell survival, dormancy, and location dependence of metastatic inefficiency. Cancer Res, 60:2541-2546.
18. Luzzi, K.J., MacDonald, I.C., Schmidt, E.E., et al., 1998, Multistep nature of metastatic inefficiency - Dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. Am J Pathol, 153:865-873.
19. Wong, C.W., Lee, A, Shientag, L., et al., 2001, Apoptosis: An early event in metastatic inefficiency. Cancer Res, 61:333-338.
20. Holmgren, L., O'Reilly, M.S., Folkman, J., 1995, Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. Nature Med, 1:149-153.
21. Cameron, M.D., Schmidt, E.E., Kerkvliet, N., et al., 2000, Temporal progression of metastasis in lung: Cell survival, dormancy, and location dependence of metastatic inefficiency. Cancer Res, 60:2541-2546.
22. Luzzi, K.J., MacDonald, I.C., Schmidt, E.E. et al., 1998, Multistep nature of metastatic inefficiency - Dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. Am J Pathol, 153:865-873.
23. Ramaswamy, S., Ross, K.N., Lander, E.S., Golub, T.R., 2003, A molecular signature of metastasis in primary solid tumors. Nat Genet, 33:49-54.
24. Nishizaki, T., Devries, S., Chew, K., et al., 1997, Genetic alterations in primary breast cancers and

their metastases - direct comparison using modified comparative genomic hybridization. Genes Chromosomes Cancer, 19:267-272.
25. Nakao, K., Shibusawa, M., Ishihara, A., et al., 2001, Genetic changes in colorectal carcinoma tumors with liver metastases analyzed by comparative genomic hybridization and DNA ploidy. Cancer, 91:721-726.
26. Redon, R., Muller, D., Caulee, K., et al., 2001, A simple specific pattern of chromosomal aberrations at early stages of head and neck squamous cell carcinomas: PIK3CA but not p63 gene as a likely target of 3q26-qter gains. Cancer Res, 61:4122-4129.
27. Wu, W.G., Tang, X.M., Hu, W., et al., 2002, Identification and validation of metastasis-associated proteins in head and neck cancer cell lines by two-dimensional electrophoresis and mass spectrometry. Clin Exptl Metastasis, 19:319-326.
28. Nakayama, T., Taback, B., Turner, R., Morton, D.L., Hoon, D.S.B., 2001, Molecular clonality of in-transit melanoma metastasis. Am J Pathol, 158:1371-1378.
29. Massi, D., Sardi, I., Urso, C., et al., 2002, Microsatellite analysis in cutaneous malignant melanoma. Melanoma Res, 12:577-584.
30. Shih, J.Y., Yang, S.C., Hong, T.M., et al., 2001, Collapsin response mediator protein-1 and the invasion and metastasis of cancer cells. J Natl Cancer Inst, 93:1392-1400.
31. Reifenberger, J., Knobbe, C.B., Wolter, M., et al., 2002, Molecular genetic analysis of malignant melanomas for aberrations of the Wnt signaling pathway genes CTNNB1, APC, ICAT and BTRC. Int J Cancer, 100:549-556.
32. Goldberg, S.F., Miele, M.E., Hatta, N., et al., 2003, Melanoma metastasis suppression by chromosome 6: Evidence for a pathway regulated by CRSP3 and TXNIP. Cancer Res, 63.
33. Kang, Y.B., Siegel, P.M., Shu, W.P., et al., 2003, A multigenic program mediating breast cancer metastasis to bone. Cancer Cell, 3:537-549.
34. Nishizaki, T., Chew, K., Chu, L., et al., 1997, Genetic alterations in lobular breast cancer by comparative genomic hybridization. Int J Cancer, 74:513-517.
35. Welch, D.R., and Wei, L.L., 1998, Genetic and epigenetic regulation of human breast cancer progression and metastasis. Endocrine-related Cancer, 5:155-197.
36. Welch, D.R., and Goldberg, S.F., 1997, Molecular mechanisms controlling human melanoma progression and metastasis. Pathobiol, 65:311-330.
37. Kauffman, E.C., Robinson, V.L., Stadler, W.M., Sokoloff, M.H., and Rinker-Schaeffer, C.W., 2003, Metastasis suppression: the evolving role of metastasis suppressor genes for regulating cancer cell growth at the secondary site. J Urol, 169:1122-1133.
38. Miele, M.E., de la Rosa, A., Lee, J.H. et al., 1997, Suppression of human melanoma metastasis following introduction of chromosome 6 is independent of NME1 (Nm23). Clin Exptl Metastasis, 15:259-265.
39. Phillips, K.K., Welch, D.R., Miele, M.E., et al., 1996, Suppression of MDA-MB-435 breast carcinoma cell metastasis following the introduction of human chromosome 11. Cancer Res, 56:1222-1226.
40. Welch, D.R., Chen, P., Miele, M.E., et al., 1994, Microcell-mediated transfer of chromosome 6 into metastatic human C8161 melanoma cells suppresses metastasis but does not inhibit tumorigenicity. Oncogene, 9:255-262.
41. van Belzen, N., Dinjens, W.N., Diesveld, M.P., et al., 1997, A novel gene which is up-regulated during colon epithelial cell differentiation and down-regulated in colorectal neoplasms. Lab Invest, 77:85-92.
42. Steeg, P.S., Bevilacqua, G., Kopper, L., et al., 1988, Evidence for a novel gene associated with low tumor metastatic potential. J Natl Cancer Inst, 80:200-204.
43. Salerno, M., Ouatas, T., Palmieri, D., and Steeg, P.S., 2003, Inhibition of signal transduction by the nm23 metastasis suppressor: Possible mechanisms. Clin Exptl Metastasis, 20:3-10.
44. Otsuki, Y., Tanaka, M., Yoshii, S., et al., 2001, Tumor metastasis suppressor nm23H1 regulates Rac1 GTPase by interaction with Tiam1. Proc Natl Acad Sci, 98:4385-4390.
45. Wagner, P.D., Steeg, P.S., and Vu, N.D., 1997, Two-component kinase-like activity of nm23 correlates with its motility-suppressing activity. Proc Natl Acad Sci, 94:9000-9005.
46. Hartsough, M.T., Morrison, D.K., Salerno, M., et al., 2002, Nm23-H1 metastasis suppressor phosphorylation of kinase suppressor of ras via a histidine protein kinase pathway. J Biol Chem, 277:32389-32399.
47. Hartsough, M.T., Clare, S.E., Mair, M., et al., 2001, Elevation of breast carcinoma Nm23-H1 metastasis suppressor gene expression and reduced motility by DNA methylation inhibition. Cancer Res , 61:2320-2327.
48. Steeg, P.S., Palmieri, D., Ouatas, T., and Salerno, M., 2003, Histidine kinases and histidine phosphorylated proteins in mammalian cell biology, signal transduction and cancer. Cancer Lett, 190:1-12.
49. Postel, E.H., Berberich, S.J., Flint, S.J., and Ferrone, C.A., 1993, Human c-myc transcription factor PuF identified as nm23-H2 nucleoside diphosphate kinase, a candidate suppressor of tumor metastasis. Science, 261:478-480.

50. Fan, Z., Beresford, P.J., Oh, D.Y., Zhang, D., and Lieberman, J., 2003, Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. Cell, 112:659-672.
51. Freije, J.M., MacDonald, N.J., and Steeg, P.S., 1998, Nm23 and tumour metastasis: basic and translational advances. Biochem Soc Symp, 63:261-271.
52. Lombardi, D., Lacombe, M.L., and Paggi, M.G., 2000, nm23: Unraveling its biological function in cell differentiation. J Cell Physiol, 182:144-149.
53. Luu, H.H., Zagaja, G.P., Dubauskas, Z., et al., 1998, Identification of a novel metastasis-suppressor region on human chromosome 12. Cancer Res, 58:3561-3565.
54. Rinker-Schaeffer, C.W., Welch, D.R., and Sokoloff, M., 2001, Defining the biologic role of genes that regulate prostate cancer metastasis. Curr Opin Urol, 10:397-401.
55. Ouatas, T., Halverson, D., and Steeg, P.S., 2003, Dexamethasone and medroxypro-gesterone acetate elevate Nm23-H1 metastasis suppressor expression in metastatic human breast carcinoma cells via glucocorticoid receptor-dependent, transcriptional and post-transcriptional mechanisms: New uses for old compounds. Clin Cancer Res, 9:3763-3772.
56. Quinn, C.C., Gray, G.E., and Hockfield, S., 1999, A family of proteins implicated in axon guidance and outgrowth. J Neurobiol, 41:158-164.
57. Dong, J.T., Lamb, P.W., Rinker-Schaeffer, C.W., et al., 1995, KAI1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2. Science, 268:884-886.
58. Phillips, K.K., White, A.E., Hicks, D.J., et al., 1998, Correlation between reduction of metastasis in the MDA-MB-435 model system and increased expression of the Kai-1 protein. Molec Carcinog, 21:111-120.
59. Takaoka, A., Hinoda, Y., Sato, S., et al., 1998, Reduced invasive and metastatic potentials of KAI1-transfected melanoma cells. Jpn J Cancer Res, 89:397-404.
60. Yang, X.H., Welch, D.R., Phillips, K.K., Weissman, B.E., and Wei, L.L., 1997, KAI1, a putative marker for metastatic potential in human breast cancer. Cancer Lett, 119:149-155.
61. Huang, C.I., Kohno, N., Ogawa, E., et al., 1998, Correlation of reduction in MRP-1/CD9 and KAI1/CD82 expression with recurrences in breast cancer patients. Am J Pathol, 153:973-983.
62. Yang, X., Wei, L., Tang, C., et al., 2000, KAI1 protein is down-regulated during the progression of human breast cancer. Clin Cancer Res, 6:3424-3429.
63. Ichikawa, T., Ichikawa, Y., Dong, J., et al., 1992, Localization of metastasis suppressor gene(s) for prostatic cancer to the short arm of human chromosome 11. Cancer Res, 52:3486-3490.
64. Odintsova, E., Sugiura, T., and Berditchevski, F., 2000, Attenuation of EGF receptor signaling by a metastasis suppressor, the tetraspanin CD82/KAI-1. Curr Biol, 10:1009-1012.
65. Delaguillaumie, A., Lagaudriere-Gesbert, C., Popoff, M.R., and Conjeaud, H., 2002, Rho GTPases link cytoskeletal rearrangements and activation processes induced via the tetraspanin CD82 in T lymphocytes. J Cell Sci, 115:433-443.
66. Lynch, H.T., Casey, M.J., Lynch, J., White, T..EK., and Godwin, A.K., 1998, Genetics and ovarian carcinoma. Sem Oncol, 25:265-280.
67. Sekita, N., Suzuki, H., Ichikawa, T., et al., 2001, Epigenetic regulation of the KAI1 metastasis suppressor gene in human prostate cancer cell lines. Jpn J Cancer Res, 92:947-951.
68. Jackson, P., Millar, D., Kingsley, E., et al., 2000, Methylation of a CpG island within the promoter region of the KAI1 metastasis suppressor gene is not responsible for down-regulation of KAI1 expression in invasive cancers or cancer cell lines. Cancer Lett, 157:169-176.
69. Duriez, C., Falette, N., Cortes, U., Moyret-Lalle, C., and Puisieux, A., 2000, Absence of p53-dependent induction of the metastatic suppressor KAI1 gene after DNA damage. Oncogene, 19:2461-2464.
70. Mashimo, T., Watabe, M., Hirota, S., et al., 1998, The expression of the KAI1 gene, a tumor metastasis suppressor, is directly activated by p53. Proc Natl Acad Sci, 95:11307-11311.
71. Lee, J.-H., and Welch, D.R., 1997, Identification of highly expressed genes in metastasis-suppressed chromosome 6/human malignant melanoma hybrid cells using subtractive hybridization and differential display. Int J Cancer, 71:1035-1044.
72. Lee, J.-H., Miele, M.E., Hicks, D.J., et al., 1996, KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. J Natl Cancer Inst, 88:1731-1737.
73. Goldberg, S.F., Miele, M.E., Paquette, C.A., and Welch, D.R., 2001, Identifying metastasis suppressor genes in human melanoma. Anticancer Research.
74. Shirasaki, F., Takata, M., Hatta, N., and Takehara, K., 2001, Loss of expression of the metastasis suppressor gene KiSS1 during melanoma progression and its association with LOH of chromosome 6q16.3-q23. Cancer Res, 61:7422-7425.
75. Lee, J.-H., and Welch, D.R., 1997, Suppression of metastasis in human breast carcinoma MDA-MB-435 cells after transfection with the metastasis suppressor gene, KiSS-1. Cancer Res, 57:2384-2387.
76. Yan, C.H., Wang, H., and Boyd, D.D., 2001, KiSS-1 represses 92-kDa type IV collagenase expression by down- regulating NFκB binding to the promoter as a

consequence of IκBα-induced block of p65/p50 nuclear translocation. J Biol Chem, 276:1164-1172.
77. Kotani, M., Detheux, M., Vandenbogaerde , A., et al., 2001, The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. J Biol Chem, 276:34631-34636.
78. Ohtaki, T., Shintani, Y., Honda, S., et al., 2001, Metastasis suppressor gene KiSS1 encodes peptide ligand of a G-protein-coupled receptor. Nature, 411:613-617.
79. Ringel, M.D., Hardy, E., Bernet, V.J., et al., 2002, Metastin receptor Is overexpressed in papillary thyroid cancer and activates MAP Kinase in thyroid cancer cells. J Clin Endocrin Metab, 87:2399.
80. Stafford, L.J., Xia, C.Z., Ma, W.B., Cai, Y., and Liu, M.Y., 2002, Identification and characterization of mouse metastasis-suppressor KiSS1 and its G-protein-coupled receptor. Cancer Res, 62:5399-5404.
81. Muir, A.I., Chamberlain, L., Elshourbagy, N.A., et al., 2001, AXOR12: A novel human G protein-coupled receptor, activated by the peptide KiSS-1. J Biol Chem, 276:28969-28975.
82. Janneau, J.L., Maldonado-Estrada, J., Tachdjian, G., et al., 2002, Transcriptional expression of genes involved in cell invasion and migration by normal and tumoral trophoblast cells. J Clin Endocrin Metab, 87:5336-5339.
83. Takino, T., Koshikawa, N., Miyamori, H., et al., 2003, Cleavage of metastasis suppressor gene product KiSS-1 protein/metastin by matrix metalloproteinases. Oncogene, 22:4617-4626.
84. Bilban, M., Ghaffari-Tabrizi, N., Hintermann, E., et al., 2004, Kisspeptin-10, a KiSS-1/metastin-derived decapeptide, is a physiological invasion inhibitor of primary human trophoblasts. J Cell Sci, 117:1319-1328.
85. Nishiyama, A., Matsui, M., Iwata, S., et al., 1999, Identification of thioredoxin-binding protein-2/vitamin D(3) up- regulated protein 1 as a negative regulator of thioredoxin function and expression. J Biol Chem, 274:21645-21650.
86. Yamanaka, H., Maehira, F., Oshiro, M., et al., 2000, A possible interaction of thioredoxin with VDUP1 in HeLa cells detected in a yeast two-hybrid system. Biochem Biophys Res Comm, 271:796-800.
87. Saitoh, T., Tanaka, S., and Koike, T., 2001, Rapid induction and Ca(2+) influx-mediated suppression of vitamin D3 up- regulated protein 1 (VDUP1) mRNA in cerebellar granule neurons undergoing apoptosis. J Neurochem, 78:1267-1276.
88. Junn, E., Han, S.H., Im, J.Y., et al., 2000, Vitamin D3 up-regulated protein 1 mediates oxidative stress via suppressing the thioredoxin function. J Immunol, 164:6287-6295.
89. Butler, L.M., Zhou, X., Xu, W.S., et al., 2002, The histone deacetylase inhibitor SAHA arrests cancer cell growth, up-regulates thioredoxin-binding protein-2, and down-regulates thioredoxin. Proc Natl Acad Sci, 99:11700-11705.
90. Näär, A.M., Beaurang, P.A., Zhou, S., et al., 1999, Composite co-activator ARC mediates chromatin-directed transcriptional activation. Nature, 398:828-832.
91. Taatjes, D.J., Naa,r A.M., Andel, III, F., Nogales, E., and Tjian, R., 2002, Structure, function, and activator-induced conformations of the CRSP coactivator. Science, 295:1058-1062.
92. Jiang, Y.F., Goldberg, I.D., and Shi, Y.E., 2002, Complex roles of tissue inhibitors of metalloproteinases in cancer. Oncogene, 21:2245-2252.
93. Chang, C., and Werb, Z., 2001, The many faces of metalloproteases: cell growth, invasion, angiogenesis and metastasis. Trends Cell Biol, 11:S37-S43.
94. Toi, M., Ishigaki, S., and Tominaga, T., 1998, Metalloproteinases and tissue inhibitors of metalloproteinases. Breast Cancer Res Treat, 52:113-124.
95. Giannelli, G., Bergamini, C., Marinosci, F., et al., 2002, Clinical role of MMP-2/TIMP-2 imbalance in hepatocellular carcinoma. Int J Cancer, 97:425-431.
96. Ylisirnio, S., Hoyhtya, M., Makitaro, R., et al., 2001, Elevated serum levels of type I collagen degradation marker ICTP and tissue inhibitor of metalloproteinase (TIMP) 1 are associated with poor prognosis in lung cancer. Clin Cancer Res, 7:1633-1637.
97. Ylisirnio, S., Hoyhtya, M., and Turpeenniemi-Hujanen, T., 2000, Serum matrix metalloproteinases -2, -9 and tissue inhibitors of metalloproteinases -1, -2 in lung cancer--TIMP-1 as a prognostic marker. Anticancer Res, 20:1311-1316.
98. Pellegrini, P., Contasta, I., Berghella, A.M., et al., 2000, Simultaneous measurement of soluble carcinoembryonic antigen and the tissue inhibitor of metalloproteinase TIMP1 serum levels for use as markers of pre-invasive to invasive colorectal cancer. Cancer Immunol Immunother, 49:388-394.
99. Chambers, A.F., and Matrisian, L.M., 1997, Changing views of the role of matrix metalloproteinases in metastasis. J Natl Cancer Inst, 89:1260-1270.
100. Yu, W.H., Yu, S.S.C., Meng, Q., Brew, K., Woessner, Jr., J.F., 2000, TIMP-3 binds to sulfated glycosaminoglycans of the extracellular matrix. J Biol Chem, 275:31226-31232.
101. Gruss, C., and Herlyn, M., 2001, Role of cadherins and matrixins in melanoma. Curr Opin Oncol, 13:117-123.

102. Berx, G., Cleton-Jansen, A.M., Strumane, K., et al., 1996, E-cadherin is inactivated in a majority of invasive human lobular breast cancers by truncation mutations throughout its extracellular domain. Oncogene, 13:1919-1925.
103. Nakayama, S., Sasaki, A., Mese, H., et al., 2001, The E-cadherin gene is silenced by CpG methylation in human oral squamous cell carcinomas. Int J Cancer, 93:667-673.
104. Graff, J.R., Gabrielson, E., Fujii, H., Baylin, S.B., and Herman, J.G., 2000, Methylation patterns of the E-cadherin 5' CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. J Biol Chem, 275:2727-2732.
105. Jiang, W.G., 1996, E-cadherin and its associated protein catenins, cancer invasion and metastasis. Br J Surg, 83:437-446.
106. Shiozaki, H., Oka, H., Inoue, M., Tamura, S., and Monden, 1996, M., E-cadherin mediated adhesion system in cancer cells. Cancer, 77:1605-1613.
107. Nam, J.S., Ino, Y., Sakamoto, M., and Hirohashi, S., 2002, Src family kinase inhibitor PP2 restores the E-cadherin/catenin cell adhesion system in human cancer cells and reduces cancer metastasis. Clin Cancer Res, 8:2430-2436.
108. Vermeulen, S.J., Bruyneel, E.A., Bracke, M.E., et al., 1995, Transition from the noninvasive to the invasive phenotype and loss of α-catenin in human colon cancer cells. Cancer Res, 55:4722-4728.
109. Frixen, U.H., Behrens, J., Sachs, M., et al., 1991, E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J Cell Biol, 113:173-185.
110. Perl, A.K., Wilgenbus, P., Dahl, U., Semb, H., and Christofori, G., 1998, A causal role for E-cadherin in the transition from adenoma to carcinoma. Nature, 392:190-193.
111. Christofori, G., and Semb, H., 1999, The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. Trends Biochem Sci, 24:73-76.
112. Mareel, M., Boterberg, T., Noe, V., et al., 1997, E-cadherin/catenin/cytoskeleton complex: a regulator of cancer invasion. J Cell Physiol, 173:271-274.
113. Kashima, T., Nakamura, K., Kawaguchi, J., et al., 2003, Overexpression of cadherins suppresses pulmonary metastasis of osteosarcoma in vivo. Int J Cancer, 104:147-154.
114. Nieman, M.T., Prudoff, R.S., Johnson, K.R., and Wheelock, M.J., 1999, N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. J Cell Biol, 147:631-643.
115. Tran, N.L., Nagle, R.B., Cress, A.E., and Heimark, R.L., 1999, N-Cadherin expression in human prostate carcinoma cell lines. An epithelial-mesenchymal transformation mediating adhesion with stromal cells. Am J Pathol, 155:787-798.
116. Pishvaian, M.J., Feltes, C.M., Thompson, P., et al., 1999, Cadherin-11 is expressed in invasive breast cancer cell lines. Cancer Res, 59:947-952.
117. Li, G., Satyamoorthy, K., and Herlyn, M., 2001, N-cadherin-mediated intercellular interactions promote survival and migration of melanoma cells. Cancer Res, 61:3819-3825.
118. Hazan, R.B., Phillips, G.R., Qiao, R.F., Norton, L., and Aaronson, S.A., 2000, Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. J Cell Biol, 148:779-790.
119. Kauffman, E.C., Robinson, V.L., Stadler, W.M., Sokoloff, M.H., and Rinker-Schaeffer, C.W., 2003, Metastasis suppression: the evolving role of metastasis suppressor genes for regulating cancer cell growth at the secondary site. J Urol, 169:1122-1133.
120. Teng, D.H.F., Perry, III, W.L., Hogan, J.K., et al., 1997, Human mitogen-activated protein kinase kinase 4 as a candidate tumor suppressor. Cancer Res, 57:4177-4182.
121. Yamada, S.D., Hickson, J.A., Hrobowski, Y., et al., 2002, Mitogen-activated protein kinase kinase 4 (MKK4) acts as a metastasis suppressor gene in human ovarian carcinoma. Cancer Res, 62:6717-6723.
122. Yoshida, B.A., Dubauskas, Z., Chekmareva, M.A., et al., Mitogen-activated protein kinase kinase 4/stress-activated protein/Erk kinase 1 (MKK4/SEK1), a prostate cancer metastasis suppressor gene encoded by human chromosome 17. Cancer Res, 59:5483-5487.
123. Yoshida, B.A., Dubauskas, Z., Chekmareva, M.A., Zaucha, M.M., Christiano, T.R., Christiano, A.P., et al., 1999, Identification and characterization of candidate prostate cancer metastasis-suppressor genes encoded on human chromosome 17. Proceedings of the American Association for Cancer Research 40, 701.
124. Chekmareva, M.A., Kadkhodaian, M.M., Hollowell, C.M.P., et al., 1998, Chromosome 17-mediated dormancy of AT6.1 prostate cancer micrometastases. Cancer Res, 58:4963-4969.
125. Kim, H..L, Van der Griend, D.J., Yang, X., et al., 2001, Mitogen-activated protein kinase kinase 4 metastasis suppressor gene expression is inversely related to histological pattern in advancing human prostatic cancers. Cancer Res, 61:2833-2837.
126. Seraj, M.J., Samant, R.S., Verderame, M.F., and Welch, D.R., 2000, Functional evidence for a novel human breast carcinoma metastasis suppressor, BRMS1, encoded at chromosome 11q13. Cancer Res, 60:2764-2769.
127. Li, Z., Zhou, Z., Saunders, M.M., Casey, G., Welch, D.R., and Donahue, H.J., 2001, Connexin and osteopontin expression correlate with breast cancer

metastatic potential. Proceedings of the American Association for Cancer Research 42.
128. Samant, R.S., Seraj, M.J., Saunders, M.M., et al., 2001, Analysis of mechanisms underlying BRMS1 suppression of metastasis. Clin Exptl Metastasis, 18:683-693.
129. Samant, R.S., Debies, M.T., Shevde, L.A., Verderame, M.F., and Welch, D.R., 2002, Identification and characterization of murine ortholog (Brms1) of breast cancer metastasis suppressor 1 (BRMS1). Int J Cancer, 97:15-20.
130. Saunders, M.M., Seraj, M.J., Li, Z.Y., et al., 2001, Breast cancer metastatic potential correlates with a breakdown in homospecific and heterospecific gap junctional intercellular communication. Cancer Res, 61:1765-1767.
131. Seraj, M.J., Harding, M.A., Gildea, J.J., Welch, D.R., and Theodorescu, D., 2001, The relationship of BRMS1 and RhoGDI2 gene expression to metastatic potential in lineage related human bladder cancer cell lines. Clin Exptl Metastasis, 18:519-525.
132. Shevde, L.A., Samant, R.S., Goldberg, S.F., et al., 2002, Suppression of human melanoma metastasis by the metastasis suppressor gene, BRMS1. Exp Cell Res, 273:229-239.
133. Locke, D., Perusinghe, N., Newman, T., et al., 2000, Developmental expression and assembly of connexins into homomeric and heteromeric gap junction hemichannels in the mouse mammary gland. J Cell Physiol, 183:228-237.
134. Monaghan, P., and Moss, D., 1996, Connexin expression and gap junctions in the mammary gland. Cell Biol Int, 20:121-125.
135. Monaghan, P., Clarke, C., Perusinghe, N.P., et al., 1996, Gap junction distribution and connexin expression in human breast. Exp Cell Res, 223:29-38.
136. Jamieson, S., Going, J.J., D'Arcy, R., and George, W.D., 1998, Expression of gap junction proteins connexin 26 and connexin 43 in normal human breast and in breast tumours. J Pathol, 184:37-43.
137. Pozzi, A., Risek, B., Kiang, D.T., Gilula, N.B., and Kumar, N.M., 1995, Analysis of multiple gap junction gene products in the rodent and human mammary gland. Exp Cell Res, 220:212-9.
138. Wilgenbus, K.K., Kirkpatrick, C.J., Knuechel, R., Willecke, K., and Traub, O., 1992, Expression of Cx26, Cx32 and Cx43 gap junction proteins in normal and neoplastic human tissues. Int J Cancer, 51:522-529.
139. Meehan, W.J., Samant, R.S., Hopper, J.E., et al., 2004, Breast cancer metastasis suppressor 1 (BRMS1) forms complexes with retinoblastoma-binding protein 1 (RBP1) and the mSin3 histone deacetylase complex and represses transcription. J Biol Chem, 279:1562-1569.
140. Nauert, J.B., Klauck, T.M., Langeberg, L.K., and Scott, J.D., 1997, Gravin, an autoantigen recognized by serum from myasthenia gravis patients, is a kinase scaffold protein. Curr Biol, 7:52-62.
141. Gelman, I.H., 2002, The role of SSeCKS/gravin/AKAP12 scaffolding proteins in the spaciotemporal control of signaling pathways in oncogenesis and development. Front Biosci, 7:d1782-d1797.
142. Lin, X., Tombler, E., Nelson, P.J., Ross, M., and Gelman, I.H., 1996, A novel src- and ras-suppressed protein kinase C substrate associated with cytoskeletal architecture. J Biol Chem, 271:28430-28438.
143. Xia, W., Unger, P., Miller, L., Nelson, J., and Gelman, I.H., 2001, The Src-suppressed C kinase substrate, SSeCKS, is a potential metastasis inhibitor in prostate cancer. Cancer Res, 61:5644-5651.
144. Nelson, P.J., and Gelman, I.H., 1997, Cell-cycle regulated expression and serine phosphorylation of the myristylated protein kinase C substrate, SSeCKS: correlation with culture confluency, cell cycle phase and serum response. Mol Cell Biochem, 175:233-241.
145. Boettner, B., and VanAelst, L., 2002, The role of Rho GTPases in disease development. Gene, 286:155-174.
146. Gildea, J.J., Seraj, M.J., Oxford, G., et al., 2002, RhoGD12 is an invasion and metastasis suppressor gene in human cancer. Cancer Res, 62:6418-6423.
147. Guan, R.J., Ford, H.L., Fu, Y., et al., 2000, Drg-1 as a differentiation-related, putative metastatic suppressor gene in human colon cancer. Cancer Res, 60:749-755.
148. Bandyopadhyay, S., Pai, S.K., Gross, S.C., et al., 2003, The Drg-1 gene suppresses tumor metastasis in prostate cancer. Cancer Res, 63:1731-1736.
149. Kurdistani, S.K., Arizti, P., Reime,r C.L., et al., 1998, Inhibition of tumor cell growth by RTP/rit42 and its responsiveness to p53 and DNA damage. Cancer Res, 58:4439-4444.
150. Motwani, M., Sirotnak, F.M., She, Y.H., Commes, T., and Schwartz, G.K., 2002, Drg1, a novel target for modulating sensitivity to CPT-11 in colon cancer cells. Cancer Res, 62:3950-3955.
151. Agarwala, K.L., Kokame, K., Kato, H., and Miyata, T., 2000, Phosphorylation of RTP, an ER stress-responsive cytoplasmic protein. Biochem Biophys Res Comm, 272:641-647.
152. Corbit, K.C., Trakul, N., Eves, E.M., et al., 2003, Activation of Raf-1 signaling by protein kinase C through a mechanism involving Raf kinase inhibitory protein. J Biol Chem, 278:13061-13068.
153. Yeung, K.C., Rose, D.W., Dhillon, A.S., et al., 2001, Raf kinase inhibitor protein interacts with NF-kappaB-inducing kinase and TAK1 and inhibits NF-kappaB activation. Molec Cell Biol, 21:7207-7217.

154. Fu, Z., Smith, P.C., Zhang, L., et al., 2003, Effects of Raf kinase inhibitor protein expression on suppression of prostate cancer metastasis. J Natl Cancer Inst, 95:878-889.
155. Castellani, V., and Rougon, G., 2002, Control of semaphorin signaling. Curr Opin Neurobiol, 12:532-541.
156. Dickson, B.J., 2002, Molecular mechanisms of axon guidance. Science, 298:1959-1964.
157. Comoglio, P.M., and Trusolino, L., 2002, Invasive growth: from development to metastasis. J Clin Invest, 109:857-862.
158. Fujita, H., Okada, F., Hamada, J., et al., 2001, Gelsolin functions as a metastasis suppressor in B16-BL6 mouse melanoma cells and requirement of the carboxyl-terminus for its effect. Int J Cancer, 93:773-780.
159. Sagawa, N., Fujita, H., Banno, Y., et al., 2003, Gelsolin suppresses tumorigenicity through inhibiting PKC activation in a human lung cancer cell line, PC10. Br J Cancer, 88:606-612.
160. Tanaka, M., Müllauer, L., Ogiso, Y., et al., 1995, Gelsolin: A candidate for suppressor of human bladder cancer. Cancer Res, 55:3228-3232.
161. Jiang, N., Meng, Y.H., Zhang, S.L., Mensah-Osman, E., and Sheng, S.J., 2002, Maspin sensitizes breast carcinoma cells to induced apoptosis. Oncogene, 21:4089-4098.
162. Zhang, M., Volpert, O., Shi, Y.H., and Bouck, N., 2000, Maspin is an angiogenesis inhibitor. Nature Med, 6:196-199.
163. Futscher, B.W., Oshiro, M.M., Wozniak, R.J., 2002, et al. Role for DNA methylation in the control of cell type-specific maspin expression. Nat Genet, 31:175-179.
164. Kirschmann, D.A., Lininger, R.A., Gardner, L.M.G., et al., 2000, Down-regulation of HP1Hsα expression is associated with the metastatic phenotype in breast cancer. Cancer Res, 60:3359-3363.
165. Gao, A.C., Lou, W., Dong, J.T., and Isaacs, J.T., 1997, CD44 is a metastasis suppressor gene for prostatic cancer located on human chromosome 11p13. Cancer Res, 57:846-849.
166. Yu, D.H., Qu, C.K., Henegariu, O., Lu, X., and Feng, G.S., 1998, Protein-tyrosine phosphatase Shp-2 regulates cell spreading, migration, and focal adhesion. J Biol Chem, 273:21125-21131.
167. Rengifo-Cam, W., Konishi, A., Morishita, N., et al., 2004, Csk defines the ability of integrin-mediated cell adhesion and migration in human colon cancer cells: implication for a potential role in cancer metastasis. Oncogene, 23:289-297.
168. Nakagawa, T., Tanaka, S., Suzuki, H., et al., 2000, Overexpression of the csk gene suppresses tumor metastasis in vivo. Int J Cancer, 88:384-391.
169. Michl, P., Barth, C., Buchholz, M., et al., 2003, Claudin-4 expression decreases invasiveness and metastatic potential of pancreatic cancer. Cancer Res, 63:6265-6271.
170. Goldberg, S.F., Harms, J.F., Quon, K., and Welch, D.R., 1999, Metastasis-suppressed C8161 melanoma cells arrest in lung but fail to proliferate. Clin Exptl Metastasis, 17:601-607.
171. Steeg, P.S., Ouatas, T., Halverson, D., Palmieri, D., and Salerno, M., 2003, Metastasis suppressor genes: Basic biology and potential clinical use. Clin Breast Cancer, 5:51-62.
172. Graff, J.R., and Zimmer, S.G., 2003, Translational control and metastatic progression: enhanced activity of the mRNA cap-binding protein eIF-4E selectively enhances translation of metastasis-related mRNAs. Clin Exptl Metastasis, 20:265-273.
173. Zimmer, S.G., and Graff, J.R., 2002, The emerging role for the mRNA cap-binding protein, EIF-4E, in metastatic progression. In: Cancer Metastasis: Biology and Treatment.Welch DR, ed. Dordrecht, The Netherlands: Kluwer Academic Publishers, 257-278.
174. Plass, C., 2002, Cancer epigenomics. Hum Molec Genet, 11:2479-2488.
175. Plass, C., and Soloway, P.D., 2002, DNA methylation, imprinting and cancer. Eur J Hum Genet, 10:6-16.
176. Imhof, A., and Becker, P.B., 2001, Modifications of the histone N-terminal domains. Evidence for an "epigenetic code"? Mol Biotechnol, 17:1-13.
177. Karpf, A.R., and Jones, D.A., 2002, Reactivating the expression of methylation silenced genes in human cancer. Oncogene, 21:5496-5503.
178. Kelly, W.K., O'Connor, O.A., and Marks, P.A., 2002, Histone deacetylase inhibitors: from target to clinical trials. Exp Opin Invest Drugs, 11:1695-1713.
179. Elledge, R.M., and Lee, W.H., 1995, Life and death by p53. BioEssays, 17:923-930.
180. Mabjeesh, N.J., Post, D.E., Willard, M.T., et al., 2002, Geldanamycin induces degradation of hypoxia-inducible factor 1α protein via the proteosome pathway in prostate cancer cells. Cancer Res, 62:2478-2482.
181. Ouatas, T., Halverson, D., and Steeg, P.S., 2003, Dexamethasone and medroxyprogesterone acetate elevate Nm23-H1 metastasis suppressor expression in metastatic human breast carcinoma cells via glucocorticoid receptor-dependent, transcriptional and post-transcriptional mechanisms: New uses for old compounds. Clin Cancer Res, 9:3763-3772.

Chapter 25

Tumor-Host Interactions at the Metastatic Site: MKK4, Signal Transduction and the Stress Response

Jonathan C. Berger[1], Victoria L. Robinson[2,3], Jonathan A. Hickson[2,4], Donald Vander Griend[2,3], and Carrie W. Rinker-Schaeffer[2-4]

[1] The Pritzker School of Medicine, The University of Chicago,USA, [2] Section of Urology, Department of Surgery, The University of Chicago,USA, [3] The Committee on Cancer Biology, University of Chicago,USA, [4] Department of Biochemistry and Molecular Biology,

Abstract: The Mitogen-Activated Protein Kinase Kinase 4 (MKK4, also referred to as JNKK1, SEK2, or MEK4) has been identified as a metastasis suppressor gene in prostate and ovarian cancer. Metastasis suppressor genes are defined by the ability of their encoded proteins to suppress the development of metastases in vivo without affecting primary tumor growth when transfected into metastatic cell lines. Studies exploring the mechanisms of metastatic colonization and metastasis suppressor genes suggest that the critical events determining metastatic efficiency occur at the secondary site. Furthermore, metastasis suppressor proteins may operate by modulating major signal transduction pathways that regulate important cellular processes such as proliferation, differentiation, dormancy, and apoptosis. These findings have at least two important implications. First, tumor-host interactions at the metastatic site play an important role in the regulation of metastatic growth. Second, developing a comprehensive understanding of the specific biochemical events regulating both metastatic progression and metastatic suppression may ultimately contribute to new diagnostic and therapeutic modalities for advanced cancers.

Key words: Metastasis, suppression, genes, cancer, tumorigenesis, KISS1, MKK4, Nm23, BRMS1, CRSP3, TXNIP, E-cadherin, SseCKS, KAI1, TIMPs, DRG-1, RKIP, RhoGDI2

1. TUMOR-HOST INTERACTIONS AT THE SECONDARY SITE

For more than two decades researchers believed that escape of a cell from the primary tumor was the rate-limiting event determining metastatic efficiency (1). This belief was based on the concept that once a cell had eluded growth control mechanisms, it had only to escape from the primary tumor and evade host defenses in order to form metastases. In support of this theory were studies showing a correlation between increased motility and invasion *in vitro* and increased metastasis formation *in vivo* (2-4). An implication of this model was that disseminated cancer cells are unable to respond to extracellular growth controls and consequently replicate completely autonomously. Recently, multiple independent laboratories studying the clinical, biological, and molecular determinants of metastasis have clearly shown that disseminated cancer cells at secondary sites remain subject to growth controls. Taken together, these studies show unequivocally that tumor cells leave the primary tumor but often fail to proliferate at an ectopic site (5-8). These findings demonstrate that cells that have acquired the ability to escape (either actively or

G. G. Meadows (ed.), Integration/Interaction of Oncologic Growth, 437-448

passively) from primary tumor, survive in the circulation, and reach secondary sites are not by definition autonomous; they remain subject to at least some of the growth and migratory controls in their immediate environment. Thus, cellular proliferation at the secondary site, hereafter referred to as *metastatic colonization*, must now be considered a potential rate-limiting step of metastasis.

These findings imply that the tumor-host interactions at the secondary site are distinct from those at the primary site and that elucidating the nature of these interactions is crucial to understanding the process of metastasis. Furthermore, this work seeks to explain the well-documented but poorly explained concept of metastatic site specificity, which was first documented by Stephen Paget over a century ago (9). In his seminal work, Paget compared a metastatic cell growing in a distant organ to a seed growing in the soil. Just as a seed can grow only in soil that it is uniquely suited for, so can a cancer cell grow in tissues that provide it with the necessary molecular environment. Our challenge is now to gain a mechanistic understanding of how disseminated cancer cells respond to foreign microenvironments.

In recent years, experiments utilizing RT-PCR and immunohistochemistry have indicated that tumor cells may escape from the primary tumor very early in the course of disease, and are often clinically undetectable at secondary sites at the time of a cancer diagnosis (10-12). Simultaneously, Chambers and her coworkers have developed novel techniques of intravital video-microscopy and cell-accounting which enable the visualization and assessment of the individual steps of metastasis (5). Their experiments have yielded *in vivo* data from experimental, real-time models which show that disseminated tumor cells often reside as dormant single cells or micrometastases at secondary sites. These data support the findings from clinical studies (13). Interestingly, functional cloning experiments aimed at the identification and characterization of the genetic determinants of metastasis have also identified metastatic colonization as a rate-determining step for formation of overt metastases (14). These studies have identified numerous *metastasis suppressor genes*, the ectopic expression of which suppress the formation of metastases without affecting primary tumor growth *in vivo*. For example, *in vivo* metastasis assays demonstrate that metastatic tumor cells expressing the metastasis suppressor genes MKK4 or KiSS1 successfully arrest in capillary beds at secondary sites but fail to proliferate (6, 7). Thus, data from three independent avenues of metastasis research have identified metastatic colonization as an important step in the development of both experimental and clinical metastases.

2. SIGNAL TRANSDUCTION, METASTASIS SUPPRESSOR GENES, AND MKK4

2.1 Signal transduction pathways associated with metastasis suppressor proteins

Biochemical data currently support a model in which certain metastasis suppressor proteins participate in conserved eukaryotic signal transduction pathways. These signaling cascades are comprised of at least three parallel modules: the Mitogen-activated Protein Kinase/Extracellular Signal Regulated Kinase (MAPK/ERK) module, the Stress-activated Protein Kinase/c-Jun N-terminal Kinase (SAPK/JNK) and the p38 kinase module (See Figure 1). These distinct but biochemically interconnected signaling modules relay extracellular stimuli through interconnected protein kinase cascades that activate transcription factors and affect cellular responses to stress, inflammation and growth factors (15, 16).

A detailed characterization of these pathways is critical to elucidating the function of metastasis suppressor proteins. Each cascade represents a series of kinase-substrate phosphorylation reactions that function to relay an extracellular signal to the nucleus. Each pathway consists of at least three members: a MAP kinase kinase kinase (MAP3K) which, when activated, phosphorylates a MAP kinase kinase (MAP2K) which, in turn phosphylates

a MAP kinse (MAPK). Extracellular signals can be communicated to the ERK pathway by cell surface receptors. The mechanism by which extracellular stress activates MAP3Ks in the JNK/p38 pathways is not as well-established.

The MAPK/ERK pathway involves the receptor tyrosine kinase (RTK) class of transmembrane proteins and serine-threonine kinases (e.g. PDGF, EGF, insulin receptors, etc.). Typically, cell surface receptors interact with adaptor molecules to recruit a protein kinase complex to the cell membrane. The prototypical MAPK/ERK pathway involves RTKs that interact with Ras through proteins with SH2 and SH3 adaptor domains. In its active form, Ras recruits Raf (a MAP3K), to the membrane complex resulting in the activation of a target MAP/ERK Kinase (MEK, MAP2K). Phosphorylation of MEK results in the activation and subsequent translocation of ERK (MAPK) to the nucleus, where it phosphorylates various transcription factors (e.g. Elk-1, c-Fos) and DNA binding proteins (15, 16).

Signal amplification and regulation may occur at each step in this cascade. Specificity of the signal is conferred through both the affinity of a kinase for a given target protein as well as protein expression levels. Additional regulation is conferred by interaction with other scaffolding or adaptor proteins. For example, the Nm23-H1 metastasis suppressor protein interacts with the MAPK module by binding to and phosphorylating the Kinase Suppressor of Ras (Ksr) protein, a scaffold protein that participates in the regulation of the activity of the MAPK/ERK pathway upstream of MEK (17). Ksr has been shown to positively regulate Ras-mediated MAPK signaling, suggesting that Nm23's metastasis suppression activity may be mediated by inhibition of Ksr-enabled MAPK signaling (18).

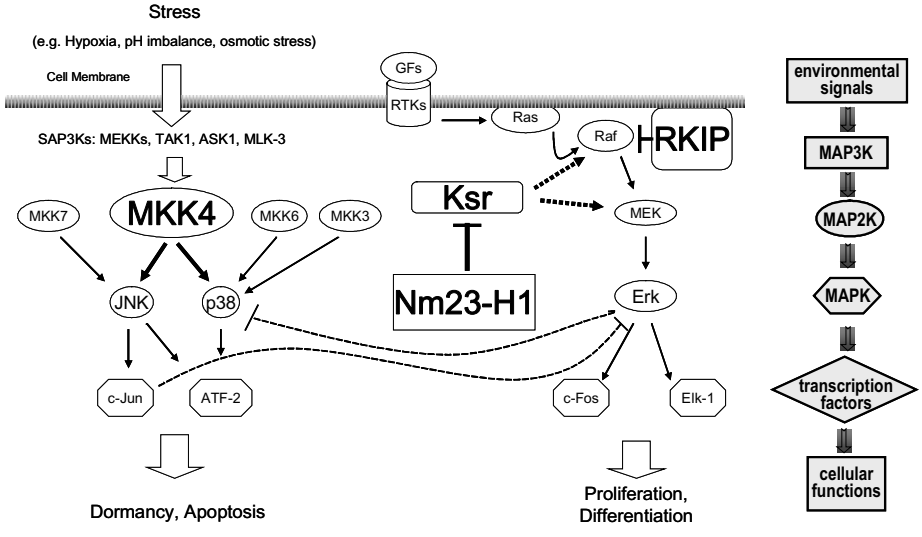


Figure 1. Signal transduction pathways associated with metastasis suppressor genes. Figure adapted from Berger, et al. Metastasis suppressor genes: signal transduction, crosstalk and the potential for modulating the behavior of metastatic cells. *Anti-Cancer Drugs. 2004. Article in press.*

Similarly, the metastasis suppressor Raf Kinase Inhibitor Protein (RKIP) functions as a negative upstream regulator of MAPK/ERK signaling. Raf and MEK interact with RKIP at overlapping sites, and binding of either molecule inhibits binding of the other. Both binding domains must be destroyed in order to alleviate RKIP-mediated MAPK inhibition. Furthermore, studies with constitutively active MEK mutants (MEK-DD) suggest that RKIP functions upstream of MEK (19). Thus, RKIP may suppress metastasis by inhibiting Raf-mediated phosphorylation of MEK.

In contrast to the association of ERK with proliferation, JNK and p38 have been classically

associated with cell cycle arrest and apoptosis in response to environmental stresses and cytokines, pH changes, UV-irradiation, hypoxia, and growth factor deprivation (20). As such, these proteins represent functionally critical components in the cellular response to environmental cues. Several MAP2Ks operate upstream of p38 and JNK. MKK7 specifically phosphorylates JNK, whereas MKK3 and MKK6 specifically target p38. Interestingly MKK4 can phosphorylate and activate both JNK and p38 (see below).

The ERK, JNK, and p38 pathways have historically been considered as separate signaling cascades mediating distinct cellular functions. It is now well-accepted, however, that these pathways interact extensively with each other as well as a number of other pathways in a complex signaling network (21, 22). In addition to biochemical studies, recent *in vivo* studies suggest an antagonistic relationship between ERK and p38 signaling that may determine whether a tumor cell proliferates or enters a state of dormancy (21, 23). Aguirre-Ghiso *et al*. demonstrated that the ERK/p38 activity ratio in head and neck cancer cell lines correlates with the balance between cellular proliferation and dormancy. Based on their data, they propose a model in which persistent ERK activity is maintained through urokinase plasminogen activator receptor (uPAR) activity and its interactions with $\alpha5\beta1$-integrin. Furthermore, they argue that shifting the ratio between phospho-ERK and phospho-p38 determines whether a cell will proliferate or remain dormant (23). In subsequent studies they showed that modulation of the ERK/p38 ratio may induce dormant (high p38) or proliferative (high ERK) phenotypes in additional models (e.g. cancers of the breast, prostate, skin, and connective tissue) (21).

Determining how the functions of recently identified metastasis suppressor proteins participate in or complement these signal transduction pathways may provide a new insight into tumor-host interactions at the secondary site. The ERK, JNK, and p38 pathways have established roles in the relay of extracellular signals to the nucleus. Furthermore, these pathways can be reliably activated in response to well-characterized stimuli (24). Because metastasis suppressor proteins modulate the function of these pathways to suppress metastasis suggests that the disseminated cancer cells are indeed responding to the environment at the secondary site.

2.2 Mitogen-Activated Protein Kinase Kinase 4 (MKK4) is a metastasis suppressor gene

Positional cloning coupled with *in vivo* metastasis assays identified the Mitogen-Activated Protein Kinase Kinase 4 (MKK4, also referred to as JNKK1, SEK2, or MEK4) as a metastasis suppressor gene encoded by human chromosome 17 (6, 25, 26). A duel specificity kinase that phosphorylates both threonine and tyrosine residues, MKK4 has been shown to phosphorylate the JNK (TPY) and p38 (TGY) MAPK activation motifs. MKK4 thus functions as a MAP2K for both p38 and JNK, representing an important point of cross-talk between the two pathways. The MKK4 protein is widely expressed throughout human and mouse tissues and has a role in hepatic, neural, and thymic development in mice (27). Functional and clinical correlative studies identified a role for MKK4 protein in the suppression of metastasis in ovarian and prostate cancers (27-30). The function of MKK4 in tumor progression and metastasis may be context-dependent, as studies examining loss-of-heterozygosity of human chromosome 17 suggest that it may function as a tumor suppressor gene in a small percentage of pancreatic, biliary, and breast cancers (31). The role of MKK4 in gastric cancer is more controversial; reports have shown that MKK4 protein expression is *increased* in invasive gastric cancer, while another study indicated no change in MKK4 protein expression but significant loss-of-heterozygosity at telomeric markers for MKK4 (32, 33). Such seemingly contradictory activities illustrate the challenges faced when trying to determine how a metastasis suppressor protein such as MKK4 functions *in vivo* and within a particular cancer type.

3. DEFINING THE MECHANISM OF MKK4-MEDIATED METASTASIS SUPPRESSION

3.1 Mechanistic signaling studies

The JNK and p38 pathways have well-established roles in the regulation of apoptosis, differentiation, and proliferation. As an upstream regulator of these kinases, it is hypothesized that MKK4 derives its metastasis suppressor function from its ability to modulate their activity. Alternatively, MKK4 may have novel function(s) which are responsible for metastasis suppression. Determining the mechanism by which MKK4 suppresses metastasis involves isolating the specific signaling events that correspond to the metastasis suppressor phenotype. Fundamental to this research is the design of rigorous *in vitro* studies which demonstrate that the JNK and/or p38 pathways are activated in cells which display suppressed metastasis *in vivo*. Such studies represent an attempt to characterize in detail the upstream and downstream participants in MKK4-mediated metastasis suppression and are currently underway. They involve modulating the activity of signaling pathways through either activation or inhibition of specific pathway components:

Pathway activation. It is often useful to artificially activate one or more members of a signaling pathway. One approach for activating a pathway is to employ chemical stimuli that are known to induce coordinated gene expression changes through a specific signal transduction cascade. For example, phorbol ester and anisomycin activate p38 (15). Alternatively, over-expression of activating proteins can stimulate a pathway. In some cases, activation of a pathway can be achieved upon over-expression of an upstream activator. However, it may not be sufficient in cell types where there is not an adequate stimulus. Constitutively active proteins are mutated such that they require no stimulus to phosphorylate their target proteins. For instance, the ectopic expression of constitutively active MKK6 (referred to as MKK6 (EE)) results in increased activation of p38 (34).

Pathway inhibition. Inhibition of one or more members of a signaling pathway can also be useful in elucidating biochemical mechanisms. Recently, efforts to identify chemical inhibitors have resulted in the characterization of specific small molecule inhibitors for multiple signaling pathways. For example, SB203580 is a small molecule that selectively binds to and inactivates p38 (35). Additionally, over-expression of dominant-negative proteins can inhibit a pathway. A dominant negative protein is mutated such that it is both inactive and interferes with the function of endogenous protein. When using a dominant negative it is critical to demonstrate that the mutant is indeed inhibiting the endogenous protein.

3.2 Development of an in vitro stress model

The hypothesis that MKK4-mediated metastasis suppression is related to the transmission of stress signals suggests that certain stimuli need to be present to activate the metastasis suppressor phenotype. Developing an *in vitro* stress model of MKK4-mediated metastasis suppression is important for several reasons. First of all, it represents an attempt to describe MKK4-mediated cellular response to extracellular stimuli that complements *in vivo* studies. Secondly, it may identify specific extracellular stresses that activate metastasis suppressor pathways. Finally, subjecting MKK4 transfected cells to extracellular stresses that have previously been shown to activate JNK and p38 may provide further evidence that MKK4 suppresses metastasis through activation of a stress response.

A working model predicts that ectopic expression of MKK4 activates a signaling cascade that suppresses metastatic growth in response to stress stimuli. Given the known biochemical functions of MKK4, metastasis suppression could be the result of a variety of cellular events (i.e. increased apoptosis, cell cycle arrest, etc.). There is growing evidence that induction of apoptosis is the predominant outcome (36, 37). Although logical, a model in which MKK4-expressing cells undergo apoptosis in response to stresses at the metastatic site is difficult to test *in vivo*. Thus, development of an *in vitro* system that can be used for mechanistic

studies to explain *in vivo* findings is crucial to confirming the mechanism of MKK4-mediated metastasis suppression (38).

Anchorage-independent growth in soft agar was used as a model for the structural changes experienced by disseminated tumor cells. This approach is supported by previous studies that established a correlation between clonogenicity in soft agar and the efficiency of metastatic colonization (39, 40). The stress-response in cells expressing ectopic MKK4 was examined using highly metastatic AT6.1-vector constructs or metastasis-suppressed AT6.1-MKK4 cells (6). As shown in Figure 2, Panel A, when cells are exposed

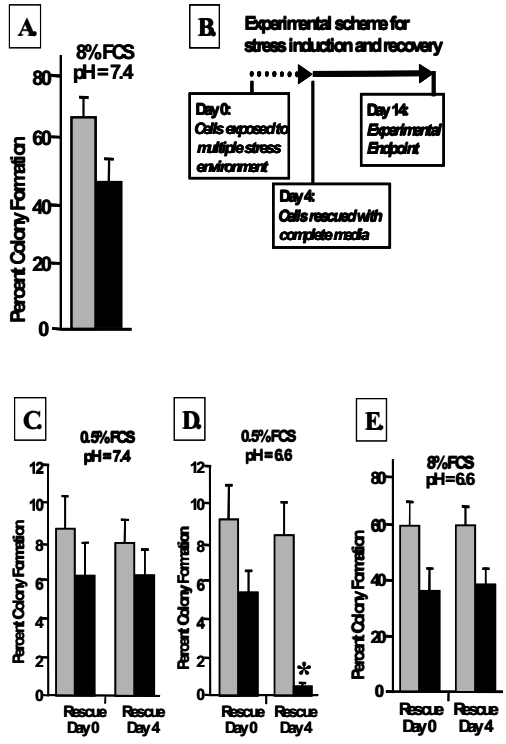


Figure 2. A combination of multiple extracellular stimuli leads to reduced colony formation of AT6.1-MKK4 cells. Gray bars represent results from AT6.1-vector cells and black bars represent AT6.1-MKK4 cells. A. Anchorage-independent growth of AT6.1-vector and AT6.1-MKK4 cells. 1x10^4 logarithmically growing AT6.1-vector or AT6.1-MKK4 cells were plated soft agar under standard growth conditions [e.g. 0.4% agarose prepared in RPMI containing 8% FCS (pH 7.4)]. Cells were allowed to grow for 14 days, after which viable colonies were identified by MTT assay and the percent colony formation was calculated. B. Experimental scheme for stress induction and recovery. The scheme for adding multiple stresses places the cells under the stringent condition for four days, after which the cells are rescued with complete media and grown under standard conditions for an additional 10 days. C. Growth of AT6.1-vector and AT6.1-MKK4 cells exposed to the combined stresses of anchorage independence and growth factor deprivation. D. Growth of AT6.1-vector and AT6.1-MKK4 cells exposed to the combined stresses of anchorage independence, growth factor deprivation, and low pH. E. Growth of AT6.1-vec and AT6.1-MKK4 cells exposed to the combined stresses of anchorage independence and low pH without growth factor deprivation. To test the assertion that all three stresses must be present to induce decreased colony formation, AT6.1-vector control and AT6.1-MKK4 cells were placed under media containing 8% FCS and low pH. Figure previously published in Robinson, et al. MKK4 and metastasis suppression: a marriage of signal transduction and metastasis research. *Clin Exp Metastasis. 2003;**20**(1):25-30.*

to the single stress of anchorage-independent growth (in complete media containing 8% FCS), there was a modest reduction in colony formation by AT6.1-MKK4 cells (*black bar*) as compared to AT6.1-vector (*gray bar*) only control cells. This suppression of colonization was not to the same degree as the suppression observed *in vivo* (77%) (6). It was then speculated that inclusion of the additional stress(es) of growth factor deprivation and/or low pH, which are known to affect MKK4's signaling cascade, would enhance the observed suppression. To test this assertion, AT6.1-vector and AT6.1-MKK4 cells were plated in the multiple stress environment for four days, after which the cells were "rescued" with complete media containing 8% FCS at pH 7.4 and colonies allowed to grow for ten days (Figure 2, Panel B). Viable colonies were detected by MTT reduction and percent colony formation was calculated as previously described (41). Growth factor deprivation (0.5% FCS) was the first stress used in combination with anchorage-independent growth. Although this combination resulted in a significant *overall* decrease in the number of colonies, the *relative* decrease in colony formation between AT6.1-MKK4 cells and AT6.1-vector was still only moderate (Figure 2, Panel C). Finally a third stress, low pH (6.6), was added. Under this multiple stress environment (e.g. anchorage-independent growth, 0.5% FCS, and pH 6.6), AT6.1-MKK4 cells showed a 90% decrease in colony formation as compared to AT6.1-vec cells (Figure 2, Panel D). This is comparable to the suppression of colony formation seen *in vivo* (6). It is important to note that exposure to the multiple stress environment did not effect colony formation by AT6.1-vector cells. These results suggested that a minimum of three stresses were needed for this *in vitro* assay. If this was true than cells exposed to the combination of anchorage-independent growth and low pH would show colony formation similar to anchorage-independent growth alone (Figure 2, Panel A). This was indeed the case as shown in Figure 2, Panel E. Taken together, the results from these experiments suggest that the stringent conditions of growth factor deprivation, low-pH, and anchorage-independent growth suppress colony formation via an MKK4-dependent signaling pathway. Efforts are underway to use this model to elucidate the potential biochemical mechanism by which MKK4 mediates metastasis suppression.

4. METASTASIS RESEARCH AND NEW CLINICAL OPPORTUNITIES

The identification of specific signaling pathways that regulate metastatic growth is an important advance, yet there are significant roadblocks to successful incorporation of this knowledge into clinical practice. First of all, the individual metastasis suppressor protein must be considered in terms of the pathway it participates in. Simply turning “on” or “off” a single gene (protein) in a signal transduction pathway will inevitably have broader affects than intended, for each pathway may affect, positively or negatively, the regulation of many as yet undocumented processes. Furthermore, the context-specific nature of signal transduction may limit our ability to generalize the affects of modulating a specific pathway to other model systems or tissue types. For instance, though high MKK4 expression has an established correlation with metastasis suppression in prostate and ovarian carcinomas, activation of the JNK/p38 pathway may have a role the malignant transformation of small cell lung carcinomas (29). Thus the effect of a specific signaling pathway may have to be determined on a case-by-case basis.

4.1 Metastasis suppressors and adjuvant therapy

In vivo models suggest that metastatic colonization, which as noted is the development of overt metastatic lesions from microscopic deposits, represents a potentially key rate-limiting step of metastatic development. The identification of a role for several of the known metastasis suppressor proteins in the regulation metastatic colonization through highly conserved signal transduction pathways represents a significant maturation in our ideas about metastasis. In order to translate these findings into tangible clinical benefit, we must first determine whether the findings from *in vivo* models correlate with the behavior of human cancers.

There is an accumulation of clinical data supporting colonization as the critical event in the development of human metastases. Historically, surgical oncologists have theorized that the removal of solid tumors releases tumor cells into the systemic circulation (42-45). Recent developments in molecular biology have confirmed that cancer cells can readily be found in the blood. Several studies using RT-PCR for expression of specific markers have shown that cancer cells are often present in the circulation at the time of diagnosis or surgery (10). As many as 25% of patients undergoing radical retropubic prostatectomy have evidence of hematogenous dissemination of tumor cells during surgery (46). Immunohistochemical studies have confirmed that tumor cells may reside as clinically occult microscopic metastases in bone marrow or other tissues at the time of diagnosis (11, 12). Collectively, these data imply that tumor cells readily escape from human primary tumors, yet fail to colonize a secondary site.

If metastatic colonization represents the rate-limiting step for human cancers, then our knowledge of metastasis suppressor genes and their encoded proteins could be used to guide clinical decision-making. Specifically, physicians typically treat patients with adjuvant regimens in order to eliminate microscopic metastatic disease before its progression into clinically detectable disease. The selection of patients for such regimens assumes that microscopic lesions will inevitably grow into clinically significant tumors. Basic metastasis research, however, suggests that a microscopic metastasis will proliferate at the secondary site only if it has undergone specific genetic and epigenetic changes that are distinct from the events of primary tumorigenesis. Disseminated tumor cells that do not possess this ability are not a clinical threat and need not be treated. Clinicians thus subject many patients to the cost and morbidity of adjuvant therapy to achieve clinical benefit in only a few patients who have disease with metastatic capability.

The identification of metastasis suppressor genes/proteins may provide an avenue by which clinicians can interrogate cancer cells for the ability to form clinically relevant metastatic disease. A molecular assay for molecular markers of metastasis would allow clinicians to stratify patients into high and low risk for metastatic disease. Such an assay would analyze the expression of the relevant tumor type-specific metastasis suppressor proteins. Clinicians and patients could then choose together whether aggressive adjuvant therapy is warranted.

Of course, significant problems need to be addressed before such an algorithm can be considered viable. First of all, we need to determine which tissue specimens to assay. A logical first choice would be primary tumor samples obtained from biopsies or surgical therapy. However, if our contention that metastasis requires molecular changes distinct from tumorigenesis is valid, than we cannot assume that the genetic profile of primary tumor cells reliably reflects that of disseminated cells. Furthermore, if only a subset of primary tumor cells have the ability to metastasize, will an assay done on a biopsy or tissue specimen necessarily contain the cells of interest?

Such questions lead us to the possibility of examining disseminated tumor cells directly. Bone marrow or other secondary site-directed biopsies combined with methodologies for appropriate cell enrichment can provide samples of disseminated disease for assay (47). However, invasive techniques introduce additional morbidity. Furthermore, it has not been shown that the genetic profile of disseminated tumor cells is stable over time. A patient that is stratified as low risk after a metastasis suppressor expression assay may need to be reassessed at some time in the future in order to reconfirm the status of their disease.

A foreseeable strategy might involve the assay of primary tumor cells at the time of surgery. If a high probability of metastasis is determined by the identification of the molecular changes associated with metastatic progression, than adjuvant therapy would be recommended. If such markers are not identified in the primary tumor, than the clinician could recommend a search for disseminated cells in the bone marrow or other tumor type-specific locations. These cells could then be assayed either once or repeatedly to determine metastatic ability and guide therapy.

4.2 Metastasis suppressor genes and novel cancer therapies

The greatest hope among metastasis researchers is that an understanding of the mechanisms of metastasis suppression may lead to targeted therapies. Tumor metastasis represents the most feared, least treatable, and ultimately most lethal consequence of malignancy. Metastatic disease often relegates patients to palliative therapy and decreased quality of life. Some of the remarkable successes in cancer drug design in the last several decades have come from an understanding of the molecular biology of disease. Selective Estrogen Receptor Modulators (SERMs) have become a cornerstone of adjuvant therapy for breast cancer, and are beginning to find a role in breast cancer prophylaxis in high-risk women (48). The phenomenal success of the tyrosine kinase inhibitor Imatinib (Gleevec) in certain patients with chronic myelogenous leukemia and gastro-intestinal stromal tumors is another example of how an understanding of the molecular biology of cancer can stimulate the development of novel therapies. Ideally, basic science research in the field of metastasis suppressor genes will similarly translate into clinical therapeutics.

Studies indicate that the majority of metastasis suppressors are down-regulated, but not mutated or deleted, in metastatic lesions (30, 49). This observation may provide a unique therapeutic opportunity because, unlike mutated oncogenes or tumor suppressor genes, exogenous DNA or a constitutively active gene product does not necessarily need to be introduced into malignant cells. Rather, it may be possible to identify compounds that restore expression of silenced metastasis suppressor genes and thus modulate important signal transduction cascades such as the MAPK and SAPK pathways to inhibit metastatic colonization. Alternatively, we may be able to manipulate the signaling pathways in which metastasis suppressor proteins participate without specifically targeting metastasis suppressor proteins.

Preliminary efforts to identify compounds that have a negative affect on metastasis growth through metastasis suppressor pathways are in progress. Currently, drugs that affect metastasis suppressor up-regulation have non-specific, pleiotropic affects on gene expression, and none can be considered a specific activator of a metastasis suppressor pathway (50-58). Such compounds include DNA methylation inhibitors, histone deacetylase inhibitors, steroid hormones, anti-inflammatory drugs, and immunomodulators. While not specific activators of metastasis suppressor genes, such compounds may yet hold promise in the clinic, and are currently being evaluated for *in vivo* metastasis suppressing activity (59).

The development of drugs that specifically target metastatic colonization may come as the functional studies describing the signal transduction pathways regulating metastatic growth are completed. Undoubtedly, there are numerous proteins that interact with SAPK, p38 and ERK pathways, each of which may have potential as a therapeutic target. Perhaps the ideal molecular targets are not the metastasis suppressor genes or proteins themselves, but their antagonists. Identifying a specific inhibitor of a functional metastasis suppressor antagonist could modulate signaling in a therapeutically beneficial manner without accomplishing the clinically difficult task of up-regulating metastasis suppressor gene expression.

ACKNOWLEDGEMENTS

This work was supported by NCI RO1 CA89569-01; ACS Grant RPG-99-068-01CSM; Fletcher Scholar Award of The Cancer Research Foundation (Chicago, IL); The University of Chicago Research Cure and Education (RESCUE) Fund; and the National Foundation for Cancer Research, Center for Metastasis Research.

REFERENCES

1. Hall, S.J., and Thompson, T.C., 1997, Spontaneous but not experimental metastatic activities differentiate primary tumor-derived vs metastasis-derived mouse prostate cancer cell lines. Clin Exp Metastasis, 15(6):630-8.
2. Nabeshima, K., Kataoka, H., and Koono, M., 1986, Enhanced migration of tumor cells in response to collagen degradation products and tumor cell

collagenolytic activity. Invasion Metastasis, 6(5):270-86.
3. Fridman, R., Scott, A.F., Muller, D., et al., 1990, The role of cell adhesion and migration in the in vitro invasiveness of mouse adrenal carcinoma cells. Invasion Metastasis, 10(4):208-24.
4. Taylor, W.R., Greenberg, A.H., Turley, E.A., et al., 1993, Cell motility, invasion, and malignancy induced by overexpression of K-FGF or bFGF. Exp Cell Res, 204(2):295-301.
5. MacDonald, I.C., Groom, A.C., and Chambers, A.F., 2002, Cancer spread and micrometastasis development: quantitative approaches for in vivo models. Bioessays, 24(10):885-93.
6. Yoshida, B.A., Dubauskas, Z., Chekmareva, M.A., et al., 1999, Mitogen-activated protein kinase kinase 4/stress-activated protein/Erk kinase 1 (MKK4/SEK1), a prostate cancer metastasis suppressor gene encoded by human chromosome 17. Cancer Res, 59(21):5483-7.
7. Goldberg, S.F., Harms, J.F., Quon, K., et al., 1999, Metastasis-suppressed C8161 melanoma cells arrest in lung but fail to proliferate. Clin Exp Metastasis, 17(7):601-7.
8. Christiano, A.P., Yoshida, B.A., Dubauskas, Z., et al., 2000, Development of markers of prostate cancer metastasis. Review and perspective, 5(5):217-223.
9. Paget, S., 1989, The distribution of secondary growths in cancer of the breast. 1889. Cancer Metastasis Rev, 8(2):98-101.
10. Raj, G.V., Moreno, J.G., and Gomella, L.G., 1998, Utilization of polymerase chain reaction technology in the detection of solid tumors.[comment]. Cancer, 82(8):1419-42.
11. Cher, M.L., de Oliveira, J.G., Beaman, A.A., et al., 1999, Cellular proliferation and prevalence of micrometastatic cells in the bone marrow of patients with clinically localized prostate cancer. Clin Cancer Res, 5(9):2421-5.
12. Melchior, S.W., Corey, E., Ellis, W.J., et al., 1997, Early tumor cell dissemination in patients with clinically localized carcinoma of the prostate. Clin Cancer Res, 3(2):249-56.
13. Cameron, M.D., Schmidt, E.E., Kerkvliet, N., et al., 2000, Temporal progression of metastasis in lung: cell survival, dormancy, and location dependence of metastatic inefficiency. Cancer Res, 60(9):2541-6.
14. Kauffman, E.C., Robinson, V.L., Stadler, W.M., et al., 2003, Metastasis suppression: the evolving role of metastasis suppressor genes for regulating cancer cell growth at the secondary site.[comment]. J Urol, 169(3):1122-33.
15. Kyriakis, J.M., and Avruch, J., 2001, Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev, 81(2):807-69.
16. Pearson, G., Robinson, F., Beers Gibson, T., et al., 2001, Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev, 22(2):153-83.
17. Morrison, D.K., 2001, KSR: a MAPK scaffold of the Ras pathway?. J Cell Sci, 114(Pt 9):1609-12.
18. Lozano, J., Xing, R., Cai, Z., et al., 2003, Deficiency of kinase suppressor of Ras1 prevents oncogenic ras signaling in mice. Cancer Res, 63(14):4232-4238.
19. Yeung, K., Janosch, P., McFerran, B., et al., 2000, Mechanism of suppression of the Raf/MEK/extracellular signal-regulated kinase pathway by the raf kinase inhibitor protein. Mol Cell Biol, 20(9):3079-85.
20. Kyriakis, J.M., Banerjee, P., Nikolakaki, E., et al., 1994, The stress-activated protein kinase subfamily of c-Jun kinases. Nature, 369(6476):156-60.
21. Aguirre-Ghiso, J.A., Estrada, Y., Liu, D., et al., 2003, ERK(MAPK) activity as a determinant of tumor growth and dormancy; regulation by p38(SAPK). Cancer Res, 63(7):1684-95.
22. Kennedy, N.J., Sluss, H.K., Jones, S.N., et al., 2003, Suppression of Ras-stimulated transformation by the JNK signal transduction pathway. Genes & Development, 17(5):629-37.
23. Aguirre-Ghiso, J.A., Liu, D., Mignatti, A., et al., 2001, Urokinase receptor and fibronectin regulate the ERK(MAPK) to p38(MAPK) activity ratios that determine carcinoma cell proliferation or dormancy in vivo. Mol Biol Cell, 12(4):863-79.
24. Ip, Y.T., and Davis, R.J., 1998, Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. Curr Opin Cell Biol, 10(2):205-19.
25. Chekmareva, M.A., Hollowell, C.M., Smith, R.C., et al., 1997, Localization of prostate cancer metastasis-suppressor activity on human chromosome 17. Prostate, 33(4):271-80.
26. Sanchez, I., Hughes, R.T., Mayer, B.J., et al., 1994, Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. Nature, 372(6508):794-8.
27. Cuenda, A., 2000, Mitogen-activated protein kinase kinase 4 (MKK4). Int J Biochem Cell Biol, 32(6):581-7.
28. Yamada, S.D., Hickson, J.A., Hrobowski, Y., et al., 2002, Mitogen-activated protein kinase kinase 4 (MKK4) acts as a metastasis suppressor gene in human ovarian carcinoma. Cancer Res, 62(22):6717-23.
29. Xiao, L., and Lang, W., 2000, A dominant role for the c-Jun NH2-terminal kinase in oncogenic ras-induced morphologic transformation of human lung carcinoma cells. Cancer Res, 60(2):400-8.
30. Kim, H.L., Vander Griend, D.J., Yang, X., et al., 2001, Mitogen-activated protein kinase kinase 4 metastasis suppressor gene expression is inversely

related to histological pattern in advancing human prostatic cancers. Cancer Res, 61(7):2833-7.
31. Su, G.H., Hilgers, W., Shekher, M.C., et al., 1998, Alterations in pancreatic, biliary, and breast carcinomas support MKK4 as a genetically targeted tumor suppressor gene. Cancer Res, 58(11):2339-42.
32. Wu, C.W., Li, A.F., Chi, C.W., et al., 2000, Human gastric cancer kinase profile and prognostic significance of MKK4 kinase. Am J Pathol, 156(6):2007-15.
33. Chae, K.S., Ryu, B.K., Lee, M.G., et al., 2002, Expression and mutation analyses of MKK4, a candidate tumour suppressor gene encoded by chromosome 17p, in human gastric adenocarcinoma. Eur J Cancer, 38(15):2048-57.
34. Zheng, C., Xiang, J., Hunter, T., et al., 1999, The JNKK2-JNK1 fusion protein acts as a constitutively active c-Jun kinase that stimulates c-Jun transcription activity. J Biol Chem, 274(41):28966-71.
35. Lee, J.C., Laydon, J.T., McDonnell, P.C., et al., 1994, A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature, 372(6508):739-46.
36. Leone, A., Flatow, U., VanHoutte, K., et al., 1993, Transfection of human nm23-H1 into the human MDA-MB-435 breast carcinoma cell line: effects on tumor metastatic potential, colonization and enzymatic activity. Oncogene, 8(9):2325-33.
37. Cifone, M.A., and Fidler, I.J., 1980, Correlation of patterns of anchorage-independent growth with in vivo behavior of cells from a murine fibrosarcoma. Proc Natl Acad Sci U S A, 77(2):1039-43.
38. Robinson, V.L., Hickson, J.A., Vander Griend, D.J., et al., 2003, MKK4 and metastasis suppression: a marriage of signal transduction and metastasis research. Clin Exp Metastasis, 20(1):25-30.
39. Browning, D.D., Windes, N.D., and Ye, R.D., 1999, Activation of p38 mitogen-activated protein kinase by lipopolysaccharide in human neutrophils requires nitric oxide-dependent cGMP accumulation. J Biol Chem, 274(1):537-42.
40. Fritze, C.E., and Anderson, T.R., 2000, Epitope tagging: general method for tracking recombinant proteins. Methods Enzymol, 327:3-16.
41. Rinker-Schaeffer, C.W., Graff, J.R., De Benedetti, A., et al., 1993, Decreasing the level of translation initiation factor 4E with antisense RNA causes reversal of ras-mediated transformation and tumorigenesis of cloned rat embryo fibroblasts. Int J Cancer, 55(5):841-7.
42. Cole, W.H., Roberts, S., Watne, A., et al., 1958, The dissemination of cancer cells. Bull N Y Acad Med, 34(3):163-83.
43. Roberts, S.S., Watne, A.L., Mc, G.E., et al., 1957, Cancer cells in the circulating blood. Surg Forum, 8:146-51.
44. Roberts, S., Watne, A., Mc, G.R., et al., 1958, Technique and results of isolation of cancer cells from the circulating blood. AMA Arch Surg, 76(3):334-46.
45. Roberts, S.S., Watne, A.L., Mc, G.R., et al., 1958, The response of cancer cells in the circulating blood to surgical stress and chemotherapy. Surg Forum, 9:595-8.
46. Oefelein, M.G., Kaul, K., Herz, B., et al., 1996, Molecular detection of prostate epithelial cells from the surgical field and peripheral circulation during radical prostatectomy.[comment]. J Urol, 155(1):238-42.
47. Gao, C.-L., Rawal, S.K., Sun, L., et al., 2003, Diagnostic Potential of Prostate-specific Antigen Expressing Epithelial Cells in Blood of Prostate Cancer Patients. Clin Cancer Res, 9(7):2545-2550.
48. Fisher, B., Costantino, J.P., Wickerham, D.L., et al., 1998, Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study.[comment]. J Natl Cancer Inst, 90(18):1371-88.
49. Dong, J.T., Suzuki, H., Pin, S.S., et al., 1996, Down-regulation of the KAI1 metastasis suppressor gene during the progression of human prostatic cancer infrequently involves gene mutation or allelic loss. Cancer Res, 56(19):4387-90.
50. Natarajan, K., Mori, N., Artemov, D., et al., 2002, Exposure of human breast cancer cells to the anti-inflammatory agent indomethacin alters choline phospholipid metabolites and Nm23 expression. Neoplasia (New York), 4(5):409-16.
51. Natarajan, K., Mori, N., Artemov, D., et al., 2000, Phospholipid profiles of invasive human breast cancer cells are altered towards a less invasive phospholipid profile by the anti-inflammatory agent indomethacin. Adv Enzyme Regul, 40:271-84.
52. Yu, H.G., Huang, J.A., Yang, Y.N., et al., 2002, The effects of acetylsalicylic acid on proliferation, apoptosis, and invasion of cyclooxygenase-2 negative colon cancer cells.[comment]. Eur J Clin Invest, 32(11):838-46.
53. Lin, K.H., Wang, W.J., Wu, Y.H., et al., 2002, Activation of antimetastatic Nm23-H1 gene expression by estrogen and its alpha-receptor. Endocrinology, 143(2):467-75.
54. Liu, F., Qi, H.L., and Chen, H.L., 2000, Effects of all-trans retinoic acid and epidermal growth factor on the expression of nm23-H1 in human hepatocarcinoma cells. J Cancer Res Clin Oncol, 126(2):85-90.
55. Hartsough, M.T., Clare, S.E., Mair, M., et al., 2001, Elevation of breast carcinoma Nm23-H1 metastasis suppressor gene expression and reduced motility by DNA methylation inhibition. Cancer Res, 61(5):2320-7.

56. Yasui, W., Oue, N., Ono, S., et al., 2003, Histone acetylation and gastrointestinal carcinogenesis. Ann N Y Acad Sci, 983:220-31.
57. Ohtsuka, T., Buchsbaum, D., Oliver, P., et al., 2003, Synergistic induction of tumor cell apoptosis by death receptor antibody and chemotherapy agent through JNK/p38 and mitochondrial death pathway. Oncogene, 22(13):2034-44.
58. Ohtsuka, T., and Zhou, T., 2002, Bisindolylmaleimide VIII enhances DR5-mediated apoptosis through the MKK4/JNK/p38 kinase and the mitochondrial pathways. J Biol Chem, 277(32):29294-303.
59. Steeg, P.S., Ouatas, T., Halverson, D., et al., 2003, Metastasis suppressor genes: basic biology and potential clinical use. Clin Breast Cancer, 4(1):51-62.

Index

C

D

E

F

G

H

I

J

K

L

M

N

O

P

R

S

T

U

V