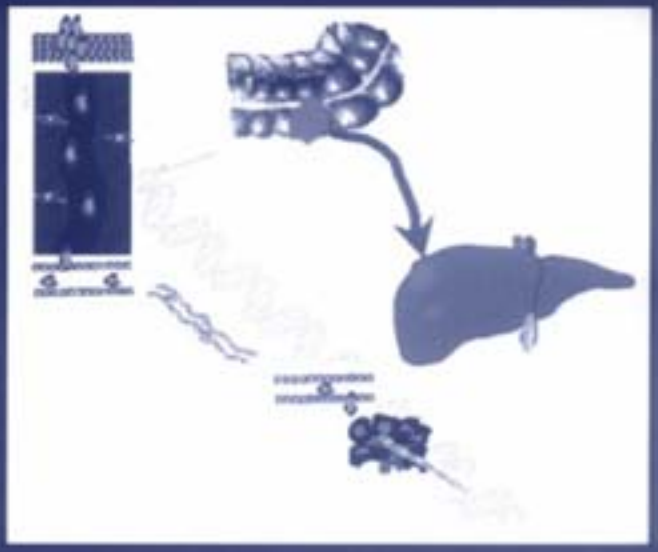
CANCER METASTASIS – BIOLOGY AND TREATMENT

Cancer Metastasis, Molecular and Cellular Mechanisms and Clinical Intervention

Edited by
Wen G. Jiang
Robert E. Mansel



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VOLUME 1

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Chapter 1

METASTATIC CASCADE, MOLECULAR/CELLULAR EVENTS AND NEW THERAPEUTIC OPPORTUNITIES

Wen G. Jiang and Robert E. Mansel

Key words: Metastasis, cell adhesion, matrix invasion, angiogenesis, motility factors, anti-motility

Abstract: Metastasis is the most life threatening event in patients with cancer. The process is composed of a number of sequencial events, of which tumour cells have to successfully complete. The understanding of the biology of the process has enabled development of certain therapeutic measures that may eventually lead to the design of new anti-metastasis therapies.

INTRODUCTION

Metastasis, the spread of cancer cells to tissues and organs beyond where the tumour is originated and formation of new tumours (secondaries and tertiaries), is the single event that results in death of most of patients with cancer. At the time of diagnosis of cancer, at least half of the patients already present clinically detectable metastasis (1). A much higher proportion of the patients will have micrometastasis that temporarily beyond conventional detection.

The process of metastasis has made cancer as a more multiplex disease. Metastasis, although sometimes simply viewed as the same tumour re-occurring in different organs, is very complex in nature. When tumour is formed, malignant cells in the primary tumour will dissociate from the mass, at an unknown rate, and travel in the body until they settle in a distant organ to develop secondary tumours. The process, known as metastatic cascade, is composed of a number of separate yet highly relevant steps, that a tumour cell must complete, in order that it can reach the target organ. The most representative steps include the loss of cell-cell adhesion capacity, degradation and invasiveness to the basement membrane and extracellular matrix, capability to successfully enter the blood stream and survive in the circulation, identification of suitable organ to settle, and eventually leaving the circulation (figure 1). The process is also heavily relies on the development of new blood vessels (angiogenesis) into the new

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site, in order to overcome the dormacy. Certain genes that are associated with metastasis have also been identified. This chapter will examine the major events in metastasis process and discuss new therapeutic opportunities that may arise as a results of the understanding of the biology of cancer metastasis.

CELL ADHESIONS

Cell adhesions play an essential role in the normal physiology as well as in the pathology of a number of diseases. From a functional point of view, there are three main types of cell junctions in epithelium, namely tight junction, anchoring junction and communicating junction (gap junction) (figure 1). The communication junction is mainly involved in the information dissemination and plays less important role in cell adhesion. Tight junctions, the topical most structure in epithelial and endothelial cells, creates a physiological intercellular barrier to maintain distinct tissue spaces and to separate the apical from the lateral plasma membranes. Its role in cell-cell adhesion is increasingly recognised. The anchoring junctions connect the cytoskeleton of cells to the cytoskeleton of their neighbouring cells or the extracellular matrix (ECM). Junctions connecting cells are adherens junctions (mediated by E-cadherin, details covered by Noe et al in this book, Chapter 5) and desmosomes and connecting cells and matrix are hemidesmosomes and cell-matrix adherens junctions (including the focal contacts).

Four groups of cell adhesion molecules form these key cell-cell and cell-matrix adhesions. These include the cadherin adhesion family, integrin family, immunoglobulin superfamily, and others, such as selectin and CD44. These molecules control the overall cell adhesion in physiological as well as pathological conditions, such as in cancer. The importance of these structure and molecules in the process of cancer invasion and metastasis have been established. Chapter 2, 3, and 4, of this book, has separately covered the aspects of these structures in cancer metastasis.

The critical role of these cell adhesion structure in metastasis has offered therapeutic opportunities in cancer intervention. Some of these options are summarised in the following.

Promotion of Cell-Cell Adhesion

Cell-cell adhesion molecules, typically cadherin family members and desmosomal members, are essential for cancer cells to adhere to each other and to remain in the tumour as a mass. Loss or dysfunction of these molecules will result in the increase of cell invasiveness and motility. These molecules have been firmly demonstrated to act as metastasis inhibiting molecules in clinical studies (2-4). Compelling evidence indicates a reverse relationship between these molecules and capability of invasion/ metastasis. Thus, agents capable of up-regulating the expression and/or the function of these molecules may have a role in the intervention of cancer metastasis. Some of the agents are known to have such properties, including gamma linolenic acid (GLA), tamoxifen, IGF-I, oestradiol, retinoic acid, and some hormones (for reviews see refs 3,5, and chapter 5).

Another critical group of adhesion molecules in endothelial cells is vascular endothelial cadherin, also known as cadherin 5. VE-cadherin is a key structure in the maintenance of the integrity and function of the endothelium. It also play a role in the angiogenesis and prevention of cancer cell penetration to the endothelium lining. Breach of intercellular adhesion structures such as VE-cadherin are known

to associate with the increased invasion of endothelium by cancer cells (6). Thus manipulation of endothelial adhesions may also a useful approach in the prevention of cancer extravasion. Little has been reported on agents that are able to up-regulate the function or the level of the molecule. A lipid, gamma linolenic acid has been demonstrated to have such a function (7).

Inhibition of Extracellular Matrix (ECM) Degradation

The hallmark of a malignant tumour is the ability of cancer cells to invade the basement membrane and subsequently blood vessels and the lymphatics. The basement membrane consists of collagen IV, laminin, fibronectin, elastin, heparan-sulfate proteoglycans and sometime collagen V. The invasion of these structures may be the results of a mechanical force and enzymatic degradation of the structure. The latter is primarily involved in the release of an array of matrix proteolytic enzyme.

The ECM proteins are degraded by extracellular or cell associated proteolytic enzymes that are secreted by both tumour cells and stromal cells and belong to two classess: *metalloproteases* (collagenases etc.) which are a family of zinc-dependent enzymes that degrade all of the major components of the extracellular matrix, and *serine proteases* (urokinase-type plasminogen activator, or u-PA) which have a highly reactive serine residue in their active site (details of these enzyme can be seen in Chapter 5 by Noe and collegues). MMP family members contain a HEXGH motif in the active site and a PRCGVPD sequence in the pro-domain that maitain enzyme latency. The activation of the enzymes is via a mechnism known as cysteine switch (8), in which cleavage of the pro-domain destablizses the inhibitory interation between the unpaired cysteine in the PRCGVPD sequence and the active site zinc atom.

In addition to MMPs and uPA system, enzymes that degrade other important components of the basement membrane, namely heparan sulfate proteoglycan (HSPG), have been recently cloned (9,10). These mammalian heparanases have been shown to play an very important role in tumour progression and metastasis and may present a new target for therapy.

Under normal circumstances, the matrix degradation are controlled by certain mechanisms including: (i). Secretion of inactive form of enzymes which are activated locally and necessarily; (ii). Regulation of enzyme activities by secreted protease inhibitors, tissue inhibitors of metalloproteases (TIMPs), which will bind to activated enzymes and block their activity; (iii). Cell surface receptors for proteases (such as u-PA) and membrane associated enzymes (such as MT-MMPs) thereby confining the enzyme to where it is needed.

Once the MMPs are activated, they will cleave a varieaty of extracellular matrix protein which will create not only a path for cancer cells, but also facilitates the migration of cancer cells over matrix (11). Of these enzymes, membrane associated MMPs (MT-MMP) are considered to be of particular interest in the metastasis (12,13), as they are involved in the activation of latent form of MMPs. MT-MMPs can also be activated by cyclooxygenases (14). Stromal MMPs, such as MMP3 can also induced highly invasive genetype of cancer cells, when over-expressed (15).

The balance between MMPs and their inhibitors (tissue inhibitors of metallo-proteinases, TIMPs) is altered in neoplasia and this contributes to the invasive and metastatic properties of malignant tumours (16,17).

Cellular invasion depends on co-operation between adhesive and proteolytic mechanisms. A single cell-surface receptor may regulate both matrix degradation and motility, thereby facilitating directed cellular invasion (18,19). For example, αvß3 integrin on cultured melanoma cells enable the binding of the cells to MMP-2 in a proteolytically active form, facilitating cell-mediated collagen degradation (18).

These MMPs and integrins are also specifically co-localised on angiogenic blood vessels and melanoma cells *in vivo.* Stromal cells also contribute to the degradation of matrix by secreting stromelysins. Intervention for this cell-matrix adhesion and subsequent degradation and invasion can therefore aim at targeting adhesion and degradation.

Matrix degradation inhibitors

The importance of proteolytic enzymes have led to numerous discoveries in order to suppress these enzymes and hitherto inhibit matrix invasion and metastasis.

Batimastat and Marimastat.

Batimastat (BB-94) and marimastat are amongst the first few synthetic metalloproteinase inhibitors for clinical use. Batimastat, otherwise known as BB94 or (4–N-hydroxyamino)-2R- iso butyl-3s-(thiopen–2-ylthiomethyl)–succinyl–1–phenylalanin–n-methylamide, is a synthetic broad spectrum metallo-proteinase inhibitor. It is a low molecular weight peptide analogue of the peptide residues on one side of a principle cleavage site in type I collagen. The peptide backbone binds to matrix metalloproteinases and a hydroxamic acid group binds to the catalytically active zinc atom (20,21).

Both inhibitors are known to act on MMPs, including MMP-1, MMP-2, MMP-3, MMP-6, MMP-7 and MMP-9. Inhibition of the activity of collagenases and other MMPs by Batimastat have been reported (22,23). These enzymes are critical in the matrix degradation and invasion by cancer cells, in the process of angiogenesis and angiogenesis switch, cytokine receptor shedding and development of cancer metastasis (24,25,26,27,28). It has no direct effect on the growth and proliferation of cancer cells. A particular interest is its effects on endothelial cells and angiogenesis (21,25), in that Batimastat had no effects on the proliferation of endothelial cells, but inhibited endothelial invasion through extracellular matrix. Indeed, some of the anti-tumour effects of Batimastat were thought to be via its inhibition on angiogenesis (25).

Furthermore, Batimastat may be more effective as a prevention and intervention means in the angiogenic switch, rather that used as end stage cancers (25). Administration of Batimastat at prevention and intervention stages results far favourable results than when delivered at progression stage (32,33,34,35,36,37).

Clinical trial to deliver these inhibitors via SC, IP, and intropleural routes have been reported and some beneficial effects were already seen (29, 31, 38,39,40,41,42)

Another metalloproteinase inhibitor, AG3340, with pM affinities for inhibiting gelatinases (MMP-2 and -9), MT-MMP-1 (MMP-14), and collagenase-3 (MMP-13) in many tumor has been shown to have clinical promise in a recent study (43). While the drgus was seen to be well tolerated in a tumour model, a groad delay in tumour growth, reduction of angiogenesis and tumour progression were seen.

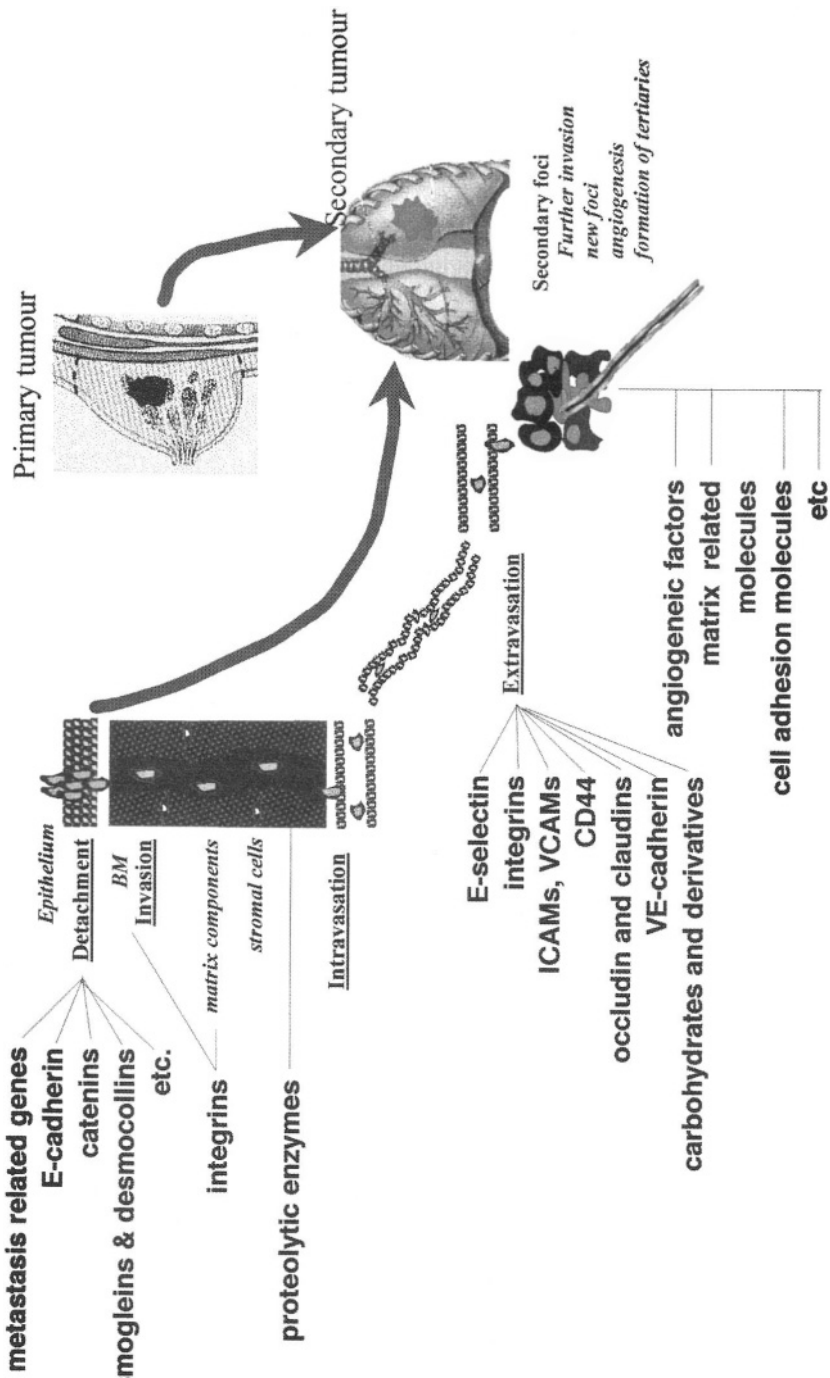

Figure 1. Metastatic cascade

To achieve inhibition of matrix degradation, the following strategies can be used: (i). The inhibitory agents directly delivered to cells/host; (ii). Antisense oligonucleotides to remove u-PA receptor(44) and MMPs (MMP-7)(45); (iii). Transfection of tumour cells with MMP inhibitors. Transfection of cells with TIMP-1 and TIMP-2 have been shown to effectively inhibit the metastatic potential of tumour cells (46,47); (iv). Antibodies to proteolytic enzymes. An antibody to uPA has been shown to inhibit the invasion and formation of metastasis both *in vitro* and in animal studies (48); (v). Inhibition of receptor bound u-PA by means of UTI, plasmin inhibitors [α2-anti-plasmin (α2AP) and α 2-macroglobulin (α2M)] (49,50).

Anti cell-matrix adhesion:

A crucial cellular behaviour during the matrix invasion is the interaction of cells with extracellular matrix. This interaction requires a group of transmembrane protein, integrins which are composed of α and ß units. Different combinations of α and ß units form different receptors for ECM. Inhibition of such interaction will stop other down stream interactions, i.e. matrix degradation and invasion (detailed structure and functions of integrins can be seen in Chapter 2).

The adhesion blockade can be achieved by: (1) antibodies to integrins which are specifically required by tumour cells to interact with ECM. Those reported antibodies include anti-α2, anti-ß1, and anti-α5 integrins (51,52,53). These antibodies block the cell-matrix interaction and reduce tumour colonisation in the lung. (2). Arg-Gly-Asp (RGD) and related peptide: RGD sequence was firstly identified as the active component of fibronectin(54). Peptides containing RGD sequence will bind to members of the integrin family and inhibit further interaction of integrins with ECM components. A variety of peptides based on RGD have been described. These have been shown not only to inhibit cell-matrix interaction, migration, matrix degradation and invasion, but also tumour cell proliferation and subsequently tumour growth and metastasis (54,55,56,57,58). (3). Peptides containing other ECM active sequences: These include laminin peptide, type V collagen peptide and thrombospondin peptide (59,60). (4). Antibodies to ECM components: monoclonal antibody to thrombospondin shows inhibitory effects on tumour-platelet aggregation (61).

ANTI-MOTILITY

Motility of tumour cells is an essential requirement for the completion of metastasis cascade. From cell-cell dissociation, matrix invasion, intra- and extravasation, to the final settling in the host organ, motility is crucial to allow cells to complete these steps. Motility of cancer cells can be stimulated by motility factors (also known as motogens) (62). Means that are anti-motility may therefore be useful options in anti-invasion and metastasis.

Rho/rac family members are among the most important signalling intermediates in the migration and motility of cancer cells. Members of the family, such as rho and p160ROCK, are known to involve in the formation of intrahepatic metastasis, prosumably due to their involvement in the highly motile properties of cancer cell under these circumstances (63).

Invasion inhibiting factor 2 (IIF-2):

Invasion inhibiting factors (IIFs) are identified from extract of liver. The protein IIF-2, which shares homology with high motility group -14/17 (HMG 14/17), has been found to inhibit motility

and *in vitro* invasion without detectable changes in E-cadherin, cell-matrix interaction, cell growth, fibronectin receptor, cytosolic free calcium; vimentin and actin and angiogenesis (64,65,66,67). A range of human and animal cell lines have been found to be responsive to IIF-2: melanoma and fibrosarcoma, colon cancer, lung cancer and breast cancer. Clinical application of the factor is yet to be explored.

MRP–1 (motility related protein-1)

MRP–1 shares similarities with CD37, ME491 (melanoma-associated antigen), TAPA-1 (antiproliferative antibody), CO-029 (a human tumour associated antigen), CD9, and sm23 antigen. MRP-1 has been shown to inhibit motility of various cell types (68,69).

Motility factor antagonists

The use of functionally inactive antagonists of motility factors has been explored in recent years. HGF antagonists have recently been reported, which compete with receptors for HGF binding but without inducing any biological effects (refer to Chaper 6). Motility factor receptor (anti-EGF receptor) antibody has been shown by Mueller (70) to suppress melanoma metastasis. Antibodies against a cell surface glycoconjugate have been shown to inhibit tumour cell motility and tumour metastasis indicating the potential usefulness of anti-motility agents in cancer treatment (71). Antibodies against integrin receptors on the cell surface have been shown to inhibit cell migration, invasion and tumour metastasis (72,73).

Anti-motlity signalling events.

Motility factors are known to stimulate cell migration via G-protein-coupled IP3. L651582 is known to inhibit IP3 and has been shown to suppress motility factor induced motility (74).

ANGIOGENESIS, TUMOUR DORMACY AND ANTI-ANGIOGENESIS OPTIONS:

New vessels originated from existing small vessels in the surrounding tissues (angiogenesis) is a prominent feature in metastatic tissue. The growth of a solid tumour and metastatic tumour can be grossly divided into the dormant phase and rapid growth phase. Dormant phase is the slow growth peroid of a tumour, which can go on for years and achieve only small size (<2mm) and in small number of cells (10^6). The turning point between the dromant phase and rapid phase is the beginning of neovasculatures and provide blood supply to tumour mass, the process known as angiogenesis.

The process is regulated by a range of factors (angiogenic factors, AFs) released from surrounding tissues, stromal cells and tumour cells. Currently, a large number of factors are know to regulate angiogensis, potential ones including VEGF (vascular endothelial growth factor, also known as vascular permeability factor or VPF), bFGF, IL-8, HGF/SF, angiopoietins, and some components from matrix proteins and their degraded products. The number of angiogenic factors are expanding an extraordinary rate, making it imposssible to provide a complete list. However, it has also be recognised that angiogenesis in cancer is not only a matter of over-expressing or over-production of angiongenic factor, there are a number of factors to be considered in angiogenesis.

1. Excess amount of angiogenic factors available to endothelial cells.
2. Reduction of inhibitors (endogenous or exogenous) of angiogenesis. This is particularly interesting as these inhibitors, together with angiogenic factors regulate the angiogenesis switch. It has been shown lately that

intervention at different stages of tumour progression with angiogenic inhibitors may have different outcome in tumour progress (25).

Tumour cells can remain invisible for many years, before suddenly switch to a phase with very rapid growth rate and become clinically visible. The trigger of the growth can be those of known procedures such as surgical removal of tumours or known. This phenomian, known as tumour dormacy, in many aspect has been recognised as closely associated with the angiogenesis process (75,76). While the detection of these micro-metastatic foci is importatant (77) and has been covered in Chapter 9, the dormancy has presented some enormous new opportunities in the treatment of cancer (78).

The progress in understanding the biology of angiogenesis has given rise to a number of opportunity to discover and re-discover new therapeutic approaches in cancer treatment. A large number of anti-angiogenic agents have been identified and the list is ever increasing. It is not possible to give a full list of these agents.

Anti-angiogenesis factors

The tranditional ones include Fumagillin and derivative, thrombo-spondin-1, interferon alpha and beta, angiostatin, platelet factor 4 and its fragments, endostatin, vasostatin, vascular endothelial growth inhibitor (VEGI).

Other anti-angiogenesis agents that have direct or indirect effects on endothelial cells include αvß5 integrin antagonists, VEGF receptor antagonists, Tie-2 receptor antagonsits, IL-12. Recent additions to the list include hyluronic acid (covered in Chapter 4), Macrolide antibiotics (including 14-membered ring members roxithromycin and clarithromycin have been demonstrated to suppress angio-genesis and reduce metastasis in melanoma models) (80), nitric oxide synthase inbitors such as N-G-nitro-L-arginine methyl ester, has been shown to inhibit tumour induced angiogenesis (81).

These agents may work on more than one stage of the angiogenesis process (79). A range of chemotherapeutic agents that are toxic to cancer cells are also known to be anti-angiogenic. The combination of chemotherapeutic drugs, with or without anti-angiogenic activities increase the efficacy of the treatment. Combinations using retinoids and IFNα; retinoid and 1,25-dihydroxyvitamin-D3; TNP-470 and 5-FU, mitomycin C, adriamycin, cisplatin, TNP-470 and minocycline, TNP 470 and IFN-α have been demonstrated to have synergistic effects on angiogenesis and therefore on tumour growth and metastasis

Anti-angiogenic factor antibody

It has been demonstrated by Arenberg (82), that IL-8 is an angiogenic factor which promotes tumour growth and metastasis. Passive immunisation with neutralising antibodies to IL-8 results in significant reduction in tumour size and lung metastasis. This reduction is associated with a decline in tumour-associated vascular density and angiogenic activity. Antibodies to vascular endothelial growth-factor (VEGF) and vascular-permeability factor (VPF) also inhibit ovarian carcinoma-associated ascites formation and tumour-growth by their inhibition of angiogenesis (83,84). Antibodies to αv β3 integrin reduce angiogenesis and tumour growth (85). Antibodies against angiogenin, a potent angiogenic factor, have also been shown to reduce tumour growth and metastasis by reduction of angiogenesis (86). Furthermore, antibodies to bFGF also exert strong anti-angiogenesis activities.

New formulation

A novel approach by chemically linking recombinant vascular endothelial growth factor (VEGF) to a truncated diphtheria toxin molecule (DT385) has been reported. The new chemical binds and thereafter inhibits the proliferation of endothelial cells due to the VEGF-receptor binding which lead to the delivery of toxin to the cells. *In vivo,* this conjugate shows marked reduction of angiogenesis in tumour (87).

Genetic approaches

Oncogenes (*ras*) are associated with increased expression of angiogenic factors and therefore can be targets in treatment. Genetic disruption of the mutant K-*ras* allele in human colon carcinoma cells results in reduction in VEGF/VPF activity. Disruption of mutant *ras* protein function in H-*ras* transformed rat intestinal epithelial cells by treatment with L-739,749 (a protein farnesyltransferase inhibitor) causes a significant suppression of VEGF/VPF (88).

It has been reported that the combination of anti-angiogenic factors, which are generally less toxic, with cytotoxic drugs and/or with more than one anti-angiogenic factor will achieve additive effect in the reduction of metastasis compared with use of either agent alone. The combination of anti-angiogenic factor such as retinoids and IFNα; retinoid and 1,25-dihydroxy-vitamin-D3; TNP-470 and 5-FU, mitomycin C, adriamycin, cisplatin, TNP-470 and minocycline, TNP 470 and IFN-α may have synergistic effects on angiogenesis and therefore on tumour growth and metastasis

Anti-tumour-endothelial interaction and endothelium invasion

Tumour cells interaction with endothelium forms the most important mechanism in extravasation. Gap junctions are assembled at focal adhesion contacts between tumour cells and endothelial cells during their interaction. These junctions mediate metabolic coupling between the junction-forming cell pair. Significantly increased adhesion and communication levels in highly lung-metastatic carcinoma cells play a role in gap junctional coupling in cancer metastasis. Antibodies to this adhesion for example anti-Lu-ECAM-1 and soluble Lu-ECAM-1 may have roles in this anti-metastasis option. Transcription factor nuclear factor kappa B (NF kappa B) controls gene expression of a number of genes including cell adhesion molecules such as E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1), which are molecules for adhesion of tumour cells to the venous or capillary bed of the target organ. Anti-NF kappa B reagents such as N-acetyl L-cysteine, N,N,N-trimethyl-sphingosine, aspirin, or pentoxifylline have been reported to inhibit tumour cell adhesion to vascular endothelial cells and therefore may interfere with this important step in metastasis formation (89,90). Binding of the tumour cells to endothelial cells can be inhibited by monoclonal antibodies specific for different VLA molecules anti-α6ß1 and anti-α5ß1 (91).

Factors, such as osteonetin, may aid the organ specific settling of metastatic cancer cells. In a recent study, Jacob et al (92) decribed that factors promoted tumour cells chemotaxix, invasion and metalloproteinase activity from bone marrow extracts is osteonectin.

CYTOKINES AND ANTI-CYTOKINES

The role of cytokines in the invasion and metastasis has been controversial. This is partly due to their diverse range of biological functions depending on the cell type, cytokine type, receptor type etc.

Those promoting invasion and metastasis include IL-6, HGF/SF (ref 93 and Chapter 6). Strategies using cytokine to combat invasion and metastasis or to reverse those promoting cytokines have been explored.

Interleukin-4

IL-4 has been shown to markedly reduce lung metastasis of renal carcinoma and extend survival in animal studies. Although CD8(+) T cells or AsGM1(+) cells are indicated to be the possible effector cells, IL-4 may also inhibit monocyte/macrophage functions, for example proinflammatory cytokine secretion (94).

Interleukin-12

IL-12 is also known as cytotoxic lymphocyte maturation factor (CLMF) or natural killer cell stimulatory factor (NKSF), which is produced primarily by stimulated macrophages. IL-12 is a disulfide- linked heterodimeric cytokine composed of a 35-kDa light chain (p35) and a 40-kDa heavy chain (p40). This cytokine has very recently been found to have anti-invasion and anti-metastasis properties both *in vitro* and *in vivo* (95-98). The mechanisms may be as follows: Up-regulation of the expression of E-cadherin; Anti-angiogenesis; Modulation of cellular immunity. It directly stimulates activated NK and T cells to produce high levels of IFN-γ, enhances their cytolytic activity, and promotes maturation of Th 1 cells as well as IL-2-activated B cells (99,100). Infusion of recombinant IL-12 or using genetically engineered cells which produce IL-12 have been explored and showed to inhibit tumour growth and metastasis and may also modify survival of tumour bearing animals. NK cells and IFN-γ play important roles in the development of the early phase of the antitumour response, but that T cells (both CD4(+) and CD8(+)) play the major role in the subsequent events, leading to long-term immunity in this response of IL-12 (97,98,101,102).

Interleukin-10

IL-10 is produced by lymphocytes and monocytes and has strong effect on monocyte/macrophage. It has been shown *in vitro* that IL-10 inhibits the invasive and metastatic parameters (103). In animals injected with IL-10 transfected cells, tumour growth/metastasis and macrophage infiltration are markedly reduced (104). This effect has perhaps best been shown by Kundu et al (105) and Huang (106).

Anti-invasion/metastasis promoting cytokine and their receptors

Since certain cytokines promote invasive and metastatic behaviour of cancer cells, strategies aimed at against these cytokines and their receptors may therefore have role to play.

Antibodies to TGFß have been shown to have strong anti-metastasis effect. This may be a mixed effect on tumour cells themselves and on immune cells (107). Anti-EGF receptor antibody shows inhibition on the metastasis in animal studies (108). A recombinant humanised monoclonal antibody (rhuMAb) to *her*2 proto-oncogene, a 185-kd growth factor receptor has been shown to exert anti-metastatic effects and at the same time be well tolerated by patients with metastatic breast cancer (109, 110). Antibody (2B1) to c-erbB-2 and Fcγ RIII extracellular domains shows beneficial effect both in animals bearing metastatic tumour and patients with metastatic colon disease (111).

Inteferons

Inteferons (−α, β, γ) have been tested for their anti-invasive properties in choriocarcinoma cells. Only IFNß shows inhibition of 72 kd gelatinase activity and reduces invasion *in vitro* (112).

ANTISIGNALLING

Intracellular signalling events may also be targets for anti-metastasis treatment and this has raised significant interest. However, the signalling events involved in invasion and metastasis are not specific to metastasis and it will be a great challenge to identify parts of the pathway which are suitable for therapeutic intervention. Efforts are being made to manipulate the signalling pathways, modify the chemical structure of mediators (G proteins for example), and geneticaly modify the mediators and receptors. Inhibitors of tyrosine kinase and other intermediates (H-7, staurosporine, calphostin, erbstatin, herbimycin) have also been explored (113, 114, 115,116). Since it has been shown that arachidonic acid lipoxygenase metabolites are second messengers for cell spreading (113,117), blocking the production of these metabolites by various inhibitors may be another approach for the treatment of cancer.

A recent study has shown that ERK1/ERK2, extracellular signal regulated kinases, regulate the trascription factor AP-1, which further participates the trascription regulation of hydrolases. An inhibitor to ERK1/ERK2, PD 098059, is able to reduce the invasiveness of cancer cells (118).

NATURALLY OCCURRING PRODUCTS:

The nature has provided us plenty of natural ingredients that may be useful in combating cancer and cancer metastasis. A list of these naturally occurring compounds have been reviewed elsewhere (119). These compounds extend from ginsen to green. They are generally less toxic than existing anticancer therapies but there clinical applications need to be further explored. Mechanisms of anti-invasion and metastasis by natural products may include:

(i). Anti-cell-matrix interaction and matrix degradation:

The anti-metastasis effect of ginsenoside-RG3 may contribute at leas, t partly to its inhibition of cell-matrix adhesion (120). Anti-invasion effect of cinnamic acid occurs by modulation of tissue inhibitor of metalloprotease 2(121). The inhibition of invasion and metastasis by ursolic acid is due to the inhibition on the expression of MMPs (122).

(ii). Anti-angiogenesis

The anti-metastasis effect of ginsenoside-RG3 may be attributed at least partly to its inhibition of angiogenesis (120). Other parameters are yet o be studied.

EGCG and green tea

Recent progress on the EGCG is worthnoting. Drinking green tea have been for years postulated to has some beneficial effect on the spread of cancers (123). Extracts from the green have been shown to inhibit angiogenesis and invasion (124, 125). A compound extracted from green tea, epigallocatechin-3-gallate (EGCG), has been shown to act as invasion inhibitor, by suppressing the synthesis of metalloproteinases (MMP-2 and MMP-9) (126).

OTHER AGENTS EXERTING ANTI-INVASION/ METASTASIS PROPERTIES

Cicaprost, a stable prostacyclin analogue, has been shown to be anti-metastatic in a series of metastasising

rodent tumours (127, 128, 129). Prostacyclin, certain polyunsaturated fatty acids also have antimetastasis activities (Chapter 7). A new lipidn know as A OM 174 , has been shown to possibly eradicate metastasis in animals (130).

While our understanding of the molecular and cellular biology of cancer metastasis have expanded at a fast pace, new therapeutic opportunities have thus arisen. Although most of the above mentioned products are at their early stage of research and development, some will undoubtedly be proved to be useful and bring benefits to clinical practice. Some of most recent available ones include matrix degradation enzyme inhibitors, anti-angiogenesis drugs, which are in their late stage trials.

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Chapter 2

THE ROLE OF INTEGRIN-MEDIATED PROCESSES IN THE BIOLOGY OF METASTASIS

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Key words: Integrin; Signalling; Adhesion; Migration; FAK; PTEN

Abstract: In order for tumour cells to metastasise they must proceed through a series of steps requiring adhesion and de-adhesion to both the underlying matrix and adjacent stromal cells. Thus understanding the molecules which mediate these adhesive processes is essential if we are to understand the mechanisms of metastasis completely. Integrins, which are one of the major families of cell adhesion molecules, mediate binding of cells to extracellular-matrix glycoproteins and also to the surface of other cells. Subsequent to ligand-binding the integrins generate intracellular signals which promote a variety of processes including survival, proliferation, migration and protease production. It is not surprising therefore that evidence accumulated over the last ten years suggests that integrins could play an active role in tumour progression and metastasis. Such a role may be direct, through promoting a better survival and invasive phenotype of the tumour cells themselves, or indirect, by promoting the growth of angiogenic blood vessels. In this review we provide an overview of integrin structure and function and discuss how integrin-dependent signals could affect the metastatic phenotype. We also discuss evidence that abnormally regulated integrin-signalling appears to be a contributory factor in the development of cancer. This is exemplified by the discovery of the tumour suppressor, PTEN, a dual-specificity phosphatase which regulates several integrin-promoted signalling pathways, whose loss is associated with the development of cancer in both experimental animals and in humans. Thus understanding integrin-regulated signalling processes, in addition to providing a deeper understanding of metastasis, may also reveal novel targets for anti-cancer therapy.

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INTRODUCTION-ADHESION AND METASTASIS

Cell adhesion plays a vital role in normal cell function, including embryonic development, immune function and wound healing as well as in pathological processes such as inflammation, thrombosis and tumour metastasis (1-4). Metastasis is the principal reason for death from cancer. This phenomenon is defined as the transfer of malignant tumour cells from the primary site of growth to another organ or part of the organ not directly connected with it. Since as many as 50% of patients who present with solid cancers already have metastases (5) it is hoped that an understanding of the mechanisms involved in the biology of the process may enable us to develop novel strategies for treatment.

While cancer can develop from many different tissues the process of metastasis seems to proceed through a consistent pattern which can be described as occurring in a series of adhesion and de-adhesion events (Figure 1).

For optimal growth normal cells, and possibly primary tumour cells, require both growth factor-mediated and adhesion-dependent signals. In order for cells to leave the primary tumour mass and disseminate there must be a reduction in their normal adhesion to other cells as well as changes in their adhesion to the surrounding extracellular matrix. The migration of cells through matrix and across vessel walls allows dissemination via the lymphatic system and the vasculature and is dependent on stable adherence and traction produced by adhesion molecules and their ligands. Arrest in distant vessels may be facilitated by homotypic adhesion of tumour cells or adhesion to lymphoid cells/platelets resulting in multicellular aggregates that increase the possibility of entrapment in capillaries (6). Alternatively, or in addition, specific adherence of the malignant cell to receptors on the lumenal surface of the circulatory system in the distant organ may promote arrest and ultimately extravasation. Development of the metastatic deposit will occur only if the local environment can provide sufficient nutrients (including oxygen and growth factors) and a suitable ECM substrate thus suggesting that some adhesion-dependent signals may need to be initiated for optimal growth of the metastases.

Cell adhesion is mediated by several families of cell-surface expressed molecules, including the cadherins (7), immunoglobulin superfamily (8), selectins (9) and the integrins (1). In this report we will only address the role of integrins in metastasis while acknowledging the other families may play an equally important role.

The integrins are the major family of adhesion molecules responsible for adhesion to components of the ECM but they also mediate cell-cell binding via interactions with members of the immunoglobulin and cadherin families (Table 1). Although the physical aspects of integrin function in which the integrin-ligand interaction provides anchorage for stable adhesion or traction for cell propulsion are essential, it also is clear that intracellular signals generated by integrin ligation can regulate cellular functions such as proliferation, apoptosis and migration; all processes which may promote metastasis. In this review we shall describe integrin structure and function briefly, provide an overview of the intracellular processes generated by integrin-dependent adhesion and how these signals may promote a more metastatic phenotype.

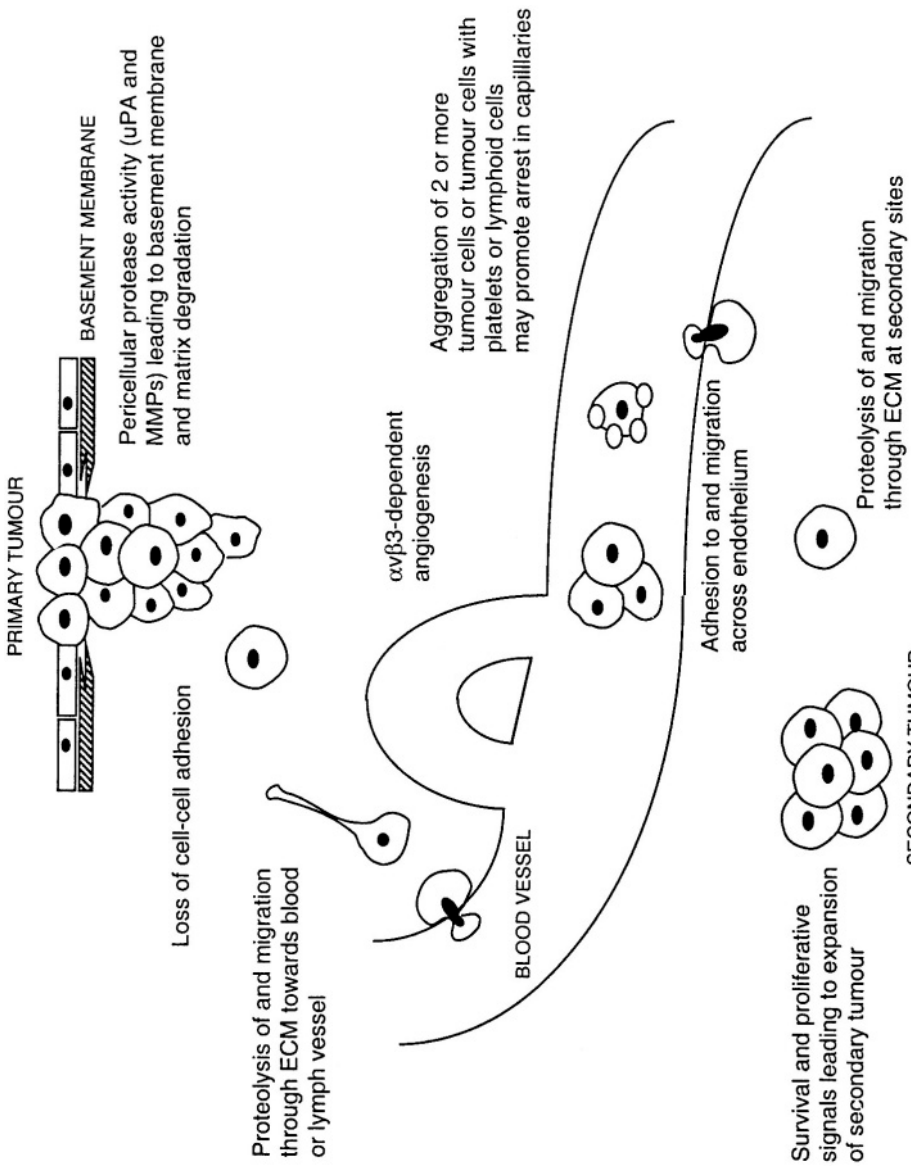

Figure 1. The metastatic process: potential integrin-dependent mechanisms

INTEGRIN STRUCTURE AND FUNCTION

Integrin Molecules

The integrins are a family of heterodimeric, transmembrane glycoproteins each consisting of a non-covalently linked α and β subunit (1). To date 16 α and 8 β subunits have been described which combine to create more than 20 heterodimers (Table 1; Figure 1). Whilst some of the subunits have so far been shown to produce only one heterodimer several dimerise with more than one other subunit and some produce many combinations including for example αv, β1 and β2 (Table 1). Most cells express at least one, and usually several, integrin heterodimers while some integrins are tissue specific, e.g. β2 integrins are expressed only on leukocytes

Each α or β subunit consists of an extracellular, transmembrane and cytoplasmic domain and both α and β subunits contain domains in the extracellular region which are involved in ligand interactions. The extracellular N-terminal domain of α subunits contain up to seven homologous repeat sequences. Three (and sometimes four) membrane proximal repeats have divalent cation binding sites (10) and bear a sequence homology to the calcium-binding EF hand motif found in calmodulin (11). Regions within these cation-binding sequences or in the proximal N-terminal repeat have been shown to interact with ligands (12-14). Several α subunits have a domain of about 200 amino acids which has sequence homology to the A-domain found in von Willebrand factor and is inserted between the second and third N-terminal repeat sequences. This domain, termed either A- or I-domain, has been shown to interact with ligand in a cation-dependent manner (15, 16). The β subunits also contain a conserved region related to the von Willebrand A-domain (17). Mutation of conserved aspartate residues in the A-domain of β1 or β3 integrin subunits results in loss of ligand binding and indicates the importance of the A-domain in integrin ligation (18, 19).

Divalent cations are essential for integrin function. In addition to the cation binding sites previously described in the EF-hand like domains of the integrin α subunit, both α- and β-A-domains contain cation binding sites (10, 20, 21). Whilst it has been proposed that the cation forms a bridge between integrin and ligand (10, 20-22)there is evidence for an alternative mechanism involving at least two cation binding sites, one of which is ligand-competent whilst the other is regulatory (23). This is believed to explain the positive and negative regulation of integrin activity exhibited by divalent cations. Thus Ca^{2+} has been shown to have an inhibitory effect on ligand binding (24) while a ligand-dependent order of preference for different cations is exhibited by certain integrins; for example α4 has a preference for $Mn^{2+}>Mg^{2+}>Ca^{2+}$ for binding to CS-1 and $Mn^{2+}>Mg^{2+}=Ca^{2+}$ for binding to VCAM-1 (10). Evidence that conformational changes are involved in integrin-divalent cation interactions has been obtained through the use of stimulatory antibodies that bind to integrins in a divalent cation-dependent manner, suggesting that masked epitopes are exposed by the cation binding (25, 26). Cation-dependent conformation was also observed in the crystal structure of the αM A-domain which varied between the Mn^{2+}- and Mg^{2+}- bound forms (27).

Integrins and their ligands

Integrin ligation is controlled by a complex system of parameters including the level of integrin expression and status

of integrin activation (see below), as well as by ligand specificity. The particular combination of α and β subunit partly determines the ligand specificity; thus the major substrate for αvβ5 is vitronectin whilst for αvβ6 it is fibronectin (Table 1). However the integrin αvβ3 binds to more than six different ECM proteins including vitronectin and fibronectin. Ligand specificity may be modulated further by associated membrane molecules (28, 29) and even by membrane lipid composition (30). Integrins bind to their ligand through the recognition of short peptide motifs, often including an aspartate residue which is thought to be important for co-ordination of cation binding (31). The best example of this is the RGD motif which was first described in the central cell binding domain of fibronectin (32, 33) but has since been found in the sequences of many ECM proteins, including fibrinogen, collagens and vitronectin. The α4β1 integrin is perhaps unusual since it recognises several different aspartate-containing motifs. The binding-site for α4β1 in its two major ligands, VCAM-1 and the alternatively spliced CSI region of fibronectin, are LDV and the homologous sequence IDSP, respectively (34, 35). There are also other α4β1 binding motifs within the fibronectin sequence, REDV and IDAPS, but these represent lower affinity binding sites (36, 37). Several ECM molecules are recognised by more than one integrin including fibronectin, vitronectin and laminin (Tab 1). Since these heterodimers can be expressed by the same cells this suggests a high degree of redundancy in integrin expression and function. Possibly this apparent redundancy represents a further level of control since different integrins can produce different signals upon ligating the same substrate resulting in distinct cell behaviour (38-40).

Integrin activation

In order for integrins to bind to their ligand they must be in a ligand-receptive state usually referred to as the activated state. Thus integrins may be in the off or on state. The transition to the activated state is associated with a conformational change in the extracellular domain of the integrin molecule which, in turn, can be promoted by binding of divalent cations to the extracellular domain or by intracellular signalling events transmitted to the integrin via the cytoplasmic domain (41). This conformational change can be detected by antibodies which recognise their epitope only when ligand is bound, so called ligand-induced-binding-site (LIBS) antibodies e.g. PAC1 (42) and 12G10 (43). These antibodies confer increased function as a result of stabilising the ligand-occupied conformation of the integrin (44). In addition several anti-bodies (e.g. 8A2(45) and TS2/16(46)) exhibit the ability to activate integrins in a ligand-independent manner. Why integrins need to regulate their state of activation is exemplified by the integrins of circulating leukocytes and platelets . These molecules need to be in the inactive state to inhibit temporally inappropriate adhesion to their ligands which could result in thrombosis. For example the integrin αIIbβ3 on resting platelets is unable to bind to its major ligand fibrinogen until activated by, for example, thrombin (e.g. from degranulating mast cells) or by adhesion to collagen (exposed at the site of a damaged blood vessel) via another integrin α2 β1 (47). This activation of the leukocyte and platelet integrins occurs through the generation of intracellular signals and is referred to as inside-out signalling (1).

Table 1. Integrin hetrodimers and their ligands

Integrins	ECM Ligand	Cell-membrane-associated ligand
α1β1	Lm, Coll	
α2β1	Lm, Coll, Tn	
α3β1	Lm, Coll, Fn, Epi, Ent	
α4β1	Fn (LDV)	VCAM-1
α5β1	Fn, FG	
α6β1	Lm, Mer, Kal	
α7β1	Lm	
α8β1	Tsp, Vn, Fn	
α9β1	Tn	
αLβ2		ICAM-1, ICAM-2, ICAM-3
αMβ2	iC3b, FG, Factor X	ICAM-1
αXβ2		
αDβ2		ICAM-3
αIIβ3	FG, Fn, vWF, Vn	
αvβ1	Fn, Vn, FG, OP	
αvβ3	Vn, FG, vWF, Fn, Lm, Tn, dColl, OP, TSP, Perl	CD31
αvβ5	Vn, OP	
αvβ6	Fn (LDV)	
αvβ8	Vn, Tn	
α6β4	Lm, Kal	
α4β7	Fn (RGD)	VCAM-1, MadCAM
αIELβ7		E-cadherin

Abbreviations:

Fn, fibronectin
VN, vitronectin
Coll, collagen
dColl, denatured collagen
Tn, tenascin
TSP, thrombospondin
vWF, von Willebrand factor
Epi, epigrilin
Op, osteopontin
Ent, entactin
Mer, merosin
FG, frbrinogen
Kal, kalanin
Lm, laminin
Perl, perlecan
ICAM, intercellular adhesion molecule
Madcam, mucosal addressin cell adhesion molecule
VCAM, vascular cell adhesion molecule

Although the mechanisms by which the integrins become activated are poorly understood two members of the family of small GTP binding proteins have been identified which influence integrin activation status. Zhang and colleagues transfected constitutively active R-Ras^{38V} into the non-adherent mouse myeloid cell line 32D.3 and showed the cells now bound well to both fibronectin and vitronectin (48). Using integrin specific blocking peptides and antibodies the results suggested R-Ras^{38V} induced activation of αvβ3, α4β1 and α5β1. The effect was not cell line specific as *de novo* expression of R-Ras^{38V} activated αIIbβ3 in Chinese hamster ovary (CHO) cells and increased the integrin-mediated adhesion of the human U937 myeloid cell line. Similarly, Hughes *et al* used a gene complementation assay to search for suppressors of integrin activation(49). Chimeric molecules composed of the extracellular domains αIIbβ3 fused to the cytoplasmic domains of α5, α6A or α6B and β1 are constitutively active when expressed in CHO cells. A panel of over 100 cDNAs were transfected into these cell lines and screened for their ability to inactivate integrin activity. The results identified that constitutively active and wild-type H-Ras or its effector kinase Raf-1 caused inactivation of these constitutively active chimeric molecules and this activation correlated with activation of the ERK2 (see below). Other molecules which modulate integrin activity have also been identified including endonexin, a 111 amino-acid molecule which binds to the β3 tail of αIIbβ3 activating this platelet integrin. However the effect is specific to αIIbβ3 as endonexin does not bind to β1 or β2 integrins (50).

FOCAL ADHESIONS AND FOCAL COMPLEXES

Integrin-dependent cell adhesion, spreading and migration on a substrate requires the dynamic formation and dissolution of sites of contact between the cell and the substrate. *In vitro,* these integrin rich sites of attachment are classified as belonging to one of two main types. When they are small and associated with the leading edge of an advancing cell membrane or when they are present at the end of filopodia they are referred to as focal complexes or point contacts (51, 52). When they are larger and act as anchorage sites for radially distributed actin microfilaments they are referred to as focal adhesions(52, 53). Focal adhesions generally are not prominent in migrating cells but are found more often in well-spread non-migratory cells(52, 53) whereas the focal complexes/point contact are more commonly associated with migration(51). Both focal adhesions and complexes are formed in response to integrin-ligand binding and in both cases aggregate together on the cytoplasmic face of the integrins. Many of the signalling molecules can mediate the changes in cell behaviour. As such they have formed the major subcellular site of study for analysis of integrin dependent signalling.

Focal adhesion formation requires the cytoplasmic tails of integrins which bind to structural proteins including α-actinin (54), talin (55) and in some cases signalling proteins (e.g. FAK(56) and integrin-linked kinase 1 (ILK-1) (57) (For additional examples of integrin cytoplasmic tail associated molecules see(58)). Moreover, the clustering of integrins seems to require contractile forces generated by the actin-cytoskeleton (58) which is controlled by the activity of

the Rho-family of small GTP-binding proteins.

The Ras-related Rho family of GTP-binding proteins includes the members Cdc42, Rac1 and RhoA which regulate actin-dependent structures. As with other G-proteins the Rho family of GTPases are in the active state when bound to GTP and in the inactive state when bound to GDP. Activation to the GTP-bound form is through the action of guanine nucleotide exchange factors (GEFs) and transition to the inactive GDP-bound form is accelerated by GTPase activating proteins (GAPs). Dominant- active or dominant-negative regulation of these three G-proteins resulted in the discovery that Cdc42 regulates filopodia formation (59), Rac1 regulates membrane ruffling (60) and RhoA regulates formation of focal adhesions and actin stress fibres (61). Since focal complexes, which are associated with filopodia and ruffling membranes, and are also linked to a more migratory phenotype (51), this might lead one to predict that Cdc42 and Rac1 promote a more migratory phenotype; as discussed below, accumulating evidence suggests this indeed is the case. The studies in Swiss 3T3 cells also revealed a functional hierarchy exists such that Cdc42 can activate Rac1 which can activate RhoA (59). Burridge and Chrzanowska-Wodnicka (1996), in response to the observation that freshly plated cells exhibit filopodial protrusions and lamellipodia prior to spreading fully and forming focal adhesions and stress fibres, suggested that this probably represents a sequential activation of the Rho-family (58).

INTEGRIN LIGATION ACTIVATES SIGNALLING PATHWAYS

A variety of intracellular signals are generated as integrins cluster (reviewed in (62, 63) including changes in intracellular calcium and pH, turnover of inositol phospholipids, activation of the serine/threonine kinase protein kinase C and phosphorylation and dephosphorylation on tyrosine of many molecules. As integrins do not possess any intrinsic enzyme activity they must recruit non-receptor tyrosine kinases and phosphatases which mediate integrin adhesion-dependent enzymatic events. Maximal integrin signalling requires both integrin receptor occupancy and aggregation of integrins (64). Miyamoto and colleagues simulated ECM-induced integrin aggregation by exposing human fibroblasts to beads coated with anti-integrin antibodies and found this was sufficient to induce accumulation of FAK and tensin and tyrosine phosphorylation of a variety of signalling molecules. However this same treatment was not sufficient to recruit the actin-binding proteins talin, vinculin and α-actinin or indeed F-actin and phosphorylated paxillin, unless GRGDSP peptides also were present or the anti-integrin antibody was a function- inhibiting antibody and thus presumably serving as a surrogate-ligand.

An early signalling event subsequent to integrin ligation is a turnover in inositol phospholipids which can modulate cytoskeletal, growth and survival signalling. Central to these processes is the production of phosphatidylinositol 4,5-bisphosphate (PIP2). McNamee *et al* (1993) noted that adhesion of fibroblasts to fibronectin caused *de novo* synthesis of PIP2 whereas loss of adhesion resulted in a significant reduction in PIP2 (65). PIP2

is produced by the action of phosphatidylinositol 4-phosphate 5-kinase (PIP5K) on phospatidylinositol 4-phosphate (PIP) and cell adhesion reversibly regulates the activity of PIP5K (and thus PIP2 levels) via a direct interaction with activated Rho (GTP-Rho) (66). PIP2 is generated from phosphatidylinositol 4-phosphate (PIP) which is in turn derived from membrane phosphatidylinositol (PI). This requires the action of phosphatidylinositol 4-kinase (PI4K) which catalyses the production of PIP from PI. Berditchevski *et al* showed that phosphatidylinositol 4-kinase (PI4K) bound to CD63, a TM4SF (transmembrane-4-superfamily) member which associates with $\alpha 3\beta 1$(67) leading to the suggestion that $\alpha 3\beta 1$ ligation is at least one integrin-dependent mechanism which can recruit and localise PI4K.

Thus integrins control the levels of PIP2 which serves as a multifunctional regulator of cell behaviour. In addition to PIP2 promoting actomyosin structures as described below it serves as a substrate for two enzymes. Phospholipase $C\gamma$ hydrolyses PIP2 into: (i) inositol 1,4,5-triphosphate (IP3), which regulates calcium release from the endoplasmic reticulum, and (ii) diacylglycerol(DAG) which, together with the increased intracellular calcium, activates PKC(68). Thus PIP2 production promotes activation of calcium and calcium/calmodulin dependent enzymes (e.g. myosin light chain kinase (58)) as well as the pleiotropic effects of PKC signalling. PIP2 is also a substrate for phosphoinositol 3-kinase (PI3K) forming phosphatidylinositol 3,4,5-triphosphate (PIP3) which recruits to the cell membrane signalling molecules which possess plekstrin homology (PH) domains (69). PH domains allow association with inositol phospholipids, particularly PIP2 and/or PIP3 over other inositol lipids (70). PH-domain containing molecules include Akt/Protein kinase B(PKB) whose activation is protective against inhibition-of-adhesion-dependent apoptosis (71) and the guanine nucleotide exchange factors (GEFs) which promote activation of Rho, Rac and Cdc42 (70).

Thus PI3K activity can modulate the actin cytoskeleton. PI3K is composed of a p85 regulatory subunit associated with a p110 enzymatic subunit. Recent data suggest different isoforms of p110s perform different functions in cells. Thus antibody inhibition of specific p110 in a macrophage cell line showed that the p85/$p110\alpha$ isoform of PI3K promoted proliferation whereas the p85/$p110\beta$ and p85/$p110\delta$ isoforms promoted migration (72).

One of the major proteins that is tyrosine phosphorylated upon integrin-dependent aggregation is focal adhesion kinase (FAK) (64, 73). When chick embryo fibroblasts (CEF) were transformed by the viral tyrosine kinase v-src, the v-src located to focal adhesions which were also the site of significant phosphotyrosine activity. FAK was identified as a major substrate of v-src and as it also located at focal adhesions was named accordingly (74). FAK is a 125kDa protein possessing a central tyrosine kinase domain, a C-terminal focal-adhesion-targeting (FAT) sequence, two proline rich regions and several tyrosine residues which may become phosphorylated upon activation (reviewed by (75, 76) (Figure 2).

Activation of FAK involves autophosphorylation of the tyrosine at position 397 (Y397) probably in trans by adjacent FAK molecules when FAK oligomerises on the cytoplasmic face of integrins as they cluster in response to ligand binding (76 , 77). Certainly, an intact cytoskeleton is essential for FAK activation (78). Thus, in Swiss 3T3 fibroblasts C3 exoenzyme inhibition of Rho blocked adhesion-

dependent acti-vation of FAK (59), whereas over-expression of Rho increased FAK-activation(79) suggesting Rho activated integrin clustering promotes FAK activation.

The Y397 site is essential for maximal FAK downstream signalling as substitution with phenylalanine (F397Y) reduces tyrosine kinase activity by 50% and inhibits phosphorylation of FAK substrates including paxillin and tensin (75). Phosphorylation of Y397 allows recruitment of src family kinases (pp60src and pp59fyn) which bind via their phosphotyrosine recognition site (the src-homology 2 domain-SH2) (80). FAK-bound src/fyn can then phosphorylate other molecules (e.g. paxillin (81) and p130CAS (82)) as well as activation and docking sites within the FAK molecule itself, the latter allowing recruitment of additional signalling molecules. Thus upon binding to FAK, src can phosphorylate Y576 and Y577 in the FAK kinase domain to fully activate its kinase function (76) and also phosphorylate Y925 thus forming a docking site for the adaptor protein Grb-2 (83). Grb-2 possesses both an SH2 and an SH3 domain, the latter mediating protein-protein interactions at specific proline-rich motifs. FAK may also bind directly to the integrin cytoplasmic tail. Schaller (1995) reported that FAK can bind to peptide sequences derived from the cytoplasmic tails of β1, β2 or β3 integrins (56). Whether this occurs within cells is not clear although microinjection of β1 cytoplasmic-domain peptides corresponding to the putative binding site of FAK induced apoptosis (84).

Upregulation of FAK has been reported in a variety of invasive and metastatic cancers including colon, breast, prostate and thyroid (reviewed in (85). Subpopulations of cells in pre-invasive oral cancer have been identified which show a significantly increased FAK expression compared with neighbouring cells (86). These data lead to the suggestion that FAK upregulation may delineate a pro-invasive phenotype. However, FAK is probably not functioning as an oncogene *per se* but rather has a central role as a major integrin-regulated signalling molecule. For example, as discussed below, overexpression of FAK can cause increased migration (87) and protection against apoptosis caused by loss of integrin-dependent adhesion (88),

ROLE OF INTEGRINS IN PROLIFERATION

Ligation of integrins can activate signalling pathways which are also activated by growth factors and mitogens. For example soluble mitogens stimulate the tyrosine phosphorylation of proteins associated with focal adhesions including FAK, paxillin and p130CAS (89, 90). The ability of integrins to promote proliferation, as discussed below, is likely to be through co-operative signalling with growth factors. However the potential level of contribution of integrin-dependent signalling to proliferation could potentially increase if there was a change in the levels of expression of a particular integrin. As changes in integrin levels often accompanies progression of cancer toward a more invasive phenotype (discussed below) modulation of integrin-dependent proliferative signalling may be a contributing factor since the ability to grow in a foreign anatomical site is an essential requirement for the metastatic cell. Before discussing integrin-dependent proliferative signalling we shall briefly review activation of the mitogen-activated protein kinase(MAPK) pathways which is promoted by growth factors and is a key event in cell regulation of growth, cyto-skeletal changes and gene expression.

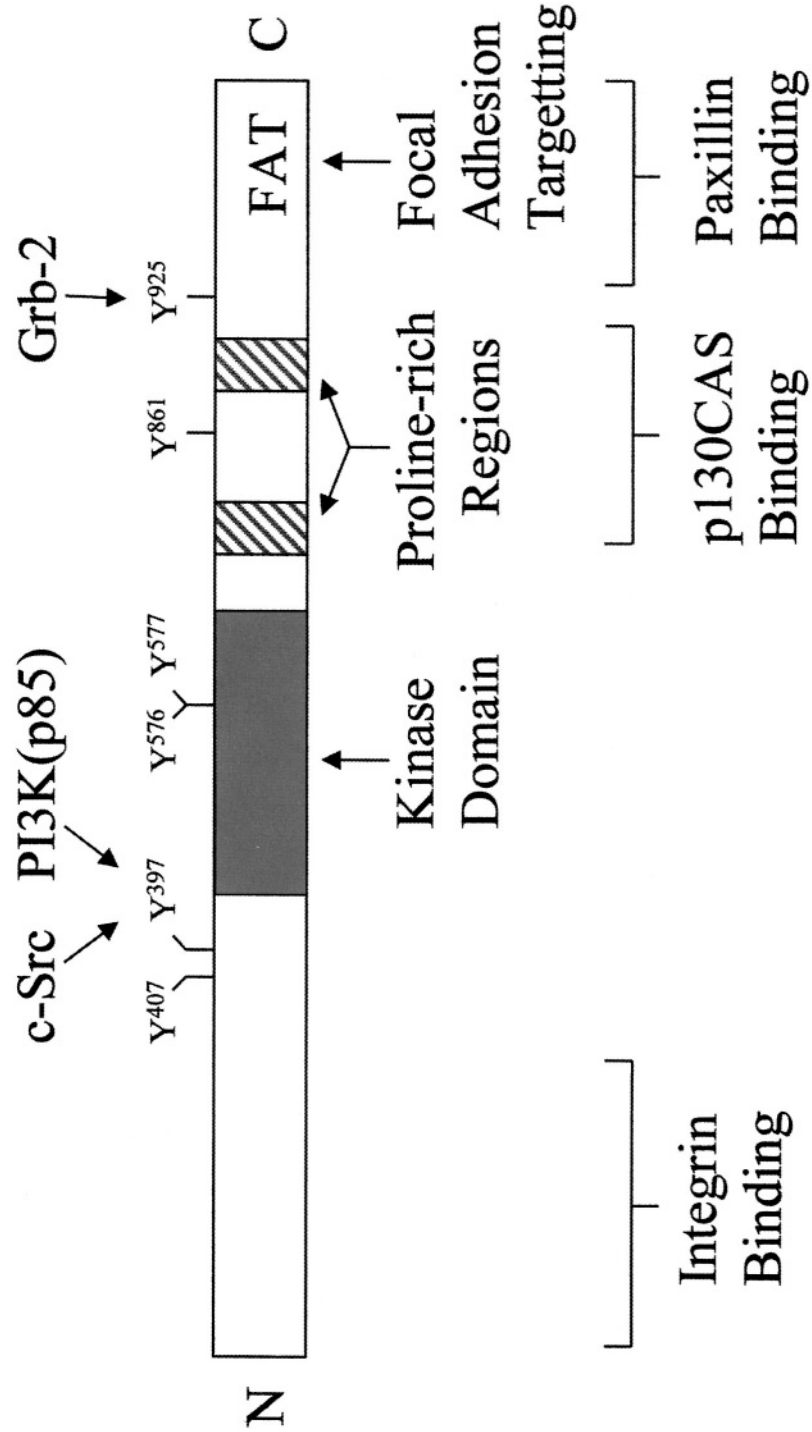

Figure 2. Schematic of p125 focal adhesion kinase (p125 FAK)

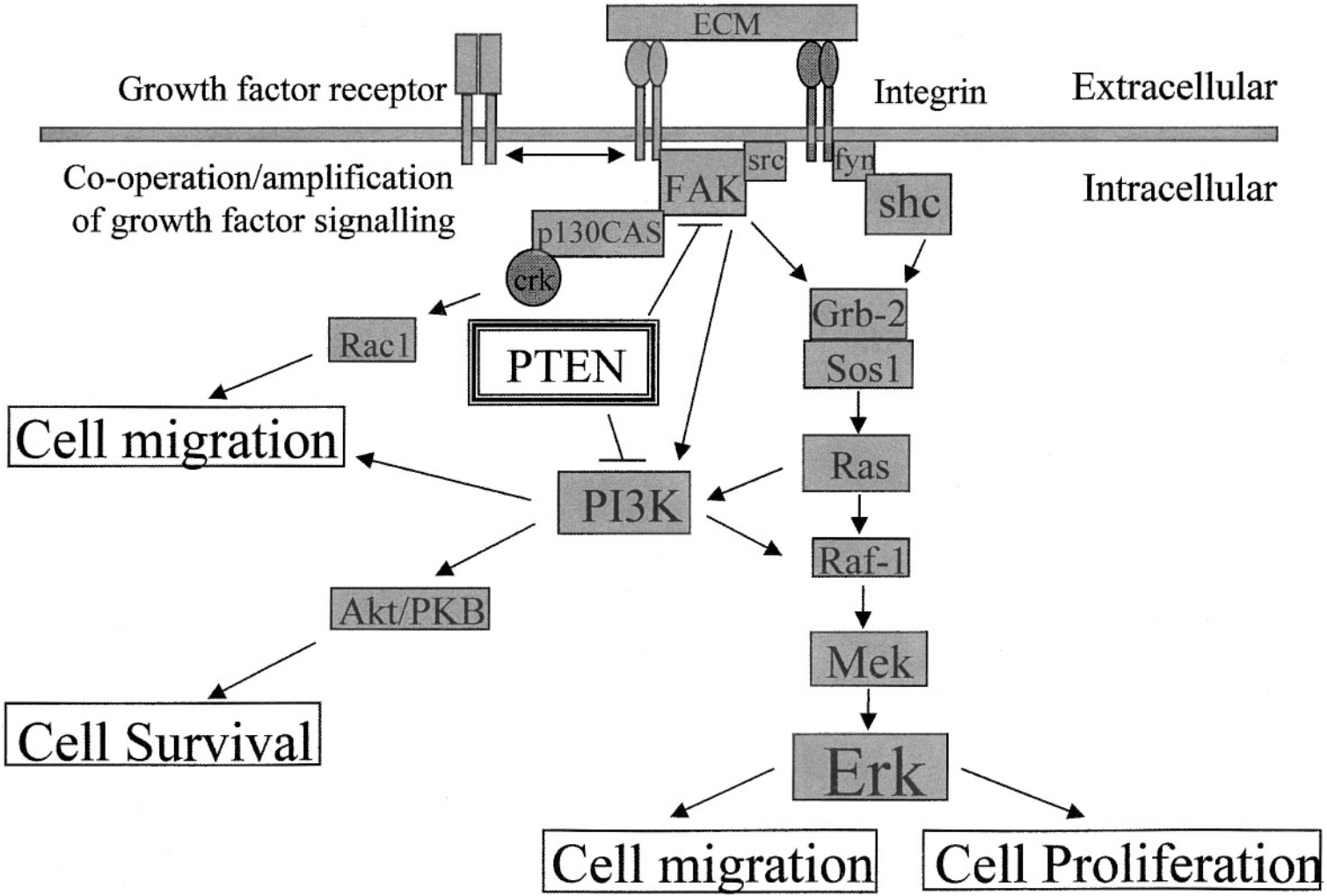

Figure 3. Integrin-ligation promotes multiple signalling pathways

The organisation of MAPK signalling is based around the sequential activation of three enzyme families (reviewed in (91). MAPKs are activated by MAPK kinases (MKK) which phosphorylate MAPKs on both tyrosine and threonine. MKKs are themselves activated by serine/threonine kinases referred to as MAPK kinase kinases (MKKK). Activators of MKKKs include the small GTP-binding proteins of the Ras superfamily. Once activated the MAPKs can translocate to the nucleus and activate several transcription factors including Elk-1, Ets-1, c-jun, c-myc and STATs (reviewed in 92) and in this way promote cell proliferation. There are several members for each of the three enzyme families(MKKKs, MKKs and MAPKs) forming at least five distinct pathways although there is crosstalk between these various pathways(91). Integrins can regulate the activity of at least two groups of MAPKs, extracellular signal regulated kinases(ERK1 and ERK2 sometimes referred to as $p42^{MAPK}$ and $p44^{MAPK}$) (93-96) and also the Jun N-terminal kinase (JNKs) (97-99).

Several integrin-dependent signalling mechanisms have been identified which can potentially activate the Ras-Raf(MKKK)-Mek(MKK)-Erk(MAPK) pathway (Figure 3). For example, Shc is an adaptor protein which subsequent to phosphorylation recruits the Grb2-Sos1 complex to the membrane. Sos1 is a Ras GEF and thus can activate the Ras-MAPK pathway. Ligation of the integrins $\alpha1\beta1$, $\alpha5\beta1$, $\alpha v\beta3$ and $\alpha6\beta4$ all activate Ras-MAPK via Shc upon adhesion(39, 40, 98). Mainiero (1997) showed that only $\alpha6\beta4$ recruited Shc directly through binding to phosphorylated sites on the $\beta4$ cytoplasmic tail whereas the other integrins utilised an indirect method requiring the membrane associated protein caveolin-1 and the tyrosine kinase pp59fyn.

Caveolin-1 is a cholesterol-binding, hairpin-shaped plasma-membrane protein whose N- and C-termini extend into the cytoplasm. It is a member of a small family of proteins involved in formation of membrane invaginations called caveolae whose functions seem to include intracellular signalling (100). Wary *et al* using rodent fibroblasts showed that a detergent soluble fraction of caveolin-1 could be co-immunoprecipitated with $\alpha1$, $\alpha2$, $\alpha3$, $\alpha5$, $\alpha6$ and αv using antibodies to these integrin subunits(101). However ligation of integrins using anti-integrin antibody- coated latex beads revealed that only $\alpha1$ and $\alpha5$ cross-linking resulted in activation of the Shc/Ras-MAPK pathway and then only in caveolin-positive cells. Thus activation of Shc-Ras-MAPK appears to operate via specific integrins only.This process required recruitment of Shc by the SH3 domain of the tyrosine kinase fyn which associates with caveolin-1. The authors proposed that fyn associates with integrin-bound caveolin and, upon integrin-ligation, becomes activated, possibly by an integrin-dependent phosphatase, so that it recruits and activates Shc which then promotes Ras-MAPK activation(101). In endothelial and keratinocyte cells Shc-linked integrins co-operated with mitogens to promote passage through the G1 phase of the cell cycle in contrast to ligation through non Shc-linked integrins which resulted in growth arrest (39, 98). Fyn-/- fibroblasts cells were also unable to enter S-phase efficiently when serum-starved and plated onto fibronectin; this was in contrast to src-/- cells which responded in the same fashion as Src+/+ cells. Thus the caveolin/fyn/Shc pathway, which is activated by specific integrins, can mediate transition through the G1-S

boundary and promote cell cycle progression (39, 98, 101).

Wary also showed that fyn-/- cells failed to activate MAPK when plated on fibronectin whereas src-/- cells did so albeit at a reduced level compared with wild type cells suggesting only a partial role for FAK-src activation of MAPK(101). In contrast studies by Schlaepfer and Hunter reported that that maximal Erk2 signalling in epithelial cells required both src and Ras activation subsequent to integrin ligation (94). Several integrin activated FAK-dependent pathways have been described which may result in MAPK activation. Grb-2 binds to the src-phosphorylated Y925 of FAK and is thus able to recruit Sos-1 and potentially activate Ras-MAPK (210). Integrin ligation and clustering results in recruitment of paxillin which binds to FAK and becomes phosphorylated allowing SH2-mediated binding of the SH2-SH3 adaptor crk (81). Crk can recruit the GEFs C3G and Sos1 (via the crk SH3 domain) which can both activate Ras. Integrin ligation also promotes binding of the p130CAS (crk-associated substrate) via its SH3 domain to FAK (possibly via src (76)) where it becomes phosphorylated creating binding sites for crk and another SH3-SH2 adaptor nck. Both crk and nck have been implicated in the activation of the JNK MAPK pathways (see (76) for references). The autophosphorylation site of FAK (Y397) is not only a recruitment site for src family kinases but also for phosphoinositol 3-kinase (PI3K) which could drive PI3K-dependent signalling (102). In studies by King (103) integrin-dependent activation of Raf-1, MEK-1 and Erk2 in Cos7 cells was inhibited by blocking PI3K activity but it did not block activation of Ras. Thus PI3K activation of Erk2 occurs downstream of Ras (and potentially, therefore, Shc) but upstream of Raf-1 (103).

Other studies have implicated FAK in regulation of the cell cycle. Zhao *et al* (1998) reported that overexpression of FAK in NIH3T3 and human foreskin fibroblasts increased the rate of transition from G1 to S phase of the cell cycle(104) and that this effect required the presence of Y397 (implicating activity of a src kinase) and the FAT (focal adhesion targeting) sequence. Thus expression of a mutant form of FAK which lacked the FAT sequence (FAK-C14) acted as a dominant negative molecule by competing for FAK substrates which would normally locate to focal adhesions in association with wild-type FAK. The FAK-C14 caused fibroblasts to arrest in G1 which was associated with an increase in the cyclin-dependent kinase inhibitor p21 and a reduction in cyclin D1. In contrast over expression of wild type FAK induced an increase in cyclin D1 and a decrease in p21(104). Thus integrin-dependent activation of FAK can have a profound affect on cell proliferation by promoting activation of MAPKs and also modulating the cell-cycle machinery. Over activity in either of these pathways could contribute to a tumourigenic phenotype. For example, expression of a membrane - bound constitutively active FAK prevented non-adherent MDCK epithelial cells from entering apoptosis (88).

As discussed above integrin occupation and clustering promotes and amplifies signalling for growth. Recent data from Aplin and Juliano (93) show that for effective integrin signalling to occur, these clusters need not be as large as a focal adhesion but may require only small actin-dependent complexes. Serum starved NIH3T3 fibroblasts in suspension or adherent to poly-L-lysine respond to exogenous epidermal growth factor (EGF) with very low phosphorylation of MAPK. In contrast, on fibronectin the EGF-dependent activation of MAPK is significantly increased. Concentrations of

inhibitors of actin filaments (Cytochalasin D or Latrunculin A) sufficient to disrupt the cortical actin cytoskeleton inhibited the fibronectin-adhesion promoted EGF activation of MAPK, whereas lower concentrations of inhibitors, sufficient only to block stress fibres, did not affect EGF-stimulated MAPK(93). Aplin and Juliano expressed constitutively active Cdc42, Rac1 or RhoA in NIH3T3 cells and showed that in suspension, the Cdc42 but not the Rac1 or RhoA constructs , enhanced EGF-dependent MAPK activation. The data suggest that the presence of focal adhesions and stress actin stress fibres is not required for integrin-dependent signalling but that smaller cortical actin complexes regulated by Cdc42 are sufficient (93). Furthermore, since large focal adhesions are not usually seen in tissues (52) their absence cannot be used to suggest that integrin-dependent signalling is not occurring *in vivo.*

In addition to promoting growth signalling directly by binding to the ECM integrins also generate proliferative signals by enhancing growth factor induced signalling. This latter signalling may be the dominant mechanism by which integrins-promote growth . Thus the ability of EGF to stimulate ERK2 activation in CHO cells is significantly enhanced if the cells are adherent to fibronectin (93). The integrin $\alpha v\beta 3$ is implicated in several pathways. The insulin related substrate-1 (IRS-1) is an adaptor molecule which is phosphorylated by activated insulin and insulin-like growth factor (IGF-1)receptors enabling it recruit other signalling molecules. A fraction of this phosphorylated IRS-1 associates with $\alpha v\beta 3$ on cells adherent to vitronectin increasing the growth promoting effect of insulin and IGF (105). Bartfeld and colleagues reported that increased proliferation of cells after treatment with the growth factor PDGF induced a 190kDa phosphoprotein to associate with $\alpha v\beta 3$(106). In subsequent studies by Schneller *et al* (1997) this protein was subsequently identified as a phosphorylated fraction of PDGF receptors (107). It seemed that $\alpha v\beta 3$ increased the phosphorylation of a small fraction of PDGFr which was responsible for the enhanced mitogenic response to PDGF of cells adherent to vitronectin. Thus integrins could promote the proliferative capacity of tumour cells by increasing the growth signals generated by activated growth factor receptors.

ROLE OF INTEGRINS IN APOPTOSIS

Normal cell growth (net sum of cell proliferation and cell death) is a consequence of signals generated by integrin-ligand interactions and soluble growth factors. Deprivation of growth factors and hormones leads to apoptosis. Similarly, disruption of cell-matrix interactions induces apoptosis, in this case termed anoikis (108). Anoikis can be viewed as a mechanism by which cells use positional information delivered from the extracellular matrix via integrin ligation in order to determine whether they are in an appropriate environment for growth while deprivation of normal cues results in programmed cell death. When cells metastasise they almost certainly encounter a foreign ECM milieu compared with the original site of growth and thus the metastatic process is likely to be at least partially due to a failure of the apoptotic pathway. For example the invading transformed melanocyte or epithelial cell must pass through their underlying dermis, which is composed of many different matrix proteins compared with the suprabasal environment, before intravasating into blood-vessels or lymph nodes and transferring to distant sites.

Subsequent extravasation and growth into a secondary tumour, particularly if it is in a different organ from the tissue of origin of the transformed cell, must occur in an environment of different ECM proteins and growth factors. Thus an understanding of how integrins control entry into anoikis or apoptosis may identify abnormalities and potentially therefore, novel targets for the therapy of cancer (Figure 3).

There are now many examples of matrix-suppressed apoptosis (38, 109). Zhang et al have shown that anoikis is suppressed in α5-transfected Chinese hamster ovary cells ligated to fibronectin via α5β1 and that this was associated with upregulation of Bcl-2 expression. This response to fibronectin also was shown to be integrin specific since cells expressing an alternative fibronectin receptor, αvβ1, underwent apoptosis when plated on fibronectin (38). Montgomery *et al* reported that expression of αvβ3 protected melanoma cells from apoptosis when growing in collagen gels (110). These data may have been related to the observation that ligation of αvβ3 is associated with down-regulation of the cyclin-dependent kinase inhibitor $p21^{WAF-1/CIP-1}$ and also p53 (111). Certainly it is consistent with the activation of the p53-$p21^{WAF-1/CIP-1}$ pathway following disruption of cell-matrix interactions (112). However, *de novo* or increases in integrin expression does not invariably lead to protection from apoptosis. For example, expression of β4 integrins in colon carcinoma cells has been shown to induce expression of $p21^{WAF-1/CIP-1}$ leading to growth arrest and apoptosis (113). Such results underlie the fact that integrin-mediated regulation of anoikis is complicated and, at present, imperfectly understood. However signalling events involved in integrin-dependent protection against apoptosis are being unravelled slowly.

Phosphoinositol 3-kinase (PI3K) has been implicated in protecting cells from entering adhesion dependent apoptosis (anoikis). Thus when MDCK cells express constitutively active Ras, protection from apoptosis was due to PI3K dependent activation of Akt/Protein kinase B promoting downstream blockade of apoptosis (71). In normal MDCK cells, adhesion via integrins activates PI3K although whether any of the other known pathways for activation of PI3K are involved is not clear. For example Shc can be activated by ligation of a variety of integrins (see above) resulting in activation of Ras. PI3K is a substrate for Ras (114) thus perhaps Shc could regulate PI3K activation upon adhesion. Alternatively PI3K may be activated subsequent to binding to the phosphorylated Y397 residue of FAK which is phosphorylated when integrins cluster(see above). In contrast to PI3K-dependent protection against anoikis, activation of the JNK/SAPK (stress activated protein kinase) pathway may promote anoikis (reviewed in (115).

Several groups have shown an involvement of focal adhesion kinase (FAK) in regulating anoikis. Xu *et al* showed that attenuation of FAK expression in tumour cells led to apoptosis (116) whereas expression of constitutively active FAK in MDCK cells blocked entry in to apoptosis even though cells were deprived of integrin ligation by being placed in suspension (88). Additionally anoikis was induced when peptides mimicking the FAK binding site on the β1 cytoplasmic tail were microinjected into fibroblasts (84). Recently Ilic *et al* have shown that survival signals from the ECM, which are transduced by integrins via FAK, suppress a p53-regulated apoptotic pathway in fibroblasts and endothelial cells (117). This pathway was blocked by inhibitors of p53 including Bcl-2 but not by inhibitors of PI3K(117).

Moreover the capacity of the ECM to inhibit anoikis varied not only between substrates but also between different cell types. Thus fibronectin was equally effective at preventing anoikis in rabbit synovial fibroblasts (RSF) and mouse endothelial cells whereas vitronectin and laminin, though very effective for endothelial cells were very poor at preventing anoikis in RSF while adhesion to collagen was not an effective protection against anoikis for either cell type (117). Since each of these substrates are recognised by different groups of integrins these data further show that protection against anoikis is dependent not only upon which specific proteins in the matrix are present but which integrins are expressed.

Small peptides containing the RGD binding motif have been used extensively in the study of the role of integrins in tumour progression and metastasis. Co-injection of small peptides, which include the RGD motif, can significantly impair the lung colonising ability of melanoma cells (118) as well as reduce the formation of spontaneous metastases (119). The principal mechanism for these results was believed to be as a consequence of the ability of RGD peptides to disrupt integrin-ligand interactions and therefore prevent integrin-dependent processes including adhesion and migration. Although these conclusions may have been true, recent data from Buckley *et al* have demanded that we may need to interpret data generated by use of RGD peptides a little more cautiously (120). It seems that RGD peptides can promote apoptosis by entering cells in an integrin-independent manner and directly activating pro-apoptotic enzyme caspase 3 (120). The authors propose that the procaspase has an RGD-dependent conformational restraint that prevents it from becoming cleaved into the active form of the enzyme. RGD peptides entering the cell disrupt this intramolecular interaction and allow activation to take place. These results do not exclude a role for integrins in apoptosis since anti-integrin antibodies that disrupt ligand interactions also promote apoptosis (121) and attachment to anti-integrin antibodies but not non-specific antibodies, prevents apoptosis in human umbilical vein endothelial cells (122).

ROLE OF INTEGRINS IN MIGRATION

Migration may be defined as the directed motility of a cell along a soluble (chemotaxis) or insoluble (haptotactic) concentration gradient. Essential requirements of the metastatic cell are that they must be motile and possess a migratory phenotype. In the early stages of metastasis there must be a reduction or loss of cell-cell cohesion between the potential metastatic cell and its immediate neighbours in the primary tumour (Figure 1). In order for a metastatic cell to take advantage from this reduction in cell cohesion it must be able to move away from the primary tumour. This may be promoted by exposure of the metastatic cell to novel ECM molecules or growth factors which could occur as the primary tumour penetrated adjacent tissues (discussed in 123) . However, migration, and thus metastasis, could not occur without expression of sufficient levels of active integrins of an appropriate ligand specificity. Thus understanding integrin-dependent migration is an important component in understanding metastasis.

Cell motility and migration require a regulated attachment to and detachment from the ECM substrate. Cells must neither be too adherent to the substrate so as to prevent detachment nor so weakly attached as to prevent sufficient traction to

be generated. Palecek and colleagues showed that maximum cell speed was generated at a median degree of adhesiveness and that cell adhesiveness was governed by a combination of three factors: the concentration of the substrate, the concentration (level of expression) of the integrin and the activation state of the integrin(124). One might also consider the speed at which cells are able to regulate the formation and deformation of the integrin-rich sites of attachment as it would also impinge upon overall cell speed. How cells regulate the formation and the deformation of focal adhesions and focal complexes still is not completely understood . Mitogens and also integrin ligation can activate the small GTP-binding proteins Cdc42, Rac1 and RhoA (discussed in 53) which are responsible for regulating actin structures in cells. The balance of activity of these three G-proteins regulates the formation of actomyosin fibre formation which is necessary for generating the contractile forces involved in cellular migration. However, regulation of phosphatidyl-inositol-4,5-bisphosphate (PIP2) levels is central to this process.

Cells in suspension have reduced levels of PIP2 (65). PIP2 releases actin monomers by binding to the actin-capping proteins gelsolin and profilin thus promoting actin filament formation (discussed by Burridge *et al* (58)). PIP2 also binds to vinculin and induces a conformational change allowing talin and actin binding (125). Activated GTP-bound Rho activates phosphatidyl inositol 4-phosphate, 5-kinase (PIP5K) which promotes the formation of PIP2 from phosphatidylinositol-4-phosphate(PIP).
Blockade of PIP2 by microinjection of anti-PIP2 antibodies blocks RhoA induced formation of focal adhesions and stress fibres (125) indicating that PIP2 mediates the Rho-dependent focal adhesion and stress fibre formation. Rho also activates Rho-kinase, which by phosphorylation, inactivates the myosin light chain kinase phosphatase (126) thus promoting accumulation of phosphorylated myosin light chains. Phosphorylation of myosin light chains (MLC) promotes actin-myosin II interaction and formation of force-generating actomyosin structures (discussed by 58). Moreover, Rho kinase may directly phosphorylate MLC (127). Thus active Rho promotes focal adhesions and actin filament formation (Figure 4).

Recently Sanders *et al* (128) have shown how Rac1 and Cdc42, by activating the p21 activated-kinase PAK1, may antagonise the activity of Rho in modulating actomyosin fibre formation (128). Myosin light chains can be phosphorylated by myosin light chain kinase (MLCK). Sanders reported that PAK1, which is activated by Racl and Cdc42, phosphorylates MLC K causing inactivation of the enzyme thus reducing the production of phosphorylated MLC. Thus it may be expected that focal adhesion formation and disassembly will depend on the relative balance between the RhoA promotion of actomyosin formation and the Rac1/Cdc42 reduction in actomyosin bundle formation. These results may explain, in part, the observations of Keeley *et al* who reported that expression of constitutively active Cdc42 or Rac in the breast cell line T47D resulted in a loss of polarisation, change in cell morphology and invasion into collagen gels. The Cdc42 and Rac effects were blocked by drug-inhibition of PI3K which was deemed necessary for reorganisation of the cytoskeleton (129) (Figure 4).

MAPK was found also to modulate actomyosin structure and, as a consequence, migration. Thus Klemke and colleagues reported that MAPK phosphorylates and activates myosin light-chain kinase (MLCK) activating its ability

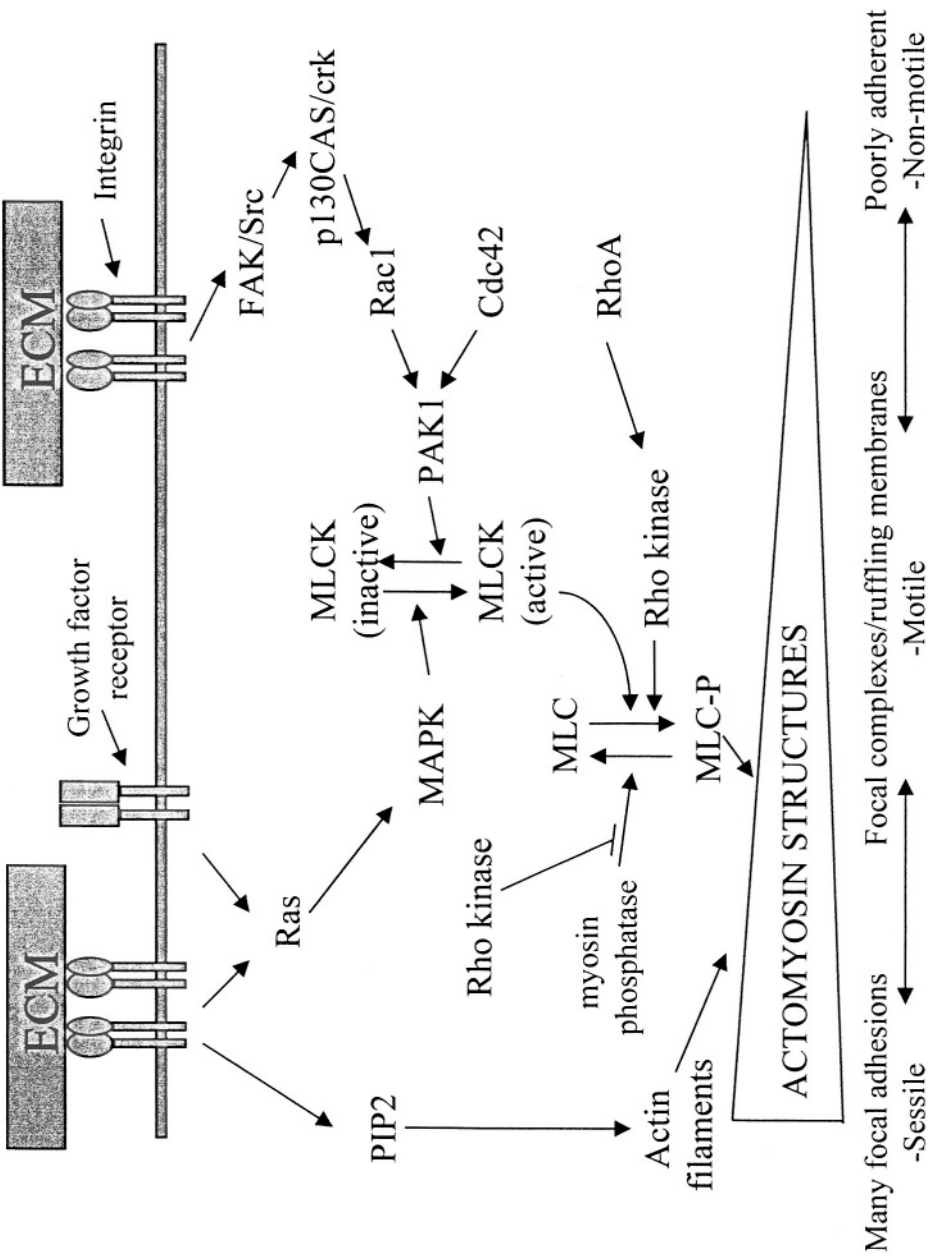

Figure 4. Integrin modulation of actomyosin migration machinery

to phosphorylate myosin light chains (MLC) (130). Klemke showed that inhibition of MAPK by a variety of methods (including drug-induced inhibition of MAPK activity, expression of MAPK antisense or blockade of its upstream activator MEK) resulted in reduced MLCK activity, reduced MLC phospho-rylation and reduced migration. In contrast expression of constitutively active MEK resulted in increased migration in COS7 cells. Thus integrin-dependent activation of the Ras-MAPK pathway could promote a more migratory phenotype. However, this is not the only integrin-activated signalling pathway which modulates migration.

Over-expression of wild-type FAK in CHO cells resulted in increased migratory capacity. This effect required the presence of the Y397 residue but not FAK-kinase activity suggesting that FAK-recruitment of src-family kinases, src and fyn, resulted in migratory signals(87). The FAK knockout (FAK-/-) mouse dies at stage E8.5 from severe vascular and mesodermal defects, a phenotype similar to the fibronectin knockout mouse, perhaps suggesting that FAK mediates fibronectin dependent signalling (131). Embryonal fibroblasts from FAK-/- mice form a large number of small focal contacts, few large focal adhesions and migrate poorly, possibly due to the large number of contacts with the substrate causing excess adhesiveness(132). Thus one of FAK's roles may be to promote deformation of focal adhesions and complexes allowing for transitory attachment to the substrate which is necessary for migration; this effect may be indirect and mediated by src (133).

In a follow up to their earlier studies, Cary *et al* showed that FAK-induced migration was not due to either FAK recruitment of Grb2 or the activity of MAPK but rather was dependent upon FAK recruitment of p130CAS (134). Integrin-dependent adhesion results in phosphorylation and association with FAK of the adaptor protein p130CAS. The p130CAS molecule consists of an SH3 domain, two proline-rich domains and a tyrosine-rich substrate-binding region of 15 potential SH2 binding motifs. Inhibition of the binding of p130CAS to FAK (by mutation of the proline-rich p130CAS binding site on FAK or competitive inhibition of the p130CAS-FAK interaction by co-expression of the p130CAS SH3 domain) resulted in inhibition of FAK induced increase in migration(134). Overexpression of p130CAS on the other hand was able to enhance migration on fibronectin while phosphorylation of p130CAS, which did not require the kinase domain of FAK but did require the Y397 residue, correlated with increased migration(134). These datasuggest that association of p130CAS with FAK results in tyrosine-phosphorylation of p130CAS by a src-family kinase bound at Y397 in FAK. This would generate a series of SH2 binding motifs in its substrate domain enabling recruitment of downstream effectors(134).

Klemke and colleagues also identified p130CAS as a migration promoter and also classified it as a metastasis promoter(135). FG pancreatic carcinoma cells attach, but cannot migrate well, on vitronectin so that a subline, called FG-M, was generated by repeatedly collecting and expanding the small number of FG cells that initially migrated toward vitronectin. FG-M cells migrated 8-times more than FG cells although the expression of αv vitronectin receptors and the ability to adhere to vitronectin remained unchanged. When plated on the chick allantoic membrane FG-M cells formed four-times as many lung metastases as equivalent numbers of cells of the parental line(135). As the growth rate, *in vitro* or *in vivo,* of these two

variants was similar these differences could not be explained on the basis of enhanced proliferation. Analysis of FG versus FG-M cells adherent to vitronectin showed that FAK phosphorylation levels were similar but that p130CAS phosphorylation was enhanced significantly in FG-M cells. Over-expression of p130CAS in COS7 cells increased their migration on Fn, Vn and Collagen , the migration correlating with phosphorylation of CAS. Expression of a mutant CAS lacking its substrate-binding domain (CAS-SD) which was not phosphorylated upon adhesion, did not increase migration but, in fact, acted as a dominant-negative regulator of migration in FG-M carcinoma cells(135). Moreover, the CAS-SD construct inhibited insulin-, EGF- and IGF-1-induced migration in serum-starved COS cells suggesting strongly that these growth factors stimulate migration in a CAS-dependent fashion. Klemke and colleagues (1998) also identified a likely mechanism of p130CAS-promoted migration (135). The p130CAS protein was originally identified as a major phosphorylated substrate in v-crk and v-src transformed cells; moreover phosphorylation of CAS is known to promote binding of the adaptor protein crk (discussed by Klemke *et a l* (135)). Crk binds CAS via its SH2 domain and recruits downstream molecules via its SH3 domain. When Klemke *et al* (1998) over-expressed crk it was sufficient to increase the migration of FG carcinoma cells on vitronectin ten-fold. Interestingly, CAS and crk associate with sites of focal contact in resting cells but translocate to the ruffling membranes in migratory cells(135). Since the small GTP-binding protein Rac regulates the actin-cytoskeleton which forms ruffling membranes, Klemke *et al* (1998) expressed dominant-negative Rac and showed that it inhibited the CAS/Crk induced migration of COS7 cells. However constitutively active Rac was not sufficient to induce migration suggesting that other pathway(s) are activated by p130CAS and /or crk. Thus in summary as integrins aggregate as a consequence of adhesion to substrate, activation of FAK can recruit and activate src family kinases which promote p130CAS/crk dependent migratory signalling (134, 135). In addition, activation of FAK may also contribute to activation of MAPK which is also implicated in generating a migratory phenotype (130). Clearly, upregulation of these signalling pathways could enhance a more migratory phenotype and thus promote a more metastatic phenotype.

ROLE OF INTEGRIN-MEDIATED PROCESSES IN METASTASIS: CLINICAL AND EXPERIMENTAL DATA

Many of the mechanisms by which integrins could promote a more metastatic phenotype (increasing growth signalling, co-operative interaction with growth factor receptors, generating migratory and survival signals) have been discussed above (Figure 5). The discovery of the tumour suppressor gene PTEN has further highlighted the potential significance of integrin-dependent signalling in pathological conditions including cancer. Deletion or mutation of both PTEN alleles occurs in a variety of cancers including those of the breast, prostate and brain (136). PTEN is a dual-specificity protein and lipid phosphatase with sequence homology to the protein tensin (136, 137). PTEN's activities include the ability to remove the phosphate from the 3' position in 3-phosphoinositides (138), dephosphorylate p125FAK (137) and inhibition of Shc activation (139).

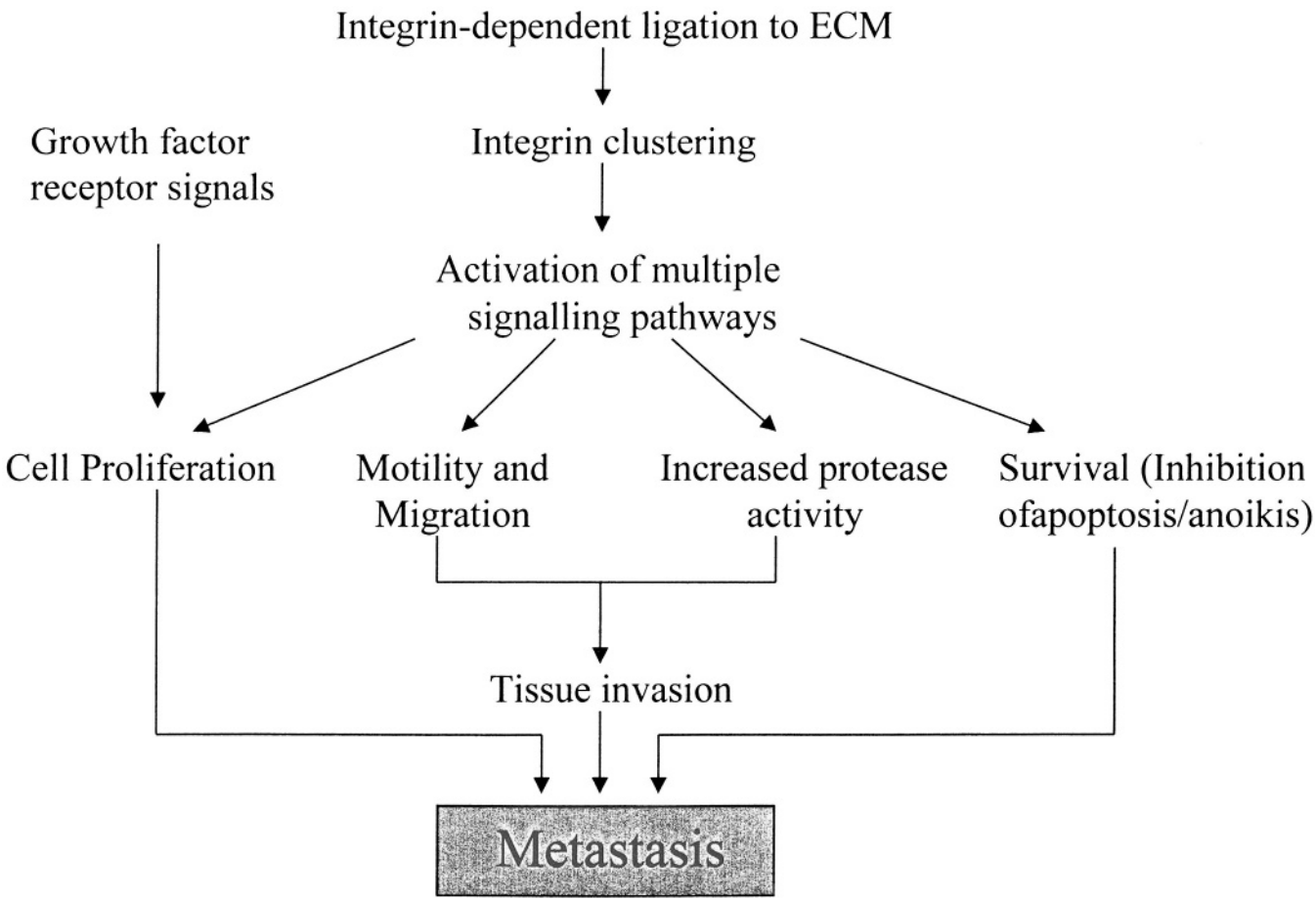

Figure 5. Integrin-dependent signalling pathways can combine to promote metastasis

Over-expression of PTEN inhibited migration, spreading and formation of focal adhesions (137) in addition to suppressing the integrin- and also the growth factor-dependent activation of the mitogen-activated protein kinase (MAPK) pathway(139). As discussed above, activation of FAK, Shc, PI3K and MAPK are critical events in regulating cell proliferation, apoptosis, adhesion and migration and thus an increased activity, or deregulation, of these proliferative and migratory pathways, caused by an absence of PTEN, would give metastatic cells a significant advantage. Whether the protein or lipid phosphatase ability is the more important function, or whether they are equally important in the tumour-suppressing ability of PTEN is still under debate (70). What is clear is that data from both human and mouse studies emphasise the central importance of PTEN in tumour suppression. Patients with Cowden's Syndrome inherit one mutant PTEN gene and have an increased susceptibility to the development of certain cancers (70) . PTEN knockout mice die *in utero* at about day 9.5 and exhibit abnormal areas of tissue patterning and hyperplasia (140). Fibroblasts from PTEN-/- mice had an increased resistance to apoptosis associated with elevated levels of Akt activity (140). In contrast mice which are heterozygous for wild-type PTEN (PTEN +/-) do survive but develop cancer in many organs including liver, prostate, endometrium, thyroid, thymus and gastrointestinal tract (141). Examination of the tumours of the thymus and liver in these mice showed loss of the single remaining wild-type allele of PTEN. Interestingly the mice also developed non-neoplastic hyperplasia of the lymph nodes caused by a defective apoptosis pathway in B cells and macrophages (141). Thus PTEN regulated pathways include suppression of cancer and also regulation of apoptosis. Since many of these pathways are driven by integrin ligation these data confirm that deregulated integrin signalling can promote the metastatic phenotype.

Although integrin-dependent generation of intracellular signals is a major mechanism of integrin modulation of cell behaviour, as discussed above, different integrins possess different ligand specificities, different signalling abilities and also different tissue-specific expression. Thus the role of integrins in cancer is likely to vary depending upon the tissue of origin of the neoplasm and also the specific integrins expressed. Immunopathology provides us with valuable clues as to the possible activity of particular integrins in specific cancers although it cannot positively identify that an integrin was actually functioning at the time of the removal of the tissue. Nevertheless the loss of or the *de novo* appearance or increase in integrins during selected stages of malignant disease has been observed for many different cancers (3, 123, 142, 143), and directs the suggestion that these changes may not be merely a consequence of tumour progression but may actually participate in or even promote tumour progression and metastasis.

There often is a loss of $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins from poorly differentiated breast and colorectal carcinomas (144-146)but an increase and/or deregulated expression of $\alpha 6\beta 4$ (see (147) for references). Since collagen and or laminin are the ligands for these three integrins they all potentially adhere cells to the basement membrane. Reduced adhesion to the basement membrane could enable cells to migrate away from their primary site of attachment and growth. Restoration of $\alpha 2$ integrin to a tumourigenic poorly differentiated breast line inhibited tumourigenicity (148). By contrast *de novo* expression of $\alpha 2$ by the rhabdomyosarcoma cell line RD resulted

in a tumour cell line with significantly enhanced metastatic potential (149). These apparently conflicting data are likely to be the consequence of the activity of different integrins in varying cellular backgrounds and highlight the potential complexity of these molecules in cancer. For example some cancers appear to exhibit an increase in integrin expression. Thus malignant melanoma progression has been associated with the upregulation of several β1 integrins including α2(150), α3(151), α4(152), α5(153) and α6(154).

The integrin α6β4 is a laminin receptor which normally is located basolaterally as a component of hemidesmosomes which attaches epithelia to basement membrane and underlying dermis. Loss of, or aberrant expression of α6β4, which occurs in many carcinomas including breast, colorectal, prostate and oral cavity almost certainly contributes to the non-polarised appearance of poorly differentiated epithelial cancers (146, 147, 155-157). As mentioned above α6β4 binding to laminin can activate the Ras-MAPK pathway via Shc and promote growth of keratinocytes(98) whereas α6β4-dependent activation of PI3K promoted a more invasive phenotype in carcinoma cells (158). Moreover in the presence of EGF α6β4-dependent migration may be induced(159). All of these functions may have contributed to the observation that α6β4 is upregulated during progression toward carcinoma in experimental carcinogenesis in mouse skin (160). In contrast *de novo* expression of α6β4 resulted in the *in vitro* growth inhibition of bladder(161) and colorectal (113) cell lines. Thus again the effect of integrin expression on various parameters of cell behaviour appears to be modulated by the cellular background.

The integrin α4β1 is expressed on lymphocytes and mediates heterophilic interaction between lymphocytes and activated endothelium prior to extravasation via binding to its ligand VCAM-1(162). However α4β1 is also upregulated on a variety of neural crest derived cancers including metastatic melanoma and has led to the suggestion that a similar mechanism to lymphocyte extravasation may mediate tumour extravasation. Cytokine pre-treatment of mice upregulates VCAM-1 expression on endothelium and also increases the number of metastases that develop after intravenous injection of mouse melanoma cells(163, 164). Antibody blockade of either the α4β1 on the melanoma or the VCAM-1 on the endothelium resulted in inhibition of the increased experimental metastasis(163, 164). However, not all studies suggest α4β1 promotes metastasis. In a report from Qian *et al* the *de novo* expression of α4β1 in a mouse melanoma line actually impaired the formation of spontaneous metastases. The suggested mechanism was through increasing homotypic adhesion thus reducing the capacity to migrate away from the primary site of growth (165).

The αv integrins provide perhaps the most consistent data relating changes in integrin expression with cancer with increased expression of this family of heterodimers often being associated with increased malignancy. Many reports document an increased expression of αvβ3 in the invasive stages (vertical growth phase and metastases) of malignant skin melanoma (152, 153, 166-168). In addition Gladson and Cheresh (1991) reported that αvβ3 was upregulated in Grade III and Grade IV astrocytoma whereas in low grade tumour and normal brain no αvβ3 was detectable(169). Comparably the fibronectin receptor αvβ6 is upregulated on colorectal carcinoma (170) and oral squamous cell carcinoma (171).

A major aspect of tumour invasion is the regulated destruction of the extracellular matrix adjacent to an advancing tumour. This destruction is the result of activation of proteases released in the pro-form from neoplastic cells or surrounding stromal cells (172-174), Accumulating evidence indicates that integrins may regulate the expression, and possibly the activity, of the two principal protease systems implicated in cancer; namely matrix metalloproteinases (MMPs) and the urokinase type plasminogen activator (uPA) system (discussed in 175).

Several integrins promote production of MMPs upon adhesion to their ligands. Thus ligation of $\alpha 2\beta 1$ regulates expression of MMP1 (collagenase type 1) (176). Huhtala and colleagues reported that when rabbit synovial fibroblasts bound to fibronectin via $\alpha 5\beta 1$ they increased their mRNA and protein for MMP9 (177). Interestingly if the cells simultaneously adhered to the alternatively spliced CS1 region of fibronectin via $\alpha 4\beta 1$ then this was antagonistic and MMP9 upregulation was inhibited significantly (177). This appears to be an example of integrin crosstalk where the activity of one integrin modulates the activity of another integrin expressed on the same cell (178). Moreover, ligation of $\alpha v\beta 3$ by addition of function-inhibiting antibodies or vitronectin increased the expression of MMP2 and also enhanced the capacity of the A375M melanoma cell line to invade the basement membrane matrix Matrigel (179).

To maximise the efficiency of protease destruction of ECM ideally it should occur immediately adjacent to the cell surface and cancer cells have evolved mechanisms to ensure that this is the case. Brooks *et al* (1996) reported that a melanoma cell line could sequester activated MMP2 at the cell surface via binding to $\alpha v\beta 3$ (180). Since this same integrin is implicated in production of MMP2 in melanoma (179) there may be the opportunity for matrix-stimulated production and sequestration of a protease at the cell surface; such a co-ordinated involvement in tumour invasion could explain the increased expression of $\alpha v\beta 3$ in the more aggressive phases of melanoma. However, integrins are probably not the principle mechanism for restricting MMP activity to the cell surface. Membrane type -MMPs (MT-MMPs) are membrane bound proteases (181) which can regulate the activity of the secreted pro-MMP molecules (182). In addition, plasmin, produced by the action of plasminogen activators, can also activate MMPs (183, 184). Since plasmin activity requires the membrane bound uPA receptor this enzyme also is located pericellularly.

Integrins are also implicated in the regulation of uPA while conversely its receptor, uPAR, may regulate integrin activities. Nip *et al* reported that antibody cross-linking of $\alpha v\beta 3$ resulted in an increase in uPAR mRNA, whereas expression of αv antisense oligo-nucleotides downregulated both αv mRNA and uPAR mRNA (185). The uPA receptor, especially if it is associated with uPA, functions as a high affinity receptor for vitronectin (186, 187)while vitronectin is the major binding protein for the uPA inhibitor PAI-1(188). Thus there exists the possibility that these components (uPA/ uPAR/PAI-1) of the serine protease pathway could all co-localise at sites of vitronectin receptor expression and raises the possibility of co-regulation of these two systems. In fact recent data suggests that vitronectin may concentrate plasminogen activator activity at the cell surface by binding to complexes of uPA and soluble uPAR, the latter produced in

small amounts by specific cell types including smooth muscle, endothelial cells and monocytic cells(189). Co-localisation of uPAR at αvβ3-rich focal adhesions has been reported in fibroblasts and rhabdomyosarcoma cells (190) and glioblastoma cells (191) although uPAR association with focal adhesions is not a universal observation (192, 193). Moreover addition of PAI-1 inhibited the αvβ3-dependent motility on vitronectin of smooth muscle cells; an activity suggested to be through steric hindrance since the binding site on vitronectin for PAI-1 is at the N-terminal domain adjacent to the integrin-binding site (194). Wei *et al* showed that over expression of uPAR in the 293 embryonal kidney cells (293-uPAR) increased their adhesion to vitronectin but decreased their affinity for fibronectin. The authors identified an oligopeptide which, they reported, bound to uPAR but did not block binding of either uPA or vitronectin to uPAR (28). Treatment of 293-uPAR with this oligopeptide reverted the adhesion phenotype to that of the parental line (high affinity for fibronectin, low affinity for vitronectin) (28). The mechanisms underlying these results are not yet clear but the authors further showed that soluble uPAR bound to purified αMβ2; an association which was enhanced by integrin activating factors. In essence these findings suggest that uPAR modulation of integrin activity in these cells may be occurring via a physical association between the uPAR and the integrin systems. Several other reports document that uPAR may be complexed with β1 and β3 integrins (195) as well as β2 integrins (196, 197) raising the possibility that uPAR-integrin co-regulation may be more widespread than previously suspected.

Cell migration is an integrin mediated process and uPA has been shown to modulate this activity. The melanoma line M24-met showed an increased ability to migrate toward Matrigel in the presence of exogenous uPA but a decreased ability to invade Matrigel if uPA activity was blocked by antibodies or addition of PAI-2 (192). The pancreatic carcinoma cell line FG migrates toward vitronectin in an αvβ5-dependent manner only if PKC is activated by phorbol esters or growth factors (198). Yebra *et al* further showed that uPA activation was also required since inhibition of uPA binding to uPAR with antibodies or blocking of uPA function by addition of exogenous PAI-2 inhibited this αvβ5-dependent migration on vitronectin (193). It appeared that the activation of PKC (by addition of PMA or TGFα) upregulated both uPA and uPAR mRNA and protein but how this actually influenced αvβ5-activity was not determined (193). However the effect was integrin-specific since neither αvβ3- nor α2β1-dependent migration was affected by the increased uPA expression (193).

ANGIOGENESIS: A TARGET FOR INTEGRIN-DIRECTED ANTI-CANCER THERAPY?

A major advance in integrin-related cancer research in the last five years has been the demonstration that it is possible to inhibit tumour angiogenesis by targeting specific integrins. Tumours grow beyond 2mm only if they have promoted angiogenesis (199). Since metastatic spread often is via the vasculature, inhibition of angiogenesis would restrict local tumour growth, and reduce significantly the potential number of cells likely to metastasise as well as removing a local 'transportation system' away from the primary tumour mass. The integrin αvβ3 is upregulated on the endothelium

of angiogenic blood vessels but is low or absent on resting blood vessels (200). Blockade of αvβ3 with antibodies or peptides inhibited the growth of tumours in the chick CAM model as well as inhibiting the growth of αvβ3-negative human tumour cells injected into human skin grafts growing on severe-combined immunodeficiency (SCID) mice (201, 202). Microscopic analysis of the transplanted skin suggested that blockade of αvβ3 induced apoptosis of the endothelial cells and thus inhibited blood vessel formation (202). Partly as a consequence of these studies a humanised form of the αvβ3-blocking antibody LM609 has been produced; termed Vitaxin it is undergoing early clinical trials and already has shown some promising effects in the treatment of vascular disease(203, 204).

Another anti-cancer, vascular-directed treatment which was developed independently of the above approach also targets αv integrins. Bacteriophages expressing peptide sequences which specifically bound to different tissue vasculature, including tumour vasculature, were identified by injecting a library of peptide-expressing phage particles into mice and then cloning the tissue-specific phages recovered from the appropriate microvessels (205). The phage that bound to the tumour vasculature included one expressing the Arg-Gly-Asp sequence which would be recognised by αv integrins. Coupling these phage particles to doxirubicin produced a drug capable of inhibiting growth of breast cancer xenografts in nude mice and of reducing drug toxicity(205); further indication that it may be possible to eradicate tumour by directing therapy at the endothelial cells in tumour blood vessels.

It should be noted αvβ3 is not the only integrin implicated in normal vascularisation. An αvβ5-dependent pathway of blood vessel formation also has been identified (206) and may partly explain why Glanzmann's thrombasthaenics who lack β3, or expression of a functional β3, (18) develop normally apart from having an absence of functional platelets and thus a dysfunctional clotting system. Similarly the β3-knockout mouse develops normally apart from a Glanzmann's like bleeding disorder (207). Surprisingly αv-knockout mice which lack all αv integrins, develop normally up to day E9.5 stage and show relatively normal organogenesis, including extensive vasculogenesis (208). Approximately 20% of these mice are born but die rapidly from intracerebral and intestinal bleeding. Thus the role of integrins in embryonal vasculogenesis is more complex than a somewhat superficial analysis of the αvβ3-data might have predicted.

SUMMARY

Integrins are essential for the normal proliferation, survival, motility and migration of cells. They perform these functions, particularly proliferation, in concert with growth factor receptor-dependent signals. Thus if tumour cells modulate the expression and/or activity of their integrins this potentially can have profound effects on various parameters of cell behaviour (Figure 5). Increased expression of a multi-functional integrin, such as αvβ3, or loss of expression or function of a position-restricting integrin, such a α6β4, clearly would offer significant advantages to a motile invading cancer cell. In addition, mutations in integrin-signalling pathways could also result in integrin-promoted increased growth, migration and survival without the need for change in integrin expression or activation state.

At the present time perhaps the most promising and most accessible integrin target for cancer therapy comprises the expression of αv integrins on tumour blood vessels and their targeting as a means of destroying tumour blood flow. However in studies by Weaver and colleagues (1997) reduction in β1-integrin signalling by the addition of inhibitory-antibodies caused a tumourigenic breast cell line to re-express E-cadherin-dependent cell adhesion, adopt a more differentiated morphotype and to lose tumourigenicity (209). These data lead to the possibility that by modulating the strength or variety of signals from the ECM it may be possible not only to inhibit tumour growth but also to revert neoplasia to a more normal phenotype. Thus, as we continue to dissect the integrin-signalling pathways it may be possible in the future to target these intracellular pathways in an entirely different and novel fashion.

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Chapter 3

THE ROLE OF HYALURONAN AND ITS DEGRADATION PRODUCTS IN TUMOUR ANGIOGENESIS AND METASTASIS

David C. West

Key words: Tumour, Angiogenesis, hyaluronan, hyaluronidase, metastasis, PH-20

Abstract: High-molecular-weight hyaluronan (HA) is inhibitory in both *in vivo* and *in vitro* models of angiogenesis. Conversely, a specific range of HA-degradation products consistently stimulates angiogenesis in these same models. Recent studies in wound healing models revealed a close temporal relationship between tissue hyaluronidase activity, tissue HA-degradation and neovascularization. It appears that HA-degradation is a prerequisite for the induction of wound angiogenesis. On going studies with transplantable tumours and tumour cell-lines indicate that both metastatic properties and angiogenesis are associated with elevated levels of hyaluronidase and HA degradation. RT-PCR analysis suggests that this is due to an aberrant, or elevated, expression of the GPI-anchored cell-surface hyaluronidase PH-20 and, in some cell-lines, the HYAL1 enzyme. The level of PH-20 expression shadows both angiogenic and metastatic potential of these cell-lines i.e. tumour progression. The role of HYAL1 is more controversial, but the present study suggests that in some cell-lines this may also play a role in the progression of tumours to a metastatic/ angiogenic phenotype.

INTRODUCTION

Hyaluronan (HA, previously known as hyaluronic acid or hyaluronate) is a high-molecular-weight unbranched polysaccharide, composed of repeating 1,4- linked disaccharide units made up of 1,3- linked glucuronic acid and N-acetylglucosamine (Figure 1). It is an honorary member of the glycosaminoglycan family, but differs from the other glycosaminoglycans as it is non-sulphated, is not synthesised covalently bound to a protein backbone and is synthesised directly through the cell plasma membrane. In common with other

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glycosamninoglycans, small amounts of HA are present in the extracellular matrix of most animal tissues, but the greatest concentrations are found in the avascular adult connective tissues, such as cartilage, synovial fluid and the vitreous humour (Table 1). This prevalence in connective tissues, combined with its high water-binding capacity and its simple structure, led to the general belief that its main function was that of an inert viscoelastic lubricant, or space-filling molecule (1, 2). Furthermore, its mechanical properties, poor immunogenicity and chemical simplicity have made it the basis for biomaterials used in viscosurgery, tissue engineering and drug delivery systems (3,4). However, over the last ten years a growing number of reports have appeared indicating that HA is not an inert mechanical molecule, but can have profound effects on the behaviour of numerous cell- types, through its interaction with several surface receptors (5-7). These effects differ depending on the cell-type concerned and appear dependent on both the size and concentration of the HA.

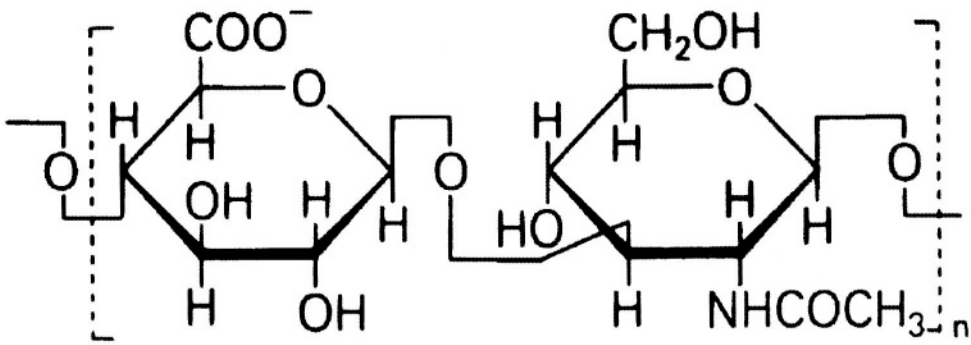

Figure 1. A schematic representation of the structure of the disaccharide unit of hyaluronan

A HA- rich matrix also develops briefly during embryogenesis, and adult tissue regeneration, or repair, coincident with rapid cell proliferation and migration. Subsequently, a decrease in tissue HA occurs concomitantly with tissue differentiation, vasculogenesis and angiogenesis (8). A localised accumulation of HA is also seen in association with tissue damage, organ rejection and many inflammatory diseases, such as psoriasis and scleroderma, and it is a major component of many tumours (9-11). The localisation and timing of HA accumulation during development and tissue repair suggests that the synthesis and degradation of HA plays an important regulatory role in these processes, especially vascularization, and certain pathological conditions such as tumour growth and metastasis (12-14).

IN VITRO EVIDENCE FOR A SIZE-DEPENDENT REGULATION OF ANGIOGENESIS

In vitro studies on the effect of macromolecular HA ($\geq 10^3$ kDa) on cultured endothelial cells have consistently shown that it significantly inhibits endothelial cell proliferation and migration (15-18) and disrupts cell/cell interactions in newly formed endothelial cell monolayers. At least *in vitro,* the HA- mediated inhibition can not be reversed by the addition of exogenous growth factors, unless the HA is degraded (16, 18). Furthermore, these effects are significant at physiologically relevant concentrations ($\geq 100\mu g/ml$) i.e. at HA concentrations found in adult avascular connective tissues and transiently during tissue remodelling and repair. In addition, the inhibitory effect decreases, as the size of HA is reduced (15). We have recently confirmed, and extended, these earlier findings using an *in vitro* angiogenesis model in which a section of rat aorta is cultured in a collagen-gel matrix, under serum-free conditions (19). We have examined the effect of HA size on both microvessel and fibroblast growth. Preliminary data suggests that macromolecular HA selectively inhibits endothelial capillary formation in a size-dependent manner. In contrast, fibroblast numbers increase as the size of the HA was increases (Burbridge and West, unpublished results). Watanabe et al. (20) have independently reported similar results, with isolated endothelial cells cultured in a 3-dimensional collagen gel system. Also, high concentrations (1mg/ml) of HA have been reported to inhibit the migration of human adipose microvascular endothelial cells into fibrin gels, *in vitro,* and blood vessel formation in fibrin-gels implanted subcutaneously in guinea pigs (21,22).

Table 1. Concentration of HA in selected tissues, in the case of tissues the concentration is expressed as mg/Kg. Taken from (9)

Source	**Concentration**, mg/L
Rooster comb	7500
Human umbilical cord	4100
Human synovial fluid	1420-3600
Bovine nasal cartilage	1200
Human vitreous humour	140-338
Rabbit renal papillae	250
Human dermis	200
Rabbit brain	65
Rabbit muscle	27
Human amniotic fluid (16 weeks)	20
Human thoracic lymph	8.5-18
Human aqueous humour	1.14
Human amniotic fluid (term)	1
Human urine	0.1-0.5
Human serum	0.01-0.1

The initial report of a discrete size-range of HA- degradation products (OHA: 2-8 kDa or 4-20 disaccharides in length), able to induce angiogenesis in the chick chorioallantoic membrane (CAM) assay (23), was followed by a series of *in vitro* studies (Table.2). These revealed that OHA could directly and specifically stimulate both the proliferation and the migration of cultured endothelial cells from several sources (16,17,24). Recently, using the rat aortic model (see above), OHA has been found to stimulate three-dimensional microvessel formation maximally at 0.1 μg/ml, compared with 1 μg/ml in the two-dimensional models. Angiogenesis in this model is thought to be dependent on endothelial migration, rather than proliferation (Burbridge and West, unpublished results).

Table 2. The anti- angiogenic actions of macromolecular hyaluronan (>500 kDa)

In vivo effect:

- inhibits granulation tissue formation and vascularization (48, 97),
- inhibits vascularization of implanted fibrin gels (21),
- inhibits cartilage vascularization (12),
- induces regression of the capillary plexus in chick limb buds (49), and
- Wound fluid HA inhibits its angiogenic activity in the CAM assay (16)

In vitro action:

- Inhibits human and bovine endothelial cell proliferation (15-18),
- Inhibits endothelial cell migration in several models (20,22,24),
- Disrupts endothelial cell- cell adhesion (17),
- Down regulates NFkB activation and IkB degradation (43), at physiological relevant concentrations i.e. 100- 200 μg/ ml.

Several reports have independently confirmed the angiogenic nature of OHA in alternative *in vitro* assays. Hirata et al (25) and Rahmanian et al (26) reported that OHA markedly stimulated endothelial tube formation, in collagen gels and on "Matrigel", and two recent studies have shown that OHA can induce endothelial cell invasion of 3-dimensional collagen and fibrin matrices (27, 28). Furthermore, Trochon et al (28, 29) confirmed the lower size-limit of the angiogenic activity as the HA octasaccharide and found that the stimulation could be neutralised by the addition of specific HA-binding proteins.

ENDOTHELIAL CELL RESPONSE TO HA SIZE: TOWARDS A MECHANISM OF ACTION

Endothelial cells appear to possess about 10^5 high affinity HA-receptors (Kd 2 X 10^{10}) on their cell surface, that both bind and internalise HA (18, 26,30-32). Analysis of HA-affinity [I^{125}-]- labelled cell-surface proteins from both human and bovine endothelial cells, originally identified five major putative cell-surface HA- binding proteins, between 90 and 125 kDa, with two minor proteins of 78 and 46 kDa (18,24,30). Western blotting and RT- PCR determination of CD44 splice-variants,

indicates that the 78-125 kDa proteins are forms of non-variant CD44H, differing in their glycosylation, and Intercellular adhesion molecule-1 (ICAM-1) isoforms (West, unpublished results). Although ICAM-1 was originally reported to be a HA- receptor (33), recent data from the same group (34) suggests that ICAM-1 does not bind HA and that ICAM-1 is not a HA-receptor. The putative 200- 400 kDa liver endothelial cell HA-receptor (35) and the well characterised mesenchymal cell HA receptors RHAMM and hyaluronectin appear to be absent from our cultured endothelial cells (5).

To date, biochemical analysis suggest that CD44 is the only HA receptor on human endothelial cells. Whilst CD44 appears to play an important role in the angiogenic process (28, 36), it also binds the HA- hexasaccharide, which is not angiogenic (23,28). Thus there may be an uncharacterised endothelial cell HA-receptor which mediates the "angiogenic" activity of OHA. However, Slevin et al (37) have reported that OHA induces rapid phosphorylation of CD44 in bovine aortic endothelial cells. Furthermore, Noble et al (38,39) and Horton et al (40,41) have reported a similar size-dependent activation of murine bone-marrow macrophages by HA-oligosaccharides, between the hexasaccharide and 440 kDa, mediated by binding to CD44.

Initial studies into the metabolic effects of OHA on cultured endothelial cells indicated that OHA could induce proliferation-related changes in protein expression and phosphorylation (16). Montesano et al (27) have reported that OHA, but not macromolecular HA, induces urokinase- type plasminogen activator (uPA) and plasminogen activator inhibitor (PAI) within a few hours. Whilst Rooney et al (42), found that OHA stimulated endothelial cell synthesis of Type I and VIII collagens. Whilst the former is an early event in the activation of the endothelial cascade, the latter relates to basement membrane synthesis and "sprout" formation. Recently, we have shown that OHA can induce, or substantially increase, endothelial cell expression of CD44, E-selectin, Flk-1 and Flt-1 vascular endothelial cell growth factor (VEGF)-receptors and interleukin-8 (IL-8) mRNA. Surprisingly, ICAM-1 is not upregulated (43). This pattern of protein induction differs from that seen with either basic Fibroblast growth factor (bFGF) or VEGF.

The induction of E- selectin by OHA, together with the rapid increase in IL-8 mRNA levels in OHA stimulated cells (West and Noble, preliminary data), suggested that activation of endothelial NFkB may be an early event in OHA inducted angiogenesis. Electrophoretic mobility shift analysis (EMSA) of cultured human umbilical vein endothelial cells, treated with OHA or macromolecular HA for various times, showed that OHA rapidly activates NFkB. Preliminary data indicates that macromolecular HA does not activate NFkB, but significantly down-regulates NFkB activation over several hours (43). Degradation of IkB parallels the changes in NFkB activation. A similar size- dependent CD44-mediated activation of NFkB has been reported for murine macrophages (38), although the exact mechanism is still unclear. In contrast, Deed et al (44) recently reported that OHA induces the immediate early response genes c-fos, c-jun, jun-B, Krox-20 and Krox-24 in bovine aortic endothelial cells, whilst high-molecular-weight HA does not. The induction of these early response genes may be mediated by the activation of protein kinase C (37). However, Laniado-Schwartzman et al (45) reported that 12(R)-hydroxyeicosatrienoic acid, an angiogenic arachidonic acid metabolite, rapidly activated of both NFkB and, to a lesser extent, the AP-1 transcription factor in cultured endothelial cells. Thus, OHA appears to bind to CD44

and activate both the NFkB and AP-1 transcription factors, by a mechanism, which may involve protein kinase C.

Several basic questions remain to be answered, such as: why are cultured endothelial cells inhibited by macromolecular HA, when many other types of cell are stimulated under the same conditions? ; why is the size-range of OHA so unique to endothelial cells and much more limited than that which activates macrophages? and, if both HA and OHA effects are mediated by CD44, what is the difference in their mechanism?

The regulation of CD44 is complex and has many cell-specific features (7). The issue of endothelial CD44 splice-variants does not seem to be a factor with endothelial cells, as several studies, including our own, have found that only CD44H is expressed on endothelial cells, even when activated (46,47). It is obvious that macromolecular HA and OHA differ in their ability to cross-link and induce multivalent clustering, or capping, of surface CD44H molecules. It seems probable the single, or even dimeric, HA-CD44 complexes formed by OHA can induce different intracellular signalling to that of the multivalent complexes formed with macromolecular HA. Thus, the surface density of CD44H, and other HA-binding CD44 variants, may be important in determining the size of HA at which such multivalent complexes form. This may account for the size-specificity observed with specific cell types. Also, CD44 associates with metalloproteinases, growth factor receptors, osteopontin and probably RHAMM. As RHAMM has not been found on endothelial cells, the lack of CD44-RHAMM interaction may differentiate endothelial cells from other types of stromal cell, e.g. fibroblasts, especially in respect of migration in HA-rich matrices.

HA DEGRADATION *IN VIVO* IS A PREREQUESITE FOR ANGIOGENESIS.

The occurrence of high levels of HA in avascular tissues, such as cartilage and vitreous humour, and at relatively avascular sites, such as the desmoplastic region of invasive tumours, suggests that extracellular matrix HA also inhibits angiogenesis *in vivo* (16). The recent report that vascularisation of cartilage, *in vivo,* requires prior degradation of its HA matrix, gives added support to this hypothesis (12). Furthermore, *in vivo* studies indicate that high concentrations of exogenous macromolecular hyaluronan can inhibit neovascularization during granulation tissue formation (21,48) or induce regression of the immature capillary plexus in the developing chick limb bud (Table.3) (49). In contrast, exogenous OHA has consistently stimulated angiogenesis in different *in vivo* models, including: the chick chorioallantoic membrane assay (23); both wound healing and graft models (50,51, West et al., in preparation), and after subcutaneous implantation or topical application (52). Using the rabbit corneal assay, Hirata et al (25) have independently confirmed the *in vivo* angiogenic activity of this range of HA oligosaccharides.

Early structural studies on the differentiation of the vasculature of the chick chorioallantoic membrane indicated that matrix HA disappeared and was replaced by sulphated glycosaminoglycans during vascularization of the membrane (53). In a series of studies we examined the relationship between angiogenesis and tissue HA metabolism, using a rat sponge-implant wound healing model (West et al., in preparation) a freeze-injured rat skin-graft model (51), and sheep foetal wounds (54). The data from all three models points to there being a close temporal association between tissue angiogenesis and the

Table 3. The angiogenic properties of OHA

In vitro effects:

Stimulates endothelial:

- Proliferation (15-17, 20),
- migration (24),
- migration into collagen/ fibrin gels (22,25,27,28), and
- tube, or lumen formation (25,26).

Induces:

- Synthesis of proliferation- / activation-associated proteins (16),
- Synthesis of type VIII collagen (42),
- Synthesis of uPA and PAI-1 (27),
- Upregulates expression of CD44H, E-selectin, IL-8 and VEGF receptors (43),
- Induces phophorylation of CD44 (37),
- Activation of AP-1 and NFkB (43,44), and
- Degradation of IkB (43)

In vivo response:

Induces angiogenesis in

- the CAM (23,245),
- the rabbit corneal assay (25),
- rat skin on topical application (52), and
- rabbit after subcutaneous implantation (Kumar & West, unpublished, 98).

Stimulates angiogenesis in:

- an impaired rat skin-graft model (51),
- a rat polyvinyl sponge implant model (West et al, in preparation; 27), and
  a pig full-thickness wound model (50).

degradation of matrix hyaluronan, as indicated by both a rapid fall in tissue HA size, and content, coinciding with an increase in hyaluronidase levels. This appears to be the case both in normal healing and when the onset of tissue angiogenesis is accelerated by exogenous angiogenic compounds. Furthermore, Mast et al (55) have shown that the addition of exogenous streptomyces hyaluronidase to foetal wounds, decreases wound HA content and increases both fibroplasia and capillary formation. To date we have not detected HA-degradation products as small as OHA in healing wound tissue. This suggests that production of angiogenic HA-oligosaccharides is not responsible for the onset of angiogenesis and that merely removing the inhibitory effect of macromolecular HA is sufficient to allow vascularization. However exogenous OHA, which had been introduced a few hours before sampling, could not be detected in tissue samples. Which suggests that small HA-oligosaccharides are either phagocytosed and rapidly degraded, or that they rapidly diffuse out of the tissue.

TUMOUR HYALURONAN METABOLISM AND ANGIOGENESIS

Increased hyaluronan levels have often been reported to be associated with human and animal malignancies, particularly in connection with tumour invasion (10,11). In addition, most tumours also exhibit increased angiogenesis (vessel density), notably metastatic tumours (56). Given the anti-angiogenic properties of HA, these two statements appear contradictory. However, morphological investigations on the localisation of HA within tumours have shown that in many tumours hyaluronan accumulates within the tumour-associated connective tissue, with the tumour cells themselves essentially negative for hyaluronan (57-59). This demarcation is most obvious in poorly differentiated epithelial-derived tumours (59). Although this is not the case for all tumours, morphological examination of Wilm's tumour revealed that most HA is associated with the epithelial blastemal cells, rather than the stroma (60). Coincidentally, one of the first insights into this apparent enigma came from an examination of the concentration and size of circulating HA in the sera of patients with Wilm's tumour and a bone-metastasising form of Wilm's tumour, Bone Metastasising Renal Tumour of Childhood (BMRTC). The study revealed that, whilst both groups of patients had extremely high levels of circulating HA (50 mg/ L), the HA in the sera of patients with metastatic BMRTC was very small, similar in size to the angiogenic HA-oligosaccharides. In contrast, Wilm's tumours, which are rarely metastatic, produced high levels of high molecular weight HA (61). Subsequently, cultured cells from these two forms of kidney tumour were found to produce HA similar in size to that found in the patients sera i.e. cells cultured from BMRTC tissue produced low-molecular-weight HA. More recently, we have analysed the HA content/size and hyaluronidase activity of allogeneic murine and rat tumours and human tumour xenografts. Although HA levels were generally increased, there was a significant reduction in HA size, compared with normal tissues, suggesting that the tumour environment may be more receptive to vascularization than HA levels alone would suggest. We also found that those tumours with low HA content ($\leq 100 \mu g/g$) or low molecular weight HA were generally metastatic, supporting the hypothesis that tumour HA metabolism may play a part in regulating tumour angiogenesis. The size of HA in this series of tumours also exhibited a loose inverse relationship to the relative levels of tumour HA and hyaluronidase activity (62,63). Similarly, Lokeshwar et al. (64) have recently reported an association between elevated hyaluronidase levels and prostate cancer progression, whilst hyaluronidase activity has been correlated with metastatic ability in biopsies of ovarian, endometrial and breast tumours (65, 66).

It is generally accepted is that HA degradation occurs exclusively intracellularly, in the lysosomal system, after receptor mediated internalisation. In hepatocytes, kupffer cells and sinusoidal endothelial cells, catabolism appears to go to completion yielding acetate, ammonia and water (67,68). Initial extracellular depolymerization of HA or proteoglycan complexes, mediated by oxygen-derived free radicals, nitric oxide or proteinases, appears to be a necessary prerequisite for internalisation. Although this may be the case for circulating HA, our analysis of HA in wound tissues and tumours suggests that a large scale, sequential degradation of the whole population of HA molecules takes place. In addition, the extent of HA depolymerization appears to increase with increasing extracellular "acidic"

hyaluronidase activity. The most likely explanation of these data is that, in many tissues, HA degradation is mainly extracellular and is catalysed by extracellular hyaluronidase(s).

Until recently, only one such enzyme had been reported, the glycosyl-phosphatidylinositol (GPI) -anchored sperm PH-20 hyaluronidase, whose distribution is normally restricted to the testes (69-71). However, the recent finding that a second hyaluronidase (HYAL1) present in normal serum also contains the GPI-anchor, indicating that cell-surface hyaluronidase(s) may be present on many cell-types (72-75). Two other hyaluronidase genes have recently been identified (HYAL2 and 3), HYAL2 appears to be essentially lysosomal and both may be upregulated by exogenous cytokines (76-78). Thus, of the known hyaluronidases, the two best candidates for extracellular matrix degradation are PH-20 and HYAL1.

The human PH-20 gene (SPAM1) has been mapped to chromosome 7, in the 7q31 region (79). This region contains a known fragile site (FRA7G) and deletions, or loss of heterozygosity, in this region are frequent in human cancers, including squamous cell carcinomas of the head and neck, prostate cancer, renal cell carcinoma, ovarian adenocarcinoma, colon carcinoma and breast cancer (80). This may be due to the deletion of the putative tumour suppressor genes caveolin-1 and –2 which co-localise to this region (80-82). Several other genes have been localised to this region, including the cystic fibrosis gene and c-met proto-oncogene, the hepatocyte growth factor receptor (79, 83). The latter gene has been reported to be mutated and/ or amplified in many tumours. In addition to the high level of mutation associated with this region of chromosome 7, numerical aberrations of chromosome 7 are also a common feature of many tumours, with trisomy, tetrasomy and polysomy a common finding. Interestingly, chromosome 7 gain appears to be closely associated with lymph node metastasis in breast cancer (84) and is thought to be a late event in tumour progression (85).

The genes for HYAL1, 2 and 3 have been mapped to chromosome 3p21 in human and 9F1-F2 in mouse (75, 77). Small cell lung carcinomas, nasopharyngeal carcinomas and several other tumours have deletions in this region, which contains a number of putative tumour suppressor genes, e.g. LuCal, 2 and 3, and Fus2 (see references 75, 77). In fact, HYAL1 may well be identical to the LuCal tumour suppressor (75). In mice the Hyall gene is probably responsible for serum hyaluronidase polymorphism and congenic mouse strains, differing only at the Hyal-1 locus, have been used to study the effect of serum HYAL1 levels on tumour growth. Mice with higher circulating levels of hyaluronidase were found to be more resistant to tumour growth than those with low levels (86). In addition, injected hyaluronidase has been reported to decrease the induction of tumours in BALB/c mice, by dimethylbenzanthracine (87). It is thought that the higher levels of circulating hyaluronidase may degrade the pericellular matrix around tumour cells, thus making them more accessible to cytolytic leukocytes (88), but hyaluronidase digestion of the pericellular matrix has also been reported to make cells more susceptible to Tumour necrosis factor-α (TNFα) cytotoxicity (89,90). However, the evidence that HYAL1 hyaluronidase plays a role in tumour suppression is indirect.

In a recent series of experiments, cultured tumour cell lines were examined in respect of their HA production, HA size, hyaluronidase activity and angiogenic activity. The most metastatic, usually those with the highest angiogenic activity, generally produce low-molecular- weight HA, both in the

medium and on the their cell- surface, and expressed high hyaluronidase activity. A more detailed study of this relationship examined three closely related human colon carcinoma cell-lines (Table. 4.), HCT116 A(+) and HCT116 B(+), originally isolated from the same colorectal carcinoma by Dr Brattain (Houston), and 2010-1 cells, a subclone of the more invasive HCT116 B(+) line, selected by Dr Kinsella (Liverpool) for its increased invasiveness *in vitro* (91). However, in a caecal metastasis model the greatest number of liver metastases were found in animals bearing HCT116 A(+) tumours (62). Analysis of these cell-lines for HA production and hyaluronidase activity gave some interesting results. HCT116 A(+) cells produced much lower amounts of HA than either of the other two cell-lines, which was also much smaller than that produced by the other two cell-lines. The HCT116 A(+) cells also had much higher levels of hyaluronidase activity, which was mainly cell-associated, whereas little activity could be detected in the other cell-lines, suggesting a causative relationship between high enzyme levels and the reduced HA size (13, 14, 62). Furthermore, the range of HA secreted by HCT116 A(+) cells (< 30- 300 kDa), in common with other metastatic cell lines, suggests that angiogenic OHA are probably present.

Table 4. Comparison of hyaluronan content, HA size, hyaluronidase activity, PH-20 and Hyal1 expression, with the angiogenic activity of a series of related human colorectal carcinoma cell lines of increasing metastatic ability (HT116A > HT116B = 2010)

Tumour cell line	Total HA (*)	HA size medium (#)	HA size cells (#)	Hyaluro-nidase (**)	PH-20 expression	HYAL1 expression	Angiogenic activity
HCT-116 A (+)	600	300	100	0.05	++++	+	+++
HCT-116 B(+)	2,000	1,800	900	-	++	+/-	+/-
2010	3,700	2,500	1,000	-	+	+/-	+/-

*: total HA ng/10^6 cells
**: NFU/10^6 cells)
kDa

Comparison of the angiogenic activity of conditioned serum-free media from the three cell-lines, in the CAM assay, showed that only HCT116 A(+)-conditioned medium was angiogenic. Interleukin-8 (IL-8) was found to be the main angiogenic cytokine in the HCT116 A(+) and HCT116 B(+) cell lines (92), with HCT116 A(+) cells secreting three times that of the HCT116 B(+) cells. Although the difference in angiogenic activity between the cell lines was potentially due to their IL-8 concentration, removal of macromolecular HA from the HCT116 B(+)- conditioned medium, by centrifugation through a 100 kDa cut-off filter, removed both the macromolecular HA and the inhibitory activity from the HCT116 B(+)- conditioned medium (Table.4). In addition, when HCT116 A(+)- conditioned medium was mixed with HCT116 B(+)- conditioned medium the angiogenic activity was marginal, confirming that HCT116 B(+)-

conditioned medium contained an anti-angiogenic activity.

In common with earlier studies with transplanted tumours, there is a loose correlation between HA size and hyaluronidase activity. Interestingly, the relationship is improved if the degree of HA synthesis is also taken into account (i.e. the enzyme/ substrate ratio), again supporting the hypothesis that HA degradation is mainly extracellular.

Sodium dodecylsulphate (SDS)-polyacrylamide HA-gel electrophoresis of the conditioned medium, and cellular extracts, from tumour cell lines revealed two distinct patterns of hyaluronidase expression in the most active tumour cell-lines. One group, which included the Hep2 (laryngeal) and Bu25 (cervical) cells, apparently expressed only a 57 kDa hyaluronidase, active at pH3.5. The second group, including D98 (cervical), HeLa (cervical), MCF7 (breast), HT116A (colorectal) and weakly in HT116B (colorectal) cells, expressed two isoforms at 64 and 50 kDa. These patterns resemble those reported for HYAL1 and PH-20, respectively. Phospholipase C (PLC) digestion of cultured cells with phospholipase C (PLC) released hyaluronidase activity from the surface of the second group of tumour cell-lines, but not the putative HYAL1 group, consistent with their expressing PH-20 and HYAL1, respectively (73).

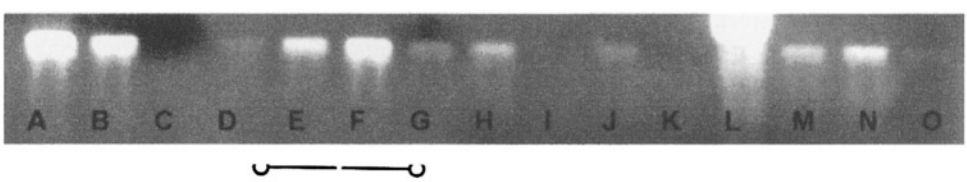

Figure 2. A graph showing the relationship between total hyaluronidase (Hase) activity and PH-20 mRNA expression by tumour cell-lines. Filled symbols represent tumour cell lines in which PH-20 expression is less than expected from their enzyme activity.

PCR analysis of the mRNA isolated from these cell lines showed a range of PH-20 expression (Table.4; Figure 3), with only 3 cell lines showing no PH-20 expression. Interestingly, the Hep2 cell-line was one of these, confirming that the 57 kDa hyaluronidase is not a form of PH-20 hyaluronidase. In most cases PH-20 expression and hyaluronidase activity showed a good correlation (Fig.2). Some cell lines, including the Hep2 and Bu25 cell-lines, expressed less PH-20 mRNA

than would have been predicted by their hyaluronidase activity. PCR analysis for HYAL1 (73) confirmed that the Hep2 cell line expressed high levels of HYAL1 mRNA. It is interesting that only a single form of the hyaluronidase enzyme (57 kDa) is expressed when multiple mRNA species were detected by PCR, in agreement with Csoka et al (73).

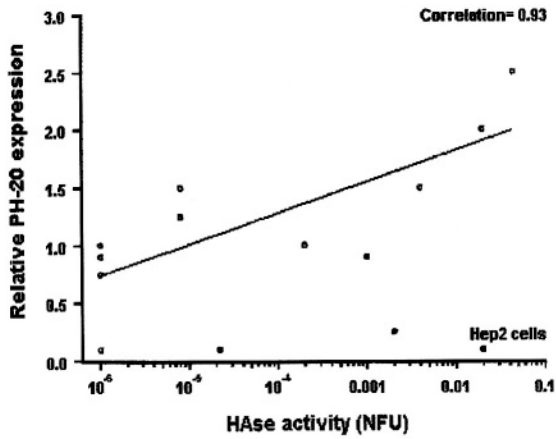

Figure 3. A representative agarose gel of RT-PCR products from tumour cell-lines analysed for PH-20 expression, using the primers outlined by Liu et al (93). Lane [A] HL60 (myeloma), [B] HeLa (cervical), [C] WiDr (cervical), [D] MCF-7 (breast),), [E] A2058 (melanoma), [F] HCT116B (colorectal), [G] KYM-1 (rhabdomyosarcoma), [H] Bu25TK- (cervical, TK-), [I] Du145 (prostate), [J] FSG (fibrosarcoma), [K] Hep2 (laryngeal), [L] RAJI (Burkitts lymphoma), [M] HCT116B (colorectal), [N] D98/AH2 (cervical) and [O] 2010 (colorectal) cells. Note artifactual band in lane K, this does not hybridize with human full-length PH-20 cDNA probe

Liu et al. (93) have reported expression of the glycosylphosphatidyl-inositol (GPI)-anchored PH-20 sperm hyaluronidase human colon carcinoma tissue, but not normal colon. Furthermore, in agreement with our own data, they found that the angiogenic activity of colon carcinoma cell lines was associated with their PH-20 expression. Addition of apigenin, a non-specific flavanoid inhibitor of hyaluronidase, to the cultured cells, prior to application, greatly reduced their angiogenic activity. However, apigenin is antiangiogenic and cytotoxic to tumour cells *in vitro,* but appears ineffective *in vivo* (94).

The size of the HA produced by the most highly angiogenic cell-lines was similar to that found previously in BMRTC cultures and patient serum. Whilst the HA-degradation products may play a role in stimulating angiogenesis and metastasis in the primary tumour, possibly amplifying the endogenous angiogenic cytokines (10,22,51), results from excised tumours suggest that the major part of this will rapidly diffuse into the circulation. Recently, Zeng et al (95) have reported that perfusion of tumour-bearing mice with HA-oligosaccharides (3-12 dissacharides in length; at approximately 0.1□g/ml circulating concentration, for 7-14 days) causes 80% inhibition of tumour formation. As higher concentrations of circulating HA-oligosaccharides were present in the sera of BMRTC patients, this suggests that circulating HA-oligoisaccharides produced by established primary tumours may inhibit the formation of metastases, in a similar way to "Angiostatin" and "Endostatin"

(96). Moreover, disruption of CD44-HA interaction also inhibits tumour formation and induces tumour cell apoptosis. In a alternative approach, Green and Underhill, have achieved similar results by perfusion with a preparation of HA-binding protein fragments, "Metastatin" (Green, personal communication).

CONCLUSIONS

Macromolecular HA inhibits angiogenesis in both *in vitro* and in many *in vivo* models, but OHA stimulates angiogenesis in these same systems. *In vitro* studies suggest that both interact with endothelial cell CD44H activating (OHA), or inhibiting (HA), the formation of the NFkB and AP-1 transcription factors. Examination of adult and foetal wound healing models indicate that high-molecular-weight HA is inhibitory to new blood vessel formation and that its degradation is a necessary prerequisite to tissue vascularisation. Subsequent study of transplantable tumours and cultured tumour cell lines, suggests that hyaluronidase degradation of extracellular matrix and cell-associated HA is related to the metastatic nature of tumours and also their angiogenic activity. The high hyaluronidase activity found in metastatic and angiogenic tumour cell-lines appears to be the result of aberrant expression of the sperm PH-20 hyaluronidase and/ or over expression of the HYAL1. Both are GPI-linked cell-surface hyaluronidases and their increased expression coincides with a greater degree of matrix and cell-surface HA degradation. It is probable that this abnormal upregulation in the expression of these hyaluronidase(s) may represent a significant step in rumour progression i.e. "an angiogenic/ metastatic switch". Nevertheless, not all cells in a particular tumour may express this phenotype, as exemplified by the three human colorectal carcinoma cell-lines mentioned above which were all isolated from the same tumour (60). Thus it unlikely that such extensive HA degradation is present throughout the tumour, but may be localised to vascular "hot spots" (49). It is also probable that in some metastatic tumours, at least, circulating levels of HA-oligosaccharides may be high enough to inhibit the growth of secondary foci until removal of the primary tumour lowers these levels (61,95).

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Chapter 4

THE E-CADHERIN/CATENIN COMPLEX IN INVASION: THE ROLE OF ECTODOMAIN SHEDDING

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Key words: Invasion; E-cadherin/catenin complex; ectodomain shedding; proteolysis.

Abstract: The E-cadherin/catenin complex is an invasion suppressor in epithelial cells. E-cadherin is a glycoprotein with an extracellular, a transmembrane and a cytoplasmic part. The cytoplasmic part is connected with the actin cytoskeleton via the catenins. ß-catenin appears to be a functional modulator of the complex, because its association is not restricted to E-cadherin only. (ß-catenin is also found in cytoplasmic (e.g. with the Adenomatous Polyposis Coli gene product, glycogen synthase kinase 3ß and axin) and nuclear (e.g. lymphoid enhancer binding factor-1 and pontin 52) complexes, that compete with its availability for the E-cadherin/catenin complex. The extracellular part of E-cadherin contains a histine-alanine-valine sequence responsible for homophilic interactions. Enzymatic cleavage of this extracellular part yield a 80 kDa fragment coined ectodomain. Ectomain shedding is a phenomenon described for many peptide receptors at the plasma membrane, and is mainly ascribed to the activity of two families of proteases: matrix metalloproteinases (MMPs) and adamilysins (ADAMs). Ectodomain shedding of E-cadherin seems to be instrumental in the functional downregulation of the E-cadherin/catenin complex: it can lead to loss of cell-cell adhesion and induction of invasion. The enzymes implicated in this process are considered as targets for anti-invasive strategies.

CANCER: INVASION AND METASTASIS

Cancer is a chronic and progressive disease characterized by growth deregulation, cellular dedifferentiation and invasion. Cancer is the result of an accumulation of genetic alterations leading to tumour progression (1, 2, 3) (Figure 1). Oncogenes and tumour-suppressor genes have been associated mainly with the regulation of growth. Activation of oncogenes and inactivation of tumour-suppressor genes lead to the transformation of a normal tissue into a benign tumour. Invasion-promoter and invasion-suppressor genes were distinguished from oncogenes and

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tumour-suppressor genes (4, 5, 6, 7). However, the distinction between both categories of genes implicated in tumour development has vanished (8, 9, 10). Although there is little doubt that these genetic alterations are the momentum of cancer development, it is our opinion that host cells are crucial for invasion and metastasis (Figure 2). Therefore, we consider invasion within a micro-ecosystem in which there is continuous molecular cross-talk between cancer cells and host elements, together establishing the tumour. Host elements implicated are : inflammatory cells, immunocytes, endothelial cells, fibroblasts, and extracellular matrix produced by them. Invasion permits the cancer cells to leave the tissue from which they originated, to enter into the circulation, to home at distant organs, to extravasate and to establish a new micro-ecosystem. There, cancer cells create a new invasive tumour, called metastasis from which a new multi-step process of invasion may start (Figure 3). Each step of the invasion process is characterized by a micro-environment containing the cancer cell and elements of the host, the latter differing from organ to organ. Several cellular activities are implicated in invasion : cell-cell adhesion, cell-matrix adhesion, motility and proteolysis. All the cells participating at these activities exhibit considerable flexibility in their interactions with other cells or with components of the extracellular matrix. Most molecules of interest, such as cadherins, integrins, proteinases, motility factors and their receptors form complexes that are sensitive to modulation at multiple levels. This is in line with the opinion that invasion is a temporary and repetitive phenomenon (11, 12, 13). Our laboratory has paid major efforts to the invasion-suppressor complex formed by E-cadherin and its associated cytoplasmic proteins, the catenins. This complex is, indeed, modulated at various levels, namely gene mutation, hypermethylation of the promoter, protein phosphorylation and protein-protein association (14, 15, 16, 17, 18). We have investigated a novel way in which the E-cadherin/catenin complex is downregulated, namely ectodomain shedding of E-cadherin leading to invasion.

THE E-CADHERIN/CATENIN COMPLEX

The E-cadherin/catenin complex is localized at the adherens junctions, it is largely responsible for the organization of epithelia.

E-Cadherin

Cadherins compose a superfamily of related cell surface molecules that require calcium for their structure and function. Type 1 cadherins show tissue-specificity as exemplified by E- (epithelial), P- (placental) and N- (neural) cadherin (19). They contain an extracellular part with a HAV (histidine alanine valine) sequence, a single transmembrane region and a genomically well conserved cytoplasmic tail. The human E-cadherin gene, cloned and characterized by Berx et al (20), is localized on chromosome 16q22.1. E-cadherin is synthesized as a 135 kD precursor molecule, glycosylated and processed to the mature protein by enzymatic removal of 127 NH_2-terminal amino acids (21).

The extracellular domain of E-cadherin

The extracellular part of E-cadherin consists of 5 repeating domains of 110 amino acids, and calcium binding sites (Figure 4). The first repeat has a HAV sequence, crucial for cell-cell adhesion (22). The extracellular domain of E-

cadherin forms an elongated monomeric structure, calcium depletion leads to globular transformation (23). The structure of the first extracellular domain of E-cadherin (ECD1) reveals 7 antiparallel ß sheets as determined by nuclear magnetic resonance (24). X-ray diffraction analysis of the first domain of N-cadherin (NCD1) reveals two dimer interfaces (25). Protomers of the strand dimer are oriented in parallel and the main feature of this dimer is the intercalation of the Trp 2 side chain into a hydrophobic pocket, suggesting that cadherins protrude from the cell surface as dimers. A second large dimer interface, called the adhesion dimer, consists of protomers with an antiparallel orientation at the interface between opposing cells. In type 1 cadherins, H (histidine) and V (valine) are found on the adhesive interface whereas the A (alanine) is buried within the hydrophobic core of the strand dimer (26).

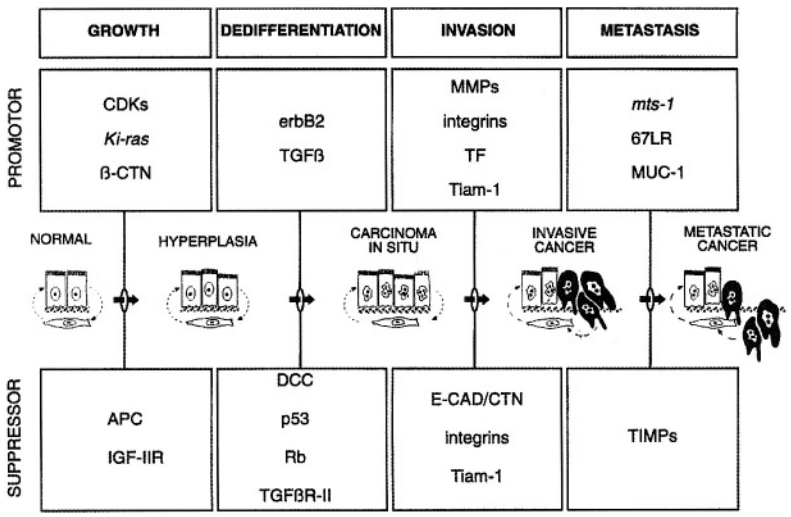


Figure 1. Schematic representation of cancer development. CDKs, cyclin dependent kinases; *Ki-ras,* oncogene from Kirsten murine sarcoma virus encoding a 21 kD guanine nucleotide binding protein; ß-CTN, ß-catenin; erbB2, oncogenic tyrosine kinase belonging to the EGFR family, TGFß, transforming growth factor ß; MMPs, matrix metalloproteinases; TF, tissue factor, invasion promoter expressed in tumour-associated myofibroblasts; Tiam-1, exchange factor for Rac, invasion promoter for lymphoma cells and invasion suppressor for epithelial cells; *mts1*, gene encoding a small calcium binding protein; LR67, 67 kD high affinity laminin receptor; MUC-1, mucin-1 or episialin, a cancer associated mucin, mediating adhesion of cancer cells to endothelial cells; APC, adenomatous polyposis coli protein; IGF-IIR, insulin-like growth factor II receptor; DCC, deleted in colorectal cancer, encoding a transmembrane glycoprotein of the immunoglobulin superfamily; p53, nuclear protein that arrests replication of damaged DNA, also called "guardian of the genome"; Rb, retinoblastoma protein encoding a phosphoprotein implicated in the regulation of the cell cycle; TGFßR-II, type II receptor for TGFß; E-CAD/CTN, E-cadherin/catenin complex; TIMPs, tissue inhibitors of metalloproteinases. Adapted from (362).

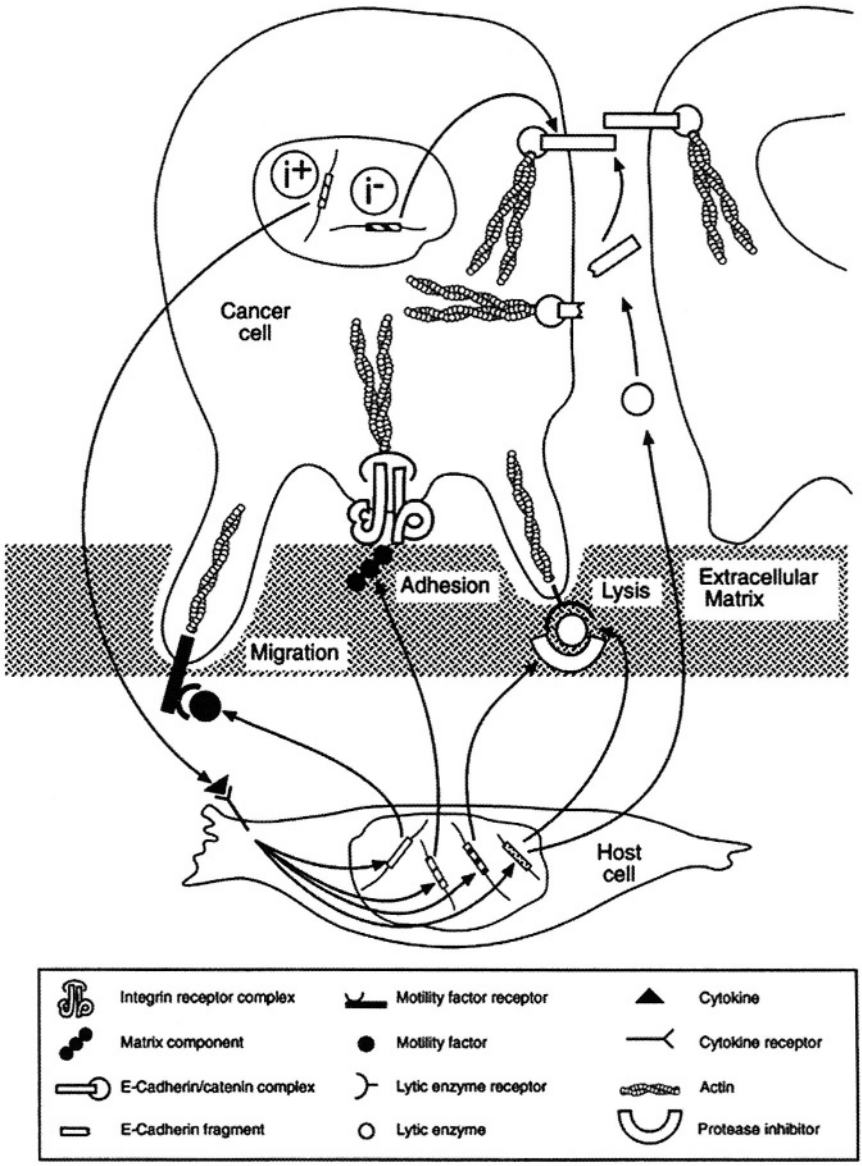


Figure 2. Micro-ecosystem of primary cancer, showing the molecular cross-talk between a cancer cell and a host cell. Symbols are explained in the box. i+, invasion-promoter gene; i- , invasion-suppressor gene. Adapted from (363).

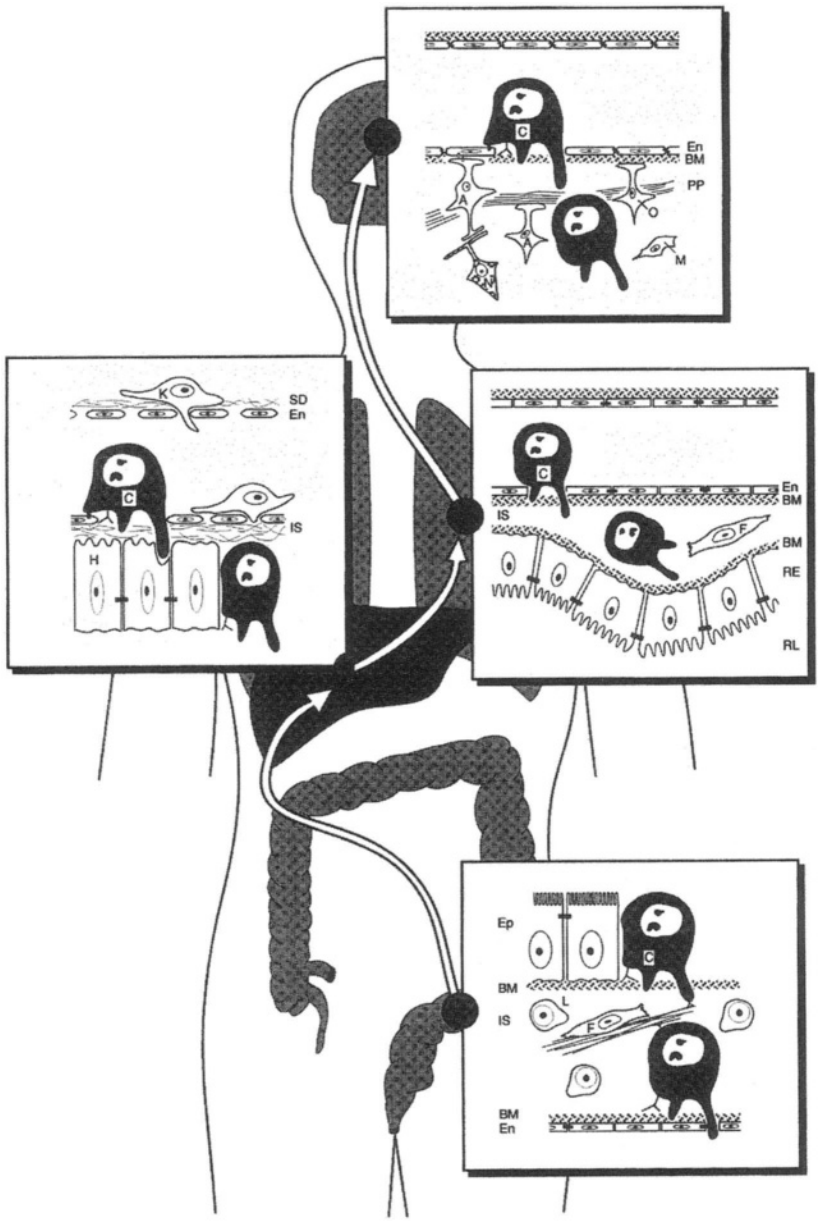


Figure 3. The metastatic spread of colon cancer to the liver, lungs and brain. En, endothelium;, Ep, epithelium; BM, basement membrane; IS, interstitial stroma; C, cancer cell; L, leukocyte; F, fibroblast; H, hepatocyte; K, Kuppfer cell; SD, Disse's space; RE, respiratory epithelium; RL, respiratory lumen; A, astrocyte; N, neural cell; O, oligodendrocyte; M, macrophage; PP, pia perivascularis. Adapted from (363).

Nagar et al. (27) published the X-ray structure of the 2 NH_2-terminal extracellular domains of E-cadherin (ECD1.2), which form also parallel dimers, but there are some differences with NCD1 fragment described by Shapiro et al. (25). Dimerization of ECD1.2 was promoted by and dependent upon 3 bound calcium ions, maintaining a rigid rod-like conformation, while the formation of parallel NCD1 dimers was independent of calcium. ECD1.2 dimers show a V-shaped arrangement with a less extensive monomer interaction near the calcium-binding sites. No adhesive interface of ECD1.2 could be observed in contrast with NCD1. However, sterically ECD1.2 could also be accommodated in a zipper-like structure. This structure of E-cadherin was confirmed biochemically by Koch et al.(28). The chimeric protein E-CAD-COMP, containing the extracellular part of E-cadherin recombinantly fused to the assembly domain of rat cartilage oligomeric matrix protein (COMP), forms a pentamer of ECD1.5 monomers with bundled COOH-termini and free NH_2-termini. Electron microscopy reveals pair formation of NH_2-terminal ECD1 cadherin domains within the same pentamer. This pair joins with a similar pair of another pentamer thus forming a tetrameric complex of two parallel and two antiparallel oriented NH_2-terminal cadherin domains (29). Lateral dimerization is required also for homophilic interactions of Xenopus C-cadherins (30). Site-directed mutagenesis of critical residues from either the donor strand or the acceptor pocket demonstrates the biological relevance of the strand dimer in N-cadherin- and E-cadherin-mediated adhesion (26).

E-cadherin was originally considered as a homophilic homotypic (interaction between E-cadherin molecules on cells of the same type) cell-cell adhesion molecule (Table 1). However, other types of interactions do exist: homophilic heterotypic (interaction between E-cadherin molecules on different cell types), heterophilic (interaction between E-cadherin and another molecule) and autotypic (interaction between E-cadherin molecules on the surface of one cell). For heterophilic binding of $\alpha_E\beta_7$ integrin, NH_2-terminal domains 1 and 2 of E-cadherin are both required and point mutation of the glutamic acid on position 31 in the first domain of E-cadherin abolishes interaction with $\alpha_E\beta_7$ integrin. This amino acid, located in a loop connecting 2 ß-strands, is unique for E-cadherin and highly conserved among species (31, 32, 33). These data show that homophilic and heterophilic binding of E-cadherin is mediated by different amino acids. $\alpha_E\beta_7$ integrin is found on a group of mucosal lymphocytes, namely intra-epithelial lymphocytes, constituting a large population of T-lymphocytes, that are responsible for attack against antigenic challenges. By these interactions E-cadherin might play a role in homing and in immune responses. Indeed, antibodies against $\alpha_E\beta_7$ inhibited lysis of allogenic renal epithelial cells possibly by interference with T-lymphocyte adhesion to the epithelium (34).

The cytoplasmic domain of E-cadherin

The 40 kD cytoplasmic domain is well conserved among type 1 cadherins. It interacts with multiple proteins either directly or via the catenins (Figure 4).

ß-catenin and plakoglobin (identical to γ-catenin) bind directly to E-cadherin (35,

18). α-catenin binds via its NH_2-terminal domain to ß-catenin or plakoglobin (36, 37, 38), linking the cadherin/catenin complex to the actin cytoskeleton, directly and/or indirectly via α-actinin (39, 40, 41). Also vinculin has been described to be part of the E-cadherin/catenin complex via association with the COOH-terminus of α-catenin (42, 43). In this way, vinculin links the E-cadherin/catenin complex to the actin filaments and the interaction of α-catenin and vinculin is crucial for tight junction assembly in epithelial cells (44).

Next to the catenins other molecules interact with the cytoplasmic tail of E-cadherin. A protein tyrosine phosphatase, PTPμ, binds directly to E-cadherin, N-cadherin and cadherin-4 (also called R-cadherin) (45, 46). The binding site on E-cadherin corresponds with 38 COOH-terminal amino acids, which overlap with the catenin binding site. Binding of PTPμ to E-cadherin was inversely correlated with tyrosine phosphorylation of E-cadherin, suggesting that PTPμ regulates E-cadherin phosporylation and in this way the adhesive properties of the E-cadherin/catenin complex (46). Another PTP (protein tyrosine phosphatase), PTP1B, was found to bind N-cadherin and to keep ß-catenin in a dephosphorylated state. Inactivation of PTP1B by mutation of the catalytic domain resulted in stimulation of ß-catenin tyrosine phosphorylation, reduced cadherin-mediated adhesion and uncoupling of cadherin/catenin complex from the actin cytoskeleton (47, 48).

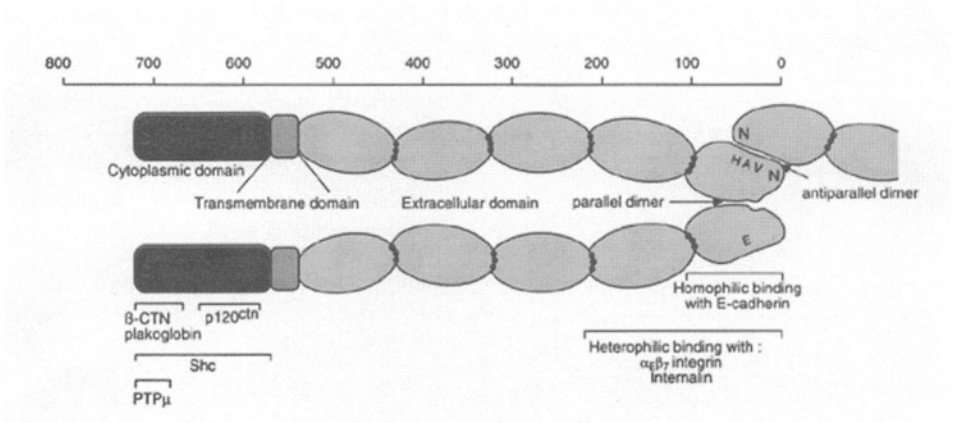


Figure 4. Schematic representation of E-cadherin mediated interactions. E-cadherin is depicted schematically with the 5 extracellular repeats in yellow, the cytoplasmic tail in green, the transmembrane domain in magenta and the blue circles represent Ca^{2+} ions. The axis indicates the number of amino acids. N, NH_2-terminus; C, COOH-terminus; ß-CTN, ß-catenin; $p120^{ctn}$, p120 catenin; Shc, Src homology 2 domain-containing α2 collagen-related; PTPμ, protein tyrosine phosphatase μ; HAV, histidine-alanine-valine; E, glutamic acid.

Table 1. Extracellular interactions of E-cadherin

Interaction		Cell type 1	Ligand	Cell type 2	Refs
Homotypic	Homophilic	Epithelial cell	E-cadherin	Epithelial cell	19
		Monocyte	E-cadherin	Monocyte	296
Heterotypic	Homophilic	Langerhans cell	E-cadherin	Keratinocyte	303
		Melanocyte	E-cadherin	Keratinocyte	304
		Thymocyte	E-cadherin	Thymic E. cell	305
	Heterophilic	Enterocyte	$\alpha_E\beta_7$	T-lymphocyte	306
		Synovium	$\alpha_E\beta_7$	T-lymphocyte	307
		Renal epithelium	$\alpha_E\beta_7$	T-lymphocyte	308
		Salivary gland	$\alpha_E\beta_7$	T-lymphocyte	309
		Enterocyte	Internalin	L. *monocytogenes*	310
		Erythroblast	?	?	311
Autotypic	Homophilic	Schwann cell	E-cadherin	Schwann cell	312

?, not known

Shc (Src homology 2 domain-containing α2 collagen-related) is an adaptor molecule and a tyrosine kinase substrate that contains an NH_2-terminal phosphotyrosine-binding domain (PTB), a central α2 collagen-like region and a SH2 domain at the COOH-terminus. Shc has no catalytic activity but mediates protein-protein interactions between different components of signal transduction pathways (49). For example, Shc links the Grb-2/Sos complex to activated growth factor receptors, resulting in Ras activation (50). By its SH2 domain Shc also interacts with the cytoplasmic domain of E-cadherin (51). This interaction might influence cadherin mediated cell-cell adhesion and link cadherin to signal transduction molecules.

Binding of E-cadherin via the catenins to the actin cytoskeleton is essential for cell-cell adhesion. Deletion or truncation of the entire cytoplasmic tail of cadherin results in loss of adhesion, in spite of continued expression on the cell surface (52, 53). Furthermore, mutant cadherins containing the cytoplasmic tail or the catenin-binding region alone exerted potent dominant-negative effects when expressed in Xenopus embryos (54), in cultured epithelial cells (55) and in mouse intestinal epithelia (56, 57).

There is evidence to accept that - next to the catenin binding domain - another region of the cytoplasmic tail, namely the juxtamembrane region proximal to the catenin-binding site, influences cadherin functions. Cadherin mutants with COOH-terminal deletions revealed that the membrane-proximal region of the cytoplasmic domain negatively regulates adhesion (58). This is in contrast with results obtained with a deletion mutant of C-cadherin, in which the juxtamembrane region of the cytoplasmic tail supports lateral clustering of the molecule and adhesive strengthening (59). Another report shows that the juxtamembrane region of E-cadherin also supports motility (60). The armadillo protein $p120^{ctn}$ is a strong candidate for regulating the functions of the

juxtamembrane region of E-cadherin since it directly interacts with this region of E-cadherin (61, 62, 63, 64, 65). $p120^{ctn}$ was originally identified as a target for Src tyrosine kinase (66) and association of $p120^{ctn}$ with the E-cadherin is stimulated upon Src activation (67). Moreover, $p120^{ctn}$ can be phosphorylated after activation of signal transduction pathways by several growth factors and oncogenes, suggesting a possible role for this protein in E-cadherin regulation (68).

ß-Catenin

ß-catenin is not exclusively bound to E-cadherin, as it also forms other complexes (Figure 5). ß-catenin is the vertebrate orthologue of the Drosophila segment polarity gene product, armadillo. The ß-catenin gene is localized on chromosome 3p22-p21-3 (69, 70, 71). The primary structure of the ß-catenin protein consists of an NH_2-terminal portion of 130 amino acids, a central region of 550 amino acids that contains 12 sequence repeats of 42 amino acids, the so-called armadillo (arm) repeats (72), and a COOH-terminal region of 100 amino acids. The three-dimensional structure of the arm repeat region of β-catenin revealed a super helix formed by helices, featuring a long, positively charged groove, which might interact with acidic proteins (73). The latter authors propose charge complementary as a mechanism of interaction with the arm repeats of β-catenin. Indeed, cadherins, APC and T-cell factors (Tcf) bind to the arm repeats of β-catenin and although their β-catenin binding regions show no significant sequence homology, they are all acidic.

The interaction of β-catenin with multiple proteins involved in several signal transduction pathways will be discussed, since in our experiments, we did see changes in the composition of the E-cadherin/catenin complex which influence probably not only the E-cadherin/catenin complex itself but also the equilibrium with other complexes.

APC, conductin, GSK-3β and β-catenin

The APC (Adenomatous Polyposis Coli) gene product is a large cytoplasmic protein of 310 kD that contains 7 arm repeats and several other protein-protein interaction domains (7). APC is a tumour suppressor molecule, which is mutated in patients with the FAP syndrome (Familial Adenomatous Polyposis) and in sporadic colorectal cancer. The arm repeats of β-catenin bind to the 20 amino acid and 15 amino acid repeated sequence in the central region of APC (74, 75). Since E-cadherin was not found in anti-APC immunoprecipitates, it was concluded that cadherins and APC form independent complexes with β-catenin. Moreover, E-cadherin competes with APC for β–catenin binding (76, 77).

Recently, mutated APC molecules were shown to loose their ability to bind or to regulate β-catenin and this was correlated with activation of β-catenin signalling (78, 79, 80). Both APC and glycogen synthase kinase 3β (GSK-3β) are essential for downregulating β-catenin levels and inactivating β-catenin signalling. APC is phosphorylated by GSK-3β in a region of the protein that is essential for its ability to regulate β-catenin levels (81). This phosphorylation also greatly increases the binding affinity of β-catenin for APC. Moreover, GSK-3β phosphorylates β-catenin on serine residues that are critical for the rapid turn-over of β-catenin by the ubiquitin-proteasome pathway (82, 83).

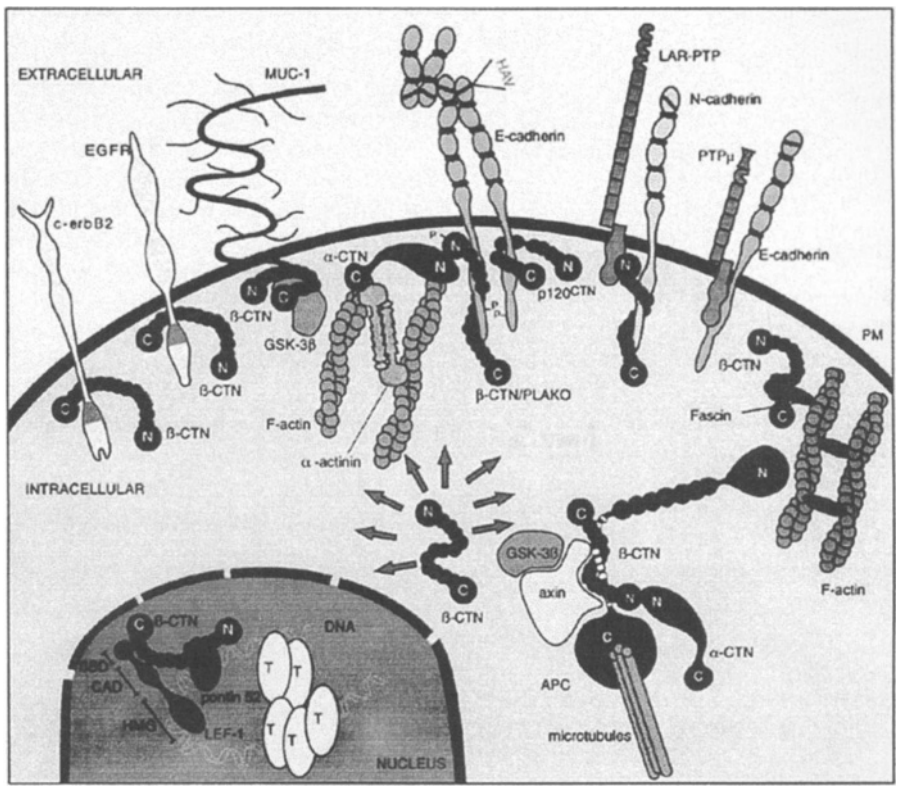


Figure 5. Schematic representation of β-catenin complexes. PM, plasmamembrane; α-CTN, α-catenin; β-CTN, β-catenin; PLAKO, plakoglobin; $p120^{ctn}$, $p120^{catenin}$; HAV, histidine alanine valine sequence, characteristic for the first extracellular domain of type I cadherins; P, phosphorylation site; EGFR, epidermal growth factor receptor; c-erbB2, oncogenic tyrosine kinase receptor from the EGFR family; MUC-1, mucin-1 or episialin, a cancer associated mucin; GSK-3β, glycogen synthase kinase-3β; LAR-PTP, leukocyte common antigen-related protein tyrosine phosphatase; PTPμ, protein tyrosine phosphatase μ; APC, adenomatous polyposis coli protein; LEF-1, lymphoid enhancer binding factor-1; βBD, β-catenin-binding domain in LEF-1; CAD, context-dependent activation domain in LEF-1; HMG, high mobility group domain in LEF-1; N and C, respectively amino- and carboxy-terminal residues; T, transcription factors. Filled circles indicate the position of Ca^{2+} ions at the cell surface proximal end of cadherin protomers. Tandemly repeated circles in β-catenin, $p120^{ctn}$ and APC indicate arm domains in these molecules. Adapted from (362).

Axin is a new potential regulator of β-catenin signalling as it inhibits dorsal axis formation in *Xenopus* that is dependent upon the Wnt-1 signalling pathway. Using the two hybrid system conductin, an axin homologue, was identified as another component of the APC/β-catenin/GSK-3β complex (84). Human axin binds directly to β-catenin, GSK-3β and APC (85). The binding site on the APC protein corresponds with a region frequently deleted in colon cancer. Axin is shown to enhance phosphorylation of both β-catenin (86) and APC (85) by GSK-3β. Overexpression of axin in cancer cells leads to downregulation of β-catenin. These results suggest that axin assembles the proteins required for β-catenin degradation in a closely associated complex.

LEF-1/Tcf, pontin 52 and β-catenin

β-catenin participates at the Wnt signalling pathway and in this way may act as an oncogene. This important development in our understanding of the functions of catenins was recently reviewed (87, 88, 89).

Failure to degrade β-catenin in the APC complex as described above leads to an increased cytoplasmic concentration. In this case β-catenin associates with members of the T-cell factor/lymphoid enhancer factor (Tcf/LEF) family and translocates to the nucleus where it participates at the regulation of gene transcription (90, 91). Tcf/LEF factors do not behave as classical transcription factors : they cannot activate transcription on their own. LEF-1 was found to participate in regulation of the enhancer associated with the T cell receptor-α gene by inducing a sharp bend in the DNA and facilitating interactions between transcription factors bound at sites flanking the LEF-1 site. So, the influence of LEF-1 on transcription activity is strictly dependent on adjacently bound unrelated transcription factors. Recently, pontin 52 was identified as another interaction partner of β-catenin in the nucleus (92).

The interaction of Tcfs with β-catenin have strong implications for colon cancer in which APC is frequently mutated. Also NH_2-terminal mutations of β-catenin, resulting in loss of GSK-3β phosphorylation sites necessary for degra-dation and stabilizing β-catenin, result in the formation of nuclear β-catenin/Tcf complexes (85,93,94,95,96). Similar muta-tions were found in malig-nant melanoma (97), in sporadic medullo-blastomas (98), in endometrioid ovarian carcinomas(99), in primary hepatocellular carcinomas (100,101) and in prostate cancer (102). In this way, β-catenin, a member of an invasion suppressor complex, serves as an oncogene.

More partners for β-catenin

The actin bundling protein fascin competes with the cytoplasmic domain of E-cadherin for binding to β-catenin (103). Overexpression of fascin, therefore, interferes with E-cadherin functions as evidenced by loss of epithelioid morphotype (104).

β-catenin is a substrate for tyrosine kinases, such as c-Met, the receptor for SF/HGF (scatter factor/hepatocyte growth factor), EGFR and Src (17, 105, 106, 107). EGFR (epidermal growth factor receptor) colocalizes with the E-cadherin/catenin complex at the lateral membrane of epithelial cells (108) and recombinant EGFR was shown to interact with the arm repeat region of β-catenin and to induce tyrosine phosphorylation of β-catenin (109). The EGFR related oncogenic c-erbB2 receptor (110) also directly associates with β-catenin and plakoglobin in gastric cell lines (111, 112) and the binding domain resides on the 3 COOH-terminal arm repeats of β-catenin

(113). By introducing NH_2-terminal deleted β-catenin, that binds to c-erbB2 but not to E-cadherin, TGF-α (transforming growth factor α) -induced tyrosine phosphorylation of β-catenin in E-cadherin immunoprecipitates was reduced together with suppression of invasion *in vitro* and metastatic potential *in vivo.* EGF (epidermal growth factor) was also shown to dissociate E-cadherin from the actin cytoskeleton, correlated with tyrosine phosphorylation of β-catenin, plakoglobin and $p120^{ctn}$ (114).

Treatment of cells with the broad spectrum tyrosine phosphatase inhibitor, orthovanadate, resulted in a higher level of tyrosine phosphorylation of the E-cadherin/catenin complex (115). Indeed, several PTPs associate with β-catenin. LAR-PTP, a member of the leucocyte common antigen related-protein (LAR) family of transmembrane tyrosine phosphatases associates with the NH_2-terminal domain of β-catenin in PC12 cells (116). Other PTPs, namely PTPK-related to PTPμ and involved in homophilic interaction (117)-PTPσ and PTP∂, also bind to β-catenin(118,119,120).

Mutations in the genes encoding presenilin 1 (PS1) and presenilin 2 (PS2) account for the majority of early-onset familial Alzheimer disease. Yu et al. (121) detected presenilin with β-catenin in a complex in the brain. Transfection of PS1 in HEK 293 cells increases β-catenin stability while mutated PS1 stimulates β-catenin degradation *in vitro* and in transgenic mice. Also reduced β-catenin levels are detected in the brains of patients with Alzheimer's disease. The results indicate that impaired β-catenin signalling, due to reduction of β-catenin levels, increases neuronal vulnerability to apoptosis (122).

The human MUC-1 (DF3, episialin) is a membrane-associated glycoprotein consisting of a protein core with highly branched carbohydrate side chains. The NH_2-terminal ectodomain consists of varying number - from 60 to 100 - of 20 amino acid tandem repeats that are subject to O-glycosylation. Direct binding of β-catenin and GSK-3β to the cytoplasmic domain of MUC-1 was demonstrated (123, 124). GSK-3β phosphorylates MUC-1, resulting in the release of β-catenin from this complex. Expression of MUC-1 decreases the amount of β-catenin associated with E-cadherin. MUC-1 is normally expressed on the apical surface of a number of secretory epithelial tissues. Many human adenocarcinomas over-express MUC-1 on the cell surface (125), which is correlated with metastatic potential and poor prognosis. The following biological functions, deduced from *in vitro* experiments, have been proposed for MUC-1 : (i) anti-adhesive activity namely suppression of cell-cell adhesion (126, 127) and cell-matrix adhesion (128) and induction of invasion and motility (129), (ii) promotion of adhesion to endothelial cells (130) , (iii) immunosuppressive activity of the soluble form of MUC-1 by induction of apoptosis of activated T-cells (131). Some of these effects might be explained by ligation of β-catenin and influencing the E-cadherin/catenin complex.

α-Catenin

α-catenin is a 102 kD cytoplasmic molecule binding to β-catenin or plakoglobin. Up to now two α-catenin genes were identified : αE-catenin, the epithelial isoform (132, 133) localized on chromosome 5q31 (134, 135) and αN-catenin, the neural isoform with a restricted expression pattern (136) localized on chromosome 2p11.1-12 (137). α-catenin, a protein of 906 amino acids, shares homologies over 3 extended

regions with vinculin (132, 138), a structural component of cell-cell as well as cell-matrix junctions that also interacts with α-catenin (42,43).

α-catenin is necessary for cadherin functions, and lack of α-catenin expression or expression of a mutated α-catenin inhibits aggregation and induces invasion (139,140,141,142,143). Reintroduction of the α-catenin cDNA restores intercellular adhesion (136,142,144,145).

α-catenin has been implicated in the linking of cadherins to the actin cytoskeleton : it binds directly to actin via 2 domains (amino acids 1-228 and 459-906) (146) or indirectly via α-actinin (amino acids 325-394) (39, 41). α-catenin establishes a connection between adherens junctions and tight junctions since it also interacts with the tight junction protein ZO-1 (147), which associates with actin. ZO-1 localizes in the adherens junctions in cardiac muscle cells, in fibroblasts and in the initial phase of junction formation of epithial cells. ZO-1 might in this way constitute another possible link from the E-cadherin/catenin complex to the actin cytoskeleton.

Functional Regulation of the E-Cadherin/Catenin Complex

It is clear that the E-cadherin/catenin functions are influenced by multiple intracellular and extracellular factors. Here, we have tabulated (Tables 2 and 3) these factors. It is interesting to note that some of these factors have antithetic effect in as much as they may stimulate as well as inhibit the functions of the complex.

PROTEOLYSIS

Cell-cell and cell-matrix adhesion, as well as proteolysis of the extracellular matrix (ECM) are vital for normal processes such as tissue morphogenesis and wound healing, as well as for pathological processes such as tumor cell invasion and metastasis. Proteolytic degradation of cell surface and ECM proteins mediates rapid and irreversible responses to changes in the cellular microenvironment. Proteinases are divided into classes, based on the catalytic group at their active center e.g. metalloproteinases, serine proteinases, cysteine proteinases. Here, the class of the metalloproteinases will be discussed. These enzymes are of particular interest since they play a role in invasion and metastasis of cancer cells, both by remodelling and degradation of the ECM and by ectodomain shedding of cell surface molecules. The latter provides a new mechanism of action of metalloproteinases.

Metalloproteinases are characterised by the presence of a zinc ion in the active site which is required for the catalytic activity (149). A minimal conserved consensus sequence HEXXH (H, histidine, E, glutamic acid , X, any amino acid) is involved in zinc ligation. Based on sequence similarities, the metalloproteinases that share the elongated zinc binding motif HEXXHXXGXXH are named metzincins and fall into four different protein families: the astacins and the serralysins (bacterial metalloproteinases), MMPs (matrix metalloproteinases), ADAMs (adamalysins), the latter two being implicated in ectodomain shedding. Matrix Metalloproteinases (MMPs).

MMPs are synthesized as zymogens (150). The 17 family members, identified so far fall into arbitrary groups that originate from considerations of their substrates (Table 4). The natural substrates of most MMPs are, however, unknown. Collagenases degrade fibrillar collagen, stromelysins prefer

proteoglycans and glycoproteins as substrate (stromelysin-3 stands as an exception) and gelatinases are particularly potent in degradation of nonfibrillar and denatured collagens (gelatin). The newest MMP group contains 4 membrane-type MMPs, characterized by a transmembrane domain, localizing the proteinases to the cell surface (151).

Domain structure of MMPs

MMP family members differ from each other structurally by the presence of additional domains determining substrate specificity, inhibitor binding, matrix binding and cell surface localization (149). Starting from the NH_2-terminus the following domains can be distinguished.

The signal peptide is a short sequence routing the protease into the endoplasmatic reticulum for secretion.

The propeptide contains about 80 amino acids, and near the COOH-terminal end of this segment, there is a highly conserved PRCGGVPD sequence the cysteine of which binds the catalytic zinc atom and maintains the zymogen form.

The primary mechanism for MMP activation is interruption of the cysteine-zinc interaction, referred to as "cysteine switch" (152). This can be achieved either by organomercurial agents or by limited proteolysis, which is followed by autoproteolysis of the remainder propeptide yielding an active enzyme.

The furin cleavage site insert contains a consensus sequence of about 9 amino acids that can be cleaved intracellularly by furin.

The catalytic domain is a 160-170 amino acids domain, including sites for the binding of calcium ions and the HEXGH motif ligating of the catalytic zinc. In this sequence the H residues bind zinc and the E is the catalytic residue.

Type II fibronectin-like repeats are inserted in the catalytic domain and facilitate the binding to the gelatin substrate.

The hinge region links the catalytic domain to the hemopexin domain and gives flexibility to the molecule.

The hemopexin domain consists of 4 repeats that resemble hemopexin and vitronectin. A disulfide bond joins the ends together so that the repeats form a four-bladed propeller. This domain serves TIMP binding. The four MT-MMPs have a transmembrane domain and a short cytoplasmic tail.

Regulation of MMPs

MMP activity is regulated at various levels: gene activation, mRNA stability, translation, binding of proenzymes to the plasma membrane and/or ECM components, proenzyme activation, inactivation of enzymes by endogenous inhibitors, degradation of active enzyme or inactive pro-enzyme (153).

At the level of transcription, many MMPs are regulated by cytokines, growth factors and hormones. In tumor associated fibroblasts, metalloproteinase expression is induced by PDGF (platelet derived growth factor), transforming growth factor α (TGFα), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) that is produced by the tumor cells, and by IL-1 (interleukin-1) and TNF-α (tumor necrosis factor α), produced by the infiltrating lymphocytes (154).

Table 2. Putative suppressors of the function or the expression of components of the E-cadherin/catenin complex.

Agent	Putative mechanism of action	Ref(s)
Cadherin-related		
sE-CAD	molecular rearrangement of the E-cadherin/catenin complex	289, 293
HAV-peptides	tyrosine phosphorylation of β-catenin, molecular rearrangement of the E-cadherin/catenin complex	22, 293, 294
Antibodies against E-cadherin	inhibition of homophilic interaction	313
Proteoglycan-related		
GalNAcPTase-binding PG	tyrosine phosphorylation of β-catenin	314
Proteoglycans	sterical hindrance	315
Muc-1	sterical hindrance	127
Epiglycanin	sterical hindrance	316
Polypeptide factors		
AMF/gp78	downregulation of E-cadherin	317, 318
EGF	tyrosine phosphorylation of β-catenin, dissociation of E-cadherin/catenin complex from actin cytoskeleton	106, 107, 114
HGF/SF	tyrosine phosphorylation of β-catenin	106
PTH	internalization of E-cadherin	319
TGF-ß	downregulation of E-cadherin	320, 321
TNF-α	disorganization of the E-cadherin/catenin complex	322
Trefoil peptides	tyrosine phosphorylation of β-catenin	323
Stromelysin-1	downregulation of E-cadherin	324
Oncogene related		
Erb-B2	downregulation of E-cadherin, tyrosine phosphorylation of β-catenin	113, 325
Ras	tyrosine phosphorylation of β-catenin and $p120^{ctn}$	326
v-*src*	tyrosine phosphorylation of β-catenin	17, 105, 327
FER	tyrosine phosphorylation of β-catenin and $p120^{ctn}$	328
SV-40 LT	downregulation of E-cadherin	8
c-fos	downregulation of E-cadherin	329
c-jun	dissociation of β-catenin and E-cadherin	330

Table 2 to be continued..

Table 2 continuing

Agent	Putative mechanism of action	Ref(s)
Miscellaneous		
TPA	Downregulation of E-cadherin	331
IQ-GAP	Inactivation of the E-cadherin/catenin complex	332
Ep-CAM	Inactivation of the E-cadherin/catenin complex	333
17-β estradiol	Downregulation of E-cadherin, α-catenin and β-catenin	334
Calyculin A	Inactivation of the E-cadherin/catenin complex	335
Okadaic acid	Inactivation of the E-cadherin/catenin complex	33
UV radiation	Downregulation of E-cadherin	336
H. pylori	Downregulation of E-cadherin	337

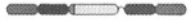
Abbreviations for table 2

sE-CAD, soluble E-cadherin fragments; HAV-peptides, histidine-alanine-valine containing peptides; GalNAcPTase, *N*-acetylgalactosaminyl-phosphotransferase; AMF/gp78, autocrine motility factor and its 78 kD receptor; EGF, epidermal growth factor; HGF/SF, hepatocyte growth factor/scatter factor; PTH, parathyroid hormone; TGF-β, transforming growth factor-β; *erb*B2, oncogene encoding a tyrosine kinase receptor from the EGF-R (epidermal growth factor receptor) family; FER, nonreceptor tyrosine kinase related to the fes/fps oncoprotein; SV-40 LT, large T-antigen of the SV40 virus; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; IQ-GAP, target of the small GTPases Cdc42 and Rac1; Ep-CAM, epithelial cell adhesion molecule; *H. pylori*, the bacterium *Helicobacter pylori.*

Table 3. Putative promoters of the function or the expression of components of the E-cadherin/catenin complex.

Agent	Putative mechanism of action	Ref(s)
Proteoglycan-related		
MuβX	inhibits anchoring of sterically hindering glycosaminoglycans	315
PKC regulators		
DiC8	PKC activation	338
PDBu	PKC activation	338
TPA	PKC activation	338, 339, 340
Polypeptide factors		
insulin-like growth factor-I	IGF-I receptor activation	313
interleukin-12	upregulation of E-cadherin	341
relaxin	upregulation of E-cadherin	342
Wnt-1	stabilization of the E-cadherin/catenin complex	343, 344
Tiam-1	activation of the E-cadherin/catenin complex	345
Rac, Rho, Cdc42	activation of the E-cadherin/catenin complex	346, 347, 348
Steroid related		
17-β estradiol	upregulation of E-cadherin	349, 350
dihydrotestosterone	upregulation of E-cadherin	351
tamoxifen	activation of the E-cadherin/catenin complex	352
ICI-182,780	upregulation of E-cadherin	351
R1881	upregulation of E-cadherin	351
hydroxyflutamide	upregulation of E-cadherin	351
Miscellaneous		
M3 receptor activation	activation of the E-cadherin/catenin complex	339
dibutyryl cAMP	upregulation of E-cadherin	353
ionizing radiation	upregulation of E-cadherin and α-catenin	354
docetaxel	upregulation of E-cadherin	355
azacytidine	demethylation E-cadherin promotor	6, 356
all-*trans*-retinoic acid	upregulation of the function of E-cadherin	357, 358
γ-linoleic acid	upregulation of E-cadherin and α-catenin	359, 360
tangeretin	activation of the E-cadherin/catenin complex	361

MuβX, 4-methylumbelliferyl β–D-xyloside; PKC, protein kinase C; DiC8, 1,2-dioctanoyl-*sn*-glycerol, PDBu, phorbol-12,13-dibutyrate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; IGF-I, insulin-like growth factor-1; Wnt-1, protooncogene-encoded protein homologuous to the product of the *Drosophila* segment polarity gene *wingless;* Tiam-1, GDP/GTP exchange factor for Rac, Rac, Rho and Cdc42, small GTPases, tamoxifen, anti-estrogen; ICI-182,780, anti-estrogen; R1881, synthetic androgen, hydroxyflutamide, anti-androgen, M3 receptor, muscarinic acethylcholine receptor; cAMP, cyclic adenosine monophosphate, docetaxel, semisynthetic taxol analogue.

Name	MMP (number)	MW (kD) (latent/active)	Activators	Substrate		Domain structure
				ECM	Other	
Collagenases						
collagenase-1 (intersitial collagenase)	MMP1	52/42	plasmin, kallikrein, chymase, MMP3	fibrillar collagens	L-selectin; TNFα	
collagenase-2 (neutrophil collagenase)	MMP8	85/64	plasmin, MMP3, MMP10	fibrillar collagens		
collagenase-3	MMP13	52/42	plasmin, MMP2, MMP3, MMP14	fibrillar collagens		
collagenase-4	MMP18	53/42				
Gelatinases						
gelatinase A	MMP2	72/66	MMP1, MMP7, MMP14, MMP15, MMP16, thrombin	gelatins, collagen IV, collagen I	FGFR1 galectin-3	
gelatinase B	MMP9	92/84	plasmin, neutrophil elastase, MMP3, MMP2, MMP13	gelatins, collagen IV, collagen I	galectin-3	
Stromelysins						
stromelysin-1	MMP3	57/42	plasmin, kallikrein, chymase, elastase, cathepsin G	PG, ECM glycoproteins, collagen IV, gelatins	α1 PI; E-cadherin; L-selectin; TNFα	
stromelysin-2	MMP10	54/44	plasmin, kallikrein, chymase, elastase, cathepsin G	PG, ECM glycoproteins, IV collagen, gelatins	α1 PI;	
stomelysin-3	MMP11	64/46	furin	laminin and fibronectin (weakly)	IGFBP-1	
Membrane-type matrix metalloproteinases						
MT1-MMP	MMP14	66/54	furin, plasmin	PG, ECM glycoproteins, fibrillar collagens		
MT2-MMP	MMP15	72/60				
MT3-MMP	MMP16	64/53				
MT4-MMP	MMP17	57/53				
Others						
matrilysin	MMP 7	28/19	plasmin, MMP3	PG,ECM glycoproteins, collagen IV, gelatins, elastin,	α1 PI; E-cadherin; $ß_4$ integrin; TNFα; uPA	
metalloelastase	MMP12	54/22		elastin	plasminogen	
	MMP19					
enamelysin	MMP20					

MW, molecular weight; ECM, extracellular matrix; PG, proteoglycans; PI, proteinase inhibitor; FGFR, fibroblast growth factor receptor; TNF, tumor necrosis factor, IGFBP, insulin-like growth factor binding protein; uPA, urokinase plasminogen activator; red, predomain; green, prodomain; yellow, catalytic domain; orange, furin cleavage site; blue, hemopexin domain; brown, gelatin-binding domain; magenta, transmembrane domain and short cytoplasmic tail.. Adapted from [150]

Table 4: Name, molecular weight, activators, substrates and domain structure of matrix metalloproteinases.

Table 5. The ADAM family of metalloproteinase : nomenclature and presence of functional domains

ADAM number	other names	Metalloproteinase activity	Fusion peptide	SH3 binding domain
ADAM1	Fertilin α	+	+	
ADAM2	Fertilin β			
ADAM3	Cyritestin, tMDCI		+	
ADAM4				
ADAM5	tMDC II			
ADAM6	tMDC IV			
ADAM7	EAP I			
ADAM8	MS2	+		+
ADAM9	MDC9	+	+	+
ADAM10	MADM	+		+
ADAM11	MDC		+	
ADAM12	Meltrin α	+	+	+
ADAM13				
ADAM14	adm-1			
ADAM15	Metargidin	+		+
ADAM16				
ADAM17	TACE	+		

ADAM, a disintegrin and metalloproteinase domain; MDC, metalloproteinase/ disintegrin/cysteine-rich; SH3, Src homology 3; +, the domain is present. Adapted from (175).

MMP production is regulated also by the pericellular environment, namely cell-cell and cell-matrix interactions. Indeed, matrilysin protein levels are correlated with the degree of E-cadherin mediated cell-cell contacts (155). Transfection of E-cadherin in a colon carcinoma cell line results in a decreased expression of gelatinase A (156). Gelatinase A expression is higher in melanoma cells selected for low levels of $\alpha_v\beta_3$ integrin (157).

Protein cascades involving MMPs as well as serine proteinases participate at activation of the proenzyme form of MMPs. MT-MMPs act as receptors and activators for gelatinase A and collagenase 3 and cell-associated plasmin activates the collagenases, the stromelysins, gelatinase B and matrilysin.

Activation by membrane associated enzymes restricts MMP activity to the pericellular environment.

Once the enzymes are active, they are susceptible to inhibition by the broad spectrum proteinase inhibitor α2-macroglobulin and by a family of specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). There are four members of the TIMP family. TIMPs regulate metalloproteinase activity at several levels by forming a complex with the enzymes. Interaction of TIMP-1 with active gelatinase B results in protease inhibition, whereas interaction with latent gelatinase B blocks activation of this enzyme (158). Introduction of TIMP-1 antisense mRNA made mouse fibroblasts metastatic in nude mice (159). Next to inhition of MMPs, TIMP-2 at low concentrations stimulates activation of progelatinase A. TIMP-2 forms a complex with MT-MMP1 that serves as a cell surface receptor for progelatinase A. Then, progelatinase A is activated by a TIMP-2-free MT-MMP1 molecule. At high concentrations, TIMP-2 blocks all the MT1-MMP molecules on the cell surface and inhibits progelatinase A activation (160, 161, 162).

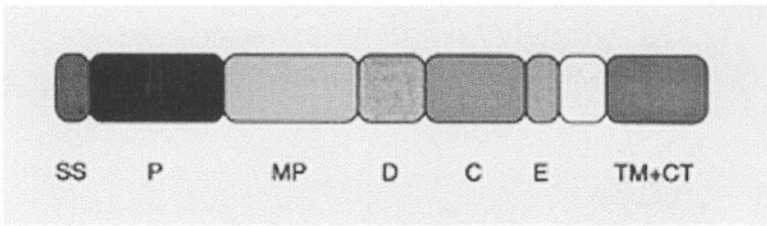


Figure 6. Domain structure of ADAMs. SS, signal peptide; P, prodomain; MP, metalloproteinase domain; D, disintegrin domain; C, cysteine-rich domain; E, EGF-like repeat; TM+CT, transmembrane and cytoplasmic domain. Adapted from (163).

ADAMs

ADAMs constitute a novel family of membrane glycoproteins with A Disintegrin And Metalloproteinase domain. ADAMs, also called adamilysins or MDCs (metalloproteinase / disintegrin / cysteine-rich), comprise several protein modules as presented in Figure 6 and Table 5.

Most ADAMs contain a metallo-proteinase domain, but only some of them are thought to be catalytically active namely the ones having the active site consensus sequence HEXGHNL-GXXHD (163).

Domain Functions of ADAMs

The metalloproteinase domain of TACE (TNFα converting enzyme or ADAM 17) was identified as the enzyme responsible for cleavage of the proinflammatory cytokine TNFα from the plasma membrane (164, 165). Another member of the ADAM family, namely ADAM 10, also has TNFα converting activity (166). Mutation of the catalytic domain of TACE revealed other functions of this ADAM, all related to ectodomain cleavage. TACE is involved in the ectodomain cleavage of several other molecules like EGFR ligands, p75 TNF

receptor, L-selectin and ß-amyloid precursor protein (167), Lethality of mice lacking TACE highlights the importance of TACE induced ectodomain shedding during development. In *Drosophila* processing of the extracellular domain of Notch depends on the ADAM 10, also called Kuzbanian and is necessary for lateral inhibition (168) and the ADAM 10 orthologue in *Caenorhabditis elegans,* namely SUP-17, might have a similar role (169).

The disintegrin domain of ADAMs is a candidate ligand for integrins (170). This domain interacts with integrins through a disintegrin loop, a thirteen amino acid motif containing an integrin-binding sequence (163). The disintegrin domain is found also in soluble snake venom, a potent anticoagulant toxin, that acts through binding of its RGD sequence with the platelet integrin $\alpha_{IIb}ß_3$ (171). Peptides, homologous to the disintegrin domain of cyritestin or fertilin ß, sperm disintegrins involved in fertilization, or antibodies against the disintegrin domains inhibited adhesion of the sperm to the egg (172). Since antibodies against $\alpha_6ß_1$, an integrin expressed on mouse eggs, block fertilization in mice (173), this integrin is a candidate receptor for sperm ADAMs.

The cysteine rich domain of the ADAMs is probably involved in membrane fusion, e.g. in sperm-egg fusion (174), as suggested by the finding that a peptide homologous to the ADAM1 cysteine rich domain interacts with the membrane and induces fusion. However, a role of ADAMs in membrane fusion can also be explained by establishing a critical binding with an integrin via the disintegrin domain, necessary for fusion (175).

The cytoplasmic domain of ADAMs have no sequence similarity with other proteins. The cytoplasmic domain of several ADAMS are rich in proline and have serine phosphorylation sites. The proline residues suggest binding sites for SH3 (Src homology 3) domain-containing signalling proteins (49). It has been suggested that the cytoplasmic domain of ADAMs binds to members of the Src family of kinases thus conveying signalling functions upon ADAMs (27).

Regulation of ADAM Activities

Activities of ADAMS are regulated both temporally and spatially. Mechanisms proposed are: alternative splicing, proteolytical cleavage of the prodomain leading to the cysteine-switch described above for MMPs (163). TIMPs inhibit the metalloproteainse activity of ADAMs. TIMP-3 overexpression results in apoptosis by inhibiting TACE and in this way stabilizing TNFR on the cell surface which makes the cells more sensitive to TNFα (176,177,178).

ECTODOMAIN SHEDDING

The extracellular domain (ecto-domain) of many transmembrane proteins is proteolytically cleaved and released into the medium (179, 180, 181). Ectodomain shedding regulates the function and localization of transmembrane proteins like growth factors, growth factor receptors, cell adhesion molecules, mucins, proteoglycans and other proteins.

Our interest in ectodomain shedding comes from our observations about regulation of E-cadherin/catenin functions by sE-CAD, an E-cadherin fragment released from the cell surface by ectodomain shedding.

Effects of Ectodomain Shedding

Ectodomain shedding converts a protein into a soluble extracellular fragment and a transmembrane fragment. Ectodomain shedding is a mechanism by which the number of cell surface

molecules is regulated. Shedding of a receptor makes the cells less responsive to its ligand, and shedding of a cell adhesion molecule weakens or impaires the adhesion (182, 183).

The ectodomain can be a soluble competitor of the membrane-bound receptor for its ligand. This mechanism has been demonstrated for growth factors and cytokine receptors and for adhesion molecules. The ectodomains of syndecans and fibroblast growth factor receptor (FGFR) bind fibroblast growth factor (FGF) and inhibit FGF activity by diminishing the availability for the membrane-associated receptor (184, 185). The soluble platelet derived growth factor receptor (PDGFR) competes with cell-associated PDGFR for ligand binding and is antagonist of PDGF stimulated effects on full length PDGFR expressing cells (186, 187). The c-kit receptor ectodomain retains the ability to bind to the ligand and blocks c-kit induced proliferation of M-07e myeloid cells (188). The mechanism of competition for ligand binding between the soluble and the transmembrane receptor was also described for interleukin-1 receptor II (IL-1RII). The soluble E-selectin fragment has an anti-inflammatory function because it binds to sialyl $Lewis^x$ on HL-60 myeloid cells and so inhibits rolling and subsequent firm adhesion to the endothelial cells (189). Soluble L-selectin also is active and inhibits L-selectin-mediated adhesion of lymphocytes to the endothelium (190, 191). Soluble receptors may also stimulate the activity of a ligand. Soluble gp80 interleukin-6 (IL-6) receptor retains IL-6 binding capacity and, together with its ligand IL-6, it acts as an agonist on cells that express the signal transducing gp130 molecule (192, 193). By this mechanims soluble gp80 renders cells that cannot bind IL-6, but do express gp130, responsive to this cytokine. On the other hand, the soluble gp130 receptor is an inhibitor of IL-6 signals (194).

Some growth factors are synthesized as a transmembrane precursor glycoprotein consisting of a growth factor domain, a transmembrane domain and a cytoplasmic part. They are processed by ectodomain shedding to a soluble form. Most of these growth factors belong to the epidermal growth factor (EGF) family (195). Ectodomain shedding can either activate or inactivate a growth factor. (Uncleaved inactive spitz, a *Drosophila* transforming growth factor α (TGFα)-like molecule, is activated by ectodomain shedding. In contrast, active membrane anchored c-kit ligand loses its activity by ectodomain cleavage and soluble Fas ligand is less efficient in inducing apoptosis than the membrane anchored form. The less potent soluble Fas ligand competes with its more potent membrane bound congener, and behaves as an inhibitor of Fas ligand induced cytotoxicity (196, 197, 198). By ectodomain shedding other biological activities that require membrane anchorage of the growth factor are lost. Some membrane-anchored forms of growth factors mediate cell-cell adhesion. Murine hematopoetic progenitor cells adhere to and proliferate on the surface of pro-TGFα expressing stromal cells only when they express EGFR, adhesion is blocked by antibodies against EGFR or by soluble TGFα or EGF (199), Similar experiments demonstrated that the boss and sevenless proteins can mediate heterotypic aggregation (200). Uncleaved growth factors interact with their receptor on a neighbouring cell and mediate juxtacrine signalling, as documented for colony stimulating factor-1, c-kit ligand, tumor necrosis factor α (TNFα) and TGFα. Noncleavable mutants of pro-TGFα activate EGFR in adjacent cells and pro-TNFα kills surrounding target cells

(201, 202, 203). The role of the membrane-anchored c-kit ligand during development and in adult mice was understood through the characterization of the Sl^d mutation, encoding a truncated soluble form of c-kit ligand. Sl^d mice are viable but they lack coat pigment, are sterile and have macrocytic anaemia, indicating that the membrane anchored c-kit ligand has specific functions (204). Membrane-anchored HB (heparin bound) - EGF is another example of a protein with multiple functions that are modified by ectodomain shedding. The membrane anchored form of HB-EGF acts as juxtacrine growth factor, adhesion molecule and receptor for diphteria toxin. All these functions are lost after ectodomain shedding (195, 205, 206).

Proteolytic cleavage of the ectodomain affects also the function of the intracellular part of the molecule. The ectodomain of receptor tyrosine kinases acts as a negative regulator of the intracellular enzymatic activity (207). A retroviral receptor oncogene, such as the *v-erbB,* encodes an oncoprotein that lacks most of the extracellular domain as compared to its proto-oncoprotein c-erbB. The oncoprotein acts as if it was continuously ligated, v-erbB is, therefore, constitutively active and its catalytic kinase domain keeps signal transduction constantly on. Similarly, deletion of the ectodomain of the proto-oncogene receptor tyrosine kinase c-erbB causes constitutive activation of its catalytic domain, keeping the signal transduction cascade switched on and causing loss of growth control. The membrane-associated fragment of c-erbB2 from which the ectodomain was cleaved, resembles the oncogenic form of EGFR (v-erbB), a constitutive active tyrosine kinase (208). Indeed, the membrane-anchored 80 kD c-erbB4 fragment has tyrosine kinase activity and acts also as a membrane-localized docking molecule for the signalling molecules PLC-γ (phospholipase C-γ) and Shc (Src homology 2 domain-containing α2 collagen-related) (209). Ectodomain cleavage of the tyrosine kinase receptor TrkA leads to receptor activation and signalling (210).

Proteinases Involved in Ectodomain Shedding

Ectodomain cleavage of transmembrane proteins occurs mostly near the plasmamembrane and is often blocked by metalloproteinase inhibitors, suggesting that metalloproteinases like MMPs or ADAMs are responsible for the cleavage. Inhibition of ectodomain shedding by metalloproteinase inhibitors is described for L-selectin (211, 212), CD44 (213) and CD43 (214), thyrotropin receptor (215), growth hormone receptor (216), c-erbB4 (217), low affinity nerve growth factor receptor (LNGFR) (218), IL-1RII (219), IL-6 receptor (220), the 55 and 75 kD TNF receptors (TNFR) (221, 222), proHB-EGF (223), TGFα (211, 224), TNFα (225, 226, 227), Fas ligand (228) and CD30 (229). Also serine proteinases are implicated in ectodomain shedding of some molecules like CD44 (213), CD43 (230) and IL-3 receptor (231).

However, only for a few molecules proteases involved in ectodomain shedding are characterized. L-selectin is cleaved by MMP1 and MMP3 and not by MMP2 or MMP9 (212). β4 integrin is a substrate for MMP7 (232) and β1 integrin on platelets is cleaved by the snake venom metalloproteinase, jararhagin, and cleavage inhibits platelet aggregation (233, 234). The disintegrin domain of jararhagin binds to the α2 integrin subunit and subsequently the β1 subunit is

cleaved. Shedding of the $\alpha_2\beta_1$ integrin is also observed in migrating melanoma cells (235). Galectin-3 is a substrate for MMP2 and MMP9 (236). Murine recombinant FGFR1 and FGFR2 is cleaved by MMP2 and not by MMP9, releasing the ectodomain (184). MDC9, belonging to the ADAM family of metalloproteinases and MMP3 are involved in ectodomain cleavage of proHB-EGF (237, 238). ADAM17, also called TACE (TNFα converting enzyme), and ADAM 10 cleave TNFα (164, 165). L1, NgCAM and NrCAM, brain cell adhesion molecules, have a serine proteinase recognition sequence which is cleaved by tPA (tissue plasminogen activator) (239). Indeed, the addition of tPA to brain membranes increases the amount of soluble CAM molecules. Chymase, a chymotrypsin-like protease expressed by human mast cells, cleaves c-kit ligand yielding a soluble, bioactive form (240).

Regulation of Ectodomain Shedding

Protein kinase C (PKC) is a key regulator of ectodomain shedding. Activation of PKC by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) results in a rapid stimulation of ectodomain shedding. Ectodomain shedding is stimulated by TPA for the adhesion molecules L-selectin (241, 242), intercellular adhesion molecule 1 (ICAM-1) (243), CD44 (213), MUC-1 (John Hilkens, personal communication), protein tyrosine phosphatases LARPTP (244, 119, 245) and PTPσ (119), CD30 (229), growth hormone receptor (216) and the tyrosine kinase receptors c-erbB4 (209), colony stimulating factor -1 receptor (246), c-kit receptor (247), TrkA (210), c-met (248) and axl (249), the cytokine receptors IL-1RII (219, 250), IL-6R (251) and TNFR (252) and growth factors like HB-EGF (206, 223) TGFα (253, 254), c-kit ligand (255) and colony stimulating factor-1 (256).

The mechanism by which PKC stimulates ectodomain shedding is, however, not clear. PKC activation causes accelerated cleavage of a wide variety of cell surface molecules in different cell types. PKC might cause the co-clustering of enzyme and substrates, anchored in distinct domains of the plasma membrane. Alternatively, PKC could modify the cytoplasmic domains of the proteinases or their substrates, leading to conformational changes that either activate the enzyme or make the cleavage site on the substrate accessible. Recently, a direct interaction of PKCδ with MDC9, the ADAM involved in ectodomain shedding of HB-EGF, was demonstrated and, as a result of the interaction, PKCδ phosphorylates MDC9 (238). These results indicate that PKC might influence the enzymes responsible for ectodomain shedding.

Treatment of cells with calcium ionophores also stimulates ectodomain shedding and this mechanism is independent of PKC activation (253). Stimulation of ectodomain shedding by calcium ionophores is observed for protein tyrosine phosphatases (119), c-kit receptor and c-kit (247, 255), HB-EGF (223), TGFα (253).

Another argument, next to the implication of PCK and calcium, for a common mechanism of ectodomain shedding is provided by a genetic approach. In a mutagenized CHO cell line ectodomain shedding of structurally unrelated membrane proteins was inhibited both in the absence or in the presence of TPA (257).

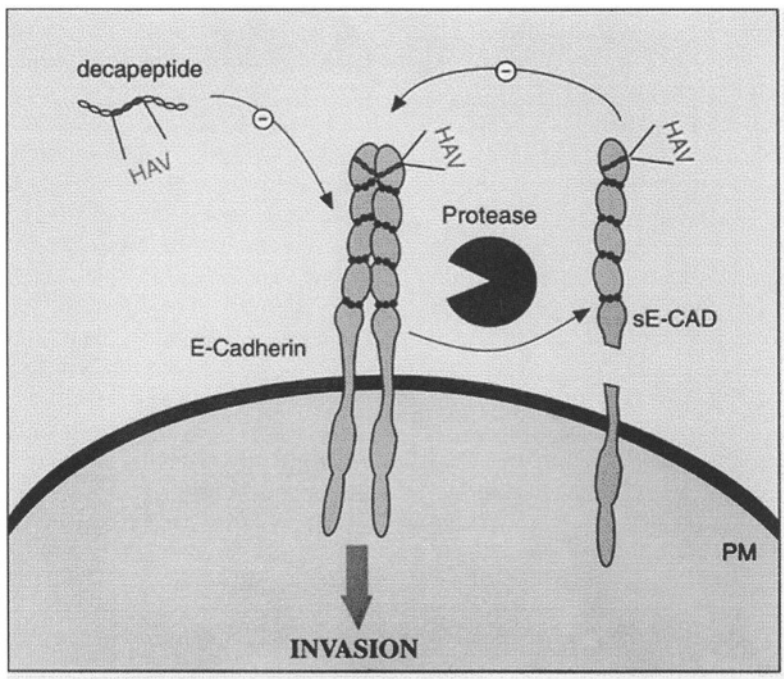


Figure 7. - Schematic representation of the functional regulation of E-cadherin by ectodomain shedding. PM, plasma membrane; sE-CAD, soluble 80 kD E-cadherin fragment; HAV, histidine alanine valine sequence, characteristic for the first extracellular domain of type 1 cadherins.

Other factors that stimulate ectodomain shedding are more specific. Activation of leucocytes promotes ectodomain shedding of L-selectin. Activation of endothelial cells leads to the shedding of E-selectin, ICAM-1 (Intercellular cell adhesion molecule 1) and vascular cell adhesion molecule 1 (V-CAM-1) (241, 258, 259, 260, 261). Activation of T-cells is known to stimulate the release of soluble CD27, TNF-R and the Fas ligand (262, 263, 264, 265, 266).

Receptor activation by a cytokine often stimulates ectodomain shedding of the receptor. This has been demonstrated for colony stimulating factor-1 receptor (246) and TrkA (210). Stimulation of syndecan-1 and -4 ectodomain shedding was observed upon treatment with HBEGF, EGF, thrombin and pervanadate (267, 268). Induction of apoptosis in epithelial cells leads to ectodomain shedding of CD44 (269). In response to high cell density, the ectodomains of protein tyrosine phosphatases are shed into the medium. Shedding is followed by a redistribution of the protein tyrosine phosphatases within the cell membrane and by an internalization of the remaining fragment, concomitant with comparable redistribution of the cadherin/catenin complex. Ectodomain shedding of protein tyrosine phosphatases might, therefore, also influence cadherin/catenin functions (119)

Detection of Soluble Fragments

Detection of the soluble fragments produced by ectodomain shedding in the serum of patients has been used as a marker for disease. Soluble L-selectin is found at high concentrations (1.5-2.0 μg/ml) in normal human serum. Levels are decreased in inflammatory diseases like rheumatoid arthritis, vasculitis and in adult respiratory distress syndrome. This may reflect reduced shedding or strong binding of the soluble fragment to cell surface ligands or both (270). Higher serum levels of soluble L-selectin have been found in sepsis and in AIDS (Acquired Immune Deficiency Syndrome), sometimes to a concentration that completely inhibits leucocyte attachment to endothelial cells in vitro (271). Serum levels of soluble E-selectin are elevated in patients with diabetes, sepsis, arthritis, lupus erythematosus or breast, gastro-intestinal and head and neck cancers, indicating that the endothelium is activated (189, 271, 272). Soluble ICAM-1 still binds to leukocyte function associated molecule 1 (LFA-1) (273) and the serum levels are elevated in patients with inflammation, infection or metastatic cancer (272, 99, 275, 276, 277).

V-CAM-1 (vascular cell adhesion molecule 1) is released from activated endothelial cells and the soluble fragment supports adhesion of a T-cell line. Higher serum levels were detected in patients with inflammatory diseases and malignancies (271, 272, 277, 278). Soluble c-erbB2 in serum of breast cancer patients is a marker of worse prognosis. Shedding of c-erbB2 results from c-erbB2 overexpression and proteolytic activity at the plasma membrane, two phenomena correlated with tumor aggressiveness (279, 280, 281). Soluble Fas ligand has been detected in the serum of leukemia (228). Increased serum levels of TNFR were found in serum of cancer patients and correlated with the stage of the disease. Other diseases in which soluble TNFR is increased are sepsis, infections and autoimmune diseases (266). Soluble IL-2Rα is a serum marker for T cell activation in immune disorders and for IL-2Rα expressing malignancies (4). IL-1RII has a membrane-bound and a soluble form, that is found in biological fluids of patients with sepsis (282, 283).

However, caution is needed when interpreting these data. Differences in the absolute values of the concentrations might be due to the specificity of the antibodies and the standards used in the ELISA (enzyme linked immunosorbent assay) kits (271). Moreover, only the free circulating molecules are measured and molecules adherent to cell surfaces or complexed with soluble ligands are missed.

Ectodomain Shedding : A Target for Therapy ?

Ectodomain shedding may provide novel targets for therapy of cancer and other diseases.

The role of proteinases in cancer has been restricted to breakdown of the extracellular matrix, so allowing cancer cells to invade. Their role in ectodomain shedding of cell-cell and cell-matrix adhesion molecules, tyrosine kinase receptors and their ligands received little attention. In vivo experiments already showed that proteinase inhibitors exert their therapeutic benefit by inhibition of ectodomain shedding.

Low affinity nerve growth factor receptor (LNGFR) on Schwann cells plays a role in the developing and regenerating neurons by presenting nerve growth factor (NGF) to the signalling high affinity receptor (218, 109). Regeneration after nerve lesion might be more efficient, if increased amounts of LNGFR are expressed on the cell-surface. Indeed, administering of 8-hydroxyquinoline, a metalloproteinase inhibitor, to rats with sciatic nerve lesion, upregulated LNGFR on the Schwann cells by inhibition of cleavage and augmented the rate of sensory neuron regeneration. Inhibition of TNFα release by Compound 1, a hydroxamic acid metalloproteinase inhibitor protected mice that were challenged with endotoxin (227).

CADHERIN ECTODOMAIN SHEDDING

Ectodomain shedding has been described for E-cadherin, N-cadherin and VE-cadherin. In the developing chick neural retina, proteolysis is one of the mechanism downregulating N-cadherin and releasing a soluble 90 kD N-cadherin fragment. Since 1,10 phenantroline protects N-cadherin in retina organ culture, it was presumed that a metalloproteinase is responsible for cleavage (285). The soluble 90 kD N-cadherin fragment is able to create an adhesive coat and promotes attachment, spreading and neurite outgrowth of embryonic chick neural retina cells (286).

Apoptosis of endothelial cells is associated with metalloproteinase-dependent cleavage of the extracellular domain of VE-cadherin and the release of a 90 kD fragment into the medium (287). Cleavage of VE-cadherin also occurs upon contact of HUVEC cells with activated neutrophils or by treatment with purified neutrophil elastase (288).

E-cadherin ectodomain shedding was first suspected from the detection of a soluble 80 kD fragment in the medium of human breast cancer MCF-7 cells (289). This E-cadherin fragment (sE-CAD) caused scattering of epithelial cells in culture and the effect was neutralised by an antibody against E-cadherin. E-cadherin cleavage and release of the 80 kD sE-CAD occurs also during the preparation phase for uterine invasion in pregnant mice and is promoted by with oestradiol (290). In contrast, neither a glycoprotein (gp84) trypsinized from murine embryonal carinoma PCC4 cells (291) nor the recombinant 80 kD fragment, harvested from a baculovirus expression system (both derived from E-cadherin), did have decompacting activity (292). We demonstrated that conditioned

medium of E-cadherin expressing MDCKts.*src*C12 cells, containing sE-CAD, stimulated invasion and inhibited aggregation of *MDCKts.srcC*12. When sE-CAD was removed from the conditioned medium by immunoadsorption, induction of invasion and inhibition of aggregation did no longer occur, showing that extracellular cadherin fragments induce loss of cell-cell adhesion and lead to tumor cell invasion (293, our unpublished results). The cadherin inhibiting activity of soluble cadherin fragments is mimicked by synthetic cadherin peptides containing the HAV sequence (Figure 7). The functions of the E-cadherin/catenin complex were inhibited by HAV-containing E-cadherin peptides in a specific way as evidenced by inhibition of aggregation, disturbance of epithelial morphology and induction of invasion into collagen type I and into precultured embryonic chick heart fragments (293). We found that only N-cadherin- and ECSODB (extracellular superoxide dismutase B)-specific HAV-containing decapeptides inhibit N-cadherin mediated aggregation and that only P-cadherin-specific HAV peptides could block the aggregation of cells expressing P-cadherin (294). An N-cadherin-specific HAV-containing decapeptide inhibited compaction of eight-cell-stage mouse embryos and outgrowth of rat neurites on astrocytes (295). N-cadherin dependent myoblast fusion was inhibited by an N-cadherin peptide (22). E-cadherin-specific 17-mer HAV peptides inhibited E-cadherin mediated fusion of monocytes during the formation of multinucleated bone-resorbing osteoclasts (296). These results show that extracellular cadherin fragments can induce loss of cell-cell adhesion and might lead to tumor cell invasion.

Cleavage of E-cadherin results also in a remaining 40 kD membrane-crossing fragment that might affect E-cadherin functions in the target cells (Figure 7). Cells from which E-cadherin is removed by trypsin do no longer aggregate. Moreover, tyrosine phosphorylation of 13-catenin is induced upon trypsinization (297). Mutant cadherins containing the cytoplasmic tail, mimicking the 40 kD remnant fragment, exert dominant-negative effects when expressed in Xenopus embryos (54), in cultured epithelial cells (55) and in mouse intestinal epithelia (56, 57).

E-cadherin cleavage might be an important and widespread new mechanism by which invasion of cancer cells is stimulated. The relevance of these in vitro observations for cancer invasion is supported by the detection of both sE-CAD and the 40 kD cytoplasmic fragment in lysates from cancer biopsies of breast and colon cancer (our unpublished results) and in gastric cancer (298). sE-CAD has also been detected in serum of patients with colon, liver, pancreas, bladder and gastric cancer (299, 300, 301, 298) and in urine of patients with bladder cancer (302). So, ectodomain shedding of E-cadherin is not only relevant for the pathogenesis of cancer, but also holds promises for diagnosis, follow-up and treatment of the disease.

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Chapter 5

CANCER AND CANCER METASTASIS-RELATED GENES

Steve Hiscox

Key words: Oncogenes, Metastasis, ß-catenin, nm-23, MTA1, CD44

Abstract: Neoplastic disease follows the acquisition of mutations within genes, a process that may span many years. When these mutations occur in proto-oncogenes that normally encode for proteins which control the cell cycle, DNA repair and transcriptional events, the result is deregulation of cellular proliferation and other intracellular pathways, ultimately leading to tumour development.

The metastatic spread of tumours is the single, most important factor that affects cancer patient mortality rate; gradually, new oncogenes are being implicated in playing a role as promotors of metastatic events rather than initial oncogenesis itself. Such genes may become potential targets for anti-metastatic therapies in the future. The aim of this chapter is thus to present a brief overview of some of the major oncogenic regulators of tumour development and spread.

DISCOVERY OF ONCOGENES

The first oncogenes were discovered in viruses that had the ability to induce tumours in infected animals. Ellerman and Bang (1) and Peyton Rous (2) demonstrated the transmitability of certain tumours at the turn of the century, although it was not until much later that the significance of their observations was realised.

With subsequent studies by Gross and others in the 1950s (3,4,5) describing murine tumour viruses, many oncologists sought to find a viral equivalent for the cause of human tumours. A number of viruses are now known to have oncogenic properties in humans and are listed in table 1 below.

This table includes the group of viruses termed retroviruses, since their genome is comprised of RNA rather than DNA. Viruses of this family may carry oncogenes that are largely similar to evolutionary-conserved genes present in

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humans, for example the retroviral oncogenes, v-src and v-myc are homologous to the human Src and Myc proto-oncogenes genes. Other retroviral vectors may insert their proviral DNA into the host geneome at sites which result in modification of host gene function or expression (by insertion of strong viral promotor elements for example).

Animal retroviruses have provided the majority of information concerning the effects of oncogenes as these viruses replicate by inserting a copy of their genome into that of the host cell and therefore have dramatic effects on the structure and expression of genes at their sites of integration. Even though there are currently no known human viruses that are equivalent to these animal viruses, studies of these acutely-oncogenic viruses in animals have lead to a wealth of information being provided concerning the mechanism of oncogenesis. In the case of humans, similar genes may become oncogenic by activation via chromosomal rearrangements, gene amplification or point mutation.

Oncogenic alterations occur mainly in five main groups of genes: protein kinases, GTP-binding proteins, transcription factors, growth factor receptors and growth factors themselves, all of which are involved in signal transduction mechanisms that inform the cell of its surrounding environment and allow it to respond by growth, division and differentiation. Loss of control of these tightly regulated pathways thus confers an environment-independent state to the cell such as a loss of contact inhibition and ultimately leads to immortalisation of the cell and uncontrolled growth.

Table 1. Some viruses that play important roles in human cancer development

Virus class	Virus	Tumour
Retroviruses	Human T-cell lymphotropic virus (HTLV-I, HTLV-II)	T-cell leukemia
Flavivirus	Hepatitis C virus	Hepatocellular carcinoma
Hepadnavirus	Hepatitis B	Hepatocellular carcinoma
Herpesvirus	Epstein-Barr virus	Nasopharyngeal carcenoma Burkitt's lymphoma
Papillomaviruses	HPV-16, 18, 33, 39	Cervical cancer Certain upper airway tumours
	HPV-5, 8, 17	Certain skin cancers

HUMAN ONCOGENE ACTIVATION / ONCOGENES

The data acquired from the study of viral oncogenesis in animals revealed that tumours could arise following induction by one or a few genetic events in particular genes. Thus it was hypothesised that cellular transformations may arise from the activation or mutation of genes that encode proteins which play a central role in the regulation of cell growth and differentiation. These altered normal genes were termed 'oncogenes' (6).

These mutations, which may build up over a period of many years, generally occur in proto-oncogenes, in genes that encode proteins with a tumour suppressor function or in genes that encode DNA repair enzymes. The effect of these

mutations is the loss of control of the normal cellular regulatory processes resulting in a transformed cell that exhibits characteristics such as deregulated growth and differentiation, loss of contact inhibition, genomic instability, loss of senescence and invasion into surrounding tissue (7, 8, 9).

The activation of proto-oncogenes in normal cells may occur by a number of mechanisms:

Point mutations in proto-oncogenes may arise as a result of exposure to external factors such as chemicals or radiation. A well-characterised case is that of Ras. The involvement of Ras proteins in human cancer was shown in 1987 when a human oncogene was identified as a mutant version of a normal H-Ras allele (10). Ras proteins are membrane bound GTPases that are active when bound to GTP and inactive when bound to GDP. A change in active state is mediated by guanine nucleotide exchange factors (GEFs) which switch Ras proteins 'on' and GTPase activating proteins (GAPs) which switch them 'off' (10,11). Active Ras proteins can interact with the serine protein kinase, Raf, which, in turn causes the activation of other proteins including members of the mitogen-activated protein (MAP) kinase family (12). MAP kinases in turn may activate a number of other proteins, some of which are transcription factors resulting in regulation of cell growth and differentiation. However, many amino acid substitutions can arise following point mutations within the Ras, resulting in a highly oncogenic Ras protein. The transfection experiments that originally described activating Ras genes in human tumours led to the finding that the transformation from normal proto-oncogenes to oncogene arose from a substitution of a single base, commonly resulting in the change of glycine 12 to lysine 12 or glutamine 61 to lysine 61 1(13, 14).

Gene amplification occurs frequently in human tumour cells and can lead to an increase in the intracellular concentration of the normal gene product. The amount of DNA that is amplified can be large, up to a region of several megabases in length and often these portions of DNA include proto-oncogene sequences. Increased amounts of these proto-oncogenes subsequently leads to the excess production of oncogenic mRNA and protein. The phenomena of DNA amplification is generally thought to occur during the later stages of cancer development, particularly in pre-metastatic or metastasising cells. Consistent with this is the observations that loss of the tumour suppressor gene, p53, also a generally late event in cancer development, may lead to a cell state in which amplification may occur. Other families that have been reported to be amplified in human tumours include EGFR, Myc, Ras and genes at the chromosomal locus 11q13 (15, 16, 17).

The expression of new proteins can arise following chromosomal translocation where in-frame fusion of coding sequences from separate genes occurs. This exchange of genetic material may occur between homologous or non-homologous chromosomes and can be either a balanced, reciprocal event or can involve the loss of material from one or both junctions. Other mechanisms may give rise to altered genes such as segment inversions occurring within the chromosome or interstitial deletions, giving rise to shortened chromosomes. More that 60 genes have now been identified that are involved in chromosomal translocations, the majority of which encode for transcription factors. The activities of these genes are thus altered following the formation of novel, chimeric proteins or overexpression due to the fusion of the gene with an anomalous regulatory sequence.

DEREGULATION OF THE CELL CYCLE

The cell cycle is a co-ordinated series of events that allows DNA replication and chromosomal separation, ensuring that the genome is replicated completely, and a single copy is inherited by each daughter cell. Each cell cycle consists of four stringently regulated phases termed G_1 (1^{st} gap phase), S (DNA synthesis), G_2 (2^{nd} gap phase) and M (meiosis/meitosis). During the gap phases, the cell prepares for either DNA synthesis or mitosis be transcribing and synthesising components of the replication or mitotic machinery. Some cells may leave the cycle temporarily and enter what is termed the G_0 phase from which they recover following the appropriate mitogenic stimulus.

Within the cell cycle are a number of checkpoints – pauses where the integrity of the DNA duplication and chromosomal separation are monitored by a family of cyclin-dependent kinases (CDKs) and their regulatory subunits, the cyclins (18, 19).

Under normal conditions, these cell cycle checkpoints exert no effect, but specific signals activate them and stop the cell cycle typical through modulating specific cyclin-CDK complexes. If cell DNA is damaged, for example, the cell arrests before entry into S phase so preventing mitosis. The damaged DNA is either subsequently repaired or the cell to undergoes apoptosis thus preventing DNA mutations from being replicated and damaged DNA from interfering with the separation of sister chromatids in mitosis.

Another major checkpoint is activated when chromosomes are incorrectly aligned in metaphase. The result of this is that the cell stays in metaphase until the chromosomes are correctly configured. Since one of the major characteristics of cancer cells is their genetic instability, with chromosomes being frequently rearranged or even lost following division, it is likely that some of the protective checkpoints controlling cell cycle progression must be inoperative. Tumours cells must have lost, or be able to ignore those checkpoints that normally prevent the replication of damaged DNA and those checkpoints that prevent cells from carrying out mitosis with misaligned chromosomes. The basis for these changes at a molecular level lies in the differences in the regulation and composition of the cyclin-dependent-CDK complexes.

In tumour cells, these checkpoints may become deregulated or completely inhibited if a positive CDK regulator such as a specific cyclin is overexpressed, or a negative regulator is removed. Some tumours have been shown to overexpress certain cyclins, particularly the D-type cyclins and other tumours have been demonstrated to lose expression of the negative cell cycle regulators (20, 21). Cyclin D1 (originally identified as the PRAD1 proto-oncogene) is overexpressed in parathyroid adenomas due to a chromosomal inversion such that it comes under the control of regulatory elements of the parathyroid gene on 11p15. The cyclin D1 locus is also amplified and overexpressed in a wide variety of tumours including breast cancer, oesophageal carcinoma and squamous cell carcinoma (22)

METASTASIS AND INVASION

The expression of a certain number of genes in a variety of different experimental systems has been shown to result in a positive modulation or gain in function that can affect the metastatic capacity in vitro and in vivo (23). It is likely, though, that the majority of these

effects are indirect and thus attention has been focussed on changes specific to tumour cells themselves. Such changes include altered expression of cell surface adhesion molecules (cadherins, integrins and immunoglobulin superfamily members) and the expression of tissue-degrading enzymes (matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs)).

CELL SURFACE ADHESION MOLECULES

Many stages of the metastatic cascade depend upon adhesive interactions between tumour cells and other tumour cells, normal host cells, basement membrane components and the components that comprise the extracellular matrix. A wide range of cell-surface adhesion molecules facilitates these interactions, each with their own ligand specificity (24, 25, 26, 27). These adhesion molecules can be generally separated into four families: the cadherins, the immunoglobulin superfamily, the integrins and the selectins.

Cadherins

The cadherins are a group of calcium-dependant cell surface adhesion molecules whose role is to mediate homotypic cell-cell adhesions (25). At least 11 different types of human cadherins have been identified according to their tissue distribution including epithelial (E-), neural (N-), and placental (P-) cadherin. E-cadherin plays a central role in maintaining the integrity of cell-cell junctions, cell and tissue morphology and cell sorting during development. Most importantly, several studies have shown a correlation between loss of E-cadherin within tumours and a gain in tumour cell invasiveness (28, 29).

A significant amount of evidence demonstrates the role of E-cadherin as a regulator of cell-cell adhesion (30, 31, 32, 33) and may be summarised as follows:

- cells lacking E-cadherin expression do not aggregate/adhere to each other whereas cells possessing E-cadherin strongly adhere together.
- E-cadherin is mainly distributed at the cell border, particularly at regions of cell-cell contact.
- neutralising the function of E-cadherin with anti-E-cadherin antibodies or deletion of its encoding gene causes cells to dissociate from each other,
- mutation of the E-cadherin protein or depriving the molecule of extracellular Ca^{2+} leads to cell dissociation,
- transfection of E-cadherin negative cells with E-cadherin DNA reverses their non-adhesive status.

The function of E-cadherin as a mediator of cell-cell adhesion is dependent upon its intercellular interactions with a family of protein molecules termed catenins. Studying proteins that co-immunoprecipitated with E-cadherin initially identified the catenins as integral components of the cadherin adhesion system. E-cadherin and catenins form complexes in the cellular membrane that ultimately serve as cell-cell adhesion complexes. The role of catenins in cadherin function has been further demonstrated in studies that show that cadherin mutants generated by deletion of their catenin-binding domains fail to mediate cell-cell adhesion (32, 34, 35).

β-catenin

β-catenin was originally described as an intracellular protein essential for the correct function of the E-cadherin cell-cell adhesion complex. However, a number of recent studies have implicated this molecule in the process of tumour progression via its involvement with the APC, Wnt and Tcf-Lef proteins. The

adenomatous polyposis coli (APC) gene is frequently mutated or deleted in colon cancers. In addition to being involved in non-inherited cases of this common cancer type, inherited mutations of the APC gene are responsible for a rare hereditary form of colon cancer, termed familial adenomatous polyposis (FAP). Individuals with this condition develop hundreds of benign colon adenomas or polyps, some of which will progress to malignancy (36). Little is known about how the APC protein functions within the cell. However, studies have shown that APC, following phosphorylation by glycogen synthase kinase-3 β (GSK-3β), binds directly to β-catenin via its NH_2 terminus (37). Thus APC, together with the GSK-3β, regulates the levels of free β-catenin within the cell. A possible role for this may be that the APC protein acts to sequester negative regulatory variants of β-catenin within the cells and thus prevent their interaction with E-cadherin (38, 39). Under normal circumstances, free β-catenin is bound by GSK-3β and APC and targeted for destruction (39, 40). However, in tissue obtained from polyposis patients and in colorectal cancer cells, the APC product is frequently mutated and fails to bind β-catenin. This results in a dramatic increase in the amount of free β-catenin in the cell. The possibility therefore arises that an increase in β-catenin may play a role in the development of benign colon polyps.

The process of APC-mediated β-catenin degradation is antagonised by Wnt-based signalling, possibly via inhibition of GSK-3β function (40). In mammalian cells, Wnts are secreted signalling proteins that become associated with the cell surface or extracellular matrix (41), although the exact mechanism by which this occurs is not yet known. Wnt appears to function in intercellular communication by interaction with serpentine receptor homologues of the *Drosophilla* frizzled family (42). Upon Wnt signalling, β-catenin is stabilized and accumulates in the cytoplasm in a monomeric form (43,44). Wnt signal transduction also promotes the association of free β-catenin with proteins of the T cell factor-lymphoid enhancing factor (Tcf-Lef) group of DNA-binding transcription factors. Studies have shown that DNA transcription occurs only if β-catenin is bound to Tcf-Lef. These observations suggest that β-catenin interacts with Tcf-Lef and activates gene expression within the cell (45, 46). Korinek *et al* (47) observed that the nuclei of colon carcinoma cells contained a β-catenin-Tcf complex that was constitutively active. Interestingly, when APC was introduced into these cells, the result was a removal of β-catenin from Tcf and loss of this transcriptional stimulus. These results further implicate APC in the regulation of normal cell function by interacting with β-catenin and suppression of β-catenin-Tcf4 signalling. Together, these observations suggest that β-catenin is involved not only in cell-cell adhesion but also the activation of gene transcription and Wnt-1 signalling. This provides compelling evidence for the role of β-catenin as a mediator of tumour development.

E-cadherin and catenins may act as tumour suppressors

Whilst up-regulation of the expression of certain cell adhesion molecules (mainly those involved in cell-matrix interactions) may promote their motility and invasion and thus enhance the metastatic potential of tumour cells, elevation of other adhesion molecules, particularly those involved in cell-cell adhesion, may inhibit

tumour cell invasion. The function of E-cadherin is to mediate tight cell-cell adhesions within tissues. As tumour cell metastasis depends in part on the loss of cell-cell adhesion within the tumour mass, factors that promote up-regulation of this molecule may act to suppress the early stages of this process. Studies on several human carcinomas have shown an inverse relationship between E-cadherin levels and tumour invasiveness (48,49). Transfection of a highly invasive tumour cell line with E-cadherin mRNA has been shown to reduce the invasiveness of tumour cells (36). This data suggests that up-regulation of E-cadherin within the primary tumour mass would enhance the capacity of tumour cells to bind to one another and thus inhibit their invasion into the surrounding tissue. The function of E-cadherin as a suppressor molecule of tumour development has thus been proposed.

Expression of E-cadherin and catenins in tumour tissues

From the evidence discussed so far, it would be natural to assume that well differentiated, non-invasive and non-metastatic carcinomas will express normal or relatively high levels of cadherins, whereas tumours that are poorly differentiated and possess a high metastatic potential will not. This correlation has been shown to hold true for several tumour types including squamous cell carcinomas of the head and neck (49), lung cancer (50), prostate and bladder carcinomas (51, 52), pancreatic cancer (53) and lobular breast cancer (29).

Mutations of E-cadherin have been recently reported by a number of different groups, which may bear importance in tumour spread (54,55). Becker *et al* (53) show that there is in-frame skipping of exon 8 or 9 together with deletion of exon 10 in patients with diffused type gastric cancer, a mutation seen in over 50% of the diffuse type carcinomas and 14% of cancers of mixed origin. Aberrant alpha catenin mRNA expression in cancer cells has also been reported by Oda *et al* (56) and this may lead to impaired E-cadherin function. An increase in urinary excretion of soluble E-cadherin has also been observed in some cancer patients (57, 58), suggesting that this molecule may be shed from the cell surface in these cases. This observation has been confirmed in bladder cancer by Banks *et al* (59).

The interaction of E-cadherin and catenins in rumour tissue needs to be explored fully. Whilst the loss of E-cadherin and/or catenins leads to increased cell dissociation and motility/invasion, certain factors may cause cell dissociation and enhance motility without apparent change of E-cadherin/catenin levels. These factors may exert their effects by modifying the interaction between catenins and cadherins/cytoskeletal proteins. Recent work by Brady-Kalnay *et al* (60) indicates that the phosphorylation state of this adhesion molecule complex can be regulated by cytokines and protein tyrosine phosphatases (PTPs). The role of PTPs as regulators of cadherin adhesive function via modulating the phosphorylation status of the complex thus presents an interesting area for future research.

Due to its potential role as a metastasis suppressor molecule and the relationship between E-cadherin levels and metastatic potential, its use as a prognostic indicator has been speculated. However, studies indicating a correlation of this type are very limited, mainly because of the short history of the molecule since its discovery. Some studies have shown that E-cadherin mRNA levels in patients surviving longer than five years are significantly higher than those with survival rate of less than 5 years. Such a relationship has also been reported in

patients with head and neck squamous cell carcinoma (61), bladder cancer (51), gastric tumours (62) and prostate cancer (63, 64).

These observations thus show the important consequences of E-cadherin loss in tumour tissue. Established cell-cell adherens junctions may prevent initial stages of tumour dissemination and local invasion and the studies above have demonstrated an inverse correlation between cadherin molecules and metastatic spread in some human cancers. Further investigations will determine the value of the molecular components of the cadherin adhesion complex as indicators of patient survival.

THE IMMUNOGLOBULIN SUPERFAMILY

The immunoglobulin superfamily of adhesion molecules comprises over 70 members and as such, represents one of the most diverse families of receptors known. Along with the immunoglobulin type receptors, this family includes the major histocompatability receptors and T-cell receptors together with proteins associated with specific tumour types such as DCC (deleted in colorectal carcinoma) (65, 66). The most well known members of the immunoglobulin superfamily involved in cell adhesive interactions are the ICAMs (intercellular adhesion molecule) and VCAMs (vascular cell adhesion molecule). This class of molecule are able to interact with integrins and mediate the firm attachment of circulating leukocytes to the endothelium allowing their subsequent movement into the stroma of a particular organ during the inflammatory response (65, 67).

Most members of this family exist as transmembrane molecules and interact with the cell cytoskeletal system. Also associated with the cytoplasmic region of these receptors are several tyrosine kinases. Thus occupation of the immunoglobulin receptors by ligand may initiate a chain of intracellular second messenger pathways (68).

The immunoglobulin superfamily receptors of most interest in tumour cell metastasis are those that are likely to be involved in tumour cell-endothelial cell interactions such as the ICAMs and VCAM-1. ICAM-1 is expressed at a low level on most tumour cells and it is interesting to note that melanoma tumour progression and an increased risk of metastasis has been correlated with ICAM-1 expression (69). It should be noted, however, that a definitive link between such factors is not clear as other studies have shown that ICAM-1 expression does not significantly contribute to tumour progression (70). VCAM-1 may also be involved in tumour spread. Tumour cells bind endothelial VCAM-1 via $\alpha_4\beta_1$ integrins and thus increased expression of VCAM-1 on endothelial cells may promote tumour cell binding (71, 72). Neural cell adhesion molecule (NCAM) expression modulates the adhesive phenotype of glioma cells. Cells lacking NCAM expression display marked invasive capabilities in vivo, migrating along the vascular elements of the brain and not displaying a well-defined tumour mass whereas in NCAM expressing cells, cell invasion involves degradation of normal tissue and replacement by tumour cells. The reduced invasiveness in NCAM positive cells may be attributed to the observation that cells in which the β-unit of NCAM molecule is expressed show a reduced expression of MMP-1 and MMP-9 (73).

Selectins

The selectin family members, L-selectin, P-selectin and E-selectin, are involved in heterotypic cell-cell adhesions between blood cells and endothelial cells.

The selectins mediate leukocyte rolling along the surface of stimulated endothelium. Although cells are capable of adhering to numerous ligands under static conditions, under the conditions of blood flow, only selectins interacting between the circulating cell and the endothelium can slow the cell down sufficiently to allow other receptors to be presented to their respective ligands (74).

The adhesion of both human colon and gastric cancer cells to the endothelium has been shown to involve the interaction between E-selectin and sialyl-Lewis x (75). The expression of sialyl-Lewis x by colorectal cancer cells has been linked to poor patient survival thus implicating the E-selectin-sialyl-Lewis x adhesion system in cancer progression (76).

Integrins

The integrins are a large family of heterodimeric membrane proteins that facilitate cell-cell and cell-matrix adhesion (77, 78). At least 14 α and 8 β units have been identified which can combine to produce at least 20 different integrin molecules, each containing an α and a β subunit. The majority of integrin proteins bind to extracellular matrix proteins and promote cell-substratum adhesion, however, some integrins recognise integral membrane protein members of the immunoglobulin superfamily on other cells and mediate cell-cell adhesion (79).

While the extracellular portion of the integrin molecule binds to extracellular ligands, the intracellular region of this receptor interacts with cytoskeletal elements. Thus the integrins serve as transmembrane linkers between the extracellular matrix and the cell cytoskeleton. In addition, emerging evidence has implicated integrins as active transducers of molecular signals (for a review, see 78). Following integrin binding, a number of intracellular changes are observed including elevation of Ca^{2+} (80) and intracellular pH (81) together with the activation of protein tyrosine kinases including $pp125^{FAK}$ (82, 83). $pp125^{FAK}$ (focal adhesion kinase) co-localises with the integrins to structures that form at regions of close contact between the cell and ECM components known as focal adhesions (84). Focal adhesions also contain the protein paxillin, a cytoskeletal molecule that becomes phosphorylated on tyrosine as a direct result of the activation of $pp125^{FAK}$. Paxillin also offers a mechanical linkage for the β_1 integrin to the cell cytoskeleton. Thus integrin-ligand interactions result in both the generation of cytoplasmic signals and the anchoring of components to the cytoskeletal network.

During the malignant transformation of tumour cells, their integrin expression pattern has been reported to change. These alterations may thus contribute to tumour cell-specific features such as anchorage-independent growth and invasiveness (85, 86). In colonic epithelial cells, a loss of or reduction in fibronectin-binding integrin expression has been associated with malignant progression (87). β_1, β_3 and α_v based integrin receptors have also been implicated in tumour progression (88, 89).

Many integrin molecules recognise and bind to the tri-peptide sequence, arginine-glycine-aspartic (RGD), in extracellular matrix proteins. Co-injection of short, RGD-containing peptides with melanoma cells in a mouse model decreases the formation of metastatic lung colonies (90) further implicating these adhesion molecules in tumour spread.

CD44

CD44 is a widely distributed adhesion molecule not classified as a member of the above families. Substantial evidence has accumulated indicating that CD44 promotes both homotypic and heterotypic cell adhesion acting directly through ligand-receptor binding and indirectly

through the activation of other adhesion molecules. CD44 has also been shown to function as a lymphocyte homing molecule and as a receptor for hyaluronate (91).

In addition to promoting the adhesive behaviour of normal cells, CD44 may impinge on pathologic processes. The passage of white blood cells across the endothelial lining of circulatory vessels during inflammation can be considered to be similar to the invasion of tumour cells through the endothelium in metastasis and it is thus possible that similar adhesive molecules are involved in both processes.

It has already been shown that adhesion molecules such as VCAM-1 and VLA-4 play a role in both lymphocyte and tumour cell adhesion to the endothelium. CD44 is known to be up-regulated in some transformed cells and studies of non-Hodgkins lymphomas have demonstrated that highly metastatic tumours typically bear higher levels of CD44 than do tumours not exhibiting vigorous spreading (92). More than 10 forms of CD44 have now been identified, occurring by alternative splicing events and CD44v6, a variant form of CD44 that contains the v6 exon, has been implicated as playing an important role in tumour metastasis (93, 94, 95). Studies concerning CD44 function have also demonstrated that it mediates cellular uptake and lysosomal degradation of hyaluronan, a component of the ECM (96) thus suggesting that CD44 may also be involved to some extent in cellular invasion.

CD44 ligands include addressin, hyaluronate, osteopontin and gp600 and the finding that anti-CD44 antibody can inhibit metastasis in vivo suggests that interactions between CD44 on tumour cells and its ligands may be necessary for tumour growth and metastasis.

Over-expression of CD44 has been reported in many primary tumours, and this elevated expression appears to correlate with high metastatic (97, 98, 99). It has also been shown that the serum concentrations of soluble CD44 are significantly raised in patients with gastric or colon cancer and this increase correlates with tumour burden and metastasis. An increased expression of this molecule is also reported in metastasised large cell lymphomas (100, 101) and gross overproduction of alternatively-spliced isoforms occurs in breast and colon cancers and is associated with poor prognosis (102)

PROTEOLYTIC ENZYMES

The extracellular matrix is composed of a variety of molecules with the major components being fibronectin, laminin, vitronectin, collagen type IV, thrombospondin, elastin, hyaluronic acid, von Willebrands factor and heparin sulphate proteoglycan. In order for tumour cell invasion to occur, extensive degradation of these components is required. Cells accomplish this by releasing a battery of metalloendopeptidases that are able to digest a wide range of proteins of the extracellular matrix.

Matrix metalloproteinases

The matrix metalloproteinases (MMPs) are a family of at least 14 zinc-dependent proteolytic enzymes and can be divided into four groups as shown in table 2. This table includes the membrane type MMP (MT-MMP), a recently discovered membrane-bound MMP implicated in the activation of gelatinase A (103, 104). MMPs are secreted from the cell as inactive forms that are subsequently activated in the pericellular and extracellular environment (105).

In normal adult tissues, these endopeptidases are expressed at a relatively low level and their expression is

a stringently regulated process, controlled by growth factors such as IL-4 (down-regulation) and IL-1, TNF-α, EGF and PDGF (up-regulation) (106, 107). MMP expression is elevated in a number of normal and pathological processes including inflammation wound repair and tumour metastasis. It is through the action of these enzymes that cells are able to break down the components of the tissue surrounding them and thus remodel the environment within which they exist.

As mentioned above, the cellular secretion of these ECM-degrading enzymes clearly presents a potential danger to the host if it is left unchecked. There is thus strict regulation of their secretion, together with the presence of matrix metalloproteinase inhibitors within the blood and tissue known as tissue inhibitors of matrix metalloproteinases (TIMPs, see below). The presence of inhibitors of these enzymes within the extracellular matrix itself implies that proteolytic activity is the balance between the local concentration of activated enzymes and their endogenous inhibitors.

Table 2 Members of the matrix metalloproteinase (MMP) family

Enzyme		Molecular weight	Substrate
Gelatinases			
Gelatinase A	(MMP-2)	72 kDa	Collegens IV, V, VII, X,
Gelatinase B	(MMP-9)	92 kDa	elastin, fibronectin
Collagenases			
Fibroblast type	(MMP-1)	52 kDa	Collagens I, II, III, VII, VIII, X
Polymorphonuclear leukocyte type		kDa	
	(MMP-8)	75 kDa	
Collagenase 3	(MMP-13)		
Stromelysins			
Stromelysin-1	(MMP-3)	55 kDa	Fibronectin, laminin, collagen IV, V,
Stromelysin-2	(MMP-10)	55 kDa	IX, X, elastin
Stromelysin-3	(MMP-11)	61 kDa	
Metalloelastase	(MMP-12)	54 kDa	Elastin
Membrane MMP	(MT-MMP)	~63 kDa	Progelatinase A

It has been observed in several cases that the level of expression of MMPs in malignant tumours are significantly greater than that observed in their normal tissue counterparts and that cell lines with a high metastatic capacity synthesisze abnormally large amounts of MMPs (108, 109, 110, 111, 112).

MMPs are secreted as inactive zymogens; activation of these proteins then occurs by the action of plasmin. Plasmin is itself secreted in its inactive form and is activated in the plasma by a serine protease, which may be either tissue plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA, also known as PAI-1).

Over-expression of uPA or its receptor, uPAR, have been reported in tumour cells of mesenchymal or epithelial origin and these molecules are required for tumour invasion and metastasis. uPA upregulation has also been reported in a number of malignant tissues including

breast tumours and melanomas and the expression of uPA is an important prognostic indicator for breast cancers (113, 114, 114). Inhibition of the uPA receptor has also been demonstrated to inhibit in vivo tumour cell invasiveness (115).

Interestingly, it is now known that mitogenic signalling pathways including those that involve Ras/PKC-Erk or Rac-JNK can be activated by oncogenes with a resultant enhancement of uPA and uPAR expression (116).

Several other studies have implicated metalloproteinases in the process of tumour invasion and metastasis (117, 118, 119). It is interesting to note that transfection of cells with the oncogene, H-*ras,* has been demonstrated to enhance the secretion of this enzyme (120, 121). Growth factors such as EGF and PDGF are also known to stimulate the production of MMPs. Since *ras* transfection also enhances MMP production, it may be the case that MMP production as a consequence of growth factor activation occurs via activation of intracellular *ras*-dependant pathways.

MMPs have been shown to be involved in a number of neoplastic processes by evidence supplied from experiments using synthetic inhibitors of MMPs. Wang and co-workers (122) have shown that the MMP inhibitor, batimastat, is successful in preventing malignant tumour growth in animal models. A similar approach led to the demonstration that MMP activity is required for angiogenesis and that this process can be blocked by TIMPs and MMP inhibitors (123).

Tissue inhibitors of metalloproteinases (TIMPs)

TIMPs are secreted by a wide variety of cell types and are present in normal bone and cartilage (124). TIMPs inhibit the action of MMPs by directly binding to them, in a 1:1 ratio. Since the action of these proteins is to inhibit MMP action, it is not surprising that TIMPs are potent inhibitors of several stages of the metastatic cascade including degradation of the extracellular matrix, invasion of surrounding tissue and extravasation and tumour growth (125, 126).

It has been demonstrated that TIMP-1 and TIMP-2 can block cell invasion in vitro (127, 128) and that TIMP-1 inhibits lung colonization in vivo. Observations such as these suggest that MMPs play an important role in tumour progression. The importance of TIMPs as tumour suppressors has indeed been shown in transfection studies using vectors expressing TIMP antisense DNA (129). Following transfection of 3T3 cells, it was observed that these cells became tumourgenic and produced metastases when introduced into mice. Conversely, transfecting cells with a vector expressing TIMP-2 decreases the activity of secreted MMPs and the growth rate of the cells in vivo as well as reducing the capability of the cells to invade into the surrounding tissue (129).

NME1 AND NM23

NME-1 (non-metastatic cells 1, expressed) and NM-23 (non metastatic-23, also known as NME-2) are nucleoside diphosphate kinases (NDKs) that are known to express on the surface of the cell (130).

It has been shown both *in vivo* and *in vitro* that the nm23 gene and protein product correlate with non-metastatic behaviour of cancer cells (131). *In vitro,* the motile and invasive nature of tumour cells inversely correlates with the level of nm23; transfection of highly invasive cells with nm23 cDNA results in a reduction or

complete inhibition of invasiveness (132,133,134).

Recent studies by Hsu et al (135) have further implicated nm23 in the disruption of signal transduction pathways utilised by motility factors. The case of nm23 as a candidate metastasis suppressor gene is not clear however, since other studies suggest that elevated levels of nm23 accompany tumour progression (136, 137).

Both animal and clinical studies have shown the levels of nm23 to be decreased in tumour cell/tissues and this has been shown to be closely related to disease stage, metastasis presence and prognosis. Reduction of nm23 has been seen in patients with many different types of tumour including colorectal cancer (138, 139, 140), breast cancer (141, 142, 143, 144), liver cancer (145), melanoma (146), esophageal cancer, bladder cancer (147), ovarian cancer (148, 149). Interestingly, however, this relation-ship is not seen in thyroid cancer (150).

In the early stages of colorectal cancer, it seems that there may be an overexpression of both nm23-H1 and H2, but at advanced stages there is a marked reduction of nm23-H1 protein (151). Tumours of this type are also associated with mutations of nm23 (122).

Miss-sense mutations and loss of heterozygosity of the nm23 gene have also been reported in both ovarian serous carcinoma (149) and primary breast cancer (152).

In ovarian tumours, nm23 is related to the lymphatic dissemination of tumour cells (148) and in breast cancer, nm23 has been suggested to correlated with the lymph node involvement (143).

Interestingly, upregulation of the expression of NM-23 by essential fatty acids has been shown to correlate with a reduction of *in vitro* invasion of breast cancer cells in vitro (153).

OTHER METASTASIS-PROMOTING GENES

Oncogene transfection models have shown that some of the genes that mediate metastasis can be regulated independently from those that confer tumorogenicity (154). Transfection of NIH 3T3 cells with activated Ras oncogenes results in the production of a number of metastases (155). An enhanced metastatic potential is also reported following introduction of H-Ras family members into rodent and human cells (120,121,156,157,158). A number of candidate effector molecules have been implicated as mediators of these metastatatic phenotypes including type IV collagenase (121) and the lysosomal cysteine proteinase, cathepsin L (159). Recent studies have shown that the cathepsin L gene is activated by a number of oncogenes and that the mRNA level of cathepsin L correlates with the metastatic potential in vivo of transformed cells (160). The metastatic phenotype of cells has also been reported to be induced via transfection of cells with other oncogenes including v-mos, v-raf (serine threonine kinases) , v-src, c-fms and v-fes (tyrosine kinases) (23).

MTA1 is a recently described gene that has been implicated in mammary cell motility and growth regulation (161, 162,163). Since the product of this gene contains an SH3 domain binding motif, a zinc finger motif and potential phosphorylational sites, it is plausible to suggest that the MTA1 protein may be involved to some extent in signal transduction pathways or regulation of gene expression. Levels of MTA1 have been shown to be raised in a number of tumour types including oesophageal carcinoma (164) and gastrointestinal tumours (165) and the expression of MTA1 mRNA correlates with tumour cell invasion and metastasis (164,165).

In conclusion, it can be said that many of the genetic events that activate cellular oncogenes occur in several major ways. The simplest is where the expression of a normal gene product occurs at an inappropriately high level as happens in the case of DNA amplification. Another mechanism is where a normal gene is expressed in an inappropriate cell type, as can occur following the translocation to an active chromosomal locus, or where the function of a gene product is perturbed due to single point mutations or gene fusion products.

It is well reported that cell-cell adhesiveness is generally reduced in human cancers. Interactions between tumour cells and the tissue/cells of their immediate environment play a central role in the process of invasion and metastasis, as does the expression of matrix metalloproteinases and their activators in tumour cells. Alterations in the genes encoding such proteins may well be responsible for the up/down regulation of various proteins ultimately facilitating tumour cell invasion and metastasis.

As the field of cancer research expands, the genetic alterations that underlie tumour formation and progression are defined at a molecular level and such analyses of tumours may reveal potential targets at which anti tumour therapies may be aimed. Similarly, as the genetic predisposition to certain tumours is defined, screening tests for patients with a greater risk of developing these tumours may be conducted to predict tumour occurrence with more certainty and thus aid an earlier diagnosis of neoplasia.

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Chapter 6

HEPATOCYTE GROWTH FACTOR AND MET IN TUMOUR INVASION-METASTASIS: FROM MECHANISMS TO CANCER PREVENTION

Kunio Matsumoto and Toshikazu Nakamura

Key words: c-met, hepatocyte growth factor, HGF-antagonist, scatter factor, tumour angiogenesis, tumour-stromal interaction

Abstract: Hepatocyte growth factor (HGF), a ligand for c-met proto-oncogene product of receptor tyrosine kinase, exhibits powerful motogenic and angiogenic activities. The utilization of the HGF-Met system in cancer cells confers invasive and metastatic potentials. HGF potently enhances dissociation of cells, cell-matrix interaction, extracellular matrix breakdown, invasion, and angiogenesis, all critical events in the metastatic cascade. Tumour-stromal interaction mediated by HGF, aberrant expression of Met, autocrine or mutational activation of Met are tightly associated with carcinogenesis and malignant progression in a wide variety of tumours. Notably, NK4, the four kringle-antagonist for HGF-Met signaling inhibited tumour invasion and metastasis. The possibility has arisen that an HGF-antagonist can serve as a new therapeutic strategy for treatment of cancer patients. In normal tissues, HGF supports dynamic tissue remodeling for regeneration and the clinical application of HGF for treatment of organ failure/fibrosis will be initiated within a few years. These "two-pronged approaches" regarding HGF will lead to tissue repair and perhaps even the prevention of cancer.

INTRODUCTION

Hepatocyte growth factor (HGF) was originally identified as a potent mitogen for mature hepatocytes in primary culture (1-3). HGF was first purified from rat platelets (4) and subsequently from human plasma (5, 6). In 1989, HGF was molecularly cloned and HGF has proven to be a novel growth factor distinct from other known growth factors (7, 8). Subsequent studies done in the early 1990's revealed unanticipated biological activities of HGF. Scatter factor, originally identified as a fibroblast-derived epithelial cell motility factor (9), was purified in 1989-1990 (10, 11) and

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subsequent characterization and cloning of this factor showed it to be identical to HGF (12-15). Likewise, molecular cloning of tumor cytotoxic factor (16) and fibroblast-derived epithelial growth factor (17) independently showed these polypeptides to be identical to HGF. Fibroblast-derived epithelial morphogen also proved to be HGF (18). Moreover, the cell surface receptor for HGF was identified as a c-met protooncogene product (19, 20). The oncogenic met gene was first found to be an activated oncogene in chemically transformed human osteosarcoma cells, based on its potential to transform NIH 3T3 cells (21), and cellular protooncogene, c-met was cloned in 1987 (22). The Met/HGF receptor is a transmembrane protein containing the tyrosine kinase domain in its intracellular domains.

Subsequent studies on HGF and the Met receptor include diverse aspects (23-27). Biochemical and molecular biological studies revealed a structure-function relationship in the HGF molecule and intracellular signaling mechanism responsible for multipotent biological activities driven by HGF-Met receptor coupling. HGF is involved in embryogenesis and organogenesis during development (23-27). As HGF was initially considered to be a hepatotrophic factor which supports the vital ability of the liver to regenerate, hepatotrophic functions of HGF have been extensively studied. HGF is now known to support regeneration of various tissues and organs (24, 26). Moreover, pharmaceutical application of HGF and its gene for treatment of subjects with various diseases is likely begin within a few years (28).

As scatter factor was identified to be the same as HGF, possible involvement of HGF in cancer invasion and metastasis has been extensively examined. Among many polypeptide growth factors and cytokines, HGF is now considered to be a critical molecule which confers malignant behavior in a wide variety of cancers. In this chapter, we review roles of the HGF-Met receptor system in malignant progression of cancers and introduce new approaches for cancer prevention, including molecule antagonistic to the HGF-Met receptor system.

GENERAL ASPECTS OF HGF AND MET: STRUCTURE AND ACTIVITIES

HGF is a heterodimer composed of a 69kDa α-chain and a 34kDa β-chain (4, 7) (Figure 1). The α-chain contains the N-terminal hairpin domain and the subsequent four kringle domains and the β-chain contains serine protease-like domain (7). The kringle domain was initially found in serine proteinases involved in blood coagulation or thrombolysis and HGF has 38% amino acid sequence homology to plasminogen. Plasminogen is composed of a five kringle-containing A-chain and B-chain of the serine protease catalytic subunit, whereas HGF has no serine proteinase activity because of amino acids substitution in the catalytic center. HGF is biosynthesized as a biologically inactive single chain precursor and processing by specific serine proteinases into the two chain form is coupled to conversion to its biological activation. Met receptor is composed of a 50kDa α-chain and a 145kDa β-chain (22). The α-chain is exposed extracellularly, whilst the β-chain is a transmembrane subunit containing an intracellular tyrosine kinase domain (Fig. 1). Binding of HGF to the Met receptor induces activation of tyrosine kinase, which results in the subsequent phosphorylation of C-terminally clustered tyrosine residues (29, 30).

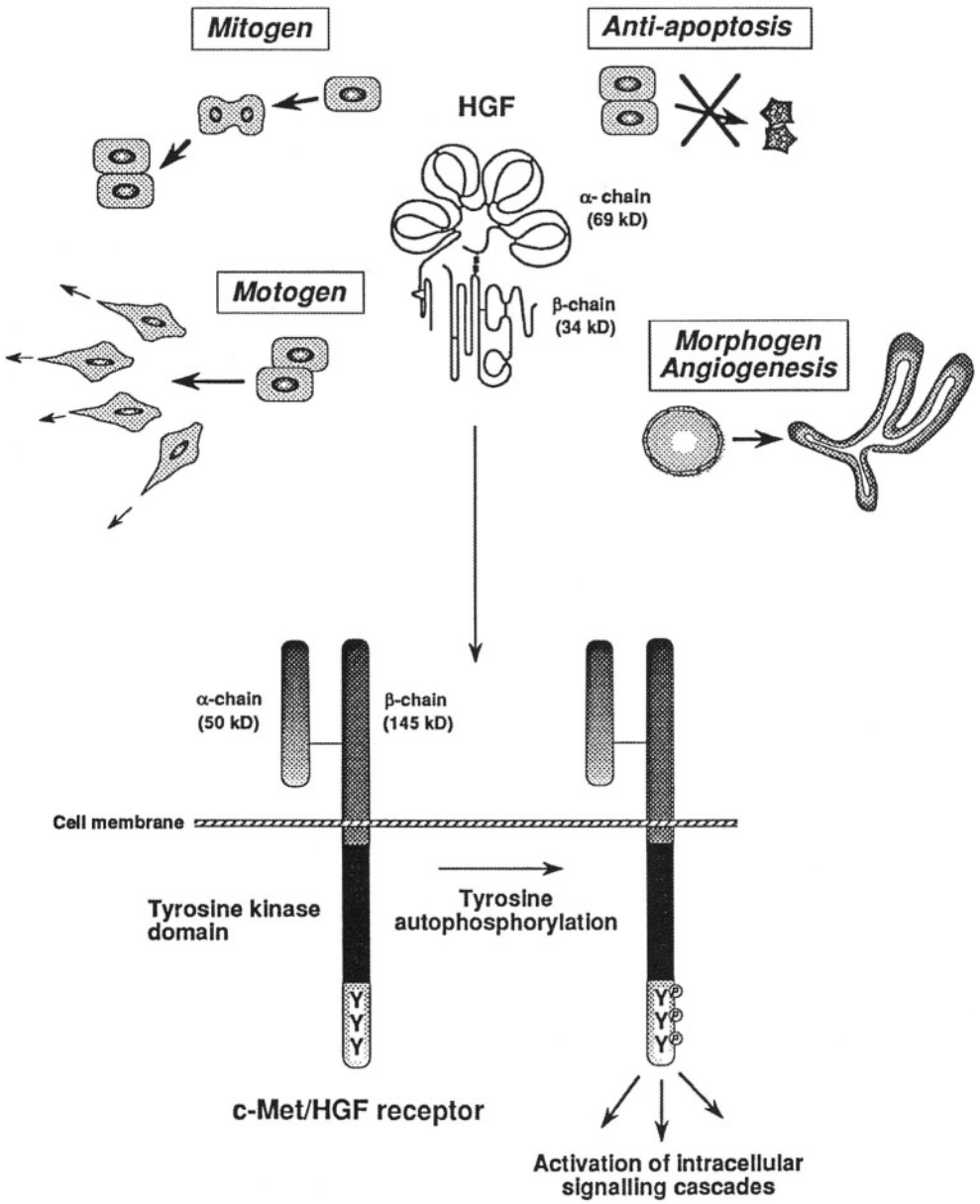

Figure 1. Schematic structures of HGF and Met/HGF receptor and typical biological activities of HGF.

Phosphorylation of these tyrosine residues recruits intracellular signaling molecules containing the src homology (SH) domain, including phospholipase C-γ (PLC-γ), Ras-GTPase activating protein (Ras-Gap-1), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI-3 kinase), c-Src, and Grb-2. The distinct combination of phosphorylation/activation of these molecules is considered to specify the subsequent intracellular signaling cascade, leading to diverse biological effects driven by HGF-Met receptor coupling.

Although HGF was originally identified as a powerful mitogen for hepatocytes, this factor has exhibits multiple biological activities for a wide variety of cells (23-27). In normal tissues, most epithelial cells, vascular endothelial cells, and some kinds of stromal cell (cells of mesenchymal origin) are target cells of HGF. Fig. 1 schematically describes typical biological activities of HGF. In addition to mitogenic action to enhance cell proliferation, HGF prevents apoptotic cell death in several types of cells (31, 32). HGF strongly stimulates cell motility, including a wide variety of cancer cells (see below). HGF exhibits morphogenic activity, inducing branching morphogenesis in ductular epithelial cells (e.g., renal tubular cells, mammary gland epithelial cells, and bile duct epithelial cells) (18). HGF possesses a potent angiogenic activity to induce new blood vessel formation (33-35).

Based on its activities to promote cell growth, migration, morphogenesis, and angiogenesis, HGF functions to organize and reorganize tissue structures during organogenesis and tissue regeneration. During embryogenesis, interaction between epithelial and mesenchymal tissues mediates crucial aspects of normal tissue morphogenesis (36). The growth and morphogenesis of developing epithelia are regulated either inductively or permissively by neighboring mesenchyme in various organs. In parenchymal organs, HGF is expressed predominantly in mesenchymal or stromal cells while Met/HGF receptor is expressed in epithelial cells (24, 27, 37, 38). HGF induces branching tubulogenesis of epithelial cells, as a mesenchymal-derived factor in several tissues, including kidney, lung, and mammary gland. Targeted mutation of HGF or the c-met gene results in impaired development of the liver, placenta, and skeletal muscles and diaphragm (39-41). HGF is essential for long-distance migration of myogenic precursor cells during development of skeletal muscles and the diaphragm. This is a pertinent example of the motogenic activity of HGF during normal biological processes. Physiologically, expression of HGF is regulated in response to tissue injuries and HGF supports regeneration of various organs such as the liver, kidney, lung, and vascular tissues (24, 26, 28). Thus, HGF exerts biological activities to construct or reconstruct normal tissue architecture during development and tissue repair, through a paracrine manner, not exclusively, but it does do so in many cases. Administration of recombinant HGF into experimental animals with tissue injuries or diseases has profound effects to enhance regeneration of organs and angiogenesis (28, 35). Moreover, HGF has therapeutic effects on acute organ failure and notably on tissue fibrosis (e.g., liver cirrhosis, chronic renal failure, and lung fibrosis) caused by chronic tissue injuries (28).

HGF IN CANCER INVASION AND METASTASIS

While HGF exhibits multiple biological activities for remodeling and maintenance of tissues, HGF greatly affects behaviors of a wide variety of cancer cells. Biological activities of HGF

on various types of cancer cells are summarized in Table 1. Consistent with Met receptor expression in normal epithelial cells, HGF targets many types of carcinoma cells (tumour originating from epithelial cells), whereas aberrant expression of the Met receptor has been noted in tumours originating from mesenchymal cells and hematopoetic cells. HGF is involved in malignant behavior of these tumour cells and exhibits distinct biological activities on cancer cells, depending on the cell type. It is noteworthy that HGF has bi-directional effects on proliferation of cancer cells. Consistently, tumor cytotoxic factor, originally identified as fibroblast-derived cytotoxic growth inhibitor for some types of cancer cells, was shown to be identical to HGF (16). Particularly, HGF inhibits proliferation of about 90% of hepatoma cells in vitro, while the number of cancer cells in which HGF induces growth inhibition is lower in other tumour cells (42-44). Bi-directional growth regulation by HGF suggests genetical alteration in intracellular signal transduction pathways from the Met receptor in cancer cells, however, molecular mechanisms responsible for the growth inhibition by HGF are as yet unknown. On the other hand, among the biological activities of HGF on cancer cells, motogenic activity of HGF to stimulate tumour migration and invasion is notable in most tumour cells (Table 1).

Induction of tumour migration and invasion by HGF

Invasion of cancer cells is regulated by distinct cellular functions, including cell-cell adhesion, cell-matrix interaction, proteolytic breakdown of extracellular matrix components, and cell migration. Although several growth factors and cell motility factors such as epidermal growth factor (EGF), transforming growth factor-α (TGF-α), TGF-β1, basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF)-I, IGF-II, platelet-derived growth factor (PDGF), autocrine motility factor (AMF) affect motility of cancer cells. HGF has profound effects on migration and invasion of a wide variety of cancer cells, as summarized in Table 1.

Figure 2 shows typical examples of tumour cell scattering and the migration induced by HGF. In monolayer culture, HGF stimulates dissociation and migration of cells, the result being a marked scattering of tumour cells (Figure 2 left). In Boyden's chamber assay, HGF exerts chemotactic or chemokinetic effects and strongly stimulates migration of tumour cells through the membrane (Figure 2, right). Although dissociation of cell-cell adhesion and subsequent migration are essential components for tumour invasion, tumour cells particularly require the potential to breakdown the scaffold of extracellular matrix components, in terms of invasion into host tissues. Figure 3A shows an example of in vitro tumour invasion induced by HGF. When tumour cells (human gallbladder carcinoma cells) were cultured onto a membrane coated with Matrigel basement membrane components, tumour cells did not invade through the membrane, yet the addition of HGF strongly stimulated invasion of the cells. Other growth factors (EGF, TGF-β1, bFGF, PDGF) were either much less potent than HGF or had no effect on invasion of tumour cells (Figure 3B). Likewise, when the tumour cells were cultured on a collagen gel matrix, they did not show invasive characteristics, however, tumour cells did invade the collagen gel matrix in the presence of HGF but not EGF, TGF-β1, PDGF, or bFGF (Figure 3C). These results indicate that HGF induces breakdown of extracellular matrix components by tumour cells, in addition to tumour cell

dissociation and migration. Similar findings that HGF induces/stimulates invasion of tumour cells into/through extracellular matrix components were noted in many types of tumour cells (Table 1).

Table 1. Biological effects of HGF on tumour cells.

Tumour cell types	Biological effects	References
Bladder carcinoma	stimulation of motility & invasion and induction of β-catenin & desmoplakin phosphorylation	45
Breast carcinoma	stimulation of motility & invasion	46, 47
	stimulation of adhesion to ECM	48
	stimulation of invasion & uPA production	49
Cholangiocarcinoma	stimulation of growth, motility & invasion	50, 51
Colorectal cancer	bi-directional growth regulation	44, 51
	stimulation of adhesion to ECM	48
	stimulation of membrane ruffling, motility & invasion	44, 53, 54
	stimulation of growth, migration, invasion, and MMP-1, -2, -9 production	55, 56
	stimulation of E-cadherin tyrosine phosphorylation	56,57
	stimulation of phosphorylation, of E-cadherin-ß-catenin complex, reduction of α-catenin-E-cadherin complex	57
Esophageal cancer	stimulation of invasion	58
Gallbladder cancer	stimulation of growth, motility & invasion	59, 60
	stimulation invasion, uPA & MMP-2 production	61
Gastric cancer	stimulation of growth, motility & invasion	62-64
	reduction of E- and P-cadherins	65
Glioblastoma	stimulation of growth, motility and colony formation	66-69
Glioma	stimulation of tumour angiogenesis	70
	stimulation of motility	71
	stimulation of growth, motility & invasion	61-73
Hepatocellular carcinoma	inhibition of growth	42, 43
	stimulation of motility	74, 75
	stimulation of adhesion to ECM & invasion	76
	inhibition of metastasis	77
Kaposi's sarcoma	stimulation of growth & angiogenesis	78, 79
Leukemia	stimulation of growth & motility	80, 81
Lymphoma	stimulation of adhesion to ECM, motility & invasion	82, 83
Lung cancers		
-Adenocarcinoma and squamous cell carcinoma	stimulation of growth, motility & invasion	50, 84, 85
-Non-small cell carcinoma	no effect on growth or growth stimulation, but stimulation of motility & invasion	86
Small cell carcinoma	stimulation of motility & invasion	50, 87

Table 1 to be continued..

Table 1 continuing

Tumour cell types	Biological effects	References
Melanoma	growth inhibition	42
	stimulation of invasion	88-90
	stimulation of uPA & gelatinase production	91
Uveal melanoma	stimulation of motility & invasion	92
Mesothelioma	stimulation of growth and motility	93
Myeloid leukemic cells	stimulation of growth, colony formation & motility	94
Neuroblastoma	stimulation of motility	67
Oral squamous cell carcinoma	inhibition of growth	42
	stimulation of motilty & invasion; induction of FAK tyrosine phosphorylation	95, 96
Ovarian cancer	stimulation of growth & motility	97, 98
Pancreatic cancer	stimulation of growth, motility & invasion	99-102
	stimulation of uPA production & adhesion to ECM; induction of E-cadherin redistribution	103
Prostate cancer	stimulation of growth, motility & invasion	104-106
Renal carcinoma	stimulation of growth, motility & MMP production	107-109
	stimulation of invasion	110
Rhabdomyosarcoma	stimulation of growth & invasion	111
Sarcoma	stimulation of motility	112
Thyroid carcinoma	induction of recruitment of $\alpha v\beta 3$ integrin to focal contact	113

Dissociation of cell-cell adhesion

Cell-cell associations tightly coupled by adhesion and junctional complexes are responsible for construction of organized multicellular tissue structures, while adhesiveness is decreased in various types of tumour cells as the result of genetic or epigenetic events (114). The dissociation of cell-cell adhesion results in increased invasiveness in tumour cells. Epithelial cadherin (E-cadherin) is involved in the tight association of epithelial and carcinoma cells through adherens junction and the desmosomal complex. Down-regulation of E-cadherin-mediated cell-cell adhesion is widely considered to contribute to the invasive characteristics of tumour cells (114, 115). Extracellular domains of cadherins mediate homophilic interactions with adjacent cells, while their cytoplasmic domains form a complex with proteins called catenins, i.e., α-catenin, β-catenin, plakoglobin/γ-catenin and $p120^{ctn}$. The catenins transmit signals and anchor cadherin to the actin cytoskeleton (Figure 4). E-cadherin directly binds to β-catenin, plakoglobin/γ-catenin, and $p120^{ctn}$, and the complex associates with α-catenin, which is thought to link cadherin complexes with the actin cytoskeleton, either directly or indirectly via α-actinin (115). E-cadherin-

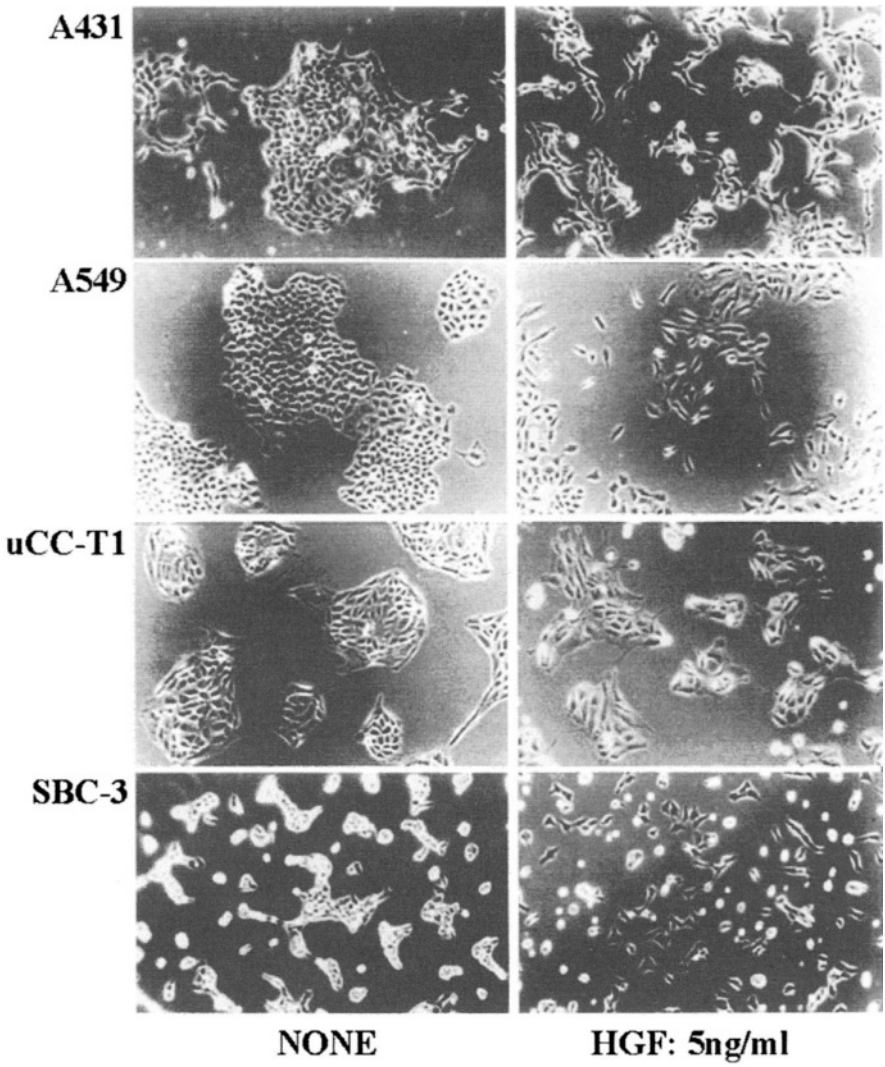

Figure 2. Enhancement of cell motility by HGF. **Left:** Scattering of human carcinoma cells induced by HGF. A431, epidermoid carcinoma; A549, non-small cell lung carcinoma, HuCC-T1, cholangiocellular carcinoma; SBC-3, small cell lung carcinoma. . **Right:** Enhancement of human tumour cell migration by HGF. Tumour cells were cultured on Transwell membrane and appearances of cells migrated through the membrane were shown. SAS, human tongue squamous cell carcinoma; T98G, human glioblastoma; Bows, human melanoma.

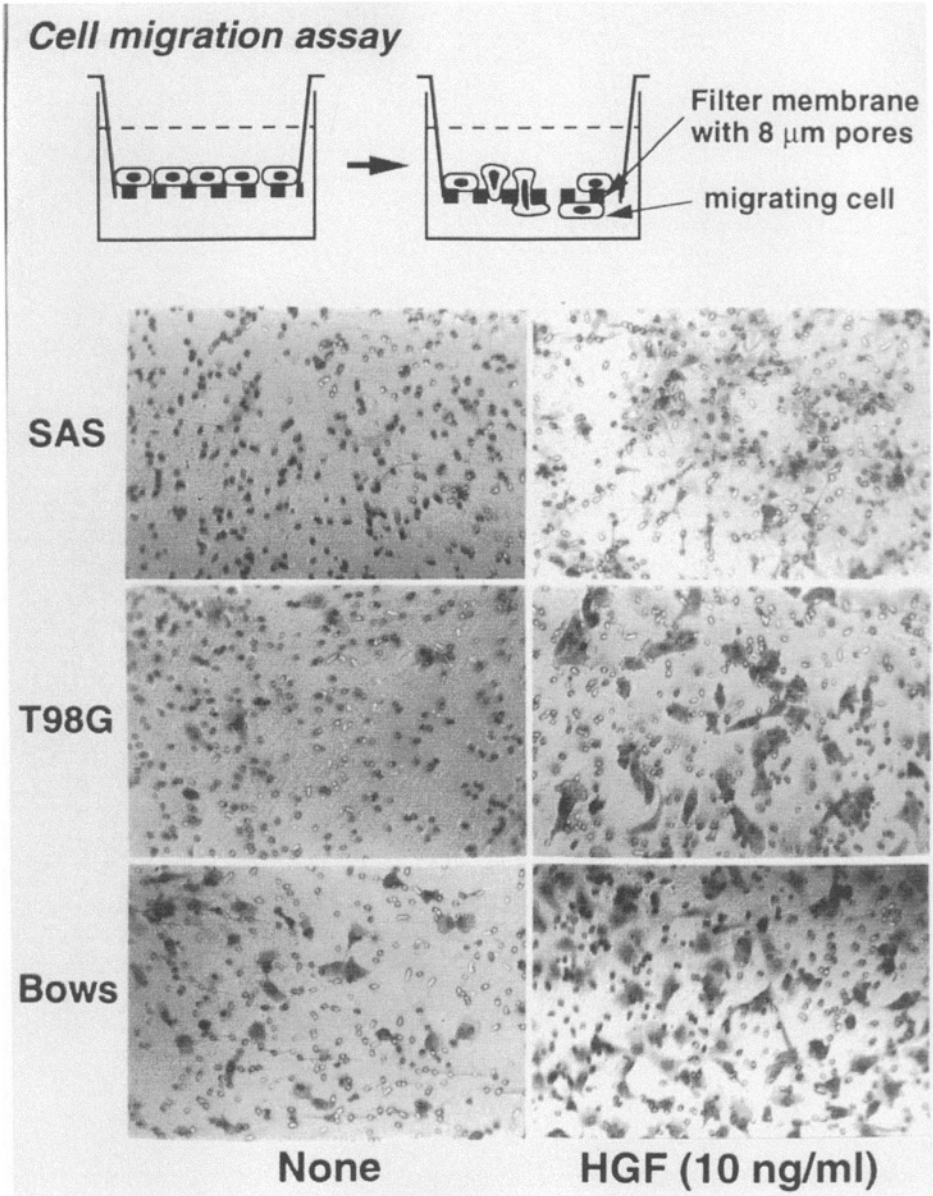

Figure 2. continuing

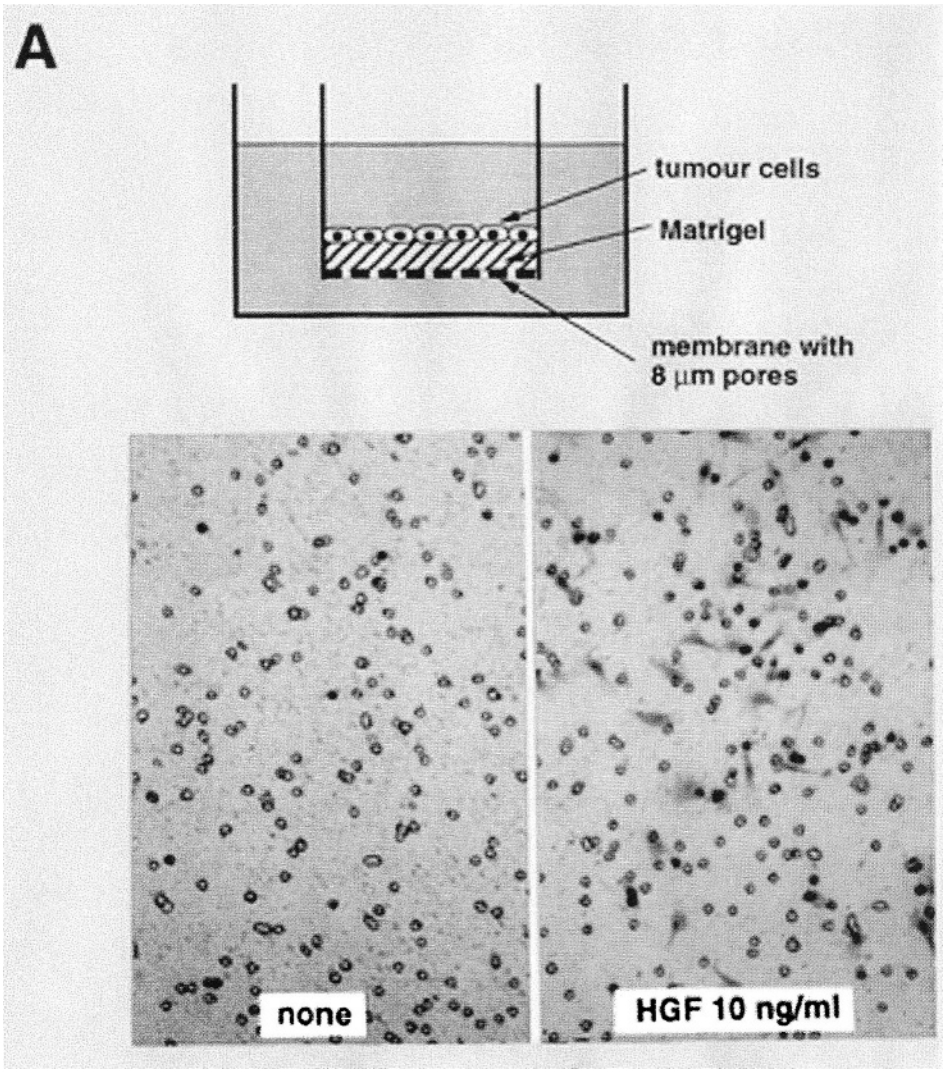

Figure 3. Enhancement of in vitro invasion of tumour cells by HGF. (**A**) Enhancement of invasion of human gallbladder cancer cells by HGF in Matrigel invasion chamber. Appearance of cells invaded through a membrane coated with Matrigel basement membrane components was shown. (**B** and **C**) Effect of several growth factors on invasion of human gallbladder cancer cells in Matrigel invasion chamber (**B**) and type I collagen gels (**C**). HGF potently stimulated invasion of the cells through both Matrigel (**B**) and collagen gel (**C**). Growth factors added to cultures were 10 ng/ml in (**C**).

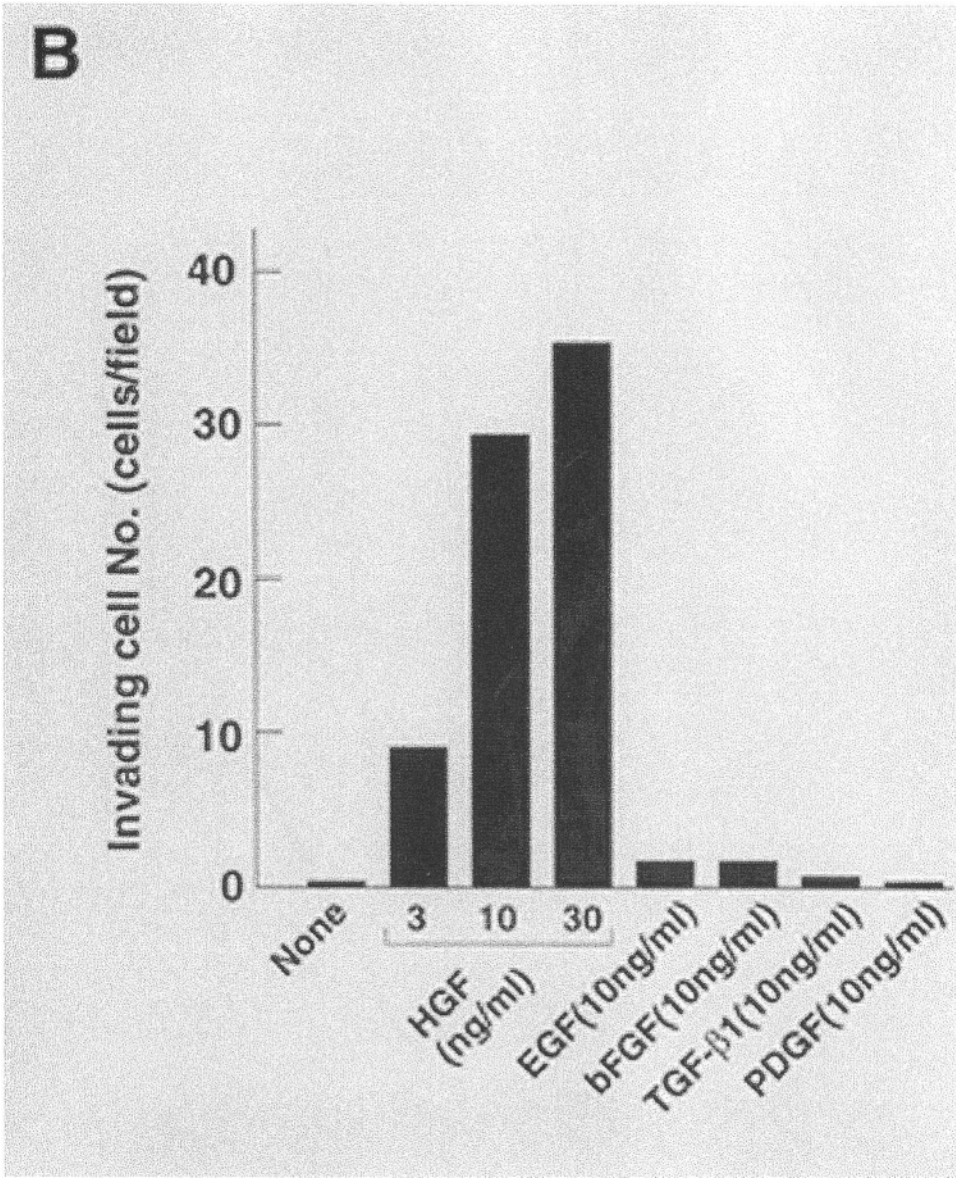

Figure 3B. continuing.

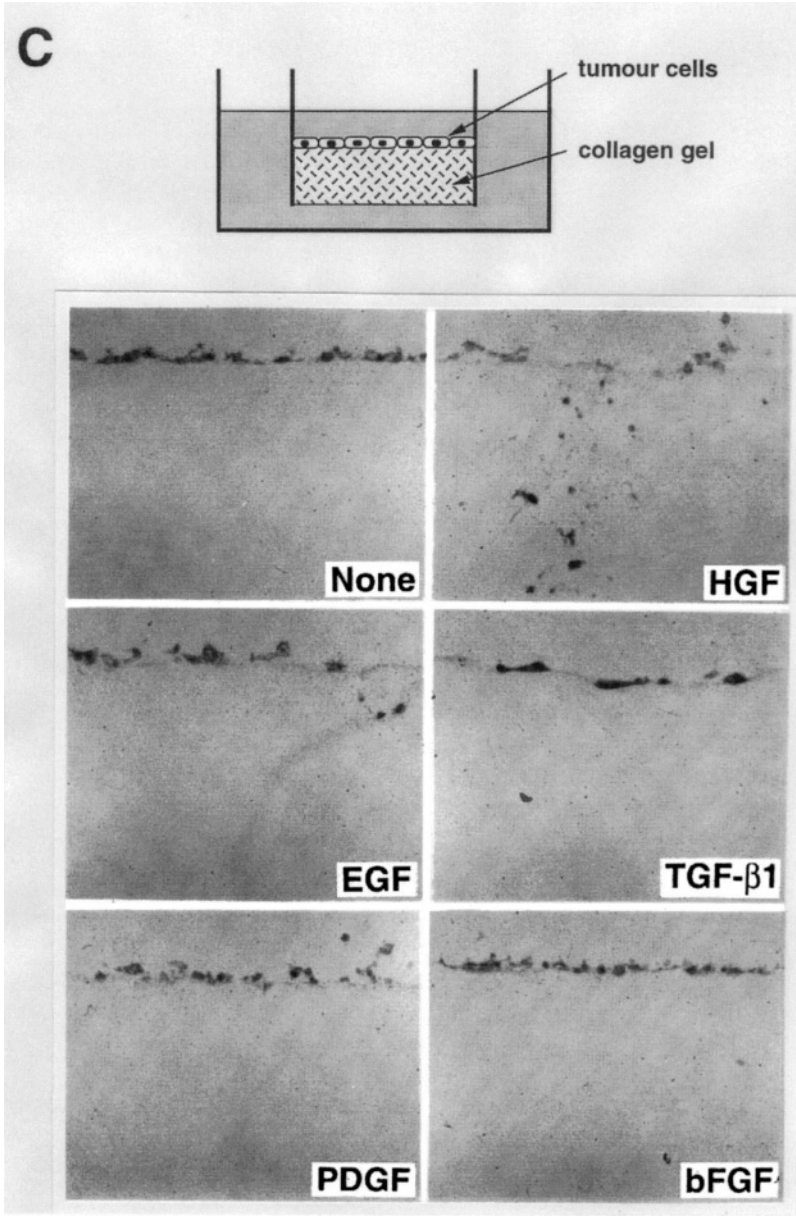

Figure 3C. continuing..

mediated cell-cell adhesion is regulated through localization and by protein phosphorylation of catenins. In addition to regulation of cadherin-mediated cell-cell adhesiveness, β-catenin plays a crucial role in the Wnt-1 oncogene signaling pathway, directly interacts with the Axin/APC complex, and its complex with T-cell factor-lymphoid enhancing factor (TCF-LEF) translocates to the nucleus, where they are believed to activate genes involved in cell proliferation (116).

HGF stimulates dissociation of cell-cell adhesion through both tyrosine phosphorylation of catenins and by regulating expression and distribution of the cadherin-based adhesive machinery. HGF induces tyrosine phosphorylation of β-catenin, plakoglobin/γ-catenin and $p120^{ctn}$ (117-119). HGF reduces the amount of E-cadherin/α-catenin complex in human colorectal cancer cells (57), while HGF decreases the expression of E- and P-cadherins in gastric cancer cells (65). Moreover, the Met receptor localizes at the basolateral plasma membrane domain of polarized cells (120), thereby suggesting rapid and preferential tyrosine phosphorylation of the E-cadherin/catenin complex through activation of the Met receptor. HGF also inhibits intercellular communication mediated by gap junctions in rat hepatocytes (121). Jiang et al (253) showed that HGF decreases the expression of occluding, a protein involved in tight junction, and increases paracellular permeability in endothelial cells.

Several hypotheses have been proposed to explain the mechanism for adhesive changes associated with tyrosine phosphorylation of catenins, but none have yet gained wide acceptance (115). Regardless of the mechanism the basic observations consistently indicate that tyrosine phosphorylation of catenins decreases adhesiveness while tyrosine dephosphorylation has the opposite effect. The mechanism probably involves the dynamic regulation of interactions between the cadherin/catenin complex and the actin cytoskeleton, and is likely to be fundamental to integration of signals that promote cell proliferation in response to growth factors versus signals that inhibit cell growth and motility as a result of cell-cell contact. In conclusion, HGF decreases cadherin-mediated adhesiveness between carcinoma cells, through tyrosine phosphorylation of catenins and down regulation of cadherin/catenin complexes. The possible involvement of HGF in β-catenin-mediated Wnt-1/Axin/APC signaling pathways remains to be addressed.

Enhancement of cell motility and cell-matrix interaction

Cellular locomotion is regulated by multiple extracellular and intracellular processes which are successively organized by a complex signaling cascade. While the entire picture for molecular mechanisms for cell movement are unknown, regulation of cell-extracellular matrix adhesion and concomitant change in cell shape and cytoskeletal rearrangement are likely to be major components involving cell movement. Focal adhesions, adhesive complexes for anchoring of cells to extracellular matrix components, are newly formed in frontier regions of migrating cells, whereas they are dissociated in terminal regions. Coupling with active turnover (association and dissociation) of focal adhesion complexes, rearrangement and retraction of cytoskeletal network occur in cells. Cells adhere to the extracellular matrix components via the integrin family receptors which serve as transmembrane linkers predominantly between the extracellular matrix and the cytoskeletons. Extracellular domains of the integrin

molecule binds to extracellular ligands such as fibronectin, laminin, and collagens, while the intracellular domain interacts with cytoskeletal elements. In addition, emerging evidence has implicated integrins as transducers of extracellular signals involved in extracellular matrix-dependent cell shape arrangement and cell movement, $p125^{FAK}$ (focal adhesion kinase) co-localizes with integrins in focal adhesion complexes. Paxillin provides a mechanical linkage between integrin to the cell cytoskeleton and it is tyrosine-phosphorylated as a direct result of the activation of $p125^{FAK}$.

ERM (ezrin/radixin/moesin) proteins which cross-link actin filaments with plasma membranes are localized at microvilli, ruffling membrane, and cell-cell/cell-matrix adhesion sites, such as cadherin- and integrin-based adherens junctions. The C-termini of ERM proteins bind actin filaments, while the N-termini bind plasma membranes using a binding partner such as CD44 (122). CD44 plays the role of receptor for cell-associated hyaluronic acid and extracellular matrix proteins such as fibronectin, collagen, and serglycin, thus CD44 is involved in cell adhesion, migration and metastasis in cancer cells (123). N-termini and C-termini of ERM proteins are associated with each other (head-to-tail interaction) in their inactive state, so that binding to plasma membranes and actin filaments, respectively, via N-termini and C-termini of ERM proteins does not occur. Specific signals activate ERM proteins by disrupting head-to-tail interaction to bind actin filaments and plasma membranes.

On the other hand, the Rho subfamily of small GTP-binding proteins (Rho, Rac, and Cdc42) play crucial roles in cell shape and cell movement by remodeling the actin cytoskeleton and their interaction with plasma membranes. Different members of this family affect the polymerization of actin to produce filapodia (Cdc42), lamellipoda/membrane ruffling (Rac) and focal adhesions and stress fibers (Rho). A signal transduction pathway mediated via activation of Rho plays a critical role in regulation of both ERM proteins and focal adhesion (122, 124). Rho-GDI (GDP-dissociation inhibitor) is likely to be associated with the CD44-ERM complex, suggesting that it might recruit cytoplasmic Rho to the plasma membranes to be activated. Rho-kinase was shown to directly phosphorylate ERM proteins and interfere with head-to-tail association in ERM proteins, thereby resulting in activation of ERM proteins as actin filaments/plasma membrane cross-linkers (125). Rho-kinase also phosphorylates the myosin-binding subunit of myosin phosphatase and suppresses the activity of myosin phosphatase, an event which results in promotion of actomyosin contractility (126). Rho-kinase is thus tightly involved in stress fiber formation. Furthermore, activation of Rho results in tyrosine phosphorylation of $p125^{FAK}$, indicating $p125^{FAK}$ is downstream of Rho activation (124), and Rho-Rhokinase activation is essential for formation of focal adhesions (127). In addition to the Rho-Rho kinase pathway, Rho activates phosphatidyl-inositol 4-phosphate 5-kinase (PIP4-5K) (128). The enzymatic product, phosphatidylinositol 4,5-bisphosphate (PIP2) binds to vinculin and promotes interaction with both talin and actin (129). PIP2 also can induce the release of actin monomers from profilin and gelsolin complexes (130). These events are likely to participate in assembly of actin filaments at focal adhesions.

Activation of Rho plays an essential role in HGF-induced cell scattering and migration. When Rho was inactivated in MDCK renal epithelial cells by microinjection of a Rho-GDP dissociation inhibitor or by C3 toxin, a specific inhibitor of Rho, cell scattering and

migration induced by HGF were inhibited (131). In addition to the activation of Rho, HGF regulates tyrosine phosphorylation of key proteins involved in cell-matrix and plasma membrane-cytoskeleton interactions. HGF induces tyrosine phosphorylation of $p125^{FAK}$ and paxillin, and HGF affects the initial recruitment of integrins, $p125^{FAK}$ and paxillin into focal adhesion complexes (48, 95) (figure 4).

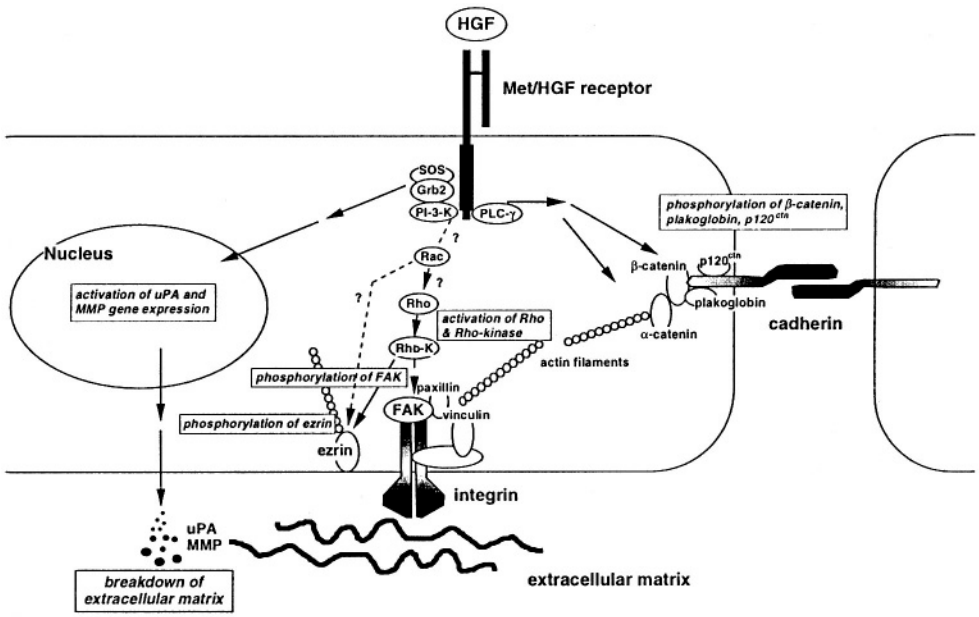

Figure 4. Possible mechanisms for signal transduction pathways responsible for enhancement/induction of tumour invasion by activation of Met receptor. Activation of Met signaling pathways leading to migration/invasion were analyzed in various cells and assay systems,

Although the mechanism by which $p125^{FAK}$ regulates formation of focal adhesions and integrin-mediated cellular anchorage on extracellular matrix proteins is not fully understood, tyrosine phosphorylation of $p125^{FAK}$ may possibly affect rapid turnover, i.e., both association and dissociation of focal adhesions and subsequent recruitment of actin stress fibers to focal adhesion complexes, since decreased ability in cellular locomotion was noted in cells deficient in $p125^{FAK}$. Likewise, HGF induces tyrosine phosphorylation of ezrin (53, 132). Tyrosine phosphorylation of ezrin may be involved in the rapid induction of HGF-induced lamellipodia/membrane ruffling, the formation of focal adhesions together with phosphorylation of $p125^{FAK}$, and may regulate rearrangement of actin stress fibers to ruffling membranes and focal adhesions.

Taken together with current notions that the Rho-Rho-kinase pathway plays a crucial role in cell-matrix and plasma membrane-cytoskeletal interactions, tyrosine phosphorylation of $p125^{FAK}$, paxillin, and ezrin, and their recruitment to plasma membranes, these events induced by HGF are mediated, at least in part, through activation of Rho and its effector Rho-kinase. In addition to a key role of Rho-Rho-kinase pathway in motogenic response by HGF, involvement of PI-3K and other small GTP-binding proteins, Rac and Ras was noted in HGF-induced cell motility response (133-135). Activation of PI-3K subsequently results in activation of Rac, and Rac is known to induce lamellipodia/-membrane ruffling and activation of Rho (124).

It is noteworthy that interactions between tumour cells and the extracellular matrix or endothelial cells is altered during malignant progression. (136). A important characteristic of metastatic tumour cells is the ability to invade through the vascular endothelial cell barrier (intravasation) with subsequent attachment at a distant site, and extravasation. β1, β3 and αv based integrin receptors have been particularly implicated in tumour progression, and interaction of tumour cells and endothelial cells is likely to increase tumour cell entry into the circulation and subsequent extravasation. HGF stimulates adhesion of B-lymphoma cells to the extracellular matrix mediated via α4β1 and avbl integrins, and promotes migration and invasion of the tumour cells (82). In non-transformed human thyroid epithelial cells, αvβ3 integrin is diffusely distributed, disconnected from the cytoskeleton, and unable to mediate adhesion to the substratum, however, in their counterpart papillary thyroid cancer cells, HGF induces recruitment of αvβ3 integrin molecules at focal adhesions and enhances attachment and spreading of cells via αvβ3 integrin (113). Likewise, HGF stimulates the expression of α2β1 integrin in hepatoma cells with subsequent increases in adhesive properties of the cells to endothelial cells and to the extracellular matrix (76). On the other hand, HGF stimulates attachment of tumour cells to endothelial cells by increasing CD44 expression in endothelial cells, a molecule which may play a critical role in tumour-endothelial interactions and establishment of metastasis (137).

Enhancement of proteolytic breakdown of the extracellular matrix

Extracellular proteolysis of extracellular matrix proteins plays a critical role in tumour invasion and metastasis. Among various types of proteinases, the urokinase type plasminogen activator (uPA)-dependent proteolytic network and matrix metalloproteinases (MMPs) affect

invasiveness of a wide variety of tumour cells. uPA anchors to cell surfaces through the uPA receptor, cell surface-anchored uPA activates plasmionogen to plasmin, and plasmin subsequently activates some types of MMPs. HGF induces uPA gene expression and strongly stimulates uPA production in many types of normal and tumour cells, including MDCK renal epithelial cells (138), gallbladder carcinoma cells (51, 61), pancreatic carcinoma (103), human squamous carcinoma (139), and mammary carcinoma (49). Similarly, establishment of an autocrine activation loop of HGF-Met in leiomyosarcoma cells resulted in marked activation of uPA and the uPA receptor system and concomitant acquisition in invasiveness and metastatic potential of the cells (140). In addition to uPA induction, HGF stimulates production of some MMPs, membrane-type MMP-1, collagenase-1, and stromelysin in distinct types of cells. HGF stimulates collagenase-1, MMP-3 (stromelysin-1) and MMP-9 in keratinocytes (141, 142), and MMP-2 and MMP-9 in gallbladder carcinoma cells (51, 61). In human colon cancer cells, HGF stimulates MMP-1, MMP-2, MMP-9 production (55). Rosenthal et al. (139) found that HGF stimulates expression of uPA, uPA receptor, MMP-9, membrane type MMT-1, collagenase-1, and stromelysin-1 in a human squamous carcinoma cell line, and HGF triggers invasion of the cells through type I collagen. They also found that inhibitors for MMPs but not for the uPA-plasmin system inhibited HGF-induced invasion of cancer cells.

HGF in tumour angiogenesis

Angiogenesis, the formation of new blood vessels from preexisting blood vessels, is a critical process involved in embryonic development, tissue regeneration, and pathological conditions such as tumourigenesis. Many investigators noted the essential role of angiogenesis during tumour progression (143). Angiogenesis is critical for tumour growth (144), and increased angiogenesis coincides with increased tumour cell entry into the circulation and thus facilitates metastasis (145). Vascular endothelial cell growth factor (VEGF) and bFGF have been particularly strengthened to be involved in tumour angiogenesis, whilst HGF has potent angiogenic activity in vivo as well as in vitro (33, 34) Potent angiogenic activity of HGF over VEGF was demonstrated in vivo (35).

Although a role of HGF in tumour angiogenesis has yet to be extensively investigated, there are reports of involvement of HGF in tumour angiogenesis. When the HGF gene was stably expressed in human breast cancer cells and glioma cells, these tumour cells exhibited more extensive tumour angiogenesis and enhanced growth in nude mice (70, 146). Expression of HGF and the Met receptor are overexpressed and these expressions are associated with an increased microvessel density in malignant pleural mesothelioma (147). Moreover, HGF enhances expression of VEGF mRNA in cultures of human glioma cells and in vivo when administered into tissues (35, 148). These results indicate that HGF is one of the growth factors affecting tumour angiogenesis.

Together with its potent action to enhance tumour cell motility/invasion, HGF is likely to confer metastatic potential in many types of tumour cells, via enhancement of tumour angiogenesis, as well as tumour invasion. Figure 5 schematically describes participation of HGF in early steps leading to cancer metastasis. HGF stimulates 1) dissociation of cancer cells at the primary site, 2) invasion through basement membrane and

host stroma (via enhancing cell-matrix interaction, proteinase network for breakdown of extracellular matrix, and motogenic responses), 3) angiogenesis in tumour tissues, and 4) interaction with blood vessels.

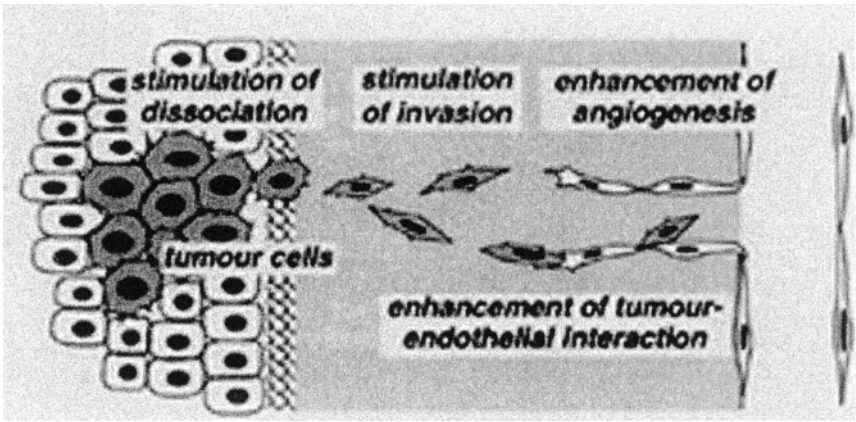

Figure 5. Involvement of HGF-Met system in tumour invasion and metastasis.. For details, see text. HGF enhances dissociation of tumour cells in primary site, invasion through both basement membrane and subsequent stroma, angiogenesis, and interaction between tumour cells and endothelial cells. Enhancement /activation of proteinase systems for breakdown of extracellular matrix, cell-matrix interaction, and cell migration are involved in tumour invasion via activation of the Met receptor.

TUMOURIGENESIS AND MALIGNANT PROGRESSION THROUGH MET/HGF RECEPTOR ACTIVATION

As described above, HGF is involved in acquisition of malignant characteristics of cancer cells. Extensive investigation has revealed that aberrant activation of the Met/HGF receptor is tightly associated with tumourigenesis and malignant progression in a variety of tumours in patients. Moreover, mutational activation of the Met receptor is the genetical disorders in a papillary renal carcinoma.

Mutational activation of the Met/HGF receptor in cancer

Hereditary papillary renal carcinoma is a inherited renal cancer characterized by a predisposition to develop multiple, bilateral papillary renal tumours. The pattern of inheritance of hereditary renal papillary carcinoma is consistent with autosomal dominant transmission with reduced penetrance. Schmidt et al. (149, 150) found that missense mutations in the c-met proto-oncogene are the causative genetical disorder in inherited and some sporadic papillary renal carcinomas. Based on genetical analysis, the causative gene was mapped at chromosome 7q31.1-34 which coincides with the chromosomal location of the c-met proto-oncogene, and

extensive sequence analysis in c-met gene revealed the existence of missense mutations in patients with hereditary and some sporadic papillary renal carcinomas. All mutations that occur in 8 distinct positions in the c-met gene are missense and are located in the tyrosine kinase domain of the Met receptor (Figure 6). Jeffers et al. (151) showed that expression of met cDNA with corresponding mutations in NIH 3T3 cells resulted in enhanced tyrosine kinase activity and that these cells were tumourigenic in nude mice. This means that Met receptor mutants originally identified in patients with hereditary and some sporadic papillary renal carcinoma are constitutively active and thus are likely to play a determinant role in papillary renal carcinoma. The possibility that activating Met receptor mutations contribute to development of other neoplastic diseases remains to be addressed.

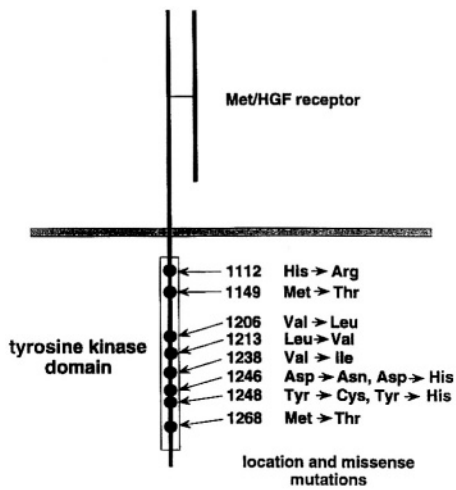

Figure 6. Localization of missense mutations in Met/HGF receptor found in patients with hereditary and sporadic papillary renal carcinoma (149, 150). These Met/HGF receptor mutations encode the constitutively activated Met receptor and thus are likely to play a determinant role for development of papillary renal carcinoma.

Carcinogenesis and invasion/metastasis via activation of Met receptor

Ligand-dependent constitutive activation of receptor tyrosine kinases through establishment of an autocrine loop in growth factors and their receptors has been found to associate with tumourigenic transformation of cells in many types of cells. Earlier studies showed that stable expression of HGF or the Met receptor gene in distinct types of non-transformed cells confers tumourigenic potential in these cells. Since the Met receptor is predominantly expressed in epithelial but not in mesenchymal cells, while HGF is expressed in mesenchymal but not in epithelial cells, two distinct types of gene transfer experiments were considerable to

establish the autocrine loop of the HGF-Met receptor: Expression of HGF in Met-positive (but HGF-negative) epithelial cells, or expression of Met receptor in HGF-positive (but Met-negative) mesenchymal cells. When the HGF gene was stably expressed in a murine hepatic epithelial cell line, the cells showed a scattered phenotype, were capable of growing in soft agar and were tumourigenic in nude mice (152). Similarly, transfection of HGF in non-parenchymal liver epithelial cells resulted in establishment of the HGF-Met autocrine loop, and importantly, these cells exhibited invasive behavior and metastasized to the lung in nude mice (153). On the other hand, stable expression of the Met receptor gene in NIH 3T3 fibroblasts conferred tumourigenic and invasive characteristics in nude mice (154-156). Similarly, expression of the Met receptor gene in mouse 127 cells and human leiomyosarcoma cells resulted in establishment of the HGF-Met autocrine loop and concomitant progression from non-tumourigenic to tumourigenic, invasive and metastatic cancers in nude mice (157). These results indicate that autocrine activation of the Met receptor confers tumourigenic, invasive and metastatic behavior in cancer cells.

Consistent with acquisition of malignant characteristics in cancer cells through experimental establishment of the HGF-Met autocrine loop, transgenic over-expression of the HGF gene in various tissues from early embryonic development in mice was found to be associated with aberrant development and tumourigenesis in several tissues and cells, including melanocytes, hepatocytes, and mammary gland epithelial cells (158-160). Melanomas in the HGF-transgenic mice were metastatic. It should be emphasized, however, that in the other transgenic mice in which the transgene of HGF was expressed in hepatocytes under control of the albumin enhancer/promoter, hepatocarcinogenesis caused by transgenic over-expression of myc gene was suppressed (161). Moreover, hepatocarcinogenesis promoted by phenobarbital was also strongly inhibited by transgenic expression of HGF in the liver (162). Although an explanation for the discrepancy in tumourigenicity has yet to be determined, in the former transgenic mice, HGF was expressed in many kind of tissues and the expression level was extremely high from embryonic to adult stages. Perhaps fractions of precursor cell (stem-like cell) populations might be aberrantly expanded in several tissues, resulting in a greater susceptibility to malignant transformation. On the other hand, inhibition of hepatocarcinogenesis in the latter transgenic mice may be related to growth inhibitory effects of HGF on hepatocellular carcinoma cells (42, 43) and HGF may possibly function as a tumour suppressor during early stages of liver carcinogenesis.

Tumourigenesis and metastatic progression in cells with the activated Met receptor via experimentally introduced HGF-Met autocrine loop suggest the existence of the HGF-Met autocrine loop in naturally developed cancers and tumour cell lines. There are several reports of autocrine activation of the Met receptor in several types of tumour cell lines, including human myeloma (163), human osteosarcoma (164), human glioma (69, 165, 166), human small cell lung carcinoma (87), and mouse mammary carcinoma (167). In these cancer cell lines, HGF affects tumour growth and confers more motile and invasive characteristics, in an autocrine manner.

Table 2 summarizes the co-expression of HGF and Met receptor in tumours, as detected by histological analysis (immunohistochemical analysis in many cases, and in situ hybridization analysis in

Table 2. **Co-expression of HGF and Met receptor in cancers.**

Type of tumour	Expression pattern	Refs
Breast carcinoma	positive for both HGF and met expression correlates stronger HGF expression; HGF-positive in stroma	168, 169
Lung adenocarcinoma	Met-positive in all cases; positive for HGF in many cases positive for both HGF and Met	85 170
Non-small cell lung carcinoma	over-expression of Met in many cases; over-expression of HGF	171
Hepatocellular carcinoma	positive for both HGF and Met in many cases positive for both HGF and Met	172 173
Pancreatic cancer	Met expression in many cases and some being positive for HGF	174
Thyroid cancer	positive for both HGF and Met in papillary cases; positive for Met but not for HGF in nonpapillary cases; HGF expression in stroma	175
Gliomas		
Glioblastoma	positive for both HGF and Met in high incidence	176
Glioma	expression of HGF and Met increases with grade of malignancy	73, 166
Schwannoma	positive for both HGF and Met in all cases positive for both HGF and Met in many cases; Met	176 177
Meningioma	expression correlates with malignancy positive for both HGF and Met in high incidence	176
Multiple myeloma	positive for both HGF and Met; HGF production in primary cultured myeloma; increased HGF level in patients	163 178 179
Hodgkin lymphoma	positive for both HGF and Met	180
Sarcomas/fibromas		
Osteosarcoma	positive for both HGF and Met in some cases, while Met- or HGF-positive in others positive for HGF; production of HGF	181 182
Chondrosarcoma	positive for both HGF and Met in some cases, while Met- or HGF-positive in others	181
Pleural mesothelioma	positive for both HGF and Met	85
Leiomyosarcoma	positive for both HGF and Met in some cases, while Met- or HGF-positive in others	181
Malignant fibrous histiocytoma	positive for HGF; production of HGF	182
Neurofibroma	positive for HGF; production of HGF	182
Synovial sarcoma	positive for both HGF and Met in biphasic synovial sarcoma high expression of Met strong Met expression in many of biphasic synovial sarcoma, but lower in monophasic	183 182 184 184
Epithelioid sarcoma	positive for both HGF and Met	79
Kaposi's sarcoma	positive for both HGF and Met; HGF production by lesion-derived spindle cells	

some cases). Co-expression of HGF and the Met receptor has been detected in many types of tumours, and the incidence of co-expression and their expression pattern varies depending on tumour types. Although most malignant neoplasms (over 90%) are carcinomas (tumours originally derived from epithelial cells), it is noteworthy that co-expression and co-localization of HGF and Met receptor have been noted particularly in sarcomas and hematopoietic tumours, originally derived from mesenchymal cells. Since Met receptor expression is negative or low in mesenchymal or stromal cells in normal tissues (in contrast to expression of HGF in these cells), aberrant induction or activation of Met receptor expression is likely to occur during development of soft tissue and hematopoietic tumours. Likewise, co-expression of HGF and Met was noted in gliomas, in a high incidence, suggesting participation of the HGF-Met autocrine loop in tumourigenesis and malignant progression in these tumours. On the other hand, co-expression of HGF and the Met receptor were noted in several types of carcinoma cells.

Since normal epithelial cells express the Met receptor but not HGF, aberrant activation of the HGF gene in these malignant carcinomas may be involved in carcinogenesis and their malignant progression. Nevertheless, it is noteworthy that the number of carcinomas co-expressing HGF and the Met receptor is relatively low, even though HGF exhibits biological activities for a wide variety of cancer cells and expression and/or over-expression of Met receptor were noted in a wide variety of tumour cells (Table 3). Since HGF is expressed in stromal cells in many types of tumour tissues, paracrine activation of the Met receptor seems to confer malignant characteristics in many cancers, as well as the autocrine activation of Met (see below).

Aberrant expression of Met receptor in cancers

As summarized in Table 3, over-expression of the Met receptor was noted in a wide variety of tumour cells and tissues, including carcinoma, lymphomas, and soft tissue tumours. Moreover, it should be emphasized that the level of Met receptor expression tends to correlate with the progression of tumours in many types of tumours; a higher expression of the Met receptor is seen in the late stage or in metastatic tumours. Over-expressed Met receptor in advanced tumour tissues may be activated by HGF, which results in acquisition of malignant behavior in many types of tumour cells. Alternatively, the Met receptor may be activated in a ligand-independent manner in some tumours wherein Met expression is extremely high, through increased susceptibility to ligand-independent receptor dimerization. In addition, the result indicate that analysis of Met receptor expression in primary tumour tissues probably relates to the prognosis of tumour malignancy (also see below).

At least two distinct mechanisms by which the Met receptor is over-expressed in cancers could be considerable, i.e., transcriptional activation or amplification of the c-met gene. In case of colorectal cancers, over-expression of the Met receptor was related to transcriptional activation in 90% of the primary tumours, whereas 8 among 9 cases of metastases of colorectal cancers accompanied amplification of c-met gene (189). Amplification of the c-met gene may possibly occur more frequently in late stage metastatic tumours than in primary tumours, presumably due to chromosomal instability in advanced cancers.

Table 3. **Expression of Met receptor in cancers**

Type of tumour	Expression of Met receptor	References
Bladder carcinoma	over-expressed	185
Bone tumours		
Osteosarcoma	over-expressed	164, 186, 187
Rhabdomyosarcoma	expressed	186
Chondrosarcoma	expressed	187
Histiocytoma	expressed	187
Breast carcinoma	over-expressed; higher levels in poorly differentiated tumours	47, 169
Colorectal cancer	over-expressed; higher in metastatic tumours gene amplification in 10% carcinomas and 8/9 in metastases	188, 189 189
Endometrial carcinoma	higher expression in late stage tumours	190
Esophageal SCC	highly expressed; higher expression in metastatic tumour	191
Esophangeal carcinoma	expressed	58
Gastric cancer	over-expressed and related to the increase in disease state; over expressed and higher in diffuse and high grade invasive cancers	62, 192
Glioblastoma	expressed	68, 72, 165
Glioma	over-expressed	170
Giant cell tumour	over-expressed	186
Hepatocellular carcinoma	over-expressed; higher expression correlated with growth correlated with poor prognosis	172, 193-199 200
Hepatoblastoma	over-expressed	201
Kaposi's sarcoma	highly expressed; induction of HGF and Met by IL-1	78, 79 202
Laryngeal carcinoma	over-expressedcorrelated with lymphonode metastasis	203
Leukemia	over-expressed	80, 81
Lung carcinoma		
Small cell carcinoma	expressed	87, 204
Non-small cell carcinoma	over-expressed and higher in late stages	171, 205
Adenoma & squamous cell carcinoma	over-expressed higher expression in metastatic cases	84, 85, 170, 206
Lymphoma	over-expressed	82
Burkitt's lymphoma	over-expressed	80, 82
Hodgkin's disease	over-expressed	80
Plasma cell	expressed	180
Melanoma	over-expressed; higher in metastatic cases	88, 207, 208
Mesothelioma	highly expressed	85

Table 3. continuing

Type of tumour	Expression of Met receptor	References
Myeloma	over-expressed	163, 178
	expressed	180
Ovarian cancer	over-expressed	97, 98
Pancreatic cancer	over-expression	100, 102
	over-expressed but lower in invasive tumour	174
	expressed	99
Peripheral nerve sheath tumour	expressed	177
Prostate cancer	over-expressed; higher in metastatic tumour	104, 105, 109, 209
Renal cell carcinoma	over-expressed	108
	higher in metastatic cases	109, 210
Thyroid cancer	low or negative expression	211
	expressed and higher in advanced cancers	212, 213
	highly expressed in papillary carcinoma	214

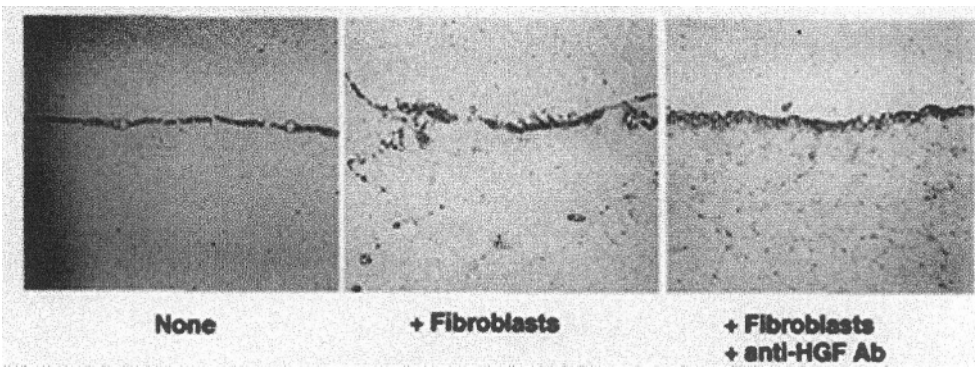

Figure 7. Invasion of human gallbladder cancer cells in co-culture with stromal fibroblasts and inhibition by anti-HGF antibody. Human gallbladder cancer cells were cultured alone on collagen gel or co-cultured with stromal fibroblasts embedded in the gel. Tumour cells did not invade the gel in the absence of fibroblasts yet did invade the gel in co-culture with fibroblasts. The invasion of tumour cells in the co-culture was almost completely inhibited by anti-HGF antibody.

HGF in tumour-stromal interactions

Host stromal influence on epithelial neoplasia and malignant behavior of carcinoma cells have been noted in various types of cancers, including cancers in prostate, stomach, skin, oral cavity, mammary gland, lung, and colon (215-217). In vivo growth of certain carcinoma cells was markedly accelerated by a broad spectrum of fibroblasts, and both in vitro and in vivo invasiveness of carcinoma cells was induced by the co-existence of stromal fibroblasts (218-221). Thus host stroma-derived factor(s) is one key molecule that regulates tumour cell invasion and metastasis. In addition to stromal involvement in tumour malignancy, stromal alterations occur during malignant progression of tumours. The presence of activated fibroblasts are seen in close proximity to tumour tissues (216). Therefore, local and mutual interactions between tumour cells and stromal cells are of particular importance in regulating extracellular matrix degradation, migration, and invasion of tumour cells. Although molecular mechanisms involved in tumour-stroma interactions are not fully understood, evidence points to HGF as being one critical molecule in tumour-stroma interactions, as it confers invasive growth potential in tumour cells

In most malignant tumours, carcinoma cells exhibit invasive potential in vivo, however, a variety of carcinoma cells in vitro do not invade into extracellular matrices such as collagen (51, 60, 96, 220, 222, 223). Fig. 7 shows in vitro invasion of human gallbladder carcinoma cells on collagen gel matrix. Although gallbladder cancer cells are highly invasive and metastatic in patients, the cells did not invade the gel, when cultured alone on the gel. In contrast, when they were cultured on the gel wherein stromal fibroblasts were embedded, they did invade the collagen gels. Importantly, an antibody against HGF almost completely abrogated invasion of the cancer cells. The result indicates that invasion of tumour cells depends on a fibroblast-derived factor and that this factor is HGF. Likewise, Matsumoto et al. (220) showed that oral squamous cell carcinoma cells acquire invasive potential on collagen gels, when normal stromal fibroblasts were incorporated in collagen gels, and the fibroblast-derived invasion factor for oral squamous cell carcinoma cells proved to be HGF (95).

Although a wide variety of carcinoma cells express the Met receptor and HGF exhibits biological activities in these cells, most carcinoma cell lines do not secrete HGF. In over 70 distinct carcinoma cells, only a few types of tumour cell lines secrete HGF (our unpublished data). However, Seslar et al (224) first noted that human breast carcinoma cells secreted a stimulatory factor(s) that enhances HGF production in fibroblasts and a similar observation was noted by Rosen et al. (225). Subsequently HGF-inducers derived from many types of tumour cells were identified (49, 50, 60, 96, 223): a variety of tumour cells secrete a variety of HGF-inducers and these include interleukin-1α (IL-1α), IL-1β, bFGF, PDGF, TGF-α, and prostaglandin E2. These molecules transcriptionally activate expression of the HGF gene. Among these inducing factors for stromal fibroblast production of HGF, IL-1α and IL-1β are secreted by various kinds of tumour cells as HGF-inducing factors (49, 50, 60, 96, 223). Taken together with the notion that tumour cells affect expression of HGF in fibroblasts, these observations suggest a mutual interaction between tumour cells and stromal fibroblasts, as mediated by HGF and HGF-inducers: tumour cells secrete molecules which induce HGF in

stromal fibroblasts, while fibroblasts secrete HGF which stimulates tumour migration, invasion, and angiogenesis.

The conceptual framework regarding mutual interactions between tumour cells and stromal fibroblasts was postulated and experimentally demonstrated, using a co-culture method of tumour cells and fibroblasts (49, 50, 60, 223).

Fig. 8A demonstrates this mutual interaction, as mediated by HGF-HGF inducer loop that confers invasive potential in tumour cells (60). Although HGF production in culture of fibroblasts alone was relatively low, HGF production by fibroblasts was strongly enhanced by tumour-derived IL-1β. In response to increases in HGF levels in this culture, invasion of tumour cells was strongly enhanced in the co-culture with fibroblasts. Importantly, invasion of tumour cells in the co-culture was strongly inhibited by antibodies against HGF and IL-1β, respectively. Similar examples of tumour-stromal interaction were noted in other tumour cells (49, 50, 223).

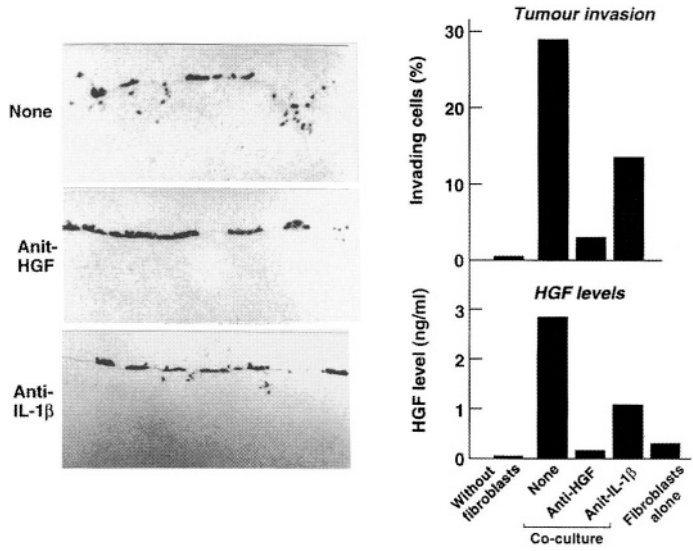

Figure 8. Mutual interaction between tumour cells and stromal fibroblasts, as mediated by the HGF-HGF-inducer loop. (**A**) Demonstration of HGF-HGF-inducer loop leading to tumour invasion. Human gallbladder cancer cells were cultured alone on collagen gel or co-cultured with fibroblasts (fibroblasts are not seen, since they were cultured in the bottom of the gel) (60). Gallbladder cancer cells secreted IL-1β as HGF-inducer and thus HGF production by fibroblasts in co-culture was much higher than that in culture of fibroblasts alone. Consistent with increase in HGF level, invasion of tumour cells was strongly enhanced in the co-culture. Importantly, tumour invasion and HGF level in co-culture were suppressed by anti-IL-1β antibody, as well as anti-HGF antibody, indicating an existence of mutual interaction between these cells as mediated by HGF-HGF-inducer (IL-1β in this case) loop. (**B**) Tumour-stromal interaction as mediated by HGF-HGF-inducer loop between tumour cells and host stromal cells. Tumour cells secrete various types of HGF-inducers such as IL-1α, IL-1β, PDGF, bFGF, TGF-α, and prostaglandin E2 (PGE2), depending on tumour types (49, 50, 60, 223).

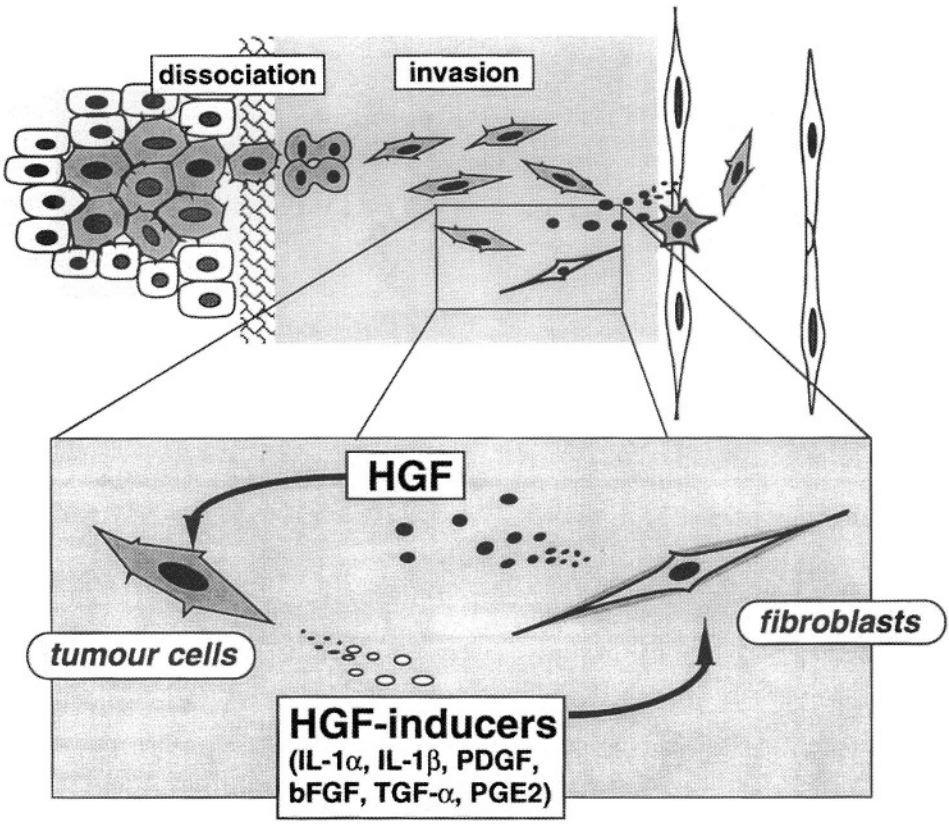

Figure 8 continuing. 8B

Fig. 8B schematically describes a molecular mechanism for tumour-stromal interaction as mediated by the HGF-HGF-inducer loop. Many types of tumour cells secrete inducing factors for stromal production of HGF, while HGF up-regulated in response to tumour-derived factors confers malignant behavior on tumour cells. In this context, it is noteworthy that HGF levels in tumour tissues correlate with the poor prognosis in several types of carcinomas in patients (Table 4). Jin et al. (226) showed that IL-1β levels in human breast carcinoma tissues is often associated with tumour invasiveness and malignant pathologic features. Interestingly, Maier et al. (202) showed that 1L-1 induced expression of HGF and Met receptor that resulted in establishment of an autocrine loop of HGF-Met in murine Kaposi's-like spindle cells. Consistently, HGF and Met were co-expressed in Kaposi's sarcoma tissues (78, 79). It may well be that several types of tumour cells utilize overlapping mechanisms of epithelial-mesenchymal interactions as mediated by HGF, but in the case of carcinomas, tumour progression and extracellular matrix remodeling occur.

CANCER PREVENTION BY HGF-ANTAGONIST

Basic knowledge on mechanisms of cancer invasion and metastasis will lead to new therapeutics for treatment of cancer patients. Close involvement of Met activation in acquisition of malignant characteristics in a variety of tumours implicates therapeutic strategies for prevention of tumour invasion, angiogenesis and metastasis, through the blockage of HGF-Met signaling. Recent studies demonstrated that blockage of HGF-Met signaling inhibits tumour invasion and metastasis.

Structural characterization of HGF-antagonist

Studies on structure-function relationship in the HGF molecule revealed functional domains for receptor binding and subsequent biological activities. Small molecules consisting of the N-terminal hairpin plus the first and second kringle domains (designated NK2) and the N-terminal and the first kringle domains (designated NK1) exist as naturally biosynthesized variant forms of HGF (227, 228). Both NK1 and NK2 can bind to the Met receptor (227-232). Together with findings that deletion of the N-terminal hairpin or the first kringle domain in HGF molecule results in impaired receptor binding and biological activities (229-231, 233), NK1 seems to serve a minimum set of domains responsible for binding to Met receptor (Fig. 9A). Interestingly, NK1 and NK2 exhibit antagonistic activity on mitogenic action of HGF in some assay systems (227, 232), while they behave as an agonist in terms of motogenic activity (87, 228, 229). Schwall et al. (234) reported that NK1 and NK2 exhibit mitogenic activity in the presence of heparin/heparansulfate through the enhanced dimer formation of ligands. Silvagno et al. (235) found that NK3 consisting of the N-terminal hairpin and subsequent three kringle domains has motogenic activity on endothelial cells. Transgenic over-expression of NK1 in mice resulted in aberrant development and neoplasmic disorder, partially as seen in transgenic over-expression of HGF (236). Thus these intermolecular fragments (NK1, NK2, and NK3) are capable of binding to the receptor and exhibit partial antagonistic and agonistic activities. On

Table 4. **HGF and Met receptor for potential cancer prognosis.**

Type of tumour	Significance/changes of HGF/Met	References
HGF as a potential indicator		
Breast carcinoma	higher tissue HGF level correlates with poor prognosis	244-246 247, 248
	higher serum HGF level correlates with poor prognosis	
Gastric adenocarcinoma	tissue HGF level is prognostic indicator	192
	higher serum HGF level correlates with shorter survival	249
Glioma	expression of HGF correlates with grade of malignancy	73, 166
Non-small cell lung cancer	higher tissue HGF level correlates with poor prognosis	250
Small cell lung cancer	higher serum HGF level correlates with poor prognosis	251
Met as a potential indicator		
Astrocytic tumour	Met expression correlates with malignancy	252
Breast carcinoma	higher Met expression in poorly differentiated tumours	47, 169, 245
Colorectal cancer	higher Met expression in metastatic cases	188, 189
Endometrial carcinoma	higher Met expression in late stages	190
Esophageal carcinoma	higher Met expression in metastatic cases	191
Gastric cancer	higher Met expression in diffuse and high grade invasive cancers	62, 192
Hepatocellular carcinoma	higher Met expression correlated with poor prognosis	200
Laryngeal carcinoma	higher Met expression correlated with lymphonode metastasis	203
Lung carcinoma		
Non-small cell lung carcinoma	higher Met expression in late stages	171, 205
	higher Met expression in metastatic cases	85, 170, 206
Adenoma & squamous cell carcinoma		
Melanoma	higher Met expression in metastatic cases	88, 207
Prostate cancer	higher Met expression in metastatic cases	104, 105
Renal cell carcinoma	higher Met expression in metastatic cases	210
Thyroid cancer	higher Met expression advanced cancers	212

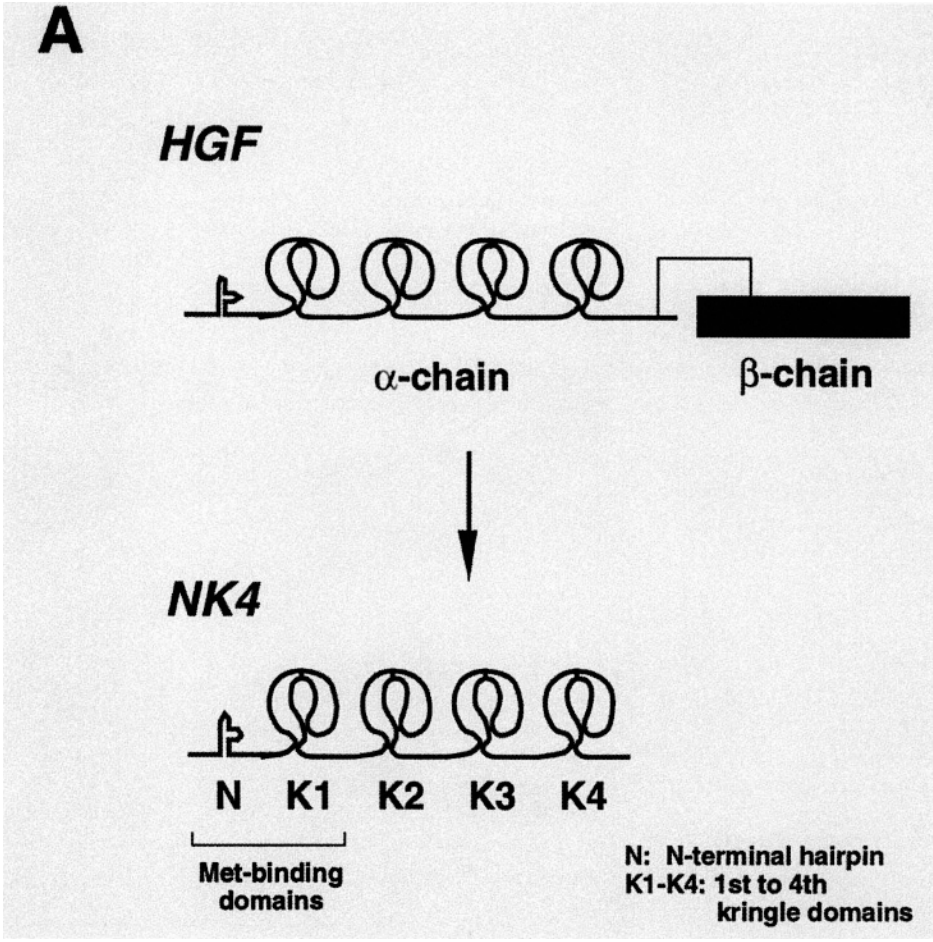

Figure 9. (**A**) Schematic structures of HGF-antagonist, NK4. (**B**) Inhibition of HGF-induced tumour invasion in collagen gel matrix by NK4. GB-d1, human gallbladder carcinoma; HuCC-T1, human cholangiocellular carcinoma; PC-3, human lung adenocarcinoma; ME-180, human cervical epidermoid carcinoma. (**C**) Inhibition of tumour invasion in co-culture with stromal fibroblasts (51). Human gallbladder cancer cells were cultured for 24 h in the absence (**a**) or presence of 110 pM HGF (**b**) without fibroblasts, or they were co-cultured with fibroblasts in the absence (**c**) or presence of 1.1 nM NK4 (**d**), 11 nM NK4 (**e**), or 110 nM NK4 (**f**).

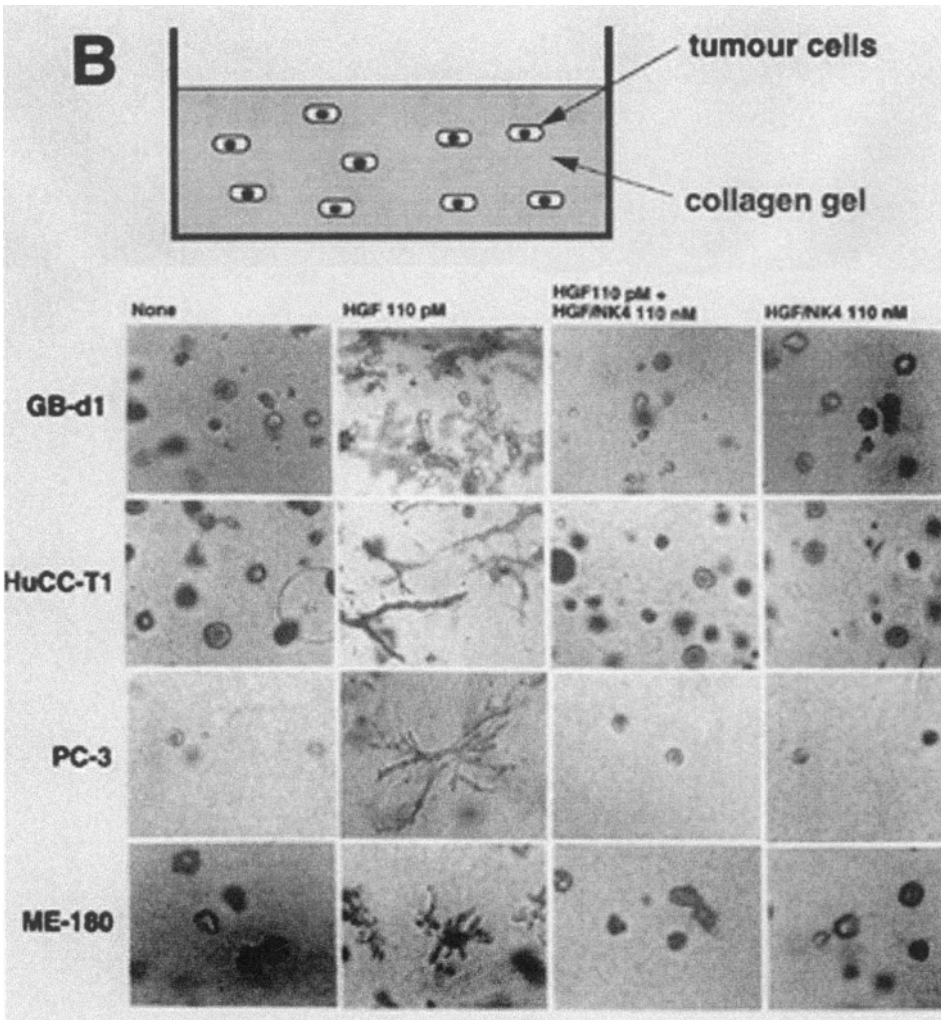

Figure 9 continuing. 9B

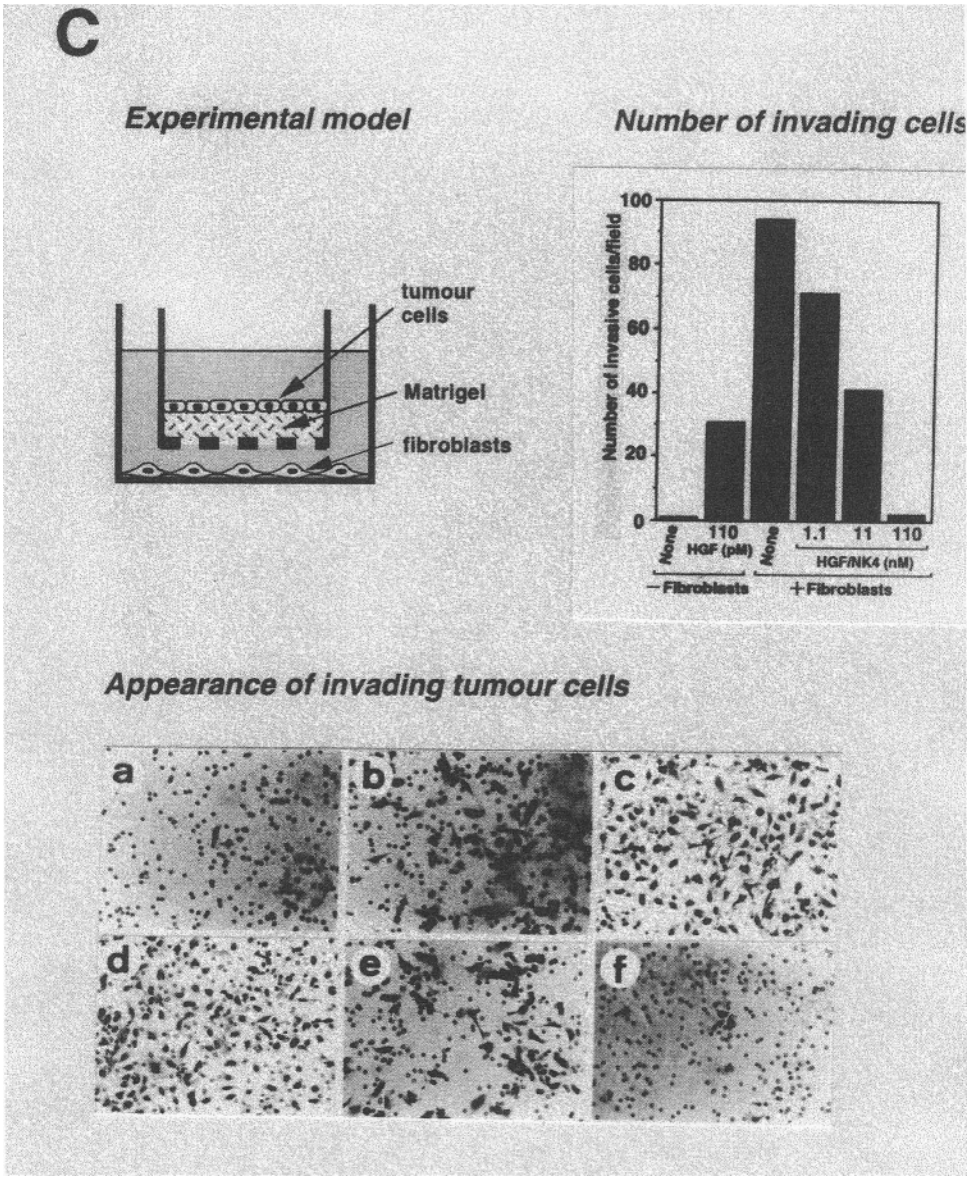

Figure 9 continuing. 9C

the other hand, Date et al. (237) isolated the intramolecular fragment (NK4) consisting of the N-terminal hairpin and four kringle domains from elastase-digested HGF. NK4 is capable of binding to the receptor, however, NK4 has no agonistic biological activities yet competitively inhibits biological activities of HGF (237, 238). Although an explanation for the lack of agonistic activity in NK4 but not in NK1, NK2, and NK3 has yet to be made, the existence of fourth kringle domain in NK4 molecule may inhibit receptor dimerization or subsequent signaling from the Met receptor. NK4 is thus the first known competitive antagonist which abrogates various biological activities of HGF.

Inhibition of invasion-metastasis by HGF-antagonist

Based on the antagonistic activity of NK4, Date et al. (51) subsequently focused on inhibitory effects on tumour invasion in vitro and in vivo. In three dimensional culture in collagen gel matrix, HGF stimulated invasion of tumour cells, whereas NK4 abrogated invasion of tumour cells (Fig. 9B). NK4 alone had no apparent effect on induction of proteinases and tumour invasiveness. Fig. 9C shows inhibitory effects of NK4 on tumour invasion through the basement membrane, in a co-culture system which mimics tumour-stroma interaction. When human gallbladder carcinoma cells were cultured alone without fibroblasts on Transwell membrane coated with Matrigel basement membrane components, the cells invaded through the membrane in the presence but not in the absence of HGF. Interestingly, invasion of the cells was strongly induced in case of co-culture with stromal fibroblasts. This invasion in the co-culture system was almost completely inhibited by NK4. Jiang et al (154) showed that NK4 inhibits in vitro angiogenesis induced by HGF in human vascular endothelial cells.

Consistent with its ability to inhibit tumour invasion driven by HGF, the local administration of NK4 into mice inhibited tumour growth, invasion and metastasis of tumours (51) (Fig. 10 and our unpublished data). The growth of gallbladder cancer cells in nude mice was strongly inhibited by the infusion of NK4 (Fig. 10B). Fig. 10C shows tumour tissues of gallbladder cancer cells implanted in nude mice, with or without NK4-administration. In control tissue, tumour cells invaded intramuscularly in host tissues, whereas NK4 inhibited such invasion. In addition, NK4 administration inhibited growth of tumours in nude mice. In tumour tissue exposed to NK4, apoptotic cell death of tumour cells occurred in central regions (Fig. 10C) and the number of apoptotic cells in tumour tissues infused with NK4 was higher than in control tumour tissues (Fig. 10D). This may be due to inhibition of angiogenesis in tumour tissues. Furthermore, in the experimental model for metastasis, using Lewis lung carcinoma cells, NK4-administration inhibited lung metastasis of Lewis lung carcinoma and Jyg breast carcinoma subcutaneously implanted into nude mice (our unpublished data). Thus NK4, an antagonist of HGF successfully inhibited tumour growth, invasion, and metastasis of some tumours.

Inhibition of HGF-induced invasion

In addition to the specific abrogation of HGF-Met coupling, using NK4, several approaches demonstrated the inhibition of tumour invasion induced by HGF. Jiang et al. (239, 240) showed that γ-linolenic acid inhibits HGF-induced membrane ruffling and invasion in human colon carcinoma cells and that it inhibits the adhesion of

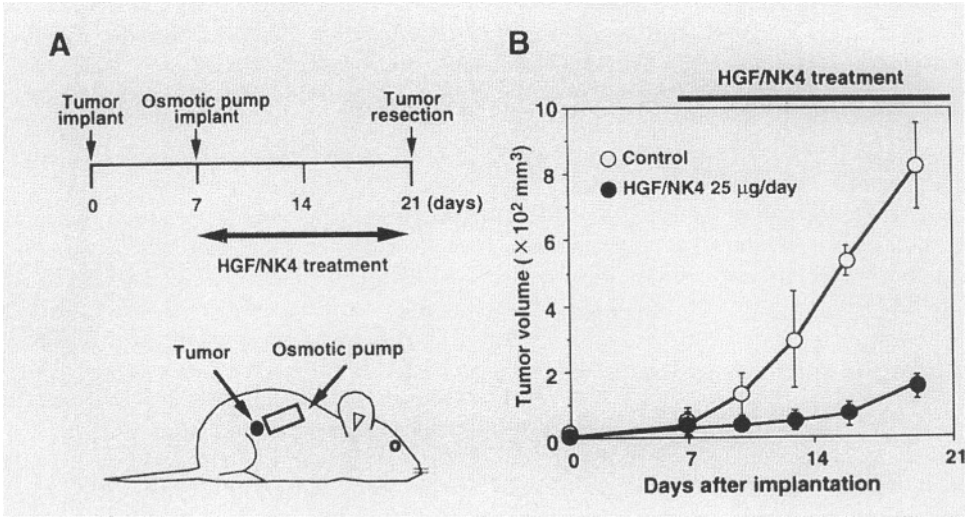

Figure 10 Inhibition of tumour growth and invasion of human gallbladder cancer by NK4 in nude mice. (**A**) Experimental schedule. NK4 was given subcutaneously, using an osmotic mini-pump. (**B**) Inhibition of tumour growth by NK4. (**C**) Typical tumour tissues in nude mice given or not given NK4. Tumour tissues (T) in a control mouse not given NK4 are shown in **a**, **c**, and **e**, while the appearance in mice given NK4 is shown in **b**, **d**, and **f**, respectively. Evidence of muscle invasion (arrow) is shown in a and **c**. Bars represents 500 μm in **a** and **b**, and 50 μm in **c**, **d**, **e**, and **f**, respectively. (**D**) Change in apoptotic cells of gallbladder cancer cells in nude mice by infusion of NK4. Distribution (left panels) and changes in the number of apoptotic tumour cells (right panel) were shown (51). Apoptotic cells were detected using the TUNEL method. The number of apoptotic tumour cells in mice given NK4-infusion was much higher than in control mice.

Figure 10 to be continued..

tumour cells to extracellular matrix via inhibition of tyrosine phosphorylation of $p125^{FAK}$ and paxillin.

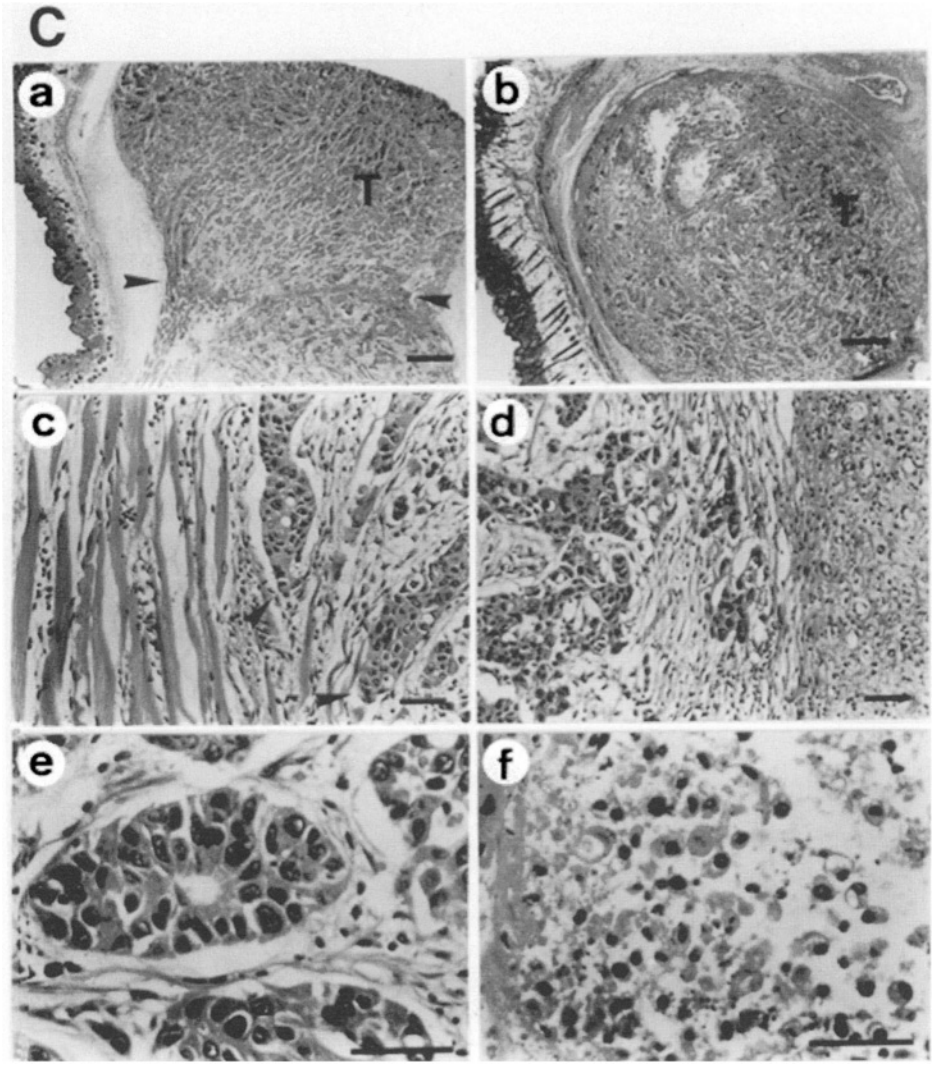

Figure *10.* Continuing. 10C.

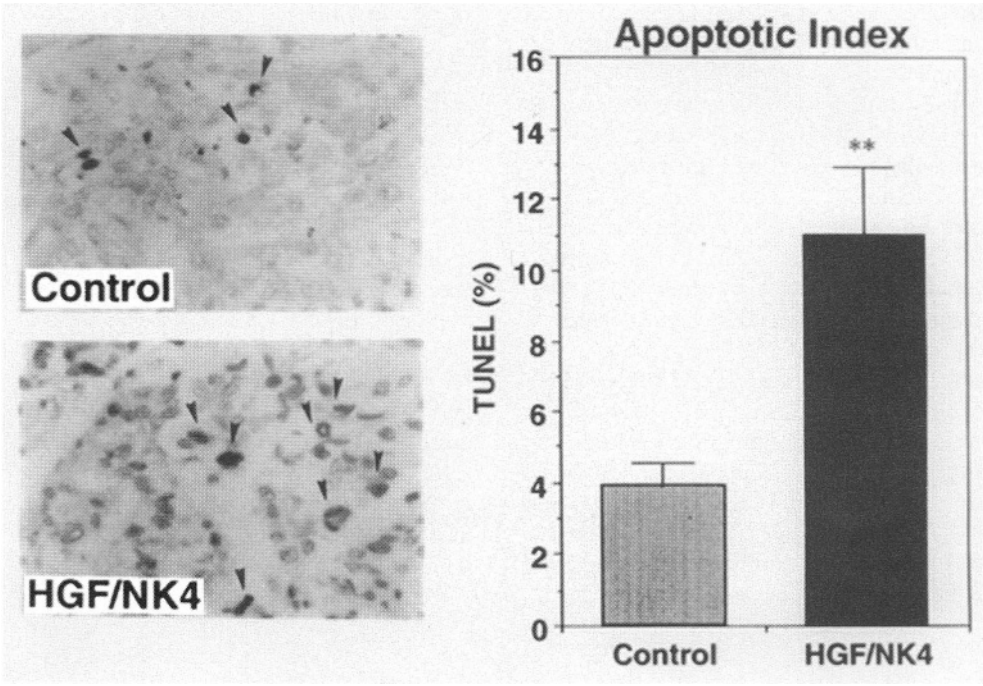

Figure *10.* Continuing. 10D.

Uchiyama et al. (55) found that HGF enhances MMP-1, MMP-2, and MMP-9 production, migration and invasion for human colon carcinoma cell lines and primary cultured colon carcinoma cells, however, IL-4 inhibited both MMP production and tumour invasion induced by HGF, in an IL-4 receptor-mediated manner. Likewise, Hiscox et al. (241) showed that IL-12 inhibits tumour cell migration and invasion induced by HGF in cultures of human colon cancer cells. IL-12 has anti-cancer action by activating natural killer (NK) cells and cytotoxic T lymphocytes. Invasion inhibitory factor-2 (IIF-2) was originally isolated from the liver with anti-metastasis activity in vivo for melanoma and lung cancer cells (242). IIF-2 inhibits HGF-induced migration and invasion of human lung and cancer cells (243). On the other hand, Kaji et al. (62) demonstrated that antisense Met oligonucleotide specifically decreased expression of the Met receptor and inhibited migration of gastric cancer cells. Although mechanisms by which these molecules exhibit inhibitory effects on migration and invasion induced by HGF are undefined except for strategies based on utilization of Met receptor antagonist and antisense oligonucleotide, these approaches are important for designing new strategy and practice of therapeutics for cancer patients.

HGF AND MET: PROGNOSTIC SIGNIFICANCE

Participation of HGF and Met in malignant behavior in a variety of tumours suggested the potential utilization of HGF and/or Met receptor expression for prognosis of patients with cancer. Several studies have shown that HGF and Met are important prognostic indicators in various tumours. Table 4 summarizes data showing that HGF and the Met receptor are potential indicators for prognosis of cancer patients. Yamashita et al (244) extensively analyzed relationships between HGF protein levels in tumour tissues and clinicopathological characteristics in patients with breast cancer. Higher HGF levels in breast tumour tissues correlated well with disease relapse and reduced overall survival, indicating that tissue HGF level is useful prognostic marker in breast cancer patients. Similar findings were noted by other workers (245, 246). Likewise, HGF levels in tissues of non-small cell lung carcinoma (250), gastric cancer (192), and endometrial carcinoma (190), were associated with poor outcome of these diseases. Thus, HGF level is a useful indicator for risk of relapse and short survival time for patients with these cancers. In addition to tissue HGF levels, serum HGF levels were significantly higher in patients with breast cancer (247, 248), gastric cancer (249), and lung cancer (251). Since HGF protein levels in tissues and sera can be readily determined by enzyme-linked immunosorbent assay (Institute of Immunology, Tokyo, Japan), HGF levels in tumour tissues and sera are useful indicator for prognosis of patients with these cancers.

On the other hand, over-expression of the Met receptor was noted in a wide variety of cancer tissues (Table 3), and importantly, expression levels of the Met receptor were higher in more advanced malignant cases in many types of carcinomas (Table 4). Comparative analysis between Met receptor expression and clinicopathological features in cancer patients indicated that higher levels of Met receptor expression are an useful indicator for relapse and a poor prognosis of patients with hepatocellular carcinoma (200), endometrial carcinoma (190), breast cancer (245), pulmonary adenocarcinoma (170), and thyroid cancer (212). Therefore, expressions of HGF

and/or Met receptor in tissues and serum HGF levels are expected to become prognostic markers in several types of cancers.

CONCLUSION AND PERSPECTIVES

HGF exhibits biological activities responsible for dynamic tissue remodeling during embryogenesis and tissue regeneration. HGF and Met are involved in organogenesis and repair, mediating epithelial-mesenchymal (or -stromal) or other heterotypic cell-cell interactions. The motogenic activity of HGF is potent and clearly a wide variety of tumour cells utilize the HGF-Met system during acquisition of invasive and metastatic potentials. Tumour-stromal interactions, as mediated by the HGF-Met system is reminiscent of epithelial-mesenchymal/stromal interactions. In normal cells, the utilization of HGF-Met signaling results in construction/reconstruction of organized tissue architecture, however, it results in invasive, angiogenic, and metastatic changes in cancer cells, presumably due to genetic disorders involved in cell-cell adhesion and cell-matrix interactions. Aberrant expression of Met in cancer tissues, up-regulation of HGF gene expression in host stroma, and mutational activation of Met receptor tyrosine kinase are associated with carcinogenesis and the malignant progression of tumours.

Invasion and subsequent establishment of metastasis are devastating events in patients with cancer. The many past approaches used to treat cancer patients (chemotherapy, radiotherapy, surgery, etc.) have often led to unsatisfactory outcomes. These approaches have disadvantages such as side effects that result in reduction in quality of life. Most past approaches did not address perhaps a most important issue in cancer treatment, i.e., prevention of invasion and metastasis. On the basis of findings that aberrant utilization of HGF-Met system is closely involved in tumour invasion and metastasis, abrogation and inhibition of HGF-Met coupling and signal transduction from the Met may prove to be therapeutic strategies to prevent cancer metastasis. The four kringle-antagonist for HGF, NK4 successfully inhibited tumour invasion, growth, and metastasis of tumours in laboratory animals. The possibility that NK4 and other inhibitors of HGF-Met signaling can serve as a therapeutic for cancer patients is now under extensive investigation.

HGF exerts dramatic effects on reconstruction of normal tissues in experimental animals with tissue injury and tissue fibrosis. Clinical application of HGF for treatment of patients with organ failure and organ fibrosis (e.g., liver fibrosis, chronic renal failure, lung fibrosis) will begin within a few years. An antagonistic molecule for HGF is expected to be effective for treatment of cancer patients. These 'two-pronged approaches' in studies of HGF are being closely scrutinized for possible application for tissue repair/remodeling and cancer prevention.

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Chapter 7

TIGHT JUNCTIONS, A CRITICAL STRUCTURE IN THE CONTROL OF CANCER INVASION AND METASTASIS

Wen G. Jiang, Tracey Martin, Iraj Llaffafian, Robert E. Mansel

Key words: Tight junction, occludin, claudin, paracellin, invasion, endothelium, brain blood barrier (BBB).

Abstract: Tight junction is the apical most structure in epithelium as well as in the endothelium. Its main function is to control the paracellular diffusion of ions and certain molecules. Although the structure has been known for decades, the molecular composition of the tight junction has only been recognised in the past decade. Molecules making up tight junctions include the transmembrane proteins occludin, claudin and paracellin, and cytoplasmic proteins, MAGUK family members. The structure has now been demonstrated as also having a role in the control of cancer cell penetration of the endothelium and in the development of cancer.

INTRODUCTION

Tight junctions (TJs) are the apical–most structure in epithelial and endothelial cells (Figure 1). The structure creates a physiological intercellular barrier and intramembrane diffusion fence, which help to maintain distinct tissue spaces and to separate the apical space from the lateral plasma (1,2).

Tight junction were first identified by electron microscopy as structures in the intercellular junction and appearing as discrete sites of apprarent membrane fusion. The past few years have witnessed a marked expansion of knowledge on the molecular structure of tight junction, with an ever increasing number of molecules identified as being involved in tight junctions. Furthermore, the role of tight junction in the development, and particularly the penetration of endothelium and mesothelium has been recognised.

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This chapter will overview the recent progress in the molecular structure of tight junctions, regulation of the function and expression of TJ molecules and the possible role of TJ in cancer.

MOLECULAR STRUCTURE OF TIGHT JUNCTION

Tight junctions appear as idenfiable ultrastructure under electron microscope. However, the molecules that constitute this structure have been known since the mid eighties. There are a number of molecules have been known to either constitute, associate or regulate the tight junctions.

ZO-1 and the MAGUK protein family.

The first protein identified in the tight junctions is ZO-1, *zonular occluden-1*. The protein was identified as a phosphoprotein of 210-225 kDa in size (3,4). The protein is a peripheral membrane protein enriched at the points of tight junctions. It was found to be phosphorylated on serine residues in quiescent conditions, becoming phosphorylated on tyrosine residues under stimulation. The protein is located in polarised epithelial and endothelial cells, in tight junctions, in the undercoat of adherens junctions, and in the nucleus under special conditions. ZO-1 can also be found in cells that lack clear tight junction structure.

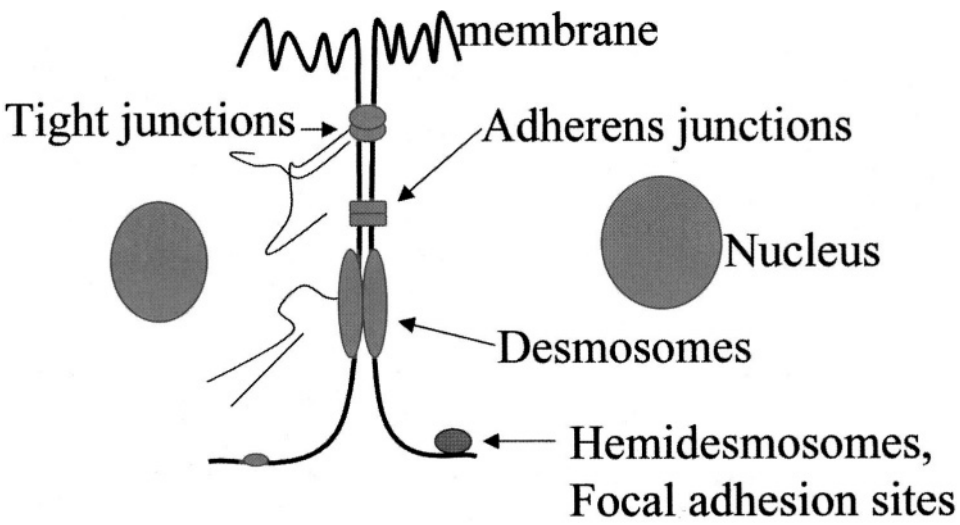

Figure 1. The main structures between endothelial (epithelial cells). Tight junction is located at the apical most location between two cells

Further analysis of the protein has revealed that ZO-1 is a member of a superfamily, members of which have a common core group of protein-protein interaction domains and localisation at the cytoplasmic surface of the plasma membrane (5). These proteins share common domains of SH3, a region homologous to guanylate kinase (GUK), and one or more PDZ domains. These proteins have therefore been named the MAGUK family, the membrane association and presence of the GUK domain. PDZ domains in these proteins may mediate reversible and regulated protein-protein interactions, through either dimerisation with other PDZ domains, or recognition of specific amino acid sequence motifs at the C termini of intergral proteins (6,7). MAGUK family members also contain a SH3 domain, which act as protein-protein interaction molecules that bind the proline amino acid motif, PXXP (8,9). The GUK domain of the MAGUK family is also involved in the protein-protein interactions (10,11)

ZO-2. ZO-2 is the second member of the MAGUK family identified in tight junctions (12). The protein was found to be a phosphoprotein of 160kDa and co-precipitate with ZO-1. There is high homology between ZO-1 and ZO-2. ZO-2 is almost uniquely located to tight junctions and contains three PDZ domains, a SH3 domain and a GUK domain.

ZO-3. ZO-3 is the third member of the MAGUK family that has been recently identified (13). This protein interacts with both ZO-1 and ZO-2, initially being identified as a protein present in the ZO-1 and ZO-2 immunoprecipitates (14, 15). The protein is 130 kDa in size and share high sequence homology with both ZO-1 and ZO-2. Similar to ZO-2, ZO-3 has three PDZ domains, a SH3 domain and a GUK domain.

Cingulin. Cingulin is a 140kDa protein found in the tight junction in epithelial and endothelial cells (16). The protein appears as two peptides interwined as a 'coiled coil'. In the tight junction complex, cingulin is more distant from the junctional membrane than ZO-1 (17).

7H6. 7H6 is a protein of 155kDa, existing in the tight junctions of hepatocytes, epithelial cells and endothelial cells (18,19).

Other molecules associated with tight junctions. A few other proteins have also been found to associate with tight junctions, including rab13, 19B1, symplekin, AF-6, rab 3B, protein kinase C and c-src. Their role in tight junctions have yet to be fully investigated (20, 21, 22, 23).

Occludin

Occludin was the first transmembrane tight junction protein identified (24,25). It has 504 amino acids and a molecular weight of 55.9 kDa (GenBank accession No D21837). Its structure is most interesting, that the molecule has 4 membrane-spanning segments, forming two extracellular loops (Figure 2 and figure 3). On SDS-PAGE, the protein migrates as a series of proteins between 62-82 kDa, as a result of its phosphorylation (on serine and threonine residues). Under different conditions, occludin was found phosphorylated, which changed the mobility of the protein under electric current.

Occludin directly associates with ZO-1 (26). The domain E (cytoplasmic domain) was found to interact with both ZO-1 and ZO-2. This interaction is critical for occludin to be localised to the tight junctions (26, 27,28,29).

Occludin has been found to a functional component of the tight junctions (30, 31). Over-expression of occludin has been shown to assist

formation of tight junctions. Modification of tight junctions with calcium and other culture conditions affects the location and function of occludin. Interestingly, peptides corresponding to the extracellular domain of occludin has been shown to damage the permeability function of tight junctions (29, 32). A synthetic peptide (SNYYGSGLSY, corresponding to residues 100 to 109 of occludin) is able to impair the resealing of tight junction, yet has no effects on the localisation of ZO-1 (33).

Occludin is widely expressed in epithelial and endothelial cells, but also at a very high level in brain endothelial cells. Occludin does not present in fibroblasts (34). It is also interesting to note that occludin does exist in cell/tissues with tight junctions, but does not in cells without tight junction. In contrast, ZO-1 exists more widely in tight junctions and other subcellular locations as well as in cells that do not have tight junctions.

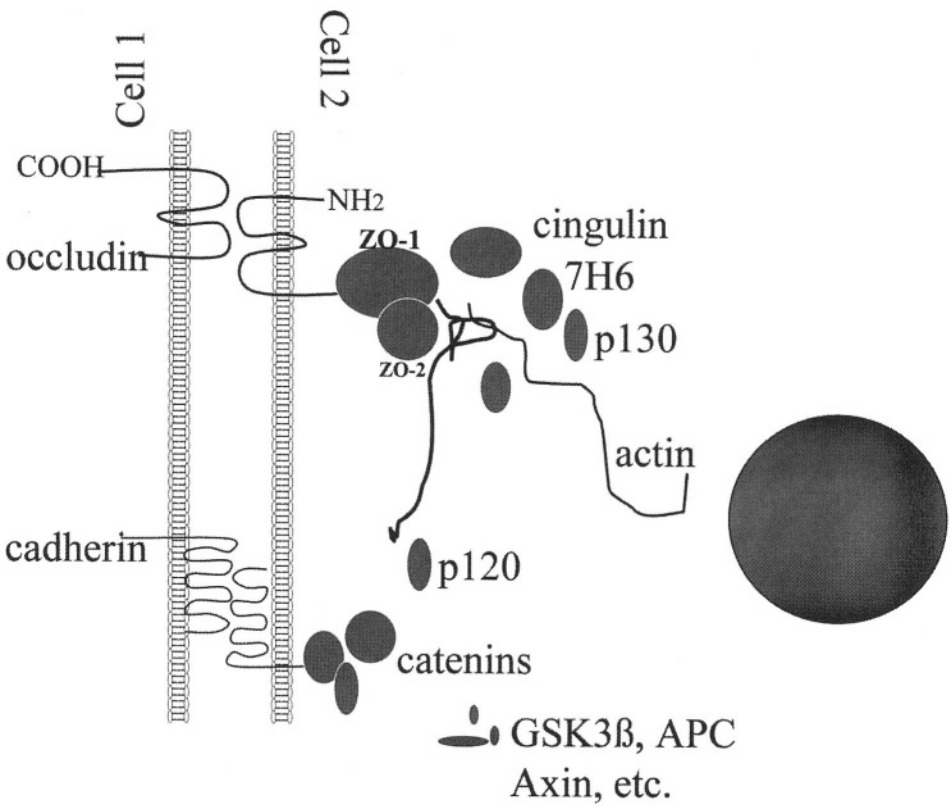

Figure 2. Interaction between integral membrane proteins (occludin) and other tight junction associated proteins

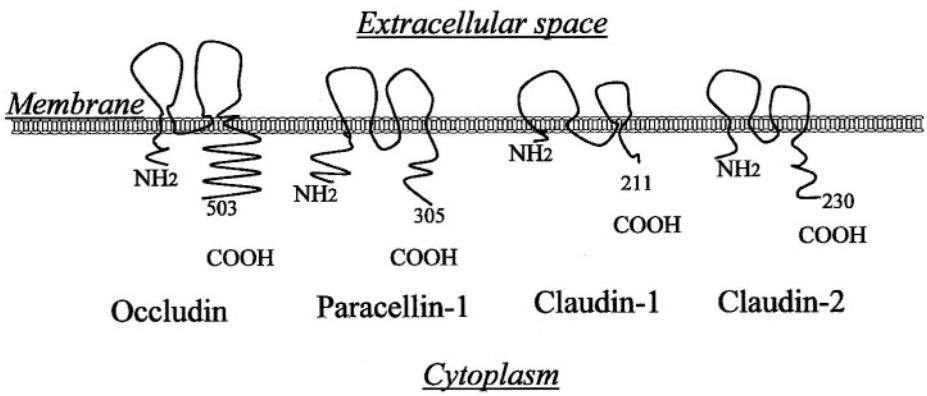

Figure 3. Molecular structure of integral membrane proteins in tight junctions.

Claudin family.

The claudin family members are among the second group of integral transmembrane proteins in tight junctions (35). The first two claudins (claudin-1 and claudin-2) were identified from isolated junctional fractions of chicken liver and found to be proteins of 211 and 230 amino acids, respectively. Claudin-1 and claudin–2 are structurally related and share 38% homology in their amino acid sequence. These proteins are approximate 23 kDa in size and although they have four membrane spanning regions, share no homology with occludin (figure 3). Claudin –1 and –2 exist at high level in tight junctions and are found in cells with or without tight junctions. Recently,

claudins has been found to be a super family that has at least 15 members (claudins –1-15) (36). Claudins are concentrated at tight junctions and may be necessary for the recruitment of occludin to tight junction areas (36,37). Furthermore, the type of claudin may be a determining factor for the tightness of the tight junctions (38).

Paracellin-1.

The most recently identified member of tight junction is paracellin. Simon and co-workers (39), have discovered this molecule when trying to identify the molecules underlying a rare autosomal recessive disease, renal hypomagnesaemia. The disorder is characterised by the constant loss of magnesium through urinary tract resulting in hypomagnesaemia that can not be corrected by oral supplementation of magnesium. Urinary stones, calcium loss and renal failure are also commonly seen. In an effort to identify the molecular mechanisms of the disease, a unique protein, termed paracellin (PCLN-1) has been identified. Human paracellin-1 is encoded by a 1024 bp gene, located in chromosome 3q (gene bank accession number AF152101). The deduced amino acid revealed that it encodes a protein of 305 amino acids. Sequence analysis shows that paracellin shares between 10-18% homology with claudin.

Paracellin-1 has a structure identical to occludin, i.e. four membrane spanning regions forming two extracellular loops (figure 3). It appears that paracellin-1 is uniquely expressed in kidney tissues, as no mRNA has been detected in other tissues, including thymus, testis, prostate, ovary, small and large bowels, and peripheral leukocytes.

The protein is an important member of the tight junction complex and plays a key role in the magnesium and, possible calcium diffusion through the tight junction. It is thus a key in the formation of paracellular channels (40).

THE ASSEMBLY OF TIGHT JUNCTIONS

The assembly of tight junctions is an extensively investigated area. However, with the increasing number of TJ molecules being identified, it becomes clear that the junctions are made of three main parts: the integral membrane proteins, peripheral proteins, and other associated proteins. Integral transmembrane proteins (occludin and claudins) are the key part in the making of the structure. These proteins interact homotypically or heterotypically with each other and control paracellular diffusion. However, the integral proteins must anchor to relevant peripheral proteins, i.e. ZO-1, which binds to the actin system. The complex also associate with other molecules, which may play a regulatory role.

TIGHT JUNCTION AND THE BLOOD-BRAIN BARRIER (BBB)

The blood-brain barrier was been recognised almost a century ago, as a structure that separates brain tissues from blood circulation and prevents hydrophilic and toxic substances from entering the brain.

While both the astrocytes and endothelial cells are important in the blood-brain barrier, it is the endothelial cells that are fundamentally important in the BBB.

The most important characteristics of the brain endothelial cells is their strong tight junctions and low endocytic vesicles (Figure 4). These endothelial cells have specific transport mechanisms for certain

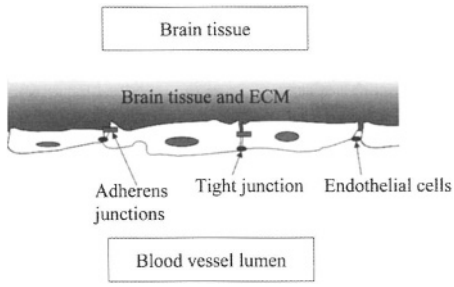

Figure 4. Possible structure of blood-brain barier.

molecules, such as L-DOPA and transferin, but otherwise reduce entry of almost all the molecules from blood to the brain.

Brain endothelial cells also have a high concentration of P-glycoprotein, which transport molecules from brain to blood(41). The structure is undoubtedly important in the control of tumour cells entering brain tissues.

Tight junctions in the brain, express far higher levels of occludin, when compared with peripheral endothelial cells (42). Higher levels of occludin in brain endothelial cells are seen at both protein level and mRNA level, suggesting a trascriptional regulation. Brain endothelial cells are derived from permeable capilaries of the perineural vascular structures that invade the embryonic neuroectoderm and from the intraneural capillaries by an angiogenic process (43). These permeable endothelial cells gradually differentiate and eventually lead to endothelial cells with characteristic blood-brain barrier functions (BBB) (43, 44, 45).

The development of high level of occludin occurs after the first postnatal week (42). Interestingly, the level of other molecules in brain endothelial cells, including alpha- and beta-catenin, ZO-1 and ZO-2 appears to be the same as that in peripheral endothelial cells.

TIGHT JUNCTION, ITS POSSIBLE ROLE IN CANCER INVASION AND METASTASIS

Interaction and subsequent penetration of the endothelium by cancer cells is a key step in the formation of distant metastasis (46,47). Once entering the blood circulation, tumour cells will face attack from the immune system. The surviving tumour cells travel in the circulation to distant tissues/organs. Once arriving at the target organ, tumour cells will undergo a series of biochemical interactions with endothelial cells. The early interactions are likely to be mediated by carbohydrate-carbohydrate reactions (locking), which occur quickly but only relatively weakly. The next stage is the development of cell-cell adhesion molecule mediated tumour-endothelial adhesion, which happens at a slower pace than the carbohydrate-carbohydrate interaction, but much stronger. Subsequently, tumour cells have to penetrate the endothelium and the basement membrane. Given the location of the tight junctions, they have to be the first structure that must be broken to allow cells to penetrate.

This is a relatively new area, due partly to the fact that the molecular structure of tight junctions is only begun to be known now. However, evidence showing that modifying tight junction

function and structure may affect tumour penetration has emerged recently.

Role of tight junction in tumour penetration of endothelial and mesothelial cells

Satoh et al (48) in their study investigating the impact of tight junction in penetration of endothelium, have used cAMP and all-trans retinoic acid (RA) as a means to regulate tight junctions in endothelial cells. Both reagents were able to reduce tight junction mediated paracellular influx and increase transendothelial resistance (TER) to electric current, indicating an enhanced tight junction function. The improvement of TJ function was the result of an increase of the level of 7H6 in these cells. While these measures had no bearing on the adhesion of mammary cancer cells to the endothelial cells, there was a significant reduction of the number of cancer cells that had penetrated the endothelium. This demonstrates the important nature of endothelial tight junctions in the control of transendothelial migration of tumour cells. What is not clear from this study, is whether the integral proteins, either the function or the level, have also be modified.

In a separate study by Tobioka et al (49), it has been shown that enhancement of the function of tight junctions also reduce the penetration of tumour cells through the mesothelial cells. Using confocal microscopy, the authors similarly demonstrated that retinoic acid increased the level of 7H6 at cell-cell border, followed by an increased of TER of the mesothelial cells. Consequently, the number of cancer cells that transmigrated the mesothelial layer was reduced. Again, it would be interesting to investigate the impact of transmembrane TJ protein in the transmigration.

While the function of endothelial and mesothelial cell tight junctions may be enhanced by therapeutic agents, cancer cells may release factor(s) that assist their transmigration in the endothelium. Conditioned medium from a highly invasive/metastatic melanoma cell (B16) is able to damage the function of tight junctions (increase transendothelial resistance). This destruction is irreversible, i.e. can not be rescued by normal medium (50). The penetration of the endothelial cells by tumour cells may be coincided with the destruction of adherens junction (65), such as the alteration of phosphorylation and loss of VE-cadherin and catenins in endothelial cells.

These observations of tumour cell transmigration appear to the in discrepancies with that seen with neutrophils (51), in which transendothelial migration is independent of the destruction of tight junction, but occurs at the tricellular regions in the endothelial cells. Clearly, there is a great deal to be learnt here. Neutrophils and cancer cells may have different adhesion profile and may adopt different mechanism to transmigrate.

Expression of tight junction molecules in cancers.

Tight junctions were noted to alter in tumour cells and tissues, by a reduction in their number, a few decades ago (52, 53, 54,55). These early studies demonstrated a correlation between the reduction of tight junctions and tumour differentiation, that lower level of tight junctions correlated with poorer differentiation of tumours.

The role of tight junction in the development of cancer and cancer metastasis has recently been studied. In breast tissues, using immunohistochemical staining, ZO-1 was found to be at high level in normal tissues. Staining, however,

was reduced or lost in 69% of breast cancer tissue (56). In infiltrating ductal carcinomas, a reduction in staining in 42% of well differentiated, in 83% of moderately differentiated and 93% of poorly differentiated tumours, was seen. Interestingly, ZO-1 staining was positively correlated with tumour differentiation and more specifically with the glandular differentiation of tumours. A further interesting observation was that the reduction in ZO-1 staining was strongly correlated with reduced E-cadherin. (56).

In the gastrointestinal tract, occludin and ZO-1 were found to co-express, at high level, in gastric and intestinal epithelium (57). However, a reduction of both occludin and ZO-1 has been seen in tumours. The marked reduction was seen in grade 2 and grade 3, which exhibited almost complete loss. This was seen together with a reduction of E-cadherin in these cells/tissues. A statistical test showed that the reduction of occludin and ZO-1 was inversely correlated with the histological grade of tumours and that the reduction of both molecules was almost parallel to each other.

In a most recent study by Chilenski et al (58), ZO-2 was found to have different isoforms, ZO-2A and ZO-2C. ZO-2C appears to be a truncated form of ZO-2A with a deletion of 23 amino acids at the N terminus of ZO-2. While ZO-2A expressed exclusively in normal epithelial cells and cell lines, ZO-2C is expressed in majority of pancreatic cancer cell lines.

Using freeze-fracture electron microscopy, CochandPriollet and colleagues (59) have recently studied thyroid oncocytic tumours. Both gap junctions and tight junction were found to be markedly reduced. The reduction of tight junction is particular obvious in malignant tumours.

Tight junction, adherens junctions and cell adhesion

Tight junction and adherens junctions are two main neighbouring structures in epithelial cells and endothelial cells (Figure 1). Evidence has emerged that both structures are linked. Itoh and colleagues (60) used recombinant technique to express N- or C- terminal ZO-1 in fibroblasts that express exogenous E-cadherin. N-terminus ZO-1 was seen to directly bind to alpha-catenin, and C-ZO-1 to actin filament. Neither bound to E-cadherin or beta-catenin. This indicates that ZO-1 is a molecule that allows binding of E-cadherin to the cytoskeleton system and may have a functional role in cadherin based cell-cell adhesion. This was further demonstrated by studies by VanItallie and Anderson (61), that expression of occludin in fibroblasts, which lack occludin, enhances cell-cell adhesion. However, this may require the presence of cadherin complex, as fibroblasts that lack cadherin based cell-cell adherens, do not increase in adhesiveness, after occludin transfection. In the process of tight junction formation, E-cadherin may interact with ZO-1 and initiate the localisation to the would-be tight junction, followed by sequestration of occludin to the same location (62).

A recent study may suggest another mechanism that tight junction and cell-cell adhesion may be co-regulated. Using an allergen, Der P1, it was found that this allergen was able to directly and indirectly cleave claudin-1, at sites such as KVFDSLLNLNS, and WYGNRIVQ. Treatment of cells with Der P1, would initially destruct tight junctional proteins (such as claudins and occludin), leading to the damage of tight junctions. This was soon followed by damage to cell-cell adhesion structure, desmosomes (63).

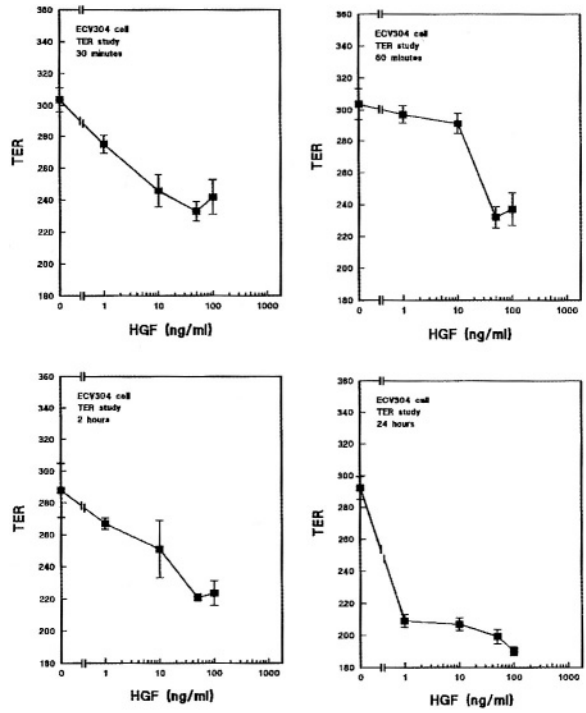

Figure 5. HGF/SF decreases transendothelial cells resistance in human vascular endothelial cells (72). ECV304 endothelial cells were treated with HGF/SF for various periods and TER measured.

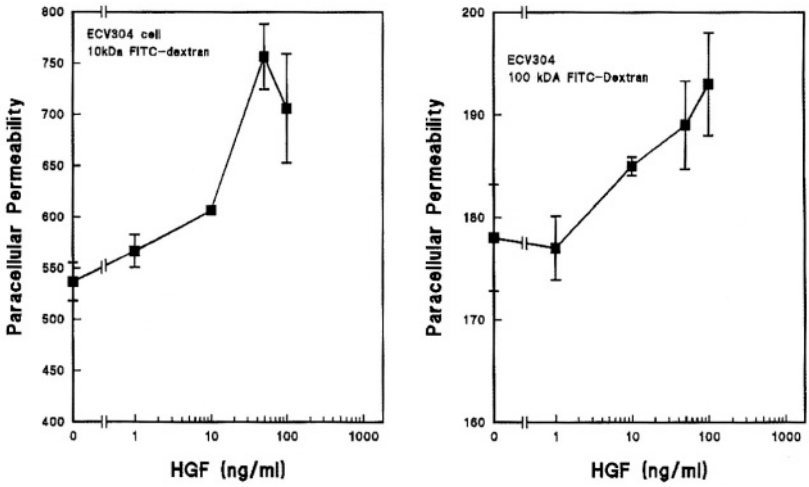

Figure 6. HGF/SF decreases paracellular permeability (72). ECV304 were treated under the same condition as in figure 5, the permeability to FITC-dextran of 10kDa (left) and 100 kDa (right) were determined. HGF/SF significantly increased the permeability to both.

REGULATION OF TIGHT JUNCTIONS

In addition to cancer cells, a number of other reagents and molecules are known to regulate the function of tight junctions.

Extracellular matrix

Extracellular matrix proteins, type IV collagen, fibronectin, and laminin have been shown to enhance the transendothelial resistance (TER) of primary cultured microvascular endothelial cell (64).

Cytokines

Tumour necrosis factor causes a decrease in the TER (transendothelial) and a fragmentation of the basal, continuous interendothelial cell zonula occludens-1 protein (ZO-1) distribution (65, 66,67). Inteferon gamma induces massive loss of ZO-1 and moderate loss of ZO-2 and occludin from T84 cell, together with a diffused localisation these molecules in the cell (66). Epidermal growth factor (EGF) induces tyrosine phosphorylation on ZO-1 and increases its affinity for interacting with the cytoskeleton (68).

HGF/SF (hepatocyte growth factor/ scatter factor) is a cytokine known to regulate a number of cellular functions, including adhesion and communication (69). It has been reported that HGF/SF regulates the paracellular permeability of epithelial cells (70), perhaps by affecting the phosphrylation of ZO-1 in the epithelial cells (71).

In vascular endothelial cells, HGF/SF markedly decreases transendothelial resistance (TER) and increases paracellular permeability to molecules (>10kDa) (Figure 5 and figure 6). The change of these tight junction functions is the results of alteration of the phosphorylation of occludin and subsequent relocation from the cell-cell border to other regions such as the cytoplasma (Figure 7). A prolonged treatment with HGF/SF may reduce the expression of occludin and ZO-1, via mechanisms yet to be identified (72) TGFB has been shown to reduce the function of tight junctions (TER) by inducing reorganization of apical F-actin and development of stress fibres, as well as the loss of normal cell border-associated ZO-1 distribution in endothelial cells. This is achieved without affecting the overall level of ZO-1 (73, 74). TGF-alpha has been shown to modify the location of ZO-1 from cell-cell border to the cytoplasm (75). Interestingly, this was seen to occur with the alteration of the growth characteristic of these cells.

Steroids

Synthetic glucocorticoid, dexamethasone, has been found to increase the function of tightjunctions. Pre-treatment of cells with dexamethasone may abolish the damaging effect exerted by TGFß (74).

cAMP

Endogenous and exogenous cAMP have been repeatedly shown to enhance the function and assembly of tight junctions. Addition of cB-cAMP to cells results in an increase in the TER (76). Induction of a rise in the intracellular cAMP also increase TER (76, 77). This effect of cAMP on tight junction may be two fold, direct effect on tight junctional molecules and indirect effect on tight junction, via mechanisms such as cell adhesion.

Protein kinase C

It has been anticipated that protein kinase C may be involved in aspects of

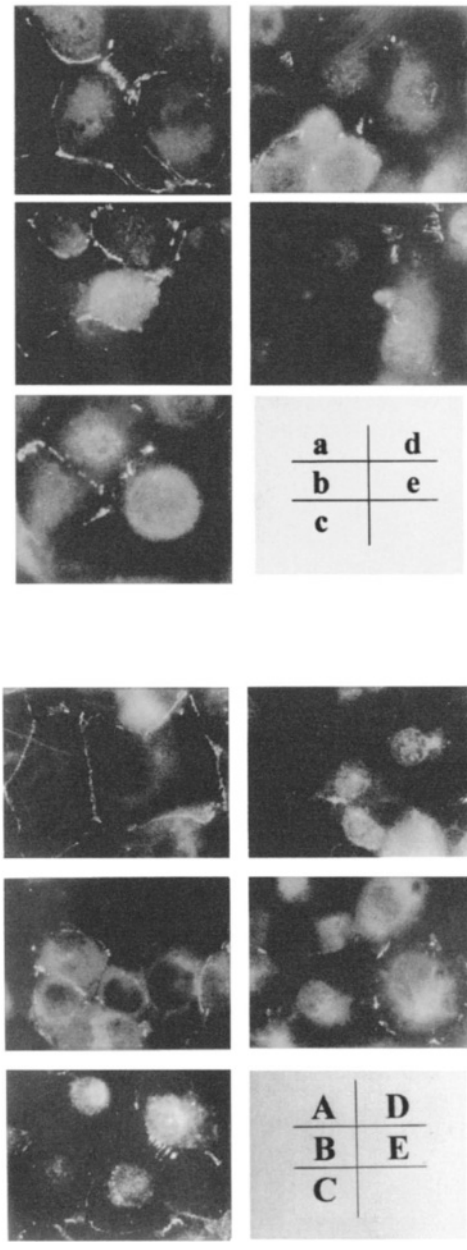

Figure 7. The effects of HGF/SF on occludin (a-e) and ZO-1 (A-E) in endothelial cells. Cells were treated with HGF/SF for 30 minutes (b and B), 2 hours (c and C), 4 hours (d and D), and 24 hours (e and E). Both molecules were stained with FITC-labelled antibodies. There was reduction occludin and ZO-1 in HGF/SF treated cells, compared with controls (a and A).

tight junctions. Recently, evidence has revealed that PKC is required for the assembly of tight junctions (78, 79, 80). Protein kinase C inhibitors inhibit the TER. This may also be the case in phorbol myristate mediated changes in tight junctions. During the assembly of tight junctions, the activity of protein kinase C markedly increases. RhoA and Rad GTPases regulate tight junction structure and function (81).

Extracellular calcium

It has been long established that paracellular permeability to ions and small molecules depends on extracellular calcium (82). Tight junction assembly occurs in the presence of extracellular calcium, but is disrupted in the presence of calcium chelators. However, the calcium dependency of tight junction assembly may be the secondary effect of calcium on structures other than tight junction, cadherin-mediated cell-cell adhesion for example.

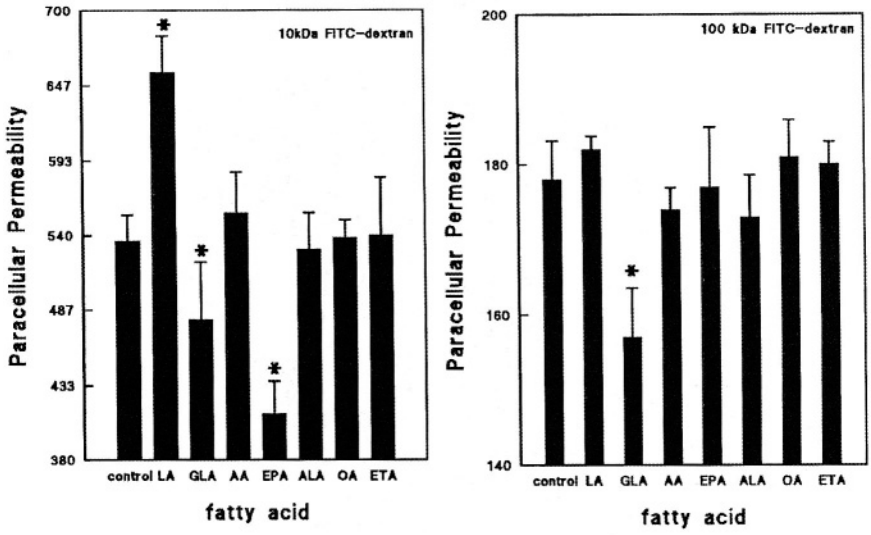

Figure 8. Effects of fatty acids on the paracellular permeability of vascular endothelial cells, ECV304. Cells were treated with different fatty acids at 50μM. The permeability to FITC-dextran (10 kDa, left, and 100 kDa right), were determined. 8 $p<0.05$ vs control.

Others

High glucose has been shown to increase the paracellular permeability of retinal capillary endothelial cell monolayers (83).

Lipids

It has been previously shown that certain lipid products, namely prostaglandin I2 and prostaglandin E2, are able to restore ischemia induced TER in epithelial cells (84). These two prostagladins exerted a synergistic effect on restoration. The effects of these eicosanoids may be via their induction on intracellular cAMP (85). Recently, another group of lipids has been reported to affect the TER (endothelial), including a range of polyunsaturated fatty acids (86,87). It has been shown that gamma linolenic acid (GLA), an anticancer essential fatty acid, can increase the transendothelial resistance and decrease paracellular permeability to large molecules. To the contrary, linoleic acid and eicosatrienoic acid, acids known to be involved in the development of certain tumours, such as breast cancer, decrease TER and increase permeability to large molecules (Figure 8). When protein from these cells were examined, it was found that these fatty acids affect the expression of occludin (figure 9).

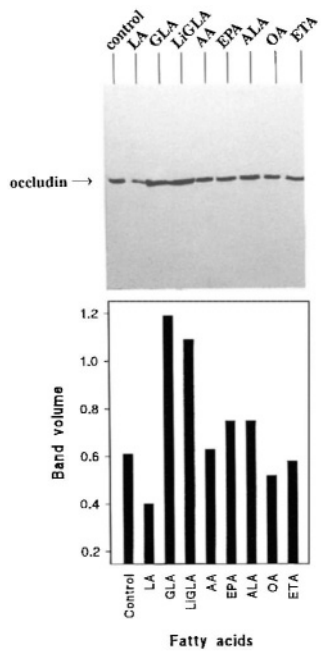

Figure 9. Effects of fatty acids on the expression of occludin in vascular endothelial cells. Gamma linolenic acid and its lithium salt were found to up-regulate the expression of occludin

The function of tight junction proteins and assembly of tight junctions are phosphorylation regulated

A prominent feature of the function of tight junction proteins is their regulation by phosphorylation. The location of occludin in a cell is dependent on its status of phosphorylation (88, 89,90,91). Highly phophorylated occludins (possibly on serine and threonine residues) are likely to target tight junctions. Hyperphosphorylation on tyrosine in ZO-1, ZO-2, ZO-3 and 130 is also necessary in the process of tight junction formation (92,93). The contrary has been reported in the development of *Xenopus laevis* (94), that dephosphorylation of occludin may play a role in the assembly of tight junction.

In summary, tight junction, the apical most structure in epithelial and endothelial cells, controls the diffusion of ions and small molecules. It is composed of transmembrane protein, including occludin, claudins and paracellin, and peripheral protein such as the MAGUK family members. The assembly and function of tight junctions are highly regulated, by events such as phosphorylation and dephosphorylation, by kinases (such as protein kinase C and GTPase). In addition to the control of paracellular permeability, it is also involved in the cell-cell adhesion.

The role of tight junctions in the development of cancer is just beginning to be realised. Although the experimental evidence showing the possible role of tight junction in the control of tumour cell penetration of endothelium and mesothelium has just begun to emerge, the pivotal role of the structure in governing the cell-cell junction and its location at the cell-cell border, clearly put tight junction at the frontline structure that has to be encountered and disrupted by tumour cells during their transendothelial migration. Further work is now required to fully establish this important aspect in cancer invasion and metastasis.

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Chapter 8

BONE MARROW MICROMETASTASES DETECTION AND SIGNIFICANCE

Hemant Singhal and Michael P. Osborne

Key words: Bone marrow micrometastases, immunocytochemistry, breast carcinoma, RT-PCR.

Abstract: Conventional clinical and radiological methods do not detect metastasis in a large majority of patients. It is accepted that this group of patients may have micrometastatic disease at presentation. The lymphovascular function of the marrow represents an ideal location to detect micrometastasis. Various immunological methods have been used to detect micrometastases. Immunochemical staining using specific polyclonal or monoclonal antibodies singly or in combination, specific for epithelial-derived cell membrane antigens yield high sensitivity in ability to detect cancer cells in bone marrow. Immunocytochemistry can detect approximately 2.5 cancer cells per $1x10^6$ normal bone marrow cells. The number of micrometastatic cells detected in bone marrow may be a measure of the systemic tumour cell burden. The majority of studies suggest that the presence and number of breast cancer micrometastases in the bone marrow predicts early distant relapse. The characteristics of the primary tumour that correlate with bone marrow micrometastases require further investigation.

INTRODUCTION

Metastasis is the major cause of death in cancer patients. Our understanding of the molecular genetic and biologic events that contribute to tumour cell dissemination has increased considerably over the last decade. There has been significant progress in the diagnosis and management of cancer and yet the impact on mortality from this disease in both the United States of America and Europe has been quite trivial. The early results of adjuvant systemic therapy suggest that the benefit is sustained for some cancers up to 15 to 20 years, which presumably accounts for the majority of the mortality reductions. It has often been proposed that the role of adjuvant systemic therapy is to eradicate or limit the progress of micrometastases. Metastasis is generally looked on as a late event in the natural history of epithelial tumours. However, the poor prognosis of some patients with apparently localised cancer indicates that micrometastases occur often before diagnosis of the primary tumour. Since the initial studies demonstrating the presence of carcinoma cells in the peripheral blood continuing attempts have been made to improve the detection of micrometastases

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and determine their significance. For the purposes of this chapter, we use the term micrometastases to mean a metastatic focus that is not detectable with conventional methods of staging including clinical examination and specialist radiological evaluation. Conventional clinical and radiological methods do not evaluate cancer cells in the blood, therefore we would use the term micrometastases for the detection of any tumour cells found in the patient's circulation.

Malignant cells shed from the primary tumor disseminate to distant sites via the bloodstream. The commonest site of metastasis is the skeletal system, where it is believed that tumor cells possessing the required adhesion molecules reside in the reticulo-endothelial system of bone marrow in the osseous medulla. These cells subsequently replicate, under the influence of growth factors not yet defined, and invade the cortex, consistent with the classical "seed and soil" hypothesis. Micrometastasis have been most extensively studied in breast cancer, we attempt to highlight features of bone marrow micrometastases and their significance using breast cancer as a model.

BONE MARROW MICROMETASTASIS IN BREAST CANCER

Ninety-five percent of patients who present with breast cancer apparently have local disease without evidence of distant metastatic spread on pretreatment staging by conventional methods (1). Despite improvements in surgical techniques, radiotherapy and drug treatment, one third of all patients relapse and die within ten years, and this proportion has not changed significantly. It is accepted that this group of patients has micrometastatic disease at presentation that cannot be detected by current standard methods (2-5). Therefore, indirect prognostic criteria, such as lymph node metastasis, tumor size, and the presence of tumor emboli in lymphatic or vascular spaces associated with the primary tumor, are used in an attempt to identify a group of patients at high risk of developing distant metastases, who would perhaps benefit from adjuvant systemic treatment. Though axillary lymph node status in breast cancer patients remains the single most important predictor of outcomes, our current methods of histopathologic analysis may be inadequate because 30% of node-negative patients recur.

The skeleton is the most common site of distant metastases for breast cancer (6) and is frequently the first site at which distant metastasis is detected. The concept of investigating bone marrow as a site for occult micrometastases has validity from two aspects. First, bone metastases start from bone marrow invasion (7) and second, the lymphovascular function of the marrow represents an ideal location to detect transient cancer cells.

Various methods have been used in an attempt to detect distant metastases at the time of initial diagnosis of the primary tumor (8) but the imaging methods currently available are too insensitive to detect micrometastatic disease. Routine radiological examination of the skeleton is almost always negative in patients with operable breast cancer (9,10). Skeletal bone scanning has a very low incidence of true positive findings in patients with Stage I and II breast cancer (11-18). Conventional techniques of examining bone marrow have a very small likelihood of identifying tumor cells at the time of initial treatment (19-22).

DEVELOPMENT OF AN IMMUNOLOGIC METHOD OF BONE MARROW EVALUATION

A group at the Ludwig Institute for Cancer Research (LICR) in London examined bone marrow aspirates using polyclonal antisera prepared against human epithelial milk-fat-globule membranes. This antigen was termed the epithelial membrane antigen (EMA) (23). Using an indirect immunoperoxidase technique, it was initially shown that EMA has a widespread but highly selective distribution in human tissues. EMA staining was observed in normal breast epithelium, primary mammary carcinomas, carcinoma cells infiltrating bone marrow, xenografts of primary carcinoma in nude mice and the MCF-7 cell line (23). Subsequent more detailed studies of breast cancers showed strong staining (24), and single metastatic breast tumor cells in bone marrow showed intense staining. Bone marrow aspirates from 20 disease-free patients treated for breast cancer 3-5 years previously were negative for EMA stained cells. An additional eight patients with positive nodes were also EMA negative. Eight of 43 (18.6%) patients with metastatic disease and negative routine marrow histology had EMA positive cells in the marrow, and it was concluded that the sampling of paraffin-embedded sections used in this study may be less satisfactory than smears (25). Continued studies using aspiration smears were more satisfactory (26,27). Early data also suggested that multiple sites yielded more information than a single site of bone marrow aspiration (28).

Other investigators have confirmed the observation of immunoreactive cells, using either EMA or anti-cytokeratin (AE1) antibodies in the bone marrow of early stage breast cancer patients (29,30). A highly sensitive immunofluorescent monoclonal antibody method has been developed and used for preliminary studies (31-34). Several mouse monoclonal antibodies specific for epithelial cells have been developed in our laboratory. These antibodies (C26, T16), along with a commercially available monoclonal antibody specific for epithelial cells (anti-cytokeratin intermediate filament antibody AE1, Labsystems, Finland) react with distinct epithelial-specific antigens. All are epithelial-specific and each reacts with most breast cancers tested (35-40). These monoclonal antibodies have not been shown to react with normal marrow components.

METHODS OF DETECTION

Various immunological methods have been used to detect micrometastases of the bone marrow. Usually, these have included a concentration of the nucleated cells in the bone marrow by centrifugation in the separation medium, with the tumour cells migrating with mononuclear population. This can then be removed and resuspended and then cytospun down to prepare slides for immunological detection. Most groups have used Ficoll-Hypaque density gradient separation (25,26, 29,30,36,41,42); to obtain the mononuclear cells. Red cell lysis has been used but has been found to be less effective than density gradient separation (26,43). Cytospin preparations of the nucleated cells obtained are usually fixed with 100% ethanol or cold acetone(29) .

A study of immunochemical staining of bone marrow biopsies, negative for metastases by conventional staining techniques, concluded this technique was not useful; however, only one biopsy was taken and one monoclonal antibody (KLI) was used.(44) Bone marrow biopsy appears to be less efficient than smears because sampling errors may be introduced

by examining tissue sections, rather than smears, where the entire specimen can be studied (26).

Immunochemical staining of epithelial cells is obtained using specific polyclonal or monoclonal antibodies singly or in combination, specific for epithelial-derived cell membrane antigens. A combination was suggested to enhance the likelihood of identifying heterogeneous tumor cells and to maximize identification of epithelial cells by their reactivity at two or more cellular sites. Monoclonal antibodies to cytokeratin are superior to other antibodies and there is general agreement that monoclonal antibodies against either CK-18 or optimally CK-19 should be used.

Immunofluorescence by indirect staining has the advantage of easy visual identification of cells exhibiting membrane and cytoplasmic fluorescence in a dark background by phase-contrast microscopy. However, this technique does not permit adequate morphologic examination of fluorescing cells. In addition, no permanent record, other than photo-documentation, is available. Immunocytochemical staining using the alkaline phosphatase anti-alkaline: phosphatase (APAAP) method permits morphologic evaluation of cells stained with Neofuchsin, or Fast Red as well as providing a permanent record. The use of an appropriate positive control cells (MCF-7 cells in bone marrow) is required. Sample size, as defined by the number of bone marrow cells examined, may be an important factor in determining the likelihood of detecting micrometastases. There is considerable variation between studies that have recorded the denominator of cells counted, ranging from $3x10^4$ to our studies of $4x10^6$ cells which can be screened in a twenty minute period. The minimum number of cells that should be examined is $1x10^6$(45).

Table 1. Antibodies used for the detection of bone marrow micrometastases in breast cancer

Antibody	Type of Antibody	Target
EMA	Polyclonal	human milk fat globule membrane
HMFG-2	Polyclonal	human milk fat globule
T16	Monoclonal	membrane glycoprotein
C26	Monoclonal	membrane glycoprotein
LICR.LON.M8	Monoclonal	membrane
MBr 1	Monoclonal	membrane glycolipid
AE-1	Monoclonal	cytokeratin
CK2	Monoclonal	cytokeratin
35BH11	Monoclonal	cytokeratin
34BE12	Monoclonal	cytokeratin
CAM 5.2	Monoclonal	cytokeratin
DF3	Monoclonal	cytokeratin
PKK 1	Monoclonal	cytokeratin
KLI	Monoclonal	cytokeratin

SENSITIVITY OF DETECTION

Immunofluorescence and immunocytochemistry yield very similar sensitivity in terms of their ability to detect cancer cells in bone marrow. Monoclonal antibody detection of single cells and clumps of cancer cells is remarkably sensitive. Light microscopy may be estimated to detect marrow involvement at the level of approximately one cancer cell in two thousand normal nucleated marrow cells. Flow cytometry has been suggested as a technique for detection of micrometastasis at the level of 1 cancer cell in 10,000 normal cells;(46) flow cytometric fluorescence-activated cell sorting has shown a sensitivity of detection of approximately 1 cancer cell in 100.(47) It was predicted, using a model system that monoclonal antibody techniques could detect approximately one cancer cell in $1x10^6$,(47) Using a similar system of metastatic tumor cells mixed with normal leucocytes it has been shown that immunofluorescence can detect one tumor cell in 200,000 normal cells.(30) Other studies have suggested a detection rate of 1 cancer cell in 1×10^5. (48) Similarly, a study of metastatic cells mixed with normal bone marrow cells showed that immunocytochemistry can detect approximately 2.5 cancer cells per $1x10^6$ normal bone marrow cells.(47,49)

Table 2. Prevalence of bone marrow micrometastases (BMM) in patients with early stage breast cancer

Study	Year	Number of patients	Antibody used
Giai	1990	45	AB/3
Manegold	1988	50	PKK 1
Ellis	1989	25	34BE12
Porro	1988	159	MBr 1
Mansi	1991	350	EMA
Schlimok	1992	187	CK2
Untch	1988	44	EMA
Ménard	1992	200	MBr 1, MBr 8
Osborne	1993	348	T16, C26, AE-1
Cote	1987	51	T16
Diel	1992	260	2E11
Kirk	1990	25	LICR.LON.M8.4

SIGNIFICANCE OF BONE MARROW MICROMETASTASIS

The majority of patients developing distant relapse do so in the skeletal system. Skeletal metastases are considered to originate within the medullary bone marrow prior to invading the cortex and the bone marrow is considered an important site for tumour cell growth

It is now possible to look at tumour cells in the bone marrow which may well represent a part of the tumour that does remain in the body after surgery and that can provide direct evidence of the tumour's disseminative potential. The presence of detectable micrometastatic disease would imply the eventual clinical appearance of obvious metastatic disease. However, the degree to which micrometastases represent true residual disease or cell shedding and metastatic potential remains controversial. Studies

continue to evaluate bone marrow micrometastasis as a prognostic variable, however the reported prevalence of micrometastases ranges from 4% to 48%.(30,36,41,42,51-53,58-62) These differences may be attributed to case selection, number and sites of aspiration, number of cells examined, type and number of antibodies used and variations in technical aspects of the method. No correlation was seen, between the prevalence of bone marrow micrometastases and standard prognostic variables such as tumor diameter or lymph node status was in some studies, in contrast to other studies which show a correlation.

Several centers have presented data on recurrence and have shown that the presence of bone marrow micrometastases identified by monoclonal antibodies predicts early relapse. (Table 3) (52,55,61,63) The largest series with longest follow up, from the Ludwig Institute group, reported that the presence of EMA staining cells correlated with the pathologic size of the tumor ($p<0.005$) and the presence of peritumoral vascular invasion ($p<0.001$).(52) At a median follow up of 76 months the presence of bone marrow micrometastases correlated with distant metastases ($p<0.005$), relapse in bone ($p<0.01$), relapse at other sites excluding bone ($p<0.001$) and shorter overall survival ($p<0.005$). In one study the presence of bone marrow micrometastases was the most powerful independent predictor of relapse in multivariate analysis.(58). *Table 3.* Relapse rate in studies reporting a follow up.

Table 3. Relapse rate in studies reporting a follow up

Author	Number of patients	BMM positive patients	Follow up in months	Relapse rate in BMM positive	Relapse rate in BMM negative
Mansi	350	89	76	48	25
Osborne	348	111	30	20	7
Diel	211	81	24	27	4
Salvadori	121	20	48	30	21

Studies have also used methods to quantify the number of cancer cells in the bone marrow. The quantification of these cells have been shown to be of significant prognostic predictor of recurrent disease. In a pilot study Cote showed that the ratio of cancer cells to nucleated cells was a significant variable for prediction of early recurrence. The presence of greater than 10 micrometastatic cells in a bone marrow sample correlated with early relapse.(63) The number of micrometastatic cells detected in bone marrow may be a measure of the systemic tumor cell burden. It has been shown, that the number of micrometastatic cells is important in multivariate analysis and is highly predictive of early relapse when combined with either a maximum tumor diameter >2.0 cm or axillary lymph node metastases.(61,62)

RT-PCR FOR DETECTION OF MICROMETASTASIS

RT-PCR is the most widely used molecular method for detection of tumour RNA following separation of mononuclear layer similar to that for immunological studies. The mRNA is extracted and converted to cDNA with the

use of specific primers and the enzyme reverse transcriptase. Amplification of the cDNA is then obtained through the use of nested RT-PCR. The amplified product can then be identified using gel electrophoresis of the PCR product by staining with ethidium bromide or by southern transfer analysis with labelled hybridisation probes.

RT-PCR has been used to amplify the mRNA of cytokeratin 19 and then used for the detection of micrometastatic cells within the bone marrow or peripheral blood in breast cancer patients. The detection of other epithelial cancers has been facilitated by the detection of mRNA of CEA for breast, colorectal gastric and oesophageal cancer and mRNA of surfactant proteins A, C and D for non small cell lung cancer. A diverse number of applications for this technology remain under intense investigation.

In immunological studies there remains the difficulty of the certainty in cytological identification of tumour cells, particularly with artefacts resulting from different methods of cell concentration and the difficulty experienced by the pathologist in definitely differentiating the precursor haemapoeitic cells from tumour cells. It is possible that RT PCR will show a promise of potentially improved methods of detection. In a comparison of immunological verses mRNA detection, the quantification that the immunological method gives, may be of more clinical significance than the pure judgement of the absence or presence of micrometastatic cells. Newer techniques of quantifying the tumour mRNA and RT PCR are under development but may suffer from the variable expression of mRNA in each tumour cell, and therefore may not be a true reflection of the tumour burden.

Recent studies have tried a combination of the immunological technique and RT-PCR using immunobeads. Isolation of carcinoma cells by immunomagnetic separation followed by reverse transcription polymerase chain reaction (immunobead RT-PCR) has been developed as a method for identifying very small numbers of breast cancer cells in blood. The expression of cytokeratin 19 (K19) was used as the marker by which the isolated tumor cells were identified. The immunobead RT-PCR technique allowed detection of one tumor cell per 10^6 leukocytes in whole blood.

CONCLUSIONS

The majority of studies suggest that the presence and number of breast cancer micrometastases in the bone marrow at the time of diagnosis or initial treatment predicts early distant relapse.

A number of issues remain unanswered. The criteria for determining a marrow to be positive have not been fully defined since there is insufficient data available concerning the prognostic significance of small numbers of micrometastatic cells. Studies reported to date do not address the utility of this method, in patients with small tumors and negative axillary lymph nodes, who have a good prognosis.

It is unclear whether all patients with micrometastases will ultimately develop clinical recurrence. It is possible the beneficial effects of adjuvant systemic therapy, may be further enhanced by using this technology combined with standard prognostic factors of tumor diameter and axillary lymph node status to identify patients at substantial risk of systemic disease.

It can also be argued that at the present time the majority of patients with invasive breast cancer are eligible for systemic therapy if the invasive tumor exceeds 1.0 cm (or in the case of special tumor types such as medullary, colloid and tubular > 3

cm). The practicality of demonstrating a beneficial effect of systemic therapy in those patients with bone marrow micrometastases who are not currently recommended to receive systemic adjuvant chemotherapy remains. The relapse rate in these patients is about 10% at 10 years, and presents the problem of the large sample size required to demonstrate a significant relapse, or mortality reduction, by adjuvant systemic therapy. In addition micrometastatic cells may spontaneously disappear as evidenced by repeated examination(64).

FUTURE DIRECTIONS

The characteristics of the primary tumor that correlate with bone marrow micrometastases require further investigation. Additional studies are required to determine the proliferative, endocrine, molecular biological and immunologic characteristics of micrometastases that correlate with recurrence. This technology may lead to the development of immunotherapeutic strategies using an immunocytotoxic approach as well as techniques for purging bone marrow of patients undergoing autologous bone marrow transplantation.

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Chapter 9

POLYUNSATURATED FATTY ACIDS AND THEIR ROLE IN CANCER INVASION AND METASTASIS

Wen G Jiang, Amanda Redfern, Aldo Eynard and Robert E Mansel

Key words: Essential fatty acids, gamma linolenic acid, eicosapentaenoic acid, cancer treatment, invasion, metastasis, cell adhesion, angiogenesis

Abstract: Highly unsaturated fatty acids (HUFAs) have been demonstrated to have anticancer functions in the past decade. Recently, these fatty acids have been shown to exert a regulatory action on tumour cell motility, invasion and metastatic behaviour via such mechanisms as regulation of cell-cell adhesion, tumour suppressor molecules and motility related signal transduction pathways, and angiogenesis. Clinical studies based on laboratory results have recently been carried out and early indications show promise in extending the survival of patients with cancer.

The relationship between diet lipids and the incidence of cancer has been established. This is best demonstrated in Eskimos, who consume essential fatty acid (EFA)-rich diets and enjoy a low incidence of cancer (1). Similarly, other populations with high EFA intake also show a low incidence of female breast cancer (2). The early eighties saw the establishment of the knowledge that EFAs also exerted toxic effects on malignant cells (3-5). The role of EFAs in cancer has since been extensively explored. Recently, another important aspect of fatty acids on cancer emerges, i.e. the anti-invasion and anti-metastasis property of these lipids.

ESSENTIAL FATTY ACIDS AND POLYUNSATURATED FATTY ACIDS

There are four families of polyunsaturated fatty acids (n-9, n-7, n-6, and n-3) (figure 1), of which only the n-6 and n-3 are essential fatty acid (EFA) series. Thus all the EFAs are polyunsaturated and only include linoleic acid (LA) (n-6) and alpha-linolenic acid (ALA) (n-3), due to the fact

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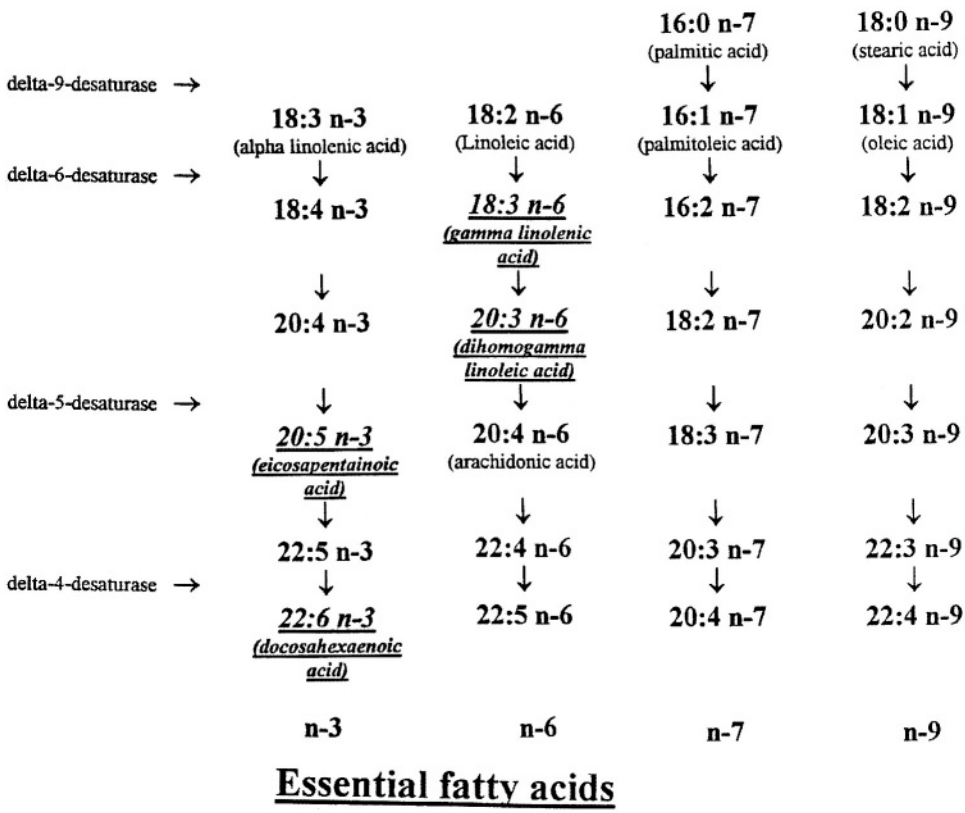

Figure 1. Polyunsaturated and essential fatty acids (n-3, 6, 7, 9). There exist four series of PUFAs, with n-3 and n-6 being essential. The n-3 series starts from alpha linolenic acid (ALA) and the n-6 from linoleic acid (LA). Both LA and ALA are obtained from the diet. These two fatty acids are firstly desaturated by delta-6-desaturase and then elongated by elongase. n-7 and n-9 series are non-essential and their parent fatty acids are firstly converted by delta-9-desaturase as opposed to delta-6-desaturase in the essential series. The desaturated steps are slow compared with elongation and thus are rate limiting in their metabolism. Those EFAs toxic to cancer cells are highly unsaturated with more than two double carbon-carbon bonds and have been underlined.

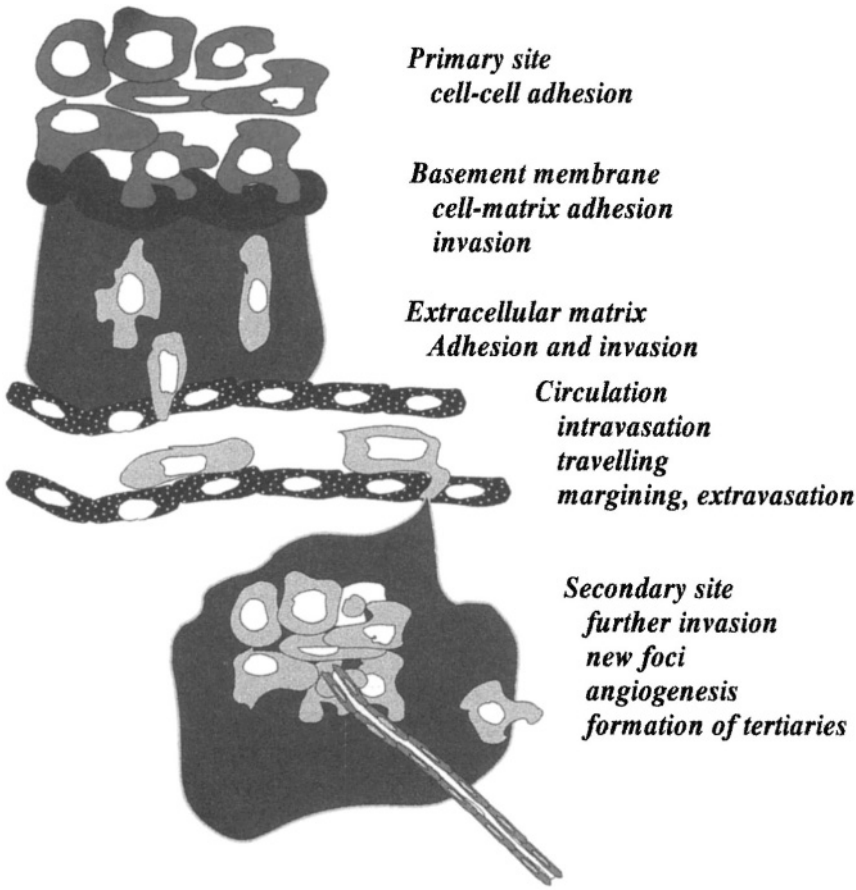

Figure 2. Cancer metastasis process

that these fatty acids can not be synthesised by humans and must be obtained from dietary sources. Other polyunsaturated fatty acids in the n-6 and n-3 series are either converted from their parent EFAs or directly from the diet. Three main metabolic pathways convert the unsaturated fatty acids to a large number of downstream metabolites, collectively known as eicosanoids. EFAs are key elements in our body, playing important physiological roles. Abnormalities of PUFAs and EFAs are also associated with heart and other vascular conditions.

The main interest in the role of EFAs in cancer has been on the cytotoxic impact of these fatty acids on cancer cells. Several PUFAs beyond the first delta-6-desaturation step in the pathways, namely gamma linolenic acid (GLA), dihomogamma linolenic acid (DGLA), eicosapentaenoic acid (EPA) and

docosahexaenoic acid (DHA) are particularly active in this aspect. However, with the discovery that these fatty acids also affect the adhesiveness of cancer cells to the excellular matrix, their role in cancer metastasis has since been extensively explored.

CANCER INVASION AND METASTASIS, A MULTIPLE STEP AND COMPLEX PROCESS

Local and distant metastases are the most life threatening event for patients with cancer. At the time of diagnosis, a large proportion of patients already display detectable micro- and/or macro-metastases. The last decade has seen a marked expansion in the knowledge and understanding of the molecular and cellular mechanisms underlying tumour invasion and metastasis. The process of formation of tumour metastases, now referred to as the metastatic cascade (6,7), is composed of a number of separate but essential steps, including the loss of cell-cell adhesion at the primary site, adhesion to and invasion of the basement membrane and extracellular matrix (ECM), intravasation, travel in the host circulation and survival of host immune responses, adhesion to the endothelium and extravasation, and establishment of secondary foci with the formation of neovasculature and angiogenesis (figure 2). Measures against these events thus bear a promising future in cancer treatment.

EFAS AND CELL-MATRIX ADHESION:

The basement membrane and the extracellular matrix is the first barrier that a cancer cell encounters before they metastasise. The adhesion of tumour cells to the extracellular matrix is a crucial event that facilitates matrix degradation and invasion, cell survival and migration of tumour cells. Tumour cell-matrix attachment is mediated by integrin molecules on the cell surface. The integrins are a group of heterodimeric proteins each comprising an α and a ß-subunit. Different combinations of α and ß-units form individual integrin molecules, each interacting with specific matrix proteins.

Binding of integrins with extracellular matrix proteins activates a series of intracellular signalling events, which are centrally mediated by focal adhesion kinase (FAK) and transduce signals for cell growth, morphology changes and migration (8,9,10). Changes of integrins, FAK and capacities of tumour-matrix adhesion are associated with the invasive and metastatic properties of cancer cells (11-14). Regulation of these events may thus have major effects on the function of integrins (15,16).

The involvement of PUFAs in tumour-matrix adhesion has been recently reported. GLA, EPA, DHA and LA all reduced tumour adhesion to a range of matrix components, including collagen type IV, fibronectin, laminin, vitronectin, and the basement membrane (17-23). These studies also show that PUFAs are less likely to affect the level of integrin, but may affect the signalling events associated with integrin. For instance, GLA inhibits the tyrosine phosphorylation of FAK and paxillin, key regulatory mediators in cell-matrix adhesion signalling (figure 3).

EPA and GLA have also been shown to induce apoptosis (24-27). Experimental inhibition of tumour cell-matrix attachment using ROD peptides, integrin/matrix antibodies or interfering with matrix adhesion signalling events (such as the phosphorylation of FAK or other mediators) results in apoptosis. The phenomena of loss of capacity to adhere to matrix, subsequent detachment from it and

development of apoptosis are now known as *anoikis* (28). The effect exerted by PUFAs on matrix adhesion together with the observations that these fatty acids also inhibit FAK phosphorylation (17,18,21, 29,30,31), indicate that EFAs are candidate molecules for the induction of anoikis. Whether these fatty acids are involved in the induction of other apoptosis-related molecules such as BCL family members remains to be elucidated. It has been long proposed that there is a relationship between cell-matrix adhesion and cell cycle progression (32). The effects of PUFAs on cell-matrix adhesion in tumour cells suggest that they may also function as regulators of cell cycle progression and future work will have to determine this relationship.

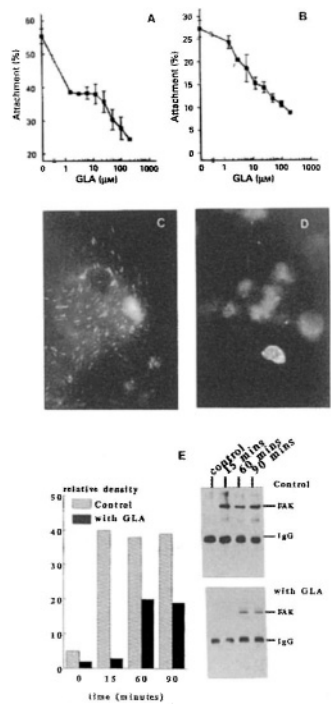

Figure 3. Effect of GLA on cell-matrix adhesion and the possible mechanisms. A and B: Adhesion of a human colon cancer cell to the basement membrane (Matrigel) (A) and fibronectin (B) coated surfaces; C and D: human breast cancer cells immunofluorescently stained with anti-FAK (focal adhesion kinase) antibody after they adhere to extracellular matrix surface in the absence (C) or presence of gamma linolenic acid (D); E: tyrosine phosphorylation of FAK after adhesion to Matrigel, study carried out with immunoprecipitation and Western blotting (right-top: control cells, right-bottom: cells with gamma linolenic acid, left: protein band densities representing the degree of tyrosine phosphorylation of FAK). Adhesion of tumour cells to both the basement membrane (Matrigel) and fibronectin was significantly reduced by GLA (A and B). This was associated with the disappearance of focal adhesion complex (cell stained with anti-FAK antibody) (D-GLA treated cells when compared with control-C) and with the inhibition of tyrosine phosphorylation of FAK (C) (21,30). The phosphorylation of paxillin, a down-stream mediator of FAK, was also been inhibited by GLA (30)

EXTRACELLULAR MATRIX DEGRADATION AND PUFAS

Matrix degradation and invasion by cancer cells represent one of the key events in the metastatic cascade (figure 2). After tumour cells have adhered to the extracellular matrix, proteolytic enzymes, either secreted from the tumour cells, bound to the tumour cell membrane or from other sources will degrade the matrix to clear the way for tumour cells to migrate. Proteolytic enzymes involved in this matrix degradation mainly include metallo-proteinases (collagenases, and stromelysins) and serine proteinases (plasmin, trypsin, thrombin, urokinase-plasminogen activator (uPA). Their activities are controlled by their activation from pro-enzyme forms and deactivation by the relevant enzyme inhibitors. Thus, the balance of degradation is maintained by the participation of proteolytic enzyme inhibitors, including plasminogen activator inhibitors (PAIs), tissue inhibitor of metalloproteinases (TIMPs), and antiplasmin. The effects of experimentally modulating the level or function of these endogenous proteolytic inhibitors in tumours may assist to reduce invasion and metastasis.

Both proteolytic enzymes and their inhibitors can be regulated by PUFAs. It has been shown that the level of plasminogen activator inhibitor I, a member of the serpin class of protease inhibitors, was enhanced by DGLA and DHA (33). This enhancement further inhibited the activity of both tissue type and urokinase type plasminogen activators (tPA and uPA). EPA, GLA, and AA have been shown to inhibit uPA and collagenase IV production from cancer cells (34,35). Such regulation occurred at a transcriptional level (33). Other unsaturated fatty acids, such as 9-octadecenoic acid, have also been shown to influene MMP-2 expression (36). Interestingly, this appeared to be the response of another recently identified tumour suppressor and member of the serpin family, maspin (37-39). GLA and EPA selectively increased the protein and mRNA level of maspin in a range of cancer cells (40). It has been reported that LA stimulated tumour cell invasion and 92-kDa type IV collagenase production in vitro; GLA however inhibited invasion and did not induce activity of the proteolytic enzyme (17,19,41,42). In vivo, such an effect was not seen in terms of the tumour growth and metastasis formation, which may be due to the metabolism of the fatty acid in vivo to arachidonate-derived eicosanoids that may be involved in the metastatic process.

EFAS AND CELL-CELL ADHESION:

Cell-cell adhesion plays a key role in the maintenance of tissue/organ integrity and almost all the physiological aspects (figure 2). A range of cell adhesion molecules have been identified and can be grouped into four superfamilies: cadherins, integrins, the immunoglobulin superfamily and others (6,7). Although it has been long suspected that EFA may be involved in cell-cell adhesion, it was not until recently that this has become evident.

E-cadherin dependent regulation of cell-cell adhesion by EFAs

E-cadherin is one of the best studied cell-cell adhesion molecules in cancer. The molecule is anchored to the cytoskeleton via catenins (figure 4). The E-cadherin complex has been known as a tumour invasion and metastasis suppressor. Both in vitro and in vivo studies have revealed that expression of E-cadherin is inversely

correlated with the motile and invasive/metastatic behaviour of a tumour cell and also inversely correlated with metastasis in cancer bearing patients. The function of E-cadherin is highly dependent on the function of catenins (figure 4) (43,44). Regulation of the expression of E-cadherin thus presents an attractive way by which the metastatic properties of cancer cells may be regulated both in vitro and in vivo (45,46).

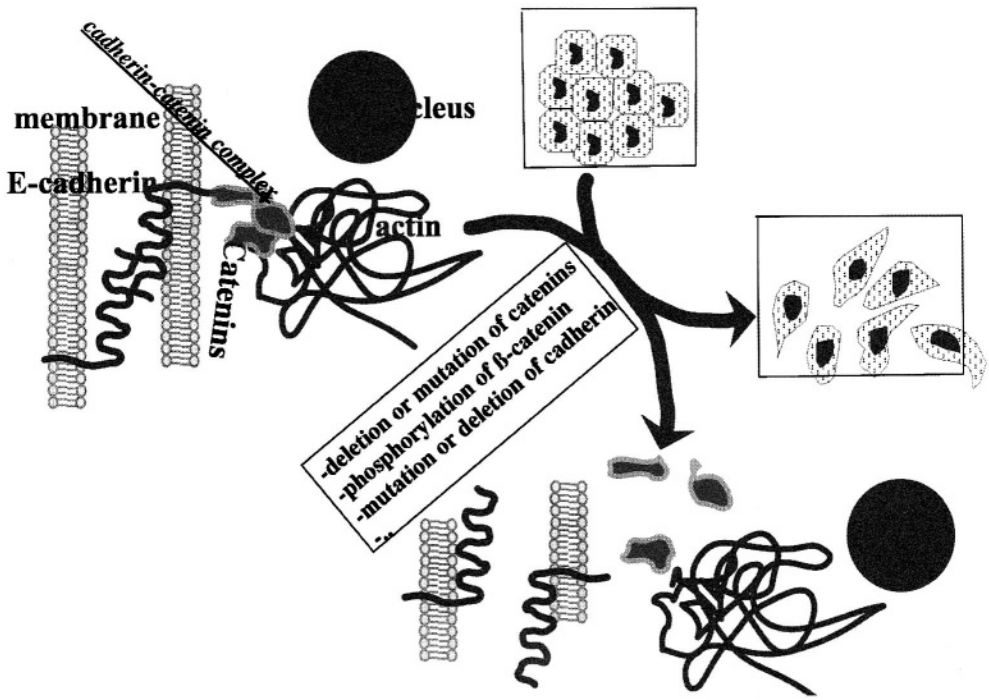

Figure 4. E-cadherin and association proteins, catenins in cell-cell adhesion. E-cadherin is a transmembrane protein. In the presence of extracellular calcium, the extracellular domain of the molecule binds to another E-cadherin on another cell, thus forming a strong cell-cell adhesion mechanism. The function of E-cadherin is regulated by a group of intracellular proteins, including catenins. Cells with normal cadherin-catenin function strongly adhere to each other. When the complex is damaged or functionaly impaired, for example reduction in their levels, mutation, deletion, and tyrosine phosphorylation of ß-catenin, the cell adhesion is damaged, which results in cancer cells less or no adhesive, but more invasive.

The culture of cancer cells with EPA and GLA, particularly the latter, resulted in a time and concentration dependent enhancement of the expression of E-cadherin in a range of cancer cells (21,47) (figure 5). These changes were associated with an increase in cell-cell aggregation and reduction of invasiveness of these cells. Importantly, these effects were achieved without causing cytoxicity.

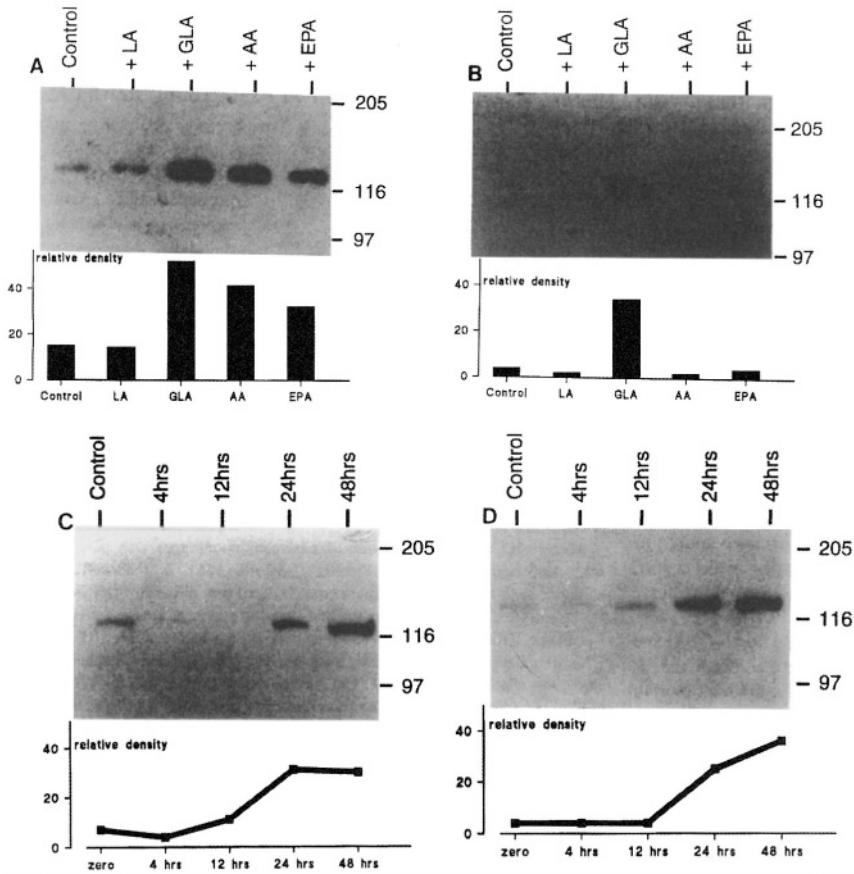

Figure 5. Expression of E-cadherin by GLA in cancer cells. Tumour cells were treated with either a range of PUFAs (A and B) or with GLA for upto 48 hours (C and D) and then proteins were extracted, separated with SDS-PAGE and molecule visualised with enhanced chemiluminiscence. The relative level of the molecule is indicated in the figure as relative protein band densities from the corresponding gel photo. GLA, among the fatty acids tested, selectively up-regulated the expression of E-cadherin, a 120kDa cell-cell adhesion molecule (A and B). This effect was seen 24 hours after treatment (C and D). A: HRT18 human colon cancer cell; B and C: PLC/PRF/5 human liver cancer cell; D: Cor L23 human lung cancer cells (47).

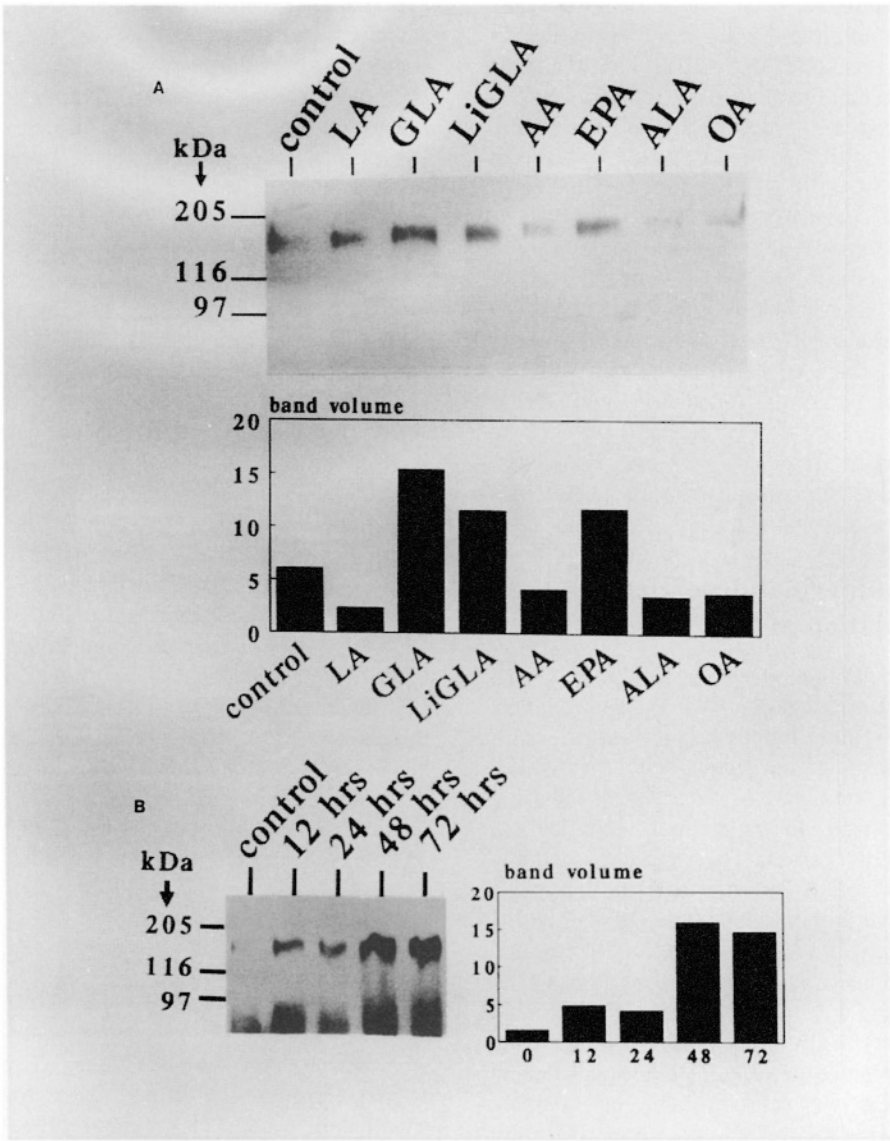

Figure 6. Effects of GLA on the expression of desmosomal cadherin, desmoglein, in HT115 cell. Cells were treated with either a range of PUFAs (A) or with GLA for upto 72 hours (B). GLA, its lithium salt (LiGLA) and EPA selectively up-regulated the expression of desmoglein with an effect seen after 24 hours treatment. Others, including LA, ALA and OA were less effective (61).

Association of E-cadherin with catenins is essential for the normal function of E-cadherin (figure 4). Of these three catenin molecules, ß-catenin has been the focus of

much recent research (48). Although the molecule has been suggested to play an important role in the integrity of the E-cadherin complex, it has recently been shown that the molecule also plays a role in the signal transduction of E-cadherin complex formation and is also involved in the wnt, LEF, TCF, and APC-mediated cellular events, including cell morphology, proliferation, tumorigenesis, and embryo development (43,44,49,50,51). Tumour cells, when cultured with GLA, showed a decrease in the level of ß-catenin (52), an event which appeared at the same time as the changes of E-cadherin and alpha-catenin. This further indicates GLA as an important regulator of the E-cadherin adhesion mechanism and thus early stages in the metastatic cascade.

E-cadherin independent regulation of cell-cell adhesion

In addition to the regulation of the E-cadherin complex, PUFAs also regulate desmosome functions in cancer cells. Desmosomes are unique cellular structures which form part of the cell-cell adhesion mechanism in epithelium, muscle and lymphoid tissues. The structure is mainly comprised of desmoglein (Dsg) 1,2 and 3 together with desmocollins (Dsc) 1,2 and 3, collectively known as desmosomal cadherins. The extracellular domains of desmogleins and desmocollins bind heterotypically and possibly homotypically in the presence of extracellular calcium to stabilise the cell-cell adhesion complex (52,54). Desmoglein and desmocollin are linked to intermediate filaments within the cell by desmoplakin and plakoglobin (55). Furthermore, formation of desmosomes in cells requires E-cadherin and related catenins. This involves interplay between E-cadherin, desmoplakin and plakoglobin (56). Both in vitro and in vivo studies have shown that a reduction of desmosome number in cancer cells correlated with an enhanced invasiveness and tumorigenesis in transitional cell carcinomas (TCC) and squamous cell carcinomas (SCC) (57,58). Reduced expression of desmoglein has also been shown to correlate with lymph node metastases in patients with cancer. Its expression in patients with poorly differentiated and highly invasive, metastatic carcinomas was markedly reduced compared with normal tissues (59,60). The regulation of desmosomal protein expression and the assembly of desmosomes themselves may thus present an important approach to influence cancer cell behaviour.

An E-cadherin negative cancer cells, HT115 cell line, exhibited an increase in the cell-cell adhesion when cultured with GLA and also in the formation of desmosomes (61). Expression of desmoglein, a desmosomal cadherin, was markedly increased after treatment with GLA (figure 6). This was accompanied by reduced cell invasiveness in vitro. Thus, EFA-mediated cell adhesion may occur via two independent but similar molecular mechanisms, involving either E-cadherin or desmosomal proteins. It may also be possible that both of these mechanisms operate at the same time within the cell.

EFA AND TUMOUR-ENDOTHELIAL INTERACTION

Adherence of tumour cells to and subsequent migration through the vascular endothelial lining is central to the metastatic process. Tumour cells initially loosely adhere to the endothelium via carbohydrate-carbohydrate interactions and then would firmly 'lock' to the endothelium before migration and invasion of the endothelium layer. The latter process requires certain adhesion molecules including selectins, ICAMs, VCAM and integrins (7,62,63,64). The adhesion of

tumour cells to endothelium also results in a reduction of gap junction communication, an increase in motility and retraction of endothelial cells thus allowing the tumour cells to transmigrate the endothelial layer (62,65)

DHA has been reported to inhibit cytokine-induced expression of adhesion molecules in endothelial cells, including VCAM-1, ICAM-1 and E-selectin (66,67,68). Similar inhibition has been reported with the LA metabolites. Interestingly, short term culture of endothelial cells with GLA (<20 minutes) resulted in a marked reduction of tumour cell adhesion to the endothelium (69). This phenomenon may be associated with an improvement of gap junction communication of endothelium by GLA. LA, however, inhibited the communication both in endothelial cells (69) and in tumour cells (70,71). The regulation of adhesion molecules in endothelial cells by EPA and LA has been demonstrated in vivo studies, which will be discussed in later parts.

EFAS AND ANGIOGENESIS:

The progression and metastasis are angiogenesis dependent (72,73,74). The central players in this process are the vascular endothelial cells, which proliferate, migrate and then assemble into tube-like structure with tight cell-cell connections to contain blood. The ECM and peri-endothelial cells are required to support these tubule structures. These structures remodel to form mature blood vessels. Endothelial cell functions, such as their motility, migration and adhesion to each other and to matrix components are essential for angiogenesis. This process is also regulated by a number of growth factors (angiogenic factors) released from both surrounding tissues and tumour cells. Formation of new vessels in tumour tissues lies upon a balance between angiogenic factors and anti-angiogenic factors. In progressive tumours, the balance shifts toward AFs. A shift toward anti-angiogenic mechanism may have an important implication in anti-cancer strategies. A range of angiogenic factors have been identified. These include vascular endothelial growth factor (VEGF), basic and acid fibroblast growth factor (FGF), transforming growth factors (TGFs), platelet derived growth factor (PDGF), hepatocyte growth factor/scatter factor (HGF/SF), platelet-activating factor (PAF), interleukins (ILs), tumour necrosis factor (TNF), angiogenin, soluble vascular cell adhesion molecule and granulocyte-colony stimulating factor (G-CSF). Anti-angiogenesis research and treatment have therefore gained much attention in the last decade.

The fact that PUFAs may be involved in the regulation of angiogenic processes, has been demonstrated previously (75,76). In vitro, the presence of GLA reduced the number of new vessels (77) (figure 7). Supplementation of GLA, EPA, or their combination greatly reduced the number of new vessels formed both in vivo (78) and in vitro (79).

A few lines of mechanisms have bee nsuggested for the anti-angiogenesis action of PUFAs. Firstly, PUFAs suppress the motility of endothelial cells (69). Secondly, PUFAs affect cell adhesion molecules in vascular endothelial cells which are required in angiogenesis (7,62-66,80,81,82). The notable one is VE-cadherin. VE-cadherin is a member of the cadherin family and uniquely expressed in endothelial cells. Its level, location and function govern the formation of endothelial tubules. Supplementation of GLA to normal human vascular endothelial cells significantly reduced the level of VE-cadherin in the endothelial cells (figure 8). This appear to be a transcriptional event, as the mRNA level of VE-cadherin was markedly reduced by the fatty acids (Cai J,

Jiang WG, and Mansel RE. unpublished data) (83). Some of these effects may be attributed to the metabolites of fatty acids (84).

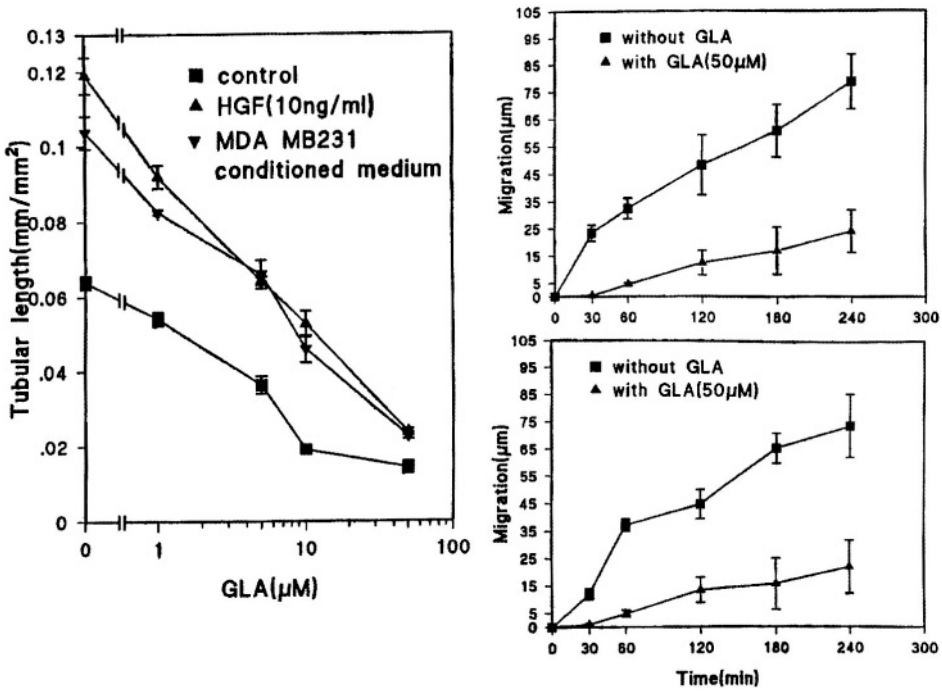

Figure 7. Inhibition of angiogenesis by PUFAs (77). GLA was found to reduce tubule formation of human umbilical vein endothelial cells (HUVECs) (left). This was associated with a reduction of migration by GLA, in both HGF/SF (top right) and tumour cell (bottom right) treated HUVEC cells.

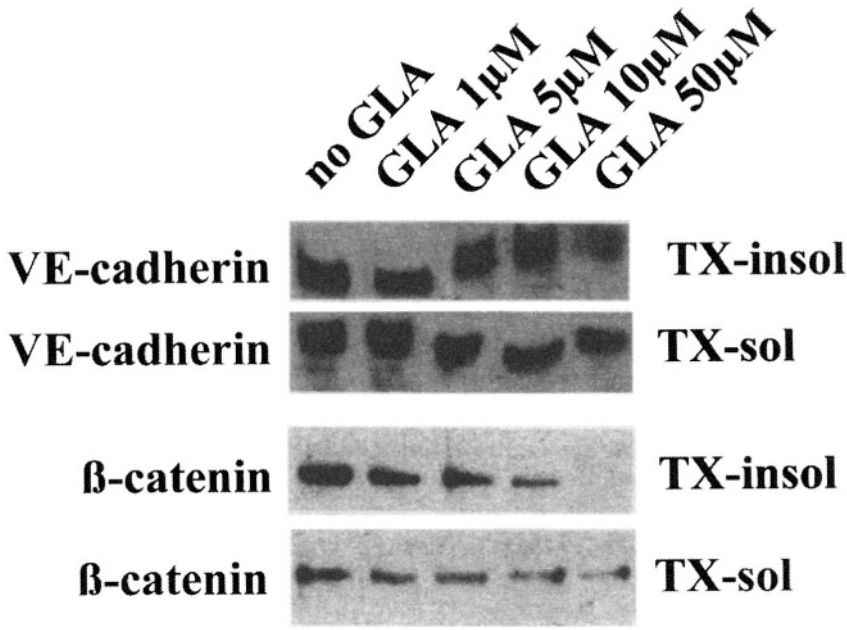

Figure 8. Effects of GLA on the expression of VE-cadherin (83). Treatment of HUVEC cells with GLA markedly reduced the level of VE-cadherin both in Triton soluble and Triton insoluble fractions

EFAS AND CANCER CELL MOTILE BEHAVIOUR:

The motility of tumour cells is required in the whole metastatic process (figure 2). The motility increases when cancer cells are stimulated by a number of motility factors (also known as motogens). These motogens are known to increase in hosts bearing tumours (85). The effect of PUFAs on tumour cell motility and motogen-stimulated motility has been recently reported.

GLA, at non-toxic concentrations, reduced both spontaneous and motogen-mediated cell motility and migration on matrix-coated surfaces (21,86). Other fatty acids, such as AA, also modified cytokine-mediated cell morphology and migration (87,88). Cell motility is a complicated process involving a number of events including plasma membrane and cytoskeleton reorganisation and numerous intracellular signalling pathways. Although firm mechanisms are yet to be established, the following have been proposed: (1). PUFA may affect the tumour cell membrane ruffling process, a dynamic parameter in tumour cell motility, by inhibiting the tyrosine phosphorylation of ezrin, an intracellular molecule implicated in this process (30,89); (2). Their metabolites may also act as regulators of cell motility (90). (3). PUFAs modify those molecules involved in cell motility, such as maspin (figure 9) (39,40).

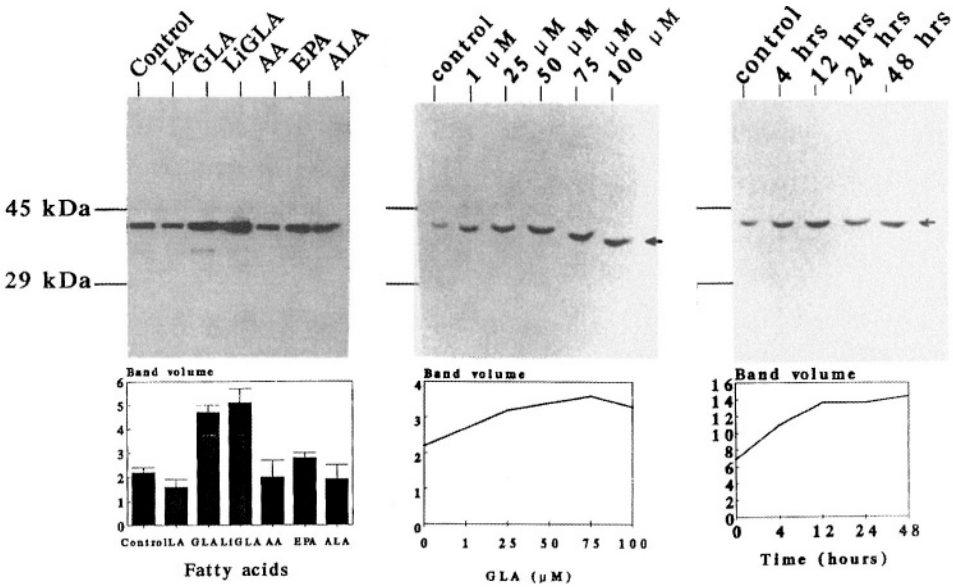

Figure 9A. Effects of GLA on the expression of maspin and motility of cancer cells. Human colon cancer cells were treated with different fatty acids (left), GLA at different concentrations (middle), or with GLA for different time periods. The level of maspin protein was determined using Wetern blotting. GLA increased the level of maspin .

Figure 9 continued.

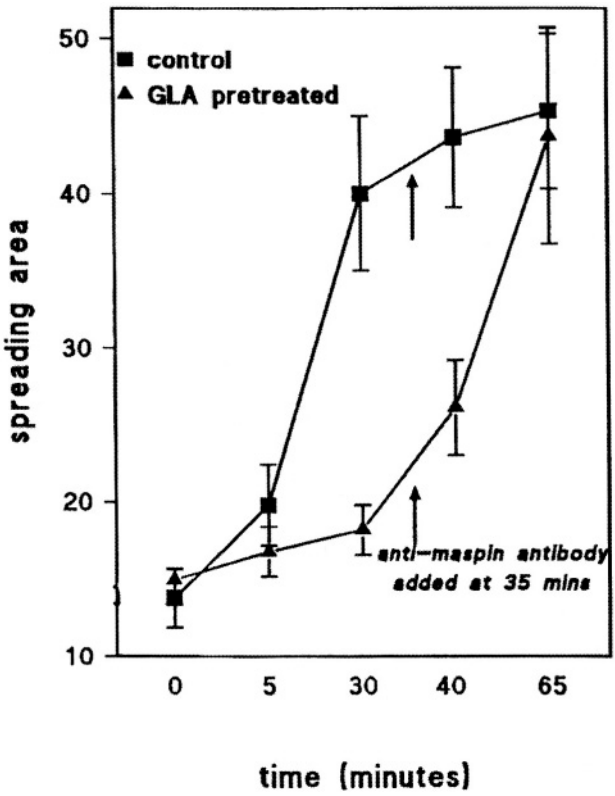

Figure 9B. Colon cancer cells were pre-treated with GLA and then added to a matrix coated culture surface. Cells without any treatment were used as control. Control cells showed a rapid spreading over the matrix. However, cells pretreated with GLA exhibited a slower spreading. Addition of an anti-maspin antibody restored the spreading of GLA-treated cells to a control level.

ANIMAL STUDIES ON PUFAS AND CANCER METASTASIS

Over the past decades, there have been a series of reports, whether intentionally or incidentally, showing that manipulating EFAs in cancer bearing animals may have important impact on the development of cancer metastasis. This has yielded some interesting, but sometimes controversial results

Breast cancer

There have been extensive research on the possible role of fatty acids in the development of breast cancer. However, the conclusions have been controversial. Fritsche and Johnston (91) determined whether plant or marine dietary (n-3) fatty acids would affect mammary tumour

growth and metastasis on weaning female BALB/c mice in comparison to corn oil fed animals. An inhibitory effect of dietary linolenic acid (i.e., 18:3 (n-3)) but not of fish oil on mammary tumour growth and metastasis was observed. High oleate and low linoleate are seen in EFA-deficiency, a condition which may favour tumorigenesis (93,94). The total amount of fat in the diet also contributed to the development of mammary tumour (95). With an increase in the dietary fat, there is an increase in experimental mammary gland tumorigenesis. The longer the duration of the high-fat diet, the greater the enhancement of tumorigenesis. These animal studies demonstrated that the amount, type, and the degree of saturation are important factors that determine their role in breast cancer development. But confusion arises from other studies. For instances, Karmali et al. (96) tested the effect of dietary n-3 and n-6 fatty acids on metastases in an experimental model of metastasis (13762MAT:B mammary adenocarcinoma cells) in weanling female Fischer 344 rats. A diet containing 23.5% corn oil can enhance experimental metastasis of mammary adenocarcinoma cells, compared with diets with low-corn oil diet or a high-fat diet containing a mixture of marine and plant n-3 fatty acids plus n-6 GLA. The total number of metastatic foci and tumour volume were the smallest in the group receiving a combination of plant and marine n-3 fatty acids.

Rose and coworkers (17,23) obtained contradictory results with n-6 PUFAs. Athymic nude mice were fed isocaloric diets containing 20% (wt/wt) fat but providing 8% GLA or LA for 7 days. Breast tumour cells were then injected into a thoracic mammary fat pad. The diets were continued for a further 11 weeks. The primary tumour growth rates were similar in mice from the two dietary groups. However, a higher incidence of macroscopic lung metastases, although not statistically significant, and higher volumes of total lung metastases were seen in the GLA-fed, than in the LA-fed mice. Zymography showed higher 92-kDa type IV collagenase activity in tumours from 8% GLA-fed mice. Interestingly these authors observed a bi-phasic response, i.e. low concentrations of GLA and LA stimulated whereas higher dose mildly inhibited the growth of MDA-MB-435 cells. In addition, LA stimulated tumour cell invasion and 92-kDa type IV collagenase production in vitro whereas GLA inhibited invasion and did not induce activity of the proteolytic enzyme. The same authors (97, 98, 99,100) further compared the effects of diets containing LA with those of diets containing EPA and DHA on the growth and metastasis of MDA-MB-435 human breast cancer cells in a nude mouse model. A stimulatory effect of n-6 polyunsaturated fatty acids on both primary tumour growth and metastasis was demonstrated (101). In contrast, the long-chain omega-3 fatty acids were inhibitory.

A recent study (102) also revealed the effects of fish oil and safflower oil on the growth and metastasis of an animal model of breast cancer. Tumour latency, growth and metastasis were assessed in mice fed diets that contained either 10 or 20% total fat containing either menhaden fish oil (FO), safflower oil (SO) or a 50/50 mixture of the two. Tumour latency was significantly longer and tumour growth significantly slower in mice fed the 20% FO diet. When spontaneous metastasis was assessed, mice fed diets containing FO had significantly decreased numbers of pulmonary nodules and total metastatic load. Likewise, mice fed FO diets had a lower level of implantation and survival of pulmonary metastases. Thus, diets containing n-3 fatty acids in fish oil significantly decrease primary breast tumour growth and its metastasis. Taken

together, these data suggest that experimental metastases of mammary gland tumours can be suppressed by n-3 fatty acids whereas the role played by n-6, especially 18:2 n-6, remains to be established.

Colorectal cancer

Several epidemiological and experimental studies have indicated a strong relationship between different types of dietary fats and the risk of colon cancer. However, the modulating effects of these nutritional factors on the metastases formation are not fully elucidated. Griffini et al (103) analysed the effects of n-3 and n-6 PUFAs on the development of experimentally induced colon carcinoma metastasis in rat liver. Animals were kept on either a low-fat diet or on a fish oil (n-3) or safflower oil (n-6) diet for 3 weeks before the injection of colon cancer cells to the portal vein. At the end of the first week after transplantation, the group with fish oil diet had significantly more metastases, both in number and size, when compared with groups receiving low-fat diet. However, the safflower oil diet had no effects on the number and total volume of metastases. At 3 weeks after tumour transplantation, groups receiving fish oil diet and the safflower oil diet had 10- and 4-fold more metastatic foci, respectively. The sizes of metastatic tumours in these two groups were much larger than groups on low-fat diet. These differences appears to be sex independent. Immunohistochemical analysis further revealed that the immune system in the liver (Kupffer cells, pit cells, T cells, newly recruited macrophages, and the activation state of macrophages) did not play a significant role in this diet-dependent outgrowth of tumours. The study thus indicates that n-3 and n-6 PUFAs promote colon cancer metastasis in the liver without down-regulating the immune system. However, one must take into consideration that the experimental approach (directly infusing tumour cells into the portal veins) was completely different from the previously mentioned studies. However, a recent study revealed that animals receiving EPA and LA have different effects on the liver metastases of colorectal cancer cells, ACL15. The study by Iwamoto and colleagures (104) demonstrated that in EPA treated group, there was a reduction of liver metastases, when compared with those fed with LA and saturated fatty acid. It has been further revealed that in EPA treated animal, there was a reduction of VCAM in endothelial cells, suggesting a possible reduction of tumour-endothelial adhesion by EPA.

Skin tumours

In an animal model of the benz-a-pyrene (BP)-induced skin papilloma, it has been shown that mice fed on 10% corn oil had the longest latency period and among the lowest incidence of skin papillomas when compared to those receiving a lower percentages of this dietary oil (105,106). The study suggests that tumour promoting activity of dietary linoleic acid may have "target tissue specificity". However, the animal studies are made complicated by the fact that in EFA deficient animals, there were less lung colonisation from melanoma than normal controls (107)

Prostate cancers

The antimetastatic effects of PUFAs in prostate cancer cells have been demonstrated, in vitro. Oleic acid (OA) and EPA enhanced DU-145 prostate tumor cell proliferation at 0.004 and 0.04 mM for up to 4 days (108). However, alpha-linolenic acid (ALA), linoleic acid (LA), GLA and arachidonic acid (AA) suppressed cell proliferation under the same conditions, possibly as a result of inhibition of DNA and protein synthesis. Therefore, the absence of EFAs and/or the excess amount of non-EFAs, such as oleic

acid, may affect invasion and metastasis of prostate cancer (93,94). In vivo, the matter appears to be complicated. In animal studies, groups receiving 23% fat diets containing 18% corn oil (CO)-5% linseed oil (18:2n-6 FA-rich), or 18% linseed oil (LO)-5% CO (18:3n-3 FA-rich), had a higher tumour grow rate that the group receiving 18% menhaden oil (MO)-5% CO (20:5 and 22:6n-3 FA-rich). This inhibitory effect of the high-fish diet was due, at least in part, to a reduction of arachidonic acid available for prostaglandin biosynthesis. Furthermore, groups fed with a linoleic acid rich diet had a higher growth rate that that fed with a low-fat diet (100).

Lung cancer

Data regarding the role of dietary fats in lung tumorigenesis are scarce and inconsistent. Diets supplemented with 18% corn oil enhanced carcinogen-induced tumorigenesis in hamsters (109). Mice receiving diets containing up to 31% of cottonseed oil, however, did not exhibit any increase of spontaneous lung tumorigenesis (110). A low tumour promoting activity in the lungs was seen in C57BL6J mice fed with corn oil diet enriched with n-6 FAs (94). A variant of the Lewis lung carcinoma (H59) that metastasizes preferentially to the liver, was investigated by Coulombe et al. (111). C57BL/6 female mice were fed different diets containing either no fats (TEK), 8% of fish oil (POL), linseed oil (LIN), safflower oil (SAP) or beef tallow (BT) and were subcutaneously injected with H59 cells. The n-3 PUFA-rich diets (LIN and POL) elicited more metastases than the n-6 PUFA-rich (SAP), fat-free (TEK), or saturated fats (BT) diets. However, dietary fat did not influence the ganglioside composition of either the primary or the metastatic tumours.

Another development in this tumour type has been the use of conjugated linoleic acid (CLA) (112). CLA has been demonstrated to inhibit proliferation of a number of human malignant tumour cells including melanoma, colorectal, breast and lung cancer cell lines. In animals, CLA reduced the incidence of epidermal tumours and forestomach neoplasia in mouse, aberrant crypt foci in rats colon and also mammary tumorigenesis. Further study has demonstrated an effect of CLA on prostate tumour (113). SCID mice were fed with either a standard diet, diets supplemented with 1% linoleic acid (LA) or 1% CLA for 2 weeks prior to subcutaneous (s.c.) inoculation of DU-145 cells. Mice receiving LA-supplemented diet displayed significantly higher body weight, lower food intake and increased local tumour load as compared to the other two groups. Mice fed the CLA-supplemented diet displayed not only smaller local tumours, but also a drastic reduction in lung metastases.

EPIDEMIOLOGICAL DATA

Mammary gland

Bougnoux et al (114) studied the relationship between the levels of various fatty acids in adipose breast tissue and visceral metastases in a cohort of 121 patients with an initially localised breast cancer. Adipose breast tissue was obtained at the time of initial surgery, and its fatty acid content analysed by capillary gas chromatography. A low level of alpha-linolenic acid (18:3n-3) in adipose breast tissue was associated with positive axillary lymph node status and with the presence of vascular invasion, but not with tumour size or mitotic index. After an average 31 months of follow-up, 21 patients developed metastases. Large tumour size, high mitotic index, presence of vascular invasion and low level of 18:3n-3 were the single factors significantly associated with an increased risk of metastasis. Low 18:3n-3 level and

large tumour size were the two factors predictive of metastases, using the Cox proportional hazard regression analysis. Their results suggest that host alpha-linolenic acid has a specific role in the metastatic process in vivo.

Prostate

Differences in the metabolism of EFA between patients with benign or malignant prostatic disease may help to elucidate their role in the development of the tumours. Chaudry et al (115) demonstrated that the phospholipid components of total lipid was greater in malignant (P less than 0.04, unpaired t-test) than in benign prostate tissues. The concentrations of linoleic acid (LA) and di-homo gamma linolenic acid (DGLA) in plasma and tissue were not different between the two groups. However, a significant reduction of arachidonic acid (P less than 0.002, Mann-Whitney U-test) and docosapentaenoic acid (DPA) (P = 0.009) concentrations was observed in malignant tissue as compared to benign. Patients with malignant prostatic disease also had a significantly higher concentration of oleic acid in phospholipids of both plasma and prostatic tissues. The stearic to oleic acid ratio was similar in plasma but was significantly reduced in malignant tissue (P = 0.006). It was suggested that the decreased arachidonic acid concentration in malignant tissue may be due to an increase of conversion to eicosanoids, rather than an impairment in desaturase activity in situ.

In a randomized control study, the effect of dietary n-3 polyunsaturated fatty acids plus vitamin E on the immune status and survival of well-nourished and malnourished patients was investigated (116). Sixty patients with generalised solid tumours were randomized to receive dietary supplementation with either fish oil (18 g of n-3 polyunsaturated fatty acids, PUFA) or placebo daily until death. Each group included 15 well-nourished and 15 malnourished patients. Total T cells, T-helper cells, T-suppressor cells, natural killer cells, and the synthesis of interleukin-1, interleukin-6, and tumour necrosis factor by peripheral blood mononuclear cells before and on Day 40 of fish oil supplementation, were determined. Karnofsky performance status, nutritional state, and survival were also estimated. The ratio of T-helper cells to T-suppressor cells was significantly lower in malnourished patients. N-3 PUFA had a considerable immunomodulating effect, as seen by an increase of this ratio in the subgroup of malnourished patients. There were no significant differences in cytokine production among the various groups, except for a decrease in tumour necrosis factor production in malnourished cancer patients, which was restored by n-3 fatty acids. The mean survival was significantly higher for the subgroup of well-nourished patients in both groups, whereas n-3 fatty acids prolonged the survival of all the patients. Hence, malnutrition appears to be an important predictor of survival for patients with late stage malignancies. Whereas n-3 polyunsaturated fatty acids had a significant immunomodulating effect and seemed to prolong the survival of malnourished patients with generalized malignancy.

CLINICAL IMPLICATIONS

Clinical considerations

From the in vitro and animal studies discussed above, it is clear that some properties of HUFAs make them attractive options in the treatment of cancer: 1. HUFAs modify cell membrane phospholipids; 2. HUFAs modify cellular functions which may reduce tumour motile/invasive potential; 3. HUFAs are directly toxic to tumour cells; 4. HUFAs modify the sensitivity of tumour cells to

chemotherapeutic agents and to radiation; 5. HUFAs exert a protective role towards normal tissues (in radiation); 6. HUFAs are low in cytoxicity to normal cells (117,118). These reasons have prompted clinical studies in patients with cancer. However, most studies reported so far are at early stage, on relatively small scale (small number of patients and limited tumour types), and use rather simple strategies, i.e. by supplementing fatty acid alone.

Clinical studies on patients with cancer

Patients with the following tumours have been tested using fatty acids:

1. Gliomas and astrocytoma:

Local delivery of GLA (intra-cerebral) in 15 patients with malignant gliomas resulted in regression when evaluated by computerised tomography and increased survival of the patients by 1.5-2 years (119). Patients with cerebral astrocytoma showed improvement in general condition as well as tumour (120).

2. Breast cancer:

As already discussed, there have been significant interests and controversies in the effects of PUFAs on breast cancer. A recent clinical study has tested 32 patients with breast cancer with tumour spread to the lymph nodes in the axilla, by giving patients a combination of nutritional antioxidants (Vitamin C: 2850 mg, Vitamin E: 2500 iu, beta-carotene 32.5 iu, selenium 387 μg plus secondary vitamins and minerals), essential fatty acids (1.2 g gamma linolenic acid and 3.5 g n-3 fatty acids) and Coenzyme Q(10) (90 mg per day). It is very interesting to note that none of the patients died during the study period (18 mths) (the expected number was four). None of the patients showed signs of further distant metastases and quality of life was improved (no weight loss, reduced use of pain killers). Further to this, six patients also showed apparent partial remission (121).

3. Pancreatic cancer:

A recent multicenter study of 48 patients with advanced pancreatic cancer showed (127) that Infusion of GLA increased survival. Eighteen patients with unresectable pancreatic cancer receiving dietary supplementation orally with fish oil capsules containing eicosapentaenoic acid 18% and docosahexaenoic acid 12% also had an improvement in weight loss and cachexia (122). It has been suggested that this anti-cachectic effect of EPA was due to its regulation of acute phase proteins and inflammatory cytokines such as IL-6 and TNF.

4. Colorectal cancer.

60 patients with sporadic adenomas receiving fish oil which was high in EPA showed a decrease in the proliferative indices and mucosal arachidonic acid levels (123). However, the beneficial effects seen in these studies have not been repeated in a controlled trial with late stage colorectal cancer, in which patients with Dukes's C colorectal cancer receiving GLA did not have benefit in terms of survival (124).

5. Liver cancer:

Van der Merwe (120,125,126), in a double blinded trial in patients with liver cancer, indicated an improvement in survival (42 days in control group and 90 days in GLA treated group) and retardation of tumour growth in these patients. In these studies, GLA was delivered in the form of evening primrose oil (120). This may reflect the observations made in in vitro studies that liver cancer cells are amongst the most sensitive cells to GLA.

6. Other tumour types.

Fatty acid (GLA) has also been given to patients with malignant mesothelioma,

renal cell carcinoma, lung and gastric carcinomas (120). Although clinical improvement and weight gain were observed in these patients, the sample number was too small to make a fair assessment and more extensive studies are required.

Polyunsaturated fatty acids have been demonstrated to play a role in the regulation of the invasive and metastastic behaviours. So far, most evidence has come from in vitro studies. However, animal studies and certain epidemiological studies have revealed a possible link between the amount, and the type, the purity of these lipids with cancer metastasis. Early clinical studies have shown signs of promises in using PUFAs as a possible means of treatment of cancer and cancer metastasis. However, there remains a large number of un-answered questions and controversies, as already discussed in the previous sections. Some of the key issues are high lighted in the following. 1 .The key mechanism, by which PUFAs regulate invasive/metastatic process. We yet have to develop a core explanation to the diverse effects exerted by PUFAs in the metastatic process. 2. The potential mechanisms by which PUFAs govern gene transcription. It is unlikely that any one mechanism can fully elucidate the nuclear actions of PUFA. As regulators of gene expression, PUFAs (or metabolites) are thought to affect the activity of transcription factors, which in turn target key cis-linked elements associated with specific genes. This will be a potential interesting area to explore. 3. Narrowing the gap between in vitro and animal studies, between animal study and human studies. 4. Improve formulae that may allow more effecient delivery to patients. 5. Development of effective combination between other forms of therapy, such as chemotherapy, radiotherapy and hormone therapy.

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Chapter 10

METASTATIC ENDOCRINE CANCER

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INTRODUCTION

Malignant endocrine tumours although rare present unique problems and challenges to the clinician. These tumours often have a high potential for local and distant metastatic spread and give rise to dramatic clinical syndromes by virtue not only of the local, mechanical and anatomical features of the disease but also by the unwanted humoral effects resulting from secretion of a wide range of hormones and polypeptides. It is undoubtedly true that the treatment of many endocrine malignancies, even when there are lymph node metastases, is extremely successful and rewarding. This is well illustrated by papillary thyroid carcinoma in a child where in spite of cervical lymph node spread thyroidectomy and lymphadenectomy result in long term survival approaching 100% (1). This very high success rate for the treatment of malignant endocrine disease is in marked contrast to that seen with many most other malignancies. However some endocrine organs display a less favourable picture perhaps well shown by adrenocortical adenocarcinoma with a five year survival rate of only 16% (2).

Many treatment modalities are available for the management of metastatic endocrine disease and these include chemotherapy, somatostatin analogues, tumour embolisation and surgical resection of metastatic disease. The adverse effects consequent upon excess humoral secretion are often such a dominant clinical feature that tumour debulking is capable of inducing excellent palliation and even contributing to long term survival.

In a chapter dealing with the management of metastatic endocrine disease the essential problems and concepts can only be fully discussed in the light of a complete understanding of the nature of the primary tumour and the optimum treatment which should be applied to that stage of the disease in its various forms. This is well illustrated by the treatment of the different types of thyroid cancer. Although thyroid cancer is a relatively rare disease (incidence of

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3.4 – 4.7 per 100,000 population) it is nevertheless the commonest endocrine malignancy (3). Therefore it is entirely appropriate that a large section of the chapter will be devoted to the consideration of this disease.

The management of endocrine tumours also well illustrates the need for a multidisciplinary approach to the patient with excellent co-operation between endocrine surgeon, endocrinologist, oncologist, radiologist, pathologist and biochemist.

Table 1. Type and Incidence of Thyroid Carcinoma.

Carcinoma	Age
Papillary (DTC)	70-80
Follicular (DTC)	10
Hürthle Cell (DTC)	3
Medullary	5-10
Lymphoma	1
Anaplastic	1

DTC= Differentiated Thyroid Carcinoma

THYROID CANCER

Incidence and Pathology

Thyroid cancer is a relatively rare malignancy accounting for less than 1% of all malignancies. Only 0.5% of cancer deaths are due to thyroid cancer with one death per million in the United Kingdom and surprisingly six deaths per million in the United States (4-6). Approximately 90-95% of thyroid cancers are *differentiated thyroid cancers* (DTC) originating from thyroid follicular cells. A classification of thyroid cancer is shown in Table 1.

Papillary Carcinoma

This is the most common thyroid cancer, responsible for approximately 80% of cases. Patients tend to be in the 30-40 year age group with a female to male bias of 2:1 (7). Papillary cancer is the predominant thyroid malignancy in children and patients who have previously been exposed to ionising radiation of the head or neck (8).

Macroscopically tumours are hard and grey/white in colour and lack a tumour capsule. Multifocal disease is common (80-85%). Tumours may erode through the thyroid capsule to invade adjacent structures such as oesophagus, trachea and recurrent laryngeal nerves. Histologically a '*fern like*' papillary pattern is usually seen, although tumours may exhibit a mixed papillary/follicular or a pure follicular pattern (follicular variant) but always behave like papillary. Occasionally, calcium deposits in a characteristic fibro vascular Stroma (*psammoma bodies*) a feature unique to papillary thyroid cancer may be identified. Diagnosis is confirmed by pathognomic, cytological features which include nuclear grooving, intranuclear cytoplasmic inclusions and *Orphan Annie* cells. *Tall cell, columnar, diffuse sclerosing* and *clear cell* are all described as histological variants of papillary cancer and are reported to be associated with a poorer prognosis (9).

Metastatic spread in papillary thyroid cancer is usually via intra-thyroidal lymphatic channels, paratracheal and cervical lymph nodes. Distant metastases

are a late feature, lung and bone being the most common sites.

Three forms of papillary thyroid cancer are recognised based on tumour size, macroscopic and histological invasion (Table 2). Classification is important and has a bearing on the management of both primary and metastatic disease.

Rarely, papillary thyroid cancers may be encapsulated and this is reported to be associated with a better prognosis. Lymph node metastases, vascular and lymphatic invasion may be seen in all types of papillary thyroid cancer. Factors associated with a poorer prognosis include tumour size, vascular invasion, cell type (see above) and the older patient (>40 years). Coexisting thyroid lymphocytic infiltration may be associated with a better prognosis.

Table 2. Histological Classification of Papillary Thyroid Carcinoma

Type	Size (cm)	Thyroid Capsular Invasion	Regional Lymph Node Metastases	Distant Metastases
Microcarcinoma	< 1	No	Rare	Rare
Intrathyroidal	> 1	No	Yes	Rare
Extrathyroidal	> 1	Yes	Yes	Yes

Table 3. Histological Classification of Follicular Thyroid Carcinoma

Type	Invasion of Tumour Capsule	Angioinvasion	Regional Lymph Node Metastases	Distant Metastases
Minimally Invasive	Minimal	No	No	Rare
Frankly Invasive	Yes	Yes	Rare (<10%)	Yes

Follicular Carcinoma

Approximately 10% of thyroid malignancies are follicular carcinomas. Mean age at presentation is 50 years, compared with 35 years in patients with papillary carcinoma. Women are affected three times more commonly than men. Overall, follicular carcinoma carries a slightly worse prognosis than papillary carcinoma (10).

Cancers are usually solitary and encapsulated. As with papillary cancer, local invasion into adjacent structures may occur. In contrast to papillary cancer, local lymph node metastatic spread is encountered in < 10% of patients and usually a feature of advanced disease. Blood borne metastases to bone, liver and lung are more common. Histologically, follicular and colloid formation is dependent on the degree of tumour differentiation present. Irrespective of

size, follicular cancers are divided into two main types depending on the degree of tumour invasion encountered histologically (Table 3).

Hürthle Cell Cancer

These tumours account for approximately 3% of thyroid cancers. The oxyphilic Hürthle cell cancer is composed mostly of cells with eosinophilic granular cytoplasm and large clear nuclei. Pathologically cancers are classified and behave in the same fashion as follicular cancers but with increased incidence of multifocality and lymph node metastases. Some authors believe this cancer to be associated with a slightly worse prognosis than follicular cancers. This belief, however, is not universally held (11).

Medullary Carcinoma

Originating from the calcitonin secreting C cells, medullary thyroid cancer (MTC) is responsible for about 5%-10% of all thyroid malignancies. Tumours are usually located in the superior thyroid poles. They may occur in isolation (sporadic), be familial associated with multiple endocrine neoplasia (MEN) Type 2A (MTC, hyperparathyroidism and phaeochromocytoma) or Type 2B (MTC, phaeochromocytoma, mucosal ganglioneuromatosis and Marfan like habitus) or be familial with no MEN association (FMTC) (12-15).

In hereditary cases the development of C cell hyperplasia always precedes the onset of malignant change. Tumours therefore are frequently multiple and bilateral tumour formation is common. In contrast, sporadic MTC is usually unilateral (though 25% of cases are bilateral). The increased C cell mass is associated with raised circulating levels of serum calcitonin. This may be measured by radioimmunoassay and provides an excellent tumour marker for MTC (16,17). Positive immunohistochemistry for calcitonin and the presence of amyloid in tissue sections is confirmatory of the tumour.

As with all thyroid cancers, local invasion of adjacent structures may occur. Metastatic spread occurs initially via local lymphatics to paratracheal and cervical lymph nodes and progresses into the superior mediastinum. Haematogenous spread follows at a later stage, usually to liver, bone and lung.

Anaplastic Carcinoma

This highly aggressive thyroid cancer is uniformly fatal. Death often occurs within six months of initial presentation and almost always by one year. Tumours usually occur in the elderly female population and are rare before 50 years of age. Anaplastic change in a previous DTC is probably responsible for most cases (18,19).

Cytologically cells show marked heterogeneity and are usually identified on fine needle aspiration cytology (FNAC). Tumour growth is extremely rapid, enveloping and invading local structures such as trachea, recurrent laryngeal nerves, oesophagus, carotid vessels and jugular vein at an alarming rate. Metastatic spread occurs early to local lymph nodes and though haematogenous spread.

Lymphoma

This disease is frequently mistaken for small cell anaplastic carcinoma, presenting most commonly in elderly female patients. Most cancers are of the non-Hodgkin B cell type. Pre-existing chronic lymphocytic thyroiditis (Hashimoto's thyroiditis) with or without hypothyroidism necessitating thyroxine

treatment is usually present (20). Chronic antigenic lymphocytic stimulation leading to the development of carcinoma is suggested as a possible cause.

CLINICAL PRESENTATION OF METASTATIC DTC

Thyroid carcinoma usually presents with a palpable thyroid nodule in a euthyroid patient (papillary microcarcinomas being the exception). Following primary treatment, patients should be closely monitored at regular intervals. Assessment should include clinical examination and measurement of serum tumour marker levels.

When clinical examination reveals a palpable mass, suggestive of local recurrence in the thyroid bed and/or regional metastases (cervical lymphadenopathy), diagnosis can usually be confirmed by fine needle aspiration cytology (FNAC).

Distant spread is usually to lung, bone and liver. Symptoms are not specific but may include lassitude, loss of appetite, weight loss or general malaise. Dyspnoea or haemoptosis suggest pulmonary disease, jaundice, hepatomegaly or hepar pain, indicate liver disease and bone pain with or without a palpable mass suggest bone secondaries.

DIAGNOSTIC CONFIRMATION OF METASTATIC DTC

Thyroglobulin (Tg)

Normal and neoplastic thyroid tissue, produce a unique tumour marker, thyroglobulin (Tg). Following total thyroidectomy for DTC, serum Tg levels should theoretically fall to zero, although levels of <3 ng/ml are deemed acceptable. As TSH is the best stimulus for Tg production a baseline Tg level should be obtained just prior to ^{131}I scanning when the patient is hypothyroid following total thyroidectomy. Thyroglobulin levels may then be monitored routinely at regular intervals in patients who have undergone total thyroidectomy or checked when recurrent or metastatic disease is suspected clinically. Rising Tg levels are suggestive of recurrent or metastatic disease and ^{131}I scanning should then be performed (21,22). Tg measurement has virtually replaced ^{131}I scanning in the preliminary screening for metastatic disease, although thyroglobulin antibodies may lead to falsely low Tg levels in the presence of metastases (23).

Suspected metastatic bone disease, where possible, should be biopsied and histological specimens stained for thyroglobulin. Positive immunocytochemistry for Tg is diagnostic of metastatic DTC.

Chest X-Ray

Patients with DTC should have a regular chest X-ray every 3rd year as an integral part of a routine follow up. Pulmonary metastases may be evident even in the absence of any clinical symptoms. When the chest X-ray is suggestive of pulmonary metastatic disease in DTC then ^{131}I scanning should be performed to confirm the diagnosis.

Radioiodine Scanning (^{131}I)

Radioiodine is a powerful tool in both the detection and treatment of DTC. Almost all follicular thyroid cancers and most papillary cancers concentrate ^{131}I. In papillary cancer, however, the proportion falls in the older age group (>50) to about 80%. In contrast, only 10% of Hürthle cell cancers concentrate ^{131}I but still retain the ability to produce thyroglobulin.

Identification of metastatic DTC is greatly facilitated by initial total or near total thyroidectomy. Removing all normal thyroid tissue prevents any residual thyroid tissue from competing for ^{131}I uptake, enhancing the diagnostic potential.

Following initial or completion total or near total thyroidectomy for DTC the patient is allowed to become hypothyroid over the course of 6-8 weeks. This may be achieved either by completely stopping all thyroid replacement if the scan date has been pre-arranged, or by placing the patient onto T3 (triiodothyronine) which is discontinued approximately 2 weeks prior to the scan. A low iodine diet may also enhance ^{131}I uptake. In most medical centres a small screening dose of ^{131}I is administered (2mCi) and uptake at 24 hours measured. This should be <1% following total thyroidectomy. '*Hot spots*' detected in the neck are usually residual thyroid bed tissue, although metastases in the cervical nodes or adjacent tissues may also be displayed. Larger doses of ^{131}I for screening purposes are unnecessary and may have the unwanted effect of suppressing the uptake of subsequent therapeutic ^{131}I (*stunning of thyroid tissue*) (24). Radioiodine is far more sensitive in detecting micrometastatic disease than chest X-ray or CT scanning (25).

Ultrasound, PET, Thallium-201, CT and MRI Scanning

All of these modalities have a role in the detection of recurrent local or distant metastatic disease. Ultrasound or CT guided FNAC or needle core biopsy may be particularly useful in providing tissue for cytological or histological assessment. MRI assessment of cervical and mediastinal disease is of benefit in many cases in evaluating the degree and extent of local invasion and in distinguishing between postoperative fibrosis (low intensity T2 images) and recurrent disease (high intensity T2 images). A study comparing MRI with thallium-201 scintigraphy in 39 patients who had undergone thyroidectomy and modified radical neck dissection for DTC, demonstrated that MRI (39/51) was more sensitive than thallium-201 (24/51) in detecting nodal tumour recurrence sites (26). However, patients with DTC where recurrent or metastatic disease is suspected but not demonstrated on ^{131}I scanning, thallium-201 scintigraphy should be considered.

Positron Emission Tomography (PET) using 18F-fluorodeoxyglucose (FDG) has been employed to identify pulmonary and mediastinal DTC metastatic disease. Whether this modality will prove useful in the continued management of metastatic thyroid cancer has yet to be shown (27).

MANAGEMENT OF METASTATIC DTC

The initial surgical management of the primary tumour in DTC is fundamentally important with respect to 1) Control of local recurrence, 2) Removal of regional metastatic disease and 3) Treatment of distant metastatic disease with ^{131}I.

Papillary Cancer

Intrathyroidal Spread.

Papillary thyroid cancer may spread via intrathyroidal lymphatic channels and at the time of the initial operation, co-existing, contralateral, intrathyroidal disease has been reported in 30% to 87.5% of patients with papillary thyroid cancer (28,29). Analysis of 576 patients followed for a median of 10 years demonstrated a recurrence rate of 11% in patients undergoing total thyroidectomy compared to 19% in patients undergoing

subtotal resection (30). Local recurrence is a serious complication with a mortality rate of 50% (30-32). An exception to total or near total thyroidectomy may be advocated for the management of papillary microcarcinoma or the rare, encapsulated papillary carcinoma, when intrathyroidal spread is rare and unilateral total thyroid lobectomy deemed adequate.

Local Invasion and Recurrence

In patients where extrathyroidal papillary cancer invades local structures, it may be necessary to include part of the trachea or oesophagus, recurrent laryngeal nerve or jugular vein in an en-bloc excision with the thyroid gland in an attempt to eradicate local disease. Recurrent local disease is best dealt with by repeated surgical resection. In experienced hands this has been shown to be associated with low mortality and morbidity with respect to RLN damage and hypoparathyroidism (33).

Nodal Metastases

Lymph node metastases are common in papillary thyroid cancer, often occurring at an early stage in the disease with relatively small primary tumours and frequently present at the initial presentation. Cervical lymphadenopathy is therefore not a sign of advanced primary disease and its presence does not appear to adversely affect prognosis. On occasions the initial clinical presentation may be of cervical metastatic disease alone (*lateral aberrant thyroid*).

Palpable cervical lymphadenopathy, confirmed as papillary thyroid cancer by FNAC, should be treated with modified radical neck dissection preserving the sternocleidomastoid muscle, internal jugular vein and accessory nerve.

Cervical micrometastases have been found in 30% to 90% of patients with papillary thyroid cancer. Lymph node involvement is particularly common in children. At primary operation, therefore, thyroidectomy should be accompanied by ipsilateral lymph node biopsy and frozen section histology. When lymph nodes are found to be positive for metastatic cancer, modified radical dissection in association with total or near total thyroidectomy should be performed. Prophylactic cervical lymph node dissection has not been shown to improve overall survival and is not necessary (34,35).

In contrast, central compartment lymph nodes should always be cleared as a matter of routine in papillary cancer (taking great care to preserve the recurrent laryngeal nerves and parathyroid glands).

Distant Metastases

Radioiodine Therapy (^{131}I)

Initial surgical management of the primary cancer has a profound bearing on subsequent management of any co-existing or subsequent metastatic disease. Metastatic DTC can be detected and treated by radioactive iodine in about 75% of patients (36). Total or near total thyroidectomy facilitates both diagnosis and treatment of metastatic disease by removing all normal thyroid tissue, thus preventing competition for iodine uptake and is probably the most powerful argument in favour of total thyroidectomy for DTC (30,37)

Following surgical resection or in patients where surgery is deemed not suitable, either because metastatic disease is occult (associated with raised Tg levels but not clinically palpable disease), medical or technical reasons, patients should be treated with ^{131}I ablation therapy. The treatment rational follows that for ^{131}I screening, with patients with patients being rendered hypothyroid and a therapeutic treatment dose of ^{131}I (150-200

mCi) administered. The maximum cumulative dose is 800-1000 mCi. Patients may therefore undergo repeated treatment with ^{131}I.

Results from ^{131}I ablation therapy for DTC are reproduced in several series, with complete response being reported in 35-45% of patients (25,38,39). Patients where raised Tg levels suggest occult disease and ^{131}I localising studies fail to identify any recurrent or metastatic disease, further studies should include chest X-ray, CT and thallium-201 scan. If disease is not localised administration of a therapeutic dose of ^{131}I may demonstrate and treat micrometastases in approximately 30% of patients (36,40)

The effectiveness of ^{131}I in treating pulmonary metastases has been demonstrated by Schlumberger et al who reported a 75% success rate in treating micrometastatic disease in DTC following total thyroidectomy. In macroscopic disease the response rate falls to < 10% (25,41).

Mazzaferri and Young detailed the use of ^{131}I as an adjuvant therapy following total thyroidectomy for DTC reporting a decrease in the incidence of loco-regional recurrence to 6.4% compared to total thyroidectomy alone (11%), an observation supported by other authors (32,42).

Recombinant TSH has been used to improve diagnostic ^{131}I scanning for metastatic disease prior to ablative therapy (43).

Radiotherapy (DXT)

In contrast to lung metastases, which concentrate ^{131}I and often respond well to treatment, bone metastases do not exhibit a similar propensity for ^{131}I uptake. External beam radiation controls associated pain more effectively. For patients with bone metastases a treatment protocol should be ^{131}I therapy and if uptake/response is poor, external beam radiotherapy (25,44). External beam radiotherapy may also be used to good effect in controlling unresectable, recurrent loco-regional disease which fails to concentrate ^{131}I.

TSH suppression therapy

Following treatment for recurrent disease, patients should be placed onto T4 (Thyroxine) at levels which suppress TSH (Thyroid Stimulating Hormone) to below 1.0 mU/L in patients at '*low risk*' and <0.1 mU/L in '*high risk*' patients (TSH suppression therapy). The effectiveness of this treatment in decreasing the incidence of regional recurrence has been demonstrated by Mazzaferri et al (30).

Chemotherapy

Chemotherapy has not been demonstrated to have any role in the management of metastatic papillary thyroid cancer.

Surgical Excision

When technically possible, well localised, isolated metastases of the liver, lung and bone may be surgically excised. Brain metastases are rare in DTC and associated with a very poor prognosis. Surgical resection is occasionally indicated in these patients to control neurological symptoms, which may often be severe.

Follicular Thyroid Cancer

Intrathyroidal Spread

This does not tend to occur as frequently as in papillary cancer. Tumours tend to be isolated, encapsulated (90%) and unilateral. Total or near thyroidectomy is advocated to facilitate ^{131}I screening for metastatic disease and increase the efficacy of therapeutic ^{131}I. An exception to total or near total

thyroidectomy may be made in minimally invasive follicular where total thyroid lobectomy and isthmusectomy are regarded as sufficient surgical treatment.

Local Invasion

Extrathyroidal invasion in follicular thyroid cancer is present at initial procedure in about 25% of patients (45). As with papillary thyroid cancer local invasion into adjacent structures should be managed where technically feasible by primary en-bloc excision to include the affected organs. The RLN on the affected side may need to be sacrificed if it cannot be separated or shaved from the tumour mass. On rare occasions, follicular thyroid cancer may invade directly into the jugular vein. In this situation it may be necessary to open the vein and retrieve the metastatic tumour which may extend as far as the right atrium.

Nodal Metastases

Regional lymph node metastases are rare in follicular thyroid cancer, occurring in only 10% of patients and are associated with advanced primary disease. Cervical lymphadenopathy present at initial presentation should be managed by modified radical neck dissection, in company with total thyroidectomy. Recurrent regional metastases should likewise be treated by modified radical neck dissection. Reduction in tumour load will increase the effectiveness of ^{131}I therapy.

Distant Metastases

Haematogenous spread to lung, liver and bone is more common in follicular than papillary thyroid cancer. Approximately 10%-30% of patients have distant metastatic spread present at initial presentation (45). Distant metastases may be treated by:-

Radioiodine

The treatment rational for radioiodine in DTC has already been outlined above. Tumour differentiation probably has a significant role in control or ablation of recurrent disease as more differentiated tumours are likely to concentrate ^{131}I. Overall, follicular thyroid cancer tends to concentrate ^{131}I better than papillary thyroid cancer. In contrast, Hürthle cell cancers as discussed above are an important exception.

Radiotherapy

As in papillary thyroid cancer, DXT may offer a role in the control of unresectable, residual primary tumour, locally recurrent disease, regional lymph node metastases and for the control of pain in bone metastases.

Chemotherapy

This treatment modality has little to offer in the management of metastatic follicular carcinoma of the thyroid.

MANAGEMENT OF METASTATIC ANAPLASTIC THYROID CANCER

Locally invasive with early lymphatic and distant metastatic spread, management of metastatic disease in this uniformly fatal condition is particularly difficult. Diagnosis can usually be obtained by FNAC. Incisional biopsy should be avoided as this can result in metastatic tumour in the incision (46). Surgery should be reserved only to relieve airway obstruction or in certain instances to debulk tumours (47). Tracheostomy may be necessary to relieve airway obstruction.

Local Invasion

Tumours invade quickly and aggressively into adjacent structures in the

neck and frequently involve the overlying skin resulting in ulceration. Thyroidectomy to gain local control is rarely feasible.

Nodal Metastases and Distant Spread

Lymphatic and haematogenous spread occurs early in anaplastic thyroid cancer. At presentation, cervical lymphadenopathy is present in 85% of patients and distant metastases in 75%. Metastases to lung are the most common (>80%), followed by the adrenals (30%), liver (17%), bone and brain (15%) (48,49). Historically the most favoured method of treating the primary cancer and associated regional lymph node metastases has been with external beam radiotherapy. In early series this was coupled with thyroidectomy in an attempt to control the local disease. Both response to treatment with DXT and control of local disease following surgery are extremely disappointing with local recurrence rates between 30 and 80% (50,51).

Attempts to improve survival with a variety of chemotherapeutic agents including doxorubicin, cisplatin and 5 Fluorouracil have also failed to produce any significant benefit, even when used in conjunction with DXT (52-54).

LYMPHOMA

Thyroid lymphoma is best managed by radiotherapy and/or chemotherapy, which also simultaneously treats any metastatic disease present (55,56). Thyroidectomy is necessary only in relieving obstructive symptoms and surgery should be limited to incisional biopsy for diagnosis.

MANAGEMENT OF METASTATIC MEDULLARY THYROID CANCER

Clinical Presentation

The signs and symptoms of loco-regional recurrence or distant metastatic medullary thyroid cancer (MTC) are essentially the same as those for DTC as outlined above. In patients with metastatic MTC, diarrhoea and/or flushing may be particularly troublesome and unpleasant features, probably due to prostaglandin secretion.

Diagnostic Confirmation of Metastatic MTC

Calcitonin

In patients with MTC, calcitonin provides a unique tumour marker and levels should be monitored regularly following total thyroidectomy. In a report of 123 patients with MTC treated by total thyroidectomy, postoperative calcitonin levels were low or non-detectable when MTC was confined to the thyroid gland (67%). In contrast only 8% of patients had low calcitonin levels when extrathyroidal spread was present (57). Further reports suggest that calcitonin levels rarely return to zero following total thyroidectomy in familial disease (58-60).

CEA measurements should also be performed in patients with MTC. Though not as sensitive as calcitonin in detecting recurrent or persistent disease, raised CEA levels are associated with a poorer overall prognosis (61).

Chest X-ray, Ultrasound, CT and MRI Scanning

The above radiological modalities can be used to locate metastatic disease. Ultrasound or CT guided FNAC or needle biopsy allow cytological confirmation of non-palpable recurrent, cervical or hepatic metastatic disease. MRI is potentially helpful in delineating scar tissue from recurrent disease in the neck and mediastinum.

In patients with raised calcitonin levels, in whom CT, USS or MRI have failed to localise metastatic disease, diagnostic laparoscopy may be employed. Hepatic metastases in MTC are frequently small and on the surface of the liver, allowing direct visualisation and biopsy (62).

DMSA, MIBG and Monoclonal Antibody Scanning in MTC

Nuclear imaging studies with ^{131}I metaiodobenzylguanidine (MIBG), dimercaptosuccinic acid (DMSA) and 99mTc-sestambimi have all been utilised in detecting metastatic MTC disease. Although useful on occasions, overall results have been disappointing and distant MTC metastases remain difficult to detect.

PET scanning in selected patients has also shown to be of benefit. A recent study by Juweid et al reports the use of radiolabelled anti-CEA monoclonal antibodies to detect residual MTC in patients with raised calcitonin levels following total thyroidectomy. They reported positive scans in 13 of 16 patients (81%) but whether this technique has a significant role to play in the management of metastatic MTC remains to be determined (63).

Management of Recurrent or Metastatic MTC

Multifocal Intrathyroidal Disease

Multifocal disease is present in 90% of patients with familial MTC and 20% with sporadic MTC (57). In contrast to papillary thyroid cancer this is not only due to intrathyroidal spread but is primarily a consequence of diffuse premalignant C cell hyperplasia. Medullary thyroid cancer also follows a more aggressive course than DTC with a higher propensity for local recurrence. Total thyroidectomy is therefore advocated in an attempt to eradicate the primary disease, decrease the incidence of local recurrence and allow the use of calcitonin as a postoperative tumour marker.

Nodal Metastases

Metastatic cervical lymph nodes are present and clinically apparent at initial assessment in 25%-60% of patients. This incidence is increased in familial disease compared to sporadic (64,65). Even when RET proto-oncogene screening has identified children at risk of MTC and thyroidectomy has been performed 'prophylactically', positive lymph nodes are found in 8.6% of cases (66,67).

Dralle and colleagues have described a technique of four compartment microdissection for clearing regional lymph nodes in MTC (65). Compartment A includes central and paratracheal nodes which should be cleared routinely in association with total thyroidectomy, taking care to preserve the RLN's and parathyroid glands. Compartments B and C include right and left lateral cervical nodes and compartment D, superior mediastinal nodes. In patients with palpable cervical lymphadenopathy,

unilateral or bilateral modified radical neck dissection should be performed. Prophylactic modified radical neck dissection should be performed in patients with tumours >2 cm in size, as 60% of these patients have microscopic metastatic disease present (68).

Distant Metastases

Rising serum calcitonin levels suggest recurrent disease and the localisation procedures outlined above should be performed. Sites for recurrent and metastatic disease include the thyroid bed, cervical nodes, superior mediastinal nodes, liver, lung and bone. When localisation techniques fail to identify recurrent or metastatic disease amenable to resection, following definitive surgery (total thyroidectomy, central compartment and modified radical neck dissection) a conservative approach has been advocated with good effect (69).

i) Surgical Excision

Repeated surgical excision or tumour debulking is of considerable benefit in controlling symptoms associated with local recurrence in the neck and selected distant metastatic disease (70). Procedures reported for distant disease include pneumonectomy or lobectomy for isolated pulmonary metastases, hemi-hepatectectomy or segmentectomy, oesophagectomy, mastectomy, pituitary resection or total hip replacement for isolated femoral head metastases (71,72).

ii) Radioiodine and Radiotherapy

Medullary thyroid cancer does not concentrate ^{131}I and attempts at treatment with radioiodine have failed to demonstrate significant response (73). Radiotherapy treatment results in significant fibrosis and scarring, rendering further surgical resection difficult and hazardous. Studies on the effect of radiotherapy in medullary thyroid cancer indicate a poor overall response (74,75). Use of DXT should therefore be limited to patients with unresectable residual disease, controlling pain associated with unresectable bone metastases or recurrent loco-regional disease not amenable to surgical excision.

iii) Chemotherapy

Chemotherapy with doxorubicin (adriamycin), 5-fluorouracil, dacarbazine (DTIC) and cisplatin, either as isolated agents or in varying combinations have all generally resulted in high toxicity and poor tumour response (76-80).

PARATHYROID CARCINOMA

Incidence and Pathology

Parathyroid carcinoma is one of the rarest endocrine cancers, responsible for less than 1% of all cases of primary hyperparathyroidism (HPT) (81). The rarity of the tumour has meant that large series and information on treatment modalities other than surgery are scarce. Factors which should raise the preoperative suspicion of parathyroid carcinoma in patients with primary HPT include corrected serum calcium levels in excess of 3.5 mmol/L, markedly elevated serum parathyroid hormone (PTH) levels (often 10 times upper limit of normal), severe symptomatic hypercalcaemia (including coma), bone disease, renal disease and a palpable neck mass.

At operation macroscopic findings include a firm, greyish white tumour surrounded by a fibrous capsule. Adherence and invasion of adjacent structures such as the thyroid lobe, strap muscles, oesophagus or recurrent laryngeal nerve is common (82). The diagnosis should be made by the surgeon on the basis of these clinical and operative

findings but requires a high index of suspicion.

Histological diagnosis of parathyroid carcinoma may be difficult. Schantz & Castleman outlined a set of criteria for making the diagnosis though rarely are all present (83). These include a fibrous capsule and/or fibrous trabeculae through the tumour, a rosette-like or trabecular cellular architecture, mitoses and capsular or vascular invasion. Many authors suggest that parathyroid carcinoma can only be confirmed by demonstrating distant metastatic spread.

Clinical Presentation of Metastatic Disease.

Long term follow up with periodic calcium and PTH measurement is essential in patients with parathyroid cancer. Following successful surgery for the primary tumour, serum calcium and PTH levels fall and hypercalcaemic symptoms should abate. Recurrence of hypercalcaemic symptoms (which tend to be more severe), include lethargy, malaise, polydypsia, polyuria, myotonia, bone pain, loss of appetite and psychiatric disturbance suggest recurrent or metastatic disease (84). Non-functioning cancers are reported but are rare (85).

Diagnostic Confirmation of Recurrent or Metastatic Disease

Recurrent or metastatic parathyroid carcinoma is confirmed by documenting hypercalcaemia and an elevated serum PTH.

Localisation of Recurrent or Metastatic Disease

Surgical resection remains the best method of controlling symptoms. It is essential therefore to accurately localise recurrent or distant metastatic disease.

Ultrasound CT and MRJ are all potentially helpful in the localisation of metastatic disease. Cervical and upper mediastinal disease may also be identified by ^{201}Tl-chloride scintiscanning (86-88). In the event that recurrent or metastatic disease cannot be accurately located, selective venous catheterisation coupled with sampling and PTH measurement, may localise disease to the neck, lungs or liver (84,86).

Management of Recurrent or Metastatic Disease

Local Recurrence

i) Surgery

Cervical tissues are the most common site for recurrent disease in parathyroid carcinoma. Local recurrence may result from the tumour capsule having been breached at the primary procedure. Undoubtedly the best way of decreasing the incidence of recurrent local disease rests with the surgeon recognising the possibility of parathyroid carcinoma preoperatively, confirming the diagnosis macroscopically at the initial operation for primary HPT and performing an adequate operation at the first surgical episode. This should be by performing an en-bloc excision of the tumour with the ipsilateral thyroid lobe and removing the tumour and all adherent or invaded structures without breaching the tumour capsule (81,88,89). On occasion this may necessitate sacrifice of the recurrent laryngeal (87).

Symptoms and metabolic complications are best palliated by an aggressive surgical approach to recurrent disease (90). Modified radical or radical neck dissection is advocated to clear metastatic regional recurrence.

ii) Radiotherapy

This treatment modality has not been shown to be effective in controlling symptom in patients with loco-regional recurrence of parathyroid carcinoma. In one reported series only one patient in a group of six receiving radiotherapy showed a significant response to treatment with radiotherapy (91).

Nodal Metastases

Lymph node metastases are uncommon and tend to occur late in parathyroid carcinoma. Prophylactic radical neck dissection is therefore unnecessary (92-94). Patients with palpable cervical lymphadenopathy should be treated by modified radical or when indicated, radical neck dissection.

Distant Metastases

i) Surgical Excision

Distant metastases occur in parathyroid carcinoma, having been reported in liver, mediastinum, pancreas, ribs, lumbar spine and lungs (87,95,96). This disease is best managed by surgical excision when technically possible.

ii) Chemotherapy

No response to chemotherapy with methotrexate, adriamycin, cyclophosphamide, fluorouracil and dacarbazine either alone or in combination has been demonstrated in parathyroid carcinoma (97-99).

In advanced cases of recurrent or metastatic parathyroid carcinoma severe hypercalcaemic may be difficult to control. Intravenous saline with frusemide to induce a diuresis may improve hypercalcaemia temporarily. Use of biphosphonates which bind to hydroxyapatite, inhibiting osteoclastic bone reabsorption has been used with varying success in lowering serum calcium and improving symptoms. The effect, however, is short lived and of limited use in terminally ill patients (100). Other drugs have also been used, including calcitonin, mithramycin and steroids all with disappointing effect (101-103).

Prognosis

Five year survival rates in parathyroid cancer differ in reported series between 29% and 50% (81,83,104). In a series of 11 patients reported over a 19 year period at the University Hospital of Wales, the authors reported a 90% survival rate at mean follow up of 9.6 years (105).

ADRENOCORTICAL CARCINOMA

Incidence and Pathology

Adrenocortical carcinomas are rare malignancies, accounting for approximately 0.02% of all carcinomas with a reported incidence of 2 cases per million population (106). Tumours most commonly present in the 30-50 year age group with a female to male ratio of 2:1. Most lesions are large (> 5 cm) and occasionally palpable. Local invasion into adjacent structures, local lymph node and distant metastases are frequently present at initial assessment.

Macroscopically areas of haemorrhage and necrosis are present in a firm, lobulated tumour which is grey/pink in colour on cut section. Microscopic features include numerous mitosis and vascular invasion. These features, however, may also be present in benign adrenal tumours, thus histological diagnosis of malignancy is often difficult.

Approximately 60% of adrenocortical carcinomas are functioning, secreting

steroid hormones which lead to a wide spectrum of clinical conditions.

Clinical Presentation

Non Functioning Tumours

Patients may present with an abdominal mass (tumours can grow to >20 cm), symptoms of weight loss, lassitude, fatigue, flank pain, a low grade pyrexia of unexplained origin. Despite the term non-functioning, subtle clinical changes, including mild hirsuitism can be evident. These changes may result from dehydroepiandrosterone production which has some value as a marker of malignancy. On rare occasions patients have presented with acute haemorrhagic shock from a sudden bleed into the tumour.

Functioning Tumors

Functioning adrenocortical carcinomas are responsible for Cushing's syndrome in 10% of patients presenting with the syndrome. In contrast to benign secreting tumours, onset of signs and symptoms is frequently rapid and distressing. Women are affected four times as commonly as men. The onset of Cushing's syndrome in a child or young adult should raise the suspicion of malignancy. Secreting tumours produce a diversity of clinical syndromes including hypercortisolism (30%), virilisation (22%), feminisation (10%). Mixed hormonal secretions resulting in a varied clinical picture are present in 35% of tumours and when present are almost always associated with malignancy. As with non-functioning carcinomas, tumours can be large at initial presentation, illustrated by the fact that over 15% of tumours > 6 cm are malignant (2).

Initial Surgical Management of Adrenocortical Carcinoma

Aggressive surgical resection is the treatment of choice for stage I and II cancers and in selected patients with stage III cancers (Table 4). Unfortunately, 45%-70% of patients have either stage III or stage IV disease at presentation, reflected in the very poor overall survival figures of this highly malignant cancer. Wide resection with en-bloc tissue removal and lymphadenectomy is necessary in an attempt to gain local control of the cancer. Adjacent structures, including spleen, kidney and pancreas may also be sacrificed to achieve adequate clearance.

Imaging of Local Recurrence and Distant Metastases

Suspected recurrent loco-regional disease or distant metastases to the liver and lung may be imaged with chest X-ray, CT or MRI. Tumour spread into the inferior vena cava may also be accurately assessed by MRI, making caval venography obsolete. T2 weighted MRI images are useful in differentiating recurrent tumour from scar tissue in the operative field. USS or CT guided FNAC or needle biopsy provides histological confirmation of local recurrence, hepatic or other metastatic disease.

Table 4. Staging of Adrenocortical Carcinoma

Stage	Size (cm)	Local Extension		Regional Lymph Nodes	Distant Metastases
I	< 5	No		No	No
II	> 5	No		No	No
IIII	-	Yes	Or	Yes	No
IV	-	Yes	+	Yes	(Yes)

MANAGEMENT OF RECURRENT OR METASTATIC ADRENOCORTICAL CARCINOMA

Local Recurrence

Survival rates in patients with adrenocortical carcinoma are abysmal. Careful assessment is therefore necessary to identify at an early stage patients with locally recurrent or distant metastatic in whom repeated surgical resection may be of benefit in alleviating symptoms and controlling local disease (2,107,108). Few patients are suitable for repeated surgical resection. Only 7 of 41 patients in one series and 3 of 19 in another report were considered candidates undergo repeated surgical excision (109,110).

Nodal Metastases

Nodal metastases are present in 10% to 45% of operative patients. Lymph nodes should be included in the primary surgical procedure as an en-bloc excision. Lymph node metastases in adrenocortical carcinoma are associated with a poor overall survival.

Distant Metastatic Disease

Patients Stage IV adrenocortical carcinoma have very short life expectancies. Surgical excision is not warranted if widespread disease is present. Symptomatic, solitary hepatic or pulmonary metastases, however, may be considered for surgical resection in suitable patients (111).

Radiotherapy and Chemotherapy

Adrenocortical carcinoma is not particularly radiosensitive and radiotherapy has had little effect in controlling local disease or decreasing local recurrence (112).

Mitotane (o,p' DDD) is the only agent which has been shown to be of some value in patients with advanced disease. It is most effective when used in patients prior to the development of metastatic disease following surgical resection for stage I and II disease and in patients undergoing repeated surgical resection for recurrent disease (113). This drug destroys the contra-lateral adrenal gland and steroid replacement is therefore essential.

Mitotane has been shown to decrease multidrug resistance *in vitro* in adrenocortical carcinoma. Recent reports, however, describing mitotane used in association with combination chemotherapy including etoposide, doxorubicin and cisplatin have described high toxicity and morbidity with no significant improvement in either overall or disease free survival (114, 115).

Prognosis in Adrenocortical Carcinoma

Survival rates for adrenocortical carcinoma are dependent upon disease

stage at presentation. A recent, multicentre French study of 156 patients reported an overall 5 year survival of 34% but only 9% 1 year survival in patients undergoing palliative resection (109).

MALIGNANT PHAEOCHROMOCYTOMA

Incidence and Pathology

The incidence of phaeochromocytoma is approximately 1-2 cases per 100,000 population. Malignant phaeochromocytomas accounts for only 10% of all phaeochromocytomas but in extra-adrenal tumours, located along the course of the sympathetic chain the incidence of malignancy rises to 40-50% (116). There is a higher incidence of extra-adrenal tumours in children compared with adults.

Tumours are usually cystic with areas of haemorrhage and necrosis. Vascular invasion is a histological feature of phaeochromocytomas seen in benign as well as malignant tumours. Diagnosis of malignancy is therefore difficult and may only be confirmed by establishing the presence of distant spread.

Localisation

Recurrence or persistence of symptoms in patients who have previously undergone surgery for phaeochromocytoma may indicate malignancy. Symptoms include hypertension (sustained or paroxysmal), headaches and palpitations. Recurrent or distant metastatic disease may be confirmed by measuring urinary catecholamines and metabolites (metanephrines and vanillyl mandelic acid) levels in an acidified 24 hour collection.

MRJ, which has a characteristic signal in phaeochromocytoma, is the best method of identifying recurrent local disease (117). Distant disease may also be identified by MIBG scan, though this has a false negative rate of approximately 10% (116).

Treatment of Recurrent or Distant Malignant Phaeochromocytoma

Recurrent disease should be resected when ever feasible. ^{131}I-MIBG has been shown to have a therapeutic as well as diagnostic role but treatment is palliative (118). Chemotherapy using combination cyclophosphamide, vincristine and dacarbazine has produced a partial response in controlling symptoms in 57% of patients but overall results are disappointing (119). Radiotherapy can be employed to control pain from bone metastases (120). Symptoms of catecholamine excess may to some extent be alleviated by combination therapy with alpha and beta blocking agents with or without α-methyl-ρ-tyrosine an agent which inhibits catecholamine secretion.

Treatment of hypertension by conventional antihypertensives will also be appropriate when there is persistent metastatic disease.

Prognosis

Five year survival rates in malignant phaeochromocytoma are 35-40% with extra adrenal tumours carrying a less favourable prognosis (121).

CARCINOID TUMOURS

Incidence, pathology and clinical features

These are rare tumours with an incidence of 1 per 100,000 per year and

arise from the enterochromaffin cells. Carcinoid tumours were first described during the last century but Oberndorfer in 1907 was the first to apply the term carcinoid indicating that the lesions were small, resembled carcinoma but in his opinion often had a benign course (122). The enterochromaffin cells otherwise known as Kulchitsky cells may be stained with chromium salts, have an affinity for silver salts and contain neurosecretory granules. Carcinoid tumours are classified according to their location as foregut, midgut and hindgut (123). Foregut tumours most frequently involve bronchus but may also be present in thymus, stomach, duodenum and pancreas. The midgut lesions are found in the small bowel, appendix and right colon. Hindgut disease affects the left colon, rectum or ovaries. Typically carcinoid tumours are multifocal, have frequent association with other concurrent malignancies and have a malignant potential which is related to the site of origin of the tumour (124). Perhaps less than 2% of appendicular carcinoids are malignant in contrast to colonic carcinoids where more than 50% will have demonstrable metastases at the time of presentation. The primary tumour size is also an important predictor of the risk of metastatic spread this occurring in 70% of tumours 2 cms or more in diameter (125). Carcinoid tumours secrete a range of amines and polypeptide hormones, some of which are capable of producing striking clinical symptoms. The most dramatic of these is the classical carcinoid syndrome caused by tumour humoral secretions of serotonin and bradykinin (126). Midgut carcinoids may also be capable of secreting substance P, tachykinin, neuropeptide K and Y, and prostaglandins. The carcinoid syndrome is characterised by the features of flushing, diarrhoea and carcinoid heart disease with valve lesions affecting the right side of the heart. These features occur typically when there are liver metastases from a small bowel tumour. Intraabdominal fibrosis may cause bowel obstruction and even intestinal ischaemia and infarction resulting from vascular constriction.

Foregut tumours are also capable of initiating the syndrome often in the absence of metastases but the flushing tends to be prolonged rather than transient and is frequently associated with periorbital oedema and lacrimation suggesting the possibility that histamine like substances may be involved in the causation (127).

In the remainder of this section the therapeutic options available for treating local spread and distant metastases from malignant midgut carcinoids will be considered in detail. The clinical presentation should suggest the diagnosis and the point has already been emphasised that the classical carcinoid syndrome arising from a midgut lesion is likely to be associated with metastatic nodal and hepatic spread. Biochemical confirmation of the disease is achieved by measurement of urinary 5HIAA (5 hydroxyindole acetic acid). The primary tumour is usually too small to be shown on barium follow through examination but small bowel entrapment by the extensive fibrosis with mesenteric shortening is a diagnostic feature. Ultrasound and CT are employed to diagnose lymph node and hepatic metastasis. Angiography was previously employed for defining vascular anatomy, often showing segmental occlusions and tortuosity of mesenteric vessels, but this modality is now employed infrequently. Most carcinoid tumours possess somatostatin receptors and this has allowed the development of scintigraphy with radiolabelled somatostatin analogues. This technique is excellent for demonstrating both metastases and even locating the primary tumour (128, 129)

Surgery

The majority of patients with midgut carcinoid disease should undergo laparotomy and appropriate bowel resection in order to remove the obstructing primary tumour, resect associated mesenteric lymph node metastases and relieve the profound mesenteric fibrosis so frequently a marked feature of the disease. The latter will encase intestinal blood supply often giving rise to incipient intestinal ischaemia. The surgical resection of this disease with mesenteric metastases may well involve a difficult dissection taking care to preserve the main vascular supply to the intestine and at the same time achieving adequate resection without creating short bowel syndrome or intestinal devascularisation (130). Intestinal bypass procedures should be avoided whenever possible and reserved only for those patients where extensive tumour spread precludes adequate treatment of obstruction.

It should be remembered that as in the case of many endocrine tumours where humoral effects are as important as mechanical and anatomical consequences that the principle of major debulking, even leaving significant residual tumour, may have considerable therapeutic benefit and facilitate the subsequent treatment of residual disease by medical and other methods. Further surgical resections may sometimes be indicated when there is disease progression or recurrent disease.

Table 5. Islet-cell tumours and associated symptoms

Islet-cell Tumour	Symptoms
Insulinoma	Hypoglycaemia, neuroglycopenia
Gastrinoma	***Zollinger-Ellison Syndrome.*** Peptic ulceration, oesophagitis, diarrhoea
Glucagonoma	Necrotising migratory rash, diabetes, diarrhoea
VIPoma	Diarrhoea, hypokalaemia
Somatostatinoma	Diarrhoea, gallstones
PPoma	None
Non functioning	None

Liver Metastases

Liver metastases are common in patients with carcinoid syndrome due to midgut tumours. Solitary deposits and small appropriately positioned multiple lesions may often be successfully enucleated. Larger lesions may be amenable to a formal hepatic lobectomy. This is likely to be a debulking exercise but as already described may have profound benefits for the patient with respect to unwanted humorally mediated symptoms.

Other patients, particularly those with bilateral hepatic disease, may benefit from staged hepatic embolisation performed several weeks post laparotomy (131). This technique may be refined as chemoembolisation with good response and tumour control (132). It is a sensible precaution to perform a cholecystectomy at the time of the initial laparotomy in order to avoid the possible complication of gallbladder ischaemia and necrosis following embolisation. Carcinoid crisis may also be a complication of the embolisation procedure due to death of tissue and release of serotonin and

bradykinin. These effects can be minimised by prophylactic treatment with somatostatin analogue (Sandostatin during and after embolisation).

Liver transplantation has also been considered when metastatic disease is confined to the liver.

Medical Therapy

The mainstay of chemotherapy for unresectable symptomatic disease has traditionally been streptozocin and 5 fluorouracil (133). The objective of such treatment is primarily to achieve symptomatic benefit and this has been demonstrated in up to 30% of patients. Numerous other agents have been used to control the symptoms of carcinoid syndrome including cyproheptadine (a serotonin and histamine antagonist), methysergide, and the 5HT-2 antagonist ketanserin, but most of these agents are now of historical interest only.

There has been considerable support for the use of alpha interferon, Oberg and colleagues reporting symptomatic improvement in 60% of patients with carcinoid syndrome (134). There are side effects of interferon and development of antibodies may limit the long term efficacy of the agent.

The most significant advance in the treatment of metastatic carcinoid syndrome has undoubtedly been the introduction of the somatostatin analogue Sandostatin (135,136). It will produce symptomatic relief in the majority of patients. Minimal reduction in tumour size is to be expected although tumour regression has been reported. Doses of Sandostatin required usually range from 200-600 mcgs per day and the agent is administered by 8 hourly subcutaneous injection. Recently long acting analogues have been developed which can be administered either two or four weekly. Furthermore the likelihood of a satisfactory response to somatostatin therapy can be predicted from the somatostatin receptor status demonstrated by scintigraphy.

PANCREATIC ISLET CELL TUMOURS

Incidence, pathology and clinical features

These tumours are rare with insulinoma being the most common islet cell tumour followed by gastrinoma. A classification of functioning and non functioning islet cell tumours with their clinical features is shown in Table 5. Most islet cell tumours which secrete an active hormone or peptide will give rise to an identifiable clinical picture. The protean characteristics of hypoglycaemia are seen with insulinoma and invariably severe peptic ulceration with gastrinoma. Non functioning islet cell tumours are not associated with any recognisable hormonal syndrome. Islet cell tumours generally speaking have a high potential for local growth and invasion, metastases to regional lymph nodes and to the liver. The incidence of malignancy in the various tumour types is very different with perhaps only 10% of insulinomas being malignant in contrast to more than 60% of gastrinomas (137,138). The malignancy rate for glucagonoma and VIPoma is approximately 50%.

The diagnosis of malignancy may not be easily made by routine histological criteria and the only absolute proof of malignancy will be confirmed metastatic disease and spread to distant sites.

Insulinoma

Surgery

Because the majority of insulinomas are entirely benign enucleation of a single primary tumour results in both biochemical and clinical cure. The principal challenge in this condition is the identification of the lesion which may be less than 1 cm in size. Octreotide scintigraphy (139) and endoscopic ultrasound have made a significant contribution to the preoperative localisation of insulinoma (140). Intraoperative ultrasound is also of enormous value in the identification of pancreatic islet cell tumours (141). The malignant insulinoma may be apparent by virtue of its size, evidence of invasion and metastases to lymph nodes and liver. If possible resection of the primary tumour with adjacent nodes should be performed. Even if only incomplete excision is feasible the debulking procedure may provide excellent palliation and improvement of symptoms.

Medical Treatment

When there is clearly unresectable disease medical therapy and chemotherapy may offer some benefit.

Diazoxide is a non diuretic benzothiadiazine and will reduce circulating glucose levels. The agent has now been replaced by somatostatin analogues which are very much more effective in controlling hypoglycaemia.

Previously chemotherapy with streptozocin used either alone or in combination with 5 flurouracil and doxorubacin were the main stays of chemotherapy for metastatic or unresectable disease.

Hepatic artery embolisation may also be considered as an adjunct to the above measures.

Gastrinoma

Sporadic tumours

Most patients with the Zollinger-Ellison syndrome have the sporadic form of gastrinoma which is a solitary tumour. Although most primary tumours are localised in the pancreas in 20-40% of cases the primary lesion will be within the duodenal wall (138). Surgical exploration should only be performed in gastrinoma cases if liver metastases have been excluded. When the primary tumour is identified within the pancreas it should be excised along with regional lymph node clearance. If the primary tumour is not found within the pancreas a duodenotomy should be performed and a search conducted for the tumour which may be a small microadenoma less than 0.5 cms in diameter. Tumours in the wall of the duodenum are invariably smaller than pancreatic primaries and are more likely to be associated with lymph node metastases which should be included in the resection. Pancreatic primary tumours are usually larger than 1 cm in diameter and have a higher potential for metastasising to the liver

Familial (MEN I) tumours

The need for surgical exploration in this variety of gastrinoma has until recently remained somewhat controversial but there is a growing consensus that providing liver metastases have been excluded a laparotomy and resection of disease should be performed. By definition these tumours are multiple within pancreas and duodenal wall and require a thorough exploration of both organs. More than 70% of patients will have tumours within the duodenal wall (138). These lesions are excised via a generous duodenotomy and the regional lymph node metastases very commonly

present excised. Lesions in the pancreatic head are enucleated and a distal pancreateclomy as far as the superior mesenteric vein is also performed in order to remove significant intrapancreatic gastrinomas. With these strategies long term clinical cures are recorded although biochemical cure is less satisfactory.

Treatment of Unresectable Metastatic Disease

Chemotherapy is unlikely to be of value for regional lymph node metastases but can be used for tumour which has metastasised to the liver or distal sites. Streptozocin and doxorubicin are reported to be the most effective agents.

Somatostatin analogues are less effective in gastrinoma than insulinoma. However the powerful proton pump inhibitors such as omeprazole (Losec) are extremely effective in controlling the undesirable gastrointestinal effects of the excess gastrin secreted by unresectable metastatic disease.

Hepatic artery embolisation has also been employed in selected cases. Although hepatic transplantation has been reported in ZE syndrome enhanced survival has not been demonstrated (142).

Non Functioning Islet Cell Tumours

Curative resection should be the main objective, but even when only palliative debulking is possible long term survival has been reported.

Occasionally adequate resection will require a Whipple's procedure which may even be appropriate when there are local lymph node metastases.

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Chapter 11

PROSTATE CANCER

Malcolm D Mason

Abstract: Prostate cancer is a major cause of death from cancer among men in the Western world. It's most striking clinical peculiarity is its tendency to metastasise to bone, resulting in osteoblastic (sclerotic) deposits. This phenomenon may be related to anatomical factors, or to specific biological factors. These include alterations in the expression of adhesion molecules by prostate cancer cells (such as reduced E-Cadherin expression or function, loss of integrins such as $\alpha 4$, $\alpha 5$, and αv). The production of growth factors and cytokines such as TGFβ or IGF may mediate cross-talk between bone cells and prostate cancer cells. An understanding of these various factors may result in novel approaches to the treatment of metastatic prostate cancer. Other factors which impact on earlier stages of metastasis, such as expression of the collagenase uPA, might also be amenable to therapeutic intervention, but the applicability of such interventions in patients with already established metastatic disease remains to be demonstrated.

INTRODUCTION

Prostate cancer is one of the leading causes of death from cancer in males in the Western world (1). In many ways, it is unique. Its natural history is dominated - at any rate from the clinical point of view - by its bizarre and striking propensity to metastasise to bone, resulting in sclerotic deposits (in addition to lymphatic spread, which generally occurs at an earlier stage). From a therapeutic point of view, it is also unique, in that it is highly sensitive to androgen ablative hormonal therapy (2). At the same time, it has proven more resistant to basic scientific investigation, because of the relative difficulty in establishing prostatic carcinoma cell lines in the laboratory. However, techniques that are more recent are yielding data, which, in addition to illuminating the biology of prostate cancer, are likely to indicate routes for future therapeutic exploitation.

Here, we will consider the natural history of prostate cancer, from both biological and clinical viewpoints, and those therapeutic aspects that particularly impact on the metastatic process or subsequent growth of established metastatic disease.

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PROSTATE CANCER NATURAL HISTORY AND PATTERNS OF METASTASIS

Clinical Aspects

Prostate cancer has a slow natural history. This has a number of consequences, and is responsible for one of the unique therapeutic features of the disease – that some patients can be left untreated because the disease does not progress during their lifetime (3). Many years ago, autopsy data showed that the true incidence of prostate cancer, as defined on histological grounds, greatly exceeded the observed incidence of clinically significant disease (4). The prevailing concept resulting from this was that a form of prostate cancer existed, which inherently lacked the biological potential to spread locally and to metastasize - so-called "latent" prostate cancer. This, in turn, implies that in lesions which microscopically look like small cancers in the prostate gland, a specific event or series of events is required for a tumour to attain metastatic potential (and therefore clinical significance). In support of this hypothesis is the observation that, although the incidence of prostate cancer differs greatly worldwide, the incidence of "latent" prostate cancer (defined from autopsy studies) is the same. For example, Japan has a very low incidence of prostate cancer, but a similar (30-40%) incidence of incidentally discovered prostate cancer at autopsy (5;6). However, attempts to discover the biological basis for progression from a latent to potentially metastatic form have largely been unsuccessful. It seems plausible that the condition labeled "latent" prostate cancer includes a spectrum of disease, in which some tumours have higher metastatic potential than others - either by virtue of their size, or possibly due to differences at the genetic level (7). An alternative hypothesis might be that, although the disease is fundamentally the same in latent and metastatic forms (from the standpoint of molecular carcinogenesis), the speed at which it progresses is different in so-called latent and significant cases. The various environmental, genetic and other factors responsible for the differing incidence of clinical disease, may merely act by influencing the growth rate of an already metastasis-competent tumour.

An added dilemma for the clinician is that the true effectiveness of radical therapy is fundamentally unproven. There is a tendency for clinicians to harbor particular allegiance to their own therapeutic modality. Surgeons with expertise in the operation of total prostatectomy often strongly defend it as a powerful means of effecting cure, as do radiotherapists with similar professional interests in radical radiotherapy (8). There is, however, a conspicuous lack of randomised trials, which is a serious drawback in a disease where the latent form will colour the results of treatment so strongly. The American Urologist Whitmore (9) summed up the dilemma thus:

> *"Is cure necessary in those in whom it may be possible? Is cure possible in those in whom it may be necessary?"*

In assessing whether or not to treat a patient with prostate cancer, the clinician will take account of several factors:

1. The biological potential of the tumour, as judged by its histological grade (10). This is usually documented using the Gleason grading system, which is based on the

premise that most prostate cancers exhibit a major and a minor pattern histologically. The pathologist assigns a score (1-5) to the major and minor patterns, and thence a sum score which will lie between 2 and 10. Tumours with a sum score of 2-4 are, generally, well differentiated, those with a score of 5-7 are moderately differentiated, and those with a score of 8-10 are poorly differentiated.

2. The biological potential of the tumour, as judged by the serum level of Prostate Specific Antigen (PSA) (10). This glycoprotein, produced by both normal prostatic epithelium, and by prostatic adenocarcinoma, is uniformly found to be a prognostic indicator. The risk of harboring bone metastases (occult or otherwise) increases as serum PSA increases; conversely, it is uncommon to find bone metastases in patients with a PSA of less than 20 ng/1 (11).
3. The patients age (both chronological age, and biological age). Many clinicians will not treat patients with slowly growing tumours (as defined by low histological grade and/or low PSA levels) in patients with a life expectancy of less than 10 years(12).
4. Clinical evidence that the disease is confined to the prostate. This is based on imaging (Computerised tomography, or magnetic resonance imaging of the prostate and pelvis, plus isotope bone scanning). Subsequently, in some patients, pelvic lymph nodes are sampled, and examined histologically, radical treatment being restricted to those patients who have no evidence of metastatic disease on these criteria. Alternatively, use may be made of retrospective data from radical prostatectomy series, which allow a prediction to be made of the likelihood of organ-confined disease and pelvic lymph node involvement based on the clinical extent of the primary tumour, the serum PSA level, and the histological grade of the tumour. (13).

The recent retrospective report from the US SEER programme has been invaluable in providing the largest published database on the outcome of patients with early prostate cancer treated by surgery, radiotherapy, or watchful waiting (14). While in no way does it replace a randomised trial, it does provide strong circumstantial evidence that, at least in patients with poorly differentiated tumours, radical treatment might lead to improved survival at ten years, compared with patients managed by surveillance. This would imply that, in at least some cases, prostate cancers are truly confined to the prostate gland at the time of diagnosis, and that treatment can eradicate some tumours. Rightly or wrongly, many clinicians - the present author included — are reluctant to withhold radical therapy from a younger, fitter patient, particularly if he has high-grade disease.

Clinical aspects of metastatic disease

Lymph node metastases

The utility of pelvic lymph node sampling in the assessment of patients is a testament to the biological significance of lymphatic metastases. From a therapeutic viewpoint, the presence of lymph node metastases has serious implications. Lymphatic metastases are frequently correlated with the subsequent development of bone metastases, and are therefore a biological marker of occult disseminated disease (15). Patients with pelvic lymph node metastases are often considered to have incurable disease, and

are therefore usually managed by hormonal manipulation, or with other modalities that are with palliative intent (12).

Although lymphatic metastases are commonly found in patients assessed for radical treatment, they do not invariably give rise to clinical problems. When they do, patients usually suffer from the effects of lymphatic obstruction, resulting in swollen legs. It is relatively unusual for para-aortic, or mediastinal lymph node metastases to be of clinical significance, although, presumably, they are frequently involved pathologically (16).

Are patients with pelvic lymph node metastases really incurable? Patients with lymph node metastases from some other cancers, notably squamous carcinomas of the head and neck and cervix uteri, can undoubtedly be cured. Recent data suggests that the same might be true of some patients with breast cancer. In older series of prostate cancer patients treated with radiotherapy, the technique traditionally included pelvic lymph node irradiation (17). Nonetheless, a prevailing view is that, in prostate cancer, the true incidence of disseminated metastases in patients with lymph node metastases is sufficiently high as to render such an approach unjustifiable as a routine measure (12). However, it is important to remember that a small minority of patients with histologically proven lymph node metastases, treated with curative intent, have achieved long-term survival in a RTOG randomised trial, even though the overall results of that trial did not appear to show an overall survival benefit for routine lymph node irradiation (18;19).
Finally, the frequency with which prostate cancer metastasises to regional lymph nodes must imply something about the limitations of an immune response to such tumours, which will be discussed later.

Bone metastases

Bone metastases overwhelmingly dominate the clinical picture of advanced prostate cancer. In no other malignancy are the complications of bone metastases, and in particular bone pain, so much more significant than the complications of disease at other sites. Bone metastases from prostate cancer are typically *osteoblastic* or sclerotic, in contrast to the predominantly lytic deposits seen in other solid tumours, or the exclusively lytic deposits seen in myeloma. In this respect, prostate cancer really is a model system, which illustrates how organ-specific the process of metastasis can be, both in terms of site and in terms of the biological appearance of such metastases. This subject also, therefore, implies that some important lessons about host-tumour cell interactions could be learned from the phenomenon of prostate cancer-bone interactions.

However, before expanding this argument, is all really as it seems? At the beginning of the century, it was argued that anatomical features accounted for much of the apparent specificity of tumour metastatic sites. Of relevance to prostate cancer was the description by Batson of the vertebral plexus which bears his name and which is said to provide a particularly rich and accessible route for metastasising prostate cancer cells from their organ of origin to the vertebral column. (20;21).

Furthermore, are clinicians correct in implying that metastases to sites other than bone are rare? To older pathologists, whose expertise in highly accurate and detailed observational studies could not be rivaled to-day, prostate cancers were characterised by widespread dissemination, even though most clinical problems arose from bone metastases (22). These observations are almost forgotten today, and yet they are surely crucial to our biological understanding of

the basis of prostate cancer metastases. Patients with soft tissue metastatic disease, and (at any rate, clinically) a paucity of bone metastases *are* seen, albeit infrequently. Anecdotally, they are felt by clinicians to differ from patients with predominantly bone disease, though they have similar response rates to hormonal manipulation (23,24). However, they are felt to have an even slower natural history, and to be responsive to chemotherapy in ways that bone-metastatic disease is not. Whether all such cases are pure adenocarcinomas, or whether some are, in reality, small cell carcinomas, with a natural history similar to neuroendocrine tumours, is unknown. (The interrelationship between epithelial and neuroendocrine pathways of differentiation may be of the utmost importance, although it will not be further discussed here).

Nonetheless, the majority of cases of advanced prostate cancer are characteristically dominated by bone metastases. Of the usual complications of bone metastases – pathological fracture, bone pain, and hypercalcaemia, bone pain is almost invariable at some stage, while pathological fractures are relatively less common than in other solid tumours, and hypercalcaemia is excessively rare (25). The vertebrae and pelvis are frequently involved, although metastases can occur in almost any bony site. However, for some reason, metastatic deposits in some areas, for example in the bones of the hands, are comparatively rare. Immunohistochemical staining can reveal clinically unsuspected micrometastases in the bone marrows of patients with clinically localised disease, but the significance of this is as yet unknown (26).

Bone metastases are incurable. Unless he succumbs to intercurrent illness, every man with prostate cancer metastatic to bone will eventually die of his disease. However, hormone therapy is an extremely useful palliative measure, which can produce dramatic, though sadly temporary, responses in patients with symptomatic bone metastases.

Biological Aspects

The causes of prostate cancer are unknown. Dietary, genetic, and other environmental factors are all likely to play a part (27), but their relative contributions are unclear, and they are likely to interact in a highly complex manner. The problem of latent prostate cancer, discussed in the previous section, highlights the need to understand the natural history of the disease in terms of the biological factors that influence proliferation, invasion, and metastasis. Indeed, a complete understanding of this would eliminate the clinical uncertainty, over which patients do and do not require treatment. The common aspects of the metastatic cascade, and more general features of metastasis, are covered elsewhere in this book and will not be discussed in any detail here. Instead, those aspects which are specifically pertinent to prostate cancer will be considered.

Chromosomal markers as predictors of metastatic disease in prostate cancer

Many studies have attempted to identify genes that suppress - or enhance metastasis, either by simultaneously measuring their presence or absence in primary tumours and metastases, or by correlating their presence in primary tumours with the subsequent development of metastatic disease. Both approaches have their strengths and limitations. Abnormalities in a number of chromosomes have been associated with prostate cancer, including gains of 11p, 1q, 3q, 2p, 8q, and loss of 8p, 9p, 10q, 13q, 16q, 17p, 10q, 2q, 5q, 6q, 7q and 15q

(28-30). In-situ hybridization has revealed significant gains in chromosomes 7 and 8 in association with progressive disease, a gain in chromosome 8 in association with local progression, and over-representation of 8q sequences in association with metastatic disease to bone, but not to other sites (31). The over-representation of 8q might result from an isochromosome.. Conversely, some workers have found regions of the genome that may suppress metastasis in prostate cancer. Using microcell-mediated chromosomal transfer, human chromosome 12 was introduced into the highly metastatic Dunning prostate cancer cell line AT6 (32). Cells that took up a conserved 70-cM portion of chromosome 12 were more than 30 times less likely to metastasize to the lungs. Subsequent experiments suggested that this gene did not prevent the metastatic process *per se,* but that, once arrested at a metastatic site, tumour cells were unable to survive and proliferate further (33). Other chromosomal sites at which metastasis-suppressing genes might be located include 16q24, and the DCC locus (29;34).

Adhesion Molecules

Cell-cell adhesion molecules.

Investigations into the role of adhesion molecules in prostate cancer progression have been spurred on by observations that the E-Cadherin/catenin system may be malfunctioning in prostate cancer. Most of the evidence links reduced expression of E-Cadherin with enhanced invasive behaviour (29;35-37). However, there are several other ways in which the E-Cadherin complex can be upset in prostate cancers reviewed as in (37). These include mutations, or altered expression of the catenins, molecules that associate with cadherins. Particular attention has been focused on β-catenin, because it associates with other important regulatory molecules, such as APC and Frz (the receptor for wnt-mediated signaling (see chapter 4). β-catenin is translocated to the nucleus via its interaction with the transcription factor LEF, and its biology suggests that it is in a key position to mediate important effects of a number of tumour suppressor genes (37). Other catenins may, however, also be important in prostate cancer: restoration of E-cadherin function, by transfecting PC3 cells with the α-catenin gene (on chromosome 5) resulted in the abrogation of their tumorigenicity (38). Clinical studies, which attempt to correlate patterns of expression of E-cadherin or associated molecules such as β–catenin in tumour biopsy samples with subsequent clinical behaviour need to be expanded, ideally in conjunction with a major clinical study such as a screening trial.

Cell-Matrix adhesion molecules.

Adhesive interactions between tumour cells and extracellular matrix may be as important as those between tumour cells Some existing therapeutic agents might interfere with tumour cell adhesion. Notable among these are the bisphosphonates, which, when used to pre-treat breast and prostate cancer cells, have been shown to inhibit their adhesion to mineralised and to unmineralised bone matrices (39). As yet the clinical significance of this is unclear, but is discussed in more detail below.

The integrins are a family of adhesion molecules which predominantly mediate cell-matrix adhesion, but which also influence cell behaviour via several pathways of signal transudation (see Chapter 2). Normal prostatic epithelia express a variety of integrins, which are polarised to the basal surface of cells. Although many integrins are lost in invasive prostate cancers (including α4, α5, αv, and β4), α6β1 and α3β1 are

retained, and it has been suggested that $\alpha 6\beta 1$ in particular is a mediator of an invasive phenotype (40). A model is proposed whereby prostatic carcinoma cells secrete laminin and express $\alpha 6\beta 1$, with invasion occurring on the surfaces of laminin coated structures such as nerve, blood vessel, and muscle after intraperitoneal injection of tumour cells (40). This is an interesting observation given that perineural spread is a well-recognised histological feature in prostatectomy specimens, and may be associated with a poor outcome. It is also noteworthy that, in normal prostatic epithelia, $\alpha 6\beta 4$ associates with hemisdesmosomal structures, and this, too, is lost in invasive disease. A second integrin studied in prostate cancer is $\alpha IIB\beta 3$, which is expressed on DU145 cells which metastasise following intraprostatic injection (41). Blocking antibodies to $\alpha IIB\beta 3$ inhibit lung colony formation after tail vein injection. It is possible, therefore, that this integrin is also of importance in mediating prostate cancer metastasis. A third integrin, $\alpha 2\beta 1$, may be important in medicating the attachment of prostate cancer cells to bone matrix, and might partly explain the avidity of prostate cancer for bone (42).

Other components of the extracellular matrix may also affect prostate cancer cell behaviour. One study has correlated the levels of chondroitin sulphate in cancer-bearing prostates with a poor outcome (43). Other proteoglycans may inhibit growth. The semisynthetic, glycosaminoglycan-like molecule pentosan polysulphate (PPS) is a potent inhibitor, not only of cell adhesion, but also of proliferation in anchorage dependent prostate cancer cell lines such as LNCaP and DU 145(44).

Other adhesion molecules.

Among other molecules that have been implicated in the metastatic behaviour of prostate cancer cells are CD44, other members of the immunoglobulin superfamily, and selectins.

Reduced expression of the adhesion molecule CD44 is associated with increased metastatic potential in the rat Dunning model. This effect can, however, be reversed by transfection-enhanced forced expression of CD44, suggesting that in prostate cancers as with other cancers this is an important molecule (see Chapters 1 and 5) (45).

A number of members of the immunoglobulin superfamily have been studied in prostate cancer, including I-CAM and V-CAM. The C-CAM molecule was transfected into PC3 prostate cancer cells, where its expression resulted in reduced tumorigenicity, suggesting that it may function in a similar way to CD44 (46).

Other surface molecules are expressed on prostate cancer cells, which may have an adhesive function. An example is the oligosaccharide Sialyl Le(X), which is associated with hormone-refractory, metastatic disease. However, its biological function is unknown. Surprisingly, it does not appear to mediate binding to selectins (47).

Matrix Degradation

It is axiomatic that, in order to be competent for metastasis, tumours must be able to degrade extracellular matrix. It is logical, therefore to ask whether the expression or control of matrix-degrading enzymes is involved in some way in the pathogenesis of prostate cancer. It is known that prostate cancers express matrix metalloproteinases, and other enzymes such as the urokinase-type Plasminogen Activator (uPA). It has been suggested that the levels of such enzymes, particularly uPA, have a tendency to correlate with metastatic potential (48-50). In PC3 prostate cancer cells engineered to

overexpress uPA, a higher rate of bone metastases was seen, in contrast to control cells. Conversely, cells engineered to under-express uPA showed a lower rate of metastasis than controls (51). Similarly, replacing wild-type uPA in PC3 prostate cancer with a mutant form defective in enzymatic activity resulted in a reduced metastatic capability when injected subcutaneously into nude mice (52). However, the relationship between expression of uPA and other factors involved in metastasis may be more complex than first envisaged. For example, the angiogenesis inhibitor angiostatin is generated from plasminogen by uPA produced by prostate cancer cells. Why a metastasis-favouring substance should also inhibit angiogenesis is unclear (53).

Other secreted proteinases are also likely to be involved in the process of tumour cell invasion. It has been suggested from human biopsy specimens that higher levels of the matrix metalloproteinase MMP-7 are correlated with an increased frequency of metastases, particularly when balanced against lower expression of the MMP inhibitor TIMP-1 (54). In support of this, transfection of poorly metastatic DU145 cells with MMP-7 significantly increases their metastatic potential in SCID mice (55). In a similar study, expression of the activated form of MMP-2 appears to be more pronounced in lesions with a higher Gleason score, and in lymph node metastases (56). Whether abnormalities of the specific genes regulating such enzymes is more directly involved is unclear, but, presumably, some of these are controlled downstream of androgen receptor signaling.

Motility factors

Cell motility is an essential component of invasion and metastasis. Intuitively, it would be logical for it to be coupled with other aspects of the metastatic cascade, such as matrix degradation, and, like the latter, probably also has its normal counterparts in embryogenesis and in wound repair. Surprisingly, among the mechanisms whereby motility is enhanced is signaling via the EGF receptor (57). DU145 cells that express a truncated form of the EGFR, are competent for proliferation, but nor for metastasis. Furthermore, the pharmacological agent U73122 is able to block EGFR-mediated motility, but not mitogenesis, and treatment of DU145 cells with this agent also reduced invasiveness (57). Other factors which appear to stimulate prostate cancer cell motility are bombesin (58), Autocrine Motility Factor (AMF) (59) and hepatocyte growth factor (60).

Angiogenesis

The importance of angiogenesis in the metastatic process in general has been highlighted extensively elsewhere. It is likely to be as important in prostate cancer as it is elsewhere, with regard to the genesis of a primary tumour, the metastatic process, and the maintenance of established metastatic deposits. It is also, hopefully, a process just as amenable to therapeutic intervention in prostate cancer as it might be in other cancers. This topic is discussed further in Chapters 1 and 4.

With regard to prostate cancer, several observations are worthy of mention:

1. Histomorphometric studies have suggested that prostate cancers associated with micrometastases in bone marrow have a higher blood capillary density ratio than those without micrometastases (61).
2. Similar results have been seen correlating microvessel counts with metastases (62)

3. As previously discussed, PC-3 prostate cancer cells are able to generate the angiogenesis inhibitor angiostatin, by serine protease cleavage of plasminogen (53)

KAI1

The KAI1 protein is expressed on prostate epithelium, though not stroma. Its gene has been located to the short arm of chromosome 11, but its function is unknown. It is, however, a membrane protein that is also expressed in rat prostate cells. Its expression is reduced in some prostate cancer cells, but in particular it is reduced or absent in metastasising tumours in patients failing androgen ablation therapy (63). The basis for this association needs further explanation, but KAI1 may prove to be an important factor in prostate cancer metastasis and progression.

Interactions between prostate cancer and bone

Our understanding of the interactions between normal bone cells, bone marrow cells, and tumour cells has improved vastly in recent years. In the case of prostate cancer, the sclerotic reaction in bone implies that tumour cells stimulate osteoblasts, and indeed such seems to be the case. A number of cytokines have now been identified which do this. There is abundant cross talk between osteoblasts, osteoclasts, and other cells, and in recent years much has been learned about the basic biology of metastatic disease in bone (64). Similarly, prostate cancer cells respond to cytokines produced by bone cells, of which TGF-β may be of particular importance, stimulating both collagenase secretion and cell adhesion to bone matrix, while no longer inhibiting proliferation (42;65). Interestingly, PSA may potentiate the action of IGF, by reducing the binding of IGF to the binding protein, IGFBP-3 (66).

Clarke and colleagues have carried out detailed histomorphometric examinations of bone metastases in prostate cancer patients (67). Using this technique, very small micrometastases can be identified, and the earliest recognisable histological feature of a host reaction is a layer of osteoblasts around the tumour, which appear to be laying down osteoid. At this stage, it is extremely difficult to recognize osteoclasts, but at later stages of metastatic disease, when there has been extensive but disorganised bone formation, additional osteoclasts are frequently seen, in association with resorption cavities. Biochemical investigations have left no doubt that there is excessive bone breakdown, even at the early stages of the disease, and this is the basis for investigating the use of bisphosphonates, inhibitors of osteoclast activity (see below). The nature of the "soil-and-seed" relationship between prostate cancer and bone is highly complex. Indeed, in its early stages, when micrometastases are present in the bone marrow, it has been suggested that adhesion of prostate cancer cells to bone marrow endothelium is an important interaction, and, in the case of highly metastatic Mat-LyLu cells, is more pronounced than adhesion to other bone-derived cells, including osteoblasts and non-endothelial bone marrow stromal cells (68). In agreement with this, bone marrow stroma (or conditioned media from such cells) was unable to stimulate the growth of prostate epithelial cells and prostate carcinoma cells (69). On the other hand, osteoblasts appear to produce factors that, in some way, are survival-promoting for prostate cancer cells, as demonstrated by the response of prostate carcinoma cells to Adriamycin in the presence or absence of osteoblasts (70).

Furthermore, bone matrix may regulate key genes in prostate cancer cells: LNCaP cells grown on bone extracellular matrix were found to express multiple, unique transcripts which contained homeobox motifs. Cells expressing these transcripts were more likely to be androgen independent (71).

THERAPEUTIC APPROACHES

Standard therapeutic approaches

Introduction

Unfortunately, most of our therapeutic efforts have been directed against already established metastases. However, in recent years, there has been more interest in the use of adjuvant therapy, either in the form of hormonal manipulation, or other measures such as bisphosphonates.

Hormonal manipulation

It has been known that prostate cancers are sensitive to hormonal manipulation since the classic studies of Huggins in the 1940s. Approximately 85% of patients with established disease respond to some form of hormonal manipulation, by either bilateral orchidectomy, or treatment with LHRH analogues, oral anti-androgens, or oestrogens. Often, such responses can be dramatic, with complete resolution of bone pain from metastatic disease, and a fall in serum PSA levels to normal ranges. Unfortunately, however, such responses are always temporary, relapse occurring after a median time of approximately 18 months. The response to second-line hormonal manipulation is considerably inferior, both in terms of its quality, and its frequency, which is only around 5-15%. There are no satisfactory treatments for hormone refractory disease.

In an attempt to improve the results of hormonal manipulation, many investigators studied the effects of so-called Maximal Androgen Blockade, in which blockade of testicular androgens (by orchidectomy or LHRH analogues) is combined with blockade of adrenal androgens (by oral anti-androgens). Despite initial reports, which were encouraging, a recent meta-analysis of all randomised trials of MAB versus monotherapy has shown that any benefit in terms of absolute survival rates is likely to be of the order of a few percent (72). It is becoming more widely felt that MAB cannot routinely be justified.

There is now no doubt that adjuvant hormone therapy prolongs survival in some patients with breast cancer (see Chapter 13). It is entirely logical, therefore that adjuvant (or early) androgen deprivation should be investigated in prostate cancer. The results of randomised trials of immediate versus deferred hormone therapy in prostate cancer have been conflicting. One of the largest recent studies was performed by the UK Medical Research Council, and this study did, indeed, suggest that immediate hormone therapy improved survival, in both metastatic and non-metastatic patients (73). However, a meta-analysis of all available randomised trials of immediate versus deferred hormone therapy by the Prostate Cancer Trialists' Group has suggested that, although immediate hormone therapy has a substantial effect on prostate-cancer specific mortality, its effect on overall mortality is very small (unpublished data, presented to the Royal Society of Medicine, London, & O. Dalesio, personal communication).

Radiotherapy

It is widely known that radiotherapy, administered in low doses, relieves bone pain due to metastatic disease, and this is as true of prostate cancer as it is of other cancers. However, the mechanism by which this is achieved is unknown. It is unlikely to be an effect due solely to tumour cell killing, since doses as low as 4Gy in a single fraction can be effective, and there is probably only a very limited dose-response, since fractionated radiotherapy appears to be no more effective than a single fraction (74). External beam radiotherapy is highly effective, with responses seen in up to 80% of patients.

Strontium-89

Strontium, when administered intravenously, is taken up avidly by bone, preferentially at sites of new bone formation. In normal bone, which though turning over is relatively quiescent, the total amount of strontium taken up is relatively small, compared to areas of pathological new bone formation, such as at the sites of sclerotic metastases. Strontium-89 is a beta-emitting isotope, which when taken up at the site of bone metastases from prostate cancer, will be retained for many months, resulting in the local delivery of a high dose of radiation. At the same time, the radiation dose received by neighboring tissues is very small due to the low penetration of beta particles.

Intravenous strontium-89 was shown to reduce bone pain in prostate cancer patients as long ago as 1976 (75). A review of the literature has suggested that around 80% of patients will respond to strontium-89 (76), with 10% of patients achieving complete pain relief. One randomised trial comparing strontium-89 with external beam radiotherapy suggested that their efficacy and bone marrow toxicity was similar, but that strontium-89 delayed the onset of pain at untreated sites (77).

The logistical advantages of this form of therapy are such that it should be further investigated in earlier disease, such as in men with asymptomatic bone metastases.

Bisphosphonates

Bisphosphonates are analogues of pyrophosphate, in which the P-C-P backbone is retained, but the side-chains are altered, rendering them insensitive to cleavage by alkaline phosphatase.

They have a number of important effects on bone, the most relevant being that they are potent inhibitors of osteoclast activity. This is relevant to the therapy of bone metastases, which are characterised by an increase in osteoclastic activity with a consequent increase in bone resorption. This is thought to be responsible for two of the major complications of bone metastases in cancer – hypercalcaemia and pathological fractures.

Furthermore, it has recently been shown that bisphosphonates inhibit the adhesion of breast and prostate cancer cells to bone matrix (39). It is conceivable that such an activity could have biological effects in prostate cancer, though tumour cells can presumably achieve anchorage-independence by circumventing normal control mechanisms.

The use of bisphosphonates in breast cancer and in myeloma has resulted in demonstrable clinical benefit, in terms of reducing these complications – and additionally impacts on bone pain (78). However, at first sight, the relevance of bisphosphonates to prostate cancer appears counter-intuitive.

In both breast cancer and myeloma, bone metastases are characteristically lytic, whereas in prostate cancer, they are

predominantly sclerotic, suggesting an increase in osteoblastic activity, as discussed earlier. However, histomorphometric and biochemical studies have demonstrated that in prostate cancer, as in other cancers, there is excessive osteoclast activity, albeit in association with a more generalised increase in osteoblast activity (67;79).

Large-scale studies are lacking, but there is some evidence that bisphosphonates may improve bone pain in patients with metastatic prostate cancer (78; 80, 81, 82, 83, 84), though this has not always been demonstrated (85). One histomorpho-metric study concluded that bisphosphonate therapy in prostate cancer might be associated with impaired bone mineralisation (86), but this has not yet been confirmed by other studies.

The UK Medical Research Council (MRC) has recently completed two large double-blind, placebo-controlled randomised trials in prostate cancer (87). Both studies randomised patients to oral clodronate, 2080 mg daily, or to placebo for 5 years. In the PR04 study, 500 patients with locally advanced, but clinically non-metastatic disease were entered, the rationale being to treat the occult bone metastases that must be present in a proportion of such patients. In the PR05 study, 300 patients with established bone metastases in a hormone-responsive phase were randomised to the same drug or placebo, for a duration of 3 years. Both studies require long-term follow-up, and the final results may, therefore, not be available for some years. Their preliminary analysis is due in 2002.

Until then, the use of bisphosphonates as an adjuvant therapy to prevent or delay the onset of clinical bone disease in prostate cancer remains experimental.

Novel therapeutic approaches

Immunological

Given the resurgence of interest in tumour immunology, it is not surprising that attention should have been given to prostate cancer immunotherapy. PSA itself is a potential immunological target, since its expression is almost exclusively restricted to prostatic tissue and prostate cancer. Experimental approaches to prostate cancer immunotherapy have included

- *immunoconjugates,* in which monoclonal antibody is combined with a toxin (88),
- *cytokine therapy* (including antibody-cytokine fusion proteins) (89),
- *lymphokine activated killer cells* (90;91),
- *dendritic cells,* professional antigen-presenting cells, which are pulsed ex-vivo with tumour or tumour antigen, and re-infused to induce a specific cytotoxic T-cell response. Such an approach, using two prostate-specific membrane antigen peptides, has recently been reported to produce clinical and biochemical responses in patients with metastatic prostate cancer (92).

Inhibitors of matrix degradation

The importance of matrix degradation in the metastatic process has naturally led to attempts to modify it. The principle that interference with uPA reduces metastatic potential has been tested using transfectants of mutant uPA which lack catalytic activity, and which when expressed do, indeed, inhibit metastasis in experimental systems (52). One major disadvantage of such treatments is that they can only be applied clinically in patients with tumours that have not yet

established distant metastases (otherwise it is a case of shutting the stable door after the horse has bolted). Most patients present at a time when metastases, if they are going to develop, are already present at the time of diagnosis. Nonetheless, other attempts have been made to target matrix degradation. This has included the use of existing drugs such as tetracycline to inhibit MMPs and amiloride to inhibit uPA (93;94), thought with variable success. The pharmacological agent B-428 appears to have similar effects to truncation of uPA in the Dunning rat model, but in addition to reducing metastases, appeared to reduce the volume of the primary tumour. If such agents had intrinsic antiproliferative activity, they could, after all, find their way into the clinic (95).

Vitamin D analogues

1,25-dihydroxyvitamin D is known to be an inhibitor of proliferation in prostate cancer cells, and to promote their differentiation. Its use as a therapeutic agent is limited by its tendency to cause hypercalcaemia, but an analogue, EB1089, has the same antiproliferative effects as the native compound, but lacks its hypercalcaemic action. In addition, it significantly inhibits the development of metastases in rats bearing tumours of MAT LyLu prostate cancer cells.(96). This compound merits further investigation as a potential therapeutic agent.

CONCLUSIONS

In recent years, great strides have been made in our understanding of metastasis as it relates to prostate cancer. This can only improve our clinical management of early, organ-confined disease, as we increasingly understand the biological determinants of significant as opposed to latent disease. More problematic will be the effective treatment of established metastatic disease, but several avenues of exploration have been discussed here. Effective anti-angiogenic agents, modulators of hormone responsiveness, modulators of growth factors, and perhaps agents which modulate cell adhesion and matrix degradation will all find a clinical role, but it is essential that such agents are studied in a systematic and coordinated manner. It is to be hoped that the next decade will yield some dramatic advances in such therapeutic applications.

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Chapter 12

GASTROINTESTINAL TUMOURS: CANCER METASTASIS, MOLECULAR AND CELLULAR BIOLOGY

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INTRODUCTION

Tumour biology and mechanism of metastasis

The basic molecular mechanisms employed by the invasive and metastatic cells are similar among the various types of carcinoma. Hence, the ability of tumour cells to disseminate from the primary tumour mass and to spread via blood vessels or the lymphatic system is governed by similar factors in differing tumours. The extracellular matrix (ECM) forms a barrier to the progression of tumour cells and its various constituent molecules impede this cellular movement. In order to invade and metastasise, the tumour cells must overcome these obstacles. Secretions of proteases including aspartate, serine, cysteine and metalloproteinases will assist the progression through this barrier. The cellular adhesion molecules or receptors mediate cell-to-cell and cell to ECM interaction during invasion and metastasis. The more aggressive invasive gastrointestinal carcinomas are more likely to produce liver metastases than those are which are less invasive in vitro (1). Gastrointestinal cancers can spread by direct invasion into adjacent organs, embolisation to local lymph nodes or by venous drainage from the bowel through the portal vein into the liver.

Adhesion molecules

The role of adhesion receptors on the surface of cancer cells in the mediation of tumour cell migration, invasion, and metastasis remains poorly understood. A number of specific cell surface-associated molecules which mediate cell-matrix and cell-cell interactions have been characterised, including the family of integrin receptors, the cadherins, the immunoglobulin (IgG) superfamily, a 67-kDa laminin-binding protein, and the CD44 receptor. Changes in the expression and function of these adhesion molecules

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are important in the development of gastrointestinal malignancies. They have potential clinical application as a diagnostic tool, prognostic marker or as new targets in the anti-cancer therapy.

Integrin constitutes a large and diverse family of receptors involved in adhesion between cell membrane and ECM. They are composed of two non-covalent molecules α and β subunits (total of 14 α and 8 β) and in combination creating over 20 heterodimers. Their role is complex but a reduction or change is observed in pancreas (2) and colon (3,4), suggesting a loss of attachment to the basal membrane.

Downregulation of the E-cadherin receptor and the cytoplasmic protein alpha-catenin is associated with tumour dedifferentiation, infiltrative growth, and lymph node metastasis in oesophageal cancer. There are similar correlations between the loss of E cadherin and malignancy in the stomach (5), colo-rectum (6,7), and pancreas (8). In gastric cancer, under-expression of E-cadherin due to gene mutations is restricted to diffuse-type tumours. In pancreatic cancer the expression of integrin adhesion receptors is significantly altered during the malignant transformation of the pancreatic tissue while a loss of the E-cadherin receptor can generate dedifferentiation and invasiveness of pancreas carcinoma cells. Overexpression of the E-cadherin receptor in colorectal carcinoma correlates with a poor prognosis. It may serve as an independent prognostic marker in Dukes' stage colon cancer and helps in the selection of patients for adjuvant therapy following curative surgical resection (9).

In a study of stage II and IIIA in gastric carcinoma. CD44 expression was reported to be a poor prognostic factor (10). The expression of the CD44 standard and the CD44-9v isoform on the surface of gastric cancer cells correlate with higher tumour-induced mortality and poor survival (11,12). The CD44-6v isoform is predominantly expressed by intestinal-type gastric carcinomas. The alteration in the integrin and isoforms of the CD44 receptors following malignant transformation of the colonic mucosa may influence the metastatic potential of cancerous cells (9).

Role of proteolytic enzymes

Experimental systems and clinical trials have demonstrated that matrix metalloproteinase enzymes play a key role in tumour invasion and metastasis. Evidence for this was obtained from studies with tissue specific inhibitors of metalloproteinase (TIMPs). In vitro study of gastric carcinoma, TIMPI has been shown to be a negative regulator of metastasis (13). Invasion assays and experimental metastases assays have shown inhibition of invasion and metastasis by TIMPI (14,15) Plasminogen activator urokinase has also been implicated in the process of invasion, and involved in the degradation of basement membrane laminin. Collagenase IV and cathepsin D are other proteolytic enzymes shown to be important in invasion and metastasis. Cathepsin D expression in the primary tumour correlates with a poor prognosis. The coagulation system that made up of complex series of proteinases plays an important role in blood borne metastasis. Animal studies have supported a critical role for coagulation in determining the success of circulating tumour cells in seeding as new deposits. Activation of the coagulation system is observed in most types of human cancer. Early trials of batimastat, a matrix metalloproteinase inhibitor, in patients with malignant ascites have reported a clinical response in about half the patients (16).

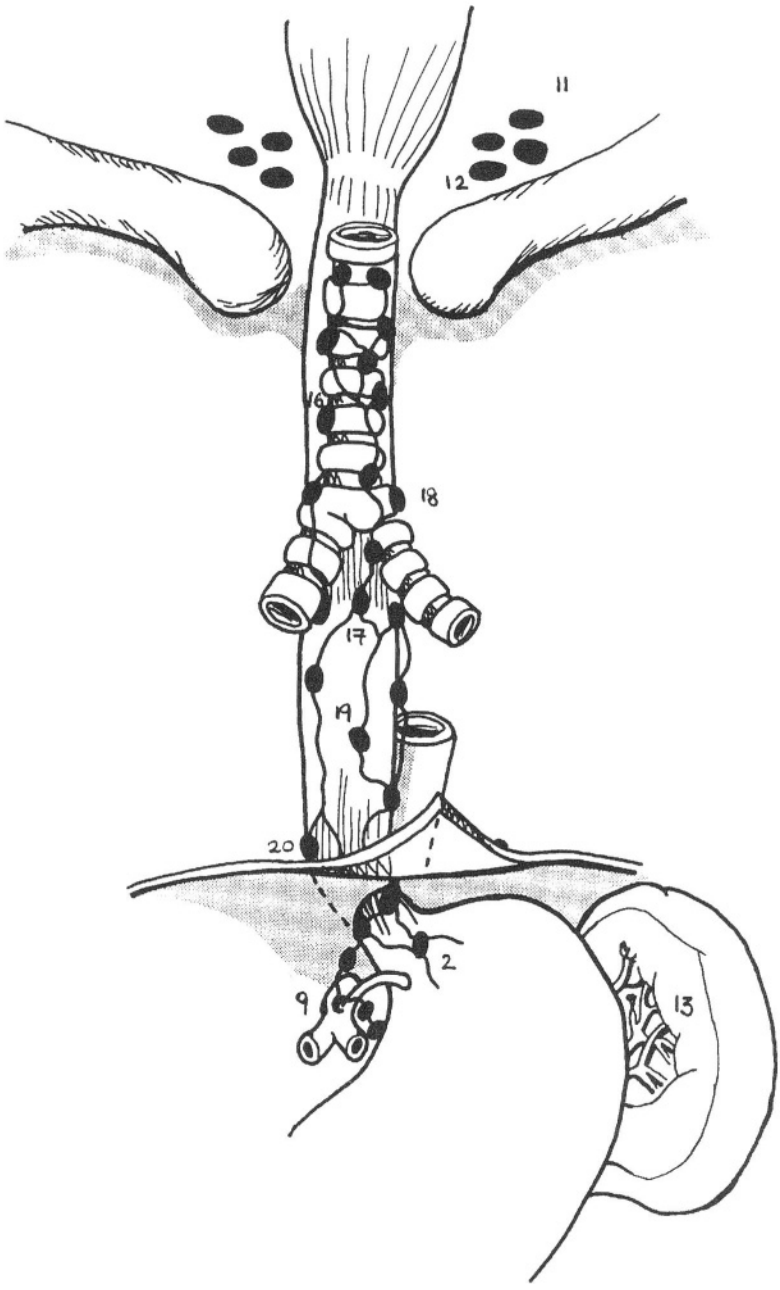

Figure 1. Lymph node stations in the oesophagus and their boundaries that are involved during the spread of the tumour.

STAGING OF DISEASE

The concept of staging has been developed since 1920. Clinical and pathological stage is a snapshot in the life of a tumour, and it provides the most accurate prognostic index, combined with histological features. Pre-treatment staging of cancer for assessing the extent of spread is extremely important to allow selection of appropriate treatment and for monitoring treatment response. Where consensus exists on the staging of a particular tumour, different regimens can be properly compared within and between institutions. Several staging methods are in use throughout the world, and each has its strengths and weaknesses. In the colorectal tumours, the Dukes Classification, Astler- Coller and Australian classification are used. A number of authors prefer the WNM system for staging oesophageal cancer described by Skinner et al (17) (figure 1). It is also important to note that definition of 'carcinoma' in Japan is by virtue of its structural and cytological features, but by invasion in the Western system. These differences in definitions made it difficult to compare studies and results on gastrointestinal 'cancer' originating from Japan in Western journals (18).

The Union International Contre Cancer (UICC) introduced TNM classification, to standardize and unify the staging to apply to all tumours. The older staging methods had the advantage of simplicity but lack of precision. The TNM system is more complex and includes prognostic indicator such as the depth of tumour penetration, extend of spread extra-luminally, number of lymph nodes affected and the presence of metastases in other organs. This system requires surgical and pathological information, such as primary growth, and its invasion, nodal status and the presence of metastases (table 1). The extent of spread is graded quantitatively, on a scale of 1-4. TNM results are then stage graded into 1 - 4, where the best chance of a cure is in stage 1, early disease, and stage 4, as in late disease the treatment is mainly palliative.

Investigation and staging of the gastrointestinal cancer.

Modern management of gastrointestinal cancer depends on the diagnosis. Various benign lesions may be misdiagnosed as malignant, such as polyps, and malignant lesions may be underestimated. Barium studies and endoscopy remain the main stay of diagnosis. However not every patient requires both investigations, as each has a different purpose. Barium is mainly used to define the anatomical problem, often giving the diagnosis as well as helpful information on the extent of the problem. However, contrast studies have several limitations. The true extent can often be greater than apparent from barium examination. Normal barium investigation does not exclude advanced disease, for example in linitis plastica, a variant of stomach cancer. Flat lesions and lesions less than 5mm (flat adenomas), early gastric cancers and small polyps is poorly demonstrated and often missed. Diagnostic endoscopies have the advantage of endoscopic tissue biopsy and cytology, and accurate visual examination of small lesions. Endoscopies also allow for therapeutic intervention eg polypectomty, stents and dilatation of lesions.

Selections of diagnostic and staging investigations depend on the primary site and the likely pattern of metastatic spread from the cancer. Cancers metastases reflect both the biology of the individual tumour and the topographic anatomy of the organ involved.

Table 1. Staging of oesophageal cancer (UICC 1987)

TNM staging		
T	T1	Tumour limited to the mucosa or submucosa
	T2	Tumour involving the muscularis propria
	T3	Tumour involving the adventitia
	T4	Extension to extraoesophageal structures
N	N0	No nodes
	N1	Regional nodal involvement
M	M0	No distant metastases
	M1	Distance metastases

Staging grouping	
Stage 1	T1 N0 M0
Stage 2A	T2-3 N0 M0
Stage 2B	T1-2 N1 M0
Stage 3	T3 N1 M0, T4 Nx M0
Stage 4	Tx Nx M1

Skinner classification of oesophageal cancer	
Wall penetration	W0 = Tumour limited by the muscularis mucosa W1 = Tumour penetrating through the submucosa and into but not through muscle layers W2 = Tumour penetrating through muscle layers
Lymph node involvement	N0 = No lymph node involvement N1 = 1-4 lymph node involved N2 = 5 or more lymph node involvement
Metastasis	M0 = Absence M1 = Present

Staging modalities

The main flaw in gastrointestinal tumour assessment is the staging accuracy of the disease. This is further complicated by the use of different staging classifications. Accurate diagnosis and staging with a common standard of treatment is important, especially patients entered into clinical trials. Emphasis should be put on the prognostic value of local tumour extent and lymph node metastases in all malignancy.

Liver metastases are the most common site of spread in for gastrointestinal tumours. Various modalities such as ultrasonography, computerize tomography and laparoscopy helps to localise these metastases. Bronchoscopy is essential in oesophageal tumours especially in the upper two thirds, to exclude direct invasion or fistula formation into the lung or bronchial tree. Success in the

management of cancer patients partly depends on accurate staging.

Ultrasonography

Ultrasonography is useful, cheap and widely available investigative modality. It has become a non-invasive extension of the physical examination and gives valuable information such as size and location of organ metastases, gross ascites and lymphadenopathy. Unfortunately operator dependence limits its use and requires a high level of expertise.

Computed Tomography (CT)

Computed Tomography (CT) scanning is the commonest modality used to investigate the extent of the disease. The overall accuracy of CT in assessing loco-regional disease of gastric and gastro-oesophageal carcinomas was disappointing. The accuracy of preoperative CT for pancreatic cancer in determining the perigastric tumour spread were reported 73% with sensitivity and specificity of 33% and 97%, respectively.

The accuracy for lymph node involvement and liver metastasis were 70% and 96% with sensitivity of 36% and 89% and specificity of 97% and 98%, respectively. Peritoneal dissemination was not detected in 27%, and stage IV disease was not diagnosed correctly in 45%. Comparing computed tomography with hepatic perfusion scintigraphy and abdominal ultrasound, CT is more sensitive in detecting over 90% of lesions, while intra-operative ultrasound and static scintigraphy were 80%. CT angiography is currently the most sensitive technique for reliable imaging liver metastases below 0.5 cm in diameter (19).

Laparoscopy

Laparoscopy has proved to be a more accurate and sensitive staging modality in gastrointestinal cancers than others. Consequently, better selection of patients with disease amenable to resection allows patients with advanced disease to be spared unnecessary laparotomies by discovering spread not seen by other modalities. With laparoscopy, laparotomy could be avoided in more than half of patient with primary liver tumours, liver metastases and proximal bowel tumours. The preoperative staging has been altered in as many as 40% of these patients. Laparoscopy, however, was found less useful in oesophageal and gastric tumours - laparotomy was only avoided in 5% and preoperative staging changed in 17%. Laparoscopy necessitates a general anaesthetic but it has a low morbidity, is associated with a rapid recovery, and it avoids the need for laparotomy in many patients. Palliative laparoscopic procedures are being developed for treatment of patients with advanced gastrointestinal malignancies.

Intraoperative ultrasound (IUS)

Intraoperative ultrasound (IUS) during either laparotomy or laparoscopy can assist the surgeon in directing appropriate therapy for intra-abdominal disease. This is particularly the case for primary or metastatic malignancies involving the liver and primary malignancies of the pancreas and upper gastrointestinal tract. It is the most sensitive imaging technique for detecting small intraparenchymal lesions of the liver, pancreas, and other solid organs. Its increased sensitivity has been responsible for the change the intraoperative treatment plan in a significant percentage of patients, especially with respect to resectability. Laparoscopic surgery may have replaced the surgeon's hand as assessor of the liver and other organs during surgery.

Laparoscopic ultrasound (LUS)

Laparoscopic ultrasound (LUS) is another important modality for the staging of hepatic, pancreatic, and gastrointestinal

malignancies. Laparoscopy and LUS can be used in diagnosing and staging intra-abdominal malignancies; furthermore, it provides an important role in the palliation and treatment of these malignancies. In recent years, using laparoscopic ultrasonography, as an adjunct to diagnostic laparoscopy for staging of tumours of the upper gastrointestinal tract, liver, biliary tree, and pancreas, has made it possible to visualize most anatomical structures in the upper abdomen consistently and in detail. Laparoscopic ultrasonography is able to detect small liver metastases, lymph node metastases, and small primary tumours of the pancreas and bile ducts. It can assist in the assessment of local extension of tumours of the pancreas and stomach. LUS has a higher accuracy (of over 90%) for assessing the primary tumour and nodal status compared with the conventional CT and ultrasonography (less than 64%) (20). By combining LUS with laparoscopy, it is possible to identify liver metastases with a sensitivity approaching 100%, from 60%, and an overall improvement in sensitivity to over 95% and specificity of up to 70% for nodal status (21). This improves the overall accuracy of the staging with the preoperative tumour stage changed between 15 and 60% for the different gastrointestinal malignancies and laparotomy could be avoided in 5-55% (22).

Endoscopic ultrasound (EUS)

Endoscopic ultrasound (EUS) provides a more accurate assessment of local invasion of the cancer and lymph node metastasis, and is now widely used in the staging of oesophageal and gastric cancer. EUS provides a better resolution than other imaging techniques, including spiral CT scan, MRI and transabdominal ultrasound (23). Numerous centres in France (24), Germany (25), Netherlands (26,27) and the United States (28) have all demonstrated that EUS is better in loco-regional staging of patients with oesophageal carcinoma, with superior accuracy in detecting tumour invasion. EUS has also provided better accuracy in determination of nodal status, by demonstrating the size and echogenic pattern of the nodes (29). The accuracy of EUS in assessing the extent of cancer invasion is more than 80% for ampullary carcinoma, and more than 70% for extrahepatic bile duct carcinoma. The accuracy of EUS in predicting regional lymph node metastasis is about 60% for ampullary carcinoma, and similar for extrahepatic bile duct carcinoma. Invasion of portal vein can sometimes assessed by EUS in 2 of 3 patients, but it is not as sensitive for liver metastasis. It may be able to detect small liver metastases of 0.5cm in the left lobe of the liver. Endoscopic ultrasonography (EUS) can be useful in the diagnosing submucosal tumours.

A further adjunct to the use of endoscopic ultrasonography is endoscopic ultrasonographically guided fine needle aspiration biopsy (EUS-FNAB). EUS-FNAB may be able to establish a histological diagnosis. EUS-FNAB may increase the diagnostic capability of EUS in differentiating between submucosal tumours (30).

Transrectal ultrasonography (TRUS)

Transrectal ultrasonography (TRUS) is useful in assessing the depth of rectal wall invasion and in assessing extra-rectal spread. TRUS is statistically superior to digital rectal examination and has a sensitivity of 82.9%, a specificity of 97.5% and an accuracy of 90.7% (31). TRUS is also better than conventional ultrasonography at detecting a small amount of ascites, with sensitivity, specificity and accuracy of 75%, 93% and 86.6%, respectively. TRUS however is limited in accuracy in staging miliary

extrarectal tumours compared to nodular and thickened types. TRUS is unable to fully assess up to 25% of rectal tumours due to pain or stenosis.

Angiography

Angiography provides an anatomical arterial map of the liver, confirming the involvement of the vascular stem of the hepatic artery or portal vein or bilateral involvement of vasculature to preclude curative resection. Unfortunately, as a diagnostic modality, it has a low sensitivity for liver metastases and often misses tumours in the left lobe and extrahepatic disease. It can however detect extremely small metastases, less than 5 mm. In view of it poor sensitivity it is recommended only in selected cases.

Magnetic Resonnance Imaging (MRI

Magnetic Resonnance Imaging (MRI) is useful in assessing invasion beyond the gut wall or metastases to other organs. It is however limited in the routine use of investigating gastrointestinal tumours because of motion artefact and poor spatial resolution. MRI however is particularly good in assessing oesophageal and rectal tumour extension where there is less spatial resolution with more fat surroundings. The multiplanar capabilities allow sagittal sections to delineate the rectal anatomy, especially the rectal sphincters, hence allowing accurate assessment of the feasibility of sphincter saving operations. Furthermore it can detect borderline pelvic lymphadenopathy, detecting nodes ranging from 12mm to 20mm. Brown et al have reported in their study that preoperative thin-section MR imaging can accurately identify tumour stage of rectal cancer and depth of extramural tumour infiltration (32). Patients staged with T3 tumours are at high risk of failure of complete excision, hence will benefit from preoperative adjuvant radiotherapy.

Hepatic metastases will give a well-defined margin and homogenous internal morphology with hypoechoic T_1 and T_2 weighted images. MRI has a higher sensitivity in detection of individual metastatic liver tumour than CT, 64% over 51% respectively. It has a high sensitivity and specificity in identifying individual patients with liver metastases. It can assess the tumour involvement in hepatic veins with sensitivity of 75% and specificity of 98% (33). However the results are comparable with ultrasonography (81% sensitivity, 97% specificity, and 87% positive and 95% negative predictive values) for assessment of hepatic vein involvement by tumour.

MRI use may be limited in pancreatic cancer. It may have difficulty in diagnosing cancer and pancreatitis. Tumour invasion and extension to adjacent structure may become ambiguous because of poor spatial resolution. In this instance CT is superior to MRI.

Peritoneal lavage

Peritoneal lavage is a simple procedure that can be performed during laparotomy for GI tumours. A number of centres advocate the use of peritoneal lavage as an adjunct to diagnostic laparoscopy. Tumour cells in the lavage fluid are thought to indicate intraperitoneal tumour seeding and to have a negative effect on survival. For this reason, peritoneal lavage is frequently added to diagnostic laparoscopy for staging GI malignancies. However, the lavage result have altered the assessment of tumour stage and adequately predicted inoperable disease in less than 2% of the patients. Furthermore, it has limited prognostic value for survival.

Role of tumour markers

In the general work up of a patient with a suspected malignancy, routine blood tests (full blood picture, urea and electrolyte, liver function tests, bone profile) are taken following clinical consultation. It may reveal anaemia in occult caecal or stomach cancer. Abnormal liver function test suggests liver metastases. During cancer development and progression, certain carbohydrate chains are expressed 'as a marker' on the cell. A tumour specific marker will prove invaluable in cancer management. An ideal markers should be tumour type specific, detect all tumours of that type, be a good indicator of tumour bulk, be produced equally by metastases and primary tumour, easy to quantify, sensitive at low levels and inexpensive. The commonly used serum tumour markers in gastrointestinal cancer are CEA and CA 19.9. Tumour markers may also be useful in patient selection for chemotherapy regimes.

Carcino-embryonic antigen (CEA) is a cancer-associated 'oncofetal' protein predominantly found on cancer cells of the colon and stomach. CEA is not exclusive to gastrointestinal carcinomas and the foetal digestive tract. It is also present in other tumours such as pancreatic cancer and cholangio-carcinoma. In a recent colorectal cancer study, CEA was found to have a sensitivity of less than 65% and a diagnostic accuracy of only 76% (34). Similar results are found in pancreatic cancer. Unfortunately, CEA is also increased in a small number of benign inflammatory bowel and diverticular disease and 10% of chronic pancreatitis. It is present at a low level in the normal colon.

CA 19-9 is an oligosaccharide antigen identical structurally to silaylated Lewis A (35). It is detected by using murine monoclonal antibody against human colonic adenocarcinoma cell line SW1116 (36). Raised levels of CA 19.9 are found in colorectal tumours as well as pancreatic and gastric cancer. It can detect 87% of pancreatic tumours at levels of greater than 37 μ/ml. However at this level 15-36% of patients with benign pancreatic, liver and biliary disease (37) are positive. Levels of >100μ/ml are more specific but less sensitive and rarely found in patients with benign disease. Metastatic disease patients also present with higher levels of CA19-9.

The search for the ideal tumour marker has remained elusive. Serological tumour markers do not have sufficient sensitivity and specificity to be used as a screening tool for colorectal, gastric, or pancreatic cancers. Most tumour markers are likely to be positive in advanced stages of the tumour and negative in small tumours. Hence, their use is unlikely to influence outcome in advanced or recurrent disease, since no curative treatment is available at present for most advanced GI tumours. Elevated pre-operative levels of CEA and CA 19-9 correlate with advanced disease and poor clinical outcome. Monitoring of serum CEA after primary resection of colorectal cancer may identify a small number of asymptomatic patients with recurrent disease who are amenable to a second surgical procedure with curative intent. Monitoring CEA levels also provides a measure of clinical response, with a sensitivity of 85% and specificity of 90% respectively when objectively evaluated with CT scan. Over 50% of patients with rising CEA have unresectable disease. Thus CEA level monitoring does not improve the resectability rate or survival.

Table 2. Chemotherapy family

Type of the drugs	Name
Antimetabolites	methotrexate and 5-Flurouracil
Alkylating agents	Cyclophosphamide, Ifosfamide, Busulphan
Antibiotics	Doxorubicin, Mitomycin C, Bleomycin
Platinum	Cisplatin
Plant products:	Vinca alkaloids
Taxanes	aclitaxel, Docetaxel

TREATMENT

Chemotherapy

The principle behind chemotherapy is the reduction of occult cellular or metastatic disease with potential improvement of in survival. Despite tremendous strides in cancer chemotherapy in the management of leukaemia, children's solid tumours, and lymphoma, the impact of chemotherapy is not reflected in gastrointestinal carcinomas. Less than 40% of all carcinomas of the gastrointestinal tract will be controlled by the current modalities of treatment. Several new agents have reported promising activity in gastrointestinal malignancies in the past several years. Currently there is no cure for advanced gastrointestinal malignancies with the available agents or approaches as a single modality treatment. Understanding the mode of action of these drugs allows the rational use of different combinations. Similarly, when combination therapy is used, the drugs of the different mode of action may enable enhancement of the effect of the other drug groups. Chemotherapy drugs are divided into six classes (Table 2).

Single agent

5 fluorouracil (5FU)

5 fluorouracil (5FU) remains the most effective anticancer agent available either as a single or combination therapy agent for the treatment of patients with gastrointestinal tract cancer. Intravenous or intraarterial continuous drip infusion is the most effective if administered over the long term (if possible, 4 weeks or more). The drip infusions are restricted by the dose-limited toxicity with gastrointestinal disorders such as stomatitis and diarrhoea. Colorectal cancer has also been successfully treated with several direct thymidylate synthase inhibitors, oral 5-fluorouracil analogues, irinotecan, and oxaliplatin, alone and in combination regimens. The response rates to single agent 5FU are in the range of 9.5-23% while in combination, 32% with methyl-CCNU, 37% with mitomycin-C. Upper gastro-intestinal malignancies have shown some response to combinations including irinotecan and the taxanes. Pancreatic cancer, while remaining relatively chemoresistant, is proving treatable with the new direct thymidylate synthase inhibitors, docetaxel, and gemcitabine, with improvements in quality of life being an important outcome..

Cisplatin

Cisplatin is administrated intravenously, and is effective in oesophageal cancers. It binds to DNA, forming adducts resulting in DNA links hence distorting the shape of DNA and inhibiting DNA replication. It is commonly used in combination with 5-FU in oesophageal tumours.

Mitomycin C

Mitomycin C is an antibiotic, active as a free radical forming agent and as a bifunctional alkylating agent. The drug possesses two alkylating groups which enables it to bind to two nitrogen atoms. They form interstrand DNA links, cross linking the two strands of DNA, hence, interfering with normal DNA function of replication and repair.

Taxoid

The taxoid class of antitumour compounds, most notably paclitaxel, was originally extracted from the Pacific yew, and its semisynthetic analog docetaxel, represent significant advances in the treatment of patients with a variety of malignancies. Although Paclitaxel and docetaxel have a similar chemical root, important biological and clinical differences exist between the two compounds. The mechanism of mitosis disruption and cell replication is novel and unique to taxol compounds, but there are small but important differences in the formation of the stable, non-functional microtubule bundles and in the affinity of the two compounds for binding sites. This lack of complete cross-resistance was observed between docetaxel and paclitaxel in preclinical and clinical studies (38).

Tumour resistance to chemotherapy may result from either the poor cellular uptake of chemotherapy drugs or the fact that the cells are intrinsically resistant or have developed resistance to cytotoxic drugs. Overexpression of cellular enzymes such as the 'multidrug resistant' (MDR) P-glycoprotein may inhibit cytotoxic drugs or be inhibited by them. The MDR protein functions as an efflux pump for cytotoxic drugs (including mitomycin C and vincristine), thus overexpression of MDR decreases intracellular drug concentration. Drug resistance remains a significant clinical problem to be solved.

Radiotherapy

Radiation therapy has played an increasing role in the improving local control in certain gastrointestinal cancers, especially rectal and oesophageal cancer. The principle behind radiotherapy is that at a given dose, a certain fraction of malignant cells will be killed. However the normal gastrointestinal mucosa is relatively sensitive to irradiation and hence, increasing radiation dose is limited by the tolerance of normal surrounding tissues. Furthermore the radiation oncologist is faced with difficulty in localization and immobilization in treating many gastrointestinal cancers. These problems are minimized in the rectum, where radiotherapy is now standard in the treatment of carcinoma, both as an adjunctive therapy and neoadjuvant therapy.

Hormonal manipulation

Expression of sex steroid receptors by gastrointestinal (GI) tumours may mean that there is a role for hormonal manipulation in their management. Jacob reported very low levels of both ER and PgR in the GI cell lines using enzyme immunoassay (EIA), with levels of PgR generally higher than those for ER. Neither ER nor PgR were detected in cytosols made from the GI cell lines (39). Currently there significant response with hormonal manipulation.

Principle of surgery

The aim of potentially curative surgery is to remove all the malignant tissues. Cure however depends on a number of factors. Evaluation and true staging of the disease prior to surgery is the most effective way of ensuring that the surgeon can remove all the cancer tissues. However, a significant proportion of the patients with no evidence of disseminated malignancy and who have had removal of all gross disease, harbour occult metastases. This may not be declared until months or even years after surgery. In general for these patients, no modification to the surgical technique will enhance their curative resection, and survival will depend on combined modality treatment. In some cases, however, prognosis may be greatly influenced by the surgeon. Adequately excision of all apparent disease, as indicated by the pre-operative staging, may cure the patients. But if the disease is inadequately excised, there is an increased likelihood of local recurrence and perhaps, impaired survival. Histological confirmation of adequacy of local excision relates more to local recurrences rates while raw survival data may be more pertinently relate to occult metastatic disease.

Curative

Curative surgery consists of en bloc resection of the primary tumour together with the draining vasculature and regional lymph nodes and offers the best hope for cure at the present time. Long-term survival has not changed significantly over decades, despite sometime aggressive extended resections. Such extended resections are performed in the belief that when the highest dissected lymph node (farthest from tumour) is free of tumour, a better chance from cure is achieved. However, the value of extended procedures is still controversial. Advocates of more limited procedures argue that tumour stage more than surgical technique predicts ultimate outcome, and that therapy should be as minimally invasive and mutilating as possible to ensure the best quality for remaining life.

Palliative

With adequate staging, cancer surgeons today can with a degree of confidence select patients who will benefit from the more radical curative surgical approaches. This allows aggressive palliation and palliative care for those who cannot gain cure from heroic surgical approaches.

TUMOUR TYPES

Carcinomas

Oesophagus

The incidence of oesophageal cancer, ranges from 5 per 100 000 in the USA to 100 per 100 000 in China. Almost 2 decades ago, 90% of oesophageal tumours were squamous cell carcinoma and 10% were adenocarcinoma. Since then, there has been a steady increase in the incidence of adenocarcinoma of the oesophagus, with some reports suggesting that adenocarcinoma now accounts for more than 50% of oesophageal tumours. It is commonest at the narrowing of the oesophagus especially at the arch of aorta, level of transit of left main bronchus, at the diaphragm and at the cardia. Adenocarcinoma at the cardia is, mainly, from gastric origin. Most oesophageal adenocarcinomas are thought to arise from Barrett's metaplasia. The variant, oat cell carcinoma has poor prognosis. Current debate is on the increasing incidence of distal oesophageal tumours and proximal gastric tumours. A number of centres have

disagreed with arbitrary separation of oesophago-gastric tumours. They have argued that the etiology and clinical presentation are often the same, supported by the similarity of the cell type and differentiation.

Clinical picture

The most common and most important presenting symptoms in patients with oesophageal cancer is dysphagia. Unfortunately this accounts for 80-90% of the patients and a large proportion of these patients are incurable and inoperable at time of presentation. Pain is also an ominous symptom and may indicate penetration of the tumour through the wall of the oesophagus. Productive cough especially at night may be caused by aspiration, or more seriously, an oesophago–tracheal fistula.

Pattern of metastases

Within the wall of the oesophagus, cancer cells tend to spread submucosally both longitudinally and circumferentially in advance of direct mucosal disruption. These tumours penetrate the serosally deficient wall of the oesophagus with ease and metastasise via lymphatic permeation. Oesophageal cancer usually spreads early by direct invasion and becomes adherent to adjacent structures.

The extent of tumour involvement is often underestimated. Lymphatic spread develops early (Figure 1) and lymphatic metastases can be identified in the neck in more than 30% of patients at presentation. In the Western series, up to 80% of the patients may have lymph node involvement at the time of surgical resection. Draining lymph nodes for the oesophagus include local intrathoracic paraoesophageal nodes, supraclavicular nodes, nodes surrounding the left gastric artery and coelic axis within the abdomen. In squamous cell carcinoma, perioesophageal and paratracheal nodes are most frequently involved, 28.6% in both followed by recurrent laryngeal nerve, deep cervical and perigastric nodes. In the middle third, the perioesophageal nodes, followed by perigastric and subcarina nodes are involved in 27.3%, 18.2%, 10.6% respectively. In the lower oesophagus, the perigastric lymph nodes again are the most common followed by coeliac and recurrent laryngeal nerve nodes. The depth of tumour invasion was also found to correlate with lymph node metastasis. Haematogenous spread is unusual in the early stages of oesophageal cancer.

Surgery

Curative

Age alone is not a contraindication to surgery. Patients aged over 70 years have benefited following oesophageal resection and have a postoperative mortality rate similar to that of other age groups (40,41) . Several surgical approaches are available for oesophageal resection. The standard approach is either by a two stage total thoracic oesophagectomy (Ivor Lewis) procedure or the three stage transthoracic oesophagectomy (McKeown) procedure (TTO). Transhiatal oesophagectomy (THO) has been proposed as an alternative to TTO. Other less common resection are the endothoracic endoesophageal resection, video-assisted oesophagectomy and laryngo-oesophagectomy. Often the main determinants of the operation chosen depend on the surgeon's expertise in a single technique of oesophagectomy. Transthoracic oesophagectomy, transhiatal oesophagectomy, and total thoracic esophagectomy had similar morbidity, mortality, and recurrence rates (42,43) . Survival and sites of recurrence reflected disease stage, not the technique of oesophagectomy used. One study reported no differences in long-term

survival between TTO and the Lewis procedure (44). The 5-year survival rate was 20% with the overall operative mortality of 5%. Many centres similarly reported mortality rates under 5% and a 5 year survivals around 20% with surgery alone for squamous cell carcinoma.

Although radical surgery may be considered curative for early-stage disease, surgery cannot completely control systemic spread or wide lymphatic spread. Surgeons undertaking combined extended upper abdominal and posterior mediastinal lymphadenectomy have shown consistently superior 5 year survival rates, stage for stage, compared to patients in whom lymphadenectomy is not undertaken. The abdominal dissection should include removal of splenic artery nodes combined with splenectomy. Whether upper mediastinal and cervical nodal dissection is appropriate remains to be shown. Lerut reported that 20% have nodal involvement even for oesophageal adenocarcinoma (45).

Palliative

Surgical resection for palliation is controversial. While surgical resection does provide effective palliation of malignant dysphagia, it is associated with high mortality and morbidity in patients who are in the last year of life. Effective palliation can be achieved by one of the various modalities such as endoscopic oesophageal dilation, oesophageal intubation and Nd-YAG laser.

Endoscopic dilation, although less effective in the treatment of oesophageal carcinoma, has a low complication rate (perforation) of 5% with short hospital stay. Dysphagia recurs in all the patients and repeats dilations are needed at 4-week intervals. Most patients require three or fewer dilations during their remaining life span (46).

Self expanding metal stents (such as the polythene covered Gianturco stent, polyurethane covered Wall stent and the knitted metallic alloy (Stecker) stent have recently been widely used. They can either be introduced under fluoroscopic control or endoscopically. Oesophageal intubation may be helpful in patients with tracheo-oesophageal fistula to prevent or limit the soiling of the tracheobronchial tree (47). They may reduce the need for repeated endoscopic oesophageal dilatation.

Nd-YAG laser system is effective in palliating more than 80% of patients with obstructing oesophageal carcinoma in most studies and has been shown to have a lower mortality rate and fewer complications than tube insertion. In a prospective non-randomised trial, Loizou et al compared palliative treatment with laser and with intraluminal intubation (48) Relief of dysphagia (80%) and survival rates (5 - 6 months) were similar between the two groups. Laser-treated patients did better over the remainder of their lives but required more procedures.

Chemotherapy

The response to a single agent is poor in oesophageal carcinoma, with about 14-42% responding (49,50) with no reported survival benefit. Bleomycin, 5FU, mitomycin and cisplatin are the most commonly used agents either as single agents or in combination therapy, with a further advantage gained utilising the synergistic effect with radiotherapy. A number of the early studies have reported responses with squamous cell carcinoma. Bleomycin can pulmonary fibrosis and is no longer preferred. 5-FU is efficacious when given as a continuous infusion and demonstrates synergism with cisplatin. 5-FU as a single agent is reported to have a response rate of 14% in the ECOG trial[50]. Cumulative response with Cisplatin was about 25% (49,51). In an EORTC study a response rate of 18% for single agent therapy was reported but combination

with 5-FU achieved a response rate of 36% in 47 patients (52). There has been no single agent cisplatin trial in adenocarcinoma. Vinorelbine, a semisynthetic vinca alkaloid, has been studied in squamous carcinoma with a response rate of 20% (53) but there is no report in adenocarcinoma

Recent use of paclitaxel had a radiosensitising effect radiation but was concentration and schedule dependent. Ajani et al reported 34% response in adenocarcinoma and 28% response with squamous cell carcinoma (54). Unfortunately this study is small with 33 patients in the former and 18 patients in the latter groups. Other drugs have been reported in squamous cell carcinoma but produce a poor response rate of less than 5%. These drugs include Methotrexate analogue dichloromethotrexate and trimetrexate, etoposide, ifosamide and carboplatin. Carboplatin has also been studied in adenocarcinoma, but poor results of response rate. Vinca alkaloid vindesine and polyamine synthesis inhibitor mitoguazone (methyl-GAG, MGBG) have also been tried but toxicity has limited usage.

A number of combination regimes have been evaluated primarily in squamous carcinoma. Kelsen et al at MSKCC reported a 17% response rate with Cisplatin and infusional bleomycin (55). While a three-drug regime of cisplatin, vindesine with either bleomycin or MGBG yield a response rate of 31% and 40% respectively (56,57) . Other combinations of cisplatin based three drug regimens also reported similar response rates of between 30-40%.

With combined cisplatin and infusional 5FU in squamous cell carcinoma, response rate of 35% was reported in metastatic, recurrent or locally advanced incurable squamous cell carcinoma (52,58). Neoadjuvant therapies with the combination have been reported in 40-60%. Replacing Carboplastin for cisplatin did not achieve the same result. A multicentre study with combination of intravenous cisplatin, infusional 5FU and paclitaxel, reported a 45% response rate (58,59) but the study was limited by stomatitis and neutropenia which occurred in most patients. Currently cisplatin and 5FU are the standard first line treatment for patient with adenocarcinoma or squamous cell carcinoma.

Radiotherapy

Radiotherapy has been a standard traditional treatment arm of oesophageal cancer, either alone or as an adjuvant to surgery. There is no randomised trial comparing radiotherapy and surgery alone. Retrospective non-randomised trials have supported surgery over radiotherapy. The rational for this modality is that squamous carcinoma is radiosensitive. Adjuvant radiotherapy with surgery failed to report any significant survival benefit over surgery alone. Unlike rectal carcinoma, neoadjuvant therapy has not been reported as producing any improved results (60). Radiotherapy alone however is also found to be inferior to combine radio-chemotherapy with 5FU and cisplatin in a phase III trial (61). Recent trials suggest that preoperative chemoradiotherapy may be superior to surgery alone but trials are ongoing to better define the role of combined modality therapy in the surgical management of oesophageal cancer (62).

Prognosis

For oesophageal cancer, the result of surgery and radiotherapy are both very poor, but broadly similar in the long term. One-year survival rate is approximately 20% and the five-year survival rate of fewer than 10%. In the Italian multi-centre study, only 18% of the patients who undergone resection were considered early oesophageal cancer. Overall 5-year

survival rates for intraepithelial, intramucosal and sub mucosal tumours were 92.8%, 72.8% and 44.3%. Even with limited wall infiltration there is significant lymphatic spread. There was a higher recurrence of disease with sub mucosal lesions.

Stomach

Gastric carcinoma is common with an incidence of 13 per 100 000. The incidence is highest in Japan followed by Chile, Austria and Finland. Adenocarcinoma is the most common cancer of the stomach, being responsible for more than 90% of gastric tumours. There is a higher incidence of adenocarcinoma of the stomach arising from adenomatous polyps, with polyps composed of dysplastic glands having malignant change occurring in 18-75%, especially those that are greater than 2 cm. There is a strong association between chronic gastritis and gastric cancer. A total of 94% of the superficial cancers occur in areas of gastritis, and carcinomas have been found also in intestinal metaplasia. The tumours are classified according to differentiation into well, moderate and poorly differentiated. Lauren classification divides them into intestinal, composed of malignant glands, and diffuse type, make up of small groups or single malignant cells. The prognosis of intestinal type is better than that of diffuse type. The diffuse type is not accompanied by gastritis. Dysplasia of the mucosa represents important premalignant mucosal change.

Clinical picture

The principle symptoms are anorexia and dyspepsia associated with mild abdominal discomfort and belching. The finding of anaemia is also a common initial presenting mode. Patients with advanced gastric cancer may present with Virchow's node, acanthosis nigricans, Blumer's shelf, ascites, gross cachexia and a palpable mass. Their presence indicates incurability and short median survival. The main hope for improved long survival is to detect early gastric cancer, where only surgical resection can offer a cure. Early gastric cancer however, can be easily missed.

Pattern of metastases

Early gastric cancer denotes a stage at which the gastric cancer is curable. Even in these cases, there are 10% reported to have venous metastases. Gastric cancer is most commonly spread by either lymphatics or direct invasion. There is also haematogenous spread as well as tumour implantation. It may spread luminally into the duodenum and oesophagus. Duodenal involvement occurs in 24-30% of the cases. The extent of spread is the most important prognostic determinant. Lymphatic flow (figure 2) around the upper portion of the greater curvature of the stomach, is toward the nodes around the splenic hilus and splenic artery. The spread of carcinoma is often to the lymph nodes around the splenic hilus and splenic artery when the primary tumour is located along the greater curvature of the upper third of the stomach. More than a quarter of the patients with gastric carcinomas has positive hilar nodes metastasis located in the upper third of the stomach. The incidence of microscopic lymph node metastasis around the splenic artery was about 15% in patients who underwent distal pancreatectomy simultaneously with total gastrectomy for gastric carcinoma. Lymph node metastases are common. Extramural lymphatics carry deposits to lymph nodes in more than 50% of operative cases. Those adjacent to the tumour are the most commonly invaded, but several reports show that up to 25% have initial lymphatic spreads to nodes

distant from tumour. Hepatic metastases are obvious in 10-20% and indicate incurability, but the incidence of undetected metastases is of course unknown. Palliation only is offered in 50-70% of the patients.

Surgery

Japanese centres report excellent results when wide local excision is combined with systematic extended lymph node dissection, especially in gastric and oesophageal cancer (Table 3). The overall 5-year survival of over 50% for the large number of patients undergoing gastric resection for cancer seems to demonstrate convincingly the value of the extended lymphadenectomy. All oriental studies are uncontrolled, as are most reports from Western countries. The role of extended lymphadenectomy is therefore far from certain. The results from two randomised studies (British Medical Research Council and Dutch Gastric Cancer Trial) are awaited.

Chemotherapy

Complete responses are uncommon with single agent therapy. Responses are usually brief and without any significant impact on survival. 5-FU is the most commonly used single agent in gastric cancer, with an overall reported response rate of 21%. Mitomycin C has also been extensively used with a reported objective response of 30% (63,64).

Doxorubicin (adriamycin), an anthracycline antibiotic, is used in patients with advanced disease. It has a lower response rate (17%)(63,64) compared with the former. Cisplatin produced significant response in 19% including patients previously treated with chemotherapy (65,66) . Responses to Taxotere were seen in 14% of those with adenocarcinoma (67).

Most combination chemotherapy regimes have reported response rates between 30–50%. 5-FU based combinations are most widely used with response rates of 19-53%. With etoposide based combination regimes the response rates are reported between 18-53%. FAM (5-Fluorouracil, Doxorubicin, Mitomycin) combination was the most widely used in the 1980's. with partial response rates reported in 42%. A larger study reported a culmulative response rate of 30% with complete remission of 2%. North Central Cancer Treatment has reported no significant difference with single agent 5FU compared to 5FU plus doxorubicin and FAM (68).

In the cisplatin based chemotherapy (FAP: 5-Fluorouracil, Doxorubicin, Cisplatin) where Cisplatin replaced mitomycin, the overall cumulative response rate for the FAP regime was 34%. The overall response rate of 51% was reported for Cisplatin-5FU combination compared with 25% for FAM and 26% for 5FU alone. Progression free survival was reported to be longer but there was no significant difference in overall survival. In a prospective randomised trial comparing FAP with FAMe (5FU, doxorubicin and methyl-CCNU) and FAT (5FU, doxorubicin and triazinate), the Gastrointestinal Tumour Study Group reported response rate of 19%, 15% and 20% respectively. The median survival rates were 31 weeks, 24 weeks and 30 weeks respectively. In another variant study where epirubicin replaced doxorubicin, Cunningham et al reported an objective response of 71% in a small study of 14 patients. A second trial reported a response of 37% with a 17% complete remission rate. The Italian Oncology Group reported a 47% response rate with 11% of the untreated patients having complete remission, compared a phase III trial with PEFE (cisplatin, epirubicin, leucovorin and fluorouracil) and FAM. They reported response rate of 43% with PEFE and 15% with FAM.

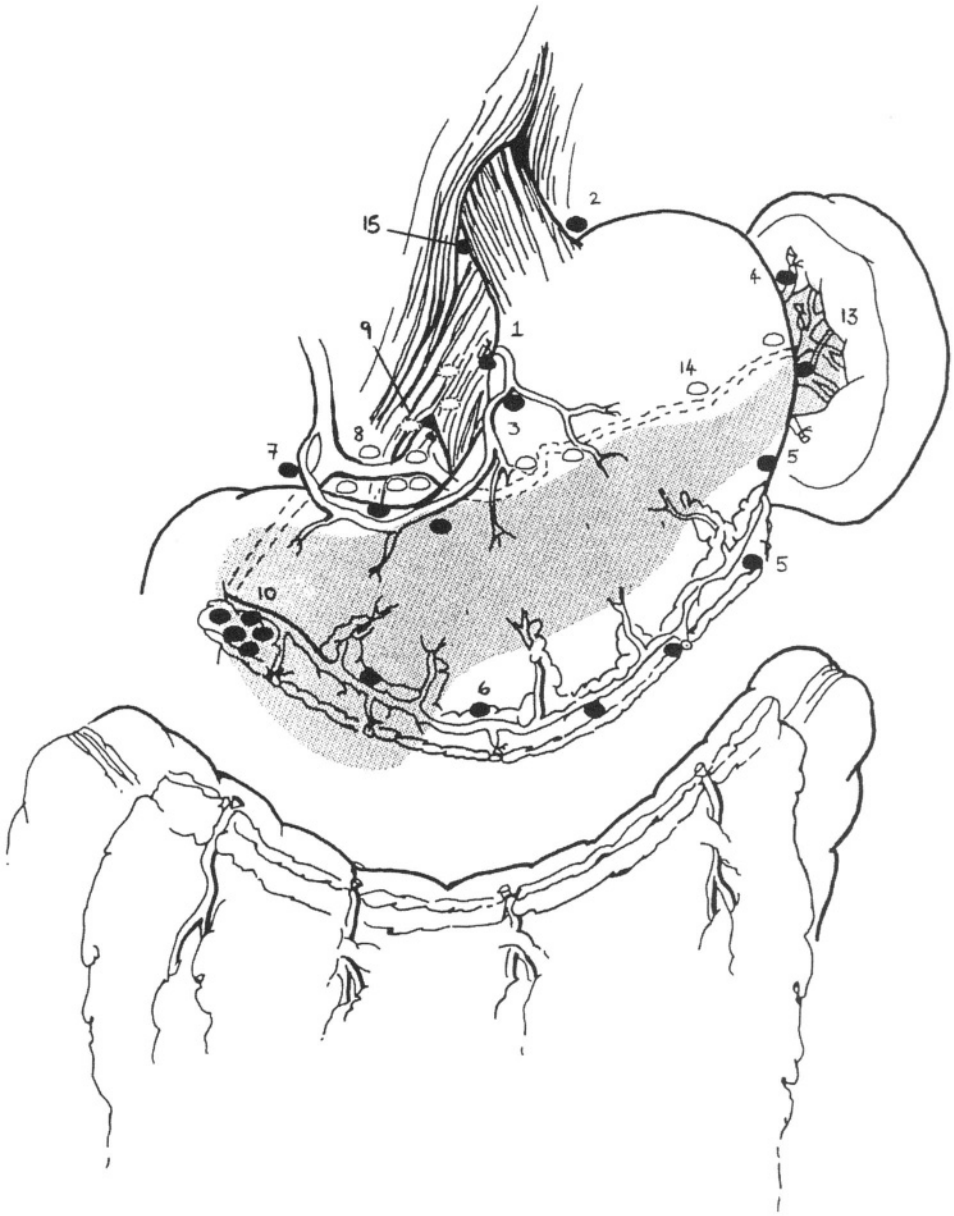

Figure 2. Lymph node station in the stomach that are involved in spread of tumour

Table 3. Japanese calssification of stomach resection

D Resection	Description
D0	Does not remove any lymph node group
D1	Removes nodes in group I (N1): perigastric lymph node, but leaves a larger portion of omentum
D2	Adequate gastrectomy with Group II (N2) nodes en block, Usually include entire greater omentum, superior leave of transverse mesocolon, pancreatic capsule and lesser omentum. Lymph node removed from gastroduodenal artery at the hepatic origin and common hepatic artery, porta hepatitis, splenic artery to splenic hilum of the spleen
D2	Attempts to remove Group III (N3) nodes include pancreas and spleen

*Previous R resection is replaced by D resection in line with (UICC)

Radiotherapy

There is a limited amount of data to suggest that radiation therapy has a major impact on the outcome of patients with gastric cancer. Very few studies have evaluated radiation therapy alone (with no concomitant chemotherapy) as an adjuvant to surgical resection. Although others have evaluated other combinations of chemotherapy with radiation therapy, there has not been any advantage shown to date with any drug regimen, besides 5-FU, when combined with radiotherapy. Data suggests that for patients with node positive disease, serosal involvement, or close or positive surgical resection margins, postoperative radiation therapy may be of value. Early data on radiation therapy for gastric cancer comes from the Mayo Clinic, where studies were performed in the 1960s on the use of radiation therapy and 5-FU in a variety of gastrointestinal malignancies. Although these reports were based on patients with locally advanced tumours, they lay the groundwork for the subsequent adjuvant studies. Childs' group (11) reported a study of patients with advanced gastric cancer who were randomised to radiation therapy alone to a dose of approximately 4000 cGy or radiation therapy combined with 5-FU as a radiation sensitiser (bolus 5-FU for 3 days, 15 mg/kg/d). This study showed a significant improvement in the survival with the combination of low dose 5-FU and radiation compared with radiation alone. A randomised study by the British Stomach Cancer Group shows no significant difference in survival between postoperative radiation therapy, postoperative chemotherapy with 5-FU, adriamycin and mitomycin C, or surgery alone, but there was a decrease in local recurrence rate.

A randomised Mayo clinic study comparing radiation therapy (3750 cGy in 24 fractions) plus 5-FU (15 mg/kg x 3) versus surgery alone for poor prognosis patients, produced a 5-year survival of 20%, compared with a 4% 5-year survival in the surgery only controls. With intraoperative electron beam radiation therapy, in a non randomised trial, where patients received a single dose of high energy electrons delivered to the tumor bed at the time of gastrectomy, there was an improved 5-year survival in patients with locally advanced disease (20%). However, a small-randomised trial at the National Cancer Institute did not report any significant survival advantage, but there was an improvement in local control as compared with surgery alone.

Prognosis

Approximately one quarter of patients with resectable carcinoma of the stomach

will be alive five years after treatment, though only two thirds of the patients undergoing surgery prove to have resectable cancer. If vigorous screening programs are undertaken, as for example in Japan (where the incidence is highest in the world), there is no doubt that these cancers are generally identified earlier whist still operable, and the surgical resectibility and the long term results are correspondingly better. The Japanese Research Society for gastric carcinoma have sub-divided the nodes according to involvement and location of main tumour Figure 1. That group of node involvement indicates an increase in advancement of disease which correlates with decreased survival. N1 is within 3cm of primary tumour, N2 is greater than 3 cm and N3 and N4 are distant nodes, and they correlate with survival of 92.8%, 58.5%, 11.1% and 8.5%. The Japanese have demonstrated the value of extended lymphadenectomy in that it correlates with increased survival. R2 resections with N1 and N2 achieve a 49.5% 5-year survival resection compared with R1 resection and R0 of 42.4% and 26.0%. In comparison with Europe and the USA, the Japanese 5-year survival is between 14.4-33.3%, while the former two were 3.7% - 10.8% and 7.0-15.1% respectively. Stage I tumours accounts for 30% of the gastric tumours.

Colorectum

Adenocarcinoma is the commonest type cancer in the colon and rectum. It represents 98% of colonic tumours.

Clinical picture

The commonest presentations of colorectal tumours are change in bowel habit, PR bleeding and approximately 30% present as acute bowel obstruction. Rectal tumours may present with tenesmus or a PR mass on routine examination, and caecal tumours often present simply with anaemia.

Pattern of metastases

The single most powerful prognostic factor in apparently localized carcinoma of the colon and rectum is the presence or absence of nodal metastases at the time of surgical resection of the primary tumour (69,70,71). In addition to the prognostic significance of nodal metastases, the presence of tumour in the regional node basin is an important criterion for a recommendation for adjuvant systemic therapy (72).

Carcinoma of the large bowel can spread via extramural lymphatic, the blood stream, by transcoelomic implantation, and by direct invasion of adjacent structures.. The tumour penetrates the muscularis mucosa and infiltrates outwards with little longitudinal spread along the wall. Primarily, the regional lymph nodes are in the vicinity of the named arteries and veins. When lymph nodes become involved, they are not necessarily along the most direct route between the tumour and origin of the mesenteric vessels. Curative resection, therefore, requires a fan shaped mesenteric resection, to encompass all regional avenues of lymphatic spread (Fig 3), so that regional lymph nodes are removed. Lymph node and vascular spread can occur without full thickness invasion of the intestinal wall. These skip lesion can lead to distant metastases without the local tumour being advance. This has prognostic significance. Lymph nodes removed from lateral pelvic wall show involvement by tumour in 10-15% of cases with low rectal cancer. Distal rectal cancers are also likely to have isolated lung metastases, because the venous drainage of the distal rectum is through the inferior and middle haemorrhoidal veins and not the portal system. At the time of diagnosis of

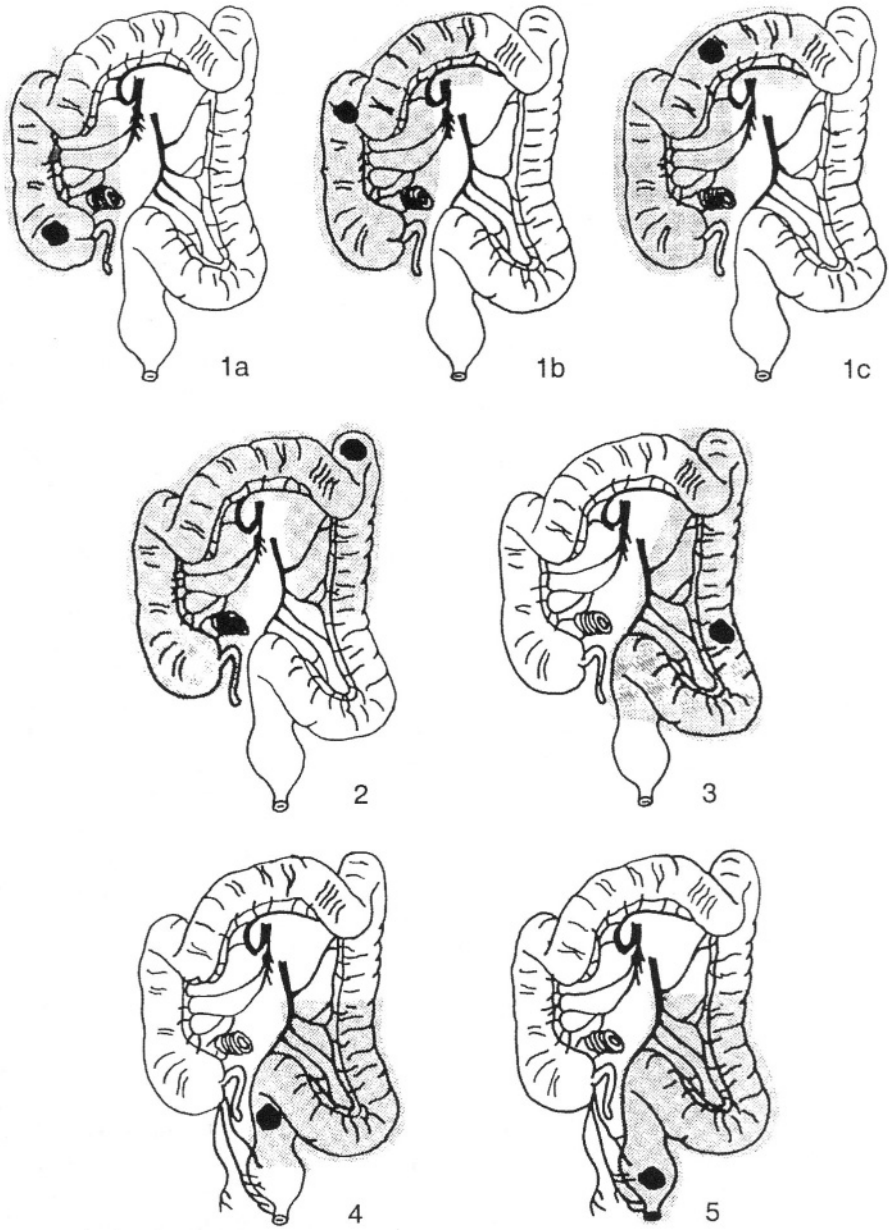

Figure 3. The limit of colonic resection and its vascular pedicles, depending on the site of tumour. 1A, B, C: Right hemicolectomies; 2: right extended hemicolectomies; 3: left hemicolectomies; 4: anterior resection; 5: abdominal-perineal resection.

colorectal cancer, 25% of patients will have liver metastases, and a similar proportion will manifest with liver recurrences isolated after curative resection. Nearly 10% of patients with colorectal cancer will have pulmonary metastases detected during life, and 10% of these metastases are isolated. These latter patients have a 20 - 30% chance of long term survival with resection. Bone, adrenal gland and brain metastases are rare but associated with disseminated disease, and isolated brain deposits are rarely resectable. Another route is the spread via peritoneal cavity resulting in intestinal obstruction. The spread to ovaries is known as the Krukenberg tumours.

Surgery

The principle of curative surgery for colorectal cancer is adequate excision of the tumour and regional lymph nodes, leaving normal colon proximally and distally and leaving disease free circumferential margins. A 3 cm margin is often more than adequate as the tumour rarely spreads more than 1.2 cm longitudinally beyond the macroscopic area of involvement (73).

Patients with colon cancer often metastasise to regional lymph nodes, but they may still be cured with surgery. Hence, it is essential to remove the intermediate and more central (principal) lymph nodes with ligation and division of multiple main vascular trunks (figure 3) demonstrates appropriate resections for the common colon cancer sites. Rectosigmoid resections include the inferior mesenteric nodes and leaving the left colic artery intact to assure adequate blood supply at the anastomosis. It has also been shown that, in rectal cancers, excision of the mesorectum is necessary to minimize the chance of local recurrence. Total mesorectal excision reduces the recurrence rate from 20 - 30% to approximately 5% (74).

Extended lymph node resection

Adequate regional lymph node dissection is part of effective therapy for colon cancer. Given the potential surgical, anatomic, and pathologic variability involved in recovering lymph nodes, it is not uncommon to experience broad variability in the number of lymph nodes histologically examined after resection of carcinoma of the colon or rectum. Because the presence of or absence of nodal metastases is of critical prognostic importance, determining the number of lymph nodes that need to be examined to accurately reflect the status of the regional node basin after curative resection of a colorectal carcinoma is of considerable interest. The Working Party Report to the World Congress of Gastroenterology (Sydney, Australia, 1990) (75) recommended that a minimum of 12 lymph nodes be examined before the patient is considered free of lymph node metastases. The recommended minimal number of nodes that need to be examined to accurately stage the regional lymph node basin has varied from a minimum of 6 (76) to 17 nodes (77).

Chemotherapy

In colon cancer, the first therapeutic approach is surgery, but the important role of adjuvant chemotherapy in these patients, in terms of disease-free survival and overall survival benefit, is now well established. Until today, standard therapy was represented by 5-fluorouracil plus levamisole and/or calcium folinate (folinic acid). In Duke's C carcinoma, a survival benefit has been demonstrated of 22% with 5FU and folinic acid, and thus chemotherapy is standard in these patients. The benefits in Duke's B disease and the best exact combination and dose of chemo are still being investigated.

Other strategies include monoclonal antibodies (mAb), which improve survival, (with a decrease in mortality by 32%), and portal vein 5-fluorouracil, alone or in combination with systemic therapy. In rectal cancer, the best results have been obtained with a combination of radiotherapy and chemotherapy. In advanced colorectal cancer, a standard treatment has not yet been established. This disease is usually considered as chemoresistant and for more than 30 years 5-fluorouracil has been the standard drug. Tumour response rates (partial & complete) for patients treated with bolus intravenous 5-fluorouracil are 10 to 15%, with a median survival of about 1 year. Many attempts have been made to improve these results. Biochemical modulation of 5-fluorouracil is one of the most interesting strategies developed in the last few years in an attempt to increase the therapeutic index of this compound. Another way has been to administer 5-fluorouracil by continuous infusion. Further innovative compounds such as irinotecan and raltitrexed are now being evaluated in clinical trials. Preliminary data from phase II and III studies have provided encouraging results on the use of these new drugs. In metastatic disease confined to the liver, the possibility of locoregional therapy through implantable pumps should be taken into consideration. Other agents Cisplatin and CPT-11 are effective for use in the gastrointestinal tract, and much work is going on now to allow these to be taken orally.

Radiotherapy

The anatomical position of the rectum, ie in the pelvis, with the majority of it below the peritoneal reflection, makes it amenable to the use of radiotherapy. This is for two reasons, (1) It is not on a mobile mesentery, thus being easy to localise, and (2) It is not surrounded by small bowel, which would be at high risk of developing radiation enteritis if in the radiation field, thus allowing generous dosage of radiation. The rest of the large bowel is not suitable for radiotherapy. In patients with Dukes' B or C rectal cancer, combined radiation and FU increase survival and decrease local and distal recurrence (78). In North America, adjuvant postoperative radio-chemotherapy is used as a standard treatment for pathologic T3/4 or pathologic N1/2/3 rectal tumours, whereas in Europe and particularly in France, preoperative radiotherapy is preferred. The European Organization for Research and Treatment of Cancer and Stockholm randomised trials have shown a significant reduction in the local recurrence rate with preoperative radiotherapy. More recently, the Swedish Rectal Cancer Group has demonstrated an improvement in 5-year survival (58% in the radiotherapy-plus-surgery group *v* 48% in the surgery-alone group) with accelerated preoperative radiotherapy (25 Gy in five fractions over 1 week) followed by surgery within 1 week of the completion of radiotherapy. The currently on-going MRC CRO7 trial is designed to demonstrate which of pre-operative and post-operative radiotherapy gives the best results and lowest morbidity. The fact that radiotherapy is to be considered in all patients with rectal cancer is now not under dispute.

Prognosis

In colon cancer, the 5-year survival rates are influenced by histological grading and Dukes Staging (figure 1). The 5-year survival rate in operated patients around 50-70%. Overall, including non-operable cases, the survival drops down to 20-25% of which 50% present with obstruction. Duke's A and B have a 70-80% 5 year survival compared with that of Dukes C, which is 30-40%. Furthermore, younger age groups and shorter history also influence poor survival. The overall

5-year survival for rectal cancers is 50% in specialist units. Discussion on the treatment of colorectal liver metastases is discussed later in this chapter.

Small bowel cancer

Tumours arising from the small bowel are rare, with the duodenum being the most common site. Adenocarcinoma is the most frequent carcinoma found in the small bowel. In the review of the literature, the upper small intestine is the most common site for carcinoma with the duodenum, jejunum, and ileum involved in 40%, 38% and 22% respectively. The 25cm loop of the duodenum contains the highest concentration of occurrence of adenocarcinomas in the small intestine. They are most prevalent in the second part of the duodenum, and jaundice may develop in 20-30% of the patients overall. They are seldom large enough to be palpable through the abdomen, though hepatomegaly or even ascites may be found if there is secondary spread. Primary carcinoma should be distinguished from cancers involving the duodenum by direct invasion or discrete metastasis.

Jejunoileal carcinoma

Most tumours lie within fifty cm of the ligament of Treitz. Unlike the duodenum, where carcinomas constitute at least 80% of malignancies, the jejunum and ileum have approximately equal numbers of carcinomas, carcinoids and sarcomas (including lymphomas). Most carcinomas of the ileum occur in association with chronic granulamatous enteritis. Nearly every type of small intestinal tumours have been reported in Meckel's diverticulum.

Clinical picture

Malignant lesions tend to have a shorter duration of symptoms than benign ones. In patients with malignant tumors, the most common symptoms are pain (not always associated with obstruction) in 32% to 86% and weight loss in a third-two thirds of cases. Bleeding is less frequent than in patients with benign small bowel tumours. Localized perforation is seen in 10% of patients, more commonly with lymphomas or sarcomas. A mass is palpable in less than 25% of those presented with dilated bowel proximal to an obstructing tumour of patients with malignant small bowel tumours. With the exception of an elevated 5-hydroxyindole acetic acid (5-HIAA) level in the presence of carcinoid syndrome, the presentation of small bowel tumours is non-specific. There is often a delay between onset of symptoms and presentation to the physician of 1 month, and a further delay of over 7 mths before the diagnosis is made.

Pattern of metastases

The lack of specific symptoms results in two thirds of the patients presenting with metastases, 32% having paraduodenal and 33-67% regional involvement (79). Carcinomas, lymphoma and carcinoid tumours can be multiple. Lieomyosarcomas spread by involving adjacent structures and mesenteric nodes, and via lymphatic or haematogenous routes to the liver. Extra-abdominal metastasis is unusual.

Surgery

The principle is similar to the treatment of colon cancer, primary control with adequate margins of bowel resection and regional lymphadenectomy.

Chemotherapy

The role of adjuvant therapy is not clearly defined because of the low incidence of small bowel tumours. A trial with 5FU may be warranted. Jigyasu have one responder in a small series of 14

patients with jejuno-ileal adenocarcinoma over 30 years.

Radiotherapy

In patients with advanced unresectable adenocarcinoma of the duodenum, it may be that palliative radiation therapy can be of some benefit in controlling chronic blood loss. Radiation therapy is difficult in these patients given the mobile nature of the small bowel mesentery and the inability to localize the target field.

Prognosis

Most recent studies have reported 5-year survival between 23-34%.

Anal cancers

These are rare, accounting for only 1-5% of all large bowel malignancy. These tumours are strongly associated with transmissible viral aetiology (80). Most anal canal cancers are epidermal in origin and are known collectively as 'epidermoid' carcinomas. True adeno-carcinomas are rare, but these arise from anal glands that radiate from the dentate line into the sphincter muscles. More often, anal canal adenocarcinomas are low rectal cancers low rectal cancers, which have spread downward to involve the canal. The types of epidermoid cancers are squamous cell, basloid and muco epidermoid. These all behave similarly.

Clinical presentation.

Benign lesions are much more common in this area. Therefore it is not surprising that the lesion is initially misdiagnosed as benign. The most common symptoms are pain and bleeding, in half of the cases, with 25% presenting with a mass and 25% with pruritis and discharge. The diagnosis usually requires an examination under anaesthesia and biopsy.

Pattern of metastases

Locally advanced tumours can invade into the sphincter resulting in faecal incontinence, vaginal discharge and fistulation. The initial lymph nodes to be involved are the perirectal nodes, then the inguinal, haemorrhoidal and lateral pelvic nodes. Up to a third of the patients present with groin lymphadenopathy, but only half of these will be confirmed to have metastatic spread on biopsy, the others having secondary infection. Biopsy or FNA is necessary if radical block dissection is contemplated. Distant spread is only found in a small minority of the cases, and the sites involved are the liver, lunds, and rarely kidneys and brain, via haematogenous spread.

Surgery

Surgery is no longer the first line treatment of most anal carcinomas, but the surgeon still plays an important role in the management. The previously performed AP resection for locally advanced tumours has been superseded by chemo-radiation (see below). Surgical input is required, however in the following circumstances (1) For the initial EUA and biopsy, (2) Small anal verge lesions, when local excision can suffice (3) For formation of temporary defunctioning colostomies, electively, before chemo-radiation, in order to control the expected local symptoms (4) For lesions too large for chemo-radiation, when AP resection is required (5) For excision of recurrent or residual tumour after chemo- radiation, or (6) For the complications of the disease and its treatment, such as, incontinence, fistulation and radionecrosis.

Chemo-radiotherapy

Radiotherapy had been the primary treatment of choice in the early century, then surgery came into vogue in the 50s.It was noted in the 90s that 5 year survival rates of between 66 and 75% could be

obtained with radiation alone. Previous studies had shown effectiveness of 5-FU mitomycin C in 1974. Combined modality surgery became standard treatment of anal carcinoma, and the recent UKCCR trial has confirmed that chemo-radiotherapy, rather than radio therapy alone, gives local recurrence rates of only 36% (as compared to radiation therapy alone which has a recurrence of 59%) (81). Chemo-radiation is now the standard treatment of anal carcinoma.

Gall bladder

Gall bladder carcinoma is the most common carcinoma of the biliary tree and the fifth most common cancer of the alimentary tract. Nonetheless they are uncommon. They are reported to be commonest among the Japanese and Americans of Chinese, Korean and Native American Indian origin. There is a strong association with gallstones in the aetiology. Adenocarcinoma of the gallbladder is by far the commonest cell type, and accounts for 80-95%, with histological forms of papillary, tubular, mucinous and signet ring cell types. Undifferentiated or anaplastic, squamous cell carcinoma and mixed adenosquamous or adenoacanthoma account for 2-7%, 2-5% and 1-3% of the cases, respectively. Carcinoid, malignant melanoma, clear cell carcinoma and spindle cell are all rare.

Clinical picture

Gallbladder cancers are rare but lethal. Those who present with early disease often are found incidentally after routine pathological examination of gall bladders removed for cholecystitis. Otherwise the disease usually presents late.

Pattern of metastases

Gallbladder cancer tends to spread locally, rather than metastasise. The problem is that local spread can occur by lymphatic, vascular, intraneural, or intraductal invasion. Multiple channels of dissemination are responsible for the limited ability to restrain or cure the neoplasm. Direct invasion of the hepatic bed in the gallbladder fossa is seen in 45-90% of patients with disseminated disease. The lymphatic drainage of the gallbladder begins in the intramural plexus, moves to the cystic nodes, into the hiatal nodes, to the superior and posterior pancreaticoduodenal nodes and finally to the periaortic chain. Lymphatic extension occurs in 20-70% of the cases. Positive paraaortic nodes are found in 20-40% of resected patients and 10-15% of those with subserosal cancer invasion. The rate of patients with positive paraaortic nodes/all patients with positive nodes was 30-50%. The number of cholestic veins on the hepatic side of the gallbladder ranges from 2-20. These drain directly into the segment IV of the liver and rarely empty into the portal veins. Blood vessel infiltration is observed in advanced gallbladder cancers. Vascular extension is seen in 10-20% of cases. Cystic vein through the liver bed is considered an important route of liver metastasis because of the high incidence of liver metastases at the liver bed. 70% are located at segment 4a and 5. Tumour spreads through the neural sheath in 24% of patients and intraductally through the biliary system in 19%.

Surgery

The extent of the surgical procedure in the treatment of gallbladder cancer is related to the depth of tumour infiltration. Extended resections are only recommended for patients with stage II to IV tumours. Paraaortic lymphadenectomy does not improve the surgical outcome, and most patients with positive paraaortic nodes die within 1 year, even after aggressive surgery with extensive lymph node dissection. Therefore it is important

to clarify the value of paraaortic lymph node dissection for patients with possibly positive paraaortic node metastasis and those with histologically positive nodes excluding the paraaortic area. Although pancreatoduodenectomy for prophylactic lymphadenectomy around the head of the pancreas has been carried out in some institutions. The procedure does not seem to be effective because the main lymphatic route from the gallbladder has a direct connection with the paraaortic nodes via the pericholedochal, periportal, and/or the posterior nodes along the common hepatic artery. A D2 plus paraaortic lymph node dissection (extended D2) is recommended as a standard surgical strategy for carcinoma of the gallbladder (82).

Chemotherapy

Most reports of the role of chemotherapy in the management of patients with gallbladder cancer have two limitations: (1) small numbers of patients & (2) a mixture of gallbladder, bile duct and sometimes, liver or pancreatic cancers. As a result, the number of published phase II trials of single agents or combinations of drugs for patients with cancers of the biliary tree is small.

Fluorinated pyrimidines have been the mainstay of treatment for cancers of the biliary tree. Response rates for 5-fluorouracil (5-FU) have been only 10% to 24%. Phase II trials with mitomycin C or cisplatin (at submaximal doses) as single agents have also reported partial response in only 10% of patients. In a phase II trial of 5-FU and leucovorin in combination with methotrexate and epirubicin there were no responses in 21 patients. No studies have been reported that clearly documents the superiority of multiple drugs over 5-FU alone.

Radiotherapy

Radiotherapy has been used in patients with resectable gallbladder cancer as well as following surgical resection. In unresected patients external beam radiotherapy may help to reduce pain or to relieve biliary obstruction. In the postoperative adjuvant setting, non-randomised data have proven a survival advantage for external beam radiation alone. Intraoperative radiotherapy (IORT) also has been used in small numbers of patients in the United States and Japan. The feasibility of IORT has been established, but the advantage of this technique, in combination with resection and/or external beam radiotherapy, has not been proven.

Prognosis

Carcinoma of the gallbladder is one of the gastrointestinal malignancies with an extraordinarily poor prognosis. The clinical course of patients with carcinoma of the gallbladder depends on the depth of tumour invasion. The overall 1 year and 5 years survival is 12% and less than 5%, respectively. Microscopic gallbladder cancer has a better survival of 64% 5-year survival and 44% for 10-year survival if the tumour extends to the serosa. However the survival rate drops to a 1 year survival of 30-40% if the tumour extends through the serosa. The curative resection rate has been only 22.2% (83). Stage-dependent surgical procedures have resulted in cumulative survival rates of 33.3% for stages II and I, 8.3% for stage III and 1.9% for stage IV (83). The overall prognosis is significantly determined by metastatic spread to the lymph nodes. Despite aggressive surgical resection there has been no significant increase in survival for the last 30 years.

Bile duct tumours

Primary extrahepatic biliary tract tumours are adenocarcinomas (cholangiocarcinomas) of varying degrees of differentiation, account for less than 0.5%

of all GI tumours. These tumours. It is common that cholangiocarcinoma present with obstructive jaundice. Lymphatic spread to the regional lymph node and blood borne spread to all parts of the liver are not infrequent. The majority are well or poorly differentiated adenocarcinoma with variable stromal component.

Pattern of metastases

The site of cancer is the most important prognostic factor in bile duct cancer. 50-75% of the cancers are located in the upper third of the extra-hepatic biliary tract at the level of the common hepatic duct or cystic duct- common duct junction. An uncommon diffuse form of bile duct cancer carries a particularly poor prognosis. The frequency of lymphatic spread of carcinomas in the proximal, middle, and distal bile ducts, is 48%, 67%, and 56%, respectively. With regard to the mode of lymphatic spread:

(1) a metastatic pathway along the common hepatic artery predominates over that to the retropancreatic area in the proximal duct carcinoma group;
(2) In the middle duct carcinoma group, metastatic lymph nodes are distributed widely, involving nodes around the superior mesenteric artery or the para-aortic area; and
(3) In the distal duct carcinoma group, metastatic nodes generally were localized around the head of the pancreas.

The incidence of local metastases approaches 50% at operation, spread to liver and hepatoduodenal ligament being most common. Metastases to distant organs are uncommon at the time the cancer is identified.

Surgery

Specific curative or palliative procedures very much depend upon the site and stage of the tumour. The mainstay of curative treatment for patients with extrahepatic biliary tumours is still surgery. In the past, cholangiocarcinoma was considered unresectable. But today, regardless of the appearance cross sectional imaging, it may be prudent to refer to the hepatobiliary surgeon before assuming the lesion is inoperable. However, primary tumour resection is not warranted if there is peritoneal or liver metastases. The surgical management for intrahepatic cholangiocarcinoma is similar to that for hepatocellular carcinoma (84) and perhaps with hepaticoduodenal lymphadenectomy. Resection entails anastomosis of the proximal ductal system to a loop of small bowel. Two thirds of middle and distal bile duct tumours are resectable, but only less than a third of the proximal lesions are deemed resectable. Surgical mortality is now less than 5%. Aggressive surgical approaches to obtain curative resections are advocated to bring about a better prognosis in hilar cholangiocarcinoma, independently of whether hepatic resection or local resection is performed (85,86).

The primary aim of palliative procedures is to relieve biliary obstruction. The choice of operative procedure e.g. bilio-enteric bypass for obstructed segment or non-operative palliation depends on accurate staging, diagnosis and quality of life. Patients with proximal bile duct cancer have the worst prognosis compared to patients with middle and distal cancers. Surgery still offers the best prognosis for resectable cancers. The prognosis for resectable intrahepatic cholangio-carcinoma is somewhat more favorable than for extrahepatic cholangiocarcinoma tumours.

Chemotherapy

Most of the trials of chemotherapy for biliary tract cancers have included patients with both gallbladder cancer and cholangiocarcinoma because of the

limited numbers. The use of 5-FU, alone or in combination with other agents, has not been proved to enhance survival in patients with cholangiocarcinoma. The agents that have been evaluated clinically were also ineffective in human cell lines. The combination of cisplatin and 5-FU was reported to be most effective, but clinical experience with this combination for cholangiocarcinoma has been limited. Only cisplatin, 5-FU, and vindesine were active as single agents, and carboquone, doxorubicin, epirucin, etoposide, and mitomycin C had no activity.

Radiotherapy

Numerous reports have suggested that radiation therapy improves survival for patients with cholangio-carcinoma, especially when resection is impossible. External-beam radiotherapy has been delivered most frequently through multishaped ports using three or four fields with standard fractions (1.8 to 2.0 Gy/day) to a total dose of 45 to 60 Gy. Custom blocking is used routinely to limit the dose to the liver, right kidney, duodenum, spinal cord, and small intestine. Non prospective, randomised trials have been reported, and patients who have been chosen to receive radiation therapy have been healthier and have had localized or resectable tumours. Moreover, a recent well-controlled, but not randomised, trial from the John Hopkins reported no benefit for postoperative adjuvant radiation.

Prognosis

Survival in patients with cholangiocarcinoma is influenced by the site and stage of the tumour as well as by its resectability. Intrahepatic cholangiocarcinomas often present in an advanced stage of disease, with only a minority of these patients having resectable tumours. The prognosis for resectable intrahepatic cholangio-carcinomas is somewhat more favourable than that for perihilar tumour. The latter has median survival has varied between 18 and 30 months, and the 5-year survival rate has been as high as 35% to 45% for the former. Compared with patients with resectable perihilar cholangiocarcinomas, median survival is 18 to 24 months but the 5-year survival rate is only 10% to 20%. When hepatic and caudate lobe resection has been added for these patients, operative mortality usually has increased, but 5-year survival rates also have increased to 20% to 35%. Patients with distal cholangiocarcinoma have the highest rate of resection. Those with resectable distal bile duct cancer have a median survival of 32 to 38 months and a 5-year survival rate of 35% to 45%.

Pancreas

The incidence of pancreatic cancer was 9.2 per 100 000 in 1987. It is the second most common GI cancer after colorectal. Malignant tumours can be exocrine or endocrine in origin. The former is more common, with 80% of the tumours being adenocarcinoma of ductal origin. Giant cell, adenosquamous and acinar cell varieties are rare but carry a similar if not worse prognosis than adenocarcinoma. Primary lymphoma accounts for 5% of pancreatic tumours. Malignant cystic tumours including cystadenocarcinoma and papillary cystic tumours behave differently and carries a very good prognosis if they are completely resected.

Clinical picture

The lack of obvious clinical signs and symptoms often delays diagnosis in most patients. Jaundice, due to extrahepatic biliary obstruction, is present in approximately 50% of patients at diagnosis and is associated with a less advanced stage of disease than are other signs or symptoms. The pain typical of

locally advanced pancreatic cancer is a dull, fairly constant pain of visceral origin localized to the region of the middle and upper back. The pain is due to tumour invasion of the celiac and mesenteric plexus. In the absence of extrahepatic biliary obstruction, few patients present with potentially resectable disease.

Pattern of metastases

Local nodal spread is extremely common because of the extensive lymphatic drainage of the exocrine pancreas. The paraaortic lymph nodes are the highest level of lymph nodes that can be resected safely in the abdomen for pancreatic and other gastrointestinal tumours. Kayahara reported that in patients with invasive ductal carcinoma of the pancreas who underwent pancreatectomy, 76% of pancreatic head carcinoma and 83% of the pancreatic body and tail carcinoma had lymph node involvement, with 18% of the former and 17% of the latter having paraaortic lymph node involvement (87). Tumour size did not correlate with paraaortic lymph node involvement. These tumours also tend to metastasise early to the liver. Two thirds are located in the head of the pancreas, producing obstructive jaundice and epigastric or back pain as the chief symptoms. Other local sites commonly involved by direct extension include the duodenum, bile duct, spleen, transverse colon and retroperitoneal sites. Distant metastases are common.

Surgery

Pancreatic resection

Surgical technique evolved from simple gastroenterostomy to several specific pancreatic resections. These include the standard Whipple's procedure, the pylorus-preserving resection, and total and subtotal pancreatectomies. Despite various modified pancreatic resections, the 5-year survival rate is about 15-36% after resection. The outcome is still less encouraging when all the tumour stages are considered. Most radical resections become palliative procedures because of the high recurrence rates

Extended lymph node resection

Regional pancreatectomy presented by Former in 1973 popularises the two distinct strategies (88). Type I is where an en bloc resection of the pancreas is performed with the intrapancreatic segment of the portal vein, base of transverse mesocolon, soft tissues around the pancreas and regional lymph nodes. The regional lymph node dissection includes the retroperitoneal lymph nodes from the diaphragm to the origin of the inferior mesenteric artery. Type II includes the additional removal of the hepatic artery and coeliac axis or a segment of the superior mesenteric artery. This method is rarely performed in Europe and North America. It has failed to show significant advantage in long term survival compared with the classic Whipple's procedure (89).

The Japanese preferred resection consists of regional lymphadenectomy and dissection of nervous plexus around the retroperitoneum followed by resection and reconstruction of the portal vein system with extended proximal pancreatectomy (90). This practice is the standardized by the Japanese Pancreatic Society. They report a 5 year survival rate of 28-46% (89). We are still awaiting a controlled clinical trial to reach a reliable conclusion.

Chemotherapy

Most studies in the treatment of pancreatic cancer, single agent or combination, have been disappointing with low response and little survival benefit. Some studies have reported a 15-30% response rate but these results were not reproducible. Most studies have been

small. 5FU is the most commonly used single agent with a response rate of 28% in a study of 212. Despite significant activity of semi-synthetic taxane docetaxel in pancreatic cell lines and 29% objective response in hepatic metastasis, the joint study between MDACC and MSKCC could not confirm these findings. Early results with gemcitabine have been promising. There is a small but significant improvement in response rate and median survival compared with 5-FU. In addition there is also an improvement in disease related symptoms. Unfortunately median survival for these patients is less than 6 months. Modulation of 5FU has not shown significant benefit and has no advantage over single agent 5FU. Hormonal manipulation to date has also been disappointing despite laboratory evidence of gastrointestinal hormone, sex steroids and growth factors sensitivity in pancreatic carcinoma. Results from combination chemotherapy have been poor and ineffective to support clinical studies evaluating novel single agents. Several 5-fluorouracil based regimens have been tried without a significant impact on palliation or survival (91).

Radiotherapy

There is not significant benefit in receiving radiotherapy alone. Even with chemo-radiation, the mean survival was reported to be only 10.4 months compared with and 6.3 months in the group that received radiation alone. Clinically matched, untreated patients with locally advanced pancreatic cancer were also found to have a median survival of approximately 6 months.

Prognosis

The majority of adenocarcinomas of the pancreas are non-resectable at diagnosis due to locally advanced or metastatic disease. Surgical resection offers the only potential cure for pancreatic carcinoma. Histological origin determines prognosis after curative resection. Non-functioning islet tumours of the pancreas will have 50% 5 years survival, with ampullary tumours having a 40% 5 year survival. Acinar carcinoma has a median survival of 18 month, and only 1% of the patients are alive after 5 years. Mean survival after diagnosis is less than 6 months. Tumour size is of less prognostic relevance. Nodal status was the only single factor significantly affecting survival. The 3-and 5-year survival rates were respectively 35% and 19% for node-negative patients and 7% and 0% for node-positive patients. Ozaki reported 5-year survival rates for stages I, II, III, and IV disease being 41%, 17%, 11%, and 6%, respectively. With multivariate analysis, he found that lymph node metastasis, intrapancreatic perineural invasion, and portal vein invasion were significant prognostic factors (92). There is a high operative mortality, of 21%. Complete resection of papillary cystic tumour result in 95% cure and 5-year survival for cystadenocarcinoma is 50%. Blaszkowsky reported from data collected from 1986-1993, the five-year survival of all stages combined was 4% (91).

Metastatic liver tumours

Metastatic adenocarcinoma is the most common hepatic malignancy in Europe and North America.

Liver metastases occur in 40 % of all malignancy. Most of the patients referred for liver resection with metastatic tumours are notably those with colorectal cancer. At the time of diagnosis of colorectal cancer, 25% of the patients will have liver metastases, and a similar proportion will manifest with recurrences isolated after curative resection, from rumour emboli via the portal vein.

Clinical picture

Right upper quadrant pain, abdominal mass, weight loss and anorexia are late indicators of liver metastases. Therefore, to increase the likelihood for resectable liver metastases being discovered, one has to have a high degree of suspicion supported by various radiological imaging techniques and tumour makers.

Investigation

It is recommended that all patients with cancer should be properly staged with liver imaging before undergoing surgical resection. Patients should then have regular follow-up after curative surgical resection with tumour markers and radiological imaging of the liver. Ultrasonography is probably of adequate accuracy to allow detection of occult liver tumour at an early stage. Raised tumour markers such as CEA should be highly suspicious of metastases, and therefore warrant further investigation.

Contrast enhanced dynamic CT is utilised to confirm the presence of the tumour as well as the position, number and localisation of metastases. This could further be enhanced by arterial portography and angiography, with or without CT assistance. The role of MRI is still being defined.

Two prospective comparative studies have reported sensitivity and accuracy for detecting liver metastases. In the first study, liver enzymes, scintigraphy, ultrasonography and computed tomography for detection of liver metastases were performed in 113 patients who were eligible for operation for a carcinoma in the gastrointestinal tract. Twenty-three of the 113 patients (20 per cent) had liver metastases. The accuracy of scintigraphy was 79 per cent, of ultrasonography 85 per cent and of computed tomography 88 per cent. The results of these tests showed no statistical differences. Ultrasonography and computed tomography were significantly more accurate than alkaline phosphatase and gamma-glutamyl transpeptidase (respectively P less than 0.01 and P less than 0.05). Taking into account cost effectiveness, simplicity and patient tolerance, ultrasonography is the test of choice for the detection of liver metastases (93).

In the second study, the conventional methods of CT scan (CT), ultrasound (US), scintigraphy (SC), and laboratory tests (LDH, AP, 5-Nt) were prospectively compared in 135 patients with gastrointestinal carcinoma to define the most useful test to detect hepatic metastases. Thirty-six patients (26.7%) had hepatic metastases at laparotomy. Sensitivities were low: 46% for SC, 58% for US, 68% for CT and 63% for LDH. Accuracies ranged from 62% (LDH) to 78% (SC). No significant differences were found. The relatively low sensitivity, specificity and accuracy of conventional imaging and laboratory tests hamper accurate and efficient detection of hepatic metastases (94).

Surgery

Surgical resection is currently still the best treatment modality for a prospect of long term survival and improved prognosis. Despite numerous studies on liver resection for colorectal metastases, the selection of patients that would benefit from the resection is still not established. All the liver metastases must be remove to have a change of long-term survival. The number of hepatic metastases and the surgical margin are important prognostic factors. Liver resection therefore depends on correctly identifying the tumour and the intrahepatic vascular system in order to retain an adequate surgical margin. Intraoperative ultrasonography is therefore essential to detect deposits not identified preoperatively, and to detect the relationship with the vascular supply.

The prognosis of patients with four or more metastatic nodules in the liver remains poor even with clear surgical margin (95). The overall survival rate after liver resection for metastatic colorectal cancer is 20-40% (95,96). Contraindications to surgery include the presence of metastases in the hepatic or coeliac lymph nodes and tumour invading the inferior vena cava or the hepatic hilus. Repeat liver resection is currently being evaluated and is believed by many surgeons to have acceptable mortality.

The indications for repeated resections are: recurrent cancer in the liver which should be localized, with absence of extrahepatic metastasis, good functional condition of the liver and absence of concomitant diseases (97). Intensive adhesions with distorted anatomical interrelations after previous extended resections make it technically more complicated, and then there is the deteriorated functional condition of the liver (97). Repeat liver resection should be considered as it does not increase mortality nor morbidity rate (98), but will improved survival to a certain extent (95).

Chemotherapy

The most important positive predictor for overall response to chemotherapy is the presence of liver metastases (99). Chemotherapy can be administrated directly into the liver in the treatment of liver metastases - hepatic intrarterial therapy. Argument for this is the high hepatic extraction and drug concentration, with minimal system exposure. Initial phase II studies reported objective response of 30-80%. Randomised studies supported these findings (40-60%) but again no substantial survival benefit between studies has been shown. Only one study, the French cooperative group, reported a significant benefit in survival. The cancer and leukaemia group b phase II randomised trial of HAI FUDR, leucovariin and dexamethazonehave reported a 78% response rate with improve median survival in patients with metastatic disease. Despite the earlier enthusiasm, the technique is limited by liver toxicity and development of systemic disease.

Prognosis

Colorectal liver metastases have a more favourable 5 year survival rate compared with those lung, breast and other primaries from the gastrointestinal tract (100). The overall survival of patients with colorectal liver metastases is still abysmal, with median survival for widespread and solitary nodule of 2.8-10 months and 4.5-21 (101,102) , 5 year survival between 7-16% (103,104) . Recent several large series have reported a 30-48% 5 year survival rate after hepatectomy with operative mortality of 1-6% (105, 106, 107, 108). The predominant site of tumour recurrence after liver resection is the liver remnant (105,107) and usually occur within 18 months (108).

Secondary bowel tumours

The gastrointestinal tract is not infrequently involved by metastatic disease. The most common tumour metastasising to the gastrointestinal tract is melanoma, with 60% of patients who die of melanoma having autopsy evidence of metastatic disease involving the gastrointestinal tract. Other less common tumours involved are the cervix, lung, breast, ovaries kidney and thyroid. Symptoms of small bowel metastases most commonly include bleeding and obstruction and, less commonly, perforation. Obstructing metastatic lesions are rarely solitary. Surgical treatment of these patients with local small bowel invasion is almost always palliative, and will be either local resection or bypass.

The outcome in patients undergoing small bowel resection for metastases is very poor with only a few long-term survivors. There are reported good results in secondary melanoma to the small bowel. The mean survival in a study was reported at 31 months in patients who underwent complete resection, compared with 10 months in patients who underwent no curative procedures[109] another reported a 28% 5-year survival rate in 12 patients with no other site of metastatic melanoma who underwent complete surgical resection (110). There are also some reports of prolonged survival in patients treated for GI metastases of breast carcinoma (111,112).

Carcinoid

Carcinoid tumours are enigmatic, slow growing malignancies, which occur most frequently (74%) in the gastrointestinal tract. In recent years, it has become apparent that the term 'carcinoid' represents a wide spectrum of different neoplasms originating from a variety of different neuroendocrine cell types.

Clinical picture

Clinical manifestations are often vague or absent. Nevertheless, in approximately 10% of patients the tumours secrete bioactive mediators, which may engender various elements of characteristic carcinoid syndrome. The carcinoid syndrome results from secretion of 5HT (5-hydroxytryptamine) and bradykinnin and prostaglandins and clinically manifests itself with abdominal pain, severe diarrhoea and flushing. In many instances the neoplasms are detected incidentally at the time of surgery for other gastrointestinal disorders.

Pattern of spread

The tendency for metastatic spread correlates with tumour size, and is substantially higher in lesions larger than 2.0 cm. An association with noncarcinoid neoplasms is ascribed in 8-17% of lesions (113). The major sites of spread include the liver and mesenteric lymph nodes. Carcinoids are curious, yellow coloured tumours arising mainly from the neurosecretory cells in the appendix and caecum. Only in 25% of the carcinoids is the tumour is functional, particularly where hepatic metastases are present.

Investigation

Carcinoid lesions are usually identified histologically by their affinity for silver salts, by general neuroendocrine markers, or more specifically by immunocytochemistry using antibodies against their specific cellular products. Within the gut, the most frequent sites are the small bowel (29%), the appendix (19%) and rectum (13%).

Surgery

Treatment consists of radical surgical excision of the tumour, although gastric (type I and II) and rectal carcinoids may be managed with local excision (113).

Chemotherapy

Carcinoid tumours are not uncommon. Single active agents with 5FU, streptozotocin, mitomycin C, cyclophosphomide, methotrexate, A interferon and doxorubicin have been disappointing although studies have claimed a response rate of 30-55%. Combination chemotherapy has equally been disappointing. Medical therapies with somatostatin analogues, omeprazole, and locoregional tumor ablation have made a positive impact on curative and palliative therapy (114). Long-acting somatostatin analogs, such as octreotide, comprise the therapeutic modality of choice for the symptomatic relief of flushing and diarrhoea in patients with carcinoid syndrome. In patients with

gastrin-producing duodenal carcinoids (gastrinoma), gastric acid hypersecretion are perfectly controlled by proton pump inhibitors. Antiproliferative medical strategies to control the growth of metastatic carcinoid tumours include long-acting somatostatin analogs, interferon alpha, and the combination of the two. However, the success rate is less than 50%, and it is questionable whether true tumour regression can be expected (115). Chemotherapy, including etoposide and cisplatin, has been shown to be effective only for purely differentiated neuroendocrine carcinomas and not for slowly growing carcinoids (115).

Prognosis

Overall 5-year survival is excellent for carcinoids of the appendix (86%) and rectum (72%), whereas small intestinal (55%), gastric (49%) and colonic carcinoids (42%) exhibit a far worse prognosis (113).

Primary Gastrointestinal Lymphoma

Primary gastrointestinal lymphomas make up about 25% of all small intestinal malignancies, but account for just 4-9% of all non-Hodgkin's lymphoma. Many cases are secondary as the gut is the commonest site for extranodal disease. Approximately 15% of patients with nodal disease also have GI involvement (116).

Where GI involvement occurs as the only evidence of lymphoma, it is referred to as primary gut lymphomas. The most frequent site of primary GI lymphoma is the stomach, followed by the small intestine, rectum and colon (117,118). The British National Lymphoma Investigation (BNLI) published a series of 175 patients with gut lymphomas-approximately half were in the stomach and half in the intestine (Table 4).

Clinical features

Patients may present with abdominal swelling or pain, weight loss, anorexia, nausea and vomiting. Less common features are GI bleeding, intestinal obstruction, altered bowel habit or perforation. Ascites typically develops late in the disease and is most commonly associated with secondary GI lymphomas.

MALT lymphomas

MALT stands for **M**ucosa-**A**ssociated **L**ymphoid **T**issue. The tumour invades locally at sites where it has arisen such as stomach, small bowel, lung, salivary gland and thyroid. These patients have indolent disease with only local symptoms. If dissemination occurs, it typically happens late in the course of the disease. It has been hypothesised that this disease may be multifocal (119). Staging studies are usually negative.

Abnormal karyotypes are common, especially rearrangements of chromosome 1p and numeric abnormalities of chromosome 3 and 7. The tumour cells express monoclonal immunoglobulin and pan B markers CD19 and CD 20. Monoclonal light and heavy chain rearrangements are also identified.

Considerable interest has been raised following reports from Isaacson et al that Helicobacter pylori is present in at least 90% cases of stomach MALTomas, and that lymphomas could regress with anti-Helicobacter therapy. However, high grade MALTomas are chemo-radiosensitive and combination chemotherapy should not be delayed by trials of anti-Helicobacter treatment.

Table 4. Classification of primary gut lymphomas

Lymphoma	Classification
B-cell	MALT lymphomas
	Low grade
	High grade
	Immunoproliferative small intestinal disease, IPSID (Mediterranean lymphoma)
	Mantle cell lymphoma (Lymphomatous polyposis)
	Burkitt-like lymphoma
	Peripheral lymph node equivalents
T-cell	Enteropathy-associated T-cell lymphoma
	Others

Enteropathy-associated T-cell lymphoma

Patients suffering from coeliac disease are predisposed to developing small intestinal lymphomas. It is often associated with small bowel ulceration. Some patients may not have previous documented history of coeliac disease as 'latency' is a recognised feature. The cancer cells express pan-T markers. It has been suggested that a gluten free diet may protect against the development of lymphoma (120). The prognosis is poor in most cases.

HIV associated lymphomas

HIV related lymphomas are usually high grade B-cell malignancies, frequently involving extranodal sites such as the GI tract. Although patients do respond to multiagent chemotherapy, pre-existing immune-suppression and cytopenias complicate their treatment.

Treatment

Experience in the management of GI lymphomas is limited by the rarity of this disease. Assessment of each patient by staging, histology with close medical and surgical consults remains important for determining the most appropriate treatment option. This includes surgical resection, chemotherapy or radiotherapy, often in combination.

Surgical intervention may be necessitated by intestinal obstruction or perforation, or when chemo-radiotherapy could lead to gut perforation as a result of rapid response to therapy.

For high-grade lymphomas, a standard chemotherapeutic regimen such as CHOP (Cyclophosphamide, Doxorubicin, Vincristine and Prednisolone) with the appropriate adjuvant radiotherapy to the site of disease is appropriate. In contrast, treatment of low grade disease remains controversial and is best conducted within the context of a well designed clinical trial.

Sarcomas

Leiomyoma and lieosarcomas are capable of metastatic spread, and equally distributed throughout the small intestine. The tumours comprise relative circumscribed whorls of smooth muscle that lack a proper capsule with variable degrees of hyalinization. As the smooth muscle enlarges, they tend to expand the serosal aspect of the intestine and may stretch and even ulcerate the mucosa.

Sarcomata of the oesophagus are rare. Most are limited to single case reports. Leiomyosarcoma and rhabdomyosarcoma have been reported.

Chemotherapy

There is no evidence that after complete resection in sarcoma, adjuvant chemotherapy or radiotherapy have shown to reduce the risk of recurrence.

FUTURE

The management of GI cancers is forever changing, as advances in molecular biology, radiological imaging, surgical technique, radiotherapy and chemotherapy continue to provide new opportunities for further improvement in the outlook of patients with these diseases. Also, novel therapies such as cancer vaccines and protease inhibitors hopefully will give us new methods of fighting GI cancers, while advances in screening and further understanding of the risk factors may cause further reduction in the incidence of the conditions

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Chapter 13

BREAST CANCER METASTASIS

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Abstract: Distant metastases from breast cancer are common, especially in patients with positive axillary nodes. They often develop late and are the predominant cause of mortality from the disease. The natural history of breast cancer remains controversial. It is unclear whether metastasis has already occurred by the time of presentation, especially in the case of small, screen-detected tumours. The commonest sites of distant metastasis are bone, liver and lungs. Bony metastases are commonest in the axial skeleton, but also frequently occur in the long bones. Their complications include spinal cord compression, pathological fracture and hypercalcaemia. When there is clinical suspicion of distant metastasis, investigation consists predominantly of imaging techniques, such as bone scans, plain films, computed tomography and magnetic resonance imaging. After surgery to the primary tumour, adjuvant chemotherapy and hormonal therapy is used to treat occult micrometastatic deposits. Clinically apparent metastases may be treated systemically, using hormonal therapy or chemotherapy, or locally, using radiotherapy or surgery. Patients with metastases confined to bone have a better prognosis than those with visceral metastases.

INTRODUCTION

Breast cancer is common. The lifetime risk of the disease for women in the general population is 1 in 10. In developed countries, it is the commonest cancer in women and its incidence is increasing (1). Despite good local control with surgery, radiotherapy and hormonal therapy, long-term mortality from the disease remains high because of distant metastasis. 7% of women with breast cancer present with widespread metastatic disease at initial diagnosis. Of those with no evident metastases who undergo potentially curative surgery, 20-30 % of patients with negative axillary nodes and 50-60 % with positive axillary nodes will develop widespread metastases (2). Kryj and colleagues recently carried out a retrospective review by of 309 patients with operable breast cancer with a minimum 10-year follow-up. Distant

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metastases were found in 49 % of all patients, but only 25 % of node-negative patients, and 25 % of patients developed metastases during the first 18 months of follow-up (3). This implies that subclinical micrometastatic deposits were already present. Indeed, it has been argued that it is always a systemic disease by the time of presentation (4). This chapter looks at all aspects of breast cancer metastasis. It will firstly consider the mechanism and timing of metastasis. Then it will describe the frequent sites of metastasis, and the tumour characteristics associated with them. Methods of diagnosis and treatment of metastases will be discussed. Finally, it will consider the prognosis of patients with metastatic breast cancer.

TUMOUR BIOLOGY

The tumour biology of breast cancer is a subject steeped in controversy. Two areas will be covered in this section. Firstly, the theories of the kinetics of tumour growth will be outlined. Secondly, the longstanding debate as to whether breast cancer is a systemic or local disease at its inception will be discussed.

The kinetics of tumour growth have direct therapeutic implications. They are classically described by the Gompertzian equation, which describes continuous growth, the rate of which decreases as the size of the tumour increases (5). This is due to large tumours having retarding factors, which result in a greater number of cells being in a dormant state. It can be applied to both the primary and metastases, and an extension of this theory is that the rate of growth of metastases may increase when the primary tumour is surgically removed. This would make metastatic foci particularly vulnerable to chemotherapy after surgery.

A recently proposed alternative model is one of micrometastatic dormancy, with a stochastic growth pattern (6). This is supported by the late presentation of both local recurrences and distant metastases that is often seen. If this is the case, the object of adjuvant hormonal and chemotherapy is to maintain this dormant state for as long as possible, ideally for the lifetime of the patient.

The original concept of the natural history of breast cancer, championed by the American surgeon, William Halsted, is that breast cancer remains localised at its site of origin, until a certain time when lymphatic invasion takes place and the disease becomes loco-regional. After a further interval, vascular invasion and dissemination occurs, resulting in metastatic disease. This concept was the basis for radical local surgery for breast cancer, as the chance of cure was thought to increase with the amount of tissue excised.

The alternative hypothesis, subscribed to by Fisher and others, is that breast cancer is a systemic disease by the time of clinical presentation (4). Indeed, it has been proposed that delay in the diagnosis of breast cancer does not reduce survival (7). A 1cm tumour has already undergone 30 doublings, compared to the lethal tumour burden of 40 doublings. Thus, it is argued that dissemination is likely to have already occurred. Evidence for this is the failure of more extensive loco-regional surgery to prevent disseminated disease (8).

In apparent conflict with the systemic hypothesis is the data from recent screening trials, which show a 30 % decrease in mortality in screened patients (9). This may mean that the primary tumour is being removed before viable metastases have developed. An alternative interpretation is that micrometastases are present, but the tumour burden is comparatively small and thus survival is

prolonged. This question will only be answered when long-term data from screened patients becomes available.

SITES OF METASTASIS

In this review nodal involvement is regarded as loco-regional disease and is not dealt with further. Seven percent of women with breast cancer will present with distant disease at initial diagnosis[10]. The most common sites of metastasis are bone, liver and lungs. Less common sites that may be involved are the peritoneal cavity, brain, adrenal gland, ovaries and thyroid. Risk factors for metastasis include premenopausal status, tumour stage, and axillary node involvement.

Bone metastases

The skeleton is the most common site of metastatic disease in breast cancer and the site of distant relapse in almost one half of the cases (3). Bony metastases from breast cancer have a predilection for the axial skeleton, but long bone metastases are also common. In the United Kingdom 9000 women with breast cancer develop bone metastases each year. Despite this the BASO guidelines have recommended that there is no role for routine skeletal surveys or bone scan screening of asymptomatic women with breast cancer, as available evidence seems to suggest that early detection of metastatic disease in asymptomatic women does not improve survival (10) and bone imaging techniques have a very low positive rate in asymptomatic patients.

The mechanism of tumour spread from the primary breast cancer to bone has been extensively studied and the cellular basis of this spread has been discussed in Chapter 8. However it will suffice to state at this point that proteolytic enzymes, cell adhesion molecules like E-cadherin, laminin and integrins, along with neovascularisation all play an important part in the metastatic process. The cellular mechanism by which tumour cells cause bone destruction in metastases from breast cancer has been shown to be due to the increased osteoclastic activity at the site of metastasis. There are 2 theories by which tumours are believed to cause an increase in osteoclastic activity. The first is that there is a local peptide produced by the tumour cells at the site of metastases. In breast cancer metastases, this has been shown to be Parathyroid Hormone Related Peptide (PTHrP) (11) which in turn is regulated by one of the growth factors, TGF-β (12). This local production of PTHrP stimulates osteoclastic activity at the site of bony metastases (13). The second theory is that tumour cells themselves may destroy bone directly (14).

The clinical presentations of patients with skeletal metastases are bone pain, pathological fractures, nerve compression syndromes and hypercalcemia.

In some patients a pathological fracture may be the first presentation of metastatic disease from breast cancer. Long bone pathological fractures occur in 10-20 % of patients with bony metastases, and hypercalcaemia occurs in 10-15 % (10).

Coleman and colleagues have recently published a major study of patients with bone metastases from breast cancer. They looked specifically at the associations between characteristics of the tumour and the development and subsequent prognosis of bone metastases. Their study showed that those patients who had disease confined only to the skeleton were more likely to be older, with lobular carcinoma and to have presented with little or no axillary node involvement. Those who developed extra skeletal disease were more likely to have had

poorly differentiated ductal carcinomas with heavy axillary lymph node involvement at the time of diagnosis. An interesting finding of the study was that patients having bone disease at the time of initial presentation with breast cancer had a better survival than those who developed skeletal disease later (15).

Liver metastases

Liver metastasis from breast cancer usually carries with it a poor prognosis. The mechanism by which breast cancer spreads to the liver is not as clearly understood as for certain malignancies, such as colorectal cancer. These spread commonly to the liver because the liver is the first capillary bed encountered by circulating malignant cells. However, as for distant metastases elsewhere, it is believed that local growth factors or hormones may induce preferential growth of malignant cells. Indeed insulin-like growth factor has been shown to be present in the liver and is a growth and motility factor for both breast and lung cancer (16).

Some of the tumour characteristics in patients developing liver metastases have been studied and, like isolated bone disease, visceral metastases also seem to occur more commonly in lobular carcinoma than infiltrating ductal cancer (17). Visceral metastases have also been reported more commonly with oestrogen receptor (ER) negative tumours, postmenopausal and older aged women. One other important characteristic is the disease free interval (DFI) which is the time period between the initial diagnosis of breast cancer and the first appearance of metastases. Studies have shown that patients who develop visceral metastases have a significantly shorter DFI compared to those with skeletal disease (18).

Brain and lung metastases

Metastases to the brain and lung are less frequent than the above two sites and seem to be associated with similar tumour characteristics to those mentioned above. Again, the disease free interval seems to play a part in the prognosis.

DIAGNOSIS OF BREAST CANCER METASTASES

At the time of presentation, routine investigation in an asymptomatic patient for the detection of metastases has a very low yield and is not recommended. The exception is the patient who presents with locally advanced disease (T3-4 or N2). There is a 30% yield of metastases in this group (19). During follow-up, investigation of asymptomatic patients for metastatic disease is also unnecessary. It does not alter the clinical course of the disease and causes unnecessary anxiety, especially as false-positive results commonly occur. Most metastases are diagnosed on the basis of symptoms and clinical signs, and are usually found by the patient herself first. There is little benefit from routine imaging or measurement of serum markers (20). However, these may be used to monitor patients and their response to treatment once the diagnosis of metastatic disease has been made. A recent study (21) showed that a combination of two serum markers, carcinoembryonic antigen and carbohydrate antigen 15-3, and erythrocyte sedimentation rate could be used to direct systemic therapy. This section considers the modalities for investigation of those patients presenting with locally advanced disease or clinical features of metastasis. Each common site of metastasis will be considered in turn (table 1).

Table 1. Investigations for diagnosis of breast cancer metastases

Site	Modality	Investigations
Non-specific	Serum markers	Carcinoembryonic antigen Carbohydrate antigen 15-3 Erythrocyte sedimentation rate
Bone	Serum markers	Alkaline phosphatase (bone isoenzyme) C-terminal telopeptide of type I collagen
	Imaging	Plain film radiography Radioisotope scanning Computed tomography (CT) Magnetic resonance imaging (MRI)
Liver	Serum markers	Liver function tests
	Imaging	Ultrasound CT MRI
Brain	Imaging	CT MRI
Lung	Imaging	Plain film radiography CT MRI
	Biopsy	Percutaneous biopsy Video-assisted thoracic surgery resection

Bony metastases

The British Association of Surgical Oncology has recently published detailed guidelines for the management of metastatic bone disease in breast cancer, which covers the investigation and treatment of metastatic bone disease in great detail (22).

There are several recently developed biochemical markers for bony metastasis in breast cancer. The most promising of these are the serum bone isoenzyme of alkaline phosphatase and the serum C-terminal telopeptide of type I collagen, which are indices of bone formation and resorption respectively. However, the sensitivity and specificity of these tests is currently inadequate (23). It is likely that their main role will be in monitoring of bony disease rather than its diagnosis. Thus, imaging remains the gold standard for diagnosis of bony metastases.

There are four main imaging modalities used to detect bony metastases: plain film radiography, radioisotope scanning, computed tomography (CT), and magnetic resonance imaging (MRI).

The radioisotope bone scan is the standard initial imaging modality. The standard isotope used is 99m-technetium methylene diphosphonate (^{99m}Tc MDP). The tracer accumulates in any site with increased osteoblastic activity or blood supply. The advantages of the bone scan are its high sensitivity and its whole-body coverage. Its main disadvantage is its low specificity, as tracer uptake may represent infection, trauma or arthropathy. Thus, in patients with cancer, only 50 % of solitary foci represent metastases (24). Difficulties

can also arise when aggressive lytic lesions have no osteoblastic repair response and when diffuse metastases produce generalised increased uptake, known as a malignant superscan. However, multiple foci strongly suggest metastatic disease.

The plain radiograph is a useful adjunct to the bone scan. It has poor sensitivity, but a positive result confirms the bone scan findings. It may also distinguish the benign conditions listed above from true metastasis. It can also be used to assess the risk of a pathological fracture, which is high if greater than 50 % of the cortex is destroyed.

A CT scan can be used in a similar manner and can detect bone marrow metastases before the onset of bone destruction.

MRI can also demonstrate metastases not seen by bone scans, because of its ability to detect bone marrow changes. It is particularly sensitive for spinal metastases (25). This makes it a useful technique in breast cancer, which commonly metastasises to the axial skeleton. However, specificity can again be a problem, with difficulty differentiating tumour from fractures and changes due to treatment. The malignant superscan, due to diffuse marrow involvement, can also occur.

Two recently developed techniques are single-photon-emission computed tomography (SPECT) and positron emission tomography (PET) using 18-fluoro-2-deoxyglucose (FDG) (26). SPECT combines the use of radioisotope with CT imaging. The resulting improved contrast resolution both increases sensitivity in the spine and increases specificity, by defining the position and pattern of vertebral anomalies. PET using FDG may have a more prominent role in the future. FDG is a radioisotope taken up by areas of abnormal glucose metabolism. It can therefore detect areas of bone marrow involvement before bone reaction takes place, whilst providing the increased resolution of PET.

Where the above investigations are inconclusive and a high clinical suspicion still exists, a bone biopsy may be warranted

Liver metastases

Liver function tests are quick and cheap to perform, and strongly suggest metastasis, if elevated. However studies have shown normal liver function tests in 33-38 % of patients with liver metastases diagnosed by imaging (27,28). Therefore, some form of imaging must be used if liver metastases are suspected.

Imaging modalities for liver metastases include ultrasound, CT scanning, and MRI. The ideal imaging technique should have three main attributes. Firstly, it should detect any metastasis present- ie it should be sensitive. Secondly it should differentiate benign liver lesions, which are commonly seen in breast cancer patients- ie it should be specific. Finally, it should provide adequate resolution and anatomical detail to determine resectability. Ultrasound is relatively cheap and accessible and has a reasonable sensitivity and specificity at around 80 % and 85 % respectively (29). CT scanning is more expensive but provides better sensitivity and specificity, both at around 90 %. A recent study indicates that MRI may have even better sensitivity and specificity (30), although it is less accessible and there have been reports of metastases treated by chemotherapy mimicking haemangiomas (31). CT or MRI should be used if there is a possibility of liver resection, as they provide better anatomical detail preoperatively.

Brain metastases

Brain metastases are diagnosed by CT or MRI scanning, following clinical suspicion, which is usually raised by new neurological symptoms or headaches of recent onset. Both are usually performed with contrast enhancement. CT is relatively inexpensive and readily available, but MRI with gadolinium enhancement has been shown to detect smaller lesions (32). Therefore, although CT is likely to be adequate for diagnosis of a symptomatic metastasis, MRI is essential if surgical resection is being considered.

Lung metastases

Lung metastases can be detected by plain film radiography, CT scan, or MRI (33). A plain chest radiograph is cheap, quick to perform and is often adequate to diagnose lung metastases. Spiral CT can acquire an image in a single breath and provides increased sensitivity. It also detects a larger amount of nodules when multiple metastases are present. However, differentiation of benign conditions can be a problem, especially in the case of a solitary nodule. The diagnosis of lung metastases obviously has profound implications for the patient and her management. A histological diagnosis can be obtained in equivocal cases, either by percutaneous biopsy or using video-assisted thoracic surgery resection. Until recently, MRI has been too slow to accurately image the lungs. However, the advent of rapid gradient echo sequences allows imaging of the lung during a breath. As MRI provides excellent differentiation of parenchymal lesions and vascular structures, it may provide increased specificity in the diagnosis of lung metastases. Thus it is likely to have an increasing role in the future.

TREATMENT OF METASTATIC BREAST CANCER

There are two broad strategies to treat breast cancer metastases. Firstly, in early breast cancer, treatment can be aimed at occult micrometastatic deposits to prevent the development of clinically apparent metastatic disease. This explains the survival advantage obtained by systemic adjuvant therapy in selected patient groups. Secondly, in advanced disease, treatment is aimed at established metastases. These can be treated locally or systemically.

Adjuvant treatment

Adjuvant therapeutic options in breast cancer consist of chemotherapy, hormonal therapy and radiotherapy. Adjuvant radiotherapy is used to prevent loco-regional recurrence and will not be considered further.

Most women with early breast cancer will benefit from some form of systemic adjuvant therapy, be it chemotherapy, hormonal therapy, or a combination of both. There have been around 400 trials of systemic adjuvant therapy over the past 25 years involving more than 220,000 women (34). When added to loco-regional treatment, chemotherapy and hormonal therapy reduce recurrence and mortality for more than 15 years.

Combination chemotherapy is more effective than single agent chemotherapy and reduces annual mortality by around 20% (35). It has been shown to be of benefit in women up to 69 years of age, although the effects are most marked in pre-menopausal women. This may be due to the ovarian suppression induced by chemotherapy. Alternatively, older patients may receive a reduced dose of chemotherapy, resulting in less effective

treatment (36). The standard regimen of chemotherapy is cyclophosphamide, methotrexate and fluorouracil (CMF) for 4-6 months. This was established after publication of the first overview of adjuvant treatment in 1988 (37). Recent trials indicate that regimens containing anthracyclines such as doxorubicin are more effective (38) and additional treatment with paclitaxel can further improve survival (39).

Table 2. Treatment of breast cancer metastases

Clinical setting	Site	Modality
Treatment of micrometastases (Adjuvant)	Systemic	Hormonal therapy Chemotherapy
Clinically apparent metastases	Systemic	Hormonal therapy Chemotherapy
	Bone	Hormonal therapy Chemotherapy Bisphosphonates – eg pamidronate, clodronate Radiotherapy – eg 30 Gy over 2 weeks Surgery
	Liver	Chemotherapy Hormonal therapy Surgery
	Brain	Radiotherapy – Whole-brain irradiation (25Gy) Chemotherapy Hormonal therapy Steroids Surgery
	Lung	Chemotherapy Hormonal therapy Surgery

Many breast cancers are oestrogen dependent. These oestrogen receptor (ER) positive tumours respond to the anti-oestrogen tamoxifen. The drug can suppress the growth and cause regression of both primary tumours and metastases. Although initially thought to be effective adjuvant therapy in postmenopausal women only, the most recent overview (40) has shown reduction in recurrence and mortality in all age groups, providing the tumour is ER positive. Treatment for 5 years is more effective than a shorter duration, but whether treatment for longer than 5 years provides further benefit is not yet known. Equivalent benefit can be obtained in premenopausal women by ovarian ablation, either by surgery or irradiation (41). The ATAC trial is currently evaluating the role of anastrozole in the adjuvant setting. Anastrozole is a new aromatase inhibitor, which blocks the rate limiting enzyme in oestrogen production without the toxicity of the original aromatase inhibitor, aminoglutethimide.

Treatment of established metastatic disease

The overwhelming majority of women with established metastatic breast cancer will die of their disease (42). Therefore, treatment aims to prolong life and prevent complications, whilst providing optimal palliation by controlling symptoms and preserving quality of life. The overall treatment strategy for a patient depends on age, hormone receptor status, and extent of disease. Overt metastases may be treated locally, systemically, or a combination of both. An overview of systemic treatment will be followed by discussion of treatment options for each common site of metastasis. The majority of women with metastases will die of their breast cancer within two years although patients with hormone-sensitive breast cancer may have many years of high quality life.

Systemic treatment

There are two main forms of systemic treatment for metastatic breast cancer: hormonal therapy and chemotherapy. For women with ER positive tumours and disease that is not life-threatening in the short term, hormonal therapy is usually the initial treatment of choice. This is especially true for the elderly or asymptomatic patient. Women with metastases refractory to hormonal therapy or imminently life-threatening disease should receive chemotherapy. Survival benefit from chemotherapy must be balanced against its toxicity. This should be discussed with the patient so an informed choice can be made. Toxicity may be less of a consideration if the patient has symptomatic disease. Truly ER negative tumours are unlikely to respond to hormonal therapy. However, there are often degrees of ER positivity. In addition, the assay methods used on the original tumour may be less reliable than current ER assays. Therefore, a trial of hormonal therapy may be considered in selected patients.

Hormonal manipulation can be achieved by ovarian ablation, but specific drugs are now more commonly used. These include antioestrogens (eg tamoxifen, raloxifene), aromatase inhibitors (eg anastrozole), gonadotropin-releasing-hormone analogues (eg goserelin) and progestins. 20-35 % of women have an objective response to the initial hormone therapy, which is usually tamoxifen (43). This will eventually become ineffective. However, women who respond to one hormonal treatment will often respond to another. Thus, several courses of sequential hormonal treatments are possible, often allowing several years of good quality life with minimal side effects.

The two commonly used first-line chemotherapeutic regimens are CMF and fluorouracil, doxorubicin and cyclophosphamide (FAC). 40-60% of women have an objective response to CMF, whilst 50-80% respond to FAC (44). Anthracycline-containing regimens, such as the latter, have been shown to be more effective than CMF, with an increase in median survival from 14 to 18 months, and an increase in median time to treatment failure from 5 to 7 months (45). These benefits must be weighed against the increased toxicity of doxorubicin. When disease progresses after first-line chemotherapy, taxanes such as paclitaxel and docetaxel are usually employed. Third-line chemotherapy of choice is vinorelbine.

Bony metastases

Management of bony metastases is multidiscliplinary, involving oncologists, radiologists, and orthopaedic and spinal surgeons. Early assessment of lesions to

prevent pathological fractures or spinal cord compression is essential. The management of pathological fractures should be performed by orthopaedic surgeons with a specialist interest in this field. The commonest site for a pathological fracture is the proximal femur and warrants immediate stabilisation and reconstruction. Spinal cord compression should be recognised early and is an oncological emergency. The clinical suspicion should be confirmed by an urgent MRI and should be treated as soon as possible following discussion with the surgeon, oncologist and radiologist by either surgery or radiotherapy. When present, hypercalcemia is associated with a poor prognosis and limited survival (46). However the symptoms from hypercalcemia could be severe and in the acute case should be treated with adequate rehydration, and the infusion of bisphosphonates. In addition to surgery, bone metastases can be treated by irradiation, hormonal therapy, chemotherapy, and bisphosphonates.

Indications for radiotherapy of bone metastases include pain, risk of pathological fracture, and spinal cord compression. Treatment aims to relieve symptoms, restore function and prevent complications. Palliative radiotherapy for measurable bone metastases uses lower total doses than curative treatment of microscopic disease. Usually, high daily doses are used over a shorter period of time. This is known as hypofractionated irradiation. A typical regimen would be 30 Gy in 10 fractions over 2 weeks (47). This provides rapid relief of symptoms, but recognises that a measurable bony metastasis cannot be eradicated because the radiation tolerance of surrounding tissues, such as the spinal cord, would be exceeded. Ideally, it should prevent the metastasis from causing symptoms during the remaining lifetime of the patient.

For more widespread bony metastases, hemibody irradiation or radiopharmaceuticals can be used. Hemibody irradiation consists of a single fraction of 6-10 Gy to the upper, middle or lower body. However, it is most useful in blastic metastases, and there are concerns regarding permanent bone marrow damage. Therefore, radiopharmaceuticals such as ^{89}Sr (strontium) are gaining in popularity. ^{89}Sr combines with the calcium component of hydroxyapatite in osteoblastic lesions. Pain is relieved for more than 6 months in 60-80% of patients.

Bony metastases from breast cancer produce parathyroid hormone related peptide, which stimulates osteoclastic bone resorption (48). Bisphosphonates such as clodronate and pamidronate are non-cytotoxic drugs, which are powerful inhibitors of osteoclastic bone resorption. Pamidronate, a second-generation bisphosphonate, is more effective at inhibiting osteoclasts than clodronate. Administered by monthly intravenous infusion for 12 months, it has been shown to reduce not only hypercalcaemia (49,50), but also skeletal complications and pain (51). The advantage of bisphosphonates over radiotherapy and chemotherapy is their lack of bone marrow toxicity.

Liver metastases

Treatment of liver metastases is normally systemic, using chemotherapy or hormonal therapy. However, response to either treatment modality is not very encouraging (52). There have been a few reports of successful surgical resection of solitary liver metastases from breast cancer though these reports are with small numbers of patients. Although this is rarely undertaken, due to the presence of disease elsewhere, it should be considered in cases of isolated liver metastases. In a

recent study (53) of 34 patients, of whom 59 % had a solitary metastasis, curative resection was possible in 86 %, with a 5-year survival of 22 %.

Brain metastases

The mainstays of treatment for patients with brain metastases are whole-brain irradiation (WBRT) and / or chemotherapy. Hormonal therapy and steroids are usually used in addition. WBRT uses a total dose of around 25 Gy. A recent study (54) showed that patients with metastases from breast carcinoma had better survival with WBRT than patients with other primary tumours. Patients with up to three accessible metastases may be considered for surgical resection or stereotactic radiosurgery, followed by WBRT[55]. Recent literature indicates a survival advantage, with a median survival of 9-16 months (55-57), compared to 3-4 months in patients treated by WBRT (56,57).

Lung metastases

Lung metastases are usually treated with chemotherapy or hormonal therapy. Patients with metastases confined to the lungs may have better response rates and survival following chemotherapy than expected for patients with visceral metastases. A retrospective review (58) of 1581 patients with metastatic breast cancer treated with doxorubicin / cyclophosphamide-containing chemotherapy found an overall response of 76 % in patients with metastases confined to the lungs compared to 64 % in the remainder. Complete response was 33% compared with 14 %. 10-year survival was 9% compared to 3%. Surgical resection of metastases in carefully selected patients appears to offer a significant survival benefit (59). In a recent Italian study (60), 12 patients, of whom 9 had isolated lung metastases and 3 had multifocal lesions, underwent wedge resection or lobectomy. The median survival rate was 40 months and 5-year survival 42 %.

PROGNOSIS

The prognosis of patients with metastatic breast cancer is related to the site of first relapse. Patients with bone-only metastases have a much better prognosis than patients with visceral-only metastases. A retrospective review by Perez and colleagues involving 510 patients found that patients first developing metastases confined to bone had a median survival time of 24-28 months, whilst patients with visceral metastases had a median survival time of only 12-13 months (61).

The site of metastasis also affects response to therapy. Soft tissue and bony lesions respond better to hormonal therapy than visceral metastases. This is partly because tumours with visceral metastases are more often ER negative. A study by Parnes and colleagues showed a response rate of 70 % with bony disease but only 21 % with visceral disease (62). Similarly, visceral and in particular liver metastases have been shown to have a poor response to chemotherapy. Patients who respond to systemic therapy for their metastases have a good chance of being alive at 3 years, and 20 % will be alive at 5 years (22).

The HER-2/neu oncogene is overexpressed in 20-30 % of patients with breast cancer. These patients have a poorer prognosis and disease that is more resistant to chemotherapy (63).

THE FUTURE

Most treatments for metastatic breast cancer are cytotoxic or cytostatic in nature. In the future, agents that directly

interfere with the metastatic process may be developed. However, the best method of preventing metastatic development is to prevent the primary breast cancer. Further evidence of the chemoprevention of breast cancer with agents such as tamoxifen and raloxifene is eagerly awaited.

A monoclonal antibody to the extracellular domain of the HER-2/neu oncoprotein has been developed. In a recent randomised trial, administration of the antibody with chemotherapy resulted in 2-3 times the rate of response and increased overall survival compared with chemotherapy alone (64). Specific treatments such as this are likely to have the greatest impact on survival from metastatic breast cancer in the future.

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Chapter 14

THE MALIGNANT LYMPHOMAS

Christopher H. Poynton

"This enlargement of the glands appeared to be a primitive affect of those bodies, rather than the result of an irritation propagated to them from some ulcerated surface or other inflamed texture...." Thomas Hodgkin 1832.

INTRODUCTION:

The malignant lymphomas are a heterogeneous group of diseases of leucocyte origin that are ultimately derived from products of the haemopoietic stem cell. The breadth of lymphoma entities broadly reflect the different stages of ontogeny of lymphoid differentiation during the development of B-cells, T-cells and NK cells. Although the old term histiocytic lymphoma might suggest a derivation from cells of the monocyte-macrophage lineage, very few lymphomas (less than 1% of large cell lymphomas) are now thought to arise truly from histiocytes. Those that do (for example from Langerhans cells or dendritic cells) are best classified separately, although there is sometimes little to distinguish them pathologically or clinically with other anaplastic large cell lymphomas.

The lymphomas were first identified as a clinical entity by Thomas Hodgkin in 1832 (1) - although reanalysis of his original specimens a century later established that at least some were in fact tuberculosis rather than malignant lymphomas. Initially all such lymph gland enlargements were termed Hodgkin's disease, but by the end of the 19th century it was realised that the behaviour of some were more aggressive than others and the term lymphosarcoma was coined to distinguish them from Hodgkin's disease. Since then a plethora of different lymphoma entities have been described, classified and reclassified, with new schemes currently emerging every 5 years or so. Thus we have seen the important contributions of Brill, Symmers, Gall, Mallory, Rappoport, Lukes, Collins, Lennest, and more recently, a committee from the International Lymphoma Sub Group (ILSG) (2). This latter group reported in 1994 and is now regarded as the basis for the modern classification of the lymphomas (both Hodgkin's lymphoma and the Non Hodgkin's

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lymphomas) - the Revised European American Lymphoma classification (REAL) although it has already undergone some modifications by the World Health Organisation (WHO) expert group in 1998 (3) (Table 1).

In this chapter we will focus on the biology of the non Hodgkin's lymphomas, which as a group account for about 7% of all malignant disease. It has, however, recently become apparent that the overall incidence is increasing at a rapid rate - by about 3% per annum for reasons that are not entirely clear (4). The REAL classification has clarified the identity and relationship of lymphoma cells to their normal counterparts in the lymph node and has enabled the immunophenotypic characteristics of these cells to be a useful part of the diagnosis and moreover to link specific entities with known molecular genetic lesions (5). A full diagnosis should now be able to link the morphology, immunophenotype, cytogenetic and molecular characteristics together increasing the number of lymphoid 'entities' that we now recognize (Table 2). Although antigen detection is vital in determining cell lineage, relatively little is known about their function. Constellations of antigens and their unique quantitative expression on a tumour cell can be used for immunodiagnosis, follow up of minimal residual disease and in assigning treatment with specific monoclonal antibody therapy. They are also important in separating out the confusing and overlapping low grade lymphomas into their REAL entities (Figure 1). Table 3 shows a list of routinely used surface, cytoplasmic and nuclear associated CD and other markers used in lymphoma diagnosis.

The malignant lymphomas are not generally regarded as tumours that metastasize in the traditional sense. Normal lymphocytes circulate widely and there is now evidence that "pre-lymphoma" cells do the same. Clonal cytogenetic lesions normally associated with follicular lymphoma have been detected in the blood of "normal" individuals-t(14;18) (6) who have no clinical evidence of lymphoma. Recently Wiemels and colleagues have shown that the specific t(12:21) DNA hybrid sequences associated with acute lymphoblastic leukaemia developing in one identical twin was present at birth (many years before) in both twins (one remaining unaffected) (7). This data would suggest that it is highly likely that cells need to undergo several mutational events before overt lymphoid malignancy becomes apparent. Cells that have already undergone one or more steps in the development of malignant lymphoma are therefore already widespread throughout the circulation. This finding is not unique to lymphoid malignancy as similar observations have been documented as age related for the bcr-abl fusion gene product of t(9;22) (8) which is associated with several forms of leukaemia.

LYMPHOCYTE CIRCULATION AND TRAFFICKING

The functional anatomy of the lymphoid system is shown in Figure 2. Normal lymphocytes have highly controlled circulation and homing mechanisms known as trafficking. The process starts with the production of lymphoid progenitors in the bone marrow which produces between 10^{10} and 10^{11} lymphocytes a day (9). Only a fraction of these attain maturity, the remainder undergoing apoptosis at the rate of about 7 million per second. Recirculation from lymphatic system to blood occurs at a rate of about 5 million lymphocytes a second. Production, selection and circulation of

Table 1. WHO revision (1998) of the Revised European-American Lymphoma (REAL) Classification (The italics show the original (1994) REAL classification terms used)

Neoplasms	Classification
B-cell neoplasmas	Precursor B-cell lymphoblastic leukaemia/lymphoma
	Mature B-cell neoplasms
	B-cell chronic lymphocytic leukaemia/small lymphocytic lymphoma
	B-cell prolymphocytic leukaemia
	Lymphoplasmacytic lymphoma (*lymphoplasmacytoid lymphoma)*
	Mantle cell lymphoma
	Follicular lymphoma (*follicle centre lymphoma)*
	Marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) type
	Nodal marginal zone lymphoma with or without monocytoid B-cells
	Splenic marginal zone B-cell lymphoma
	Hairy cell leukaemia
	Diffuse large B-cell lymphoma
	Subtypes: mediastinal (thymic), intravascular, primary effusion lymphoma
	Burkitt lymphoma
	Plasmacytoma
	Plasma cell myeloma
T-cell neoplasms	Precursor T-cell lymphoblastic leukaemia/lymphoma
	Mature T-cell and NK-cell neoplasms
	T-cell prolymphocytic leukaemia
	T-cell large granular lymphocytic leukaemia
	NK-cell leukaemia
	Extranodal NK/T-cell lymphoma, nasal-type (*angiocentric lymphoma)*
	Mycosis fungoides
	Sézary syndrome
	Angioimmunoblastic T-cell lymphoma
	Peripheral T-cell lymphoma (unspecified)
	Adult T-cell leukaemia/lymphoma ($HTLV1^+$)
	Systemic anaplastic large cell lymphoma (T- and null-cell types)
	Primary cutaneous anaplastic large cell lymphoma
	Subcutaneous panniculitis-like T-cell lymphoma
	Enteropathy-type intestinal T-cell lymphoma
	Hepatosplenic γδ T-cell lymphoma
Hodgkin's Lymphoma (Hodgkin's disease	Nodular lymphocyte predominance Hodgkin's lymphoma
	Classic Hodgkin's lymphoma
	Hodgkin's lymphoma, nodular sclerosis (grades I and II)
	Classical Hodgkin's lymphoma, lymphocyte-rich
	Hodgkin's lymphoma, mixed cellularity
	Hodgkin's lymphoma, lymphocytic depletion (includes some *Hodgkin-like anaplastic large cell lymphoma).*

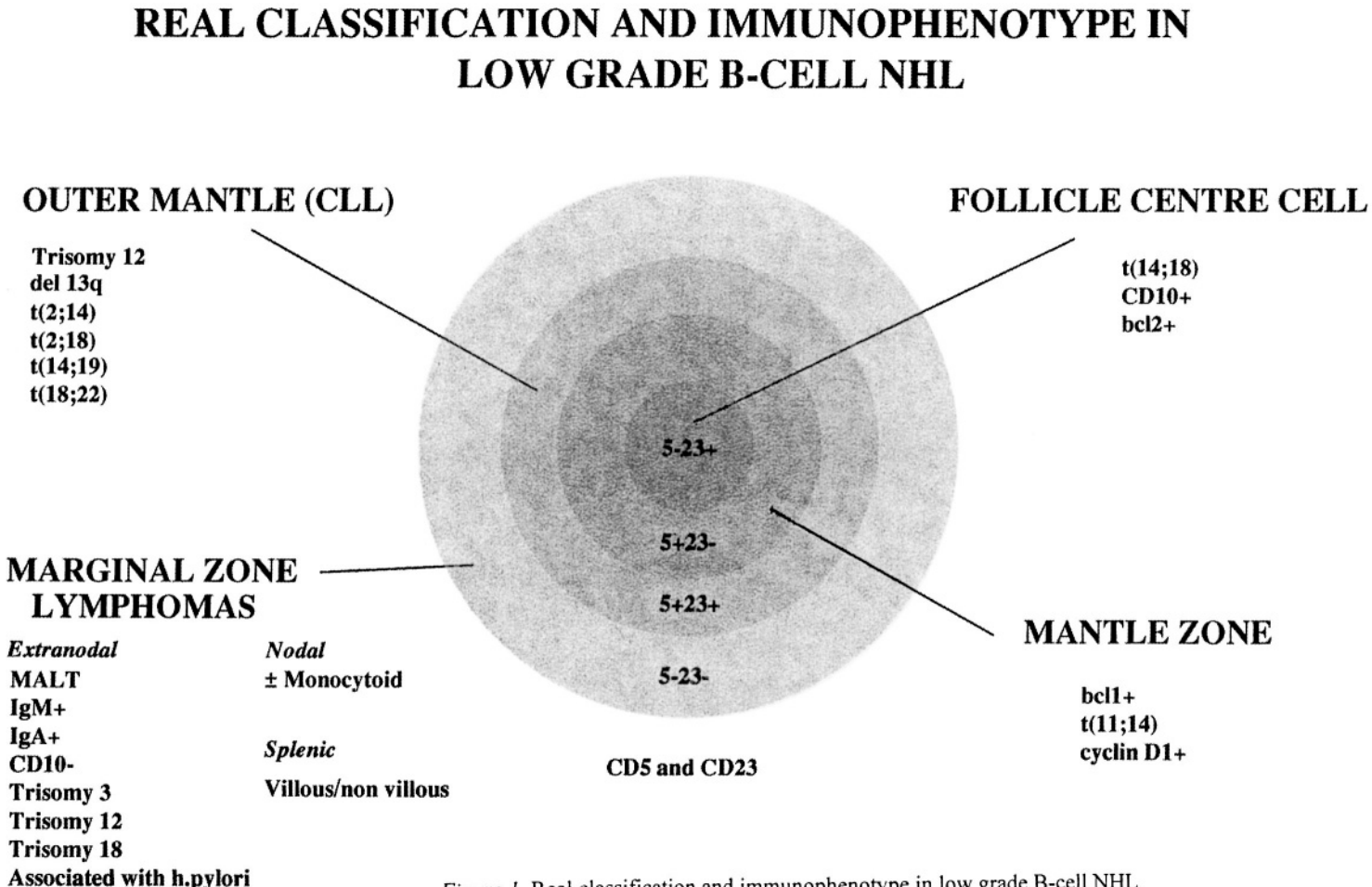

Figure 1. Real classification and immunophenotype in low grade B-cell NHL

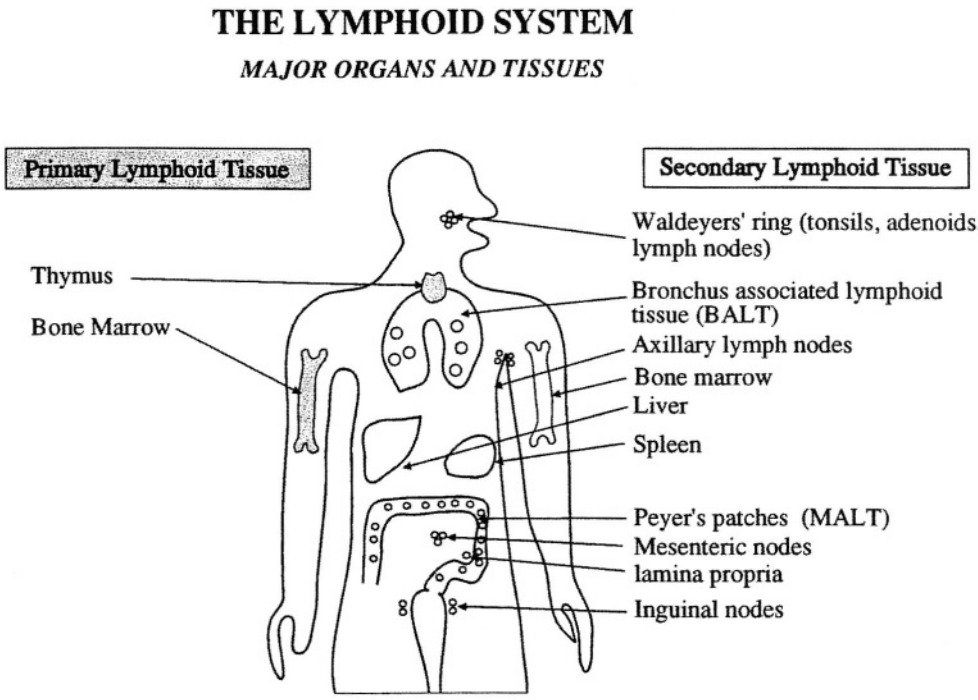

Figure 2. The lymphoid system (major organs and tissues)

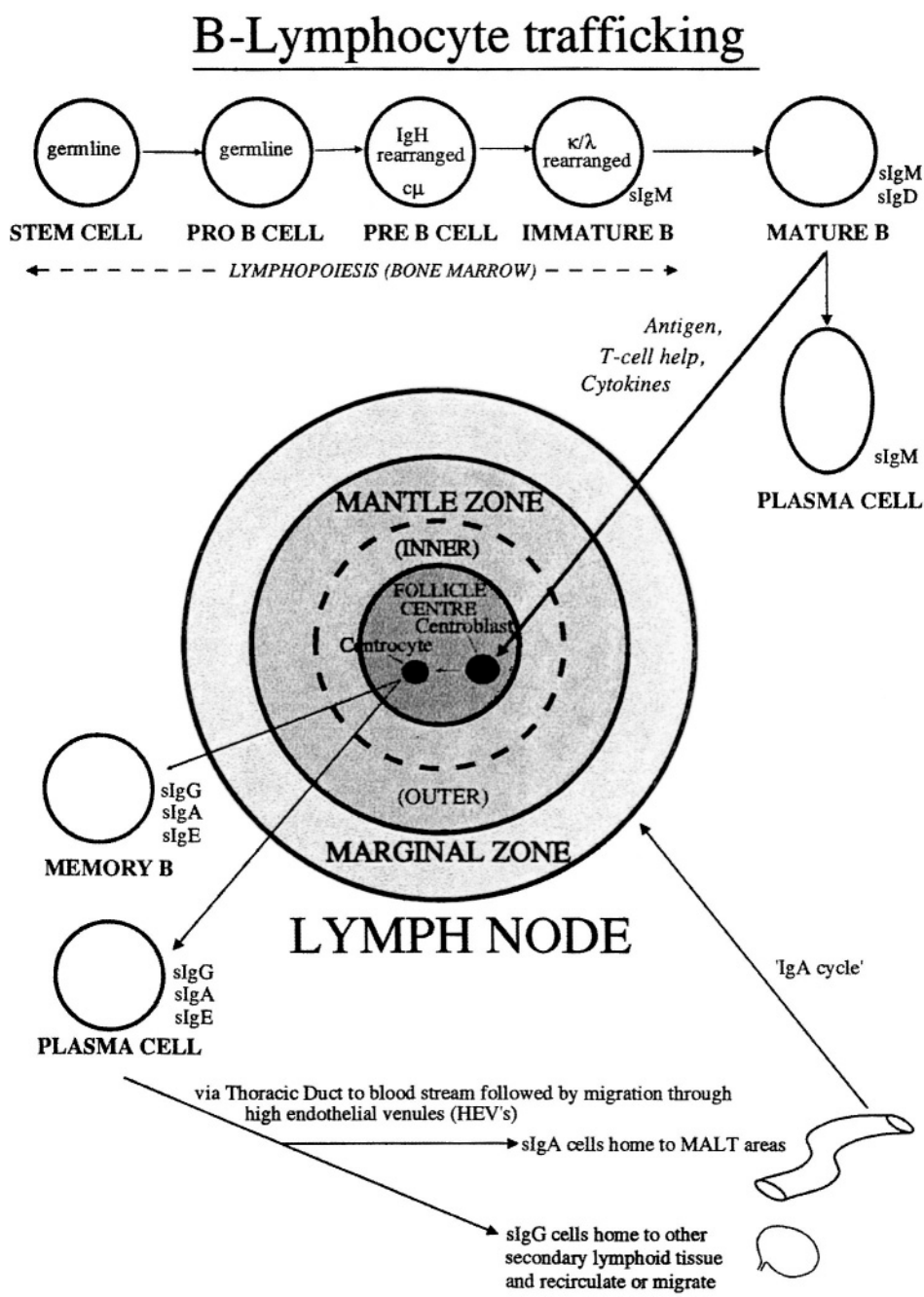

Figure 3. B-lymphocyte trafficking

lymphocytes are highly controlled and not just a series of random stochastic events and how it occurs is now leading to a fundamental change in our understanding of the behaviour of the malignant counterparts of these cells[10]. It is becoming clear that several shared ligands and their receptors are responsible for controlling the circulation and migration of lymphocytes - particularly the integrms (Table 4) and the selectins and some members of the immunoglobulin superfamily (Table 5).

B-CELL DEVELOPMENT, CIRCULATION, AND MALIGNANT COUNTERPARTS

Lymphocytes are derived from haemopoietic stem cells ($CD34^+$) in the bone marrow and the stages of development of the B-cells can be broadly divided into three phases - Lymphopoiesis (non antigen driven) in the bone marrow, immunopoiesis (antigen driven) in the lymph nodes followed by release into the circulation with homing to MALT and other areas (12) and to sites of inflammation (Figure 3). B cell development has been divided into at least six major stages of development based on expression oF B-cell receptor proteins, Ig rearrangement status -and other (CD) antigen expression-(11). The first step is the acquisition of the associated parts of the B cell receptor (CD19/CD21/CD79b) to form a 'pro-B cell' (13) - which remains anchored in the bone marrow by several adhesion molecules, particularly CD117 (c-kit), VLA-4 and VCAM-1 (14). This cell is at a stage of development before immunoglobulin gene rearrangement has taken place. The process of immunoglobulin gene rearrangement is complex involving selection of one variable (V) gene from over 300 on chromosome 14 followed by splicing to a diversity (D) gene selected from more than 30 and then selection from 6 junctional (J) genes. At each stage somatic hypermutation occurs involving deletions or additions of nucleotide bases allowing frame shifts to increase the diversity of potential immunoglobulins[15]. B-lineage lymphoid acute leukaemias are most commonly derived from cells at the pro-B (or earlier) stage. Gene rearrangement results in unique complementarity determining regions (CDR) and since they occur early in B-cell development act as clinically useful markers in lymphoma (where the phenotype is generally of a much more mature B-cell) both as a diagnostic tool to establish clonality and as a means of monitoring residual disease after treatment. At the pre-B cell stage the cell has not yet rearranged a light chain so only μ protein is expressed and is associated with a 'surrogate' light chain (λ_s) for stability (16). Conventional light chain gene rearrangement follows with kappa first and if this fails then lambda resulting in an "immature B" cell. Cells that fail to rearrange their light chain genes successfully undergo apoptosis. By this time the cell is expressing a complete immunoglobulin IgM molecule on the surface which acts as a receptor for further differentiation into "mature B" cells. Mature B cells begin to co-express IgD (using the same V gene), and interact with other surface transmembrane proteins (Ig-α, and Ig-β and Ig-γ) enabling repetitive antigen interactions with T-cells or APC's results in intracellular signalling (via the tyrosine kinase syk) (17).

Table 2. Cytogenetic, molecular and immunophenotypic associations in lymphoid malignancies

Cytogenetics	Molecular	Phenotype
t(1;19)(q23;p13)	E2A-PBX1	cμ+ preB ALL
t(4;11)(q21;p23)	MLL-AF4	CD10 negative, CD15+ ALL
t(9;22)(q34;p11)p190	BCR-ABL	mixed phenotypes, bad prognosis
t((9;22)(q34;p11)p210	BCR-ABL	mixed phenotypes, poor prognosis
t(12;21)(q13;p22)	TEL-AML1	CD13+ B-lineage ALL
TAL1 deletion	SIL-TAL1	T lineage ALL
t(1;14)(p22;q32)	TAL1-TCR δ	T lineage ALL
t(10;14)	HOX11-TCR δ	T lineage ALL
11q23-many	MLL	B lineage ALL
t(7;9	TAL2	T lineage ALL
t(8;14)(q24;q32)	cmyc-IgH	Burkitt's/ALL
t(8;22)(q24;q11)	cmyc-kappa	Burkitt's/ALL
t(2;8)(p11;q24)	cmyc-lambda	Burkitt's/ALL
t(14;18)(q32;q21)	IgH-bcl2	CD5-/CD23+/CD10+ FL
t(11;14)(q13;q32)	PRAD1(cyclinD1)-IgH	CD5+/CD23- MCL
t(2;5)(p23;q35)	NPM-ALK	ALCL (T lineage)
t(11;18)9q21;q21)	AP12-MLT	Marginal Zone Lymphoma (MALT)
t(9;13)	-	Immunocytoma
iso8q	-	Benign polyclonal B-cell proliferation
13q14	-	$CD5^+$ $CD38^+$ 'postfollicular' CLL
Trisomy 12	-	$CD5^+$ $CD38^{low}$ 'prefollicular' CLL

Key: ALL=Acute Lymphoblastic Leukaemia/Lymphoblastic Lymphoma
MCL = Mantle Cell Lymphoma
ALCL=Anaplastic Large Cell Lymphoma
FL = Follicle Cell Lymphoma
MALT = Mucosa Associated Lymphoid Tissue
CLL = Chronic Lymphocytic Leukaemia

Table 3. CD numbers in lymphoma diagnosis

CD Nos	Roles in diagnosis
CD1a	Cortical thymocyte marker, present only at a transient stage of T cell development in the thymus. The presence of CD1a+ cells in the peripheral blood or bone marrow usually suggests a T-cell at the lymphoblast stage (eg T-all/lymphoblastic lymphoma).
CD 2	T cell marker which appears very early in T cell development. Expressed on peripheral T cell lymphomas and Natural Killer cell malignancies
CD 3	Pan-T cell marker associated with the T cell antigen receptor. CD3 appears in the cytoplasm of the cell earlier than on the surface, and cases of T-ALL are usually surface CD3 negative but cytoplasmic CD3+. CytCD3 is therefore a useful and the specific marker of T cell lineage in NHL.
CD 4	Present on the helper subset of T lymphocytes. It is also found on normal monocytes. Present on Sezary/Mycosis Fungoides cells
CD 5	Pan-T cell antigen which is expressed early in T cell development and on a subset of normal B cells, the malignant expansion of which gives rise to B-CLL. Strongly expressed in (B) mantle cell NHL and in T-NHL..
CD 7	Pan-T cell antigen which appears early in T celll development, and is also present on Natural Killer cells. The antigen is expressed quite weakly on normal mature T cells, but very strong expression is seen in T-ALL and T-PLL, whereas Sezary cells are usually weak or negative.
CD 8	Found on the cytotoxic subset of T lymphocytes on most natural killer cells. Slightly waek expression is common in T-NHL.
CD 10	The Common Acute Lymphoblastic Leukaemia Antigen (CALLA). Present in many cases of follicle centre cell lymphoma (B-cell) and in lymphoblastic lymphoma and occasionally in other high grade B-NHL
CD 11	A family of cell adhesion antigens with a wide distribution on lymphoid and myeloid cells CD11a ... present on T cells, B cells, granulocytes and monocytes CD11b ... present on lymphocytes, and most granulocytes and monocytes CD11c ... present on B cells, Natural killer cells, granulocytes, and particularly monocytes.
CD 14	Monocyte/neutrophil marker. Monocytes express CD14 strongly. It is useful in assigning monocytic lineage eg Langerhans cell tumours.
CD 15	A myeloid marker which is strongly expressed on most mature neutrophils, and weakly expressed on monocytes. It is also found on Reed-Sternberg cells (Hodgkin's Lymphoma)
CD 16	This marker is found on most mature neutrophils and may also be found on Natural Killer cells malignancies
CD 19	A reliable B cell marker which appears early in B cell development and persists until the terminal plasma cell stage, where it is usually lost. This is the most widely used B cell marker and virtually all B cell malignancies (except myeloma) are positive
CD 20	A B cell marker which appears later than CD19 in B cell development, and is positive on all mature B cells and most B lineage NHL
CD 22	A B cell marker which appears in the cytoplasm much earlier than most other B cell markers, and is only expressed on the surface of relatively mature B cells.CytCD22 is therefore one of the most reliable markers of B-lineage NHL
CD 23	Activation antigen, present on a subset of normal B cells, and virtually all cases of CLL and follicular NHL

Table 3 to be continued...

Table 3. continuing

CD Nos	Roles in diagnosis
CD 25	CD25 Activation antigen, which is only very weakly expressed on activated T and B cells, but is an important marker for the diagnosis of hairy cell leukaemia (medium/strong positivity, whereas hairy cell variant and splenic lymphomas (villous and non villous) are usually negative). Often expressed in peripheral T-NHL
CD 30	Ki-1 antigen present in Hodgkin's Lymphoma and in anaplastic large cell lymphomas. It's presence in NHL may suggest a better prognosis.
CD 34	Stem cell marker, present on progenitor cells of the bone marrow. An important marker to diagnose the immature subtypes of lymphoblastic lymphomas
CD 38	Antigen present on activated T cells, some B cells, and monocytes. The antibody is very useful to demonstrate the presence of plasmacytic malignancies (multiple myeloma and some NHL).
CD 45	Leucocyte common antigen, present on all leucocytes, but absent from red cells and pl This is extremely useful in deciding whether a tumour is of haemopoietic lineage. haemopoietic tumours will not express this marker, but negativity can be seen in cases lymphoblastic leukaemia. *This antigen exists in several different isoforms*: CD45RA: in the peripheral blood found on 60-80% of T cells, all B cells monocytes, and granulocytes. Positive T cells are "naive". CD45RB: in the peripheral blood found on 80% of T cells, all B cells, an monocytes. CD45RO: in the peripheral blood found on 20-40% of T cells, also present on most monocytes and granulocytes, but absent from B cells. T cells expressing CD45RO have been antigenically stimulated (memory cells).
CD 52	A gpi anchored antigen recognised by CAMPATH-1 antibody, which has broad reactivity against lymphocytes and monocytes. The antibody is of benefit in treating some NHL and CLL cases
CD 56	NCAM is the most important marker of natural killer cells. However it is not restricted to haemopoietic cells and is found in other tumours (eg PNET and neuroblastoma).
CD 64	High affinity receptor for IgG, mediates antibody-dependent cell-mediated cytotoxicity. Weakly expressed on activated neutrophils, with monocytes and monoblasts showing stronger staining. Useful for Langherhans cell tumours
CD 79	This antigen is a dimer and is associated with the B cell immunoglobulin receptor: CD 79a: considered to be the best specific marker of the B cell lineage, present o cells at all stages of maturation, including plasma cells. This antigen is best detected cytoplasm. CD79b: present on most B cells, but absent on plasma cells. Also negative on cases of CLL, and can help to distinguish these from other B-cell malignancies.
CD 103	This antigen is present on a very small number of CD8+ T cells in the peripheral blood, and some lymph node T cells. It is also present on hairy cells, and is an important marker to distinguish hairy cell leukaemia from splenic lymphoma with villous lymphocytes
CD 138	Syndecan-1 antigen, present on some B-cells and expressed on most plasma cells.
Ki-67	A useful marker of cell cycle activity that can be used to distinguish Burkitt's and lymphoblastic NHL (high Ki67+) from mantle cell and high grade NHL (medium Ki67+) to low grade lymphomas (low Ki67+).
Cyclin D1	Another useful marker of cell cycle activity that is overexpressed in mantle cell lymphoma which is generally regarded as diagnostic of that condition.
ALK	The protein product of a gene on chromosome 5 that is not normally detectable on white cells, but is overexpressed in some T-lineage high grade lymphomas, especially those associated with the t(2;5) NPM-ALK translocation. These types of anaplastic large cell lymphoma tend to occur in young people and are associated with a good prognosis

Table 4. Adhesion molecules involved in lymphocyte trafficking

Integrins

Name	α subunit	ß subunit	ligand
VLA-1	α1 (CD49a)		Laminin, collagen
VLA-2	α2 (CD 49b)		Collagen, Laminin
VLA-3	α3 (CD 49c)		Fibronectin, laminin, collagen
VLA-4	α4 (CD 49d)		VCAM-1, fibronectin
VLA-5	α5 (CD 49e)		Fibronectin
VLA-6	α6 (CD 49f)		Laminin
ß1α7	α7		Laminin
ß1α8	α8		
ß1αv	αv (CD 51)		Fibronectin
LFA-1	αL (CD 11a)	ß2	ICAM-1,2,3
MAC-1	αM (CD 11b)		ICAM-1,3, fibrinogen
P150,95	αx (CD 11c)		Fibinogen, C3bi
CD41a (gpIIb/IIIa)	αIib (CD 41)	ß3	Fibronectin, Fibrinogen,
Vibronectin receptor	αv (CD 51)		
ß4α6	α6 (CD 49f)	ß4 (CD 104)	Laminin
ß5αv	αv (C 51)	ß5	Vibronectin, fibronectin
ß6αv	αv (CD 51)	ß6	Fibronectin
ß7α4	α4 (CD 49d)	ß7	Fibronectin, VCAM-1, MAdCAM-1
ß8αv	αv (CD 51)	ß8	

Table 5. Adhesion molecules involved in lymphocyte trafficking

Selectins

Name	CD number	Expressed on	Ligand	Ligand expression	Main role
E-selectin	CD62E	Endothelial cells	Unknown	Memory T (CD4+), granucocytes	Neutrophil and monocytes, CD4+ capture and rolling
L-selectin	CD62L	Many WBCs	MaDCAM, CD34, GlyCAM-1	HEV's	Homing of lymphocytes
P-selectin	CD62P	Platelets Endothelial cells Megakararyo-cytes	PSGL-1	Neutrophils Monocytes NK Platelets	Neutrophil and monocyte capture and rolling

Immunoglobulin superfamily

Name	CD number	Expressed on	Ligand	Ligand expression	Main role
ICAM-1	CD54	Lymphocytes and many others	LFA-1 (CD11a/CD 18) Mac-1 (CD11b/CD 18)	Lymphocytes and many others	Adhesion of WBC's to endothelium and extravasation
ICAM-2	CD102	Endothelial cells and many WBC's	LFA-1 (CD11a/CD 18)	Many WBC's	Lymphocyte trafficking
ICAM-3	CD50	Lymphocytes, neutrophils, monocytes, NK	LFA-1 (CD11a/CD 18) Mac-1 (CD11b/CD 18)	Lymphocytes and many others	Regulation of intercellular adhesion
VCAM-1	CD106	Endothelial cells, and many WBC's	VLA-4 (CD49d/CD 29) α4 ß7	Lymphocytes Monocytes	Adhesion of WBC's to endothelium and extravasation
PECAM-1	CD31	Endothelial cells, platelets, monocytes, granuclocytes T-cells	PECAM-1	Endothelial cells Platelets Monocytes Granulocytes T-cells	Extravasation and transmigration
MAdCAM-1		Mucosal HEV	L-selectin α4 ß7	Many WBC's	Lymphocyte homing to MALT areas

Further differentiation (at this stage termed "antigen dependent immunopoiesis") is largely cytokine driven and takes place in the secondary lymphoid tissue (for example mesenteric lymph nodes, mucosal associated lymphoid tissue (MALT) and the spleen).

B cells are capable of directly recognising some antigens that are able to cross link the B-cell receptor (and thereby induce B cell proliferation) independently of T-cell help. These antigens (T-independent) tend to be large and repetitive in their epitope structure (e.g. pneumococcal polysaccharide). However, most antigens are not recognized by B-cells and require preliminary recognition by T-cells. The B-cell then recognises the T-cell receptor and this is followed by several ligand interactions - LFA1 (B-cell) with CD54 (T-cell) followed by CD40 (B-cell) with CD40 ligand on the T-cell. A second series of events is triggered then - LFA3 (B-cell) with CD2 (T-cell) (18). CD2 expression appears in conjunction with CD28 on T-cells, and interacts with CD80 and CD86 on APC's. These costimulatory molecules on T-cells are the key to this phase of antigen dependent B-cell further differentiation. The antigen presenting cells involved in the germinal centre are follicular dendritic cells and the B cells (by now centroblasts) proliferate rapidly (in the so called dark zone of the follicle centre). A number of important events mark the transition from centroblast to centrocyte. Somatic hypermutation in centroblasts results in a wide and continually expanding repertoire of B-cell receptors. Selection then has to take place and many are deleted at this stage. Phenotypically, follicle centre cells at the centroblast stage do not express CD44 and L-selectins, whereas centrocytes do. Cross linking of the B-cell receptors also induces CD23 expression and upregulation of HLA class II, (hence the classic follicle centre lymphoma that is CD5-/CD23+). CD10 is also expressed by centrocytes and by this stage that immunogloblin class switching is complete.

Traditionally, lymphocyte commitment to production of a single antibody was thought to occur following a single chance encounter with correctly presented antigen (on APC) or appropriate T cell receptor (on a T cell). There is now evidence that this occurs over a period of time - the cells needing repeated encounters with antigen (multiple 'kisses'). This hones and defines both the selected cells and increases the affinity of antibody. Humoral immune responses result in a polyclonal B cell proliferation, producing antibody with varying affinity for antigen. Affinity maturation (whereby the mean affinity of the polyclonal response to an antigen increases) occurs in the germinal centre[19]. Many B-cells die at this stage through apoptosis, since despite successful gene rearrangement, their antibody is of too low affinity or they have too infrequent antigen contact to accomplish the events described above to go on to become memory cells or plasma cells. B-cells can also be rescued from apoptosis by interaction of ligands with follicular dendritic cells (CLA-4 (B cell) with 1CAM-1 and VCAM-1) and it is probably a combination of these events that operates the selection procedure[20,21].

After this, B-cells either mature further to become memory B cells (through CD40 cross linking with CD40L on T cells in the centrocytic area of the germinal centre) or (if there is CD21/CD23 cross linking) become plasma cells.

High affinity antibody producing B cells are a feature of memory cells, a subset of naive B-cells (IgD+) derived from the centrocyte which migrate to the mantle zone of the lymph node. These memory cells acquire the antigen CDS. The function of $CD5^+$ is currently

unknown. It is clear that $CD5^+$ B cells are derived separately from $CD5^-$ B cells[22] and that the former show restricted V gene usage as a result of antigen driven selection whereas the latter utilize a wider range of V genes.

The malignant counterpart of these CD5+ memory cells is chronic lymphocytic leukaemia or lymphocytic lymphoma. However, recent work has shown two CD5+/CD23+ populations of B-cells exist, one of which recirculates back to the inner mantle zone and can also form the malignant counterpart of what is recognised as a different type of CLL.

Some naive B-cells have less avid antibody affinity yet do not undergo apoptosis. They fail to differentiate fully in the germinal centre and migrate through the mantle zone to the marginal zone where further T cell interaction and B cell selection occurs. Having completed the final stage of development beyond the centrocyte stage, mature B-cells lose their characteristic clefted nucleus and are released into the lymph and enter the blood stream via the thoracic duct. In the blood stream the highly controlled migration continues. IgA secreting cells express the integrin $\alpha 4\beta 7$ (the mucosal homing receptor) which binds to the mucosal cell adhesion molecule (MAdCAM-1) in the gut mucosa, Peyer's patches and the mesenteric lymph nodes and then circulate back to regional lymph nodes (10). IgG, A and E producing cells in the blood, traffic to secondary lymphoid tissue. Lymphocytes enter the lymph node through specialised high endothelial venules (HEV's) found in the interfollicular areas where they come directly into contact with T cells. The process of travelling to sites of infection is directed by adhesion molecules (which bind to lymphocytes relatively loosely) with the result that lymphocytes roll along the blood vessels, directed by integrins and selectins to their destination. The destination is defined when a stop signal is encountered. These stop signals are delivered by the stronger binding cell adhesion molecules e.g. ICAM1 (CD54), ICAM2 (CD 102) and LFA1 (CD11a/CD18). The final journey to their destination is by local migration under the influence of chemoattractants.

Mature B cells express surface immunoglobulin. When antigen binds to it this usually results in proliferation of the cell and secretion of the immunoglobulin. This secreted Ig may exert a negative effect by virtue of cross linking the B cell receptor sites that are occupied with antigen (via tyrosine kinases of the ZAP70/Syk family), followed by binding to phosphotyrosine phosphates activated by SHIP/Shc binding), and this seems to be important for preventing excess production of immunoglobulin. A group of lymphoma related diseases are characterized by overproduction of immunoglobulin, such as MGUS (of IgM or IgG class) and in these conditions defective negative signalling by this mechanism has been shown and is thought to be due to competition between SH2 binding domains on the different protein messengers (17).

T-CELL CIRCULATION, DEVELOPMENT, AND LYMPHOMA COUNTERPARTS

The development process is also highly controlled for T cells which have specific chemokine receptors to direct them. In contrast to B-cell development which occurs widely at sites of immune recognition, T-cell development is much more restricted to T-cell areas located in the paracortical regions of lymph nodes and spleen, the thymus and the skin and gut. There is another fundamental difference between T and B-cells. T-cells

need to recognise peptide antigen in the context of MHC whereas B-cells are able to recognise soluble antigen directly with the Ig B-cell receptor complex. There are some exceptions to this such as the ability of some T-cells to recognise HLA alloantigens and some superantigens directly. For B-cells, direct antigen recognition is also the mechanism whereby autoreactive B-cells are taken up in the spleen and rendered functionally anergic since they are exposed directly to antigen there.

T cell precursors arise from a common lymphoid progenitor derived from the $CD34^+$ compartment of the bone marrow. Germ line cells that are committed T cells (prothymocytes) and express cytoplasmic $CD3^+$ and $CD7^+$ migrate to the T-cell processing areas (thymus, skin and gut). As they mature T-cells migrate from the cortex to the medulla of the thymus. An important enzyme involved at this stage is Terminal deoxynucleotidyl transferase (TdT), which is expressed by developing T-cells in the cortex of the thymus. It is here that VDJ joining and TdT activity occurs, increasing by hypermutational events the potential repertoire of T cell receptors (TCR) to be formed. At this point, T cells express both CD4 and CD8, (common thymocytes) following which they migrate to the medullary thymic areas to become $CD4^+$ or $CD8^+$ cells (mature thymocytes). Most mature T cells express the heterodimeric $\alpha\beta$ TCR protein, but a smaller circulating, yet important category of T cells carry the $\gamma\delta$ TCR heterodimer. The $\gamma\delta$ T cells are involved in first line defence immune responses (they are thought to be evolutionally primitive) and home to mucosal sites as well as the skin. Thymocytes of the $\alpha\beta$ TCR lineage undergo thymic selection to ensure autoreactive T cells are deleted, and following this are released into the circulation as mature $CD4^+$ or $CD8^+$ T cells.

T-cells account for 2/3 of the circulating lymphocytes and also have a longer life span than B cells. They circulate in a similar manner to B cells though high endothelial venules to secondary lymphoid tissue and via lymph back to the blood again.

Homing of T cells is also controlled by adhesion molecules of three main families-the selectins, the immunoglobulin superfamily, the integrins and CD44.

Another important antigen pivotal in T cell development is CD45 which exists in a number of isomeric forms at different stages of ontogeny and is hence a useful marker in looking at malignant counterparts of T-cells. Four different CD45 isoforms, recognised by monoclonal antibodies to different epitopes, are seen on T cells, ranging from 180kD to 220kD (CD45RA, CD45RB, CD45RC, and CD45RO (the lowest molecular weight one). Memory T-cells are characterised by their expression of CD45RO, L-selectin and the $\alpha_1\beta_2$ integrin LFA-1. LFA-1 (α_1 β_2) is a cellular adhesion molecule of the B_2 integrin family. In this family there is a common B_2 unit (CD 18) associated with distinct α subunits - α_1(CD11a), α_M(CD116), α_X(CD11$_C$) and α_D(CD11d). LFA-1 is involved in leucocyte migration locally (at the site of inflammation) and 3 ligands have been characterised - ICAM1 (CD54), ICAM2, and ICAM-3.

Naive T-cells are CD45RA+ and also express LFA-1 which binds preferentially to the high molecular weight forms of CD45 whereas CD2 binds to the low molecular weight forms of CD45 (CD45RO). Other integrins such as VLA-4, 5 & 6 and the CD44 family are also preferentially expressed on memory T cells. Some T-cells that home to the skin do so by expression of a cutaneous

lymphocyte associated antigen (CLA) that bind to a selection - E-selectin.

By differentially expressing different types and levels of these adhesion molecules, T cells can migrate selectively though the high endothelial venules encoutering different ligands at different HEV sites such as MAdCAM in Peyer's patches in the gut, VCAM and E-selectin in the skin and ICAM (1-3) in inflammatory areas.

There is further evidence that even at a late stage of functionality $CD4^+$ T cells that are largely responsible for the T cell help in a proinflammatory (TH_1) response express different adhesion molecules to the antibody response directed by TH_2 cells or the uncommitted TH_o cells.

To do this, TH_2 cells express CD40 ligand and bind to CD40 on the B cell. This triggers differentiation of these B cells into memory B cells producing Th_2 associated antibody subclasses (IgG4 and IgE) whereas T helper cells that are polarized to TH_1 proinflammatory cytokine production (IL-12, IL02, IFN-γ) have different receptors for chemokines (CXCR3 and CCR5) to TH_2 (IL-4, IL-13) producing cells (CCR3 and CCR4). This could be extremely relevant in the development of malignancy from these cells - for example Sezary/Mycosis fungoides CD4+ cells being the malignant counterpart of a TH_2 type cell.

These complex homing mechanisms (rolling on endothelial cells, - integrin adhesion and chemoattraction at the destination site) allow selective trafficking to specific anatomical sites which in the case of T cells is particularly the skin and areas of chronic inflammation. This has great relevance to the T-cell lymphomas which are often found (or relapse in) T-cell anatomical areas particularly the skin, and it will be important to understand more about the homing behaviour of the normal lymphoid counterparts of malignant lymphoproliferative disorders as a common cause of death is due to extranodal spread , especially to the central nervous system.

MECHANISMS OF LYMPHOMAGENESIS:

Unlike the acute myeloid leukaemias where a number of well defined aetiological factors have emerged (benzene and other toxins, irradiation), the known causative agents of the lymphomas are largely associated with infection. The Herpes viruses (especially EBV and HHV8) are associated with the development of lymphomas and this has been well documented in groups of heavily immunosuppressed individuals such as recipients of bone marrow or solid organ transplants or patients infected with HIV. However, the development of lymphoma is certainly not associated with one single mutational event and is likely to be a multi-step process. The finding of translocations such as t(14;18) and t(9;22)[6,8] in the normal population as well as the recent findings from twin studies of specific DNA sequences associated with t(12;21) in an unaffected identical twin whose sibling has acute lymphoblastic leukaemia with the same molecular change[7], suggests that these translocations are only part of the development of lymphoid malignancy (albeit an early one). A scenario for the development of follicular lymphoma is shown in Figure 4.

Most of the known molecular mechanisms of malignancy are reflected in the huge variety of lymphomas - altered tumour suppressor genes (p53 in high grade B-NHL), oncogene over-expression (c-myc in Burkitt's lymphoma), cell cycle dysregulation (cyclin D1 in mantle cell lymphoma) and disturbances of apoptotic mechanisms (bcl2 in follicular lymphomas). A particularly common mechanism of such disturbances is via

Table 6. B cell lymphoma associated genes

Name	Chromosome location	Example of a translocation in lymphoma	Disease Association
Bcl-1	11q13	t(11;14)	MCL B-CLL B-PLL
Bcl-2	18q21	t(14;18)	FL
Bcl-3	19q13	t(14;19)	B-CLL FL
Bcl-6	3q27	t(3;14)	Diffuse large B-cell lymphoma Burkitts
Bcl-8	15q 11-13	t(14;15)	Diffuse large B-cell lymphoma
Bcl-9	1q21	t(1;14)	B lineage ALL
Bcl-10	?	t(1;14)	undertain

Key: MCL = Mantle Cell Lymphoma
B-CLL = B cell chronic lymphocytic leukaemia
B-PLL = B-cell prolymphocytic leukaemia
FL = Follicle Cell Lymphoma
ALL = acute lymphoblastic leukaemia.

chromosome translocations, where the IgH promoter on chromosome 14 becomes adjacent to another gene that produces a regulatory protein (eg cyclin D1, bcl2) and upregulates it.

Several novel or overproduced proteins have been associated with lymphoma the B-cell lymphoma or bcl family now numbered up to at least 10 (Table 6). The sheer variety of mechanisms of lymphomagenesis put into focus the problems of interpreting the clinical trials of the 1980's and early 1990's in lymphoma. It is no longer useful to perform a clinical trial on "low grade lymphomas" that might include follicular and mantle cell diseases together.

An important emerging group of lymphomas are associated with immunosuppression. Solid organ transplant patients who remain on heavy immunosuppression with cyclosporin, FK506 and other suppressors of T-cell function may develop post transplant lymphoproliferative disorders (PTLD) as do HIV infected patients. These lymphomas range from relatively benign lymph node enlargements (that can revert to normal after reduction of the immunosuppression) to malignant body cavity high grade diffuse B-cell lymphomas. These latter are very often extranodal and have a propensity to arise in or spread to the central nervous system (CNS). They have a very poor prognosis once in the CNS but are often quite treatable in the early stages or if outside the CNS.

Recent work has suggested that some of these tumours may respond to a reduction in immunosuppression (where feasible) even at a late stage and that the response is due to an immunologically mediated tumour suppression by T-cells.

Another infective agent that is associated with lymphomagenesis is the bacterium *helicobacter pylorii.* These bacteria colonize the stomach and early part of the duodenum and result in a mucosal associated lymphoma (MALT) derived from the marginal zone of Peyer's patch lymph nodes. As discussed above it is precisely in this marginal zone that chronic antigenic stimulation and T and B cell cooperation takes place but as with the PTLD's, it has been shown that withdrawal of the stimulus at an early stage (in the case of *helicobacter* infection by treatment with antibiotics) results in regression of the lymphoma. At a later stage MALT lymphomas no longer behave as "pseudolymphomas" (their old name) but transform through further molecular events to aggressive high grade tumours.

THERAPEUTIC APPROACHES TO THE NON HODGKIN'S LYMPHOMAS

Chemotherapy is still the mainstay of treatment for the lymphomas. However, whereas escalation from standard doses of chemotherapy to high dose chemotherapy (requiring haemopoietic stem cell support) has shown great benefit in cases of acute leukaemia, the results in the Non Hodgkin's lymphomas have been more disappointing. Indeed the cynic might say nothing has changed in the quarter of a century since CHOP was introduced. Remission can be induced in over 80% of high grade NHL, but relapse within the first 2 years remains a very high probability, with ultimate cure achieved in less than 30%. Various prognostic indexes have been used - for example the International Prognostic Index (IPI) (23) as shown in Table 7, but the overwhelming factor in an overview of trials of chemotherapy in NHL still ´appears to be age.

One reason for these disappointing results in the unfocussed nature of clinical trials, because the heterogeneity of this group of diseases has allowed the inclusion of what we now realize are ultimately very bad diseases masquerading as low grade diseases (mantle cell lymphoma often appears similar morphologically to follicle centre cell lymphoma) and conversely very aggressive looking diseases down the microscope can melt away relatively easily (T lineage anaplastic large cell lymphoma in young people with the chromosome translocation t(2;5)). The International Lymphoma Study Group (ILSG) has broadly divided the commoner non Hodgkin's Lymphomas into 4 histological categories that have prognostic relevance - ranging from a long expected survival to a short survival (regardless of other factors such as achievement of remission, clinical stage or other scores in the IPI). These four categories are shown in Table 8. In this way it can readily be seen how easily in the past diseases in group 4 (bad prognosis) can be grouped together in the same clinical trial with diseases in group 1, making interpretation of chemotherapy trials involving 2 arm comparisons extremely difficult.

This is not the only reason for the lack of progress in the chemotherapy of NHL. The high relapse rate after achievement of complete remission is often associated with the upregulation of multidrug resistance (mdr) pumps (pgp, Irp and mrp) and further mutational changes in p53. Standard metaphase cytogenetic analysis in poor prognosis lymphomas often reveal multiple structural and numerical abnormalities in the chromosomes, leading to an unstable genome with a high

spontaneous mutation rate and selective pressure for aggressive clones. Those clones that escape and then thrive after chemotherapy are very difficult to eliminate. There is overwhelming clinical evidence that lymphomas that have failed three different types of chemotherapeutic regime have a median survival of only a few months. Consequently the notion arose in the 1980's that perhaps as much therapy as could be tolerated should be given upfront. There have broadly been three approaches to the use of high dose chemotherapy requiring stem cell support. Firstly upfront initial intensive treatment as a first line exposure of the tumour to chemotherapy. This may involve several "marrow ablative" doses of chemotherapy requiring stem cell support to allow reseeding of the bone marrow. The most promising results have been obtained by Gianni and colleagues using a high dose sequential chemotherapy regimen (4 cycles) with stem cell rescue in high grade NHL (24). However the administration of such potentially toxic therapy is limited to younger patients and is extremely expensive and resource dependent in terms of transplant beds. These young patients had a 76% event free survival over 5 years vs 49% for those on standard chemotherapy (p=0.004).

The second approach has been less successful but more widely applicable. Initial conventional chemotherapy (for example 6 courses of CHOP) is given followed by consolidation of the complete remission with one high dose module (usually BEAM, CBV or BEAC) with stem cell support. This was the approach adopted in the French LNH-87 and Italian study group trials (25-27), which both showed that benefit was seen in the high and high intermediate IPI groups.

The third approach has generally been less satisfactory (LNH-93 and the German NHL study group). In these trials limited initial chemotherapy (eg 3 courses of CHOP) was given followed by a high dose module and compared with standard therapy (eg 6 courses of CHOP). There was no survival difference between the high dose and conventional arm (28,29).

The three approaches discussed above all apply to initial therapy of newly diagnosed high and intermediate grade NHL. However in patients with high grade lymphomas who have relapsed after standard therapy, there appears to be a very definite advantage for high dose therapy. The result of a large EBMT study - the PARMA study showed a 46% 5 year survival in high and intermediate grade NHL at 5 years after 2 courses of second line chemotherapy (DHAP) and a high dose module, compared to 4 conventional DHAP courses (26%) (30).

The situation in the low grade lymphomas is even more clouded. Whilst some investigators claim a clear benefit for high dose therapy in low grade lymphomas, others suggest that the best results are obtained only when high dose therapy is given in the context of a (very) complete remission and a stem cell rescue product that is negative by PCR detection of residual lymphoma (a level of sensitivity of about 1 in 10^4 - 10^5). The latter is interesting as the data is derived from a small but very homogeneous disease group - follicle centre cell lymphomas with t(14;18) detectable at diagnosis by PCR (31). However, it is not possible to know if reason for benefit was that the selected patients were a particularly good group by virtue of the excellent remission and PCR status already achieved.

In summary the role of high dose chemotherapy as first line treatment in NHL is unclear. It is undoubtedly of benefit in some cases but the results are largely unpredictable and new trials with better characterized molecular lesions to strati´fy disease groups are cleary the next step in clinical trials.

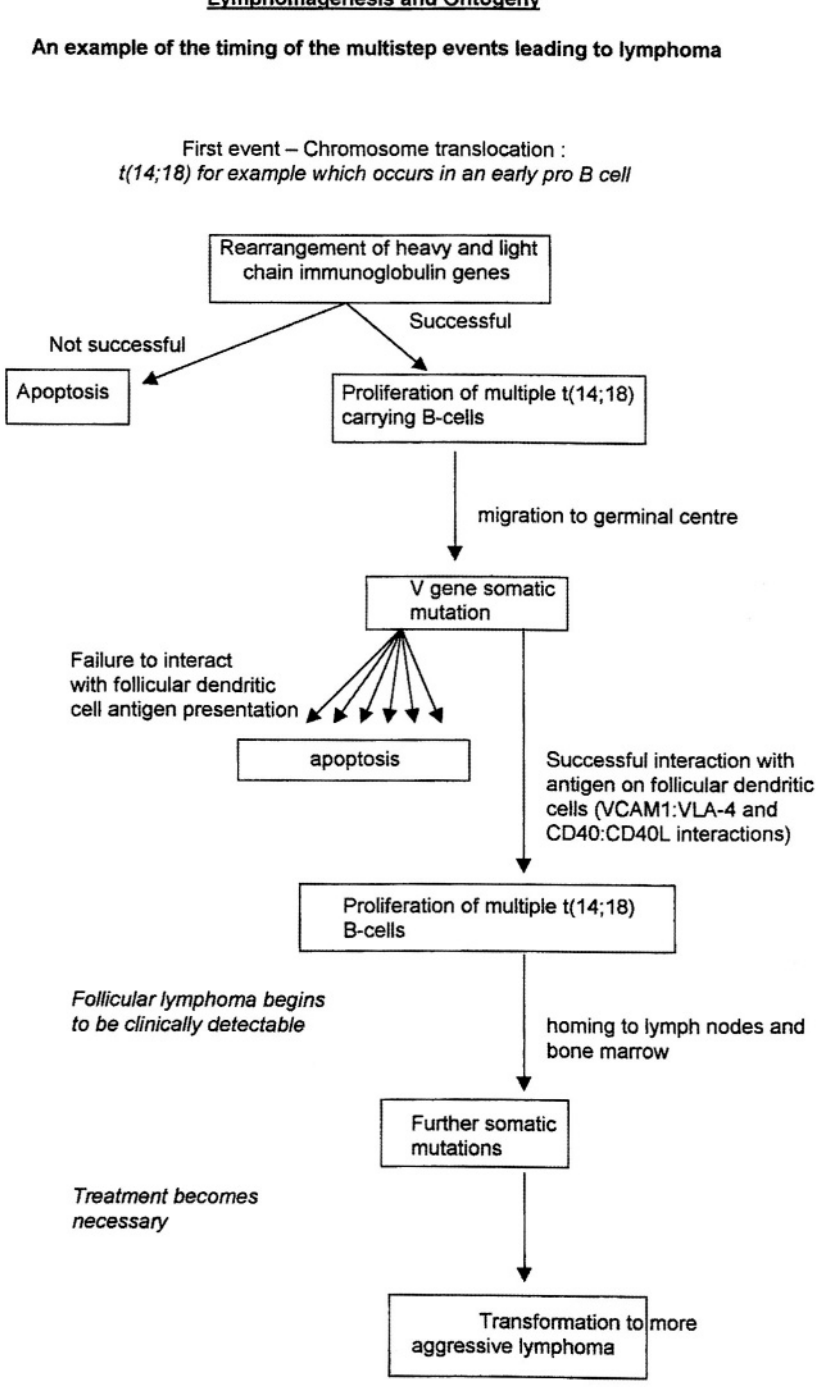

Figure 4. Lymphomagenesis and ontogeny

Table 7. The International Prognostic Index is a model for predicting the outcome in patients with NHL on the basis of the patient's clinical characteristics before treatment. It operates independently of the histological classifications.

It is calculated by assigning 0 or 1 to each of the following

Remarks	Index
Age	≤60 = 0
	>60 = 1
Performance status	0-1 = 0
	2-4 = 1
Clinical Stage of disease	I or II = 0
	III or IV= 1
Extranodal site involvment	≤1 site = 0
	>1 site = 1
LDH level	Normal = 0
	Raised = 1

Score	IPI
0 or 1	Low = L
2	Low intermediate = L1
3	High intermediate = H1
4 or 5	High

Table 8. Prognostic groups within the REAL classification – 5 year survival

Group	Lymphomas
Group I (>70%)	Anaplastic Large Cell Lymphoma (ALCL)
	Follicle Cell Lymphoma (FCC)
	Mucosa Associated Lymphoid Tissue Lymphoma (MALT)
Group II (50-70%)	Chronic Lymphocytic Leukaemia (CLL)
	Lymphoplasmacytoid Lymphoma
	Nodal Marginal Lymphomas
Group III (30-49%)	Diffuse Large B Cell Lymphoma (DLBCL)
	Mediastinal B cell Lymphoma
	Burkitt's
Group IV (<30%)	Precursor T Cell Lymphoma
	Peripheral T Cell Lymphoma
	Mantle Cell Lymphoma (MCL)

NEW APPROACHES TO THE TREATMENT OF NHL:

The failure to improve on CHOP chemotherapy in NHL over the past 25 years (and the resistance of at least 15% of Hodgkin's disease to chemotherapy) has led to a reappraisal of this difficult group of diseases with a view to characterizing them at diagnosis as far as possible immunologically, molecularly and cytogenetically. This is not to say that improvements have not occurred, but they have largely been due to better supportive care (antimicrobial agents and growth factors) rather than the chemotherapy itself.

The lymphomas were the first group of diseases to be treated with antibody infusions even before the advent of monoclonal antibodies. In 1980 Nadler and colleagues were able to demonstrate a transient reduction in circulating lymphoma cells with unconjugated anti B-cell (Bl, CD 19) McAb (32). Levy and colleagues went on to report a study of 17 patients treated with anti-idiotypic (33) antibodies to their lymphoma with overall response rates of about 50%. in lymphoma. Following these initial studies, several monoclonal antibodies have shown early promise in clinical trials in NHL. Several approaches have been used to enhance the activity of the targeting McAb by conjugation to toxins or other immune effector molecules.

Successful McAb treatment depends on many inter-related immunological factors. Clearly the cell must express the antigen on its surface, in a configuration that allows antibody binding. The resulting antibody antigen complex is then handled by the cell in a number of ways, depending on antibody affinity, antigen density, cross linking of antigen on the surface by the antibody or proximity of the binding site to the surface of the cell. Some antibody-antigen complexes are quite fluid and are simply shed from the surface (e.g. the CD 10 - J5 antibody complex). Some classes of antibody are good at ADCC (IgG1) or complement activation (IgM). Others are less able to harness immune effector mechanisms. Some of these individual features of an antibody can be used to advantage. Hence antibodies recognising a large molecule such as CD33 that is spatially some distance from the cell surface tend to be internalized by the cell (e.g. anti CD33). An anti CD33 McAb has been conjugated to the toxin calicheamicin and is currently in trials for acute myeloid leukaemia expressing the CD33 antigen (Figure 5). Another monoclonal antibody that has been quite widely used in vitro and in vivo is the humanized IgG1 McAb, anti CD52 (CAMPATH-1) which has good ADCC activity, can activate complement and has been used in several T and B cell malignancies (34,35).

Recently a series of antibodies directed against CD20 has become available for clinical use and are undergoing large scale phase III and phase IV clinical studies. Anti CD20 McAb (Mabthera, Rituximab) is a chimeric IgGl antibody whose complementarity determining region (CDR III) was originally mouse derived and was then engineered with a human Fc portion. Such an antibody would be expected to bind complement binding and allow ADCC through its human Fc receptors. The CD20 antigen (Figure 6) is a 35kD protein encoded by a gene on chromosome 11 (at 11q12) that has a very close association with other B-cell antigens in clusters on the surface of the cell. It does not extend far from the surface but remains tightly associated with the cell membrane. This physical structure means that it does not modulate

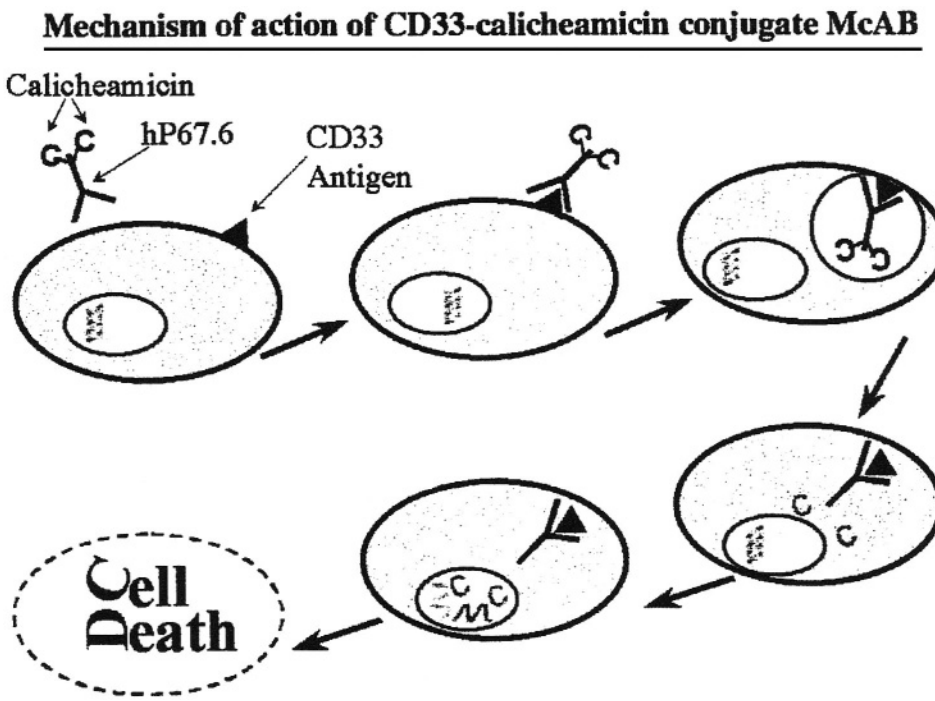

Figure 5. Mechanism of action of CD33-calicheamicin conjugate McAB

or internalize easily when attached to a monoclonal antibody. CD20 is expressed from quite early in B cell ontogeny and hence present on the vast majority of NHL but absent on haemopoietic stem cells (as well as mature plasma cells). The antigen has an important role in cell signalling and regulation of the cell cycle in B-cells. So as well as harnessing immune effector mechanisms, the CD20 - Rituximab complex does not appear to shed or internalised and appears to promote some sort of apoptotic influence presumably through an intracellular signalling, preventing entry into G1 of the cell cycle. This may well allow normal cell cycle control mechanisms such as p53 to function better in promoting apoptosis of the lymphoma cells.

A large number of Phase II studies of Rituximab have now been reported, and a picture is emerging of benefit in low grade lymphomas (36). Using PCR analysis for IgH or t(14;18) in follicular lymphoma up to 80% of patients can be rendered PCR negative in the bone marrow after treatment with Rituximab. Whether this can be translated into improved overall survival yet is unclear. Phase III studies are currently ongoing, and the long term benefits of anti CD20 therapy will be awaited with interest from a number of large trial groups (ECOG/ SWOG/CALGB/EORTC).

Rituximab is given intravenously as a weekly dose for 4-8 weeks and has very low acceptable toxicity profile and high patient acceptability. Occasional fatal tumour lysis has been reported when it is used in patients with very extensive disease, or very high circulating tumour loads (>50 x 10^9/l circulating lymphoma cells). Clinical studies have demonstrated complete remission rates in low grade lymphomas refractory to chemotherapy of less than 10% but overall response rates up to 63%. In previously untreated low grade NHL CR rates are much higher (up to 60%) and when combined with CHOP chemothearpy for untreated high grade NHL, up to 75% achieve CR. This latter figure is no different from the use of CHOP alone. However one of the diseases that expresses CD20 at a high level on the cell surface is mantle cell lymphoma which is often very refractory to chemotherapy. Several studies have confirmed early reports of high CR rates in MCL, although the durability of response data is so far lacking.

Anti CD20 has also been conjugated to radioisotopes such as ^{131}I and ^{90}Y. As an isotope ^{131}I is far from ideal as it emits gamma irradiation of high energy, (but it is easier to conjugate). ^{90}Y is a pure β emitter and hence emits energy over a short (a few cells) distance. However, With radioisotope conjugates, uptake in the bone marrow and liver is variable between patients, making scanning dosimetry essential in each patient prior to therapeutic dosing. These radiopharmaceuticals are consequently much less easy to use, and have yet to be fully evaluated in phase III studies. Early reports suggest slightly higher response rates than with unconjugated anti CD20, but with greater toxicity.

Further recruitment of immune effector mechanisms is the subject of a great research interest. Bispecific antibodies have been produced , where each half of the immunoglobulin recognises a different antigen (37). Opportunities therefore arise to attract neutrophils, lymphocytes, NK cell and complement. Several researchers have focused on attracting effector cells (especially cytotoxic T lymphocytes) to the tumour. Bispecific antibodies that recognise both tumour cell and T cell receptor/CD3 or costimulatory molecules such as CD80, CD86 and CD40L have been engineered. NK cells can be attracted with anti CD 16 and monocytic

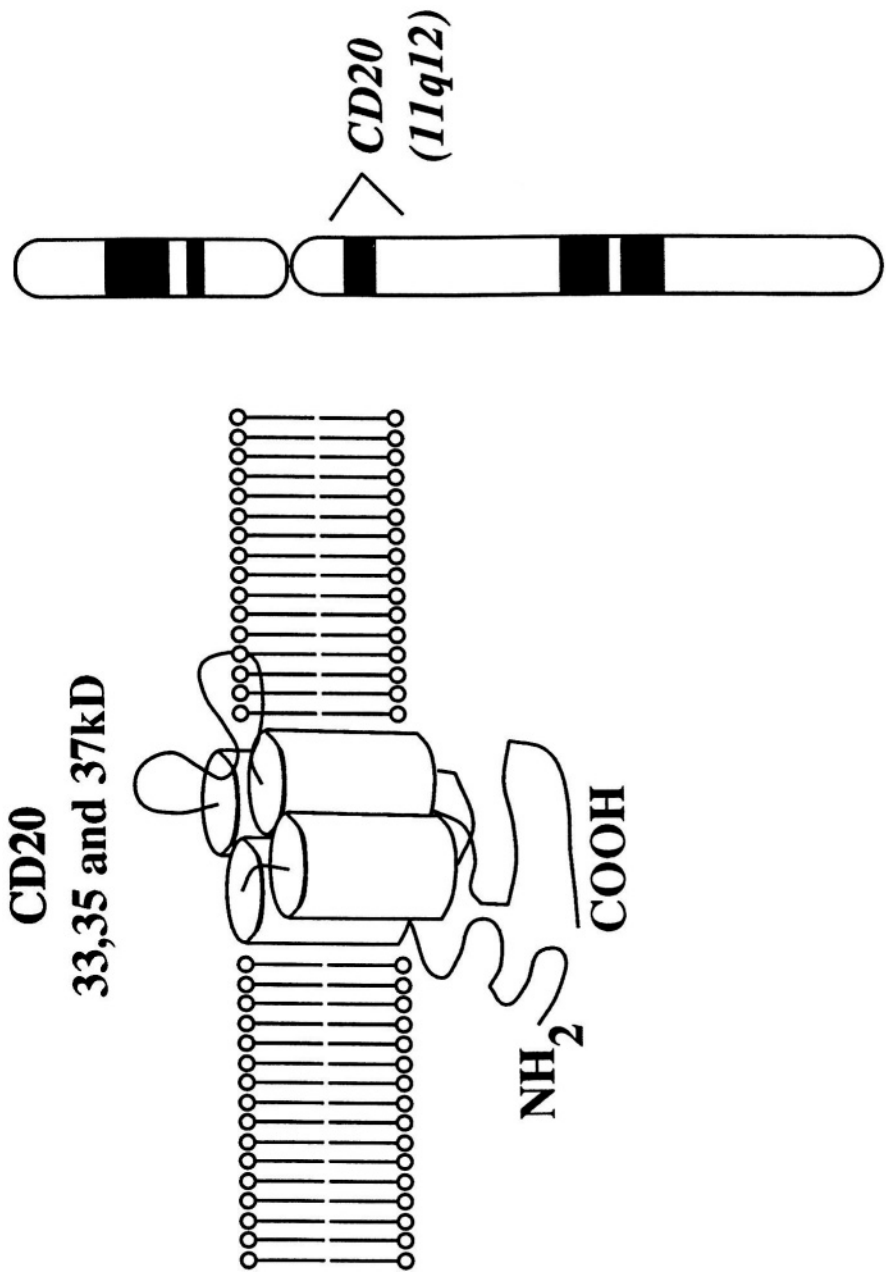

Figure 6. Structure of CD20 antigen

lineage dendritic cells with anti-CD64, which is present on monocytic lineage dendritic cells.

In a clinical context phase II studies have begun in Hodgkin's lymphoma using CD16/CD30 antibody (38,39).

CONCLUDING REMARKS

The problems of treating the lymphomas are not traditionally thought of as resulting from a metastatic process emanating from a localised tumour. T and B-cells mature 'on the streets' and the assumption must be that lymphomagenesis takes place over a period of time when the lymphocytes are already circulating from lymph nodes to blood via lymphatic drainage. Lymphocytes are critically dependent for their development on adhesion molecules to direct them to their next site of activation. One important feature of lymphocytes is their expendability. At every stage of development those that fail to achieve that phase, undergo apoptosis. A fuller understanding of these events and the control of apoptosis may allow specific triggering of these events in the malignant counterparts of these cells. At the present time although apoptotic events such as bcl2/bax ratios can be monitored they tell us little about why the cell was selected for apoptosis. Successful chemotherapy also induces many cells to enter apoptosis, so the conclusion must be that this is merely a final common pathway of lymphocyte death. One thing is certain - without a more fundamental understanding of the events that occur at each stage of normal lymphocyte development our ability to cure patients from a disease that is already widespread at diagnosis will be limited. Combinations of approaches to therapy rather than a single intervention are most likely to emerge now, such as chemotherapy combined with apoptotic promotion (antisense to bcl2) and immunotherapy all given together. Perhaps in this way we can improve on the somewhat disappointing rate of therapeutic progress in non Hodgkin's lymphoma over the last 25 years and accelerate over the next 25.

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Chapter 15

CANCER METASTASIS : BIOLOGICAL AND CLINICAL ASPECTS, GYNAECOLOGICAL CANCER

Malcolm Adams and Bharat Jasani

INTRODUCTION

Metastasis is the leading cause of treatment failure and death from gynaecological cancer, responsible for approximately 6,400 deaths 1 (see table 1) in England and Wales in 1997. Gynaecological cancer comprises three major malignancies epithelial ovarian cancer, cervical cancer and endometrial cancer, each with differing aetiology, prognosis and pattern of spread. Other gynaecological cancers are relatively uncommon.

Ovarian cancer includes a heterogeneous group of malignancy of variable behaviour. Epithelial ovarian cancer with its insidious presentation and late stage on presentation remains the most lethal. Nearly two thirds of patients with ovarian cancer present with metastases to the peritoneum and therefore have advanced inoperable disease (2). Ovarian tumours of borderline malignancy may grow very large without evidence of metastases on presentation but may metastasise late, whilst some undifferentiated malignant ovarian tumours may metastasise early and widely when the primary tumour is small. Endometrial cancer and cervical cancer usually present with early stage disease confined to the pelvis and are amenable to radical local treatment with surgery or radiotherapy.

Ovarian cancer ranks fourth after breast, bowel and lung cancer as one of the leading causes of death in women with no immediate prospect of reduction in mortality with current screening techniques although this remains an active area of research. Mortality in ovarian cancer since the 1970s has been rising in women over 55 but declining in women under 55 which may reflect a preventative effect of the contraceptive pill. Also there maybe a modest effect of treatment of metastatic disease with chemotherapy since the 1980s (3). Future reduction in ovarian cancer mortality is likely to be dependent for the foreseeable future on more effective treatment of metastatic disease. In contrast the mortality of cervical cancer has fallen in recent years

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from approximately 2,200 deaths in 1985 to 1,225 in 1997 (1), probably a result of screening rather than any improvement of treatment of invasive cancer. Endometrial cancer has the best prognosis because of early presentation and since the 1950s the mortality of this cancer has been falling in the absence of a fall in incidence (4).

Optimum treatment of gynaecological cancer depends on careful definition of the extent of the disease, the presence or risk of metastases (lymphatic and distant) which is influenced by biology of the tumour.

The quality of the treatment may significantly impact on outcome. Selection of optimum treatment for patients at risk of metastatic disease or with frank metastases is best considered under the following headings:-

- defining the risk and extent of metastases at staging
- the risk of metastatic disease based on tumour prognostic factors
- clinical relevance of biological and molecular parameters
- optimal conventional treatment determined by prognostic factors
- novel treatment targets

In recent years it has been increasingly recognised that the metastatic process in gynaecological cancers involves a complex cascade of events reflecting specialised interactions between host cells and tumour cells rather than just random events. Increased understanding of these events has lead to recognition of new prognostic factors and novel new targets for treatment of metastases. The clinical value remains to be defined as will be discussed in this chapter.

OVARIAN CANCER

Defining the extent of metastatic disease

Pattern of tumour spread

In their early development phase, ovarian cancers are usually confined to cystic growths arising within the ovarian parenchyma. With time the tumour is found to invade and penetrate the ovarian capsule allowing exfoliation of malignant cells into the peritoneal cavity. Such cells may follow the circulation of peritoneal fluid via the paracolic gutters and ultimately to the undersurface of the diaphragm. Peritoneal tumour implants thus occur characteristically throughout the peritoneum and the omentum is frequently a site of tumour implants. In nearly two thirds of patients peritoneal implants are present on presentation (2). Whilst transcoelomic spread is predominant lymphatic dissemination is also important. Lymphatic spread tends to follow the ovarian blood supply of the infundibular pelvic ligaments to terminate in para-aortic retroperitoneal lymphatics along the vena cava. Lymphatic spread may also occur laterally towards the pelvic side wall involving the hypogastric obturator and external iliac lymph nodes. Spread to the inguinal lymphatics may result from spread along the round

Table 1. Mortality from Gynaecological cancer (1)

Site	Number of registrations 1992 +	Number of Deaths 1997+	Death rate per 100,000 women 1997+	5 year survival England *
Ovary	5,338	3,985	15.0	33 %
Cervix	3,400	1,225	4.6	55%
Endometrium	3,912	774	2.9	70%

+ Source Guidance on commissioning Cancer services. Improving outcomes in Gynaecological Cancers (1)

* data extracted from Eurocare study which examined 5 year survival across Europe (3).

ligament (5,6). Peritoneal and lymphatic spread is frequently occult in apparently early stage disease as shown in restaging studies (7). The reported incidence of occult metastases varies widely in apparent stage 1 as a result of variable quality of initial surgical staging and variable tumour biology (see table 2). Increasing incidence of pelvic and para-aortic node metastases is evident with increasing stage (see table 3). Distant metastasis particularly involving the pleura and less commonly the parenchymal liver tissue is a feature of late disease in 16% on presentation (9). Brain metastases are unusual and the virtual absence of bony metastasis even in late disease is suggestive of tumour tropism to particular metastatic sites.

Table 2. Microscopic disease in patients with apparent stage 1-2 ovarian cancer (Piver 1976) (7) and recent reviews

Site of Microscopic Metastasis	Proportion with subclinical metastases with apparent stage 1-2 ovarian cancer		
	Piver 1976 (7)	Schueler et al 1998 (13)	Literature review by Schueler et al 1998 (13)
Para-aortic nodes	10 %	12 %	0-25%
Pelvic nodes	-	8 %	0-20%
Omentum	3%	5 %	0-7%
Diaphragm	11%	9 %	0-44%
Peritoneal Washings	33 %	22%	10-46 %
Pelvic Peritoneum	-	9 %	6-10%
Abdominal Peritoneum	-	8 %	7-9 %
Bowel mesentery		6 %	3-13 %

Tumour Stage

Diagnosis of ovarian cancer and FIGO staging requires a laparotomy, adequate inspection and palpation of the abdominal cavity, peritoneal cytology and appropriate biopsies (see table 2).

Laparoscopy is inadequate in diagnosis and staging as much of the peritoneal cavity is inaccessible and the retroperitoneal space cannot be explored. Pelvic and para-aortic lymph node metastases have been largely ignored in the past as it was assumed that they were not frequently involved. In fact lymph node metastases are common on presentation and the incidence progressively increases with FIGO stage (see table 4). The FIGO system does not incorporate the size of residual tumour after surgery which is a drawback as it is a key prognostic factor.

With the propensity of ovarian cancer to produce occult metastases peritoneal and lymph node metastases adequate surgical staging always requires the application of all the procedures in table 3. In the absence of adequate staging it is not possible to decide whether a patient has truly stage 1 disease or is harbouring " occult metastases" which require appropriate treatment. Adequately staged stage 1 disease is highly curable surgically and has a prognosis approaching 80% at 5 years.

Table 3. Adequate staging procedures

Procedures	Exploration
Vertical incision	adequate visualisation of structures in the upper abdomen
Peritoneal washings	pelvis, paracolic gutters and diaphragmatic surfaces
Inspection/palpation/random biopsies and biopsy of suspicious lesions	all peritoneal surfaces
Infracolic omentectomy	Palpation and biopsy of pelvic and para-aortic nodes

The presence of nodal metastases in apparent stage 1 disease is a prognostic factor and associated with a survival of 71.5% 5 year survival rate as compared to 91.6% in patients with negative nodes (10) . Multivariate analysis in a series of 242 stage 1 patients has shown positive nodes was an independent adverse prognostic factor (10). In advanced ovarian cancer the prognostic significance of nodal metastases is less clear. The 71.5% survival for FIGO stage 3C disease is higher than would be expected for stage 3C disease with intraperitoneal metastases (10). Whereas survival for patients with nodal metastases appears to be significantly worse than node negative patients for stage 1-2 disease (12). Nodal status does not appear to be an independent adverse prognostic factor for patients with advanced disease with intra-peritoneal metastases whether residual tumour is < 1 cm or absent after cytoreductive surgery (11).

Accuracy of surgical staging

The wide variation in survival in stage 1 and 2 may reflect accuracy of surgical staging (see table 4). Schueler et al (13) found that in a series 45 patients surgical restaging resulted in upstaging in 29% with 54% of those upstaged proving to have stage 3 disease. If adjuvant treatment had been based on the original diagnosis he found that 64 % with frank metastatic disease would have been denied adjuvant treatment. Survival in stage 1 and 2 has been found to be significantly better when performed by a specialist gynaecological oncologist. This probably reflects the accuracy of staging rather than a treatment factor eg. chemotherapy (see table 5).

Reviewing 351 patients with stage 1 ovarian cancer Zanetta et al (15) found that tumour grade was the single most important biological prognostic factor but the thoroughness of staging was a significant independent factor which impacted on survival particularly in poorly differentiated carcinomas.

Table 4. 5 year survival of epithelial ovarian cancer and incidence of pelvic node metastases according to stage

FIGO stage (8)	Incidence of pelvic node/ para-aortic metastases (10,11)	FIGO 1988, n=4,892 (9)	Nguyen, 1993, n=5,156
1. Confined to ovaries	13.2%, (n=242) Baoichi et al 1998 (10)		88.9
a. One ovary intracystic		62.5	92
b. Two ovaries intracystic		72	85
c. One or two ovaries with capsule/invasion/rupture/ ascites/positive peritoneal washings		57.4	82
2. Spread to pelvic organs			
a. Uterus and tubes		37.5	67
b. Other pelvic tissues		52.5	56
c. a or b/ capsule rupture/ ascites/positive peritoneal washings		37.5	51
3. One or two ovaries with peritoneal implants outside the pelvis or positive retro peritoneal or inguinal nodes	35.3% (n =456) Parrazzini et al (11)	10.8	23.8
a. Microscopic seeding of abdominal peritoneal surfaces			39
b. Implants of abdominal surfaces none exceeding 2cm			25
c. Abdominal implants greater than 2 cm or positive retro-peritoneal or inguinal nodes			17
4. Distant metastases including parenchymal liver		16	<11.6

Tumour Grade

Poorly differentiated tumours are responsible for approximately two thirds of epithelial ovarian cancers, with approximately 10% being well differentiated (16). Tumour grade is the single most important prognostic factor in stage 1 disease but has the disadvantage of considerable subjectivity and inter-observer variation (17,18). Five year overall survival is approximately 70-80 % for well differentiated tumours, 30-45% for moderately differentiated cancers and 5-25% for poorly differentiated tumours (16). Grade correlates with the incidence of nodal metastases in early (see table 6). In a series of 280 patients with stage 1 cancer (10) there was little difference in the incidence of nodal metastases in patients with grade 1-2 disease but a significant increase nodal metastases in grade 3 disease with a significant drop in 5 year survival when nodal metastases were present. Of interest in this study was the detection of pelvic nodal metastases in 7/39 (21.2 %) patients with borderline tumours. The prognostic significance of this is unclear in these tumours as lymph node status does not appear to significantly affect survival (19). In advanced disease grade appears to relate to the risk of nodal metastasis but in patients with peritoneal metastases the presence of nodal metastases does not appear to adversely affect prognosis (12)

Table 5. The effect of staging by a gynaecological oncologist on outcome in stage 1 and 2

Stage	Specialist gynaecological oncologist Surgeon	5 year survival	P value
1	Yes	83±7%	<0.05
	No	76±8%	
2	Yes	59±11%	<0.03
	No	39±11%	

Table 6. Relationship between tumour grade and nodal metastases in stage 1 (10)

FIGO stage	Grade	Proportion with nodal metastasis (%)	% 5 year survival
Stage 1A-C N=280	Well differentiated (grade 1)	3/88 (3.9)	92.7
	Moderate differentiated (grade 2)	6/92 (7)	93.2
	Poorly differentiated (grade 3)	15/46 (38.5)	76.2

Tumour residuum after surgery

The size of residual tumour remaining after surgical cytoreduction for advanced ovarian cancer may reflect tumour biology i.e. its degree invasiveness of abdominal structures as well as the degree of surgical effort. It is the second most important prognostic factor after stage. There is an inverse ratio between the size of residual tumour and patient outcome and in a small meta-analysis of 4 randomised trials of platinum based chemotherapy, residual tumour size was the major determinant of survival (20). The relative risk of dying from the disease increases with residual tumour size (see table 7). Nejt (21) showed that 3 year survival was approximately 75%, 50% and 25 % respectively for those with no macroscopic residual tumour <1 cm and >5 cm respectively after primary cytoreductive surgery.

Table 7. Relative risk of death according to residual tumour size (20)

Size of residual tumour	Relative risk of dying (95% confidence intervals)
< 2cms	1.0
2-5 cms	2.12 (1.6-2.7)
>5 cms	3.15 (2.8-4.4)

Table 8. Overall survival (all stages) between 1978 -1989 according to age group

Age	1978-1980 (n=9,996)	1987-1989 (n=10,575)	RR*
15 – 44	57%	64%	0.8
45 – 54	33%	44%	0.7
55 – 64	28%	34%	0.8
65 – 74	21%	20%	1.0
75+	20%	18%	1.1
Overall	30%	33%	0.9

** Relative risk of dying for ovarian cancer diagnosed between 1987-1989 versus 1978-1980*

Age at Presentation

Examination of data from the recently updated Eurocare study which determined the 5 year survival of 48,917 with ovarian cancer patients in 17 European countries diagnosed between 1978 and 1989 (22) reveals that age has a major effect on outcome, (see table 8). The 5 year survival for patients between 45-54 is approximately double that for over 65 who represent 50% of the patients with ovarian cancer. These data demonstrated a 30% reduction of risk of dying from ovarian cancer between 1978 - 1989 for women aged 45-54 (RR = 0.7) across Europe possibly as a result of the introduction of cisplatin in the early 1980s (see table 1). However there was no reduction in the death rate of the over 65s. This lack of improvement of survival in the older women may partly result from a nihilistic approach to treatment in this age group (23) but biological differences may also play a part. The GOG group reported that patients older than 69 years of age exhibited poorer survival than those of younger age even after correction for

stage, residual disease and performance status (24).

Patient performance status

Patients with advanced metastatic disease with minimal disease related symptoms i.e. with good performance status (Eastern Cooperative Oncology Group-ECOG 0-2 or Karnovsky score more than 70%), have a better outcome with treatment (25). Warwick et al (1995) (26) analysed the prognostic factors from two prospective randomised phase 3 trials initiated by the Midlands Ovarian Cancer Study Group in the 1980s involving 362 patients. They found the median survival of good performance patients was 27 months versus only 10 months for poor performance patients. Performance status was in this series a more powerful prognostic factor than residual tumour as indicated by the hazard ratios as shown in the (table 9).

Table 9. Prognostic value of performance status, residual tumour (26)

Prognostic Factor	Hazard ratio	P value	95% confidence limits
Performance status (<1 versus >1)	1.71	0.002	1.21 – 2.39
Residual disease (<2cm versus > 2cm)	1.56	0.005	1.13 – 2.12

The risk of metastatic disease based on prognostic factors

In early disease grade and stage, sub group are the key prognostic factors whereas in advanced metastatic disease stage, size of residual tumour and performance are the most important.

In a study of 194 patients with stage 1 disease grade, the presence of ascites and surface tumour were the only indepenent factors (27). Similarly, Vergote identified grade substage and ploidy (see later) as the only independent prognostic factors in a study of 290 stage 1 patients (28). Histological subtypes appear to have considerably less prognostic influence in stage 1 disease. Whilst serous tumours have been suggested to be more likely to give rise to metastatic recurrence. Mucinous and endometriod cancers cell type per se do not appear to be prognostic factors (27,28). Clear cell type occurs in about 10% of stage 1 and in some studies has been associated with increased risk of recurrence (29). The prognostic value of capsule rupture in the absence of surface tumour, poor grade and ascites remains doubtful as an independent factor (28).

In advanced metastatic disease the key predictors of survival are FIGO stage, residual tumour, performance and platinum based chemotherapy (see later). However, in a large multivariate analysis of 3000 patients (30) with advanced disease only cisplatin treatment and tumour residuum or cisplatin treatment were shown to be independent variables. In a similar large study of 7000 (31) patients, only stage and cisplatin was shown to be an independent prognostic factor. Various predictive models have been developed from retrospective multivariate analyses including factors such as performance status stage grade and tumour residuum but their value will remain unclear until validated prospectively (26,32-33).

Towards evidence based optimum treatment tailored by prognostic factors

Optimum management of gynaecological cancer is based on tailoring treatment according to the extent of disease and the risk of metastasis as determined by careful staging and evaluation key pathological prognostic factors. Prognostic groups are largely defined according to which define the risk of metastatic recurrence enabling adjuvant treatments to be used in a selected manner to maximise outcome and minimise unnecessary morbidity. The Caiman - Hine initiative has resulted in the production of evidence based guidelines[1] and has recognised the importance in treatment by a site specialist team in optimising outcome in gynaecological cancer. Surgery is best performed by a gynaecological oncologist, and radiotherapy and chemotherapy best administered by site specialists clinical and medical oncologists. The team needs also to include expert pathology input necessary to minimise chances of pathological diagnositic misclassification and ensure key pathological parameters e.g. depth of invasion is accurately reported. A number of audits (34,35) have emphasised the need for specialist pathology to minimise inaccurate pathological diagnosis in ovarian and particularly borderline ovarian tumours. Unfortunately most patients with ovarian cancer are liable to succumb from metastatic disease and palliation is likely be difficult with subacute intestinal obstruction. This also emphasises the further need for a palliative medicine specialist within the team as well as a specialist nurse to support the patient addressed communication issues and provide time!

Table 10. Prognostic factors for ovarian cancer

Risk of recurrence from metastasis	Key prognostic factors	% node metastases	5 year Survival
Low	Stage 1a-b GI-2	3.9-7	80 – 90%
Intermediate	Stage C tumour on surface ascites G3 ± clear cell	Upto 38.5	60 – 70%
High risk	Stage 2a 2b 2c, no residuum		38%
Very high risk	Stage 3 -4		
	Residuum 0	35.3	75%*
	< 1cm		50%*
	< 5 cm		25%*

* 3 year survival

Management of Ovarian cancer

There is increasing recognition that outcome of management of ovarian cancer is improved with treatment within a multidisciplinary team. In early ovarian cancer inadequate staging resulting in metastatic disease being inadequately treated has been a persistent feature of staging undertaken by non-specialist surgeons (13,15). (see table 5). Most patients present with advanced disease and whilst the quality of chemotherapy may be important, the quality of surgery may also play a significant role (36). The well cited audit of 533 ovarian cancer in Scotland (37) confirmed that access to platinum chemotherapy was a highly significant prognostic factor, together with

the size of residual tumour after debulking surgery. The quality factor of multidisciplinary care was also a powerful prognostic factor.

Multidisciplinary care by an expert team was the statistically the most powerful prognostic factor independent of platinum treatment and residual tumour bulk. The key factors and their significance were as follows:-

- Seen by a gynaecologist ($p< 0.05$)
- Operated on by a gynaecologist ($p < 0.05$)
- Debulking surgery to > 2 cm ($p< 0.001$)
- Platinum chemotherapy
- ($p< 0.05$)
- Specialist multidisciplinary clinic ($p<0.001$)

At a followup audit (38) it was found that treatment of stage 3 disease with surgery by a specialist gynaecologist as compared to a non-specialist surgeon reduced death rate at 3 years by 25% ($p= 0.004$). The study of ovarian cancer in Scotland found that follow up at a multidisciplinary clinic was an independent predictor of survival reducing the risk of death at 5 years by 40% (38).

Management of low risk disease (see table 10)

Appropriate surgery and accurate staging is key to the management of this group. Outcome can be expected to approach 90% 5 year survival if staging is optimal and there is no indication for adjuvant chemotherapy. Patients with stage 1 borderline tumours also do not require adjuvant treatment. The recurrence rate after unilateral oophorectomy is low for between 5-7 % (39-40) therefore conservative treatment can be justified when staging has been adequate in young women desirous of a family. The recurrence rate for borderline tumours is also very low and a conservative approach can be justified in the same circumstances. However for women who have completed their family pelvic clearance is to be preferred.

Management of intermediate risk (see table 10)

In this group which includes stage 1c, poorly differentiated tumours and clear cell cancer there may be up to a 30-40 % (27) recurrence rate and for this reason adjuvant chemotherapy is frequently given on the assumption that chemotherapy is at its most effective in the treatment of microscopic disease. However at present there no randomised evidence to indicate that adjuvant chemotherapy is more effective than a expectant approach of treating with chemotherapy at the time of recurrence. All randomised trials to date have been too small to draw any definite conclusions (41-42), and studies have been hampered by poor surgical staging. There are currently three randomised trials which are addressing this issue. It will be several years before these studies answer the question of whether adjuvant platinum therapy is indicated in these patients and interpretation may be hampered by poor standard surgical staging and the issue of whether optimum treatment in this group should include paclitaxel in combination with platinum.

Whole abdominal radiotherapy at one time was advocated for this group on the basis of a trial performed in the late 1970s (43). This data have never been confirmed in larger randomised trials Since there is a significant unpredictable hazard of major radiation induced bowel damage this adjuvant whole abdominal radiotherapy external beam radiotherapy is uncommonly used nowadays.

Randomised trials (44,45) have shown no significant survival advantage for the administration of radioactive colloidal intraperitoneally in stage 1 disease.

There is a significant risk of lymph node metastases in this group (see table 9) which would suggest a possible role for lymphadenectomy. However there is at present no evidence from retrospective study in stage 1 and 2 disease that it improves outcome(46) and it has not proved possible to evaluate the role of lymphadenectomy in a controlled trial.

For the present the optimum management of this group is optimal staging and entry into a randomised controlled trial which addresses the value of adjuvant chemotherapy.

Management of high risk groups (see table 10)

Most patients are in this group where the risk of death from metastases approaches 70-80%. Whilst optimum chemotherapy is key to improving outcome in advanced disease the importance of optimum surgery within a multidisciplinary team cannot be ignored. The size of residual tumour remaining after surgical cytoreduction is the second most important prognostic factor after stage (see table 7) and it has been unclear to what extent this reflects tumour biology or surgical skill and effort. A meta-analysis of 58 studies suggests that maximal surgical reduction of tumour bulk may increase median survival time slightly (47). In addition, a controlled trial which included 319 women demonstrated a 33 % (95% confidence intervals 10-50%) reduction in risk of death and a six months longer survival after interval debulking (48). Thus the extent of residual tumour after surgery which influences outcome with chemotherapy is probably influenced significantly by surgical skill as well as tumour biology.

What is the optimum chemotherapy for advanced disease?

(a) What is the evidence to support platinum

First line platinum based chemotherapy has become the standard therapy for advanced ovarian cancer since the 1990s as it appeared to produce modest survival. Platinum based chemotherapy improves survival among patients with ovarian cancer more advanced than stage 1. A recent update of the advanced trialist meta-analysis of 5,667 patients in 37 randomised controlled trials has shown a consistent but modest benefit for the inclusion of platinum in the chemotherapy regime (49). In addition large retrospective population based studies have shown a statistically highly significant survival advantage for platinum chemotherapy. Determination of the impact of first line ovarian cancer chemotherapy with survival as the end point has proven difficult as trials never determine the effect of primary chemotherapy per se. Overall survival is determined by the effect of first line surgery, first line chemotherapy and subsequent salvage treatments particularly second and third line chemotherapy. This salvage effect may be difficult to quantitate may have lead to an under estimate of the beneficial effect of platinum chemotherapy in metanalyses. It is frequently forgotten that prior to the introduction of platinum chemotherapy, 5 year survival in metastatic stage 3 ovarian cancer used to vary between 0-8% (50).

The meta-analysis suggested that Cisplatin and Carboplatin had a similar efficacy (50) (see table 11) but Carboplatin has a significantly less toxic profile (51) and is more amenable for out patient

therapy offering a better quality of life for these patients, and with its lack of toxicity it is suitable for elderlyand frail patients

A major debate for many years has been whether the addition of anthracycline drugs and alkylating agents to platinum improves outcome while inevitably contributing to the toxicity of treatment. However a large trial ICON2 (n = 1526) found no difference in effects on survival between CAP (cyclophosphamide, doxorubicin and cisplatin) and Carboplatin. The mean survival time for a group of patients including stage 1- 4 disease was 33 months with either treatment (HR =1.0, 95% CI:0.86-1.16) but Carboplatin was less toxic (52)

Table11. Impact of platinum chemotherapy (49)

Comparison	No.	Hazard ratio	5 year Survival Benefit for first line therapy
Single agent non platinum v platinum - based combination chemotherapy	1,329	0.93 (CI 0.83 - 1.05) p = 0.38	3% (25 to 28%)
Addition of platinum to a regimen	1,704	0.88 (CI 0.78 - 1.01) p = 0.02	5% (25 to 30%)
Carboplatin versus Cisplatin based chemotherapy	2,219	1.02 (CI 0.82-1.35) P=0.74	No significant difference

Current data thus supports the use of platinum based chemotherapy as first line therapy for advanced ovarian cancer which has improved outcome. Carboplatin appears to be equivalent to cisplatin but is less toxic and more amenable to outpatient chemotherapy. It is difficult to quantify the precise effect of first line platinum chemotherapy on improving overall survival in ovarian cancer survival as second and third line 'salvage' platinum based chemotherapy may also contribute to survival. This cross over effect has probably lead to an underestimate of platinum chemotherapy on overall survival.

(b) What is the impact of paclitaxel on outcome in ovarian cancer ?

To date 4 randomised controlled clinical trials have been performed aimed at determining the effect of adding paclitaxel to a first line platinum based regimen. Only one trial has been published so far (53). The GOG 111 (53) and OV 10 (54) trials were very similar in design and the group of patients recruited. They include over 1000 patients with largely advanced ovarian cancer and because of their similarity can be considered together below (55).

The results of both trials were similar with respect to progression free survival In GOG 111 median progression free survival increased 13 to 18 months with the addition of paclitaxel to cisplatin (Hazard ratio = 0.7 CI = 0.5 - 0.8 p < 0.001). Similarly in the OV 10 study the progression free survival increased from 12 months to 17 months with the addition of paclitaxel to cisplatin. (Hazard ratio = 0.7 CI 0.5 - 0.8 p < 0.0001). Of greater importance was the fact that both trials showed a very similar highly statistically significant improvement in survival of between 10-13 months (see table 12). Preliminary data of the another trial ICON3 which aims to test the efficacy of a paclitaxel/carboplatin combination vs single agent Carboplatin or a combination of cisplatin/ cyclophosphamide and doxorubicin (CAP) was presented in

ASCO 1999 (56). This study includes 2074 patients and includes patients from virtually the whole biological spectrum of ovarian cancer ie stage1-4 of unspecified performance status and is large enough to produce meaningful subgroup analysis. Early trends suggests a survival advantage for the addition of paclitaxel to carboplatin in patients with residual tumour after surgery (57).

Thus in 1000 patients a survival advantage of approximately 1 year was demonstrated for a population of largely stage 3 and 4 patients with residual tumour and a good performance status (ECOG 0 - 2). This represents he biggest survival advantage seen in any randomised controlled trials since the introduction of platinum. It is extremely unlikely that the results arose by pure chance as the results are highly statistically significant and very similar (58). The survival curves remain separated with a median follow up of 5 years suggesting long term survival has been improved. Ongoing clinical trials suggest that Carboplatin can be substituted for cisplatin in the combination with less toxicity better quality life and no evidence of loss of efficacy (59,60).

Therefore evidence to date suggests that paclitaxel /carboplatin is likely to be the optimal form of first line chemotherapy for advanced ovarian cancer in terms of survival duration and side effects. with the possible exception of elderly frail patients where single agent Carboplatin may be preferred (55).

Table 12. Survival data from the two randomised trials comparing cisplatin - paclitaxel (C-Pac) with cisplatin - cyclophosphamide (C-Cyclo)

Trial (ref)	Patient group stage (% sub-optimal residuum >1cm)	Median Survival		Relative Risk Survival C-pac (CI)	P - value
		C-cyclo	C- pac		
GOG 11 (53)	n = 386 stage 3 & 4 (100%)	24	38	0.7 (0.5 – 0.71)	<0.0015
INTER (54)	n = 668 stage 2 b ,c, 3,4 (65%)	25	35	0.71 (0.57 – 0.87)	0.0016
Cumulative data (53)	n = 1054				<0.000001

Second line chemotherapy

Most patients with advanced disease will relapse after initial response to platinum based chemotherapy Patients who have a progression free survival of more than six months frequently benefit from re-treatment with platinum chemotherapy and the prospect of response to treatment increases with increasing progression free survival (61). The MRC ICON4 trial is evaluating whether adding paclitaxel to platinum improves survival in the context of second line treatment this trial has not yet reported.

For patients with a progression free survival less than 6 months and who therefore can be regarded as platinum

refractory may benefit from paclitaxel or topetecan (62). Responses last a median of 20-30 weeks. Such patients should also be considered for a trial drug as the prognosis is very poor. Responses occur in about 20% and may last 20-30 weeks (62).

CERVICAL CANCER

Pattern of tumour spread

Cervical cancer tends to spread laterally and metastatic spread to lymph nodes tends to be orderly involving first the para-uterine, obturator, internal and external iliac and common iliac nodes before finally involving the para-aortic nodes. There is a consistent increase in nodal metastases with clinical stage. Morton (63) in over 5000 surgically treated patients found the incidence of pelvic nodal metastases was 16.5% of stage 1, 31.9% of stage 2 and 46.7 % of stage 3 patients. Subsequent series have suggested a similar incidence (64). Similarly, the incidence of paraaortic metastases increases with stage and the variation in incidence in different series may relate to the accuracy of lymph node sampling as well as the extent of the disease. The incidence of para-aortic node metastases ranges from 0-8% in stage 1 cervical cancer to 10- 46 % in stage 3 disease(65). It is usually associated with failure to cure pelvic tumour. The incidence of isolated para -aortic recurrence is much smaller being only 1.4-1.8% in stage 1b-3b disease (66).

The incidence of distant metastases similarly increases with pelvic stage and failure of pelvic treatment (67-68). In a series of 1200 patients distant metastases developed in 13% stage 1, 22% stage 2 and 32 % stage 3 patients with an incidence of pelvic treatment failure of 7% ,14% and 37% respectively (66-67).

Lung was a common site for metastasis and bone metastases were seen in 35% of treatment failures. Fifty-six percent of patients developed distant metastases with pelvic recurrence as compared to 18% when pelvic tumour was controlled (68). Post-mortem studies have shown that 70% of patients dying from cervical cancer will have para-aortic metastases when distant metastases are present (69). It is thus unclear to what extent distant metastases are the result of systematic lymphatic or haematogenous spread.

The presence of pelvic node metastases is a key prognostic factor. Prognosis falls from 90 % at 5 years in surgically treated patients with negative nodes to 50 -60% when pelvic nodes are positive (70). Survival at 5 years correlates with the number of pelvic node metastases falling from 50-62% to 36-37% for the involvement of 2 nodes, and 20% for 3-4 nodes, with few survivors for 5 or more nodes (70,71).

The prognosis of patients with para -aortic node metastases is poor as it is usually associated with pelvic failure and distant metastases. Nevertheless long term survival is well documented for patients with proven para-aortic node metastases when pelvic tumour is successfully eradicated being 45% for stage 1 30% for stage 2b and 22% of stage 3 patients (66) (see later).

FIGO staging (9)

FIGO stage is the most important prognostic factor in cervical cancer which is closely related to the risk of nodal metastasis. Clinical staging may be inaccurate in up to one third of patients. Inadequate staging may result in inappropriate choice of treatment particularly with surgery with reduced prognosis for patients unsuspected parametrial extension or pelvic nodal

metastases are not suspected. A metanalysis has compared lymphograpy, CT and MRI with respect to lymph node status and staging (Schneidler 1997) (72), This study found that MRI was the most accurate at determining the extent of disease whether parametrial or nodal. Similarly Hawnaur et al 1998 (73) performed a prospective study to compare tumour staging and volume assessment by examination under anaesthesia, transrectal ultrasound and MRI in patients with cervical cancer. Current clinical evidence suggests MRI offers the optimum means of staging and determining which patients are best treated with surgery or radiotherapy and should be carried out in all patients considered for surgery.

Table 13. Prognosis stage and incidence of pelvic and para-aortic node metastases in cervical cancer

FIGO Stage (9)	Definition	Incidence of pelvic node metastases range (1)	Incidence of para-aortic node metastaese (1)	5 year survival (9)
1	Confined to cervix			
1A1	Microinvasive disease <3 mm	0%	0%	<95%
1A2	Microinvasive 3-5 mm	0.6-4.8 %	0- < %	
1B	Visible tumour	15.9 %	2.3%	80%
1B1	< 4 cms	(21.2%)		
1B2	> 4 cms	(35.2%)		
2A	Extension to upper two thirds of vagina	24.5%	11%	60-70%
2B	Parametrium	31.4%	19%	50%
3	Extension to	44.8%	30%	
3A	Lower third of vagina			37%
3B	Pelvic Wall	55%		25%
4A	Extension to bladder or rectum		40%	> 10%
4B	Distant metastases eg lung			0%

Tumour volume

The relationship between tumour size and nodal metastases and prognosis is now well recognised (see table 11). The prognosis of patients with bulky stage 1B cancers (> 4 cms) is significantly worse than lesions < 4cms whether treated with radiotherapy or surgery which is related to the increased incidence of pelvic node metastases with bulky lesions (74). Consequently stage 1B tumours are now subdivided into stage 1 Bl and stage 1B2. The prognostic importance of tumour volume is also evident within stage 2A but as yet this stage is not subdivided in FIGO staging according to tumour volume.

Thus tumour volume and nodal metastasis are key prognostic factors irrespective of FIGO stage. Patients with a tumour less than 1 cm and no more than 2 positive nodes have an 80 % survival whereas those with a tumour greater than 4 cms or more than 2 positive node have a 10% 5 year survival (76).

Table 14. Relationship between tumour volume, nodal metastases and prognosis in stage I B -2 A treated surgically (modified from Piver and Chung 1975) (74)

Figo stage	Tumour size	Proportion with pelvic nodal metastases	5 year survival
1B	<4 cms	20/94 (21.2 %)	85%
	> 4 cms	19/51 (35.2%)	62%
2A	< 4 cms	8/38 (21%)	68%
	>4cms	24/57 (42%)	37%

Table 15. Relationship between stromal invasion nodal metastases and prognosis (75-76)

Depth of invasion mm	Proportion with nodal metastases(%) (75)	5 Year survival (76)
0 - 4.9	1.0	
5 - 9.9	12.4	86- 94%
10 - 14.9	26.4	
15 - 19.9	32.3	71-75 %

Cervical stroma invasion and parametrial invasion

Survival and the risk of metastases is strongly correlated with depth of tumour invasion of the cervical stroma.(see table 15) The risk of nodal metastasis appears to be virtually absent when invasion is 3 mm or less in the absence of vascular invasion (77). Averette (77) noted that with invasion of between 3-5 mm deep to the stromal surface the histological picture of microinvasive cancer changes. Tumour fingers become confluent and often separated from the epithelium and when this occurs the risk of nodal metastases commences. Similarly increasing parametrial invasion is associated with falling survival and increasing incidence of pelvic node metastasis. With 5 mm of parametrial invasion the risk of pelvic node metastasis is only 3.3 % which can rise to 17.2% with upto 15 mm of invasion (76).

Lymph vascular space invasion (LVSI)

Lymph vascular space invasion with tumour cells has been noted in 29% of surgically treated stage 1b patients but in 63% of those that recur (78). It has been commonly associated with other adverse prognostic factors such as large tumour size, deep invasion and unfavourable histologic type (79-80). There remains doubt about its true value as an independent prognostic factor for increased risk of pelvic node metastases and local recurrence and poorer survival (52,53). However, a multivariate analysis of 275 patients with stage IB - 2B treated with radical surgery with negative nodes revealed tumour size and LVSI were the only independent variables. In the presence of LVSI 11.2 % developed recurrence compared with 2% in its absence (83).

Cell type and tumour grade

There appears to be little prognostic value for tumour grade for squamous or adenocarcinoma of the cervix (84,85). Analysis of tumour grade is hampered by the variability seen between pathologists. More sophisticated automated methods for determining nuclear volume have also failed to find prognostic significance. However, in a node negative population full pathological review found that there was a four-fold increase in recurrence rate for patients with grade 2/3 disease (86).

The incidence of adenocarcinoma has increased in recent years (87). The prognostic significance of adeno-carcinoma and adenosquamous cell type has remained uncertain with some series suggesting adenocarcinomas and or adenosquamous tumours were more aggressive (88,89) whilst others reported no difference in prognosis (90,91). However in a recent multivariate analysis of 134 stage 1B or 2 surgical treated cases of adenocarcinoma and adenosquamous carcinoma and 757 squamous cancers the pattern of relapse was similar but histologic type was confirmed as an independent prognostic factor. In this study the 5 year recurrence free survival was 72.2 % vs 81.2% (92). A further multivariate analysis performed by the GOG on 168 patients with cervical adenosquamous carcinoma surgically treated also that adenosquamous carcinoma had decreased overall survival compared with squamous carcinoma although there were no differences in the pattern of relapse (93).

Small cell cancer of the cervix is rare and with a reported incidence between 0.5 -5 % of cervical cancer. Its pathogenesis is unknown but has an invariably dismal prognosis with mean survival is only 1.25 years and with virtually 100% relapse rate and few 5 year survivors patients (94).

Table 16. Prognostic groups for selective treatment of cervical cancer

Risk of Recurrence from metastasis	Key prognostic factors	% nodal metastases (1)	5 year survival
Very Low	Stage 1a1 cervical stromal invasion <3mm no vascular invasion	0%	99-100%
Low	Stage 1a 2 Stage 1b1 – 2a, tumour size < 4 cms	4.8- 15.9%	90%
Medium	Stage 1b2- 2a tumour size 4 cms nodes 1-2	20-30%	60%
High	Stage2b-4a TX node 3-4	31-44.8 %	20-40%
Very high	4b	55%	0-5%

Management of cervical cancer

A key task of the team treating cervical cancer must be accurate staging of the disease without which it is not possible to adequately select and plan appropriate therapy. All patients considered for radical surgery require a preoperative MRI scan. MRI has been proven prospectively to be the optimum method for assessment of parametrial invasion and pelvic node metastases (72,73). MRI provides accurate prognostic information on tumour volume which is difficult to obtain clinically. In a retrospective series of 44 cervical cancer patients treated with radiotherapy the 2 year disease free survival of patients with tumours > 40 mm was only 24% whilst it was 74% for tumours of AP diameter < 40 mm. Yet retrospective audits have reported that only 15.6% of cervical cancer patients are optimally staged and only 59% are appropriately treated according to treatment guidelines. Ninety-four percent have no preoperative imaging (95-97). The consequences of inadequate staging may be unnecessary death (98).

Treatment of very low risk group (see table 16)

If cervical stromal invasion does not exceed 3mm and width across does not exceed 7mm (stage 1a1) with no vascular invasion, the risk of pelvic node metastases is very low (1% or less) with a likely survival approaching 100% For most patients simple hysterectomy with removal of a vaginal cuff offers the simplest adequate treatment and morbidity is minimal. In young women who wish to retain their fertility, radical trachalectomy in expert hands is an option. The patient needs to be advised of the very small risk of recurrence and careful follow up is essential.

Treatment of low risk group (see table 16)

Once cervical stromal invasion exceeds 3 mm the risk of nodal metastases approaches 3.5%, and by the time the tumour is 4 cms the risk of pelvic nodal metastasis is likely to approach between 10-20%. Whilst the prognosis is good for patients with stage 1a2 i.e. 3-5mm invasion the preferred treatment is hysterectomy and lymphadenectomy. For the majority of patients with stage 1b or 2a lesions less than 4 cms the survival for patients treated with radical radiotherapy or radical surgery is similar (180). Surgery is usually to be preferred in younger women which will spare ovaries in post menopausal women who may not be as good a surgical risk, radiotherapy may be preferred.

Treatment of medium and poor risk groups (see table 16)

Once the AP diameter of the cervical tumour size exceeds 4 cms irrespective of stage, survival falls and the risk of pelvic node metastasis rises to 35%. For this group of patients the five recent platinum chemo/radiotherapy randomised controlled trials have had a major impact on outcome and clinical practice, in all the prognostic groups defined in the medium and poor risk groups. The results of these trials are consistent and are remarkably similar (99-103). The results suggest that the combination of cisplatin and pelvic radiotherapy improves survival for cervical cancer with adverse prognostic factors including bulky tumour 4 cm+, inoperable locally advanced disease and those with involved nodes or parametrial invasion. The improvement in survival of between 10-15%. The improved survival is associated with reduced locoregional and distant relapse. There is at present no evidence that the more toxic cisplatin /5

FU is any more effective than weekly cisplatin 40mg/m2 with concurrent pelvic radiation at 180 cGy per day which is well tolerated. There is currently no evidence of increased late radiation damage with concurrent cisplatin/radiotherapy. It is of interest to note that cisplatin/radiation was more effective than extended radiation to cover the para-aortic nodes which can be associated with severe radiation damage in some patients.

Since Cisplatin/ radiation combination appears to be well tolerated with consistent benefit demonstrated in these trials. It should now be regarded as the standard for regimen patients within this prognostic group. This approach is to be preferred to the use of neoadjuvant chemotherapy the results of which to date have proved inconclusive (104).

Very high risk group

Patients with recurrence confined to the pelvis may be treated curatively with in the absence of pelvic node metastases with pelvic exenteration. However the outlook for patients with frank metastatic disease is very poor and treatment is at best palliative with the use of palliative radiotherapy to palliate local symptoms. The most active agent remains cisplatin with a response rate of approximately 20%. There is at present no definite evidence that cisplatin combinations have any more palliative benefit than single agent cisplatin (105) although a complete and partial response rate of 64% have been recorded for paclitaxel platinum combinations in advanced adenocarcinoma of the cervix (106).

Table 17. Results of chemoradiotherapy (99-103)

Prognostic Group	Treatment Arms	No of patients	Result
Stage 1b2 (bulky) 4 cms +	Pelvic RT ± cisplatin chemo (followed by hysterectomy)	374	Projected 3 yr overall survival 83% for chemo RT vs 74% RT alone p RR 0.54 (95% CI 0.34-.86)
Ia/2,IB/or 2a with positive pelvic nodes & or pos. surgical margins	Pelvic RT ± cisplatin chemo	268	5 yr survival 67% for chemo RT vs 58% RT alone p<0.001
2b, 3, 4a or 1b 5cms +	Pelvic RT + extended RT vs pelvic RT + cisplatin chemo	403	Overall survival 67% in the chemo RT vs 40% RT (P<0.001)
2b ,3 ,4a	Pelvic RT + cisplatin vs Pelvic RT + cisplatin / 5 FU vs Pelvic RT + Hydroxyurea	575	2 yr progression-free survival cisplatin + RT 67% ; cisp/5FU+RT 64%; Hydroxyurea + RT 47% RR cisplatin vs hydroxyurea death 0.55 95% CI
2B-4A	RT + cisp /5 FU vs RT hydroxyurea		NCI report increased survival for cisplatin group but no details available

ENDOMETRIAL CANCER

Pattern of tumour spread

Endometrial cancer invades the myometrium and may spread towards the cervix and may progress laterally. The lymphatics of the myometrium drain into subserosal networks from whence lymph flows towards the infandibular ligaments from the fundus. The lymph flow from the base of the broad ligaments tends to spread towards the pelvic side wall. The risk of pelvic and para-aortic lymphatic metastases increases with stage as with ovarian and cervical cancer(see table 18). The overall risk of pelvic node metastases rises from 11 % in stage 1 to 23% in stage 2 (108). In a more recent study caried out on 621 women with Stage 1 disease by the GOG group only 12% had pelvic node metastases, 6% had cancer in pelvic nodes alone and 4% had cancer in both pelvic and para-aortic nodes with only 2 % having cancer in para-aortic nodes alone (104). The incidence varies considerably according to pathological factors eg. myometrial infiltration and grade which is now integrated into the FIGO stage (9).

FIGO Stage

Stage for stage the prognosis of cervical and endometrial cancer is similar. Since over 80 % of tumours are stage 1 (ie. confined to the uterus). Pathological factors eg. tumour grade and myometrial infiltration assume particular importance in determining treatment and on the basis of these two key prognostic factors patients at low and high risk of recurrence can be defined.

Myometrial invasion and grade

The depth of myometrial invasion is closely related to the risk of pelvic node metastases and para-aortic node metastases (see table 18). The risk of pelvic node metastases is minimal in the absence of myometrial invasion and the risk appears to increase significantly as between a 1/3 to 1/2 of the myometrial thickness is invaded. Creasman (104) found that approximately 2/3 of those who develop node metastases will also have para-aortic metastases emphasising the limitation of pelvic treatment to cure patients with pelvic node metastases.

Unlike cervical cancer, tumour grade has major prognostic influence in endometrial cancer the incidence of pelvic node metastases in well differentiated, moderately differentiated and poorly differentiated tumours is 3%, 9% and 18% respectively and 2 %, 5% and 11%, respectively, will have para-aortic node metastases on presentation (104). The high incidence of para-aortic node metastases with pelvic node metastases emphasises the limited curative value of pelvic treatments for patients with nodal metastases.

VascularLymphatic Space invasion (VLSI)

VLSI occurs less frequently in endometrial cancer than cervical cancer being present in approximately 15% patients. It was associated with a 27% incidence of pelvic nodal metastases and a 19% incidence paraaortic nodal metastases in the GOG study (104). This compares with an incidence of 7% and 9% pelvic and para-aortic node metastases respectively when VLSI is absent. A similar increased risk of nodal metastases was seen when the cancer was sited in the fundus rather than the ismthmus-cervix and also when there was adnexal

involvement. VLSI has been found to be present in 26.5% of patients who recurred (104). Gal et al (105) in a series of 122 patients with stage 1 endometrial cancer found only 10 recurrences but 9/11 (82%) patients had recurrent disease. Thus VSLI appears to be a definite adverse prognostic factor in this disease.

Table 18. Prognosis stage and incidence of pelvic and paraortic node metastases in endometrial cancer

FIGO Stage (9)	Definition	Incidence of pelvic node metastases (104)	Incidence of para-aortic node metastase (104)	5 year survival
1	Confined to corpus uteri			72% overall
1A	Limited to endometrium	1%	1%	
1B	Invasion <50% myometrium	5-6%	1-3%	
1C	Invasion >50% myometrium	25%	17%	
2	Extension to :			56 % overall
2A	Endocervical glands	16%	14%	
2B	Cervical stroma (invasion)			
3	Extension to:			31.5%
3A	serosa/adnexa/ positive cytology	32%	20%	
3B	Vaginal metastases			
3C	Pelvic and para-artic nodes			
4	Distant metastases			0%

Peritoneal spread

Positive peritoneal cytology was demonstrated in 75/537 (14%) patients in the large GOG study (104) Positive peritoneal cytology was seen with a 27 % incidence of pelvic node metastases and a 19% incidence of para-aortic node metastases as compared to a incidence of 7% and 4% in patients with negative cytology. However, it was also seen in some patients where it was the sole adverse factor. In the GOG study 29% with recurrence recurred compared with 10.5% with negative cytology. However, not all these patients received treatment for their positive cytology. Similarly in another series of 145 endometrial cancer patients it was associated with recurrence in 33% of those with positive cytology as compared with 12.6% in those with negative cytology (106). It appears to be an adverse prognostic factor associated with adverse prognostic factors including deep myometrial invasion, high grade tumour extrauterine spread and lymph vascular invasion. In patients with positive cytology half died from disease 61.1% had extrauterine involvement at initial presentation (106). It was not found to be an independent prognostic factor (106). Positive peritoneal appears to reduce survival when there is extrauterine spread from endometrial cancer but does not

appear to affect survival when there is no extrauterine disease (106).

Cell type

Serous carcinoma of the uterus is an aggressive cancer which frequently presents with extra uterine metastasis and even with intrauterine presence peritoneal metastases in a pattern similar to ovarian cancer is common. It has been reported to occur 10.5% of endometrial cancer and like clear cell cancer (3.2%) is associated with a very poor survival (107). In a series of 401 women with endometrial cancer from the USA, the 5 year survival for endometriod, papillary serous and clear cell type was 69% 18% and 25% respectively (107). In this series 88% of serous and 77% of clear cell types were seen in black women and may have contributed to the significantly poorer overall survival for black patients (109). In this series, only stage and histology were independent risk factors with multivariate analysis. In a similar series of 372 women the same association between adverse cell type was found in black women. However, in this series multivariate analysis revealed stage and grade were independent factors and race was retained as an independent prognostic factor (108). It is possible that the poor prognosis in black women reflects socioeconomic circumstances resulting in late diagnosis as well as adverse biological factors. In an analysis of 181 patients with clear cell cancer treated in Norway 5 yr progression free survival was 43% (109) in this series age over 65 years was found to be an adverse prognostic factor.

Table 19. Prognostic factors in endometrial cancer

Risk of Recurrence from metastasis	Key Prognostic factors	% incidence of nodal metastases	5 year Survival
Very Low	Stage 1a G1-2/ no myometrial invasion	0%	95 -100%
Low	Stage 1b G1-2 myometrial invasion	7%-10%	80-85%
Medium	Stage I c G1-2 Stage 1a -c G3 or vascular invasion	upto 25%	60-70%
High risk	Stage 2a - 3 b 4a extrauterine spread serous/ clear cell type Positive cytology	16-31.5 %	31-56 %
Very high risk	4 b		0%

Management of Endometrial Cancer

Treatment by a site specialist multidisciplinary team according to guidelines appears to favour outcome in endometrial. A recent audit in SE England found that when surgery was not in accordance with guidelines, survival was shorter. Most patients are treated with total hysterectomy and bilateral salpingo-oophorectomy by non-specialist surgeons, surgery being the preferred option although radiation may be curative for patients who are not considered fit for surgery. At present it is unclear whether there are a group of patients at risk of

node metastases whose outcome maybe improved with lymphadenectomy. In a large series of 649 stage 1 endometrial cancers 212 had extensive lymph node sampling produced a highly statistically significant survival advantage for high risk disease involving the cervix uterine serosa or peritoneal washings (110). This important surgical question of whether node sampling increases survival, is currently being addressed in a large multicentre randomised trial (ASTEC).

Diagnosis and staging to determined which patients are at risk of pelvic node metastasis may be carried out pre-operatively with MRI to determine the degree myometrial invasion and to look for pelvic lymph node metastases. Hysteroscopic biopsy is taken to provide the histological diagnosis, tumour grade and cell type. Using these two parameters patients can be grouped prognostically as in table 19.

1. Very low risk

Where there is no mymetrial invasion as determined by MRI and the tumour is grade 1-2, the risk of pelvic node metastases is minimal These patients may be treated with hysterectomy and bilateral salpingoophorectomy and there is no need for radiotherapy or lymphadenectomy

2. Low risk

In this group the risk of lymph node metastases is low and there is no evidence that elective pelvic radiation prolongs survival although it may reduce the chance of pelvic recurrence. In a recent large series from Denmark the recurrence rate was only 7% (111) in this group when treated only with hysterectomy and bilateral salpingoophectomy.

3. Medium Risk

This group is at significant risk of pelvic recurrence and the question as to whether such patients benefit from elective lymphadenectomy and elective adjuvant pelvic radiation is being addressed in the ASTEC trial. There is no evidence in large randomised studies to support the use of adjuvant progestogens in this group (112).

4. High Risk Group

It is unclear to what extent this group at high risk of nodal metastases benefit from lymphadenectomy. However the results from the ASTEC trial will not be available for several years For the present they are treated with hysterectomy followed by routine adjuvant pelvic radiation. However, there is a need to improve the outcome of this group. Some studies using adjuvant chemotherapy containing usually cisplatin with an anthracycline have reported prolonged progression free survival in this high risk group of patients (113,114). Such adjuvant chemotherapy needs to be tested in a randomised controlled trial and this is the subject of a current EORTC study.

Stage 3 /4a patients may be curable with radical pelvic radiotherapy and it remains to be proven whether the cisplatin chemo/radiotherapy which has proved effective in controlled studies with advanced cervical cancer will be as effective in high risk endometrial cancer. Cisplatin chemotherapy is an approach worthy of testing in a controlled study in this group.

5. *Very High risk Group*

Once distant metastases have occurred, there is no curative treatment the response rate to progestogens to be poor (115). Patients with metastatic disease respond frequently to platinum based chemotherapy (106). Response may be prolonged and patients with a long progression free interval longer than 6 months after platinum may respond to rechallenge with platinum as in relapsed ovarian cancer. Prolonged complete

responses have been reported with paclitaxel and carboplatin (117). However the true value of taxanes in this disease is established at present.

MOLECULAR AND BIOLOGICAL MARKERS IN GYNAECOLOGICAL CANCER AND THEIR POTENTIAL CLINICAL VALUE

The use of traditional pathological criteria as described above, enables the clinician to define prognostic groups according to the risk of nodal metastasis and recurrence and 5 year survival which can help the clinician to plan optimum selective treatment for ovarian, cervical and endometrial cancer as discussed above. However the overall subjectivity of important pathological factors such as tumour grade has lead in the last decade to increasing exploration of more objective prognostic factors such as ploidy and automated measurement of nuclear dimensions. Also considerable new research has begun to increasingly define genetic events which influence tumour development and progression and molecular factors controlling the growth of metastasis. The challenge at present is to define the relevance this information to optimum clinical decision making and whether new targets for novel therapies can be defined from them.

Ploidy S-phase measurement

Nuclear DNA content as determined by flow cytometry has been shown to provide a reliable method of predicting survival and recurrence in ovarian and endometrial cancer. Flow cytometry also enables measurement of the degree of proliferative activity in the tumour with determination of the S-phase fraction. In a review of 12 studies in the literature including 1358 women largely with stage 3 and 4 disease median survival of aneuploid tumours ranged from 12-24 months compared with a range of 20-60 months with diploid tumours (16). There was a significantly better survival for patients with diploid tumours in advanced disease and in most of the studies reviewed ploidy was a powerful independent prognostic factor as determined by multivariate analysis. However, in advanced disease it is not clear whether this information would help clinical management. There is less data about its prognostic value in early ovarian cancer .In one small restrospective study, in stage 1/2 disease aneuploid patients had a 50% 5 year survival as compared with 80% for diploid tumours (117). It has been shown prognostic value in borderline tumours in some studies. In a large series of 321 borderline tumours 293 (91%) were diploid and of the 26 patients who died 17/26 (65%) had aneuploid tumours (118). However there are inconsistencies. In some studies of borderline tumours it was not of clear prognostic value (119), and the highly metastatic small cell cancer of the ovary associated with its very poor prognosis tumours are invariably diploid (120). In endometrial cancer DNA ploidy and measurement of the S-phase fraction has been shown to be a reliable prognostic factor independent of grade, cell type and oestrogen receptor status in some hands (121-122). Patients with elevated S-phase fraction (>5%) gained more benefit from adjuvant endocrine treatment (123). Some investigators have suggested that the S-phase fraction is not as reproducible as DNA ploidy (79). In cervical cancer ploidy status does not appear to have independent prognostic value and has not been shown to have clinical relevance (124).

Quantitative Morphometry

In order to overcome the subjectivity and lack of reproducibility of tumour grade assessment quantitative nuclear morphometry using automated systems has been proposed. Measuring the mean nuclear axis has been found to reproducible and of independent prognostic value in stage 1 endometrial cancer (123). In a series 77 stage 1 endometrial cancer a score (ECP1) incorporating myometrial invasion, DNA ploidy and mean shortest nuclear axis overshadowed other prognostic factors including grade when assessed by multivariate analysis. They were able to define a low risk group (ECP score < 0.87) with a survival of 97% and a high risk group ECP score > 0.87) where 84.6% died within 84.6%. This has led to a prospective trial of adjuvant therapy in the high risk group which will determine whether such prognostic assessment can lead to more optimum patient treatment and better outcome. Multivariate analysis has also shown that nuclear morphometry has highly significant independent prognostic significance in advanced ovarian cancer (124).

Cell proliferation markers

For many years pathologists have counted mitoses as a means of determining the cell proliferation rate of malignant tumours. To reduce the subjectivity of determining cell proliferation two proliferation antigens proliferating cell nuclear antigen /cyclin (PCNA) and Ki-67 have been studied. PCNA is expressed during GI and the early phase of S-phase of the cell cycle and Kl-67 is expressed during the G2 and mitotic part of the cycle. Ki 67 is believed to be the most reliable indicator of the tumour growth fraction but its use was limited by the necessity for using fresh snap frozen tumour (125). In endometrial cancer PCNA has been found to correlate with adverse factors myometrial invasion tumour grade and recurrence (126), whereas results with Ki 67 have been inconclusive. There has been no conclusive evidence of reliable independent prognostic influence in ovarian or cervical cancer studies being conflicting(127). However, more recently Ki 67 has been correlated with increasing grade in cervical neoplasia and apoptotic index (128). These data have suggested that progression of neoplasia in the cervix is accompanied by cellular deletion as well as cellular proliferation and therefore these parameters need to be considered together as an index to accurately relate to tumour progression (128).

Genetic Factors

Neoplasms have been shown to be heterogeneous for the metastatic phenotype, consisting of subpopulations with diverse biology (129). This cellular diversity has been attributed to inherent or acquired genetic instability during tumour progression. Selective pressures within the metastatic cascade may select for a dominant population leaving the population of greatest metastatic potential Such cellular biological diversity in tumours was demonstrated using the B16 murine tumour (130). Subpopulations of this same tumour have a differing genetic makeup and when cloned and reinjected into animals exhibited differing metastatic potential for pulmonary metastases (130).

Genetic instability

Cellular genetic instability is important in determining the susceptibility to carcinogenic events and once transformation has occurred in determining the biological behaviour of the tumour. Cells become more susceptible to such genetic

events with aging which has been related to the activity of the enzyme telomerase which controls the length of the chromosomal telomere (131) and may be relevant to genetic instability which is a feature of maligant cells. Shortening of the cell telomere length in each chromosome occurs with each cell division and eventually results in cell death. Telomerase activity has been found in a variety of malignant tumours with variable levels of activity but only rarely in benign tumours or normal tissues. High levels of activity have been found in 14/15 ovarian cancers but lower levels of activity was also detectable in 8/10 tumours of borderline malignancy and 4/11 cystadenomas (132). Similarly, in ovarian cancers telomerase activity tended to be higher in poorly differentiated tumours (133). Increased telomeric instability has been noted in ovarian surface epithelial cells from surface epithelial cells with a family history of ovarian cancer (134) and may relate to their increased susceptibility to carcinogenic events. These findings suggest that upregulation of telomerase activation is important during malignant tumour progression and is also important in determining susceptibility to carcinogenesis in ovarian cancer. Since more than 85% of cancers including gynaecological cancers express strong telomerase activity but normal adult tissues not telomerase may represent a target for specific T cell based immunotherapy.

Genetic alterations

Transformation of cell to the malignant state is a multistep process involving a series of genetic alterations for all cancers. For gynaecological cancers the precise steps are not known. However, a series of abnormalities including over expression, point mutation and gene rearrangement of protoncogenes and tumour suppressor genes have been described.

(a) Oncogenes

Abnormalities of protoncogenes whether over amplification or gene mutation may result in abnormalities of expression of gene products and consequent disturbed control of cell proliferation. The oncogenes abnormalities which have been most studied include c-myc k-ras and members of the type 1 growth factor family such as c-erbB2, c-erb B-3 and epidermal growth factor (135).

Over expression of c-erb B2 (HER-2/neu) which results in the over production of a growth factor HER-2 has been described in 20-30% of ovarian cancers. This is of particular interest because of its relevance to novel new treatments 10-20% of endometrial cancers (9) and in up to 40 % of cervical cancer of all cell types (138,139). However whilst its over-expression in breast cancer is well documented in ovarian and endometrial cancers its prognostic significance is unclear (137-138). In breast cancer recombinant humanised monoclonal anti-HER2/neu (Herceptin Genetech USA) has now been proven to be an effective treatment for metastatic breast cancer (140) and may ultimately prove relevant to all types of metastatic gynae cancer where there is over expression of HER-2/neu. The use of HER 2/neu antibodies have been demonstrated to reduce DNA repair following cisplatin treatment as shown by a reduction in DNA adducts leading to increased cell kill (141). A phase 2 study in patients with breast cancer overexpressing HER-2 /neu refractory to chemotherapy treatment found that the combination of anti-HER-2 antibody in combination with cisplatin increased response from 24% from 8% in those patients who recieved cisplatin

alone (142). This may have relevance to ovarian cancer, a tumour much more sensitive to platinum treatment than breast cancer. C-erb B2 is over expressed as determined by immunohistochemistry in between 11-88 % of endometrial cancers. Overexpression has been associated with traditional adverse prognostic factors including myometrial invasion, poorly differentiated grade and vascular space invasion (143). It has found with multivariate analysis to be an independent adverse prognostic factor in some series but not consistently (144,145). It is commonly overexpressed in the poor prognosis papillary serous adenocarcinoma (143). Similarly, EGFR overexpression has been related to poor prognosis endometrial (147) cancer but not consistently(147). Over expression of c-erb B2 in cervical cancer orccurs in about 40% and has proved a poor prognostic factor in in some series of stage lb-2a independent of nodal metastases(136).

Amplification of c-myc gene occurs in 10% of ovarian cancer and has been associated with later stage and poor grade (148) and has similarly been associated with poor survival in ovarian cancer(148). It is over expressed in 50% of cervical cancers and is associated with poor prognosis in early (149) But not late cervical cancers (150 k ras has been demonstrated in 20-40% of ovarian cancers and and 11-20% of endometrial cancers but has not been found to have prognostic significance to date to date (127). PIK3CA however, is an oncogene which is amplified in 40% of ovarian whose ocogene product p 111α has been implicated in tumour development, ras signalling, cell proliferation and suppression of apoptosis (152). Inhibition of the gene product PIK 3CA by a specific kinase inhibitor LY94002 has been shown to decrease cellular proliferation and induce apotosis and may represent a future therapeutic target in ovarian cancer (152).

(b) Tumour suppressor genes

Normal p53 otherwise known as wild-type p53, plays a a key role in cell cycle control and cell loss by apoptosis. Approximately 50 % of cancers can be shown to have a p53 abnormality, being the most common tumour suppressor gene abnormality in all human tumours.

Mutations in the p53 gene are common and the mutant p53 protein which has a long half life accumulates and becomes detectable immunohistochemically (153). However mutation of the p53 gene does not always result in over-expression of the mutant protein (154) and over-expression of the normal wild type p53 can also occur. Unlike other tumour suppressor genes that require the inactivation of both alleles to result in loss of function, a single p53 mutation has a dominant negative effect. The product of the gene mutant forms a complex with the wild type p53 gene product with consequent loss of its function (155).

p53 is mutated or overexpressed commonly in ovarian cancers particularly in advanced ovarian cancer (156,157) and loss of function may be due to a point mutation or allelic deletion. Despite its common occurrence in ovarian cancer its clinical prognostic significance remains unclear. Some studies have found that positive p53 immunostaining is an independent prognostic factor (157). whilst other studies have failed to demonstrate its prognostic value (158). The confusion maybe the result of methodological problems. A recent study of 105 frozen ovarian cancers differentiated between over expression of p53 as determined by immunohistochemistry and p53 mutations identified by single strand conformational polymorphism (SCCP) (159). They found positive immunohistochemical staining

for p53 in 72/105 (69%) ovarian cancers and confirmed mutation in 60/105 (57%) of these same tumours. There was a significant correlation between the two p53 parameters and tumour grade, stage and poor survival. The strongest predictor of outcome was a combination of the mutations and over expression rather than either parameter individually. They also noted the types of mutation related to survival.

In endometrial cancer p53 over-expression is uncommon (160) but is nearly always present in the poor prognosis papillary and clear cell type (161). In most studies however it has not emerged as an independent prognostic factor (162).

In cervical cancer p53 is involved in the carcinogenic process through a series of interactions with human papilloma virus. Accumulation of p53 plays no part in the progression from intraepthelial to invasive neoplasia and the commonly found p53 mutations have not prognostic significance (83).

Whilst the prognostic significance of p53 abnormality remains a matter of debate the frequency of abnormality makes it a potential target for gene therapy. Wild type p53 delivered using an adenovirus vector has been shown to inhibit the growth of subcutaneous ovarian xenograft tumours and produced regression in nude mouse models (163). This new approach is currently being tested in clinical trial in combination with platinum chemotherapy (164).

Approximately 90% of familial cancers result from mutations of BRCA1 or BRCA2 genes. These genes appear to be involved in in control of cellular proliferation and DNA repair (165). Cisplatin resistant cell lines have been found to have higher levels of BRCA1 protein and may be relevant to novel therapy as anti sense inhibition of BRCA1 produces increased sensitivity to platinum chemotherapy in cell lines (166).

Angiogenesis Factors

The growth of a primary tumour and its metastatic spread is critically dependant on the development of a tumour vascular network. In the prevascular phase of development tumour growth is normally limited and the risk of metastasis is low. At this stage the tumour may measure 0.2mm (166). In the absence of an developing blood supply in the prevascular state the tumour will not proliferate and may remain dormant. The vascular phase follows and is characterised by increased growth and metastatic spread. The tumour vascular network maybe partly acquired by incorporation of existing blood vessels. However, the majority of blood vessels are newly formed in a process of angiogenesis which maybe stimulated by nearly 20 proteins including vascular endothelial factor (VEGF) and basic fibroblast growth factor (FGF) which are produced by the majority of ovarian and other gynaecological cancers (167-169). Multivariate analysis reveals that serum VEGF is an independent prognostic factor in predicting outcome in ovarian cancer (170).

Research in tumour angiogenesis has lead to the testing of a large number of anti angiogenic agents in clinical trials (171). Of interest and of promise for these clinical studies is the demonstration that immature angiogenic blood vessels are preferentially sensitive to ablation with exposure to various anti-angiogenic agents including those which block the production and function of VEGF. These immature blood vessels are characterised as being α smooth muscle actin negative with antibody stains (α SMA) may be thus demonstrated histologically and distinguished from mature blood vessels

(172). In addition a number of molecular changes are associated with th endothelial cells of newly formed blood vessels including VEGFR, tie-2 /teK receptors and integrins and E selectin which make these vessels vulnerable to a variety of drug strategies (173). The induction of of tumour angiogenesis is not only the consequence of over expression of the receptors for angiogenic stimulants eg. VEGF but the result also of the loss of protein inhibitors of angiogenesis eg angiostatin and interferon α and β (174).

A disadvantage of some angiogenesis inhibitors is the rapid emergence of drug resistance since there are so many alternative stimulants to take its place However, the some kinds of anti-angiogenic agents eg endostatin may be administered chronically without the development of acquired drug resistance (175). A further finding in animal models is that chemotherapy drugs are more effective when given in combination with various anti angiogenic drugs (176). This appears to be due to increased drug delivery. It appears that some anti-angiogenic drugs have the ability by ablating immature blood vessels to convert a tortuous deformed blood supply to a more normal circulation (177). Anti-angiogenic agents will probably proved best administered in conjunction with combination chemotherapy. An ideal setting to test the value of these drugs would ultimately be in stage 3 patients with optimally debulked ovarian cancer in combination with platinum and paclitaxel based chemotherapy.

Adhesion and Invasion

The cascade of biological events which results in metastasis can be divided into three sequential processes (178):

(1) the attachment of the tumour cell to the extra cellular matrix which is facilitated by molecules, laminin and fibronectin. These large molecules normally are contained within the matrix and are by tumour cell receptors for these adhesion molecules. Laminin receptors are altered on cancer cells in number and distribution. Large numbers of receptors on cancer cells may be unoccupied and more widely distributed than the more usual polarised distribution on normal cells (179). Tumour cells exposed to laminin molecules in animal models tend to metastasise. Whilst in animal models fragments of laminin which cannot bind the tumour cell to the ECM will inhibit metastasis (180). Increased expression of laminin-5 a key adhesion protein molecule in epithelial invasive cancers. Recently, the presence of laminin-5 was found to be related to the invasive capacity of cervical cancers. Invasiveness of cervical lesions was positively associated with immunohistochemical staining of the gamma 2 chain of laminin-5 and may prove useful as a sensitive marker of early invasion (181).

The integrins are another family of surface glycoproteins which bind to adhesion proteins including fibronectin, fibrin type 1 collagen vitronectin. The integrin receptor which consists of a heterodimer of alpha and beta chains confers a degree of specificity.Integrin and E-cadherin cell adhesion molecules have altered expression in the process of invasion and metastasis. Abnormal expression of E-cadherin can be detected in cervical dysplastic epithelium but abnormal expression becomes diffuse in the majority of invasive cervical cancers and appears to reflect progression to the invasive process. However, since such disturbed expression is a feature of most invasive cervical cancers no correlation with prognosis in invasive cancer was found (182). Peptides of the Arg-Gly-Asp (RGD) group inhibit the functions of many integrins and consequently the

process of invasion and metastasis and may find a role as a novel new treatment.

(2) Proteolysis is necessary in the vicinity of the tumour cell in order to create a defect in the basement membrane and degrade and invade the underlying matrix. Key to this process are the matrix metatalloproteinases and the metallo-prteinase inhibitors TIMPs which oppose MMP activity. MMPs have an important physiological role in remodelling extra-cellular matrix eg. morphogenesis in embryo. They are categorised by cell type of orgin in e.g. Stromal MMP-2 MMP3, MMP11 inflammatory MMP-9 MMP-7

(3) Tumour cells need to migrate through the basement membrane and may respond to growth factors eg autocrine growth factors e.g. monocyte derived monokines eg TNF and and adhesion molecules eg.laminin and fibronectin. Blocking of TNF with antibodies may reduce metastasis peritoneal adherence invasiness and angiogenesis in ovarian cancer animal models (185).

Immune System

For many years it has been recognised that tumour infiltration with cytotoxic T cells is an important favourable prognostic factor for a variety of cancers. Recent research suggests that most immune response to cervical cancer may be relevant for new treatments with anti-cancer vaccines.

There is a strong association between human papilloma virus (HPV) and cervical cancer, particularly HPV types 16 and 18 have been demonstrated in approximately 93% of cervical cancers of all cell types (186). Continued expression of HPV oncogenic proteins E6 and E7 has been demonstrated to be required for the maintenance of the transformed phenotype (187) and this, together with its high frequency of occurrence, may make it an attractive and powerful "foreign" antigenic target for immunotherapy of cervical cancer (188).

For such anti-cancer immunotherapy to be successful, it is necessary to induce sufficient numbers of HPV antigen specific CTL to kill the tumour. Experimental animal studies have established the in vivo capacity of HPV antigen specific activated CTL to mediate rejection of HPV induced tumours (189). The generation of such activated CTL has been shown to critically require stimulation of the antigen specific CTL by 8 - 12 amino acid residue peptides, which are derived from proteins intracellularly in the proteosomes and subsequently selected to bind to their appropriate surface MHC class 1 allele specific molecules (190). Activation of CTL also requires co-stimulatory molecules such as B7.1/2 (191). This combination of stimulatory signals appears to be most powerfully exhibited in man and animals by bone marrow derived dendritic cells (DC) which are highly specialised antigen presentation cells uniquely capable of priming naive T-cells in vivo and vitro (192).

The presence of HPV specific CTL capable of lysing not only target cells infected with recombinants expressing E6 and E7 but also tumour cell lines transformed with HPV has been recently demonstrated in man in the PBMC of patients with CIN3 (193) using HPV 16 E7 peptides that bind to HLA A * 0201. In this study, CTL were detected in the PBMC of all HLA*A0201 cervical cancer subjects but not in normal subjects (193). HPV specific CTL have also been demonstrated at the site of disease and draining lymph nodes and when quantitated by limiting dilution analysis such HPV specific CTL have been shown to accumulate at the tumour site (194).

Tumour specific CTL may be expanded by immunisation with a tumour antigen processed and presented via the

MHC class I pathway. Borysiewicz and colleagues (195) have shown vaccination with live vaccinia virus engineered to express the HPV 16 and 18 E6/E7 antigens (TA-HPV), to result in HPV specific CTL response in one in three evaluable patients with advanced cervical cancer. In a more recent study, HPV specific CTL have also been demonstrated in a high proportion of CIN3 volunteers vaccinated intradermally with TA-HPV (196). The approach may be less effective in advanced cancer as a result of cancer induced immunosuppressive influences, the severity of which appears to increase with increasing volume of cancer (197). For such cases, the adoptive transfer of ex vivo tumour antigen pulsed autologous APCs in the form of dendritic cells may prove to be a more powerful means of expanding tumour specific CTL and a means of overcoming cancer associated defective antigen presentation as has been demonstrated in cancer bearing animals (199). Tumour antigen peptide primed DC can produce significant antigen specific anti-tumour effect against established tumours, the effect being greatest for fractionated treatment (199). In one tumour model, the murine C3 sarcoma, which presents HPV 16 E7 antigen, treatment of animals bearing established macroscopic tumour (up to $1cm^2$) with DC pulsed with HPV 16 E7 peptide resulted in sustained complete eradication of tumour masses in 80% of mice (198). This approach has consequently been suggested as a tumour specific anti-cancer vaccine in humans (200).

A reliable method for producing large numbers of DC from peripheral blood mononuclear cells (PBMC) has recently become available (201) and the method has been successfully used to generate bone marrow derived DC with similar yield, morphological, immunohisto-chemical and FACS characteristics from normal and cervical cancer patients (202). MHC class 1 restricted CTL response have been induced in vitro against autologous immortalised B cells expressing a synthetic HPV 16 E7 peptide which was used to prime DC derived from peripheral blood progenitors of normal HLA-A2 volunteers and tumour bearing individuals. Similar responses have been observed with synthetic flu M1 and HER-2 peptides. These data suggest that autologous DC prepared from peripheral blood progenitors can expand antigen specific T-cells sufficiently to be able to demonstrate a specific CTL response to a synthetic HPV 16 E7 peptide. However, in the clinical situation, pulsing DC with peptides poses practical problems because of the strict MHC class I restriction of the response. Also, studies with synthetic peptides based on the predicted binding motif of E6 and E7 have shown that these peptides are not recognised by human CTLs known to be directed to E6 and E7 (203). One practical solution may be to load HPV antigens using an autologous tumour lysate which has been shown to be positive for the gene products of HPV with PCR analysis. Autologous dendritic cells primed with melanoma tumour lysate have recently been administered directly into regional lymph nodes in patients with advanced malignant melanoma with no evidence of autoimmune side-effects and minimal toxicity (204). In this study, objective response was observed in 5/16 evaluable patients (two complete responses, three partial responses) with regression of metastases in various organs including skin, soft tissue, lung and pancreas, following vaccination with DC antigen pulsed with tumour lysate or haplotype specific tumour antigen peptides. Both patients with a complete response are still disease free at 15+ months. In addition, 2/4 patients treated with lysate primed DCs as compared with 1/6 and 2/6 with HLA1 or HLA2 peptides pulsed DC induced anti-tumour responses.

It was noted that 4/5 responders showed significant DTH reactivity with DC-tumour lysate or DC-peptide (>10mm in diameter on skin testing) (204). There is therefore a rationale for testing this approach with other tumours including cervical cancer.

CONCLUSION

It is likely that metastasis will remain the leading cause of treatment failure and death from gynaecological cancer for the forseeable future. Key to immediate improvement in outcome is to ensure that patients receive optimum conventional management from site specialist multi-disciplinary teams working on Calman-Hine principles. Such treatment being tailored to patient needs with the use of relevant prognostic factors. Improved conventional treatments including cisplatin, chemoradiation for appropriate patients with cervical cancer and taxane/platinum based combination chemotherapy in advanced ovarian cancer is likely to lead to improved outcome. Ovarian cancer with its advanced stage of presentation is likely to remain the most lethal malignancy but may be one of the first tumour sites to benefit from the use of chemotherapy combined with novel new treatments e.g. anti-angiogenesis therapy. It remains to be seen whether cervical cancer patients at high risk of relapse from metastasis will benefit from novel specific anti-cancer immunotherapy with HPV as the target.

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