Basic and Clinical Aspects of Malignant Melanoma

Cancer Treatment and Research

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Basic and Clinical Aspects of Malignant Melanoma

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To Thomas Christopher Hall, M.D., who taught me most of what I know about oncology and first introduced me to the melanocyte and its fascinating biology.

Contents

Cancer Treatment and Research

Foreword

Where do you begin to look for a recent, authoritative article on the diagnosis or management of particular malignancy? The few general oncology textbooks are generally out of date. Single papers in specialized journals are informative but seldom comprehensive; these are more often preliminary reports on a very limited number of patients. Certain general journals frequently publish good in-depth reviews of cancer topics, and published symposium lectures are often the best overviews available. Unfortunately, these reviews and supplements appear sporadically, and the reader can never be sure when a topic of special interest will be covered.

Cancer Treatment and Research is a series of authoritative volumes which aim to meet this need. It is an attempt to establish a critical mass of oncology literature covering virtually all oncology topics, revised frequently to keep the coverage up to date, easily available on a single library shelf or by a single personal subscription.

We have approached the problem in the following fashion. First, by dividing the oncology literature into specific subdividions such as lung cancer, genitourinary cancer, pediatric oncology, etc. Second, by asking eminent authorities in each of these areas to edit a volume on the specific topic on an annual or biannual basis. Each topic and tumor type is covered in a volume appearing frequently and predictably, discussing current diagnosis, staging, markers, all forms of treatment modalities, basic biology, and more.

In Cancer Treatment and Research, we have an outstanding group of editors, each having made a major commitment to bring to this new series the very best literature in his or her field. Martinus Nijhoff Publishers has made an equally major commitment to the rapid publication of high quality books, and worldwide distribution.

Where can you go to find quickly a recent authoritative article on any major oncology problem? We hope that Cancer Treatment and Research provides an answer.

WILLIAM L. MCGUIRE
Series Editor

Preface

Basic and Clinical Aspects of Malignant Melanoma continues a series of monographs on malignant melanoma ably initiated by John J. Costanzi. Its goal is to put in one volume the latest research developments in this fascinating and perplexing disease, whether they be in basic science or clinical investigations.

Such a goal can only be accomplished by calling on multiple scientific disciplines, and this volume includes studies in somatic cell biology, molecular genetics, immunology, epidemiology, pharmacology, psychiatry, endocrinology, pathology, clinical trials, and even neutron radiotherapy. It is hoped that the collection of such diverse disciplines can result in cross-fertilization among them and the growth of new and more creative scientific concepts and research approaches.

The book has been divided into three major sections. The first introduces the reader to some new studies in the basic biology of melanoma. A.P. Albino starts off by reviewing work in his and other laboratories on the role of oncogenes in the pathogenesis of premalignant and malignant pigmented lesions. In the second chapter, the precise cellular pathogenesis of metastases in melanoma is explored by Dr. V.P. Terranova. Finally, in this section, Drs. J.M. Rosenberg and D.A. Cheresh give us insight into the role and importance of ganglioside antigens in the cell membrane of tumor cells derived from the neuroectoderm, including melanoma.

The second section of the book is devoted to the natural history of melanoma. M.C. Leske has a fascinating review of the role that demographic, geographic, and time variables, as well as host, familial, and environmental factors, may play in the development of ocular melanoma. Next, T.J. Harrist describes the pathologic factors which seem to be most important in the prognosis of human melanoma. L. Nathanson then describes clinical and biological factors in human melanoma that suggest endocrine influence, or control, of the growth of that disease. R.J. DiClemente and L. Temoshok ably address this question: 'Do psychological factors have any relationship to pathology, symptoms, tumor host response, prognosis, or ability to cope of patients with malignant melanoma?' A major cause of failure of conventional therapy for melanoma is relapse in the central nervous system. W.A. Robinson,

K. Jobe, and R. Stevens examine the patterns and potential therapy of this distressing complication in the natural history of malignant melanoma.

The third section of this volume deals with treatment of melanoma. E.T. Creagan reviews results of trials of interferon in melanoma, both from the point of view of prognostic factors such as interferon receptors and clonogenic assays for interferon activity, as well as pharmacology and therapeutic benefits achieved with this novel and interesting family of glycoproteins. Melanoma has long been known to be a relatively radio-resistant disease when treated with conventional photon radiotherapy. Melanoma cells are capable of repair of photon-induced radiation damage and protected by virtue of hypoxia. These factors may be overcome by neutrons as demonstrated by the high response rate reported by P.R. Blake in the final chapter.

In summary, it is hoped that this volume will bring together new data, both from the basic science laboratory and the clinical investigator, and will be of real usefulness to the 'melanoma maven' no matter what his or her scientific background or discipline of study may be.

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Larry Nathanson
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I

Basic Biology

1. The Role of Oncogenes in the Pathogenesis of Malignant Melanoma

Anthony P. Albino

1. Introduction

The title of this chapter, 'The Role of Oncogenes in the Pathogenesis of Malignant Melanoma,' intimates knowledge of a specific action of an oncogene(s) which is directly involved in the development of melanoma; none is yet known. As of this writing, the genetic changes that lead to malignant transformation in this disease are unknown. Moreover, there is, as yet, no direct evidence that the action of any oncogene is responsible for the initiation or maintenance of any human cancer. Yet it is a widely held belief that such evidence is not only forthcoming, but the outlines of this evidence are already discernible. Current dogma maintains that proto-oncogenes, the normal unmodified gene counterparts of oncogenes, play complex but fundamental roles in the control of normal cellular growth and in other functions unrelated to proliferation. The evidence for this is multifaceted and has been extensively reported. Oncogenes, being permuted variants of proto-oncogenes, are involved, therefore, in perturbing the normal mechanisms in which these genes are involved, with the end result being neoplasia. In this chapter we will discuss the relatively limited amount of available information on the status of both proto-oncogenes and oncogenes in malignant melanoma and in premalignant and normal prescursor tissues. Further, we will discuss the interrelatedness of melanocyte-melanoma cellular differentiation programs and the process of malignant transformation. Finally, we will discuss the ongoing experiments designed to determine the steps required for the transformation of the human melanocyte and to test the concept that multiple, cooperating, independently activated oncogenes are involved in the evolution of malignant melanoma.

2. Status of oncogenes and proto-oncogenes in melanoma

2.1. ras *oncogenes and malignant melanoma*

The suggestion that genetic sequences from normal cells could induce oncogenic transformation was first indicated in experiments showing that DNA fragments

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of normal avian and murine cells transformed NIH/3T3 cells with low efficiencies [1–4]. The presence of similar transforming genes in neoplastic cells was demonstrated by the finding that DNA from chemically transformed mouse cultures also transformed NIH/3T3 cells [3, 5]. Subsequent to these experiments it was shown by a number of laboratories that the DNAs of a proportion of noncultured and cultured human tumors have genes similarly capable of transforming NIH/3T3 cells [4, 6–11]. These abnormal transforming genes, or oncogenes, were shown to be permuted versions of normal proto-oncogenes [for reviews see 4, 7, 8, 10, 11]. To date, a number of mechanisms have been shown to be involved in 'activating' the oncogenic potential of proto-oncogenes, including mutation [12, 13], amplification [14–16], rearrangement of the encoding gene sequences and/or regulatory elements [11, 17–19], promotor insertion [20], deletion [21, 22], and enhanced transcription [23, 24]. Delineating the effects of oncogenes is acknowledged as being pivotal to the understanding of the malignant process.

Our laboratory has maintained a long-standing interest in malignant melanoma [25–28], and it was a logical extension of this interest to turn our attention to oncogenes and their relationship to melanoma. Melanoma provides a valuable model with which to study stage specific events; e.g., the possible activation of cellular oncogenes — since this disease evolves in a stepwise manner — with each step, usually, being clinically detectable. Our initial experiments utilized the NIH/3T3 transformation assay [4]. NIH/3T3 cells are aneuploid mouse cells which exhibit a contact-inhibited flat morphology in culture and which, apparently, have undergone an unknown series of pre-malignant changes, leaving the cell poised to undergo malignant transformation upon addition of at least one activated oncogene. This assay is a powerful one in that it allows a functional and structural analysis of transforming genes from the DNAs of different species, but it has several important limitations: (1) NIH/3T3 cells will only detect dominantly acting transforming genes and not recessive genetic elements, (2) NIH/3T3 cells will only detect a small fraction of a much larger set of oncogenes (with a predilection for *ras* family oncogenes) and then only with certain permutations in the gene structure, (3) the assay will only detect genes necessary for late stages in the transformation process, (4) the NIH/3T3 cell is not a normal diploid cell and, thus, not a true analogue of the *in vivo* state of a cell prior to preneoplastic or neoplastic conversion, and (5) rodent cells are less refractory to transformation (by viral, chemical, and spontaneous modes) than human cells. However, these limitations notwithstanding, the assay is an extremely useful one. When high molecular weight DNA derived from cultured malignant melanomas was applied to NIH/3T3 cells in the form of a calcium-phosphate precipitate, we discovered that malignant melanomas, like most other tumor types, contain transforming genes within their DNA [29]. Table 1 summarizes our initial efforts in the search of activated oncogenes in cultured melanomas. DNA from 4 of 34 melanoma lines yielded transforming oncogenes in the NIH/3T3 assay as evidenced by the formation of highly refractile foci of 3T3 cells. The efficiency of

Table 1. Transfection of melanoma DNA

Cell type	Efficiency of primary transfection — FFU/μg	Tumorigenicity of transfectants
SK-MEL-23, 28, 30, 37, 44,	<0.003	0
47, 64, 65, 75, 100, 113,	<0.003	0
118, 122, 129, 131, 143,	<0.003	0
153, 164, 166, 167, 169,	<0.003	0
175, 176, 180, MeWo	<0.003	0
SK-MEL-119	0.04, 0.068, 0.1	5/5
SK-MEL-146	0.04, 0.072, 0.1	8/8
SK-MEL-147	0.001, 0.012	3/3
SK-MEL-93 DX-3	0.01, 0.04	8/8
SK-MEL-93 DX-1	<0.003	0/3
SK-MEL-93 DX-2	<0.003	0/3
SK-MEL-93 DX-4	<0.003	0/3
SK-MEL-93 DX-5	<0.003	0/3
SK-MEL-93 DX-6	<0.003	0/3
DX Skin fibroblasts	<0.003	0/3
DX EBV-transformed B cells	<0.003	0/3

High molecular weight DNA (> 50 kB) was extracted from cultured melanoma cells and transfected into NIH/3T3 cells as described [29]. After two to three weeks, foci of transformed NIH/3T3 cells were isolated and grown to mass culture. Tumorigenicity of transformed cells was determined by injecting $1-2 \times 10^6$ cells subcutaneously into four to six week old NFS mice. Tumors were usually detected after one to two weeks. In the case of cultures scored negative for transformed foci, the cells were trypsinized, grown to mass culture, and injected into weanling NFS mice (5×10^6/mouse) to determine the presence of any transformed cells not identifiable microscopically. These animals were observed for at least eight weeks.

primary transfection from the positive cell lines ranged from 0.01–0.1 focus forming units/μg of DNA. This frequency is similar to that seen with the DNA from other solid tumors [8]. The mouse 3T3 cells now harboring the transfected melanoma oncogenes could produce rapidly growing solid tumors upon injection into syngeneic or athymic mice indicating the complete transformation of the recipient cells. Table 1 also a summarizes the transfection experiments with DNA extracted from six cell lines derived from six separate metastatic deposits of a single patient (DX [30]). DNA from only one of these lines, DX-3, contained a detectable activated oncogene. Normal tissues from this patient did not have a similarly activated oncogene, indicating a somatic mutation in the tumor and not a germ-line event.

This initial study extended the list of human tumor types that yielded transforming DNA to malignant melanoma. As in most other tumor types, the transforming genes from melanomas identified by the NIH/3T3 assay were found to be members of the *ras* family [2, 4, 6–8]. As determined by southern blotting experiments, two types of *ras* genes were isolated from the four melanoma lines testing positive in this assay: one Ha-*ras* allele and three N-*ras* alleles. The human *ras* proto-oncogene family consists of at least five distinct members, each with a different chromosomal location. These are: N-*ras*,

chromosome 1 [31, 32]; c-Ha-*ras*-1, chromosome 11 [33]; c-Ha-*ras*-2, X chromosome [33]; c-Ki-*ras*-1, chromosome 6 [33]; and c-Ki-*ras*-2, chromosome 12 [33]. These human *ras* genes are closely related to the transforming genes of Harvey and Kirsten murine sarcoma viruses [34]. Both the human and viral *ras* genes encode serologically and structurally related p21 proteins [35–37]. In transfection studies with NIH/3T3 cells, only three of the five human *ras* genes are biologically active: N-*ras*, c-Ha-*ras*-1, and c-Ki-*ras*-2. Other groups have also detected activated oncogenes in melanoma tissues. Padua and coworkers found activated oncogenes in two melanoma cell lines [38]. One was identified as an N-*ras* oncogene, and the second is, as yet, undefined but may be a novel oncogene localized to human chromosome 19 [R.A. Padua, personal communication]. Sekiya and coworkers determined that a melanoma cell, SK-2, contained an activated Ha-*ras* oncogene [39]. The frequency of oncogene activation in cultured lines of metastatic melanoma using the NIH/3T3 assay is approximately 13%, a value not significantly different from the 10–20% frequency observed in other solid tumors by a number of groups. What is the significance of this value? Does it mean that *ras* is involved in the pathogenesis of only a subset of melanomas (and by analogy other human cancers) or could *ras* oncogenes have a more general role in melanoma requiring approaches other than the NIH/3T3 assay to reveal? Another way to interpret the variability in *ras* gene activation in melanoma is that transforming *ras* genes are not involved in the origin and maintenance of this human cancer, but arise as a consequence of the genetic instability of cancer cells and represent a manifestation of the high mutational rates of cancer cells. The finding that only one of six cell lines originating from the same patient had an activated N-*ras* allele bears on this point. The six lines have been shown by Dr. Nicholas Dracopoli of Sloan-Kettering Institute, using restriction site polymorphisms identified by a series of X-chromosome specific molecular probes, to be monoclonal in origin [40]. Thus, it would be predicted that all six metastases, being clones of the parent neoplasm, should also have an activated N-*ras* oncogene; however, the available evidence is that they do not. The simplest interpretation of these results, therefore, is as follows: transforming *ras* gene activation occurred relatively late in the course of tumor progression in the DX-3 line and was not involved in tumor initiation, maintenance, or metastasis in this patient. According to this view, then, *ras* activation is a manifestation of tumor heterogeneity: a result rather than a cause of transformation.

There are other lines of evidence that support the conclusion that activation of *ras* oncogenes are, in general, a relatively unimportant event in the evolution of melanoma. These are (1) If *ras* gene activation were an early event, it should be detectable in the precursor lesions of this disease; i.e., in dysplastic nevi and primary melanomas. Table 2 shows the results of our search for activated oncogenes in these tissues. No specimen of precursor tissue, either cultured or noncultured, manifested an active form of a *ras* oncogene detectable by the NIH/3T3 assay. Table 2 also shows that activated oncogenes were not detected in a series of noncultured metastatic melanomas. The inability to rescue

Table 2. Search for activated oncogenes in DNA from related tissues

Cell or tissue type	Number of specimens	Oncogene activation	Efficiency of primary transfection — FFU/μg
Noncultured metastatic melanoma	6	No	<0.003
Noncultured primary melanoma	3	No	<0.003
Noncultured dysplastic nevus	1	No	<0.003
Cultured melanocytes	2	No	<0.003
Cultured primary melanoma	2	No	<0.003
Cultured metastatic melanoma	34	Yes (12%)	0.01–0.1

High molecular weight DNAs isolated from the tissues and cells listed were assayed for transforming oncogenes by the NIH/3T3 assay as described in Table 1.

activated *ras* oncogenes from noncultured primary and metastatic melanomas was confirmed in a study carried out by Dr. Marco Pierotti of the National Tumor Institute in Milan. These kinds of studies indicate that *ras* gene activation is, apparently, a rare event even in metastatic melanomas; (2) This latter conclusion is based on biological studies using the NIH/3T3 assay to identify activated *ras* oncogenes. However, this interpretation has been confirmed, in part, using a molecular genetics approach. The proto-*ras* genes in human tumors have been shown to be activated by point mutations mainly affecting two major sites [41] in the first (i.e., at amino acid 12) [42, 43] and second (i.e., at amino acid 61) [44, 45] coding exons. The activating mutations result in amino acid substitutions at these sites which distinguish normal from mutant *ras* gene alleles. These amino acid substitutions can be detected at the molecular level by direct sequence analysis, by using synthetic oligonucleotide probes to the mutated and normal codons, and by determining site-specific restriction endonuclease polymorphisms. All three approaches have been used to map the mutant *ras* genes found in melanoma DNA. To date, in the two cases of reported c-Ha-*ras* activation and in one out of the four cases of N-*ras* activation, the mutation has been localized to the second exon at position 61 of the p21 protein [46, 47]. It remains to be seen if the three other cases of N-*ras* activation in melanoma have a mutation at the same position. In independent unpublished studies in this laboratory and that of Dr. Pierotti, it was found that, as in most epithelial cancers [48], mutations affecting the 12th amino acid (in the first exon) of the c-Ha-*ras* oncogene products occur relatively infrequently in melanoma cells; (3) The c-Ha-*ras* locus in humans is known to be polymorphic, and certain rare allelic restriction fragments of this gene have recently been reported in the DNAs from patients with melanoma and other tumor types [22]. The inheritance of these rare c-Ha-*ras* alleles was concluded to be linked to cancer susceptibility. However, in an independent unpublished analysis of a large panel of matched normal and melanoma DNAs from individual patients treated at the Memorial Sloan-Kettering Cancer Center (MSKCC), no specific alleles were found to be of significance in melanoma [N.C. Dracopoli, personal communication]. This study of Dr. Dracopoli

showed that the frequency of somatically induced hemizygosity at the Ha-*ras* locus in melanomas was no higher than that seen with a large number of other alleles on many different chromosomes in melanoma cells. In a similar type of study, Thein and colleagues found that the distribution of Ha-*ras* alleles in patients with myelodysplasia (a common hematological malignancy previously reported to have a high frequency of rare Ha-*ras* alleles) was not significantly different from that seen in normal individuals [49]; (4) Finally, in studies by Greene and his colleagues, segregation and genetic analysis of a large series of melanomaprone kindreds found no linkage between the c-Ha-*ras*-1 gene and a putative melanoma specific gene on chromosome 11 [50, 51]. However, the linkage data did suggest the presence of a melanoma susceptibility gene loosely linked to the Rh blood group locus on the short arm of chromosome 1. Nonrandom abnormalities of chromosome 1 have been observed in melanoma cells [52, 53], and, therefore, it is speculated that this chromosome may play a role in the pathogenesis of melanoma. It is noteworthy that the N-*ras* proto-oncogene is also located on the short arm of chromosome 1, although not in close proximity to the putative susceptibility gene [54]. Thus, it is possible that N-*ras* oncogenes may have undergone recessive mutations which, in conjunction with nonrandom hemizygosity/homozygosity, play a role in the pathogenesis of melanoma. (The effects of recessive alleles are thought to play a crucial role in the evolution of both Whilms' tumors and retinoblastomas [55].) However, there is, as yet, no evidence of a nonrandom hemizygosity/homozygosity for the N-*ras* proto-oncogene in melanomas, nor any evidence that *ras* proto-oncogenes contain recessive mutations. It has been found that, at least for the c-Ha-*ras*-1 gene, all amino acids except glycine (which is encoded by the normal proto-Ha-ras gene) and proline at position 12, activate the transforming potential of p21, and that each of these activating mutations is dominant [56].

In summary, then, until more evidence accumulates, we are left with a minimalistic role for *ras* oncogenes in the pathogenesis of melanoma. Expanding our search we next asked if proto-oncogenes other than those of the *ras* gene family are involved in the etiology of this disease.

2.2. *Non*-ras *proto-oncogenes and malignant melanoma*

We analyzed the genomic organization of known proto-oncogenes in a large series of noncultured and cultured metastatic melanomas, several primary melanomas, and one specimen of a dysplastic nevus, to determine if any of these genes are rearranged or amplified in the DNA of these tissues. Our objective was to determine if consistent alterations in proto-oncogene encoding DNA could be detected in melanoma, and whether patterns of proto-oncogene perturbations correlate with differentiation markers that subset melanomas (see below). Table 3 summarizes the results of a southern blot analysis designed to detect rearrangements or amplifications in the DNA of melanoma tissues and precursor tissues. The organization (i.e., the restriction fragment pattern) of proto-

Table 3. Genetic organization of proto-oncogenes in melanoma

Proto-oncogenes analyzed:			
Ki-*ras*	*mos*	*raf*	*rel*
Ha-*ras*	*sis*	*src*	*p53*
N-*ras*	*fos*	*ski*	
myc	*fes*	*erb A + B*	
N-*myc*	*abl*	*myb*	

Tissues and cell lines analyzed:

1) Cultured cells
   Metastatic melanoma:
   6 lines with early differentiation characteristics
   12 lines with intermediate differentiation characteristics
   10 lines with late differentiation characteristics

   Primary melanoma:
   3 lines
2) Non-cultured tissues
   Metastatic melanoma:
   6 specimens

   Primary melanoma:
   2 specimens
3) Non-cultured normal tissue
   Dysplastic nevus:
   1 specimen

Genomic organization of proto-oncogenes in the DNAs from melanomas and related tissues was analyzed by southern blot hybridization as described [29], using the oncogene specific probes listed.

oncogenes in melanoma tissues was compared to that seen in normal cultured melanocytes, cultured B-cells from patients with melanoma, and from noncultured normal kidney tissue. DNAs from these tissues, digested with one of several restriction endonucleases, were analyzed with 17 different oncogene specific probes. While more specimens of premalignant, primary, and metastatic melanomas have to be studied, this initial study showed that, when compared to normal DNA, none of 17 proto-oncogenes analyzed were rearranged or amplified in any significant or conclusive way at the DNA level in a spectrum of disease states associated with melanoma. However, minor alterations were detected in two proto-oncogenes: c-*myb* and c-*myc*. We detected a restriction fragment length polymorphism in the c-*myb* locus using DNAs extracted from cultured metastatic melanomas. When digested with Eco-R1 endonuclease, two different restriction fragment patterns were seen; the first consisted of three bands of 2.8, 2.6, and 2.0 kB detectable in approximately 70% of melanoma DNAs, and the second consisted of four bands of 2.8, 2.0, 1.5, and 1.0 kB detectable in the remaining 30%. The significance of this polymorphism, i.e., whether these are random, natural polymorphic gene differences in the c-*myb* locus or nonrandom alterations related to the disease state, awaits further analysis with DNA from matched normal tissues and from noncultured melanoma tissues. However, it is important to note that a large study of human solid tumors (excluding melanomas) and normal tissues by Yokota and colleagues revealed a possible genetic instability of the c-*myb* locus [23]. They

found similar restriction fragment patterns in the *myb* gene (including a third pattern consisting of three bands of 2.8, 1.55, and 1.05 kB which we did not find in melanomas) and speculated that the alterations in this gene may result from a loss of one allele, or a mutation at an internal Eco-R1 restriction site in the 2.6 kB fragment and may occur concomitantly with either tumor progression or metastasis. Other studies have reported that the c-*myb* proto-oncogene is differentially expressed in a proportion of human lung tumors [57].

In addition to the possibly specific alterations in the c-*myb* proto-oncogene, we have also noted that the DNAs from a small proportion of cultured melanomas (approximately 10–15%) appear to have amplified the c-*myc* gene. The level of amplification is small, approximately 1.5–3 times normal, and its significance awaits a more detailed study of noncultured melanomas to determine if this alteration in the c-*myc* gene is simply due to events generated *in vitro* or correlate with specific clinical stages of the disease. Rearranged or amplified forms of c-*myc* are thought to play a role in carcinogenesis, although precisely how is not known [19, 20].

A number of studies have observed nonrandom cytogenetic changes on chromosomes 1, 6, and 7 in the DNA from noncultured and cultured melanoma specimens [52, 53, 54, 58, 59]. Chromosome 1 is the location of at least four proto-oncogenes: *src*, L-*myc*, N-*ras*, and *ski*; Chromosome 6 contains at least three proto-oncogenes: K-*ras*-1, *myb*, and *yes*-2; Chromosome 7 harbors one known proto-oncogene: *erb-B*. While we have not yet analyzed the genomic organization of the L-*myc* or *yes*-2 proto-oncogenes, it seems likely from our data that none of the specific chromosomal abnormalities on these chromosomes involve major rearrangements or amplifications of *src*, N-*ras*, *ski*, or *erb-B* proto-oncogenes. The two structures which represent amplified DNA sequences, homogeneously staining regions (HSRs) of centromeric chromosomes and double minute (DMs) chromosomes which lack centromeres, are rarely detected in melanoma DNAs [60, 61]. When HSRs have been found in melanoma DNA there has been no concordant amplification of any known proto-oncogene [62].

Consequently, with the limited data available to date, it can be concluded that, outside of the exceptions noted above, there are no obvious or consistently detectable abnormalities which could stimulate qualitative or quantitative changes in the expression of a wide range of proto-oncogenes in melanomas. If there are such abnormalities, the changes involved are presumably subtle and, therefore, will necessitate a more detailed study to ascertain.

3. Oncogenes and differentiation

Temporal changes in proto-oncogene mRNA transcripts are manifest during many cellular functions, including tissue development, regeneration, differentiation, and cell-cycle [37, 63, 64]. Similar changes have been seen in some types of

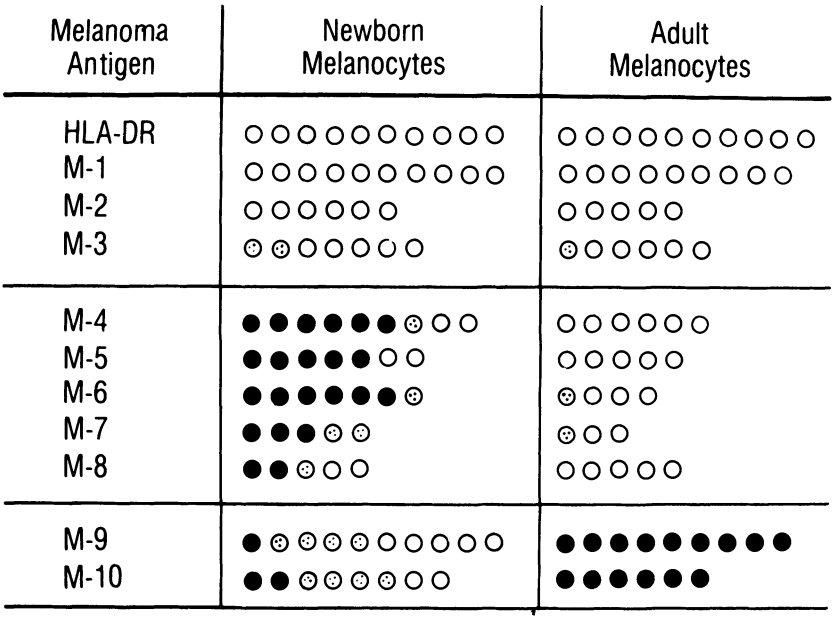

Figure 1. Serological typing of newborn and adult melanocytes for melanoma cell surface antigens: Each circle represents an individual test, and each test for a particular antigen was performed with melanocytes from a different individual. Black circles represent mouse monoclonal antibody titers $1:10^4$ to $1:10^7$, stippled circles 1:500 to 1:5,000, and open circles $< 1:250$. All antigens depicted are distinct and unrelated. HLA-DR, M-1, M-2, and M-3 antigens are early melanocyte markers, M-4 to M-8 are intermediate melanocyte markers, and M-9 and M-10 are late melanocyte markers. Adapted from Houghton et al. [67].

preneoplastic and malignant tissues and may be correlated with clinical stages of malignancy [24, 65]. However, marked heterogeneity, both qualitative and quantitative, in the expression of these mRNAs has been noted, and, therefore, it has been difficult to evaluate whether the presence of these transcripts are directly related to the malignant state or to specific differences in differentiation. Analysis of proto-oncogene specific mRNA species from tumor cells subsetted as to the differentiation stage may be more interpretable. In this regard, melanomas are a valuable model system. A large number of the over 200 melanoma cell lines established at MSKCC can be grouped into three general differentiation groups corresponding to early, intermediate, or late stages in the melanocyte developmental pathway. This grouping has been possible due to the use of a large panel of monoclonal antibodies (mAbs) which detect a wide spectrum of antigenic specificities on human melanoma cells [25, 66]. Many of these antibodies detect distinct surface antigens expressed by melanoma cells, either by all melanoma cells or by large or small subsets of melanomas [66, 67]. This diversity of melanoma phenotypes apparently reflects a corresponding diversity in the surface phenotype of normal cells undergoing melanocyte differentiation. Direct support for this idea is based on the results of serological tests with newborn and adult melanocytes [67]. Figure 1 summarizes this data.

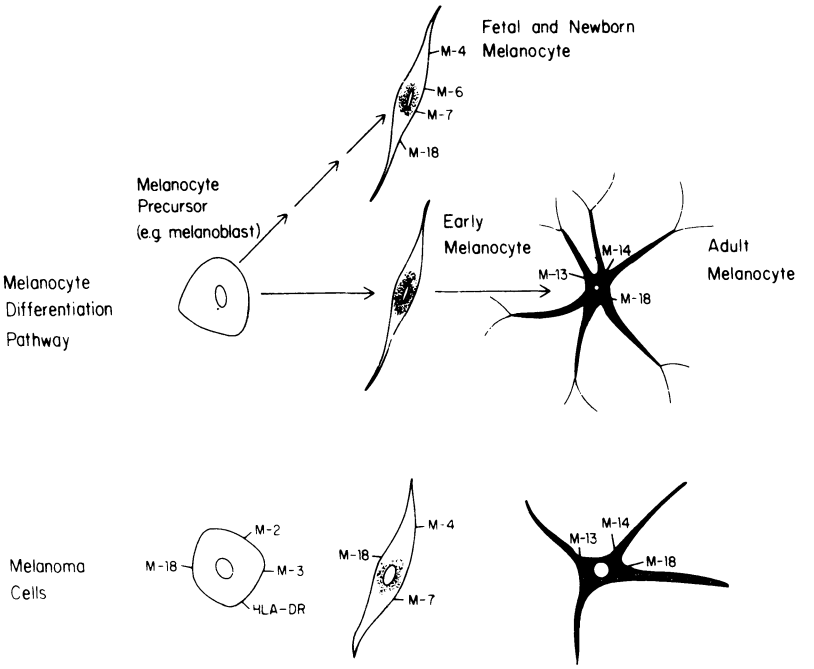

Figure 2. Proposed pathway of melanocyte differentiation based on surface antigenic phenotype and morphology: The phenotype of melanomas corresponding to early, intermediate, or late stages in the proposed melanocyte pathway is also illustrated. In this scheme, M-2, M-3, and HLA-DR antigens are early melanocyte markers; M-4, M-6, and M-7 antigens are intermediate markers; M-13 and M-14 antigens are late melanocyte markers. M-18 antigen is found at all stages of melanocyte differentiation and is present on all melanoma cells. Adapted from Houghton et al. [67].

On the basis of reactions with fetal, newborn, or adult melanocytes, antigens can be distinguished that appear to be early, intermediate, or late markers of melanocyte differentiation. Early markers are found on a subset of melanomas, but are not detected on fetal, newborn, or adult melanocytes. Intermediate markers are found on fetal and newborn melanocytes but not on adult melanocytes. Late markers are found on adult melanocytes but not on melanocytes from the fetus or newborn. Upon using mAbs identifying some of these systems to type a large series of melanoma cell lines, it was found that melanomas can be distinguished on the basis of expression of early, intermediate, or late melanocyte antigens. The ability to group melanomas allows an obvious correlation between surface antigen phenotype and other differentiation characteristics. Melanomas expressing early markers, such as HLA-DR antigens, have an epithelial morphology, lack pigmentation, and express no detectable tyrosinase. Melanomas expressing late markers have a spindle-shaped or dendritic morphology, are pigmented, and express high levels of tyrosinase. Based on these studies of melanocyte and melanoma differentiation antigens, a rudimentary surface antigenic map of the melanocyte lineage could be proposed (Figure 2). A question that could not be answered with this

evidence is whether melanomas arise at any of a number of stages throughout the melanocyte lineage or whether there is a preferential stage for malignant transformation. The finding that the phenotypes of melanoma correspond to distinct phases in the melanocyte pathway could be explained by transformation of early, intermediate, or mature progenitors, or, alternatively, by transformation of early progenitors with transformants having the ability to undergo variable but characteristic degrees of differentiation to later stages of melanocyte differentiation. The overlapping phenotypic characteristics of melanoma cell lines from different individuals are consistent with either explanation. Analyzing proto-oncogene expression in melanomas and normal melanocytes subsetted according to differentiation parameters may be instructive in determining if qualitative or quantitative changes in the expression of a particular oncogene is involved in tumorigenesis and/or differentiation. While this analysis is not as extensive as that at the DNA level, we have tested, by northern blotting, a range of cultured melanomas representing the three stages of melanocyte differentiation for altered expression of the following proto-oncogenes: c-*myc*, N-*myc*, c-Ha-*ras*, c-Ki-*ras*, N-*ras*, c-*src*, c-*fos*, and c-*sis*. Using mRNA extracted from cultured melanocytes as a baseline, we found only normal copy levels (i.e., equivalent to that seen in melanocytes) and message sizes for each of these proto-oncogenes in the melanomas examined. The one exception was c-*sis* for which no mRNA transcripts were observed. As there are no consistent alterations in the expression of oncogene mRNAs detectable in the tissues examined to date and with the probes used, this data suggests that increased or altered expression of the subset of proto-oncogenes analyzed is not part of the malignant or differentiation phenotype of melanomas. However, we are extending this study to other oncogenes and to noncultured precursor and neoplastic tissues and to noncultured matched normal tissues from individual melanoma patients. In a study by Slamon et al., it was found that the transcriptional activity of certain oncogenes (most notably c-*myc* and c-Ha-*ras*) was greater in the malignant than the normal tissues (melanomas were not analyzed [24]). However, the significance of these results is uncertain as the study could not ascertain that the same numbers and types of normal and malignant cells were being analyzed. Therefore, it remains possible that the detected differences in transcriptional activities of proto-oncogenes do not actually reflect increased expression in the malignant tissues.

One interesting observation, though, has been made with respect to melanoma differentiation pathways and activated oncogenes. As discussed above, DNA from 4/34 cultured melanomas had activated oncogenes detectable in the NIH/3T3 assay. It was logical to ask if these four melanoma lines had other phenotypic traits in common. Table 4 shows some aspects of the phenotypic variation among the melanoma cell lines tested for activated oncogene activity. Each of the four lines with detectable transforming genes had essentially the same phenotype. The lines were pigmentation negative, expressed antigens of the early melanocyte differentiation phase, and expressed Class II histocompatibility gene products, in particular HLA-DR. Therefore, these four melanoma

Table 4. Phenotypic characterization of melanomas with activated oncogenes

Cell line	Activated oncogene	HLA-DR	Early	Intermediate	Late	Pigmentation
SK-MEL-146	Ha-*ras*	+	+			—
SK-MEL-147	N-*ras*	+	+			—
SK-MEL-119	N-*ras*	+	+			—
SK-MEL-93 DX-3	N-*ras*	+	+			—
SK-MEL-93 DX-1	none	+		+		+
SK-MEL-93 DX-2	none	—			+	+
SK-MEL-93 DX-4	none	—	+			+
SK-MEL-93 DX-5	none	+		+		—
SK-MEL-93 DX-6	none	+			+	+
SK-MEL-37, 131	none	+	+			—
SK-MEL-65, 118, 129, 153, 166	none	+		+		+/—
SK-MEL-28, 64, 75	none	+			+	+/—
SK-MEL-23, 30, MeWo	none	—			+	+/—

Correlation of oncogene activation and differentiation characteristics of cultured melanomas. SK-MEL-93, DX-1 to DX-6, are six separate cell lines established from six metastatic deposits of a single patient, DX. Early, intermediate, and late antigens are markers expressed at corresponding stages of melanocyte differentiation; see Figure 2.

lines can be placed in the early to intermediate phase in the melanocyte differentiation pathway. One possible conclusion of this observation is that a subset of Ia-positive melanomas undergo activation of *ras* oncogenes. The correlation between phase of differentiation and activation of oncogenes is not an unequivocal one, though, as a small number of other melanomas with the same phenotype did not have detectable transforming activity in the NIH/3T3 assay. However, the majority of melanomas which tested negative in this assay had different phenotypes and were determined to be from the intermediate and late stages of differentiation. Therefore, it remains unclear if certain oncogenes are activated in a subset of melanoma cells from a particular phase of differentiation.

Consequently, the evidence to date shows that the only known oncogene perturbation (either at the DNA or RNA level) in a range of melanoma-related tissues examined is the activation of members of the *ras* gene family in a subset of metastatic melanomas. Thus, the role of oncogenes in the pathogenesis of melanomas is unclear and presumably complex.

4. The melanocyte as a transformation system

Our main focus is to analyze the contributing role of oncogenes in melanoma and, therefore, we have begun a series of experiments designed to determine if

deliberate introduction of oncogenes into the chromosomes of melanocytes can induce phenotypic changes characteristic of melanoma cells. A second aim of these experiments is to directly test the concept that multiple, independently activated oncogenes are involved in the etiology of neoplasms. This concept is based on the work of a number of investigators who have recently described experiments indicating that neoplastic transformation of cells can be achieved by the combined action of at least two transforming genes [37, 68–71]. A conclusion of this body of work is that the mode of action of specific oncogenes affects distinct cellular targets and that oncogenes can be classified by function into complementing groups, each of which defines a different step required for malignant transformation. For example, it seems that the mode of action of *myc* and *ras* oncogenes is qualitatively different. The *myc* oncogene induces a phenotype in rodent cells which to some degree mimics the effects of *in vitro* establishment or immortalization, while the *ras* oncogene induces traits of focus formation and tumorigenesis [69, 70]. The precise roles of complementing oncogenes, however, remain uncertain as does the role of a single oncogene. Moreover, the bulk of this type of work has been done with rodent cells, which are, for unknown reasons, less difficult to transform than human cells [72, 73]. Consequently, detailed analyses of human differentiated cell-transformation systems are necessary before the role of oncogenes in human cancers can be fully assessed.

Recent work of Eisinger and Marko has defined the conditions necessary for the growth of human melanocytes [74]. It was found that melanocytes can be selected from a mixed epidermal cell suspension by the use of 12-0-tetradecanoylphorbol 13-acetate (TPA) in the culture medium. Melanocytes proliferate extensively when the seeding density is optimal and the medium contains both TPA and cholera toxin. Under these conditions human melanocytes can be passaged serially *in vitro* for many population doublings. Long-term cultures grown under these conditions retain their differentiated properties, (i.e., antigenic phenotype, cell shape, pigmentation, etc.) and have a normal diploid karyotype [75]. The ability to grow diploid melanocytes in sufficient quantity provides unique opportunities to use these normal cells as targets for transformation and to examine transformation-related events.

We have been engaged in a long-term study designed to define the differences between melanoma cells and normal progenitor cells [27, 28, 66, 67]. In this study, a large series of melanoma and melanocyte cell lines has been characterized with respect to cell surface antigens, pigmentation, and morphology. In brief, we have found that different melanoma and melanocyte cell lines can be distinguished from one another on the basis of these characteristics, despite the fact that there exists considerable heterogeneity in these phenotypic traits. As a consequence of these kinds of data, more is known about the normal and malignant cell counterparts in melanoma than in any other solid tumor system. One major advantage afforded by this knowledge is an ability to make a reasonable prediction as to what the phenotype of a transformed melanocyte would be. Table 5 compares some of the phenotypic characteristics of mel-

Table 5. Phenotypic comparisons

	Melanocytes	Melanomas
Ia antigen	—	+
Adenosine deaminase- binding protein	+	—
G_{D3} ganglioside	low	high
Early antigens (cell surface)	—	+ or —
Late antigens (cell surface)	+	+ or —
Tyrosinase	+	+ or —
Pigment	+	+ or —
Growth factor independence	—	+
Anchorage independence	—	+
Tumorigenicity	—	+
Karyotype	diploid	aneuploid

Comparison of phenotypic characteristics of cultured melanocytes and melanoma cells. —, cells do not express this antigen of manifest this phenotypic trait; +, cells express this antigen or manifest this phenotypic trait; +/–, melanomas show heterogeneity in the expression of these characteristics. Early and late antigens are markers expressed at corresponding stages of melanocyte differentiation; see Figure 2.

anocytes and melanomas. It is immediately obvious from this chart that partially or completely transformed melanocytes would have to undergo specific changes in antigen expression, differentiation characteristics, factor dependence, immortalization, chromosomal organization, and/or neoplastic potential, in order to acquire a phenotype more resembling that seen in melanomas. The following is a brief review of some of the phenotypic characteristics which are being analyzed in transformation experiments with melanocytes.

4.1. Cell-surface antigens of melanocytes and melanomas

One class of antigens expressed by melanomas that has intrigued us has been Class II major histocompatibility antigens. Human Ia or Class II histocompatibility antigens were initially thought to be restricted to cells of the immune system, and, therefore, it was a surprise when the presence of Ia antigens was demonstrated on malignant melanomas [76, 77] and other cell types having no known immune functions [78–80]. Subsequent to these studies, it has been shown that Ia antigens can be detected on most metastatic melanoma biopsy specimens and melanoma cell lines [27, 67, 81–83]. Further, Ia antigens can also be detected on a large proportion of noncultured primary melanomas [82–84]; however, in these tissues, it is unclear what proportion of cells in the lesion are actually expressing Ia. Recent evidence suggests that cells in the advancing front of a primary melanoma are characteristically positive for Ia antigen expression [84], but it is unknown if the expression of Ia antigens is due to the transformed nature of these invading cells and/or to the inductive effects of IFN-γ produced by infiltrating lymphocytes. Normal melanocytes lack constitutive Ia expression either *in vivo* [81] or *in vitro* [67, 85]. Therefore, Ia antigens, in this system, can be considered tumor-specific as they are only expressed by neoplastic cells. Several interpretations to explain these observations can be advanced. It is possible that

Ia expression in primary and metastatic melanomas is a consequence of events occurring during or following malignant transformation of normal melanocytes [67, 77]. An alternative, but not mutually exclusive, interpretation is that Ia antigens are expressed on an early, as yet unidentified, cell in the normal melanocyte lineage and that Ia positive melanomas arise from this Ia positive progenitor [67]. In this regard, Ia expression would be an example of the persistence of a differentiation-related trait which in normal development would be extinguished. Understanding the mechanisms that control Ia expression in melanomas is an area of considerable theoretical and practical importance as a number of fundamental observations and questions exist for which we have no satisfying answers. For example, what is the biological role of Ia antigens on melanomas? As Ia molecules normally participate in the immune response (e.g., in initial antigen recognition, in providing signals for immune regulation, etc.), the induction of Ia antigen expression in melanoma cells raises the possibility that events associated with malignant transformation can induce normally nonimmune, somatic cells to function as antigen-presenting or immune regulatory cells. The finding that Ia antigen expression on primary melanomas is necessary, although not sufficient, to generate autologous T-lymphocyte reactivity supports this view [86, 87]. Alternatively, Ia antigens could function in a nonimmune context. The expression of Ia antigens during specific stages of erythroid and myeloid development suggests that Ia may have roles in differentiation and/or cell-cell interactions that are not related to the immune response [88, 89]. It has been suggested by Houghton et al., that cells in the melanocyte series are programmed to express Ia antigens, perhaps constitutively during early stages of melanocyte differentiation and as an inducible trait (e.g., by IFN-γ) during later stages. This view, however, predicts the existence of an Ia-positive melanocyte precursor (i.e., a melanoblast) which has yet to be isolated. In addition, the presence of Ia antigens on highly proliferating cell types, such as intestinal epithelial cells [90], skin Langerhans cells [91], and liver cells [92], may indicate a functional role in the cellular interactions that control proliferation and maturation [83]. Does Ia antigen expression confer any selective biological advantage on melanoma cells? This is still an open question. However, there is some suggestive evidence that HLA-DR expression correlates with an increased risk of metastasis and, therefore, with an unfavorable prognosis [84]. It has also been shown that increased expression of Ia antigens on metastatic melanoma cells (in contrast to primary melanoma cells) inhibits antitumor lymphocyte responses [93]. Finally, expression of Ia antigens may provide a basis for tissue-specific recognition signals required for cell interactions involved in the control of proliferation and maturation [94], although this would not explain the marked heterogeneity in metastatic melanoma cells which can range from no expression of Ia antigens to expression of large amounts of these antigens [30, 67, 83]. Clearly, further studies of Ia expression during normal melanocyte differentiation and during malignant transformation are necessary to resolve these issues.

A second important antigen expressed on melanomas is G_{D3} disialogan-

glioside (NeuAcα 2 $\rightarrow$ 3 Galβ 1 $\rightarrow$ 4 Glc-ceramide [66, 95]). G_{D3} has several interesting biological features. First, there is a discordance in expression by melanocytes and primary and metastatic melanomas. G_{D3} is the predominant membrane ganglioside of melanomas, being strongly expressed in most (if not all) metastatic melanomas and a majority of cells in most primary melanomas, whereas it is expressed only weakly and irregularly on the surface of melanocytes [95–97]. G_{D3} expression is localized to the cell surface in melanomas and melanocytes, whereas in most other cell types it appears to be an intracellular biosynthetic precursor to related gangliosides and, thus, in this respect, G_{D3} is a marker for cells of the melanocyte pathway [67, 95, 98]. Second, increased G_{D3} expression may be associated with the process of malignant transformation. This has been suggested by studies showing that astrocytoma cells have increased quantities of G_{D3} as compared to normal glial cells [99], and, similarly, rat fibroblasts, transfected with the adenovirus E1 transforming gene, also have augmented amounts of this ganglioside [100]. Third, while the precise role of G_{D3} ganglioside remains to be determined, accumulated evidence suggests that it is associated with both cellular adhesion [101, 102] and membrane permeability [103]. It is speculated that large concentrations of this ganglioside may be necessary for sustaining a high metabolic activity and/or rapid cellular proliferation [103]. Additional support for this idea comes from studies showing that anti-G_{D3} mAbs inhibits growth, alters the morphology, and inhibits the cellular adhesion of G_{D3}-rich melanoma cells [102]. Finally, G_{D3} is presently being clinically evaluated as a target in immunotherapeutic trials using an anti-G_{D3} monoclonal antibody (R24) [104]. Anti-G_{D3} mAbs are potent mediators of complement-mediated cytotoxicity [105] and antibody-dependent cellular cytotoxicity [106] and are, therefore, effectively able to kill melanoma target cells by immune mechanisms, which may account for the dramatic regression of tumor masses in treated melanoma patients [104]. Consequently, it is of obvious interest to determine if transformation-related events affect G_{D3} expression and/or the tissue pattern of G_{D3} and related gangliosides.

4.2. Differentiation characteristics of melanocytes and melanomas

A comparison of established melanoma and melanocyte cell lines from different individuals shows striking variation in morphology, growth rate, pigmentation, pattern of cell-surface antigens, and other distinguishing markers which distinguish these two cell types. Some of these markers are restricted to melanocytes and melanomas [27, 67, 107]; in other cases, they are shared by other cell types of neuroectodermal origin [26–28, 108]. Other markers are integrally related to the differentiated functions which distinguish pigmented melanocytes and melanomas from other cell types and include: (1) the insoluble pigment melanin, (2) the enzyme tyrosinase (necessary for the formation of melainin), (3) two structural proteins found on mature melanosomes and defined by mAbs TA99 and CF21 [107, 109], and (4) a cell surface glycoprotein, detected by mAb C350, found only on melanocytes and pigmented melanoma

cells [A.N. Houghton, personal communication]. Several markers appear to be directly related to the transformation process. One such example is adenosine deaminase binding protein (ADA bp) [110]. This protein is universally expressed on the cell surface of all melanocytes but, apparently, is extinguished upon malignant transformation as it is absent on melanomas [A.N. Houghton, personal communication]. Another transformation-related marker is Ia antigen which, as discussed above, is only observed on transformed cells. Finally, there are a number of more widely distributed cell surface proteins that we have identified, which, due to their ubiquitous expression, may have more direct roles in basic cellular functions [26, 28, 67]. The observed phenotypic heterogeneity in these melanoma markers reflects a corresponding diversity in the phenotype of normal cells undergoing melanocyte differentiation, with malignant transformation fixing a cell within a particular stage in the melanoblast-melanocyte lineage [67]. However, additional factors, either genetic or epigenetic, must also be involved in the generation of this diversity, because clones derived from the same melanoma cell line show stable phenotypic differences [A.P. Albino and A.N. Houghton, unpublished data]. This suggests two types of mechanisms: melanomas are initiated by a single transformed clone, with stable variants of the clone being generated throughout the course of the disease, and/or melanomas are initially derived from more than one clone of transformed cells. Analyzing the effect of transforming agents on the phenotypic characteristics of melanocytes and melanomas may elucidate how these mechanisms impact on tumor heterogeneity in this disease.

4.3. Growth factor dependence of melanocytes and melanomas

Proliferation of human diploid melanocytes *in vitro* depends upon the addition of exogenous growth factors to the medium [74]. Factors that support the growth of melanocytes include 12-0-tetradecanoyl phorbol 13-acetate (TPA) and growth factors derived from fetal fibroblasts, astrocytoma, and melanoma cells [111]. Melanocytes grown in the presence of TPA or melanocyte growth factors have identical phenotypes [A.N. Houghton, personal communication], suggesting that (1) TPA alone does not induce any changes in melanocytes that are characteristic of melanomas and (2) TPA and melanocyte growth factors have similar mechanisms of action in this system. In the absence of growth factors, melanocytes rapidly senesce and die. In contrast to melanocytes, cultured melanomas grow vigorously in the absence of similar growth factors, indicating that malignant transformation of melanocytes is associated with the acquisition of autonomy from exogenous growth factors. The nature and timing of cellular events necessary to the induction of this autonomy are unknown. Can the expression of single or multiple oncogenes abrogate the dependence of melanocytes on exogenous growth factors? Recent studies have shown that human and mouse cells acquiring an activated oncogene can bypass their dependence on exogenous mitogenic factors for cellular growth and tumorigenic potential [112, 113].

4.4. Chromosomal organization in melanocytes and melanomas

As with cells from most tumor types, the majority of noncultured and cultured melanoma specimens are known to have marked karyotypic abnormalities [53] with the most common perturbations being a hyperdiploid number of chromosomes, chromosomal rearrangements, a frequent loss of heterozygosity at loci on many different chromosomes [114], and nonrandom alterations on chromosomes 1, 6, and 7 [53]. However, the biological effects of any of these abnormalities is unclear. Cultured melanocytes maintain their normal chromosomal complement and structure through many passages *in vitro* [75]; therefore, these cells offer an ideal system with which to monitor progressive transformation-related chromosomal changes which are not readily discernible in aneuploid melanoma cells.

4.5. Neoplastic potential of melanocytes and melanomas

Finally, in contrast to normal melanocytes, malignant melanomas are tumorigenic in nu/nu mice and possess an anchorage independent phenotype (i.e., an ability to form colonies of growing cells in soft agar assays [A.P. Albino, unpublished data; 75]. Will the introduction of *ras* oncogenes induce in melanocytes a tumorigenic potential in animals and/or an anchorage independent phenotype? In addition, if these transformation-related characteristics are induced, can other phenotypic traits be detected which co-segregate with these biological functions?

5. Transformation of human melanocytes in vitro

Our study was designed to test the effects of introducing oncogenes into the chromosomes of human melanocytes via amphotropic retroviral vectors [116]. Our initial attempts to transform cultured melanocytes involved infecting melanocytes with transforming amphotropic pseudotypes of Harvey (Ha-MSV) and Kirsten (Ki-MSV) murine sarcoma viruses. These retroviruses contain oncogenes of the *ras* gene family and were chosen because our previous studies [29] indicated that 10% of cultured melanomas have an activated *ras* gene allele (either Ha-*ras* or N-*ras*) and no other consistent and conclusive perturbation (i.e., rearrangement or amplification) in 16 other known oncogenes. The Harvey and Kirsten strains of murine sarcoma virus (Ha-MSV and Ki-MSV) are replication-defective retroviruses that were originally isolated from mouse tumors induced by murine leukemia viruses which had been passaged through rats [for review, see 36]. Ha-MSV and Ki-MSV can transform fibroblasts in tissue culture and induce sarcomas and erythroleukemias in susceptible mice. The transforming gene of these viruses encodes a 21,000 dalton protein (p21) which is required for cellular transformation. Virions containing the transforming p21 encoding gene and amphotropic envelope proteins necessary for the

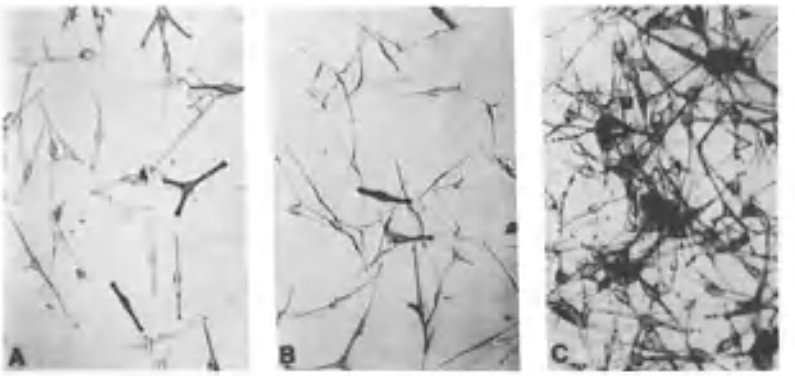

Figure 3. Morphology of cultured melanocytes: Photomicrographs of (A) uninfected melanocytes, (B) amphotropic MUIV infected melanocytes, and (C) Ki-MSV infected melanocytes. (x220). Cells shown in panels (B) and (C) are three weeks postinfection.

attachment and penetration of human cells can be readily constructed, thereby allowing efficient introduction of these oncogene containing viruses into melanocytes [117].

Initially we examined the effects of v-Ki-*ras* and v-Ha-*ras* expression on the morphology and growth characteristics of melanocytes. Figure 3 shows the results of Ki-MSV and Ha-MSV infection on the morphology and growth characteristics of human melanocytes. Two weeks postinfection, islands of Ki-MSV or Ha-MSV infected melanocytes appeared that had a distinct morphology. Uninfected melanocytes grow as bipolar, spindle-shaped cells without much intercellular contact (Figure 3A). Cells infected with the amphotropic MuLV helper virus had the same morphology as normal melanocytes (Figure 3B). Amphotropic MuLV is a replication competent retrovirus whose genome encodes only structural proteins and not an oncogene, and, therefore, it serves as a control for any effects of virus infection and replication. Melanocytes infected with either Ki-MSV or Ha-MSV, however, were more polygonal and grew as clusters of cells with a tendency to pile up (Figure 3C). Identical changes were observed in four independently Ki-MSV or Ha-MSV infected melanocyte cultures. By two weeks, 65% of the cells infected with Ki-MSV or Ha-MSV expressed viral specific mRNA and p21 protein and detectable levels of mature infectious transforming progeny virions (approximately 10^2–10^4 infectious focus forming units/ml of supernatant). Supernatants from amphotropic MuLV infected cells produced viral structural proteins and approximately 10^4 infectious non-focus forming units/ml of supernatant. Thus, Ki-MSV and Ha-MSV could infect and replicate within melanocytes and induce morphological changes. Amphotropic MuLV could also replicate in melanocytes but induced no morphological changes.

We next determined if the cell surface antigenic phenotype of melanocytes changed upon viral infection. As discussed above, we closely monitored a

number of cytoplasmic and cell-surface antigens for qualitative and/or quantitative changes. Table 6 shows the results of serological and biochemical tests for expression of these antigens in infected and noninfected melanocytes. One of the earliest detectable changes after infection with Ha-MSV or Ki-MSV was the induction of Class II major histocompatibility gene products (Ia antigens). Melanocytes showed strong expression of Ia antigens within three to five days after infection with these transforming viruses. Induction of Ia antigens by Ki-MSV and Ha-MSV was similarly observed in four independently infected melanocyte cultures. Melanocyte cultures infected with the amphotropic MuLV did not express Ia antigens. Immunological analysis by dual-labeling fluorescence indicated that the same cells which expressed Ia antigens also expressed viral p21 protein, indicating that the inductive effects were a specific action of the viral p21. In contrast to the qualitative changes in Ia expression, little or no change was detected in the normal constitutive expression of Class I histocompatibility antigens (i.e., HLA-A,B,C). To determine which Class II subloci were activated, cytoplasmic RNA was analyzed by northern blotting with Class II probes. Figure 4 shows that Ki-MSV infected melanocytes expressed RNA species specific for at least three Class II alpha gene loci, DR, DP, and DQ.; uninfected melanocytes and melanocyte cultures infected with amphotropic MuLV did not express Ia antigens or RNA specific for Class II genes. Immunoprecipitation analysis with extracts of metabolically labeled cells showed that both the alpha (34,000 dalton) and beta (28,000 dalton) chains of the Ia bimolecular complex were detectable in extracts of Ki-MSV infected melanocyte cultures and that these were indistinguishable from Ia molecules immunoprecipitated from melanoma cells (see Figure 5). Ki-MSV and Ha-MSV infected melanocytes were tested over a period of six months and showed stable and continued expression of Ia antigens.

The expression of Ia antigens can be reversibly induced in melanocytes by gamma interferon (IFN-γ, [85]) and not by a range of other substances, including IFN-α and IFN-β. Even though T-lymphocytes are the only cell type known to produce IFN-γ, we considered the possibility that melanocytes synthesize IFN-γ after infection with Ki-MSV or Ha-MSV. Using a sensitive melanocyte Ia-induction assay which detects as little as 1U/ml of IFN-γ [85], we found no detectable levels of IFN-γ or any other Ia-inducing soluble factor in the culture supernatants of Ki-MSV-infected melanocytes. Moreover, it is worth noting that the only known effect of IFN-γ on melanocytes is to induce the transient expression of Ia antigens without concomitant effects on the expression of a wide range of other cell surface antigens or phenotypic traits such as pigmentation and morphology. This is in contrast to *ras* oncogenes which have a more pronounced effect on melanocytes, affecting not only expression of Class II genes, but also morphology, antigen expression, and chromosomal structure.

While Ia expression in melanocytes is an inducible and reversible phenotype, it is characteristic of melanomas to constitutively express these antigens. Consequently, we wanted to determine if Ia induction by *ras* oncogenes was a

Table 6. Phenotypic characteristics of melanocytes and melanocytes infected with retroviruses

	Serological analysis of antigen expression (1) Reciprocal of serum titers × 10^3												
	Histo-compatibility antigens		Melanocyte antigens			Other neuroectodermal antigens						Biological properties (4)	
Melanocytes	HLA-A, B, C	Ia	C350	CF21	TA99	G_{D3}	B2.6	B5.2	MEL-1	Tyrosinase (2)	Pigmentation (3)	FFU	CFU
1346/uninfected	6250	—	3125	16	256	0.1	625	3125	2.5	42.3	+	0	$10^{-4\%}$
1346/amphotropic	6250	—	3125	16	256	0.1	625	3125	2.5	22.3	+	0	$10^{-4\%}$
1346/Ha-MSV	6250	3125	3125	64	64	25	25	3125	2.5	NT	+	3×10^4	0.04–0.1%
1346/Ki-MSV	6250	3125	3125	16	256	25	125	3125	2.5	39.2	+	3×10^3	0.04–0.1%

1. Cells were scored for cell surface antigen expression by erythrocyte rosetting assays: HLA-A, B, C, Ia, C350, GD3, B2.6, B5.2, and MEL-1; or for cytoplasmic antigen expression by indirect immunofluorescence: CF21 and TA99. Serum titers represent dilution at which 20% of the cells were positive. — indicates no positive cells.
2. Tyrosinase assays were performed as described by Pomerantz [130], as modified by Houghton et al. [67]. Tyrosinase activity is expressed as cpm × 10 + 3 of tritiated H_2O produced/mg protein. Nonpigmented renal carcinoma cells (standard) produced a background of 1 × 10 + 3 cpm of tritiated H_2O/mg protein. NT, not tested.
3. Pigmentation was estimated visually by the intensity of brown or black pigment in the cell pellet.
4. Transforming activity of virus produced by infected cells was determined by focus assays on NIH/3T3 cells [131]. FFU, focus forming units/ml of supernatant fluid. CFU, colony forming units. The percentage of cells plated per dish which gave colonies of at least 50 cells in three to four weeks. Briefly, 1 × 10 + 5 and 5 × 10 + 5 cells were plated in 100 mm dishes in 0.3% agar onto a bottom layer of 0.75% agar in quadruplicate [132].

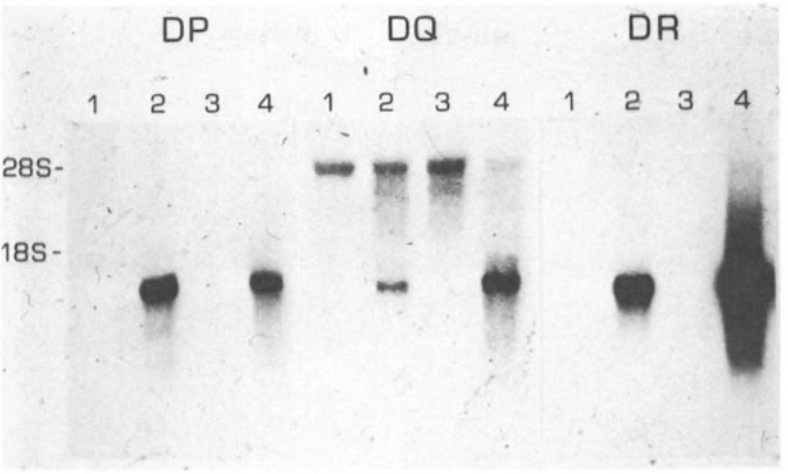

Figure 4. Northern blot analysis of Class II gene expression: Blots were hybridized to the following ^{32} P-labeled DNA fragments of between 500 and 2,500 nucleotides in length, isolated from A: DP-alpha (pDS-alpha3,8; a gift of H Erlich, Cetus Corp, Emeryville, Calif); B: DQ-alpha (pDQ10–8; [133]) and C: DR-alpha (pDRH2; [134, 135]). Positions of the 28S and 18S ribosomal RNA are indicated. Lanes — 1: uninfected melanocytes, 2: Ki-MSV infected melanocytes, 3: amphotropic MuLV infected melanocytes, and 4: SK-MEL-147 melanoma.

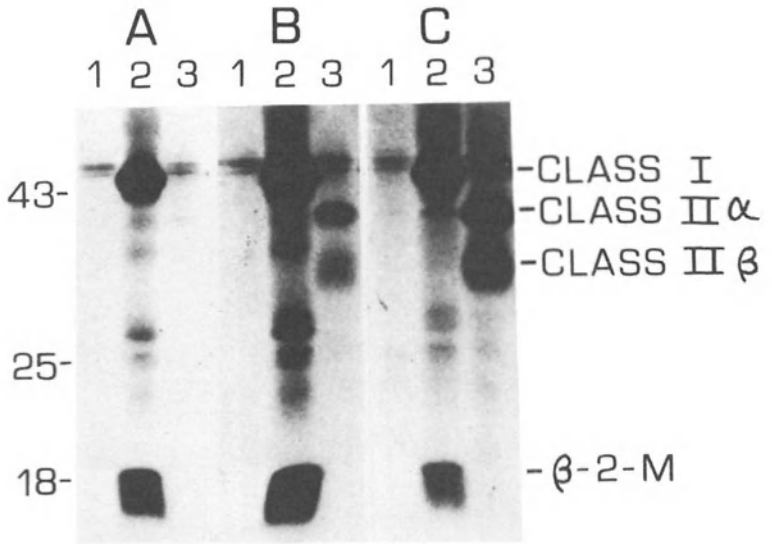

Figure 5. Immunoprecipitation of Ia antigens: Autoradiograms of immunoprecipitates obtained with extracts of [^{35}S]methionine-labeled melanocytes infected with temperature-sensitive mutant of Ki-MSV and grown at 33°C or 39°C for at least eight weeks prior to analysis by SDS-polyacrylamide gel electrophoresis. Lanes — 1,4: unrelated mouse mAb; Lanes 2,5: Y13-259 mAb (anti-p21); Lanes 3,6: L243 mAb (anti-Ia).

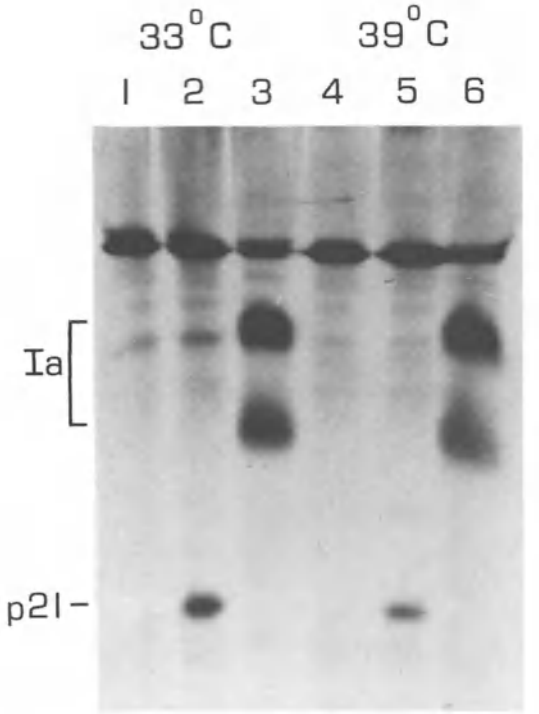

Figure 6. Immunoprecipitation of Ia antigens and viral p21 protein: Autoradiograms of immunoprecipitates obtained with extracts of the indicated cells labeled with [^{35}S]methionine for 16 hours and analyzed by SDS-polyacrylamide gel electrophoresis. A: uninfected melanocytes, B: Ki-MSV infected melanocytes, C: SK-MEL-47 melanoma. Lanes: 1, unrelated mouse mAb (control); 2, W6/32 mAb (anti-HLA-A,B;C); 3, L243 mAb (anti-Ia;). Molecular weight standards: 43 kilodaltons (kD), ovalbumin; 25kD, alpha-chymotrypsinogen; 18 kD, beta-lactoglobulin.

reversible phenomenon as in IFN-γ treated melanocytes, or a permanent one as in melanomas. To analyze this, we studied melanocytes infected with a temperature sensitive mutant of Ki-MSV [118], which has a mutation in the encoding p21 sequence. At nonpermissive temperatures the transforming functions of this virus are abrogated due to the instability of the viral p21. Figure 6 shows that, in addition to viral p21 protein, Ia antigens were expressed in equivalent amounts at both permissive and nonpermissive temperatures, suggesting that once Ia antigen expression has been established, continued expression is not dependent on functional p21. (It should be noted, however, that most TS mutants are leaky to a small degree, and it is possible that minimal amounts of functional p21 which may exist at the nonpermissive temperature are sufficient to maintain an inductive effect on Ia antigens.)

Is the Ia inductive effect of *ras* oncogenes restricted to melanocytes? Apparently, this is the case. We determined whether Ia expression can be induced in other cell types by viral *ras* oncogenes. When early passage human

fetal foreskin fibroblasts and early passage human fetal lung fibroblasts were infected with Ki-MSV, no Ia antigen induction was seen in either case even though approximately 90% of the cells were producing Ki-MSV p21 and infectious transforming virus. This data suggested that the regulatory control of Class II genes in these cells is much tighter than in melanocytes and melanomas. However, upon infecting an early passage Ia-negative melanoma (SK-MEL-93/DX-2) with Ki-MSV, we were surprised to find that viral *ras* oncogenes could not induce Ia antigen expression in these cells either. As yet, it is not clear why *ras* oncogenes fail to induce Ia antigens in this melanoma. These cells do have an intact set of MHC genes as they express Class I histocompatibility antigens and can be induced to express Ia antigens upon treatment with IFN-γ. While no specific mechanisms can be invoked as yet, it is tempting to speculate that the failure of *ras* oncogenes to induce Ia antigens may be related to the differentiated phenotype of these cells and/or to their aneuploid nature. Furthermore, like Ia-negative melanomas, foreskin and fetal lung fibroblasts can also be induced to express Ia antigens upon IFN-γ treatment. These observations suggest that the induction of Ia by *ras*-oncogenes and by IFN-γ are mediated through separate pathways. Moreover, since *ras* oncogenes and IFN-γ induce the products of Class II gene loci in a coordinate fashion, it is unlikely that differential activation of Class II genes is the basis for differences in Ia induction by IFN-γ and viral *ras*.

Expanding our analysis, we tested Ki-MSV and Ha-MSV infected melanocytes for alterations in the expression of a range of differentiation-related phenotypic characteristics (as shown in Table 6). We found essentially no change in these characteristics upon infection with *ras*-oncogene containing viruses; infected melanocytes, like uninfected control cells, (1) remained pigmented, (2) had tyrosinase levels comparable to uninfected cells, (3) expressed the cell surface glycoprotein detected by mAb C350, (4) expressed the melanosomal marker proteins TA99 and CF21, and (5) continued to express adenosine deaminase binding protein. The small quantitative differences seen were judged to be insignificant. However, we did find one melanoma marker whose expression did change — the G_{D3} disialoganglioside. Melanocytes infected with Ki-MSV showed a substantial increase in the expression of G_{D3} as measured by immune rosetting assays. Direct quantitation of G_{D3} expression using ^{125}I-labeled antibody indicated that the level of G_{D3} in Ki-MSV infected melanocytes increased by greater than five to ten fold over uninfected melanocytes or melanocytes infected with amphotropic MuLV.

As stated above, proliferation of melanocytes *in vitro*, in contrast to melanoma cells, depends upon the addition of exogenous growth factors to the medium; i.e., 12-0-tetradecanoyl phorbol 13-acetate (TPA). We determined if the expression of v-*ras* oncogenes in melanocytes affects the growth potential of these cells and/or their requirement for exogenous growth factors. In the presence of TPA, the growth rate of melanocytes infected with Ki-MSV or Ha-MSV was comparable to uninfected melanocytes and to melanocytes infected with amphotropic MuLV. In the absence of TPA, uninfected melanocytes and

MuLV-infected melanocytes died rapidly (within 6–12 days). Ki-MSV and Ha-MSV infected cultures also senesced but at a reduced rate (within 2–4 weeks). Thus, expression of *ras* oncogenes could not induce stable actively proliferating, growth factor independent melanocyte cell lines. The delay in senescence may imply that *ras* oncogenes can contribute to the acquisition of growth factor autonomy, but in combination with other, as yet, unidentified oncogenes. It is possible that TPA, required by melanocytes for growth, is complementing the function of the *ras* oncogene either directly or indirectly (e.g., by acting on some unknown cellular gene). TPA has been shown to enhance the transformation of C3H10T1/2 mouse fibroblasts transfected with a cloned activated c-Ha-*ras* oncogene [119].

Melanocytes cultured in the presence of TPA cannot form colonies in semi-solid agar. This is also true of melanocytes infected with amphotropic MuLV. However, we found that melanocytes infected with Ki-MSV and Ha-MSV acquired an anchorage-independent phenotype and could initiate colonies of proliferating cells in soft agar assays at a frequency of 0.04–0.1%, but require TPA to do so. Therefore, *ras* oncogenes can dissociate the cellular events required by melanocytes for an anchorage-independent phenotype and factor-independent growth. Moreover, this implies that alterations necessary for an anchorage-independent phenotype precede those required for factor-independent growth. These results fit the general presumption that different oncogenes affect different cellular mechanisms and that full transformation requires a synergy of effector functions.

6. Summary and conclusions

Our studies over the past several years have concentrated on the role of oncogenes in the pathogenesis of malignant melanoma and our objectives have been twofold: to analyze the status of oncogene expression during specific clinically-defined stages of melanoma; and to determine if multiple, cooperating, independently activated oncogenes are involved in the evolution of malignant melanoma.

Outside of the 10% of cultured metastatic melanomas which have mutationally activated *ras* oncogenes, there is a striking lack of evidence indicating any consistent alteration in a large series of oncogenes in a range of preneoplastic and neoplastic melanoma tissues. To date, therefore, the available evidence has yet to uncover a critical role for oncogenes in the pathogenesis of this disease. Is the lack of obvious abnormalities in proto-oncogene specific DNA and RNA from tumor tissues an unusual finding? This question has no simple answer, and it is beyond the scope of this review to attempt a detailed discussion. Interested readers can review a range of scholarly papers on this subject [4, 10, 11, 23, 24, 55, 68]. However, we shall briefly discuss this issue as it relates to melanoma. At the present level of analysis it has been difficult to pinpoint either a consistent association of a particular oncogene or consistent association of a transcription-

ally activated proto-oncogene with any specific tumor type. Further, there appears to be no specific mechanism of oncogene activation (i.e., mutation, rearrangement, amplification, elevated expression, or other genetic perturbation) common to all members of a particular tumor type, including melanoma [11]. Thus, the identification of specific cancer genes, genes, which like their viral counterparts, can initiate and maintain the malignant process, has yet to be made. A recent report by Barbacid et al., however, provides some of the clearest evidence of a causal role for oncogenes. This study showed that a majority of methylnitrosourea (MNU)-induced mammary carcinomas of rats contained a specific activating mutation in the c-Ha-*ras* proto-oncogene [120]. Due to the extreme lability of the mutagen, the mutating effects of the MNU must have occurred during the brief period of time the compound interacted with the DNA. The reproducible activation of the c-Ha-*ras* oncogene suggests that this transforming gene played a fundamental role in the development of mammary tumors in this animal model system. It remains to be seen how these results relate to spontaneous human neoplasias.

It is evident that a much more detailed study must be made to determine what specific genetic lesions are involved in the etiology of melanoma. There are any number of possible starting points. The abnormalities associated with the nonrandom karyotypic alterations involving chromosomes 1, 6, and 7 are hypothesized to involve genes which may play a role in the pathogenesis of melanoma. None of the known oncogenes are close to the detected breakpoints in these chromosomes, and it is logical to ask if melanomas contain novel, previously unidentified oncogenes at these sites, which may be involved in this disease. As discussed above, there is strong circumstantial evidence for an autosomal dominant 'melanoma' gene on chromosome 1 which predisposes people to developing a large number of dysplastic nevi and increased rate of melanoma. It is unknown if this lesion is an oncogene, in that its mere presence can cause melanoma, or some other type of gene, whose presence is intertwined in some complex series of events which lead to melanomas, perhaps by complementing an activated oncogene or transcriptionally active proto-oncogene. Analysis of these gene regions should prove fascinating. Further, Eisinger and coworkers have shown that melanomas produce one or more novel factors which support the growth of melanocytes *in vitro*. It is possible that these factors are the products of oncogenes. Sutherland and Bennett have observed that DNAs from a variety of skin cancers, including melanomas, can induce an anchorage-independent phenotype in normal human cells [121]. Each of these studies suggests the presence of genetic elements which may contribute to various attributes of melanoma cells. Biochemical and genetic analysis of these elements will be invaluable in determining their relationship to oncogenes and to melanoma.

Our experiments using melanocytes as a transformational system have proved more fruitful than our search for activated oncogenes in melanomas. To date, we have found that human melanocytes infected with Ki-MSV or Ha-MSV, but not amphotropic MuLV, undergo a series of transformation-related

changes that are characteristic of malignant melanoma. These are (1) expression of Ia antigens, in particular, DR, DQ, and DP Class II histocompatibility gene products; (2) a transformed morphology and ability to grow in soft agar; and (3) a five to tenfold increase in the cell surface expression of G_{D3} ganglioside. However, other characteristics of melanoma, such as loss of adenosine deaminase binding protein and ability to survive in the absence of specific growth factors were not observed.

What interpretations and conclusions can be drawn from these experiments? It is clear from the data that viral *ras* oncogenes have a pronounced effect on melanocytes, effecting alterations not only in morphology and antigen expression, but also in cellular mechanisms which appear to be necessary for progression to a full malignant phenotype (e.g., anchorage-independent growth and chromosomal abnormalities). How does the *ras* oncogene encoded p21 protein effect these alterations? As yet, we have no specific answers to this question, but it is likely that the induction of Class II histocompatibility genes in melanomas by viral *ras* oncogenes is via a trans activation, through p21, and not through LTR activation of nearby cellular genes. This is based on the fact that the induction of Ia antigens is an acute viral effect, with Ia antigens being expressed in over 60% of the cells within five days, and the fact that the genomes of the infected melanocytes exhibit multiple viral integration sites. Presumably, the p21 protein triggers a cascade of events which affect a wide range of cellular functions and corresponding phenotypes. While our experiments have not illuminated this cascade of events, the data has allowed some fascinating conclusions. First, these experiments are the first to show that viral *ras* oncogenes in a retroviral vector are capable of inducing, in coordinate fashion, the expression of the products of at least three Class II histocompatibility gene loci: DP, DQ, and DR. As this is the first detectable event in the transformation of melanocytes with Ki-MSV or Ha-MSV, we can conclude that viral *ras* oncogenes initiate early transformation events in melanocytes and that Ia antigen expression is an early transformation marker in this system. Thus, this supports the interpretation (but does not prove) that Ia antigen expression on melanomas is a transformation-specific marker rather than being due to the persistence of a differentiation trait of a normal progenitor cell. A precedent in experimental systems is the appearance of TL^+ leukemias in mouse strains which normally do not express this antigen. Second, a common event in cells transformed by viral and chemical carcinogens is a change in the glycolipid composition [122]. Similar types of changes are found in a given cell type even when different transforming agents are used [123]. This is thought to simply reflect a specific phenotypic alteration determined by the organization of glycolipids in the host cell. (It is still undecided if these transformation-related changes offer specific advantages to the cell; e.g., in terms of proliferation or cell-to-cell interactions.) The fact that melanocytes transformed by a viral *ras* oncogene show an increased expression of G_{D3} similar to melanoma cells, which for the most part do not have activated *ras* oncogenes, suggests that there may be a common biochemical mechanism which is perturbed by both spontaneous

transformation-related events and *ras* oncogene-induced events. This interpretation could also explain another paradox, which is the following. We have noted a lack of *ras* oncogene perturbations in melanoma and concluded that these genes are relatively unimportant in the evolution of this disease. Yet, we have also shown that *ras* oncogenes induce a number of phenotypic traits known to be associated with melanoma. The simplest hypothesis is that there are multiple pathways by which a cell can progress towards transformation. *ras* oncogenes could mimic the effects of a putative cellular gene(s) which is involved in one of these alternate pathways. Alternatively, the products of *ras* oncogenes and this unknown cellular gene(s) may intersect some common cellular function which, if perturbed, could induce transformation-related events.

Finally, the experiments outlined above have shown that melanocytes undergo a number of phenotypic alterations in response to transforming *ras* oncogenes, that these changes reflect events associated with melanomas, and, most importantly, that these changes have a temporal association or sequence. Melanocytes expressing a viral *ras* oncogene undergo transitions in their Class II gene expression, glycolipid patterns, and ability to grow in soft agar before they become factor-independent, chromosomally deranged, and tumorigenic. Determining if these events happen in a similar sequence *in vivo* is a fundamental task for the future.

7. Future studies

Normal diploid human cells, in contrast to rodent cells, have been difficult to transform with activated *ras* oncogenes as the sole transforming agent [72, 124]. However, in systems where cells have either a genetically determined chromosomal abnormality, as in Bloom's syndrome [125], or have undergone extensive aneuploidy as a consequence of prolonged passage in tissue culture [126], introduction of an activated *ras* oncogene can induce the full range of transformation characteristics. These studies suggest that a combination of an activated *ras* oncogene and aneuploidy may satisfy the minimum requirements for transformation. Melanocytes have a normal diploid karyotype *in vitro* and, therefore, as with other chromosomally normal human cells, do not undergo complete transformation by the addition of a single *ras* oncogene (i.e., these cells acquire some but not all the phenotypic characteristics of melanoma cells). We have observed that melanocytes infected with retroviruses containing a *ras* oncogene maintain a diploid karyotype during early passages. However, by eight to ten weeks postinfection, some 50% showed hyperdiploid chromosomal numbers, although no chromosomal rearrangements were observed. At present we are determining whether progressive chromosomal changes occur with prolonged passage, in melanocytes into which activated *ras* oncogenes have been introduced (either by infection with *ras*-containing retroviruses or by transfection with plasmids into which *ras* oncogenes have been cloned), and

whether such changes lead to the fully transformed features of melanoma cells. It appears, though, that expression of viral *ras* oncogenes renders melanocytes more susceptible to chromosomal instability.

In our initial studies, it could not be determined if Ki-MSV-infected melanocytes were also tumorigenic in nu/nu mice because these cells release high amounts of infectious MuLV-MSV which, due to the amphotropic nature of these viruses, causes leukemias and sarcomas in injected mice. Consequently, we are re-evaluating tumorigenic potential with melanocytes infected with defective retroviral vectors into which *ras* oncogenes have been introduced by recombinant DNA methodologies [127, 128]. The advantage of these vectors is that infected cells do not release infectious virus particles.

At present, we are examining the effects of oncogene products with intracellular locations and functions different from *ras* (either alone or in conjunction with *ras*) for melanocyte transforming activity. Moreover, to directly study the transforming effects of multiple activated oncogenes, we are infecting melanocytes with retroviruses that contain two functional oncogenes (e.g., *myc* plus *ras*). Further, it will be important to determine if complete transformation of these cells will require physical agents such as ultraviolet light or chemical mutagens acting in concert with activated oncogenes.

In addition to using melanocytes in transformation studies, we are currently attempting to culture dysplastic nevus cells from normal individuals and from individuals with dysplastic nevus syndrome. It is possible that these cells have undergone genetic alterations *in vivo* which can complement an activated oncogene introduced *in vitro*, and thereby allow the cell to complete the transformation process.

The role of oncogenes in the pathogenesis of melanoma is obscure, with melanomas showing no consistent oncogene mutations, rearrangements, amplifications, over-expression, or rare alleles. However, this conclusion is based on studies with a limited set of both oncogene probes and melanoma-related tissues. Obviously, these studies must be expanded to include the complete battery of known oncogenes and more tissue specimens representing each stage of this disease in order to establish the actual status of oncogenes in melanoma unequivocally. As of this writing, there are virtually no studies analyzing the protein expression of proto-oncogenes *in situ* in premalignant and malignant melanoma tissues. It is possible that transformation of progenitor cells in this disease involves not activation of oncogenes, but inappropriate or enhanced expression of normal proto-oncogene products. Thus we are analyzing, by immunological methods, cryopreserved precursor lesions and melanoma specimens for the presence of protein products encoded by most of the known proto-oncogenes.

Finally, we are attempting to isolate novel melanoma oncogenes by transfecting very high molecular weight melanoma DNA (>100 kB) from different phases of differentiation directly into melanocytes and, hopefully, nevocytes, then testing these cells for alterations in a wide range of phenotypic character-

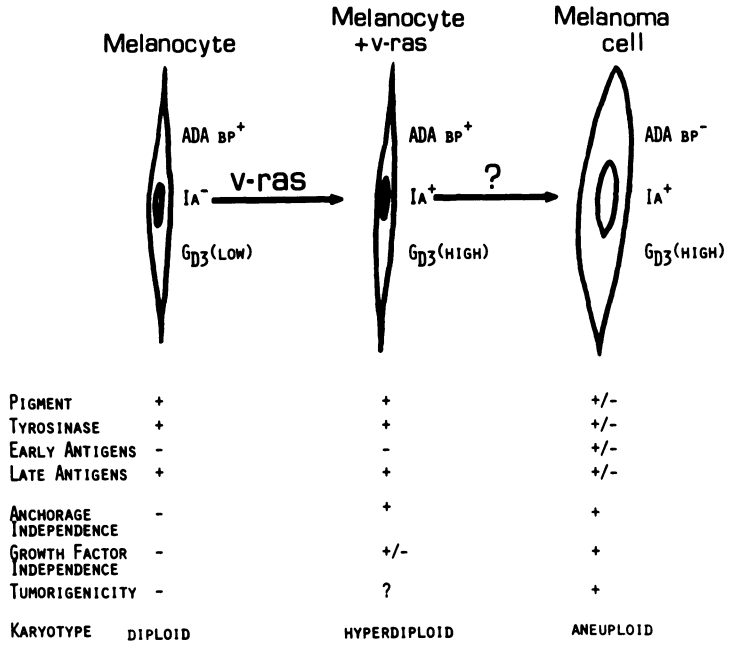

Figure 7. Cartoon summarizing phenotypic characteristics of normal melanocytes, melanocytes expressing a viral *ras* oncogene, and a completely transformed melanocyte or melanoma cell.

istics. It is possible that some transforming genes (1) have a complex structural organization requiring long tracts of DNA for encoding, (2) are activated at specific stages of differentiation within a cell lineage [129], or (3) require a cell with a compatible differentiation program to exert their transforming potential. If the transfected cells acquire transformed properties, the experiment can be repeated with DNA tagged with a marker sequence which will be useful in isolating the gene.

Thus, our approach provides a unique perspective from which to analyze transformation events in the normal human melanocyte and to more critically establish the role of oncogenes in the pathogenesis of melanoma. Our experiments to date have left us poised on a fascinating ledge as depicted in Figure 7. The introduction of activated *ras* genes into the chromosomes of human melanocytes have propelled the cell towards a malignant phenotype. The critical experiment will be to determine the nature and timing of events which must transpire in order for these partially transformed melanocytes to complete the process and become a malignant melanoma cell. Whether the completion of the transformation process will involve introduction of complementing oncogenes and/or chromosomal instability remains to be seen. The experiments that we and others are pursuing should give insight into the nature of this disease, its induction and maintenance, and the relationship of transformation to differentiation.

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2. Laminin and Fibronectin Modulate the Metastatic Activity of Melanoma Cells

Victor P. Terranova

1. Introduction

Metastasis is one of the major causes of mortality in cancer patients. Metastatic lesions arise when specific tumor cells escape from the bulk tumor, survive in the circulation, and migrate to distant sites where conditions are suitable for growth [1] (Figure 1). An important advance in our understanding of malignancy has come from the demonstration that the ability to successfully complete the metastatic cascade is not a common characteristic of all tumor cells but instead resides in a small subpopulation of cells [2–5]. Furthermore, since the migrating tumor cells often show a preferential tissue site to which they disseminate [6–9], the event cannot be random and must involve local factors which may initiate and guide tumor cell movement [6, 10].

The characterization of the metastatic process has been facilitated, in large part, by the development of specific lines of cells which exhibit different levels of metastatic propensity [11–15]. The study of these cells and their various activities is an increasing focus of research. From these studies, it appears that the metastatic process consists of several distinct but essential events. These include (1) escape of the tumor cells from the tumor proper, (2) dissemination of the cells through the lymphatics or blood vessels, (3) arrest in small vessels, (4) penetration through vessel walls and other tissue barriers, and (5) growth at the new site [16–23] (Figure 1).

During their migration, tumor cells encounter and interact with a variety of extracellular matrices [23, 24]. However, these interactions appear to be distinct from those observed with normal cells (Table 1). For example, transformed cells are generally less adherent to their substrate than normal cells [25, 26]. The tumor cells also have the capacity to secrete a variety of proteases which are able to degrade most matrix structures and thus increase the invasion and spread of the tumor [27–31]. Recently, particular attention has been directed to the role of basement membranes as discrete barriers to the passage of tumor cells [23, 31] and has allowed the identification of specific mechanisms used by the metastatic cells to adhere to [32, 33] and then degrade [34–36] these structures. Lastly, certain matrix molecules, for example, laminin and fibronectin, appear to modulate the tumor cell's metastatic activity [37–39].

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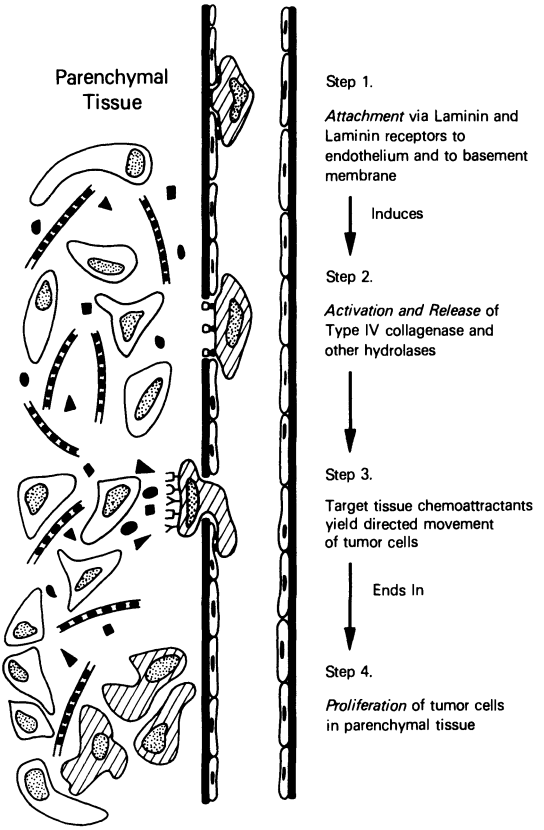

Figure 1. Proposed model for steps in tumor cell invasion of target tissue.

Table 1. Phenotypic characteristics of metastatic cells

1. Transformed
2. Subpopulation of total tumor cells
3. Many laminin receptors
4. High affinity for basement membrane
5. Synthesis and release of Type IV collagenase and other proteases
6. Highly motile
7. Survival and proliferation at distant sites.

Various *in vitro* assays have been developed for assessing specific steps in the metastatic process. These assays are directed toward testing the attachment of tumor cells to tissues [40–44], to normal cells [45–48], and to extracellular matrix [49, 50]; to measuring the penetration of tumor cells through tissue barriers [51–61]; and to measuring tumor cell mobility and response to chemoattractants [10, 62–75]. These assays have yielded valuable information regarding the biochemical events required for invasion.

2. Extracellular matrices

Considerable progress has been made in defining extracellular matrices. Each tissue-specific matrix is composed of a unique set of components which account in part for the tissue's organization and physical properties [76]. Collagens are the major structural element in these matrices. More than ten such collagenous proteins are known, and these differ chemically, genetically, and immunologically [77]. The interstitial collagens present in various fibrous structures (Types I and III) and in cartilage (Type II) are closely related in sequence, have helical domains of similar length, and form highly ordered fibrils. Other collagens have been described which are larger (Type VII), shorter (Types IX and X), or which have little helical character (type VIII). Collagen Types IV and VI are arranged in the tissues in nonfibrous structures. Such variety allows these proteins to form the scaffolding for diverse histological structures.

Type IV collagen is a unique collagen specifically and uniquely associated with basement membrane [78, 79] (Table 2). It comprises about 20% of the protein of basement membrane [80, 81] and serves as the major structural component [82, 83]. The exact size of the collagen molecules in the basement membrane is not definitively established. Some studies suggest that the biosynthetic form, Type IV procollagen (M_r = 185,000 and 170,000) is converted to a smaller molecule, Type IV collagen, (M_r = 160,000 and 140,000) which is incorporated into the matrix [83, 84]. Other studies suggest that processing of Type IV procollagen does not occur, and that the biosynthetic form is incorporated into the matrix [85–90]. Recent studies have found that the collagenous component released by reducing agents from lathyritic tissues resembles the biosynthetic protein, the procollagen Type IV molecule, in the size of its component chains [81]. The consensus of opinion favors a model for the arrangement and interactions of procollagen Type IV procollagen with laminin and heparan sulfate proteoglycan. Type IV collagen differs both chemically and structurally from collagen Types I, II, and III which form fibers in interstitial connective tissues. Unlike the Type IV collagen, the interstitial collagens are formed by proteolysis from soluble precursors and are insoluble under physiological conditions. Unlike other collagens, Type IV collagen contains nonhelical segments [91] and does not form a fibrillar matrix [92]. The

Table 2. Components of basement membrane

Component	M_r	% of BM
Type IV collagen	170–185 kd	20
Laminin	10^6 daltons	30–50
Heparan sulfate	110 kd — 10^6 daltons	1–2
Entactin	150 kd	<1
Nidogen	100 kd	<1
Fibronectin	220 kd	Variable
Type V collagen	120 kd	Variable

nonhelical segments of the molecule confer a greater degree of flexibility to the molecule and presumably create a more elastic matrix [93]. The arrangement of Type IV molecules in the basement membrane is unique; the molecules appear to be arrayed in an open network with like ends in opposition and cross-linked [94–96]. This arrangement confers tensile strength on the matrix and at the same time creates an elastic structure. Type IV collagen also contains binding sites for other components of basement membrane, including heparan sulfate proteoglycan, fibronectin, and laminin, as well as, perhaps, other specific components secreted from the underlying interstitial stroma [80, 81, 94, 97, 98].

Extracellular matrices also contain tissue-specific proteoglycans. These are glycoproteins, but so extensively modified by the addition of sulfated glycosaminoglycan chains that they are separately classified [99]. Proteoglycans may have the same type of glycosaminoglycan chains attached to different core proteins [100]. To date, eight distinct species of proteoglycans have been described whose functions vary from maintaining tissue turgor in cartilage, to creating a permeability barrier in basement membranes, and to maintaining optical transparency in the cornea.

Fibronectin and laminin are two well studied matrix glycoproteins that are the most relevant to this chapter. Fibronectin (M_r = 440,000) [25, 101] is composed of two very similar chains linked at one end by disulfide bonds. This protein is widely distributed in all fibrous tissues, in plasma (>0.5 mg/ml), and in other sites including the basement membrane zone [25, 26, 101, 102]. The fibronectin in basement membranes may be produced by adjacent stromal cells or have accumulated there from the fibronectin that circulates in the blood [103]. Fibronectin is known to bind to Type IV collagen [97, 104], heparan sulfate [101], fibrin [105], and to most other glycoproteins of the extracellular matrix [25, 26]. Recently, several reports have indicated that the shape of fibronectin molecules may vary from globular to extended forms depending on the ionic strength and pH of the extracellular environment [106–109]. This could have an effect on cell binding. The cellular receptor for whole fibronectin has not been isolated. Initially, reports of a cellular fibronectin binding domain (M_r = 85,000) in the fibronectin molecule for the cellular receptor have suggested the presence of a unique cell surface receptor [110]. More recently, a unique 140 kd fibronectin binding domain has been isolated [111].

Laminin [94] (M_r = 10^6) has a distinctive cruciform shape and is localized almost exclusively to basement membranes, with negligible levels (<30 ng/ml) in the circulation. Laminin is a major constituent of basement membranes constituting 30–50% of the protein [94] (Table 2). Laminin found exclusively in basement membranes is synthesized by cells that normally reside on these basement membranes [112, 113]. *In vitro*, laminin presents itself as a cross-shaped molecule with three short arms and one long arm [114–116]. All four arms have globular end regions, the long arm end region being different from the three short arm end regions [117]. One or more globular end regions of the short arms promote cell spreading and also bind to Type IV collagen [117–119].

The long arm of laminin contains a heparin-binding site [118, 119]. The carbohydrate composition of the globular end regions of laminin is different from that of the rod-shaped regions [116]. The long arm has recently been shown to stimulate neurite outgrowth [120]. The intersection of the short arms contains numerous disulfide bonds and is relatively protease-resistant [114, 115, 119, 121, 122]. The protease-resistant central region of the laminin molecule binds to a specific cell surface receptor (M_r = 67,000) for laminin [115, 116]. Certain types of normal and neoplastic cells contain high-affinity cell surface binding sites for laminin (kd = 2n M) [115, 116, 123–126]. Recently, specific laminin binding to sulfated cell surface glycolipids of erythrocytes has been reported [127]. Some controversy exists as to which components(s) link laminin to the basement membrane. It has been found that laminin binds to native Type IV [80, 97, 128] in a saturable manner with a dissociation constant of 5×10^{-7} M [98, 128]. This suggests that there is a single class of binding sites. However, since laminin also binds to heparan sulfate proteoglycan [83, 129, 130], multiple interactions probably account for the localization of laminin to basement membranes.

Laminin has been shown to regulate a variety of biological functions. These activities include cell attachment and spreading, tissue remodeling, embryogenesis, [90, 113, 131], cell migration [74, 132], and cell growth and differentiation [133, 134].

Part of the interest in fibronectin and in laminin derives from their substantial effects on cellular morphology, growth, and differentiation, and that in some situations, these proteins exhibit opposing activities on the same cell. Their activities are probably mediated by distinct cell surface receptors with the cell surface, protein receptor for fibronectin [111] (M_r = 140,000) being distinct from the laminin receptor [123–126] (M_r = 67,000). Highly metastatic melanoma cells and other tumor cells exhibit more laminin receptors than less metastatic cells, perhaps accounting in part for their affinity for basement membranes [37, 39, 123] (Table 1). These receptors appear to be randomly arranged on cultured tumor cells [135] while a more polarized pattern is observed with normal cells. It is possible that there is an abnormal accumulation or handling of this receptor in the malignant state, although this has not been shown directly. However, both laminin and fibronectin are multidomain proteins which bind to a variety of other ligands including gangliosides, heparan sulfate, collagen IV, and other glycoproteins [25], and these interactions could evoke diverse cellular responses.

It should also be noted that cells have receptors for various collagens. These include anchorin [136] (M_r = 131,000), which binds to interstitial collagens and is present on tendon fibroblasts and chondrocytes, and colligin [137] (M_r = 54,000), which is present on parietal endoderm cells and binds to Type IV collagen. Various proteoglycans also bind to cell surface receptors [138, 139]. Although the physiological significance of these receptors has not yet been fully evaluated, they allow for multiple, high affinity interactions between the cell and its matrix.

3. Tumor cell metastasis and basement membranes

Basement membranes are ubiquitous structures which separate all epithelial tissues, except liver, from stroma, as well as encircle nerve, muscle, fat, and smooth muscle cells. All basement membranes contain Type IV collagen, laminin, and a specific heparan sulfate proteoglycan [140]. These molecules bind to one another and form an integrated supramolecular structure of 5–10 nm cords [92]. In this structure, Type IV collagen molecules are joined at their ends in an open network [94] which is coated with the other components to form the cords [92]. Laminin binds to certain sites on the type IV collagen molecule [114, 116, 141] and through separate domains to heparan sulfate proteoglycan [142]. The glycosaminoglycan side chains of the proteoglycan are displayed along the surface of the basement membrane and create a charged barrier to the passage of proteins [143].

Ultrastructural, chemical, and biological analyses indicate that basement membrane components interact with each other to form homogeneous sheets which resist the physical penetration of cells [144]. Furthermore, due to its unique structure, Type IV collagen is not degraded by the traditional collagenases that attack stromal collagens. Rather, degradation of Type IV collagen is carried out by a specific enzyme, Type IV collagenase, produced by highly metastatic melanoma cells [34, 145], other invasive tumor cells, and probably by other cells which have the capacity to cross basement membranes. Indeed the degradation of the Type IV collagen network is likely to be one of the important steps in the lysis of basement membranes, since laminin and heparan sulfate proetoglycan are degraded by a wide variety of proteases. Recently, metastatic melanoma cells have been shown to produce a discrete heparanase [146]. The ability of melanoma and other tumor cells to degrade Type IV collagen does not insure that they will be metastatic. Similar levels of collagen IV degrading activity were found in certain metastatic and nonmetastatic lines, and the distinction in their ability to form lesions was attributed to differences in their reaction with the host immune system [147].

4. Melanoma cell adhesion to basement membranes

The breaching of basement membranes is thought to initially involve (Step 1 in Figure 1) the attachment of the tumor cells to vascular endothelial cell surfaces with subsequent passage to the underlying matrix [35, 43, 46, 50]. From studies on the adherence of tumor cells to blood vessel surfaces *in vitro*, it was found that metastatic tumor cells preferentially adhered to the basement membrane underlying the endothelial cells [148]. Based on the affinity of the cells for defined protein substrates, it appears that an important tumor cell-basement membrane attachment is mediated by laminin [33, 124] (and see below) (Table 1).

Using *in vitro* assays, it was found that metastatic tumor cells attached more

Cell Line	Type I	Type IV
BL 6	22	39
PM 2	21	44
C3H	54	29

Figure 2. Percent attachment of tumor cells to Types I and IV collagen substrates. B16-BL6 (murine melanoma bladder selected; obtained from Dr I Hart, ICRF, London, England); T241–PM2 (murine fibrosarcoma — pulmonary metastasis selected; obtained from LA Liotta, LP, NCI, NIH) and C3H (tumorigenic nonmetastatic fibrosarcoma obtained from E Schiffmann, LP, NCI, NIH) were removed from culture with 0.1% EDTA in PBS and plated on collagen substrates. Cells were incubated for 90 min in 95% air, 5% CO_2 at 37°. Attached cells were removed with 0.01% trypsin in PBS and counted electronically.

rapidly and in greater numbers to Type IV collagen than to other collagen substrates [148] (Figure 2). Because of the known activities of fibronectin and laminin in binding cells to matrix, these glycoproteins were tested on the attachment of various malignant and benign tumor cells, including murine melanoma and fibrosarcoma cell lines, adenocarcinoma cells from human breast, and adenovirus transformed cells from rat. Such observations, although still limited to a few cell lines, indicate that highly malignant cells show a distinct preference for attachment to laminin, whereas the tumorigenic but nonmetastatic cells may prefer fibronectin [32, 33, 122, 149]. These differences may be primarily quantitative, since laminin can stimulate the attachment of both high and low metastatic cells to Type IV collagen [150].

The role of laminin in metastasis has been evaluated in several systems and appears to be pleiotropic. Coinjection of melanoma cells and antibody to laminin reduces the incidence of lung tumors [33, 37, 38]. Similarly, coadministration of the cells with fragments of laminin which lack collagen and heparan binding activity, but which bind to the laminin receptor, also decreases the number of metastases [37, 38, 151, 152]. In contrast, the injection of laminin along with other melanoma cells increases the number of lung colonies [33, 37, 38] (Figure 3). Taken together, these results suggest that the binding of the cells to laminin is necessary for their adherence to exposed areas of capillary basement membrane or even to the luminal surface of the vascular endothelial cells. In this regard, it was also found that more laminin was produced by highly metastatic cells and retained on their surfaces in comparison to less malignant variants [39]. Several other specific biological properties of laminin have been reported, including an increase in the production of Type IV collagenase by tumor cells exposed to laminin [153]. It is chemotactic for certain tumor cells [152, 154–156] and promotes cell growth [157, 158]. In addition, as discussed below, melanoma cells cultured *in vitro* with laminin become more metastatic,

Additions	Pulmonary Metastases	
	Growth Media Plus Fibronectin	Growth Media Minus Fibronectin
None	38 ± 12	137 ± 20
Murine Fibronectin (50 μg/ml)	N.D.	52 ± 14
Antifibronectin Antibody (1 μg/ml)	36 ± 10	N.D.
Laminin (50 μg/ml)	134 ± 16	183 ± 34
P1 or C1 Laminin Fragment (1 μg/ml)	6 ± 4	4 ± 3
Antilaminin Antibody (1 μg/ml)	5 ± 4	5 ± 3

Figure 3. Metastasis of BL6 cells *in vivo* after culture for one week in the presence of extracellular matrix components. The standard growth medium consisted of RPMI 1640 supplemented with fetal bovine serum, which gave a fibronectin concentration of 50 μg/ml, plus glutamine, penicillin (10 U/ml), and streptomycin (100 μg/ml). Removal of fibronectin from the serum was accomplished by passing whole serum over a gelatin affinity column. Murine fibronectin was obtained from the serum of syngeneic C57/BL mice by gelatin affinity chromatography followed by heparin Sepharose chromatography. Purified laminin was added at a concentration of 50 μg/ml. In some experiments antibodies to laminin or fibronectin (1 μg/ml) were added to certain dishes on the last day. P1 or C1 laminin fragments were added at a concentration of 0.25 μg/ml. All cells were grown in culture until confluent for one passage. A suspension of cells (2×10^5 in a volume of 0.1 ml), determined to be viable by the trypan blue dye exclusion tests, was then injected into the tail veins of syngeneic, pathogen-free male mice six weeks of age. Twenty-five days later the mice were killed and their lungs were removed and immersed in Bouin's fixative. Tumors were counted with a dissecting lens (× 10). Twenty-four animals were used per assay group. Values are mean numbers of tumors ± standard deviations. The metastatic potential of cells grown with fibronectin differed significantly from that of cells grown without fibronectin ($p < 0.001$). Addition of laminin significantly increased the number of lung colonies ($p < 0.001$). N.D., not determined (37).

while those cultured with fibronectin or anti-laminin antibody express a less metastatic phenotype (Figure 3).

5. Assessment of invasive activity in vitro

Various test systems have been devised to assess the invasiveness of tumor cells. Using *in vitro* assays, metastatic cells were found to invade through isolated chorioallantoic membranes into whole tissue fragments and through tissue walls (see above). Melanoma cells which had penetrated through the bladder wall *in vitro* were subcultured and the resulting cells expressed a higher metastatic activity [55, 56]. In related work, segments of human amion [53] and lens capsule [51, 52] have been used as *in vitro* barriers to the passage of tumor cells in modified Boyden chamber devices. Over the course of several days in culture, tumor cells penetrate these barriers with the most highly metastatic cell lines penetrating the barrier in the greatest numbers. The importance of these assays is that they permit separate assessment of the invasive potential of the melanoma cells under study.

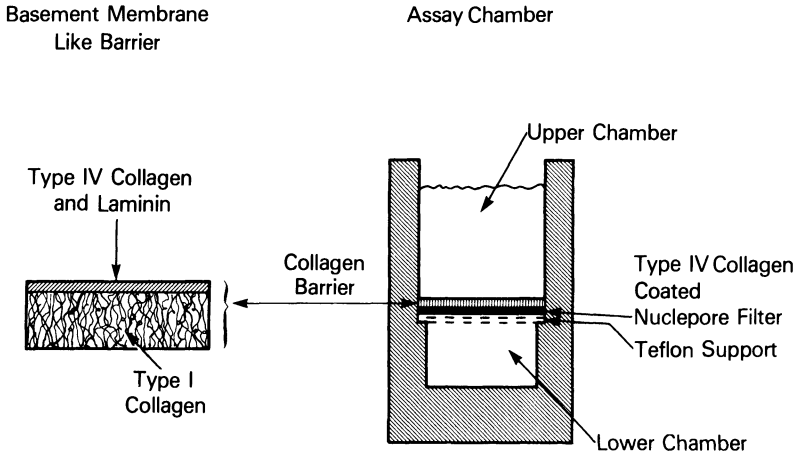

Figure 4. Diagram of a layered Laminin-Type IV collagen — Type I collagen matrix barrier and of the *in vitro* invasion assay chamber.

	Parent B16-F1	Selected B16-F1
% Attachment To Type IV Collagen	38	62
Laminin Binding (CPM/2 X 10^5 Cells)	3,700	11,500
Type IV Collagen Degradation (CPM/10^6 CELLS)	5,000	10,500
In Vitro Invasion of Barriers (Cells/High Power Field)	12	52

Figure 5. Differences observed between parental B16-F1 melanoma cells and selected B16-F1 melanoma cells. The selected cells were obtained by subculturing those cells that had penetrated the artificial basement membrane after 48 hours in culture.

Since the human amion, the lens capsule, and bladder wall contain a layer of basement membrane, an artificial matrix with Type IV collagen and laminin coated onto a pressed disc of Type I collagen was constructed [159] (Figure 4). When mounted between two compartments of a modified Boyden chamber, this reconstituted basement membrane was found to block the passage of normal fibroblasts, normal epithelial cells, and nonmalignant tumor cells. In contrast, metastatic tumor cells are able to penetrate this barrier and in numbers proportional to the metastatic activity the cells exhibit *in vivo*. Since membranes formed by Type I collagen and fibronectin were not inhibitory, these observations suggest that Type IV collagen and laminin are the components of the

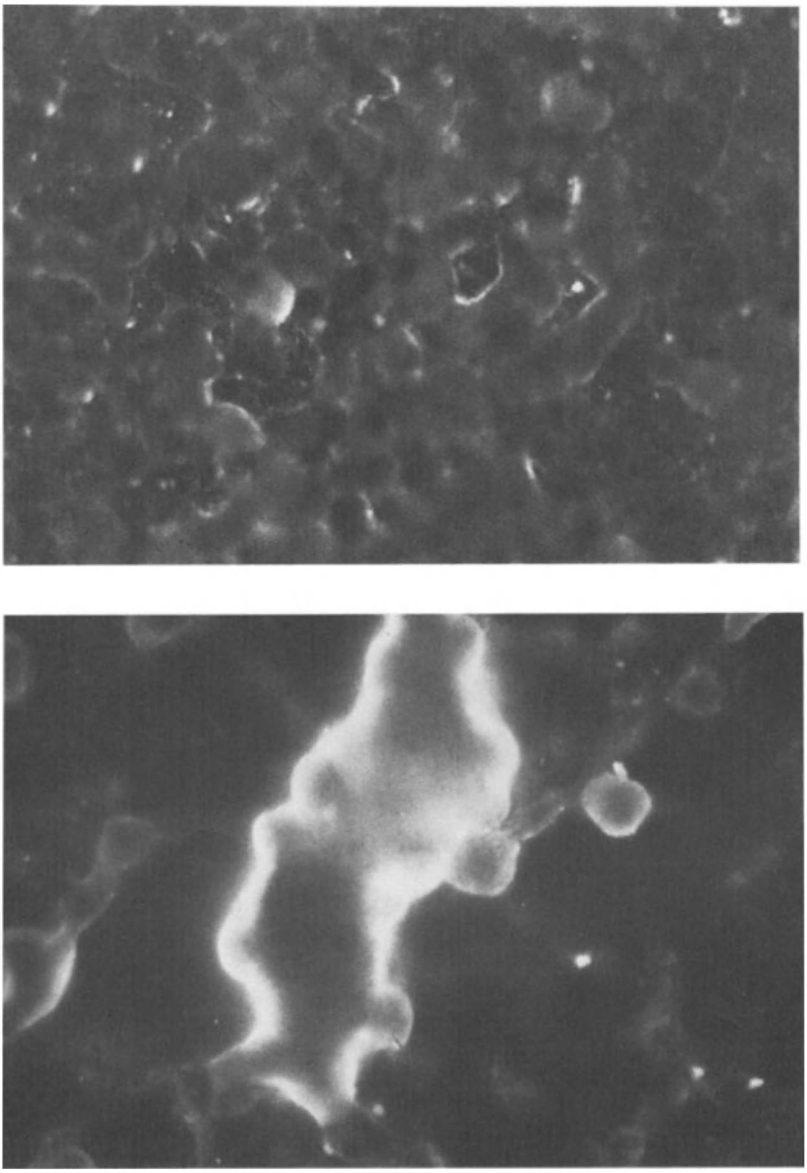

Figure 6A, 6B. Photomicrograph (× 400) of indirect immunofluorescence, stained for cell surface laminin of: A) B16-F1 parent cells and B) B16-F1 selected cells.

basement membrane that block the migration of cells. It should be noted that less than 1% of the cells added to the *in vitro* assay chamber actually cross the barrier. This data would be in agreement with other *in vivo* data of B16 tumor

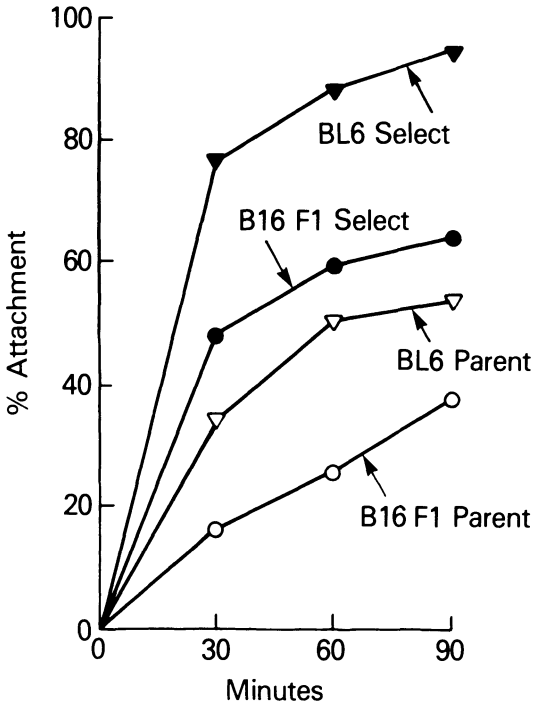

Figure 7. Kinetics of a 90 minute attachment assay of B16-F1 parent and selected cells and B16-BL6 parent and selected cells. Cells were allowed to attach to native Type IV collagen coated petri dishes.

growth and metastatic potential [160]. However, these cells can be isolated and subcultured for further studies, and such sublines of B16-F1 melanoma cells have been shown to be more invasive and metastatic than the parent line [159] (Figure 5). Interestingly, most of the phenotypic characteristics ascribed to metastatic cells (Table 1) increase when the subline is examined. Indirect immunofluorescence staining for laminin reveals substantial increases of cell surface laminin (Figure 6A, B). This data can be coupled directly to increases in number of laminin receptors — 15,000 receptors/B16-F1 parent cell as opposed to 33,000 receptors/B16-F1 selected cell. Additionally, when the number of 140 kd fibronectin receptors are examined, a concomitant decrease is observed. The increased expression of metastatic phenotypic characteristics is not limited to the B16-F1 melanoma cell line but is inducible in all melanoma cell lines examined. For example, when attachment of B16-BL6 melanoma cells to Type IV collagen is examined, the selected cells express a 65% increase in attachment over the parent cell line (Figure 7). Since in the selection process these cells are exposed to laminin, perhaps the limiting step in achieving a more metastatic phenotype is laminin exposure.

6. Local factors modulate metastasis

Highly malignant cells often express decreased metastatic activity after extended time in culture, but regain malignancy when allowed to grow in animals [161, 162]. In addition, some studies suggest that metastatic activity can be increased or induced by culturing B16 melanoma cells on less adherent plastic substrates [162], although others have observed the cells to fuse rather than to regain malignancy [163]. Even more striking evidence for the influence of membrane-matrix components on metastatic potential is the direct demonstration that membrane components, in the form of membrane vesicles from highly metastatic cells, were found to increase the malignancy of other less metastatic tumor cells [164]. The active factor(s) in the vesicles has not been determined but could possibly include receptors for matrix proteins. One reason for suspecting this is the observation that the metastatic activity of tumor cells varies when the cells are exposed to laminin or to fibronectin *in vitro* [37] (Figure 3). Laminin increases while fibronectin reduces metastatic activity. These effects are relatively rapid, requiring only one to two days' exposure to the proteins, and are reversible. These responses appear to be related to a change in the phenotype of the cells, as observed also with a variety of normal cells exposed to these matrix proteins. It is likely that laminin enhances those cellular processes that are characteristic of the metastatic phenotype, including increased expression of laminin receptors, high affinity for basement membrane, enhanced ability to degrade basement membranes, and increased motility and growth potential.

The observation that metastatic cells often colonize only certain tissues as targets suggests that factors other than binding to and degrading basement membrane are necessary for the cells to complete the metastatic cascade. Since all basement membranes contain Type IV collagen and laminin, it must be a combination of anatomical factors associated with the dissemination of the cells, plus local factors, that are the critical determinants of the site at which a secondary lesion will develop. Some recent studies suggest that the cells recognize tissue specific factors that activate their motility and direct their invasion. Brain, lung, and liver appear to contain different factors, probably small polypeptides, that attract tumor cells that metastasize preferentially to each tissue [10] (Figure 8). Such factors might diffuse into the basement membranes surrounding the tissue thus creating a unique recognition element at the level of the capillary basement membrane. Since such chemoattractants can increase tumor cell penetration tenfold in the *in vitro* invasion assay, they may play an important role in determining whether a tissue is invaded. Subsequent to invasion, other factors will determine whether a cell can survive and proliferate, and these are undoubtedly highly specific and of great importance to the development of a lesion.

Many, *but not all*, metastatic tumor cells have a similar phenotype. These are transformed cells with high numbers of laminin receptors, high affinity for basement membranes, and the ability to produce basement membrane degrading

	TISSUE EXTRACT			
Cell Line	Brain	Lung	Liver	BSA
B16BR2	41	12	8	1
T241-PM2	18	53	4	1
M-50	26	21	50	5
CRL1545	0	0	0	0

Figure 8. Directed invasion (penetration through the artificial basement membrane towards a tissue specific chemoattractant) of tumor cells. The murine B16-BR2, which has high brain colonization potential, and the murine M50–76 reticulum cell sarcoma cell line, which preferentially metastasizes to liver, were obtained from Dr I Hard, ICRF, London, England. Murine T241-PM2 fibrosarcoma cells (Dr LA Liotta, LP, NCI, NIH) have high lung-colonizing potential. CRL1545 (normal human skin fibroblasts) are obtained from the American Type Culture Collection. All cells were allowed to penetrate the artificial basement membrane for 48 hours at 37°. The data are expressed as the average number of cells per high power field (× 400) [10].

enzymes. Such characteristics help the cells traverse this critical barrier and subsequent exposure to these matrix molecules might induce a more malignant phenotype. Even with an increased malignant phenotype, colonization may be a rather rare event unless the cells respond to local factors that amplify the numbers of cells recruited to the site and induce the cells to invade. These may include tissue specific attractants and matrix molecules such as laminin.

Understanding the specific steps involved in the metastatic process should allow development of antimetastatic regimens directed at those activities specific to metastatic tumor cells. Due to the heterogeneity of primary tumors, other mechanisms for metastasis undoubtedly exist.

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3. Structure, Function, and Biosynthesis of Ganglioside Antigens Associated with Human Tumors Derived From the Neuroectoderm

Jay M. Rosenberg and David A. Cheresh

1. Introduction

The process of human tumor proliferation and metastasis undoubtedly involves surface structures that interface with the local environment, i.e., the extracellular matrix and host tissue. Therefore, it is imperative to understand the molecular and cellular events at the tumor cell surface that are associated with proliferation and metastasis. To this end, monoclonal antibodies to a variety of human tumor-associated antigens have enabled investigators to characterize molecular differences between tumor and normal tissues and thus advanced an understanding of the functional role of these antigens. Recent technological advances have now made it possible to use monoclonal antibodies (Mabs) for the characterization of a number of complex carbohydrate antigens on tumor cell-associated glycolipids [1–7]. In this regard, a number of sialic acid-bearing glycolipids, i.e., gangliosides, were shown to contain antigenic determinants highly restricted to human melanoma [2–5], neuroblastoma [6], and colon carcinoma [7]. The use of Mabs specifically directed to gangliosides may help strengthen and extend observations that implicate these molecules as putative cellular receptors for hormones [8], toxins [9], growth factors [10], and viruses [11], as well as to gain further evidence for their possible role in cell-substratum interactions [3, 12–21]. One distinct advantage of Mabs directed to carbohydrate antigens is their potential to establish a structure/function relationship for these determinants on the tumor cell surface. This is possible primarily because Mabs can recognize a carbohydrate determinant with known composition and anomeric linkages. Thus, by using Mabs to defined oligosaccharide structures on the tumor cell surface, one can pose questions regarding the functional properties of these structures as they relate to the malignant/metastatic phenotype. This is far more difficult to accomplish with Mabs directed to protein or glycoprotein antigens whose epitopes are in most cases structurally ill-defined, since they depend on unpredictable conformation and three-dimensional structure, and their complete primary amino acid sequence frequently is not available.

Malignant transformation of a variety of cell types has been associated with a change in glycolipid metabolism and expression [1, 22, 23]. Since Hakomori and

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Murakami [24] first reported characteristic changes in the glycosphingolipid compositions and metabolism of cultured cells by tumor viruses, it has been well established that malignant transformation of cells induced by either tumor viruses or chemical carcinogens is often associated with alteration in metabolism or phenotypic changes of cell surface glycosphingolipids [1, 22, 23]. These changes have been related to abnormal or disordered sociological behaviors of the transformed cells leading to the establishment of tumors.

To date, several studies have been conducted to examine the alteration in activities of the biosynthetic enzymes that catalyze reactions of the ganglioside pathways during tumorigenesis [25–28]. In this regard, work is ongoing in our laboratory to isolate and characterize the enzymes responsible for synthesizing GD2, GD3, and O-acetylated GD3 from a variety of tumor cell lines expressing these gangliosides. This review will describe some of our recent efforts to establish the structural and functional properties of ganglioside antigens on the surface of neuroectoderm-derived tumors using murine antiganglioside Mabs. The relationship of the expression of these gangliosides to the activities of their corresponding biosynthetic enzymes will also be discussed. This review is not intended to be a comprehensive review of all the extensive work accomplished in ganglioside research, but will only address studies performed by our laboratory in collaboration with several of our colleagues.

1.1 Proposed functional properties of cell associated gangliosides

The actual function of glycolipids on the cell surface has not been completely delineated, although several researchers using biochemical and immunochemical approaches have proposed that glycolipids (particularly gangliosides) are involved in cell-substratum interactions [3, 12–21, 29, 30] and thus potentially serve as cell surface receptors for the adhesive protein fibronectin [12–14]. Gangliosides have also been implicated as receptors for tetanus and cholera toxins [9], interferon [32], and as potential receptors for thyroid stimulating hormone [8] and macrophage inhibition factor [33]. The β-subunit of cholera toxin is known to bind and cross-link five GM1 molecules on the surfaces of rat thymocytes. Thereby, it actually evokes the mitogenic stimulation of such cells [34], suggesting that gangliosides expressed on the surface of a cell can be involved in the potentiation of a transmembrane signal resulting in lymphocyte activation. In addition, recent evidence suggests that cell surface gangliosides can modulate glycoprotein receptor function [10, 35]. Thus, in the presence of the disialoganglioside GD3, the serotonin receptor on neural cells shows an increased affinity for serotonin [35]. In addition, the receptor for platelet derived growth factor can be functionally modulated by gangliosides [10]. The addition of an exogenous calcium/ganglioside complex to rat brain membranes resulted in the direct phosphorylation of five proteins and the inhibition of three others [36], further suggesting that gangliosides on the cell surface are in some way involved in potentiating a transmembrane signaling event. Specific gangliosides have also been implicated as host cell receptors for viruses [11, 37]. In this

regard, Sendai virus was shown to bind to gangliosides with structures similar to GD1a, GT1b, and GQ1b, all of which contain at least one sialic acid residue attached to a terminal galactose residue [37]. However, gangliosides lacking this moiety (i.e., GM1 and GD1b) are incapable of interacting with the virus. In addition, recent evidence indicates that the monosialoganglioside GM3 serves as a specific receptor for Influenza A virus, thereby allowing for the adsorption-fusion process of viral infection [11]. In this study, evidence was presented that the critical determinant involved a sialic acid moiety connected via α 2–3 linkage to an external galactose present on a ganglioside. Thus, depending on the particular oligosaccharide composition and structure, gangliosides have a wide range of potential biological functions and are therefore useful to study as cell surface antigens.

The fact that tumor cells express altered carbohydrates on both glycolipids and glycoproteins [23] suggests that Mabs directed to such determinants may aid in elucidating some of the structural changes associated with malignant transformation. This, in turn, may also lead to an understanding of certain genetic abnormalities or modifications within the tumor cell, since the expression of carbohydrate antigens is under direct control of a variety of glycosyltransferases. In this regard, transfection of rat cells with the transforming gene of human Adenovirus Type 12 DNA and its transcriptional subunits were recently reported to induce the synthesis and expression of the disialoganglioside GD3 [31]. The synthesis of this ganglioside was subsequently shown to result from activation of a highly specific sialyltransferase, i.e., GD3 synthetase [38]. The expression of glycolipids on cell surfaces can also be under epigenetic control and thus vary according to cell cycle [39], density [39], and cell contact [40]. In some cases, ganglioside expression is directly linked to a particular stage in differentiation [22, 23, 39], suggesting that their appearance on the tumor cell surface may be 'oncofetal' in nature. Based on this hypothesis and the fact that these molecules are readily shed from the plasma membranes of tumor cells [6, 15, 22], glycolipid antigens were used successfully as markers for the immunodiagnosis of certain tumors [6, 15, 22, 41].

Recent studies in mice [15, 42] and in man [43] indicate that Mabs directed to certain tumor-associated glycolipid antigens may be particularly useful in the immunotherapeutic treatment of cancer patients. Results from our laboratory show that human melanoma cell lines derived from a patient's metastatic lesions have a marked increase in the expression of disialogangliosides GD2 and GD3 when compared to cell lines derived from primary melanoma lesions of the same patient [26]. The increased synthesis and expression of GD3 in these cells correlates directly with the increased activity of a specific sialyltransferase that converts the monosialyated ganglioside GM3 to GD3 [26]. Based on these studies, Mabs directed to glycolipid antigens, particularly gangliosides, represent potentially useful reagents for immunodiagnosis, immunotherapy, and biological characterization of malignant cells.

Most of the information regarding the functional role of glycolipids on the cell surface has come from studies involving their addition to cultured cells with

the assumption that these glycolipids appropriately embed in the lipid bilayer. However, this research is greatly facilitated by the use of Mabs that are directed to the carbohydrate moiety of the glycolipid molecule naturally exposed on the cell surface. Moreover, these Mabs provide the opportunity to learn much about the structural properties of glycolipids and their role as antigens on the surfaces of tumor cells.

Glycolipids in a pure form are relatively nonimmunogenic; however, upon appropriate antigen presentation, one can generate highly specific Mabs to their carbohydrate moiety. In fact, Mabs directed to antigenic determinants of glycolipids can have a similar degree of precision and specificity as that of Mabs to antigenic epitopes on proteins. As with many antigens, the density of a given glycolipid on the cell very much affects its antigenicity and potential immunogenicity. Other factors have also been implicated in cell surface expression of glycolipid antigens, including the chain length and ceramide composition (lipid backbone) as well as coexisting glycolipids [44]. Although the antigenic epitope of the glycolipid involves its oligosaccharide moiety, the ceramide composition (i.e., the fatty acid chain length) may also play significant roles in the antigenicity and/or immunogenicity of glycolipid on the cell surface.

1.2. Monoclonal antibodies detect ganglioside GD2 on the surface of tumors derived from the neuroectoderm

In our laboratory, Mabs were generated against intact human neuroblastoma cells (LAN-1), which are known to express an abundant level of the disialoganglioside GD2. After screening the hybridoma supernatants with a solid-phase lipid ELISA, three antiglycolipid antibodies of IgM isotype and two IgG3 isotype were isolated and later shown to react specifically with the oligosaccharide portion of GD2 [3]. These Mabs did not react with the carbohydrate portion of glycoproteins, since they failed to immunoprecipitate proteins or recognize them by western blot analysis [17].

In order to determine the specificity of GD2 expression on human-derived tumor cell lines, Mabs 126 (IgM) and 14.18 (IgG3) were applied in an ELISA assay to screen a variety of human tumor cell lines for the expression of GD2. As shown in Table 1, this antibody reacted primarily with neuroectoderm-derived tumors, particularly neuroblastoma, melanoma, and glioma, but showed little or no reactivity with a variety of other tumor cell lines. Perhaps most interesting was the detection of GD2 in small cell carcinoma of the lung (SCCL). At present, it is not certain whether SCCL arises from a common stem cell of endodermal origin or from a specialized neuroendocrine cell of the lung. It has been suggested that SCCL derives from the Kulchitsky cell of the lung which is thought to be of neuroectodermal origin [44, 45]. The presence of GD2 on SCCL cells thus lends further support to the neuroectodermal origin of this tumor type.

The tissue distribution of GD2 was determined by using these Mabs in indirect immunoperoxidase staining to screen either fresh frozen tissues or

Table 1. Elisa reactivity of mab 14.18 with cultured cell lines[a]

Neuroblastoma		*Small Cell Lung Carcinoma*	
LAN-1	+ + + +	T293	+ + + +
LAN-5 Duke	+ + + +	NIH-N417	+ + + +
SMS-KCNR	+ + + +	NIH-H-82	+ +
CHP-134	+ + + +	NIH-H-69	+ +
SMS-KAN	+ + + +	*Other tumor cell lines*	
NMB-7	+ + + +	Pancreatic carcinoma (fast growing)	—
IMR-6	+ + + +	Pancreatic carcinoma (slow growing)	—
SK-N-AS	+ + +	Pancreatic carcinoma 1320 (metastatic)	—
Melanoma		Adenocarcinoma of lung (UCLA-P3)	—
Melur	+	*Lymphoblastoid Cell lines*	
A375 P	+ + +	L-14 (B-cell)	—
A375 Met	+ + +	LG-2 (B-cell)	—
FM8	+ + +	MOLT-4 (T-cell)	—
FM9 Met	+ + +	HPB-All (T-cell)	—
M-14	+ + + +		
M-21	+ + + +		
Glioma			
U138 M6	+ + + +		

a absorbance at 492 nm: 0.15–0.3 (+); 0.3–0.6 (+ +); 0.6–0.9 (+ + +); 0.9–>2.0 (+ + + +); 0–0.050 (−).

formalin-fixed paraffin-embedded tumor, normal adult, or normal fetal tissues. As shown in Table 1, Mabs 126 and 14.18 reacted strongly with both freshly excised frozen as well as formalin-fixed neuroblastoma, melanoma, and SCCL tissues failed to show reactivity with most other tumor tissues tested. Among the normal tissues examined, only brain, nevus, and skin melanocytes showed minimal staining.

1.3. Detection of GD2 by monoclonal antibody in the sera of neuroblastoma patients serves to monitor tumor burden

Neuroblastoma, a neoplasm of the peripheral autonomic nervous system, is the second most common solid tumor in children. The majority of patients initially diagnosed with neuroblastoma already have advanced disease (Stage III and IV) for which treatment protocols have proven largely unsuccessful [47]. In fact, once detected, many neuroblastomas are quite difficult to distinguish histopathologically from other round cell tumors in children, such as lymphoblastic lymphoma, leukemia, rhabdomyosarcoma, or Ewing's sarcoma [48, 49]. Therefore, it is most useful to have access to a simple serum assay for the detection and possible monitoring of this disease. Consequently, we investigated whether detectable levels of GD2 were secreted into the sera of neuroblastoma patients. In this regard, we used Mab 126 as a probe to measure the GD2 content of lipid extracts from sera of children with active disease and compared such values directly to those of normal children obtained with this same antibody by immunostaining on TLC plates. As shown in Figure 1, all four patients' sera tested showed pronounced staining of a component that comigrated with

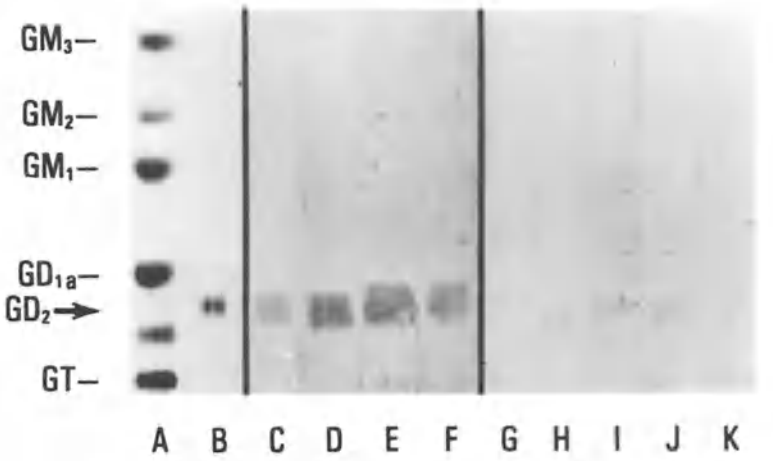

Figure 1. Reactivity of Mab 126 with GD2 in neuroblastoma sera. Immunostaining of gangliosides separated on TLC with Mab 126 was performed as described [6]: Lane A = migration of different ganglioside standards stained with resorcinol; Lane B = purified GD2; Lanes C–F = gangliosides extracted from sera of patients with active neuroblastoma; Lanes G–K = gangliosides from sera of normal children.

authentic GD2, whereas sera from normal children showed little or no staining. It was also found that after a brief deproteination of serum samples from neuroblastoma patients by chloroform: methanol (2:1), these extracts could serve as a source of GD2 and thereby effectively compete for Mab 126 binding in a solid-phase lipid ELISA [6]. The inhibitory effects of each extract obtained from 250 μl serum on Mab 126 binding was then compared to the level of inhibition achieved with a known amount of purified GD2. Such data permitted the calculation of the actual serum level of GD2 [6]. This assay made it possible to demonstrate that sera from 21/23 children with various stages (I-IV) of active neuroblastoma showed significantly elevated levels of GD2 ranging from 50–4,300 ng/ml as compared to 6/6 sera from neuroblastoma patients with no evidence of disease ranging from 5–25 ng/ml. Sera from 22/23 children with other pediatric tumors had GD2 levels ranging from 5–30 ng/ml which readily compared to 16/16 normal children having GD2 levels ranging from 1–20 ng/ml. The GD2 levels of patients with active neuroblastoma were compared to all other groups in a ranking evaluation test corresponding to Wilcoxan-Mann-Whitney and was shown to be statistically significant ($p < 0.001$). Preliminary results using blood serially drawn from these patients indicate that this assay may be effective in monitoring the level of disease prognostically (data not shown). The fact that many patients with neuroblastoma are less than one year of age suggests the obvious need for such a test that requires a minimal amount of blood. Thus, the use of a murine Mab directed specifically to the disialoganglioside GD2 made it possible to devise a simple and reproducible serum assay that could aid in the monitoring of tumor burden in patients with neuroblastoma.

1.4. Disialogangliosides GD2 and GD3 are involved in tumor cell-substratum interactions

Gangliosides have been proposed to serve as the cell surface receptor for fibronectin [12–14]. This concept was based on the fact that exogenous addition of gangliosides to cultured cells inhibited cell attachment and spreading on a fibronectin substrate [12–14]. The active moiety of the ganglioside was shown to reside in the oligosaccharide, and particularly the sialic acid moiety, since periodate oxidation of terminal sialic acid destroyed the inhibitory effects on cell attachment [12].

Initial studies from our laboratory implicating gangliosides in cell-substratum interaction were based on experiments using antiganglioside Mabs as specific functional probes. Immunofluorescence microscopy experiments were performed on M21 human melanoma cells grown on glass coverslips and removed with EDTA, a process known to generate substrate attached focal adhesion plaques [3]. Both GD2 and GD3 could be specifically localized within these structures with Mabs directed to these gangliosides [3]. Other membrane associated antigens, however, were not detected within these structures, indicating that focal adhesion plaques are probably not composed of indiscriminant membrane fragments [3]. Moreover, a biochemical analysis of adhesion plaques generated by human melanoma cells that were labeled metabolically with [^{3}H]-glucosamine demonstrated that both GD2 and GD3 are enriched at the points of melanoma cell contact with the substratum [3].

Based on the localization of GD2 and GD3 in the adhesion plaques and its surface distribution on human melanoma cells, the question arose as to whether Mabs directed to the oligosaccharide portion of these molecules could perturb cell attachment. To address this issue, M21 human melanoma cells were grown on fibronectin-coated plastic dishes, allowed to attach, spread, and reach confluency. The monolayer was then overlayed with growth media containing purified anti-GD2, anti-GD3, anti-HLA, or a nonbinding control Mab. Only cells treated with anti-GD2 or anti-GD3 in this manner showed a dose-dependent detachment and cell rounding within two to three hours of antibody exposure. The percentage of cells in the population affected was essentially identical to the relative percentage of GD2 (99%) and GD3 (62%) positive cells in the population determined by flow cytometric analysis [16]. In contrast, neither anti-HLA antibody, which binds to M21 cells, nor the nonbinding control Mabs caused any demonstrable effect on the cell monolayer. Furthermore, Mabs directed to either GD2 or GD3 had no effect on cells that did not express these antigens.

Ultrastructural analyses of the antiganglioside Mab-induced inhibition of melanoma cell attachment indicated that once the cells rounded up from the substrate, their attachment-promoting microprocesses became dislodged from the fibronectin-coated surface (Figure 2). Indirect immunolocalization with anti-GD2 as primary antibody and goat anti-mouse Ig conjugated to 30 nm gold particles, in conjunction with scanning electron microscopy, demonstrated that GD2 is most heavily expressed on these M21 melanoma cell attachment-

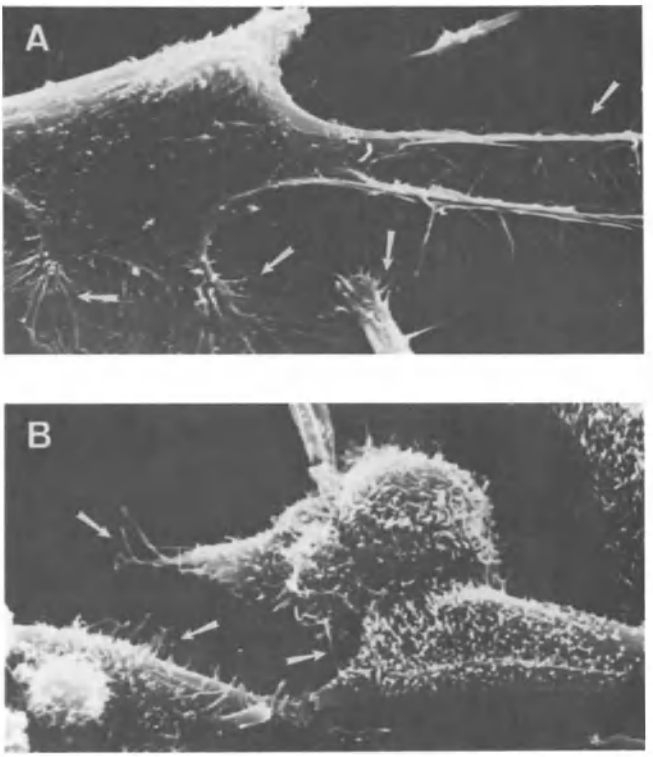

Figure 2. Detachment of M21 human melanoma cells from a fibronectin substrate by anti-GD2 Mab. M21 cells were allowed to attach and spread on a fibronectin coated coverslip for one hour. The cells were overlayed with 100 μg/ml irrelevant Mab (A) or anti-GD2 Mab 14.18 (B) for three hours at 37°C. The cells were fixed and prepared for Scanning Electron Microscopy as previously described [17]. Arrows correspond to attachment-promoting microprocesses.

promoting microprocesses as they neared the fibronectin substrate (Figure 3). In addition, immunolocalization by transmission electron microscopy on cross sections and longitudinal sections of melanoma cell microprocesses demonstrated that GD2 is enriched on these structures as compared to the cell body (Figure 4a). This technique has also allowed for the detection of this ganglioside directly as the points of cell contact (arrows) with the fibronectin substrate (Figure 4b, c). In fact, when M21 cells previously grown in suspension were allowed to adhere to immobilized fibronectin, GD2 and GD3 redistributed on the cell surface, from a random or uniform to a punctated distribution, into these microprocesses within 20 minutes at 37°C [17]. When these small cells were removed from the substrate with the divalent cation chelator EDTA, the gangliosides rapidly redistributed back to the uniform distribution on the cell surface [17]. Moreover, as M21 cells round up during mitosis, the GD2 and GD3 distribution on the cell surface becomes uniform [17], providing further

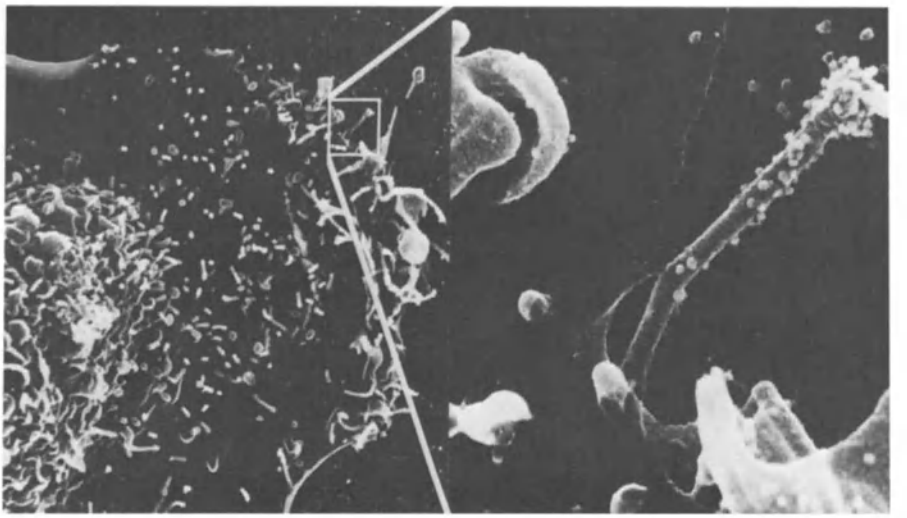

Figure 3. Immunolocalization of GD2 on M21 melanoma cell microprocesses by scanning immunoelectron microscopy. M21 cells were allowed to attach and spread on fibronectin-coated coverslips, fixed with 0.5% glutaraldehyde, and stained using Mab 14.18 as primary antibody followed by anti-mouse Ig conjugated to colloidal gold (20–30 nm) as previously described [17]. The inset of the left and right portion of the figure are magnified 2 and 10x, respectively.

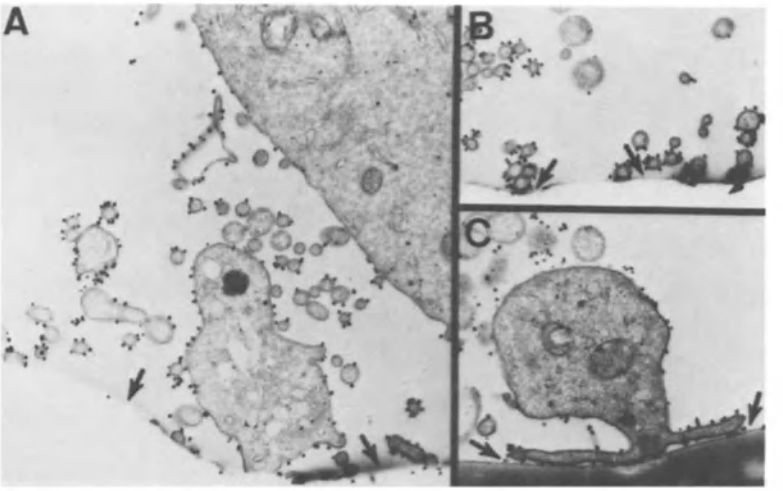

Figure 4. Immunolocalization of GD2 on the cell surface and microprocesses of M21 cells by transmission electron microscopy. M21 cells were allowed to attach to fibronectin-coated coverslips and stained with Mab 14.18 and anti-mouse Ig conjugated to gold (7–9 nm) as previously described [17]. Arrows correspond to the fibronectin substrate. A) includes a cross-section of the cell body (upper right) as well as microprocesses; B) demonstrates cross-sections of microprocesses making direct contact with the substrate; C) demonstrates longitudinal sections of two microprocesses making direct contact with the substrate.

evidence that the surface expression of these molecules correlates with their state of adhesion.

Pretreatment of human melanoma cells with Mabs directed to gangliosides could also prevent their attachment to a variety of extracellular matrix components [16]. A kinetic analysis of this effect demonstrated that the antiganglioside Mabs primarily inhibited the early stages (within five minutes) of cell attachment to either fibronectin or laminin (Figure 5). In addition to the observed effects on cell attachment, pretreatment of M21 cells within antiganglioside Mabs could also inhibit their attachment to vitronectin, collagen, and a heptapeptide (glycyl-L-arginyl-L-glycyl-L-aspartyl-L-seryl-L-prolyl-L-cysteine) which constitutes the cell attachment site of fibronectin [50]. Moreover, when melanoma cells containing GD2 and GD3 were incubated with Mabs to both of these molecules, an added inhibition of attachment was observed [16]. The

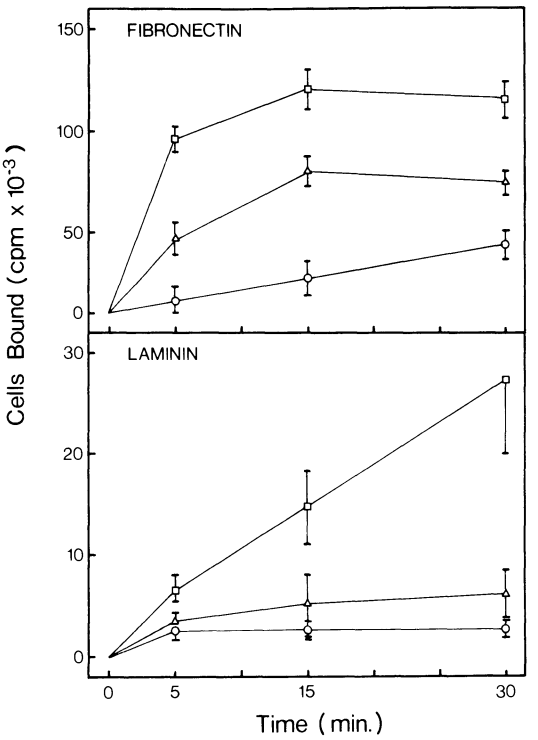

Figure 5. Effects of monoclonal antiganglioside antibodies on the kinetics of attachment of M21 human melanoma cells to fibronectin or laminin. Metabolically labeled M21 human melanoma cells (5×10^3) were allowed to attach for various times to microtiter wells coated with either fibronectin (A) or laminin (B). Prior to addition to substrate coated wells, the cells were allowed to react with Mabs W6/32 (anti-HLA□), 11C64 (anti-GD3△), or 3F8 (anti-GD2○) for one hour at 4°C and washed free of excess antibody. The data are expressed as the total number of cells bound (cpm bound) at the designated times as previously described [16]. Each point represents the mean ± SD of five replicates.

specificity of this inhibition was demonstrated, since no effect on cell attachment was observed with Mabs of various isotypes directed to either protein or carbohydrate epitopes expressed on a number of major melanoma or neuroblastoma cell surface antigens [16].

The role of gangliosides in melanoma cell attachment to a more physiologically relevant substrate has also been examined. Thus, pretreatment of M21 human melanoma cells with anti-GD2 antibodies resulted in a 92% inhibition of attachment to a matrix laid down on tissue culture plastic by bovine endothelial cells [18]. The fact that Mabs to both GD2 and GD3 can inhibit cell attachment to a number of different substrates suggests that gangliosides may play a general role in cell-substratum interactions rather than acting specifically as a surface receptor for a given substrate. In support of this hypothesis, a strong interaction could not be observed between radiolabeled fibronectin and gangliosides separated on a thin layer chromatogram [16]. Alternatively, gangliosides, being strongly anionic, may play a role in the electrostatic requirements for optimal cell-substrate interactions. In this regard, controlled periodate oxidation of the terminal, unsubstituted sialic acid residues on the cell surface not only specifically destroyed the antigenic epitopes on GD2 and GD3, but also inhibited melanoma and neuroblastoma cell attachment [16]. In fact, as shown in Figure 6, the periodate-induced ganglioside oxidation and the inhibition of the attachment of two human melanoma and one neuroblastoma cell lines were equally dose-dependent. As expected, treatment of cells with neuraminidase that results in the removal of most of the surface sialic acid, also inhibited cell attachment [16]. These data suggest that cell-substratum interactions may depend in part on the electrostatic environment provided by terminal sialic acid residues of cell surface gangliosides and possibly other anioic glycoconjugates.

The role played by cell surface gangliosides acting as specific receptors for various adhesive proteins may also be in doubt because of the recent reports indicating the presence of specific glycoprotein cell surface receptors for the extracellular matrix components, i.e., fibronectin, vitronectin, and laminin. These receptors were shown to recognize the peptide RGDS (arginyl-glycyl-L-aspartyl-L-serine), known to constitute the cell attachment site for both fibronectin [51–54] and vitronectin [55] and may thus represent a class of cell surface-associated extracellular matrix receptors. This class of cell surface receptors is also known to require the divalent cation Ca^{+2} for activity since EDTA treatment of cells eliminates their attachment activity [56, 57]. Moreover, the fibronectin receptor on the cell surface was shown to be resistant to the proteolytic cleavage by trypsin in the presence of physiological levels of Ca^{+2} [56] indicating that this divalent cation binds tightly with the receptor and may possibly help to orient its interaction with fibronectin. The role of gangliosides or other membrane components in cell attachment may therefore involve their capacity to form a Ca^{+2} dependent complex with one or more of these glycoprotein receptors in the membrane. The formation of such a complex may result in the activation of a receptor for a given substrate. It is conceivable

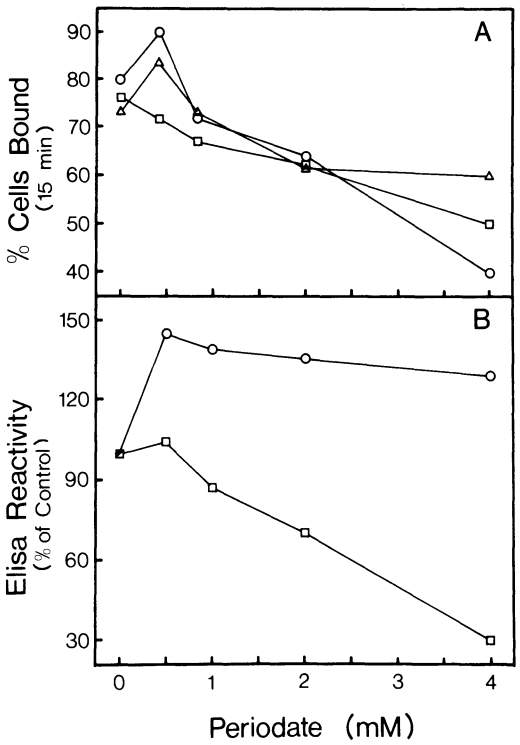

Figure 6. Effects of cell surface periodate oxidation on cell attachment and ganglioside antigen expression. Metabolically labeled M21 and 983b human melanoma cells or SK-NAS human neuroblastoma cells were treated with growth media containing 0.5–4.0 mM sodium-meta-periodate on ice in the dark for 30 minutes as previously described [16]. A) After washing, M21 (□), 983b (△), or SK-NAS (○) cells were allowed to attach to fibronectin-coated microtiter wells for 15 minutes, and the percent cells bound were determined as previously described [16]. Each point represents the mean of four replicates. B) M21 cells were treated with periodate and washed as above and then dried onto microtiter wells for antigen detection by ELISA as previously described [16] where Mabs 9.2.27 (○) or MB3.6 (anti-GD3□) were used as primary antibody. Each point represents the % of control binding, i.e., in the absence of periodate, and is expressed as the mean of duplicate values.

that Ca^{+2}, which is known to be required for fibronectin mediated cell attachment and is capable of binding very tightly to gangliosides, is within complexes containing the negatively charged ganglioside on the cell surface, thus allowing them to distribute preferentially into certain domains on the cell surface. This contention is consistent with the results of Sharom and Grant [58] who demonstrated that physiological levels of Ca^{+2} lead to cross-linking and condensing of ganglioside headgroups in membranes by complexing carboxyl residues of sialic acid. Therefore, in the presence of divalent cations, it is possible that laterally mobile carbohydrate-bearing components such as gangliosides show a tendency to cluster about complex glycoproteins containing one or several carboxyl groups. Alternatively, gangliosides may act as a secondary receptor for low affinity ionic interactions where the sum of many

such interactions may lead to a higher avidity of a cell for its substrate. Our data and the results of others [12–14] are consistent with either hypothesis.

1.5. Possible models for the role of gangliosides in cell attachment

As shown in Figure 7, four hypotheses (I-IV) are proposed to account for the potential role of gangliosides in cell attachment. In the first model (I) cell surface gangliosides and a separate fibronectin RGDS-dependent receptor are suggested as being capable of recognizing two distinct domains on the fibronectin molecule. In this case, the sum of these interactions would lead to an overall increased avidity of the cell surface for the immobilized fibronectin molecule. In partial support of this hypothesis, McCarthy et al. [59] demonstrated that a 33-kd tryptic/catheptic carboxyl-terminal heparin-binding fragment of fibronectin could stimulate murine melanoma cell attachment and spreading. This fragment, which lacks the RGDS peptide, must therefore interact with an alternative cell surface receptor. In this regard, it has been shown that cell surface proteoglycans are capable of interacting with this domain of fibronectin [60, 61]. It is not unlikely that other oligosaccharides such as those found on gangliosides may also be capable of interacting with this portion of the fibronectin molecule. The second model (II) is somewhat similar to the first; however, in this case, the assumption is made that the ganglioside binding domain is closely associated

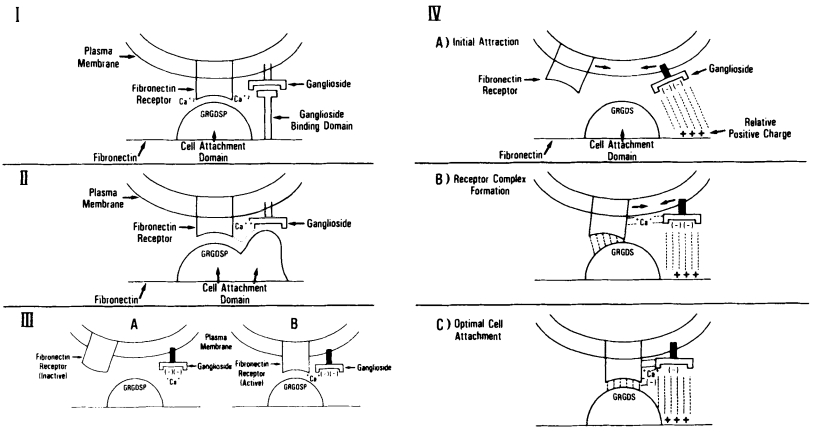

Figure 7. Schematic diagrams comparing the models for possible ganglioside involvement in cell attachment to an RGDS-containing substrate.

I) Proposes the presence of two independent binding domains on the substrate, one of which is recognized by a cell surface RGDS-dependent receptor and the other by a cell surface ganglioside. II) Postulates the presence of Ca^{+2}-dependent receptor/ganglioside complex on the cell surface that recognizes two closely associated domains on the substrate. III) Suggests that an inactive form of the receptor in A, after forming a Ca^{+2}-dependent complex in B, produces an active receptor that can now bind the RGDS domain of the substrate. IV) Proposes a time-dependent mechanism where the ganglioside provides the initial electrostatic attraction between the cell and the substrate as in A. In B, the formation of a Ca^{+2}-dependent receptor complex is postulated that allows for optimal cell substratum interaction as depicted in C.

with the actual RGDS-dependent fibronectin receptor. This receptor/ganglioside complex may be held together by a Ca^{+2} bridge and as in model I, where the sum of the two interactions would lead to an increased cell-fibronectin interaction. However, our observations indicating that antiganglioside Mabs inhibit melanoma cell attachment to the immobilized peptide (RGDS) argue against models I and II since they assume that gangliosides only interact with an independent portion of the fibronectin molecule. At present, our favored hypothesis involves a combination of hypotheses III and IV. In each of these cases, the assumption is made that in order to achieve optimal cell attachment, a complex must form, which in hypothesis III would lead to the activation of the glycoprotein receptor in the membrane for optimal interaction with its substance. Hypothesis IV, on the other hand, assumes that the charged headgroup of the ganglioside establishes the initial attractive forces in a nonspecific manner until the specific receptor can become oriented in the complex, which in time would lead to the formation of a synergistic interaction culminating in optimal cell attachment. This latter hypothesis allows for the possibility that the formation of the receptor complex may also serve to activate the specific interaction of the receptor with its substrate. In preliminary experiments, we found evidence for such a complex existing on the surface of human melanoma cells. Specifically, both GD2 and GD3 copurify with the fibronectin or vitronectin receptors when purified by affinity chromatography from a detergent lysate of M21 human melanoma cells. Moreover, immunolocalization by either indirect immunofluorescence microscopy or transmission electron microscopy demonstrated that these gangliosides codistribute with the fibronectin and vitronectin receptors on M21 cells when attached and spread on a solid substrate containing either of these adhesive proteins.

Depending on the particular oligosaccharide moiety, a ganglioside may serve to induce a preferred orientation or create a suitable electrostatic environment for optimal receptor-ligand interaction on the cell surface. In this regard, gangliosides on a number of cell types have been shown to interact with and potentially modulate the function of cell surface receptors for thyroid stimulating hormone [8], platelet-derived growth factor (PDGF) [10], and the serotonin receptor [35]. The fact that GD2 and GD3 represent the major gangliosides on the surface of human neuroblastoma and melanoma cells, respectively, and apparently play a role in their attachment to the extracellular matrix, suggests the possibility that structurally similar gangliosides on other cell types may have an analogous function. Mabs directed to other gangliosides on such cells will certainly be helpful in addressing this issue.

1.6. Ganglioside biosynthesis is mediated by specific glycosyltransferases

Based upon the structural and functional importance of GD2 and GD3 in neuroectoderm-derived tumors, studies were conducted to characterize the enzymes responsible for the biosynthesis of these gangliosides. Since GD2 is expressed on the surface of SCCL cells, enzyme assays were performed using

total membrane extracts of the SCCL cell line NIH-N417 as a source for GD2 synthetase. The GD2 synthetase activity of these cells was determined after an incubation of the enzyme source at 37°C with the GD2 biosynthetic precursor GD3, using UDP-[^{3}-H]-N-acetyl galactosamine as the glycosyl donor. Based upon the cpm incorporated into GD2 after ganglioside separation by TLC, the GD2 synthetase activity in this cell line was found to be linearly related to the membrane protein concentration with a specific activity of 0.14 pmol of GD2/hr/mg protein (Figure 8). The activity of GD2 synthetase is completely eliminated upon boiling the membranes (data not shown). A time course for GD2 synthetase activity shows maximum incorporation of radiolabel into GD2 after approximately 90 minutes at 37°C (Figure 9) after which a gradual decrease in activity is observed. While GD3 is proposed to be the biosynthetic precursor of GD2, the close structural relationship between GM2 and GD2 allows for the possibility that GD2 could be synthesized via the monosialoganglioside GM2 and CMP-sialic acid. In order to test this possibility, GD2 synthetase assays were performed using GM2 as a substrate and radiolabeled CMP-sialic acid as the glycosyl donor. As shown in Figure 10, lane B, the only visible products obtained under these conditions were GM3 and minimal amounts of GD3, whereas GD2 was readily detected whenever GD3 and UDP-N-actyl galactosamine were used in this assay (lane A). Based on the above

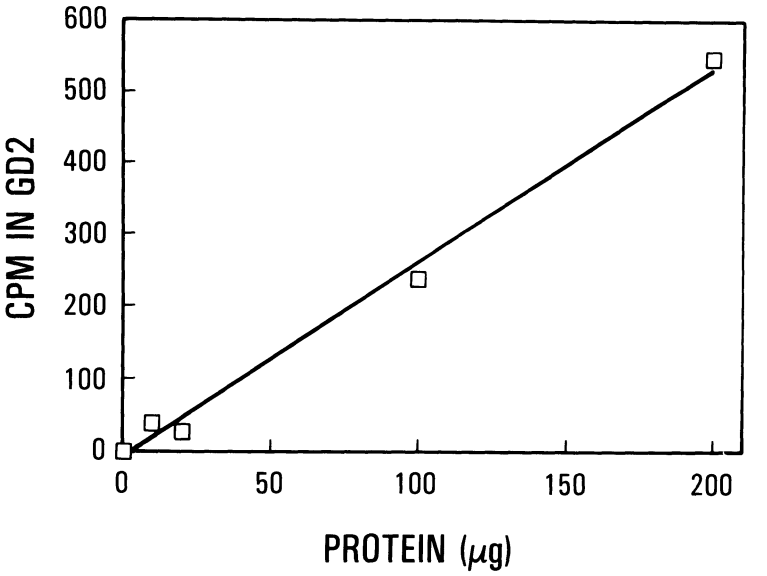

Figure 8. GD2 synthetase activity in the SCCL cell line NIH-N417 as a function of protein concentration. Total membrane extracts of NIH-N417 cells were incubated at 37°C for one hr in the presence of detergent, buffer, substrate, and glycosyl donor. Labeled ganglioside products were then isolated by extraction with chloroform/methanol (1:1) and separated by TLC using a solvent containing chloroform/methanol/0.2% aqueous CaCl2 (55:45:10). The radioactivity of each band corresponding to the migration of authentic GD2 was then quantified by scraping it from the TLC plate and counting in a scintillation counter.

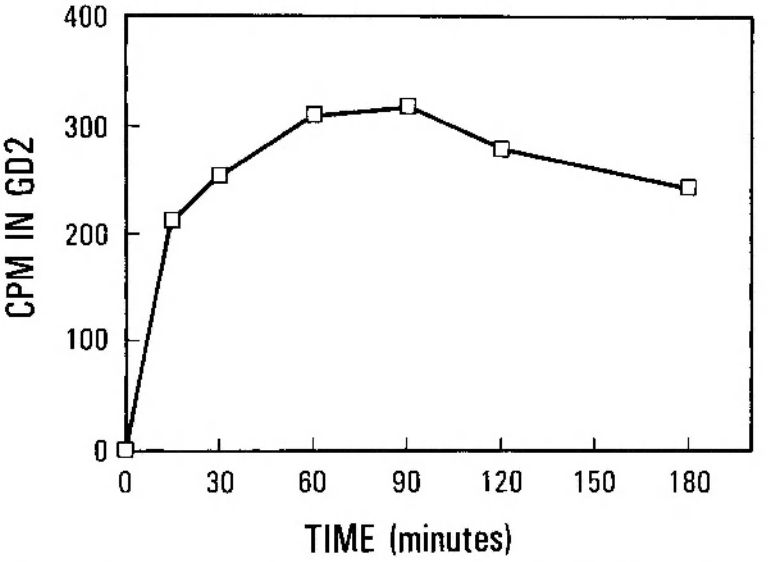

Figure 9. GD2 synthetase activity of the SCCL cell line NIH-N417 as a function of time. Total membrane extracts (100 μg) of NIH-N417 cells were incubated at 37°C for the indicated times in the presence of detergent, buffer, substrate, and glycosyl donor. Isolation of gangliosides and measurement of isotope incorporation into GD2 were performed as described in Figure 8.

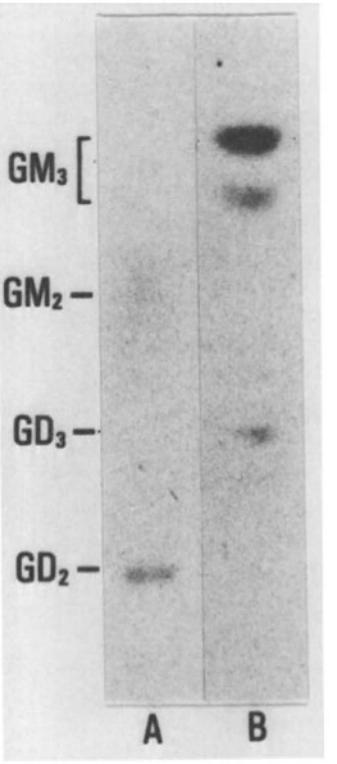

Figure 10. GD3 is a specific substrate for GD2 synthetase in SCCL. Total membrane extracts (100 μg) of NIH-N417 cells were incubated at 37°C for one hr in the presence of detergent, buffer, glycosyl donor, and 2 μg of either GD3 (Lane A) or GM2 (Lane B). Labeled ganglioside products were then extracted, separated by TLC, and subjected to autoradiography for 72 hours. The brackets correspond to the migration of GM3, GM2, GD3, and GD2 standards.

observations, a proposed scheme for the biosynthesis of GD2 in SCCL cells is shown in Figure 11.

Experiments were also performed to characterize GD3 synthetase, i.e., the enzyme responsible for the synthesis of GD3. Using the human melanoma cell line Melur as an enzyme source, total membrane extracts were incubated at 37°C with the GD3 biosynthetic precursor GM3 using CMP-[^{3}H]-sialic acid as the glycosyl donor. The GD3 synthetase activity of these cells was then determined based upon the cpm incorporated into GD3 after ganglioside separation by TLC. The activity of this enzyme was found to be linearly related to both protein concentration (Figure 12) and time (Figure 13), and activity was completely lost upon boiling of the membrane source (data not shown). In addition, GD3 synthetase showed optimal activity between pH 6.2 and 6.6, and subcellular fractionation experiments localized this enzyme within the Golgi apparatus (data not shown).

Previous work conducted in our laboratory has demonstrated that melanoma cells can specifically modify disialoganglioside GD3 by the addition of a single acetyl group to the 9-position of the terminal sialic acid [2, 62]. In this regard, Mab D1.1, which specifically recognized this 9-0-acetylated GD3 antigen, was used to demonstrate that the expression of this modified GD3 antigen was restricted to human melanoma tissue [9].

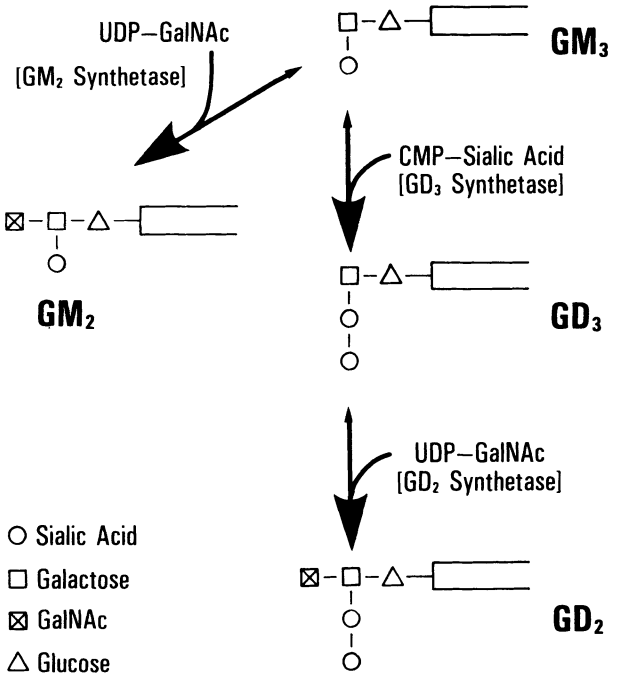

Figure 11. Proposed biosynthetic pathway leading to GD2 expression in SCCL. Key: GalNAc, N-acetylgalactosamine; CMP, cytidine 5'-monophosphate; UDP, uridine diphosphate.

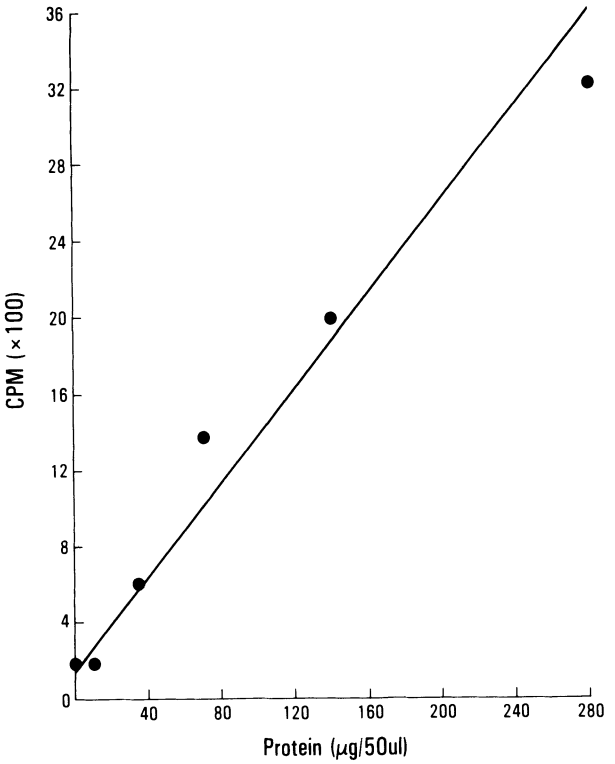

Figure 12. GD3 synthetase activity in the Melur melanoma cell line as a function of protein. Total membrane extracts of Melur melanoma cells were incubated at 37°C for one hour in the presence of detergent, buffer, substrate, and glycosyl donor. Labeled ganglioside products were then isolated by extraction with chloroform/methanol (1:1) and separated by TLC using a solvent containing chloroform/methanol/0.2% aqueous $CaCl_2$ (55:45:10). The radioactivity of each band corresponding to the migration of authentic GD3 was then quantified by scraping it from the TLC plate and counting in a scintillation counter.

The possibility that human melanoma cells contain an enzyme responsible for converting GD3 to its O-acetylated form was also examined. Lysates were prepared from human melanoma cells and allowed to react with human melanoma gangliosides which had been de-O-acetylated to destroy any pre-existing reactivity with Mab D1.1. A specific conversion of GD3 to its O-acetylated form was observed as indicated by the generation of the antigenic isotope resulting in binding of Mab D1.1 in a solid phase lipid ELISA [62]. These data suggest the presence of a specific O-acetyl transferase in these melanoma cells which, as expected, causes a concomitant decrease in the amount of native GD3 antigen, as indicated by a loss in binding of Mab MB3.6. The fact that the enzymatic activity which mediates the conversion of GD3 to the O-acetylated form was sensitive to heat (80°C for 20 min) and showed increased activity as a function of both protein concentration and time [62] further strengthens our contention that a putative O-acetyl transferase is

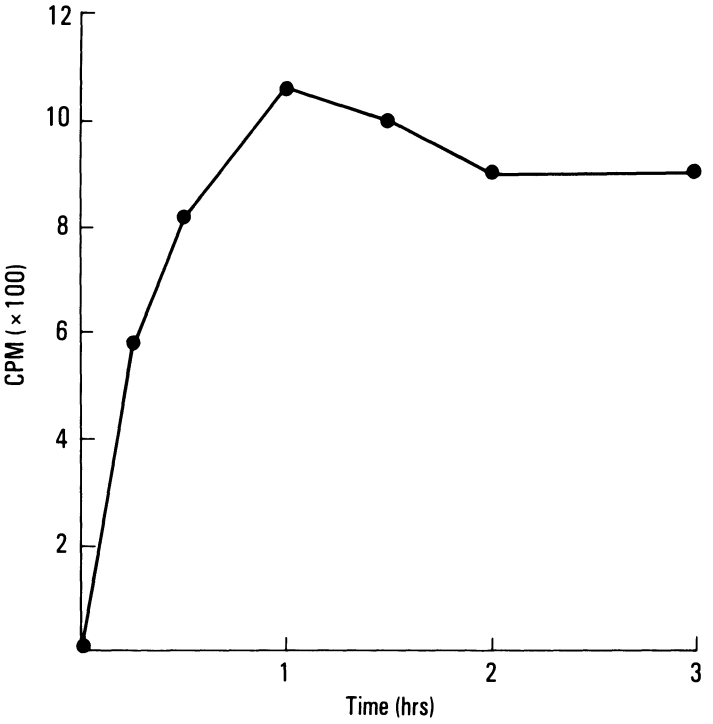

Figure 13. GD3 synthetase activity in the Melur melanoma cell line as a function of time. Total membrane extracts (100 μg) of Melur melanoma cells were incubated at 37°C for the indicated times in the presence of detergent, buffer, substrate, and glycosyl donor. Isolation of gangliosides and measurement of isotope incorporation into GD3 were performed as described in figure 12.

present in these cells. Thus, an understanding of the biosynthetic mechanisms of tumor-associated gangliosides may provide further insight into the structural and functional properties of gangliosides expressed on the surface of human tumor cells.

1.7. GD3 serves as an effective target antigen for mab-mediated cytolysis of human melanoma cells in vitro and in vivo

Melanoma represents a very invasive and deadly form of skin cancer that afflicts 17,000 individuals in the United States each year. Although horizontally spreading primary melanomas can often be successfully treated by surgery, vertically spreading tumors, when detected on the skin, have usually already metastasized to adjacent or even distant sites. This metastatic form of the disease is quite resistant to treatment resulting in 6,000 deaths per year. One approach to treat metastatic melanoma cells and attempt to slow or stop their spread is to select surface markers (antigens) expressed by these cells that may be useful targets for immunological attack. Thus, GD3, which is copiously expressed by most human melanoma tissues and, like other gangliosides, is

integrally embedded in the lipid bilayer of these cells, may be an effective target antigen for Mab mediated cytotoxicity of human melanoma cells. Based on this hypothesis, Mab MB3.6 was tested for its ability to specifically mediate the lysis of ^{51}Cr labeled human melanoma target cells. As shown in Figure 14, in the presence of human serum as a complement source, this antibody could effectively lyse 80–100% of four human melanoma cell lines expressing significant levels of GD3, i.e., FM9, FM9m, M21, and M14, within 30 minutes, whereas a melanoma cell line (A375) lacking GD3 could not be lysed within two hours. In contrast, Mab 9.2.27 (IgG2a) directed to a melanoma-associated proteoglycan antigen on these same cells was ineffective in mediating complement-dependent cytolysis of any of these cells (data not shown). These same melanoma cells were then challenged with Mab MB3.6, in the presence of normal human peripheral blood mononuclear leukocytes as effector cells, to determine whether this antigen could serve as an effective target for antibody-mediated cellular cytotoxicity (ADCC). As shown in Figure 15, Mab MB3.6 in the presence of human effector cells also mediated significant ADCC with the four melanoma cell lines expressing GD3, whereas, as expected, no lysis could be detected with the GD3-negative melanoma cell line A375. In the absence of Mab MB3.6, these same human effector cells could mediate natural killer (NK)

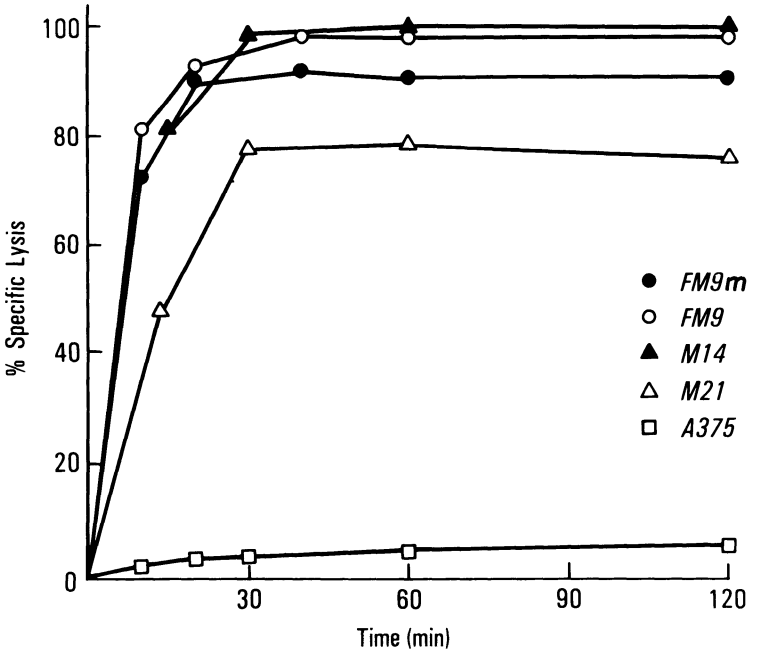

Figure 14. Mab MB3.6-mediated cytotoxicity of human melanoma cells in the presence of human complement. The specific lysis was determined for five human melanoma cell lines that were labeled with ^{51}Cr and challenged with Mab MB3.6 in the presence of human complement as described. Cell lines: FM9m (●), FM9 (○), M14 (▲), M21 (△), A375 (□).

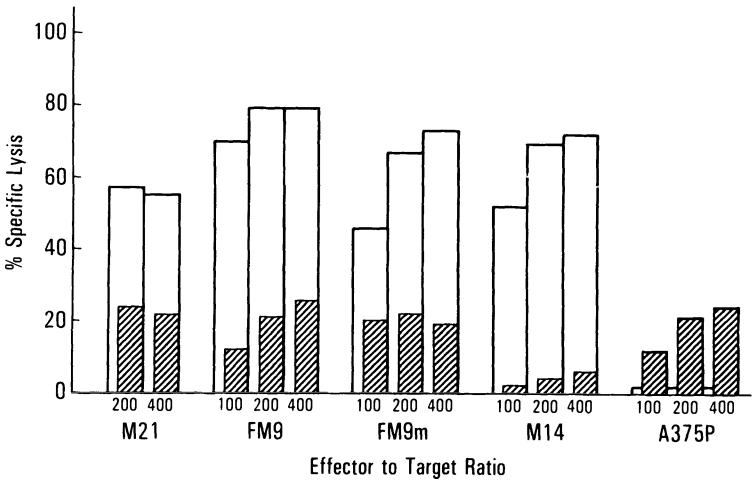

Figure 15. ADCC of human melanoma target cells mediated by Mab MB3.6 and normal human effector cells. The percentage of specific lysis was determined for five human melanoma cell lines that were labeled with ^{51}Cr and challenged with human peripheral blood mononuclear cells in the presence (open bars) or absence (hatched bars) of Mab MB3.6 as described. The effector-to-target ratio varied from 100 to 400.

cell lysis ranging from 5–25% regardless of the GD3 content of any of these cell lines.

The fact that Mab MB3.6 could induce the lysis of human melanoma cells served as a rationale to test the effects of this antibody on the ability of these cultured human melanoma cells to establish tumors in the athymic nu/nu nude mouse model. To this end, purified Mab MB3.6 was resuspended in phosphate buffered saline pH 7.2 (PBS) and injected i.p. into animals which had one day earlier received a subcutaneous injection of 10^7 M14 melanoma cells. In the control group, 6/6 animals developed sizable tumors between 30–40 days post inoculation (mean volume = 370 mm^3 + 65 S.E.), whereas only 1/6 animals treated with 70 μg/ml purified Mab MB3.6 in nine separate i.p. injections, beginning on day two post tumor inoculum, developed a small tumor of approximately 150 mm^3. Furthermore, results of more recent experiments suggest that Mab MB3.6 can effectively eradicate human melanoma tumors that prior to treatment were allowed to grow for seven days to a size of approximately 50 mm^3 in these animals (Figure 16). In control experiments, using Mab 9.2.27 directed to a melanoma-associated proteoglycan [63] and administered as above, we were unable to significantly alter the growth properties of such tumors in nude mice. The results from these experiments suggest that Mab MB3.6, which can mediate two distinct mechanisms of human tumor cell lysis *in vitro*, can also effectively reduce the growth of these same tumor cells, once established, in the athymic nude mouse model. In support of these findings is a recent report by Houghton and coworkers [43] indicating that the murine Mab R24 (IgG3), which is also directed to the GD3 ganglioside, effectively produced

Figure 16. Effects of Mab MB3.6 on human melanoma tumor formation in the athymic nude mouse. FM9m human melanoma cells (10^7) were injected into the right and left flank of 12 nude mice. Tumor formation was allowed to occur for 7 days after which 6 animals received four injections (days 7, 9, 11, and 13) of either Mab MB3.6 (70 μg in 100 μl PBS) or PBS alone. The 2 animals on the right were in the control (PBS) group and the 2 animals on the left received Mab MB3.6 treatment. These photographs were taken after tumors were allowed to develop for 23 days.

highly significant regression of metastatic tumors in 3/12 human melanoma patients in a Phase I clinical trial. These results clearly indicate, for the first time, that a mouse monoclonal antibody recognizing the appropriate target antigen, i.e., ganglioside, offers a potentially novel and effective form of immunotherapy when administered to cancer patients with solid tumors.

2. Summary

This brief review describes the use of murine antiganglioside Mabs to elucidate some of the structural and functional characteristics of the disialogangliosides GD2 and GD3 on tumors of neuroectodermal origin. Specifically, Mabs directed to GD2 react with both cell lines and tissues from neuroblastoma, melanoma, glioma, and small cell cancer of the lung. Moreover, an anti-GD2 Mab was used to develop a serum assay for monitoring the disease status of neuroblastoma patients. A functional role for both GD2 and GD3 in tumor cell-substratum interactions was demonstrated based on the surface localization of these gangliosides on human melanoma cells. In addition, inhibition of melanoma and neuroblastoma cell attachment was observed in the presence of antiganglioside Mabs. Possible models for the role of gangliosides in cell

attachment were discussed in which a favored hypothesis implicated gangliosides and divalent cations in a complex with specific glycoprotein receptors for the adhesive protein(s) fibronectin and/or vitronectin. Furthermore, the biosynthesis of GD2 and GD3, as well as 9-0-acetylated GD3, was shown to be mediated by specific enzymatic activities associated with a tumor cell membrane extract. Finally, anti-GD3 monoclonal antibodies were shown to have a pronounced effect on human melanoma tumor formation in the athymic nude mouse, indicating the possibility that Mabs directed to GD3 and possibly other gangliosides may represent an effective immunotherapeutic approach for the treatment of solid tumors in man.

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II

Natural History

4. Epidemiology of Ocular Melanoma

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1. Introduction

Ocular melanomas are eye tumors composed of melanocytic cells with malignant characteristics. Most ocular melanomas arise from uveal melanocytes distributed throughout the stroma of the choroid, ciliary body, and iris; conjuctival melanocytes may also be the cells of origin for melanomas [1]. Since conjunctival and uveal melanocytes share a common embryologic origin with melanocytes of the skin from the neural crest [2], investigators have suggested the possibility of similar etiologies for ocular and cutaneous melanoma [3, 4]. While both melanomas share certain clinical and epidemiologic characteristics, other factors demonstrate no correlation. This issue needs further investigation, as discussed in this review.

2. Occurrence

Malignancies of the eye occur at a very low rate, constituting only between 0.2–0.8% of all cancers in different parts of the world [5]. In the United States, eye cancer comprises 0.2% of all new cancer cases and 0.08% of all cancer deaths, with an estimated 1,800 cases and 400 deaths from eye cancer expected to occur in 1986 [6].

Most routine sources of cancer statistics, following the International Classification of Diseases of the World Health Organization (WHO), report data on eye malignancies as a single group [7]. This classification does not permit a separation among melanomas, retinoblastomas, and other types of eye cancer. The lack of such histologic information and the low rate of occurrence of ocular melanomas thus make it difficult to study these malignancies from available sources of cancer data. Because several studies have shown that most eye cancers in children and youths are retinoblastomas, the incidence of intraocular melanomas can be roughly estimated from data on eye cancers among adults [4, 8, 9]. For this reason, eye cancer data in adults are often used as a surrogate for ocular melanoma data. Both are not identical, however, since studies in Finland [10], Iceland [11], England [9], and the United States [8] have found that ocular

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melanomas comprise only 70–88% of all ocular tumors in persons over 15–20 years. Analyses of ocular melanoma trends based on routinely collected data on eye cancer thus have some limitations, which must be understood when interpreting the results of such analyses. A more precise estimate of incidence can be obtained from studies that separate eye melanomas from other eye tumors. Of particular interest for epidemiologic purposes are those studies that include data for the specific sites of ocular melanomas, such as choroidal, ciliary body, iris, or conjunctival tumors.

2.1. Incidence

A large number of studies conducted in Europe, the United States, and Canada (Table 1) confirm an incidence of eye cancer, eye melanoma, or choroidal melanoma ranging from about five to seven new cases per million persons per year. Incidence has been reported to vary with age, sex, race, and geographic location. In studies that have permitted evaluation of ocular melanomas by site, choroidal tumors have been found to be the most frequent by far (65–78% of all eye melanomas); ciliary body and iris melanomas appear to occur less frequently. Sixteen of the major studies that have evaluated ocular melanoma incidence are described below and summarized in Table 1. These studies have been conducted mainly in Scandinavian countries [10, 12–15], Iceland [11], England and Wales [9, 16], East Germany [17], the United States [81–22], and Canada [23]. Most of the studies are based on cancer registry data only; the others obtained their data from a variety of sources including hospital or clinic records, death certificate searches in defined populations, and published reports, as well as from cancer registries.

Since the data from the majority of the studies come from cancer registries, their interpretation is subject to the advantages and limitations of these data sources. The major advantage of using cancer registry information is its accessibility. Limitations include possible problems such as incomplete ascertainment of cases and diagnostic misclassification. Underreporting is less likely to be a serious problem in Norway where registration is compulsory and in Finland and Iceland where the registry information is routinely compared with death certificates and other data. Regarding the problem of possible diagnostic misclassification, a study addressing this question in a group of 876 eyes enucleated at the Mayo Clinic between 1954–1977 found a misclassification rate of 2.7% [24]. Other investigators have found misdiagnosis rates of 3.7–8% [25–27]. While these percentages are relatively small, this issue might be a potential problem in a condition as rare as ocular melanoma and should be taken into account when possible.

2.2. Europe

2.2.1. Scandinavia and Iceland The incidence of ocular melanomas in northern European countries is reported to be among the highest in the world. Over

Table 1. Summary of incidence studies for ocular melanoma

Author, yr	Source of data, area	Time period covered	Definition of cases	Number of cases M	F	Total	Mean annual (crude) incidence/ million/yr	Descriptive characteristics
			SCANDINAVIA					
1. Teikari & Raivio, 1985 [10]	Finnish Cancer Registry, hospital and clinic records	1973–1980	choroidal & ciliary body melanomas	125	160	285	7.6	Incidence increases with age, M/F ratio ranges from 0.37 (ages 20–29) to 1.4 (70 yrs and older)
2. Raivio, 1977 [12]	Finnish Cancer Registry, eye clinic & hospital records	1953–73	eye melanomas uveal melanomas (choroidal and ciliary melanomas)	—	—	548	5.3 5.0	Incidence increases with age; no difference in risk between sexes; no change in rates over time
3. Abrahamsson, 1983 [13]	All cases on Swedish west coast (county of Holland, city of Gothenburg) treated by enucleation	1956–75	choroid and ciliary body melanomas	—	—	91	7.2	
4. Jensen, 1963 [14]	Danish Cancer Registry	1943–52	choroid and ciliary body melanomas	—	—	302	7.5	
5. Mork, 1961 [15]	Cancer Registry of Norway	1953–60	malignant melanomas of the eye	—	—	220	M: 9.0 F: 7.0	Incidence increases with age; M/F ratio = 1.15; incidence slightly higher in urban areas
			ICELAND					
6. Gislason, et al., 1985 [11]	Icelandic Cancer Registry	1955–79	uveal malignant melanoma	—	—	33		Incidence increases with age; M/F ratio = 1.4; no change in rates over time
			choroid and ciliary body	—	—	29	5.9	
			iris melanoma	—	—	4	0.8	

Table 1. (continued)

Author, yr	Source of data, area	Time period covered	Definition of cases	Number of cases M	F	Total	Mean annual (crude) incidence/ million/yr	Descriptive characteristics
			ENGLAND AND WALES					
7. Swerdlow, 1983 [16]	Population based regional cancer registries in England and Wales; Office of Population Censuses and Surveys	1962–77	eye cancer	2,159	2,125	4,284	metropolitan counties: M: 8.3 F: 6.2 urban areas —M: 9.0 F: 7.2 rural areas —M: 9.8 F: 7.5	
8. Swerdlow, 1983 [9]	Oxford Cancer Registry	1952–78	malignant melanoma of the eye	106	101	207	M: 4.8* F: 3.9*	Mean annual age-specific incidence increases with age
			EAST GERMANY					
9. Lommatzsch, 1985 [17]	National Cancer Registry of DDR	1961–80	malignant melanoma of the eye	1,360	1,501	2,861	M: 8.7 F: 8.2	Slight increase in rates over time
			UNITED STATES					
10. SEER, 1981 [18]	Surveillance, Epidemiology, and End Results Program Sample of U.S. population	1973–77	malignant melanoma of the eye	270	257	527	5.2*	
11. Wilkes, et al., 1979 [19]	Mayo Clinic records for Rochester & Olmstead County, Minnesota	1935–74	uveal malignant melanoma	—	—	15	Total: 7.0 M: 5.0 F: 9.0	Incidence increases with age

12. Shammas and Watzke, 1977 [20]	Tumor Registry of Univ. of Iowa for Third Nat'l. Cancer Survey	1969–71	choroid and ciliary body melanomas	—	—	41	Total: 4.9 M: 4.7 F: 5.1	Study included only whites
13. Scotto, et al., 1976 [8]	Third Nat'l. Cancer Survey Sample of U.S. population (nine metropolitan areas and 25 states)	1969–71	eye melanoma	—	—	341	Total: 5.6** W: 6.0 B: 0.7	Incidence increases with age; 8-fold predominance of eye melanomas in whites over blacks; no difference in risk by sex
14. Ganley and Comstock, 1973 [21]	Cancer registry for Washington County, MD; Surveillance of death certificates	1956–65	malignant melanoma of the choroid	3	3	6	6.6 (95% C.L. 0.3–1.8)	Incidence increases with age
15. Keller, 1973 [22]	All VA hospitals in United States	1958–64	malignant melanoma of the eye	—	—	196	1.6	Incidence increases with increasing age; less frequent in blacks
			CANADA					
16. Gallagher, et al., 1985 [23]	Western Canada Melanoma Study; cancer registries of four Canadian provinces (British Columbia, Alberta, Saskatchewan, Manitoba); limited to patients age 20–79	1979–81	ocular melanoma	51	39	90	M: 8.0 F: 6.0	

*Age-adjusted rates

* 5-year incidence

**Annual age-adjusted incidence

the past 25 years, there have been six major published studies conducted to measure the incidence rates of eye melanomas in Scandinavia and Iceland. The earliest studies were conducted using the cancer registries of Norway and Denmark and covered time periods between 1953–1960 and 1943–1952, respectively [14, 15]. More recent studies have been carried out in Finland [10, 12], Sweden [13], and Iceland [11], each covering time intervals of varying duration. Although these and the other studies listed in Table 1 (to be discussed later) have been conducted over different time periods and in different populations, their results are quite consistent with regard to the magnitude of the incidence rates (Table 1).

There have been two studies of ocular melanoma in Finland. The first covered the time period from 1953–1973 [12]; the second updated the first by covering 1973–1980 [10]. By using the Finnish Cancer Registry, both studies were able to determine incidence for specific types of ocular melanomas, such as choroidal and ciliary body melanomas. Results of the earlier study indicated a mean annual (crude) incidence of 5.3 per million for eye melanomas and 5.0 per million for uveal melanomas, of which the majority were of the choroid and ciliary body. The second study demonstrated a somewhat higher crude incidence of 7.6 per million. The reasons for this slight increase were attributed mainly to the aging of the population and perhaps to improved diagnosis due to the increasing availability of ophthalmologic care. A comparison of the age-specific incidences from both time periods found no significant differences between them. Both studies reported an increase in incidence with advancing age and found similar rates for males and females [10, 12].

A study conducted by Abrahamsson identified all persons with melanoma of the choroid and ciliary body who had been treated by enucleation in an area on the Swedish west coast between 1956 and 1975 [13]. This group of patients was then used to estimate the incidence rate of such tumors for the population. This method of identifying cases of ocular melanoma was justified by the author on the grounds that enucleation was the treatment of choice during the period of the study. Patients not treated by enucleation for whatever reason (e.g., iris melanoma) were not included in this investigation. The study found an incidence rate of 7.2 per million per year, which is similar to the previously discussed results from Finland [10].

Comparable results were obtained in a study from Denmark [14]. This study was conducted by Jensen, who used the Danish Cancer Registry to identify all individuals with an ocular melanoma diagnosis between 1943 and 1952. The incidence rate calculated from these data was 7.5 per million per year. Jensen has continued to follow these patients for 25 years following enucleation; as such, this study has also provided data to evaluate prognosis and mortality in this population [14].

A study using the Cancer Registry of Norway identified a total of 220 cases of malignant melanoma of the eye between 1953 and 1960 [15]. The annual sex-specific incidence rate was nine per million for males and seven per million for

females. Incidence increased with age and was slightly higher in urban than rural areas. Data from this study did not permit a subdivision of melanomas according to primary site [15].

A recent study based on Icelandic Cancer Registry data identified all cases of uveal melanoma diagnosed in Iceland between 1955 and 1979 [11]. Incidence rates per year were age-adjusted to the mean Icelandic population from 1956–1973. The incidence for the study period was 5.9 million per year for melanomas of the choroid and ciliary body. For iris melanoma, a much less frequent type of ocular melanoma, it was 0.8 per million per year. Results of this study are consistent with others regarding the increase in incidence with advancing age, no change in sex ratio over time, and no evidence of time trends in incidence.

2.2.2. England and Wales Swerdlow has recently reported on two studies to determine the incidence of ocular cancer. One study was conducted in the Oxford region of England using data from the Oxford Cancer Registry between 1952 and 1978 for malignant melanomas of the eye [9]. The results of this study indicated no significant secular trend in the incidence of eye melanoma in males or females. Mean annual age standardized incidence rates were 4.8 and 3.9 per million respectively.

In a much larger study Swerdlow determined the incidence of eye cancer between 1962 and 1977 from 14 population-based regional cancer registries in England and Wales that submit data to the National Registry [16]. Limitations of this data source are that registration of cancers is voluntary, and data collection methods vary among regions. Because these data did not permit a separation of ocular melanomas from other types of ocular malignancies, the incidence of eye cancer in persons $\geq$ 15 years of age was used as a surrogate for eye melanoma incidence. Age-standardized rates of adult eye cancer used the 'European' population from 'Cancer Incidence in Five Continents' [5] as the standard. Incidence rates in this study varied in the registration areas; annual rates were around eight or nine per million for males and six or seven per million for females (Table 1). An interesting finding was that registration rates increased by half in males and females from 1962 to 1977 [16]. This increase is somewhat surprising, since Swerdlow's other study found no time trends in incidence rates in Oxford [9], which was one of the regions included in the larger study. Other investigators have also reported on the stability of ocular melanoma rates [28], as discussed in the section on secular trends. To explain the increase in incidence of eye cancers found in England and Wales over time, Swerdlow considered several possibilities, such as increased registration or changes in classification or diagnostic practices. The author concludes, however, that these possible reasons would not have had a major impact on the findings, since these changes were not unique to England and Wales. Possible variations in registration patterns in different regions could perhaps explain some of the increase. If real, this finding is intriguing and future time trends in these regions should be investigated.

2.2.3. East Germany A recent study from East Germany reported incidence rates for eye melanomas of 9.6 per million in males and 8.4 per million in females for 1976–1980; these rates were slightly higher than the 1961–1965 rates, which were 8.6 and 7.9 per million respectively [17]. In this study, eye melanomas were not subdivided by primary site.

2.3. North America

2.3.1. United States Incidence data on ocular melanoma are available from six different studies in the United States (Table 1). These studies provide population-based incidence rates which range from 1.6 to 7 million per year [8, 18–22]. The most recent data were calculated from the Surveillance, Epidemiology, and End Results Program (SEER), which is a National Cancer Institute program involving ten areas in the United States. The incidence rates derived during the time period 1973–1977 for individuals with malignant melanoma of the eye were 5.2 per million per year for both sexes: 5.5 for males and 5.0 for females [18]. These data show an almost twentyfold risk difference for blacks and whites with an annual rate of 5.9 per million for whites and 0.3 per million for blacks. The generally low incidence of the disease, particularly among blacks, leads to an instability of the white to black rate ratios. However, the results are consistent with an earlier study [29] which found that of a series of 2,535 ocular melanoma patients in the United States, only 0.43% (11/2,335) were black. No age- specific data are available from this report [29].

Wilkes et al. determined the incidence of uveal malignant melanoma using records from the Mayo Clinic and other hospitals in Rochester and Olmsted County, Minnesota for the period 1935–1974 [19]. They identified uveal malignant melanomas in 15 patients, which results in an annual incidence rate of seven per million. The great majority (13/15) of these tumors were located in the choroid and ciliary body. The incidence increased with age from 3 per million for persons less than 50 years to 21 per million for those 50 years and older [19].

A study by Shammas and Watzke used the Tumor Registry of the University of Iowa, as well as data from an individual practitioner, to determine the incidence of choroid and ciliary body melanomas for the period 1969–1971 [20]. Their study, which was limited to whites only, resulted in an overall annual incidence rate of 4.9 per million. Rates for males and females were 4.7 and 5.1 per million respectively. Although these results are generally consistent with others, the rates are slightly lower than those reported by other studies. These differences may be due to incomplete ascertainment of cases.

The Third National Cancer Survey, which involved a sample of the United States population in nine metropolitan areas and two states, provided data to calculate incidence rates for eye melanomas between 1969 and 1971 [8]. From these data, Scotto identified 341 cases of eye melanomas, which represented almost four-fifths of all patients with noncutaneous melanoma. Seventy-three percent of these patients had a diagnosis of melanoma of the choroid. This study reported an overall annual age-adjusted incidence of 5.6 per million, and

an incidence in whites and blacks of 6 and 0.7 per million respectively. This eightfold to ninefold higher incidence for eye melanomas in whites than blacks is less than in the SEER data, but the small number of cases in blacks may account for some of that difference. These National Cancer Survey data also indicated an increase in incidence with age and no difference in risk for males and females.

Ganley and Comstock conducted a study to evaluate the relationship between benign nevi and malignant melanomas of the choroid [21]. The incidence of malignant melanomas of the choroid in Washington County, Maryland was investigated as part of this study. These investigators used cancer registry data and reviewed death certificates for Washington County to identify choroidal melanoma cases for the time period 1956–1965. Six individuals were identified, for an annual incidence rate of 6.6 per million (95% confidence limits: 3–18). The rate was almost twice as high for individuals 30 years and older (13 per million). These findings are also consistent with the rates from other reports.

One study by Keller was conducted using data from all Veterans Administration Hospitals in the United States between January 1958 and December 1964 [22]. The relatively low annual incidence rate found by this study — 1.6 per million — may be due to underascertainment of cases or to characteristics of the male veteran population which may lead to a different risk of ocular melanomas than in the general population. The observation that incidence among veterans increases with increasing age and is much lower in blacks again is consistent with other data.

2.3.2. Canada The Western Canada Melanoma Study utilized data from cancer registries of four provinces (British Columbia, Alberta, Saskatchewan, and Manitoba) to obtain reports on all newly diagnosed ocular melanoma patients between April 1979 and March 1981 who were aged 20–79 years [23]. These study results were similar to others, with annual incidence rates of 8 and 6 million per year for males and females respectively.

2.4. International comparisons of incidence and mortality

Eye cancer data are often used as a surrogate measure for ocular melanoma data, as noted above. Hukulinen, Teppo, and Saxen compiled data from a number of sources to compare incidence of cancer of the eye in adults among 23 different countries or registration areas from 1953 to 1972 (Table 2). The authors also obtained data on adult eye cancer mortality in 33 countries or areas from 1957 to 1972. Sources of data included the WHO publications on cancer incidence, 'Cancer Incidence in Five Continents,' and published reports by individual cancer registries [4]. All of the study conclusions are subject, therefore, to the limitations of each of these data sources. With this caveat in mind, the highest incidence rates for eye cancer in males were found in Denmark, Sweden, and New Zeland, while the lowest were in Japan. The highest eye cancer rates for females were in Israel, Sweden, and New Zealand; the lowest were in India, Singapore, and Japan. Few consistent time trends were

Table 2. International comparisons of eye cancer incidence and mortality rates (per million/year)

Authors and source of data	Incidence		Mortality	
Hakulinen, Teppo and Saxen [4]	*Highest rates*		*Highest rates*	
Data from 23 countries or registration areas	*Males*		*Males*	
obtained from WHO publications on cancer	Denmark:	10.1	Finland:	3.1
incidence; Cancer Incidence in Five Continents;	Sweden:	10.9	Denmark:	3.1
published reports by individual cancer registries	New Zealand:	9.9	Norway:	3.0
	Females		*Females*	
	Israel:	9.1	Denmark:	3.0
	Sweden:	9.1	Finland:	2.6
	New Zealand:	9.0	Norway:	2.3
	Lowest rates		*Lowest rates*	
	Males		*Males*	
	Japan (Miyagi):	2.0	Thailand:	0.3
	India (Bombay):	2.0	Japan:	0.6
	Nigeria (Ibadan):	2.7	Hong Kong:	1.1
	Females		*Females*	
	India (Bombay):	1.9	Thailand:	0.2
	Singapore:	2.6	Japan:	0.6
	Japan (Miyagi):	2.7	Mexico:	0.7

identified except for an increase in incidence over time for males and females in Denmark and a decrease in incidence in the United States (Connecticut Cancer Registry) in males and females. The data on mortality were similar, with the highest rates found in Scandinavia and the lowest rates in Asian countries. No mortality data from Africa were available. Mortality rates were found to be fairly constant over the time period.

Incidence and mortality rates, adjusted for age and sex differences among the countries, showed a positive correlation in adults. In general, rates in males were higher than in females, and white populations had a higher incidence and mortality than others. Latitude was positively related to mortality and incidence of eye cancer among adults in white populations. A similar finding was reported for cutaneous melanoma. These observations could be explained at least in part by the higher reporting level in northern areas.

The summary of eye cancer data presented in Table 2 provides international comparisons which must be interpreted in light of the limitations of the available data sources. In addition, the rates reported are for eye cancer rather than for ocular melanomas. Despite these limitations, the results are consistent in showing high risk for males and for white, lightly pigmented populations.

3. Survival

In addition to providing data on the distribution and determinants of disease, epidemiologic studies are also concerned with evaluating prognostic factors and

analyzing clinical trials. Therefore, factors related to survival are of interest to such studies. Ocular melanomas metastasize by hematogenous routes to the liver, as well as to lung and other tissues [30–33]. Choroidal and ciliary body melanomas metastasize often, while metastases from iris melanomas are rare [34, 35]. According to Shields, 20–50% of patients with posterior uveal melanoma will eventually die because of a metastasized lesion [36]. Follow-up studies of various case series report similar case fatality rates [11, 13, 37].

The clinical detection of metastases occur at widely varying time intervals after the primary uveal melanoma has been diagnosed. In some cases, metastases have not developed for ten years or more [38], while about 3% of choroidal melanoma patients have metastases at presentation [39].

Survival studies of uveal tract melanoma patients have identified significant prognostic features, including presence of invasion, tumor size, histologic cell type, pigmentation, mitotic rate, and vascularity, but further studies are needed [40]. For conjunctival melanomas, little is known regarding prognostic features. Bulbar melanomas may have a better outcome than palpebral and other areas, but the effect of tumor thickness at these sites has not been controlled for. Presence of a radial growth phase does not appear to affect survival [41].

A controversy surrounds the best method of treatment for uveal melanomas in order to prevent their dissemination [42]. Enucleation — traditionally considered to be the treatment of choice — has come under scrutiny in recent years. Zimmerman and MacLean [43] noted that metastases are uncommon before enucleation and increase after such operation. This suggests that enucleation might actually lead to an increased case fatality because of dissemination of malignant cells at the time of surgery. Such concept has been challenged by Seigel and others who have shown that patterns of mortality are similar for enucleated ocular melanomas and other types of cancer [44]. The issue has not been resolved, however. To address the question of evaluating treatment, a collaborative, multicenter clinical trial is now underway [42].

4. Descriptive epidemiologic characteristics

Several epidemiologic studies have provided data to describe the occurrence of ocular melanomas according to demographic variables, geography, and time trends. Their results are summarized below.

4.1. Demographic variables

4.4.1. Age Uveal melanomas are rarely found in persons under 15 years of age and the risk increases greatly with advancing age [8–12, 15, 19, 21, 22]. This pattern of increased risk with age is similar in males and females [8–12]. The average age at diagnosis has been reported to be approximately 55–60 years. Although some reports have indicated a peak incidence around 60–69 years,

Table 3. Age-specific annual incidence of ocular melanomas (per million) in various populations, males and females, ages 20–89 years

Age group	Iceland [11] 1955–1979 (choroid & ciliary body)		Iowa, USA [20] 1969–1971 (choroid & ciliary body)		Norway [15] 1953–1960 (ocular melanoma)	
	Males	*Females*	*Males*	*Females*	*Males*	*Females*
20–29	0	0	2	2	2	1
30–39	0	0	0	2	4	3
40–49	5	10	4	6	9	6
50–59	30	10	12	11	19	15
60–69	35	25	22	19	26	22
70–79	25	25	19	14	42	31
80–89	0	4	—	8	14	14
Total	7	5	5	5	9	7
# of cases	29	12	19	22	118	102

others have found a steady increase with age and no evidence of such a peak [4, 8, 9, 45]. Age-specific incidence rates from various studies are summarized in Table 3. While the rates for some age groups are based on very small numbers of cases, these data clearly show an increasing risk with age.

Individuals with iris melanoma are significantly younger than patients with other ocular melanomas [14, 35, 46–48]. Thus, in an Icelandic study, patients with iris melanoma had an average age of 42 years, as compared to a median age of 62 years for persons with choroidal melanomas [11]. Similar age differences have been reported by Jensen [14], Ashton [49], Duke and Dunn [46], Rones and Zimmerman [50], and Raivio [12]. These results suggest that patients with iris melanomas are 10–12 years younger than patients with choroid and ciliary body melanomas. These differences may be due, in part, to diagnostic bias, because of the relative ease of detection of iris melanomas [35].

4.1.2. Sex Some studies have suggested an increased risk of uveal melanoma for males [10, 11, 15]; however, the differences in risk between males and females are not striking [8, 10, 12, 15]. For example, studies in some Scandinavian countries have found that the male to female ratios for uveal melanomas have usually been over one, but these differences are not significant [10, 15]. International comparisons of eye cancer incidence and mortality also show consistently higher rates for males than females [4]. The pattern of increased risk for males, however, is not consistent for all studies and varies according to age group and geographic location. Data from the Third National Cancer Survey (1969–1971) indicated a greater incidence among females at earlier ages and among males in mid-life [8]. A similar pattern was observed in the death rates from eye melanomas for Scandinavia and Japan, where the ratio of male to female rates in 1955–1974 was less than one until about age 40 and more than one after age 40 [51]. These differences are not marked and could be explained by chance variation [46].

4.1.3. Race All available data indicate marked racial differences in the incidence of ocular melanomas, with white populations being at higher risk than others. As seen in Table 1, the incidence rates are remarkably similar in various white populations [8–2]. Uveal melanomas, however, are extremely infrequent in other populations [4, 8, 17, 22, 29, 52]. A review of African studies shows that several investigators were unable to identify any cases over a period of several years [53]. The occurrence of ocular melanomas in Africa seems to be exceedingly rare. Racial differences are also evident in the United States, where eye melanomas occur eight to 20 times more frequently among white than black Americans [8, 18]. These marked differences in ocular melanoma risk by race suggest a possible protective effect due to or associated with increased pigmentation. Large differences in risk between blacks and whites are also seen for skin melanomas in most countries [3, 5, 51].

A study of the Maori population in New Zeland reported no cases between 1955 to 1967, while the annual incidence among the non-Maori population was six per million [52]. The apparent rarity of uveal melanomas in Maori (and Pacific Islanders) In Auckland, New Zealand, was also noted by Miller et al. [40]. Maoris are Polynesians and have intermediate skin melanoma rates [42]. Thus, these findings are also consistent with the conclusion that rates of ocular melanoma are lower among populations with normally increased pigmentation.

4.2. Geographic variations

There is a significant variation in incidence and mortality rates among different countries [4], some possible variation in incidence according to urban and rural residence [15, 16], and some questionable differences in incidence according to latitude [4]. As seen in Table 2, the incidence rates for eye cancer in adults are highest in Denmark [14] and other Scandinavian countries [10, 12, 13, 15] and lowest in Japan and India [4].

A study of eye cancer incidence in England and Wales between 1974–1977 found a gradient of increasing incidence with a decreasingly urban place of residence for both males and females [16]. Interpretation of these data regarding area of residence should be done cautiously, since methods of case ascertainment may have varied among the various cancer registration areas. In contrast to these findings, in Scandinavian countries, urban dwellers appear to be at higher risk of uveal melanomas than rural residents [15].

Because cutaneous melanoma incidence and mortality are inversely related to latitude in several countries, a similar relationship has been explored for uveal melanomas. Results of these investigations show no consistent pattern. Some data have shown that the distance of a particular country from the equator had a positive correlation with mortality and incidence from eye cancer in adults [4]; others have found no variation with latitude [8, 16]. A problem with interpreting these results is the variability in the completeness of registration in various parts of the world.

4.3. Time trends

4.3.1. Secular trends There has been a dramatic increase in the incidence and mortality of cutaneous melanoma in white populations. The rates systematically rise with each successive birth year, and the later born cohorts have the highest risk [3]. This increase raises the possibility that exposure to an etiologic agent has also increased in recent years; interest has focused on the role of sunlight exposure as a possible causative agent. Evidence for a similar increase has been explored for ocular melanomas. One study in the United States has indicated little change in incidence over time since 1947 [8]; others also suggest that changes in both incidence and mortality have been negligible over time [9, 11, 12, 19, 21]. Using incidence and mortality data on eye tumors at ages 15 and over as a surrogate measure for eye melanomas, Strickland and Lee compared time trends in several countries. Changes in rates over a 20 year period were small and inconsistent [28]. Only two investigations have indicated an increase in rates over times: one study reporting on rates in Denmark [4] and a recent study conducted in various areas of England and Wales which demonstrated a 47% increase for males and a 50% increase for females regarding incidence of adult eye cancer from 1962–1977 [16]. These increases were not consistent for all areas, since the incidence of ocular melanoma did not increase in the Oxford (England) area in 1952–1978 [9].

The apparent differences in time trends between ocular and cutaneous melanomas must be monitored carefully. Part of the rise in incidence of skin melanomas is probably due to increased awareness and reporting, as reflected by the higher incidence of early clinical stage and thin melanomas in recent years [54, 55]. A similar phenomenon for ocular melanomas apparently has not been documented. Diagnostic uncertainty regarding benign versus malignant intraocular melanomas, such as in the iris [35], make such secular trends possible and could explain temporal differences in incidence rates as well as some regional differences (as noted above). Secular trends could be also affected by recent improvements in diagnostic accuracy. Chang, Zimmerman, and McLean have shown a marked decline in the rate of incorrect diagnoses of ocular melanoma (from 12.5% to 1.4%) between 1970 and 1980 at the Armed Forces Institute of Pathology [56], probably due in large part to availability of more experienced consultants. Therefore, all of these considerations affect the interpretation of secular trends.

4.3.2. Seasonal patterns Seasonal patterns in incidence have been explored. In New York State, Polednak [57] found a statistically significant seasonal pattern for diagnosis of eye melanomas for males (and not females) with a peak after October, a time when plasma testosterone levels peak in males. This finding suggested a possible role for androgens in tumor progression.

Evidence suggests, but does not conclusively demonstrate, that skin melanoma progression may be androgen dependent [58, 59]. There is some (very limited) evidence that growth of uveal melanomas may be androgen dependent

to some degree, that is, Herbst observed that a male patient with choroidal melanoma responded to orchidectomy [60].

Swerdlow reported no significant seasonality for eye melanomas in the Oxford region for males or females [9]: data were not presented, so the seasonal curves cannot be compared with the New York State data. A study from Czechoslovakia [6] found no significant seasonality for eye melanomas, but data were not analyzed separately by sex and the sex ratio varied over time. In Polednak's study, male and female cases showed contrasting seasonal patterns so that there was no overall seasonal pattern for both sexes combined [57]. Thus, further studies are needed which examine seasonality separately by sex and by time period. Further, such seasonal patterns may not be detectable in geographic areas which differ in incidence of ocular melanoma or in climate.

5. Risk factors

Knowledge concerning risk factors for ocular melanoma is extremely limited. Some potential factors have often been explored because of their association with cutaneous melanoma. Others have been investigated because of leads from descriptive data on apparent clusters of cases. For the purpose of this discussion, these factors have been grouped into the categories of host, familial, and environmental factors.

5.1. Host factors

As discussed earlier, age and race are major risk factors. Other host factors are discussed below.

5.1.1. Hair, eye, and skin pigmentation Within white populations, the presence of fair complexion, light hair, and eye color have been found to be risk factors for cutaneous melanoma in several epidemiologic studies [3, 62–65]. Associations of these factors with uveal melanoma have also been explored, and positive findings have been reported. A study of 305 uveal melanomas occurring in Denmark from 1943 to 1952 found that the surviving patients reported a highly significant preponderance of light iris color as compared to 1,267 ophthalmologic patients aged >25 years. Thus, 82% of the cases versus 61% of the controls had light iris color. No differences were found, however, with regard to hair color [14]. Possible sources of bias in these results include the collection of data only for the surviving ocular melanoma patients and not for the total group. The information on their hair and eye color was obtained by self-report, while the data on the control group were obtained from direct observation by an unknown number of observers. In addition, the appropriateness of the control group used is unclear.

Several recent case-control studies conducted in the United States [66, 68] and in Canada [23, 67] have also reported a positive association between light

iris color and melanoma. Rootman and Gallagher reported a significant association between light iris color and iris melanoma in a case-control study that included 23 patients [67]. This result was confirmed by Kliman, Augsberger, and Shields who conducted a case-control study of 212 white patients with iris melanoma and two control groups [68]. Cases were identified from a review of 1974–1985 records of the Wills Eye Hospital in Philadelphia, Pennsylvania: one control group consisted of 76 spouses of patients referred to Wills Hospital between June 3–7, 1985; the other comprised 687 residents of four Canadian provinces who were the control group for the Western Canada Melanoma Study [23]. Iris color was determined by a variety of methods. Color photographs, chart descriptions, and drawings were used to determine iris color for the cases. Interviewers recorded eye color for the controls. While 79% of the cases had blue, grey, or green irides, the frequency of light iris color in each of the control groups was 54% or 56% respectively [68]. This association, which was statistically significant, must be interpreted cautiously given the differences in methods of iris color determination in cases and controls and questions regarding the methods of selection of the control groups.

The Western Canada Melanoma Study also reported an association between ocular melanomas and blue or grey irides [23]. This study interviewed 75% of the 87 ocular melanoma patients, aged 20–79 years, who were identified from the cancer registries of four Canadian provinces in 1979–1981. Age and sex matched controls were randomly selected from medical insurance plan lists and invited to participate. Control response rates were 59% in three of the provinces and 48% in the fourth. Skin, eye, and hair color were ascertained by direct observation in cases and controls, thus overcoming the problems of the previous studies. In univariate analyses, persons with blue or grey eyes had a higher crude risk of ocular melanoma as compared to those with brown eyes (odds ratio = 3.0). Individuals with blonde or red hair also had higher crude risks than those with dark brown or black hair (odds ratio = 7.7). These associations were not present when controlling for skin, hair, and eye color in multivariate analyses. The lack of such association is due, according to the authors, to the correlation between eye and hair color. An eye/hair color index was then constructed by combining eye and hair color categories. Persons at highest risk were those with blonde or red hair, regardless of eye color, and those with blue or grey eyes and light brown hair. Although skin color was lighter in cases than controls, these differences were not statistically significant.

A recent case-control study has reported an association between blue eye color and uveal melanomas [66]. The 497 white cases were identified from Wills Eye Hospital (Philadelphia, Pennsylvania) records from 1974–1979. Controls were patients with detached retinas matched by age, sex, race, and date of diagnosis. A second group of controls were patients referred to the oncology service for ocular tumors other than uveal melanomas. Data were collected by a telephone interview; 89% of the cases and 85% of the controls participated. Persons with brown eyes had a lower risk than those with blue eyes (odds ratio = 0.6; 95% confidence interval 0.4–0.8). Adjusting for hair and skin color

did not change the risks for eye color. As in the Western Canada Study, light hair and skin color were not associated with melanomas in the multivariate analyses. Cases reported having $\geq$ 25 freckles more frequently than the controls (odds ratio = 1.4; 1.04–2). Patients with iris melanomas were more likely to report freckles than those with other types of melanomas [66].

Despite the differences in methodology, the consistency of results among all these studies suggests that an association exists between ocular melanomas and light pigmentation. This finding is also consistent with the presence of a similar association between cutaneous melanomas and light eye and hair color.

5.1.2. Pre-existing intraocular pigmented lesions: 5.1.2a. Nevi. The relationship between uveal melanomas and pre-existing nevi has been investigated, and many investigators agree that a melanoma originates from a pre-existing nevus [69–72]. Choroidal nevi are significantly more common than are choroidal melanomas; this is also true for the frequency of nevi and dysplastic nevi of skin as compared to that of skin melanomas. Studies which followed individuals with choroidal nevi for up to nine years found no evidence of noticeable growth [14, 73]. A more recent study by Gonder et al., included follow-up of 116 patients with choroidal nevi [74]. Results of this limited study suggested that at least 4.3% of these patients progressed to choroidal melanomas with a mean follow-up period of 24.8 months. Ganley and Comstock, in a population-based study in Washington County, Maryland, found that 3.1% (nine cases) of individuals over the age of 30 had choroidal nevi [21]. The incidence rate of choroidal melanomas for persons over 30 years was only 1.3 cases per million per year. Their results suggest that only one out of approximately 5,000 persons with these nevi will develop malignant melanomas during a year.

Because of the low risk of ocular melanoma in persons with ocular nevi, it would be beneficial to identify high-risk characteristics for the development of malignant changes among these persons. For skin melanomas, melanoma risk can be predicted on the basis of dysplastic nevi and family history of dysplastic nevi or melanoma [75]. In the skin, definitive diagnosis of high-risk nevi (dysplastic nevi) requires histologic study, which is less feasible for ocular nevi. Therefore, clinical features of ocular nevi that predict malignant change should be explored further. Visual loss is not a reliable indicator of the transformation of a small choroidal melanotic tumor into a malignant melanoma [74], and other indicators need to be explored. Persons with oculodermal melanocytosis (nevus of Ota) and ocular melanocytosis, discussed below, should be examined periodically for malignancies [76].

5.1.2b. Oculodermal melanocytosis. One established risk factor for ocular melanomas is the nevus of Ota or oculodermal melanocytosis. This association was described as early as 1861 and noted in numerous case reports beginning in the early 20th century. Most recently, it has been confirmed by larger-scale studies [76, 77]. Virtually all uveal melanomas reportedly have arisen in the

ipsilateral eye and from areas of melanocytosis. Their location has been in the choroid or ciliary body but rarely in the iris.

The magnitude of the increased risk of uveal melanoma associated with the nevus of Ota is difficult to estimate. The prevalence of nevus of Ota in Gonder et al.'s [77] series of 1,250 white uveal melanoma cases, from the Massachusetts Eye and Ear Infirmary (1957–1980) and the Wills Eye Hospital (1974–1980), was 0.24% (i.e., 3/1,250). By comparison, no cases of nevus of Ota were found in a series of 13,150 white patients examined at ophthalmology services at Wills Eye Hospital in a seven month period (1980–81) [78]. Thus the increased risk of uveal melanoma in persons with nevus of Ota is large but uncertain. None of the 1,250 cases of uveal melanoma collected by Gonder et al. [77] were black, while one nevus of Ota did occur among blacks included in the clinic population at Wills Eye Hospital [78].

Velazquez and Jones [76] had a series of 1,210 uveal melanoma cases from the ES Harkness Eye Institute (New York City). Only three of these patients were black and one of these three had oculodermal melanocytosis [76]; the authors cited another reported case [79]. Velazquez and Jones [76] had no control population of nonmelanoma cases, but data on black controls from two other studies (reviewed by Gonder et al.) [77] suggest that the nevus of Ota may occur in about 0.01–0.02% of the black population seen at eye clinics [78]. Using these data, one would conclude that there may be a large, but statistically uncertain increased risk of uveal melanoma in persons with nevus of Ota.

Interestingly, the prevalence of nevus of Ota is relatively high among Japanese, but ocular melanomas are relatively rare; however, as we have noted, only a small percentage of uveal melanomas are related to the nevus of Ota, and Japanese persons may be protected by other factors such as eye color.

5.1.2c. Ocular melanocytosis. Ocular melanocytosis is histologically identical to the ocular component of oculodermal melanocytosis. The prevalence rate of ocular melanocytosis in a white population during screening of 13,150 consecutive patients at Wills Eye Hospital was 0.04% (2 of 5,241 white persons); no cases of oculodermal melanocytosis were found among these white patients [78]. Although the increased risk of uveal melanoma in ocular melanocytosis patients may be thirtyfold to thirty-five fold (Table 4), only a small proportion (i.e., 1.24–1.36%) of uveal melanomas are related to ocular or oculodermal melanocytosis [76, 77].

Ocular melanocytosis is reportedly rare among blacks (Table 3) and difficult to distinguish from normal pigmentation [78]. However, Wilkes et al. [80] reported what is believed to be the first case of orbital melanoma in a black patient with congenital ocular melanocytosis. Thus, blacks with ocular melanocytosis are apparently not excluded from increased risk of ocular melanoma. Melanoma was not originally suspected in Wilkes et al.'s [80] patient because he was black (and only 18 years old).

In conclusion, oculodermal and ocular melanocytosis are established risk factors for intraocular melanomas. The magnitude of increased risk in un-

Table 4. Ocular and oculodermal melanocytosis prevalence and estimated increased risk of uveal melanoma

	Prevalence (%) in eye clinic populations		Estimated increased risk of uveal melanoma
	Whites	*Blacks*	
1. Oculodermal melanocytosis (Nevus of Ota)	<0.01	0.01–0.02	Large but uncertain[a]
2. Ocular melanocytosis	0.04	<0.01[b]	30–35 fold[c]

Source: References [76–78].
a: See text for explanation.
b: Ocular melanocytosis is reportedly difficult to distinguish from normal intraocular pigmentation in blacks [78].
c: These estimates are uncertain because both cases and controls were from clinic populations; also, they are based on small numbers of events for numerators in calculations of prevalence.

certain, but these conditions probably account for only a small proportion of melanomas.

5.1.2d. Neurofibromatosis. Finally, neurofibromatosis (Von Recklinghausen's disease) may be a risk factor for uveal melanoma [81]. This autosomal dominant disease, which occurs in about one in 3,000 births, is characterized by at least five or six café au lait spots on the skin, along with uveal nevi [81].

5.1.3. Pre-existing dysplastic nevi and melanomas of skin Uveal melanomas have been documented in patients with B-K mole syndrome, which is also called 'dysplastic nevus syndrome' and 'familial atypical multiple mole/melanoma syndrome' [82–85]. Reviews of the literature on case reports [86] and studies of kindreds suggest that the association of intraocular melanoma with cutaneous melanoma and the dysplastic nevus syndrome may be coincidental [87–88]. The common occurrence of dysplastic nevi of the skin in the population may explain previous concerns that these nevi could be related to susceptibility to intraocular melanoma.

The conclusion that intraocular melanoma and cutaneous dysplastic nevi do not appear to be etiologically associated was based on a study of the prevalence of dysplastic nevi among uveal melanoma cases and cases of cutaneous melanoma [88]. An intriguing finding of this study, however, was the coexistence of conjunctival and cutaneous melanoma in one patient who also had a family history of 'multiple abnormal moles.' Taylor et al. [88] also cited a case of conjunctival melanoma in a patient with dysplastic nevus syndrome, and conjunctival melanomas have been reported in patients with skin melanomas (lentigo maligna melanoma, superficial spreading melanoma, and nodular melanoma). Thus, these associations require further study.

5.1.4. Laterality The question of whether melanomas have a predilection for the right or left eye has been investigated in a number of studies with

inconclusive and varying results. Some studies in the United States suggest that males have a high frequency of melanomas on the left eye while melanomas of the right eye predominate among females [22]. Subsequent studies have not corroborated these findings [8, 9, 11].

5.2. Familial factors

While most patients with uveal melanomas have no family history of such tumors, a few familial cases have been observed [89–93]. As discussed earlier, the association between uveal melanoma and the dysplastic nevus syndrome is believed to be coincidental and not of etiological significance for uveal melanoma [87, 88].

Several studies have evaluated associations of various antigens with uveal melanomas. A study from Germany reported a higher frequency of HLA (human leukocyte antigen) AW32 in 81 patients (11%) than controls (3%) [94]. The issue of the antigenic and cellular heterogeneity of melanomas is under active investigation [95–98] and is not yet fully understood.

5.3. Environmental factors

5.3.1. Sun or other light-related factors Because of the relationship between sun exposure and cutaneous melanomas, a similar association with uveal melanomas has been explored. Since much of the uveal tract is protected from exposure to ultraviolet light [3], such an association could not be easily explained by a direct effect of sunlight. In the Western Canada Study, sun-related factors were investigated by asking about skin reaction to the sun and sunlight dose, as determined by occupational, recreational, and vacation exposure. No associations were found with any of these variables [23].

A number of sun-related factors were investigated by Tucker, et al. in their recent case-control study [66]. Their results suggest an association between ocular melanoma and being born in the southern part of the United States, living in the tropics or subtropics for at least five years, sunlamp use, sunbathing, not using sunglasses, and spending more time in the sun during vacations than in daily living practices [66]. These findings are intriguing, but only suggestive at present because corroboration is needed from other investigations. However, the findings are consistent with studies concerning cutaneous melanoma and seem worthwhile for further study.

In interpreting the discrepancy in findings on sunlight exposure variables in these two case-control studies [23, 66], differences in design of the two studies should be noted. The larger sample size of the American [66] versus the Canadian [23] study (i.e., 444 cases vs. 65 cases) indicates increased statistical power to detect associations. On the other hand, the control group of the American study [66] consisted of patients with other eye diseases, while the Canadian study [23] involved randomly selected population controls; the latter design makes bias less likely. As noted by Tucker et al. [66], further studies are needed using other control groups.

In interpreting their case-control study results, Tucker et al. [66] noted that early life exposures to sunlight may be more important because most ultraviolet light is screened from the posterior eye by the lens after childhood [99]. The increased risk of iris melanoma with less eye protection also may support this interpretation [66]. In contrast, part of the secular trend for skin melanomas (versus eye melanomas) may be due to recreational sun exposure throughout life, although adolescent sunburns are also a risk factor [100].

The apparent lack of a strong secular trend for increased incidence of eye melanoma, or an age peak suggestive of a cohort effect, contrasts with the epidemiologic patterns for skin melanoma (discussed above) which have been interpreted as reflecting sun exposure effects. Thus, these contrasting epidemiologic features of skin and eye melanomas do not support the sunlight exposure hypothesis for eye melanomas. However, these findings cannot rule out the hypothesis; e.g., factors such as use of eye protection (sunglasses), childhood versus adult sun exposure trends, and possible differences in secular trend by regions of the eye which differ in ultraviolet exposure (iris and conjunctiva versus choroid) should be examined. Additional case-control studies of eye melanomas, by anatomic site and histologic type, are needed which address issues such as age, patterns of sun exposure throughout life, and use of eye protection. As noted above, large sample sizes and population controls would be advantageous in future studies.

The rarity of uveal melanomas in blacks and other races with more heavily pigmented eyes (e.g., Japanese) supports the possibility of a role of sunlight exposure in intraocular melanomas. It is not known whether some part of the uveal tract or conjunctiva shows less racial differences, as with the well-known preference for melanomas of the soles of the feet in blacks and Japanese [101, 102].

Results of animal studies strongly support the idea that ultraviolet-induced tumors are of clonal origin — i.e., derived from the progeny of a single transformed cell [103]. However, the mechanism for carcinogenic influence of UV-radiation could be direct (e.g., somatic mutations causing cell mitosis) or indirect (e.g., escape from immune surveillance via immunologic effects of ultraviolet light).

Direct effects of sunlight may depend on the penetration of ultraviolet light to different parts of the eye. Darker iris pigment may result in reduced transmission of ultraviolet light to the uvea, but this requires investigation, as noted by Gallagher et al. [23].

Indirect effects of ultraviolet light on melanogenesis are evident in the skin. Rosdahl [104] reported that skin melanocytes in areas both shielded and exposed to UV-B light displayed increased melanocyte numbers. Indirect effects of sunlight exposure on uveal melanoma risk that require consideration include the immunologic system, by analogy with effects on the skin; animal studies have shown an immunosuppressive effect of ultraviolet light, which permits development of skin tumors [105, 106].

The report that ocular (uveal) melanoma is reactivated after renal transplantation, and specifically after immunosuppressive therapy, in a case history [107]

is provocative. The frequency of uveal melanomas after immunosuppressive therapy in cancer patients also should be examined. This need is emphasized by a report of an unusual frequency of cutaneous malignant melanoma among patients treated for Hodgkin's disease, and the histologic characteristics indicated that an immunosuppressive mechanism was involved [108].

Reported immunological effects of commercial solarium exposure on humans acquiring a suntan, with reduced helper T-cell numbers, as well as the increased use of solaria [109], underscore the need for continued monitoring of uveal melanoma incidence. Additional case-control studies on ultraviolet exposure are also needed. A major problem in evaluating this issue, however, is the lack of a valid and reliable method to document lifetime sunlight exposure. Epidemiologic studies presently rely on self-reported history of exposure to ultraviolet light and this approach has many limitations, such as incomplete recall.

5.3.2. Socioeconomic factors The association of cutaneous melanomas with high socioeconomic status has been consistently reported [3, 110–114]. Limited data are available regarding the role of these factors in uveal melanoma. Studies in Scandinavian countries have found that the marital, socioeconomic, and occupational status of uveal melanoma patients are similar to those for the general population [14]. A recent study in England and Wales [16] reported the incidence of eye cancer (in 1974–1977) to be generally higher in nonmanual social classes than manual, but these findings are based on small numbers. The higher rate was found more specifically for administrators and managers, professionals, technical workers and artists, and electrical workers [16]. Of these findings, only the increased risk for indoor, managerial occupations (odds ratio = 3.5) was confirmed by the Western Canada Study [23].

5.3.3. Occupational and other environmental factors One recent report has described an increased incidence of choroidal melanomas in workers of a chemical plant, raising some questions about the role of occupational exposure to carcinogens (i.e., organic chemicals) [115]. Organic chemical exposure among chemists may be a risk factor for skin melanoma [116]. Another study, which identified a cluster of three cases of choroidal melanoma in a small community of 3,592 persons, has suggested contamination of the water supply as a possible cause for these cases [117]. These findings, however, are inconclusive. Animal studies of feline uveal melanomas induced with feline sarcoma virus suggest a possible viral etiology [118], and virus-like particles have been isolated from some uveal melanomas [119], but further studies are needed. Nutritional factors (e.g., vitamins, fat) have only recently been studied with respect to skin melanomas [120] and should be explored in case-control studies of ocular melanomas.

Another factor for investigation is possible exposure to certain drugs and heavy metals, such as those which have hyperpigmentation effects on the skin [121]. Phenothiazines, for example, preferentially bind the melanin-containing

eye tissues (retina and uveal tract) and accumulate in uveal tissue [122], with toxic (photosensitizing) effects and proliferation of pigment.

5.3.4. Hormonal factors Hormonal factors may involve both host (endogenous hormones) and environment (exogenous hormones, such as use of oral contraceptives and anabolic steroids). Several lines of evidence suggest that hormonal factors should be considered in the etiology of ocular melanomas. Known precursors of uveal melanoma, ocular and oculodermal melanocytosis, reportedly can develop or increase in pigmentation during puberty, the menstrual cycle, and pregnancy [123].

The appearance of skin melanomas around puberty and their higher incidence in females suggests an etiologic role for female sex hormones. Ocular melanomas are also rare before puberty, but a female excess in incidence does not occur except, perhaps, before middle age (see above).

The role of sex hormones in malignant melanomas of the skin is also uncertain. Estrogen and progesterone receptors have been identified in variable percentages of skin melanomas, including superficial spreading melanomas, and in patients with dysplastic nevi or family history of these nevi [124]. Long-term use of oral contraceptives has also been associated with superficial spreading melanomas in one case-control study [125]. Comparable studies need to be conducted for intraocular melanomas. Some aspects of endogenous hormones were discussed above with reference to seasonal patterns and male sex hormones.

5.4. Comparison of risk factors for ocular and cutaneous melanomas

While discussing the risk factors for ocular melanomas we have compared the findings with those for skin melanomas throughout the text. These comparisons are summarized in Table 5. Studies of the epidemiology of skin melanomas have provided clues to be explored in epidemiologic studies of ocular melanoma. Some of these clues have not proved rewarding, but others require further exploration. While there are many similarities in risk factors, a major difference remains regarding secular trends. As discussed above, the increased incidence of cutaneous melanomas over time has not been paralleled by changes in the incidence of ocular melanomas. This discrepancy suggests that different etiologic factors may have a role in each of these melanoma sites or that the etiologic factors are the same but the eye is protected and at lower risk.

6. Summary and conclusions

This review of the epidemiologic features of ocular melanomas suggests various conclusions which are outlined below.

— Several studies have been conducted to determine the incidence of ocular melanomas in Europe and North America. These studies consistently

Table 5. Comparison of risk factors for ocular and skin melanomas

Host factors	*Evidence for association*	
	Skin	*Eye*
Age (over age 15)	Yes	Yes
Sex (female)	Yes	No
Race (Whites)	Yes	Yes
Hair, skin, eye color	Yes	Eye color
Pre-existing nevi	Yes	Nevus of Ota and ocular melanocytosis
Familial factors	Yes	Questionable association
Environmental factors		
Latitude	Yes	Questionable association
Sunlight exposure	Yes	Suggestive, but needs further study
Socioeconomic factors (high)	Yes	Yes
Occupational factors	Yes	Unclear, needs further study
Nutrition (vitamins, fats)	Suggestive	Unexplored
Other factors		
Sex hormones (exogenous and endogenous)	Suggestive	Needs further study

report annual incidence rates between five and seven cases per million.

— In contrast to the rise in incidence of cutaneous melanomas, no consistent increases in incidence of ocular melanomas have been observed.

— The risk of ocular melanomas increases with age from 5–10 cases per million around age 40 to 20–40 per million in the seventh decade.

— No major differences in risk by sex have been observed, but males tend to have higher rates than females. Although the risk in women may be increased at younger ages, this pattern has not been observed consistently.

— Race is a major risk factor. Ocular melanomas are very rare in the more pigmented races.

— Light iris color has been associated with increased uveal melanoma risk in several studies. Light skin and hair color have not been independent risk factors in all studies.

— The presence of certain pre-existing pigmented lesions of the eye increases ocular melanoma risk.

— The association of increased sunlight exposure to ocular melanoma risk is unclear. Incidence is not conclusively related to geographic latitude, and the history of past sunlight exposure has not always been associated with melanoma risk.

— Most epidemiologic studies of ocular melanoma have been directed to measuring incidence rates, and few have explored specific etiologic hypotheses.

Future studies should evaluate the roles of sunlight exposure and of occupational and chemical exposures, as well as delineate the importance of precursor lesions as risk factors. These future studies should consider separately the various sites of ocular melanomas — i.e., uveal tract (iris, ciliary body, and choroid) and conjunctival tumors, since risk factors for each of these sites may not be the same. The studies should include careful analyses of secular trends.

Any future case-control studies should include a sufficiently large number of cases to give adequate statistical power and, preferably, include population controls.

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5. Malignant Melanoma: Prognostic Factors

Terence J. Harrist and Calvin L. Day, Jr.

1. Introduction

Understanding prognosis of malignant melanoma is becoming increasingly important. Firstly, the incidence of cutaneous malignant melanoma is rising rapidly [1]. There has been an 80% increase in the incidence of melanoma in the United States between 1973 and 1980 [2]. Up to one in every 200 citizens of the United States may expect to develop a melanoma during the next generation. Secondly, understanding the interplay and importance of individual prognostic factors may allow insight into the biology of the tumor. Thirdly, understanding of prognosis allows a general outline of therapy for subsets of patients with melanoma and explicit tailoring for therapy of individual patients. Lastly, although not of least importance, is the ability to give the patient some understanding of the gravity and expected outcome of his particular case. This last factor is of extreme importance because many patients and physicians equate the diagnosis of malignant melanoma with the grim prognosis and outlook for patients as cited in the older literature.

In order to understand prognosis in malignant melanoma, the following schema will be followed: Firstly, clinical staging of malignant melanoma patients will be considered. Secondly, the length of follow-up time needed to assess ultimate outcome is considered. Thirdly, the clinical and pathologic parameters included in a prognostic model are presented. Fourthly, a prognostic model using Cox multivariate analysis and the data derived from the Massachusetts General Hospital-New York University Clinical Cooperative Study Group (MGH-NYU) is developed in combination with the findings of others. Lastly, new clinicopathologic parameters that have been studied will be presented in a preliminary fashion.

2. Clinical stages

The criteria for clinical staging is presented in Table 1. Virtually all Clinical Stage III malignant melanoma patients die of disease [3]. Those patients with distant metastases that, when resected, appear to be clinically free of disease,

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Table 1. Clinical stages

Stage 3	Distant metastases
Stage 2	Regional lymph nodes suspicious for tumor (palpation)
Stage 1	No clinical evidence of metastasis

had a median survival time of 16 months versus five months for patients with unresectable distant disease. [3]

In patients who are clinically suspected of having regional lymph node metastases by palpation (Clinical Stage II) virtually all undergo regional lymph node dissection. In the clinical and pathologic Stage II patients, virtually all eventually die of malignant melanoma. Indeed Mundth and his colleagues reported a 10-year survival of 3% [4] while Balch and associates had *no* 10-year survivors [5]. Thus, in Clinical Stage II disease, the outcome is uniformly poor. Because the number of patients who get 'good outcomes' and the number of patients with 'poor outcomes' are so radically different in number, the latter markedly greater than the former, it is difficult to analyze the prognostic factors in this group.

As well, it is really the Clinical Stage I patients that are of most importance. In the MGH-NYU melanoma study in which the patients were observed within 30 days of diagnosis, 606 of the total 644 were Clinical Stage I [6]. Therefore, clinical stage I patients are encountered much more frequently and, as previously shown, virtually all Clinical Stage II–III patients die of disease. Thus it is Clinical Stage I malignant melanoma patients from which the prognostic factor models have been developed.

3. Final outcome versus time from diagnosis

From Sugarbaker and McBride's work it is clear that disease-free survival and overall survival are very highly correlated (Table 2) [7]. The ten-year results show only a 4% difference between survival and disease-free survival. However, at seven years after therapy there is only 4% difference between overall survival and disease-free survival. Indeed, in one study, all patients who were destined to die of metastatic malignant melanoma had evidence of recurrent or metastatic disease by six years [8]. Therefore, disease-free survival at six to seven years appears to be a good predictor of long-term survival with malignant melanoma. As well, the Melanoma Clinical Cooperative Group has a minimum of seven years follow-up on all patients entered between 1972 and 1977 and as much follow-up as 12 years on some. The conclusions of the studies using disease-free survival have held up on re-analysis using actual survival as the outcome. Thus, those clinical and pathologic factors that are associated with disease-free survival at six to seven years appear to be associated with long-term good results.

Table 2. Actuarial survival and disease-free survival for 128 patients with clinical stage I melanoma of the trunk [7]

Years after therapy	% Survival	% Disease-free survival
1	92	74
2	84	62
3	78	60
4	72	58
5	65	57
6	63	57
7	61	57
8	59	55
9	59	55
10	55	51

4. Clinical and pathologic factors studied (see Table 3)

Because the MGH-NYU study was prospective with patients observed within 30 days of prognosis, the exact site of the primary tumor is known. The surgical treatment variables included wide local excision versus wide local excision and elective regional lymph node dissection [9].

With respect to histologic variables, the exact mode of ascertainment must be understood. The histologic type was defined according to the criteria of Clark as was the level of invasion [10]. The measured depth was taken according to Breslow [11]. Lymphocytic response was judged at the base of the tumor at its most deeply invasive point [12]. Histologic regression was judged according to the criteria used by Gromet et al. [13]. Histologic ulceration was judged according to criteria by Balch et al. [14] and measurements were taken according to Day et al. [6, 15]. The number of mitoses per millimeter square were determined in the following manner [12]: The tumors were scanned to define zones in which there was an apparent increase in mitotic activity. In this zone the number of mitoses were counted over a millimeter square area using an ocular micrometer. Microscopic satellites, a new parameter, were defined as nests of tumor greater than 0.05 mm in diameter, deep to the principal mass of invasive tumor, and separated from it by normal collagen [15, 16].

The Cox multivariate analysis was used to statistically examine the prognostic value of each of these parameters to assemble a prognostic model [17]. It is a method of obtaining the best combination of variables to predict outcome and to correlate with biologic behavior of the tumor. It uses a process called stepwise regression. In this procedure, single factors and combinations of factors duel in a 'statistical tournament.' In general, the best individual prognostic factor is selected first and then joined by complimentary factors to complete the model.

Table 3. Clinical and Pathologic factors studied

Clinical variables
Sex
Age
Primary tumor location
Surgical treatment
Histologic variables
Histologic type (Clark)
Level of invasion (Clark)
Measured depth (millimeters) (Breslow)
Lymphatic invasion
Lymphocytic (host) response
Histologic regression
Histologic ulceration
Mitoses per millimeter square
Pathologic stage
Microscopic satellites

Table 4. Prognostic models for clinical stage I malignant melanoma

Authors	Qualifying factors	Important prognostic variables
Eldh et al.[18]	All sites	1. Thickness 2. Location 3. Ulceration 4. Size 5. Level of invasion
Balch et al.[20]	All sites	1. Thickness 2. Ulceration 3. Surgical treatment 4. Location
Cascinelli et al.[19]	All sites	1. Thickness 2. Sex
Day et al.[21, 22, 28]	1. Lower extremity 2. Upper extremity 3. Trunk	1. Thickness 2. Subsite

4.1. Tumor thickness (Breslow)

In the previous studies of the important prognostic factors for Clinical Stage I melanoma patients, thickness was the dominant variable (Table 4) [18–20]. In multiple analyses of the MGH-NYU data, thickness has been determined to be the dominant variable [21–23]. Thus, even across continents and in the hand of different investigators, thickness is reproducibly the dominant variable in predicting outcome. Breslow had previously shown that assessment of thickness in millimeters of the primary tumor had high interobserver congruence and high single observer reproducibility [11].

Table 5. Five year survival rate comparisons with respect to primary tumor thickness in clinical stage I malignant melanoma

Thickness (mm)	ELDH et al. [18]	Melanoma clinical cooperative group
≤ 0.75	98%	99%
0.76 to 1.50	90%	94%
1.51 to 2.25	83%	84%
2.26 to 3.0	72%	77%
> 3.0	46%	46%

More importantly, the survival results for given thickness ranges obtained by different centers appear to be reproducible as well. The data from Table 5 show that the survival results in given thickness ranges of the primary malignant melanoma are similar from center to center. Especially in the thicker tumors, there is good agreement. In the thin group, the World Health Organization study showed a lower survival rate [19], but they had more patients with lesions near 1.5 mm in thickness and the MGH-NYU group had more patients near 0.75 mm in thickness.

In summary, thickness is the best single prognostic variable as shown in several studies. Its ascertainment in any individual case is highly reproducible with good interobserver congruence, and the survival results for given thickness ranges are reproducible from center to center. This last observation is important in that it allows comparison of therapeutic modalities in the given thickness ranges from center and center.

4.2. Thickness ranges (Table 6)

It was assumed initially that malignant melanomas would behave more aggressively as the thickness of the primary tumor increased [24, 25, 26]. Along with this assumption, it was decided arbitrarily to separate malignant melanoma patients according to primary tumor thickness. Lesions were separated into those less than or equal to 0.75 mm in thickness, 0.75 to 1.5 mm, 1.51 to 2.25 mm, 2.26 to 3.0 mm, and greater than 3.0 mm in thickness [18–23]. These arbitrary 'cut points' have been shown to be useful. It had been previously assumed that the mortality rate increased in linear fashion with thickness. However, using logistic regression, new 'cut points' were determined using the MGH-NYU data and were corroborated by a complimentary statistical technique. The analysis suggests that the mortality rate increased with respect to thickness in 'stair step' fashion with the boundaries being less than 0.85 mm, 0.85 to 1.69 mm, 1.70 to 3.60 mm, and greater than 3.60 mm. The eight-year survival rates for patients in each category are $99 \pm 1\%$, $93 \pm 2\%$, $69 \pm 5\%$, and $38 \pm 6\%$ respectively [27].

Indeed, among patients with thin Clinical Stage I malignant melanomas less than 0.85 mm in thickness, only one of 202 patients died. In the next thickness group (0.85 to 1.69 mm), there were 12 deaths. When the plot of the locations of

Table 6. Important prognostic factors for clinical stage I malignant melanoma patients in given primary tumor thickness ranges

Thickness	Prognostic variables
0.76 to 1.69 mm	Subsite Level of invasion
1.51 to 3.99 mm	Mitoses per mm square Subsite Ulceration Microscopic satellites
> 3.65 mm	Subsite Lymphocytic response Histiocytic type Pathologic stage

these melanoma was examined, 11 of the 12 were on the upper back, posterior arm, posterior and lateral neck and scalp [28]. Indeed, the twelfth death occurred in a patient who had a melanoma just inferior to the clavicle. In addition, the one patient who died of metastatic melanoma in the thin group (less than 0.85 mm in thickness) also had his melanoma located on the posterior arm. Therefore, there seemed to be localization of lethal melanomas within this category in the so-called BANS area (see 4.3) [28]. Multivariate analysis of malignant melanomas from 0.85 to 1.69 mm in thickness showed subsite to be a dominant variable, with level of invasion second [28, 29].

In patients whose melanomas were 1.5 to 3.9 mm thick, the mitotic rate and subsite were most closely associated to outcome [12]. This thickness range was used because one group of investigators showed that elective regional lymph node dissection prolonged survival in such patients [20]. In our experience elective regional lymph node dissection was used as a variable in all multivariate analyses [12, 21, 22, 23, 28]. In none of them did elective regional lymph node dissection appear a significant variable. Thus the importance of subsite as a prognostic variable is clear. Other prognostic variables are also of importance.

4.3. Location

The effects of location on prognosis have previously been studied considering the following different areas: (1) axial versus extremity, (2) head and neck versus trunk versus extremities, and (3) head and neck versus trunk versus upper extremity versus lower extremity. In the MGH-NYU study, however, detailed body maps were used that allowed specific designation of the primary melanoma site. While the subsite concept has emphasized so-called 'BANS' area (*B*ack, posterior lateral *A*rm, posterior lateral *N*eck, *S*calp), the most striking effect of subsite on prognosis occurs when examination of the data on primary malignant melanomas of the extremities is undertaken [21, 22].

If the prognostic model derived from Cox multivariate analysis of data for Clinical Stage I malignant melanoma patients who had lesions of the upper

Table 7. Prognostic model for clinical stage I melanoma patients with primary sites on the upper extremities and lower extremities

Upper extremity	Lower extremity
1. Thickness	1. Thickness
2. Subsite	2. Subsite
3. Ulceration	3. Mitotic rate

extremity and lower extremity are examined (see Table 7), the two dominant variables are thickness and subsite [21, 22].

The previously reported prognostic models also agreed that location of the primary tumor had a significant effect on survival even after correcting for thickness. It should be noted, however, that two studies have not listed location as an additional prognostic factor. The World Health Organization (WHO) study selected only thickness and sex [19]. However, in the multivariate analysis of the MGH-NYU group, it appears as if most of the observed effects of sex on prognosis are secondary to exact site of the primary tumor. As well, a study of Clinical Stage I, Pathologic Stage II patients did not include location in the prognostic model [30]. However, virtually all patients with hand or foot melanoma die of disease when regional lymph nodes contain tumor. Indeed in the analysis of extremity melanomas, the subsites that imparted a worse prognosis were those of the hand or foot [21, 22]. It is important to emphasize that this observation does not reflect a specific histologic subtype, i.e., acral-lentiginous melanoma. Superficial spreading and nodular melanomas also occur on the hands and feet. Thick melanomas, regardless of histologic subtype, do very poorly. For example, nearly all patients with melanoma of the hand or foot died of disease if the primary tumor was greater than or equal to 2.75 mm in thickness [21, 22]. Conversely, none of the 63 patients with melanoma of the forearm or antero-medial arm died of malignant melanoma [21].

Table 8 lists the important prognostic factors for melanomas of the trunk, head, and neck. In these analyses, melanomas in the thickness range from 0.85 to 1.69 mm located on the upper back, posterior lateral upper arm, posterior lateral neck, and posterior scalp (BANS) had a worse prognosis than those melanomas at other subsites (see Table 9) [29].

Of 203 patients with Clinical Stage I melanoma and primary tumors in the 0.76 to 1.69 mm thickness range, 12 deaths occurred; 11 occurred in patients with primary tumors located in the BANS area [28]. There has been only one death from melanoma in 136 patients with melanoma located at other sites [28].

However, it should be noted that other studies have not concluded that location in the BANS area indicates a worse prognosis for intermediate thickness melanomas [31]. However, this study variably included thick melanomas (greater than 1.69 mm) and did not reflect the exact BANS area studied. The WHO study revealed a 27% difference in survival in the thickness range, 0.76 to 1.69 mm, when BANS is compared to non-BANS subsites [28, 32].

Table 8. Prognostic models for clinical stage I malignant melanoma in patients with lesions on the trunk, head and neck

Trunk	Head and neck
1. Thickness	1. Thickness
2. Subsite	2. Subsite
3. Mitotic rate	
4. Lymphocytic response	

Table 9. Clinical stage I malignant melanoma $7\frac{1}{2}$-year survival

Thickness	Non-BANS (extremities except hands & feet)	Non-BANS (head & neck)	Non-BANS (trunk)	Non-BANS (heads & feet)	BANS
<0.85 mm	100	100	100	100	98
0.85 to 1.69 mm	100	100	97	100	78
1.70 to 3.64 mm	86	64	77	60	58
≥3.65 mm	83	65	22	0	33

4.4. Ulceration

Presence or absence of ulceration is now generally accepted as having added prognostic value [6, 12, 14, 18, 21, 22, 33, 34]. In studying the parameter in more detail, ulceration width also appears to correlate with survival rate (see Table 10 and 11) [35], indicating that melanomas with only focal areas of ulceration (less than 3 mm) may behave similarly to those melanomas without ulceration.

4.5. Other variables

The data derived from assessment of other variables appear to be much less important in yielding added prognostic information.

Level of invasion as originally defined by Clark and his colleagues [10] was the critical first step in assessing and developing useful prognostic information with this capricious tumor. However, with the advent of the Breslow measurement, [11] the added information of anatomic level to prognostic model is considerably less and does not appear regularly in prognostic models.

The importance of regression is debated. Gromet et al. [13] said that thin lesions with regression (less than 0.76 mm) have a worse prognosis. Although evidence of histologic regression was present in over 50% of 202 thin melanomas (less than 0.85 mm), only one of these patients died [12]. Thus, the presence of regression in melanomas does not indicate a poor prognosis in the MGH-NYU study.

4.6. New variables

A new histologic parameter is the assessment of the presence of microscopic satellites, which are deposits of tumor, greater than 0.05 mm in diameter,

Table 10. Five-year survival rates of malignant melanoma patients by ulceration width at 3 mm increments

Ulceration width	Survival rates (%)
None	89
0.1 to 0.3 mm	86
3.1 to 6.0 mm	45
> 6.0 mm	45

Table 11. Five-year survival rates for patients with clinical stage I melanoma when stratified by primary tumor thickness and ulceration width

Thickness	Ulceration width (< 3 mm) survival rate (%)	Ulceration width (< 3 mm) survival rate (%)
≤ 0.75 mm	99	*
0.76 to 1.50 mm	91	*
1.51 to 3.99 mm	76	47
≥ 4 mm	50	34

*Too few of these melanomas had ulceration for proper evaluation

separated by the normal reticular dermis or subcutaneous adipose tissue from the principle tumor mass in the sections in which the Breslow measurement is taken [6, 16]. These lesions may represent true metastases. As well, because serial sections were not made, it may be that these deposits do not represent true 'satellites.' However, the five-year disease-free survival rate of 95 patients with microscopic satellites studied was 36% in contrast to that of 89% for 501 patients without these satellites [6]. As well, the presence of 'microscopic satellites' may be the best single predictor of occult regional lymph node metastasis [16]. However, confirming data from other studies is needed.

Clearly the most important parameter with respect to prognosis relates to the vertical growth (nodular growth) of the tumor [36]. Whether or not there is radial growth phase disease appears unimportant prognostically. Indeed, the plaque component melanomas are in many ways similar to dysplastic nevi as shown by the fact that melanocytic density, cytologic features, and size in dysplastic nevi are similar to the intraepidermal component of superficial spreading malignant melanoma [37]. As previously shown, tumor thickness and survival have an inverse (not perfect) linear relationship. In previous studies, we have shown plaque melanocytes are different from nodular melanocytes with respect to surface phenotype [38].

Therefore, it is important that further evaluation of the nodular/vertical growth phase be undertaken, for it is here that keys to the important information relative to metastasis and subsequent survival are present. In a preliminary study we reviewed the cytologic features in the vertical growth phase. We have found that patients with vertical/nodular growth phase composed of well-differentiated spindle cells had a 100-month survival rate of 100% [36]. When epithelioid or small cells with granular cytoplasm arranged in

prominent nests were present, the 100-month survival was 49%. In those patients with mixtures of spindle and epithelioid cells the 100-month survival rate was 0%. This data implies that the epithelioid cell subtype in the vertical growth phase conveys a poor prognosis. Relative evidence supporting this is a finding that epithelioid melanocytes taken from cultures of malignant melanoma cell lines have surface phenotypes and morphology more similar to primitive embryologic precursors (melanoblasts) than to fetal, neonatal, or adult melanocytes [38].

5. Summary

Thickness of the primary tumor and its exact location (subsite) appear to be the most important variables with respect to prognosis in Clinical Stage I disease. Ulceration (and ulceration width) also is a crucial variable.

While controversy still exists concerning the exact high risk subsites and the importance of other variables, *no* potential variable studied is likely to replace tumor thickness as the dominant variable.

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6. Endocrine Influences on the Natural History of Human Malignant Melanoma

Larry Nathanson and Gajanan A. Kulkarni

1. Introduction

The normal counterpart of the malignant melanoma cell is the melanocyte. This cell has been widely included in the Pearce classification of the APUD (amine precursor uptake and decarboxylase) category of cells. Without debating the validity of the APUD hypothesis, the melanocyte shares with these cells an embryologic origin in the neural crest and a role as a microendocrine system in which a cell product (the melanosome) is transported across cell membranes and intercellular space and into adjacent cells (epidermal keratinocytes). The major precursor of its biochemical product, melanin, is a substituted amino acid (3, 4-dihydroxyphenylaldinine), and the melanocyte has particular histochemical staining properties which relate to its biochemical activity. Thus, it is not surprising that malignant melanoma exhibits features of its natural history which strongly suggest endocrine influence on either the initiation of the neoplasm or on its natural history.

2. Endocrine features of the natural history of melanoma

A variety of features of the natural history of human melanoma suggest endocrine influence [1, 2, 3, 4, 5, 6]. At any level of thickness of primary melanoma, or at any stage of development of metastatic melanoma, there appears to be superior female survival [1, 7]. Secondly, the incidence of melanoma rises markedly after puberty; true malignant melanoma is rare in prepubital children [4]. The survival rate in postmenopausal women appears to be significantly inferior to that seen in premenopausal women [1]. Reports of the effect of pregnancy on malignant melanoma are confusing [8, 9, 10, 11]. Studies of large numbers of women in the reproductive age suggest that women with node positive melanoma have inferior prognosis if the disease is diagnosed during, or before, pregnancy [8]. Although it is true that women tend to present with thinner lesions, with more prognostically favorable primary melanoma, and with earlier stage metastatic melanoma than do men, this favorable survival advantage of women still persists [1, 7] when adjusted for level and stage. Women appear to have longer mean survival than men, even when incurable

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metastatic disease is present; they appear to progress through stages of metastatic disease more slowly than do men [1]. Multiparity also appears to convey a somewhat superior prognosis perhaps due to immunization against tumor-associated fetal antigens [12]. The distribution of primary melanoma is different in men than women, with the lower extremities the predominant single site of cutaneous melanoma in women, whereas the trunk is the predominant primary anatomic site in men. Although it is true that this could simply reflect clothing styles in men and women, the lack of frequency of face and neck, and arm lesions in the male, a site of sun exposure, seems to belie this hypothesis. Clinical chemotherapy and/or radiotherapy response rates may also be somewhat higher in men [13].

Recent studies have yielded conflicting data on the effect of oral contraceptives as a risk factor for melanoma [14, 15, 16, 17, 18, 19]. More recent studies [15] suggest no detectable risk for melanoma associated with oral contraceptive use, with the possible exception of women who have used such medication for over ten years [19]. The possibility that estrogen, either taken exogenously or produced endogenously (as in late pregnancy), would adversely affect female survival seems unlikely, as it is inconsistent with the higher premenopausal estrogen levels associated with favorable survival. Because some studies of melanoma have shown that only Stage II melanoma seems to be adversely affected by pregnancy [8], for Stage I disease it would appear that advice against pregnancy is unwarranted. An alternative hypothesis that melanoma is androgen dependent, explaining adverse survival in males, has been proposed [6]. No good experimental data, however, supports this hypothesis, nor does the difference in risk among various subgroups of women seem consistent with it.

3. Experimental studies

A variety of studies of hormonal factors have been carried out either *in vitro* or in animal systems [20–33]. The majority of these studies have been in a series of spontaneous experimental animal melanomas in the mouse, B16 [25], Cloudman S-91 [29], Harding-Paisey [28]; the hamster, Fortner, Greene, Bominski; the horse, Levene; the pig, Sinclair Swine; the fish, platyfish-swordtail (Gordon), etc.

Although these tumors are of spontaneous origin, the relevance of these animal models for human clinical melanoma is questionable. Still further removed from the human clinical situation are animal tumors which are chemically or virally induced. In some studies, testosterone and 17-beta estradiol have been shown to inhibit the growth of animal cells [23], and, in others, melanoma has been shown to grow more slowly in the female than in the male [24]. In the latter model, oophorectomy appeared to equalize the growth rates in males and females. Other investigators have found that estrogens stimulate the growth of animal tumors [25, 26], whereas oophorectomy and antiestrogens inhibit it.

4. Hormone receptors

The hope that melanoma cells may, under some circumstances, be hormone-sensitive stems from previously mentioned observations of apparent remission of melanoma in pregnancy following term delivery. A prerequisite for hormone sensitivity would be identification of a specific hormone receptor. Attention was first drawn to this possibility by a study in which 43% of melanoma patients were found to have estrogen receptor protein in tumor cells [34]. However, reports which suggested receptors might be present both in benign nevoid cells, as well as human melanomas [35, 36], were contradicted by a subsequent analysis [37] reporting the binding of estrogen to the enzyme, tyrosinase (the rate-limiting enzyme in melanogenesis), when dextran-coated charcoal technique was utilized. The possibility that previous observations has mistaken nonspecific estrogen binding to tyrosinase was reinforced by subsequent observations that no estradiol binding was detected in cytosols prepared from amelanotic melanomas. This suggestion was confirmed by use of sucrose density gradient analysis and DOPA inhibition studies [37]. Two studies of androgen receptors reported that, respectively, 81% [38] and 41% [39] of melanoma biopsies were positive for this receptor. Further study [35] showed a generally low range of receptor levels (peak 57.9 fm/mg cytosol protein) and of dissociation constants (peak 30 nM-K_D).

Of greater interest, however, has been the more recent reports of glucocorticoid receptors in both animal and human melanoma cells. Such receptors have been found in 28% [35], 86% [40] of melanoma cytosols tested. Such glucocorticoid receptors have also been found in mouse melanomas (B16) [41], Syrian hamster melanoma cells (RPMI3460) [42], and, more recently, in human malignant melanoma cell lines (NEL-M1) [20]. Receptor levels in this latter human melanoma cell line were 170 fm/mg tumor tissue and suggest the presence of physiologically significant levels of such glucocorticoid hormone receptors.

Plasma membrane receptors for polypeptide hormones, including melanocyte stimulating hormone (MSH), have been described in mouse melanoma cells (Cloudman S91) [21]. In this latter study, the demonstration of binding of MSH exclusively in G_2 phase of cell cycle was of great interest, for it suggests that synchronization of cells might be just as important to achieve hormone induced growth inhibition as it would be for the use of phase specific and cycle specific cytotoxic antitumor agents. Demonstration of MSH binding to plasma membrane of human melanoma cell line (HM6A) has also been reported [22].

5. Glucocorticoids

DiSorbo et al. [20], showed that *in vitro* treatment of human melanoma cells with the synthetic glucocorticoid (triamcinolone acetinide) resulted in significant growth inhibition *in vitro*. These studies also demonstrated that glucocor-

ticoids increase the activity of tyrosinase in human melanoma cells [27], and the authors suggested that the increase in tyrosinase activity, with resultant increase in melanogenesis, may be one mechanism by which glucocorticoids inhibit melanoma cell growth. Contradictory data exist in murine melanomas [28, 29], in which glucocorticoids stimulate tyrosinase activity. This, once again, emphasized the questionable relevance of experimental animal melanoma studies for the human situation. Some studies of growth inhibition induced by glucocorticoids [30, 31] suggest that this growth inhibition is accompanied by a reduction in the activity of enzymes hexokinase and G6PD, which are responsible for glucose utilization.

6. MSH

The polypeptide α-MSH may elevate intracellular cyclic AMP, tyrosinase activity, and increase melanogenesis in some cell systems [32, 33, 43]. This growth inhibition, however, may be as a result of autointoxication of the cell with melanogens, precursors of melanin pigment which are cytotoxic [43]. However, in addition, α-MSH-induced increments in tyrosinase activity may inhibit DNA replication in cell growth in human melanoma cells [22]. Wick has used the combination of MSH and theophylline, and observed a synergistic growth inhibitory effect [44]. This study, which also demonstrated significantly decreased tumorigenicity, was, however, carried out using murine (Cloudman S91) melanoma cells.

7. Other growth factors

Certain autostimulatory polypeptides have been found to be secreted by melanoma cells [45]. These factors may change the growth characteristics of tumor cells, including their anchorage independent growth, loss of contact inhibition, and growth in serum-free media. Transforming growth factor (TGF) is a polypeptide hormone which interacts with epidermal growth factor (EGF) receptors and is capable of conferring the properties of malignancy on nonmalignant cells [45]. A low TGF has not been shown to directly stimulate melanoma cell growth. Melanoma growth stimulatory protein has been extracted from melanoma cells *in vitro* [46].

Human melanoma cells have been shown to have specific receptors for nerve growth factor (NGF) [47], and NGF production by melanoma cells have also been demonstrated [48]. Thus, autostimulatory effects of melanoma cells might be mediated by NGF production given the presence of NGF receptors. Clinical studies of humans with melanoma do not appear to demonstrate any abnormality in circulating NGF levels [49], but characterization of various growth stimulatory and growth inhibitory peptides — their cloning and production by recombinant techniques — is necessary before precise physiologic studies can be

carried out. The production by melanoma cells of plasminogen activator is of great interest [50]. This factor has procoagulant activity. Its high level in tumor cells may make possible lysis and subsequent invasion of the tumor stroma, while lower levels in the plasma may help initiate settling and invasion of the tumor embolus [51].

8. Pigment metabolism

A number of analogs of melanogens, intermediate products in the biochemical pathway to melanin synthesis, may have significant effects on melanoma cell growth. Those that have been explored include Dopamine (3, 4-dihydroxybenzylamine), alpha methylparatyrosine (DEMSER), pimozide (a dopamine antagonist), 4-hydroxyanisole (a tyrosinase inhibitor), L-alanosine, and azoleic acid (a tyrosinase inhibitor). Because these factors have been recently reviewed elsewhere [52, 53] and are not true hormones, they will not be discussed in detail. Suffice it to say that they appear to exhibit inhibition of DNA polymerase [54], inhibit melanoma cell growth [55], and improve survival in animals bearing melanomas [56].

Other hormonal agents including melatonin, FSH, and LHRH may modulate melanoma cell growth [52], but their activity in humans have not been studied in detail.

9. Clinical trials

The first report of a patient who demonstrated a definite tumor regression following orchiectomy was that of Herbst, who indicated that at least some melanoma patients may exhibit hormone responsiveness [57]. Other clinical data suggest the possibility that melanoma may be an androgen-dependent tumor [6]. Estrogenic agents, including estramustine phosphate, have been studied in human melanoma with disappointing results [58]. A summary of these clinical trials is shown in Table 1. Two out of 35 patients were reported to respond to DES by Neifeld [35]. Beretta reported treatment of chemotherapy resistant melanoma patients with medroxyprogesterone acetate [59]: 2 of 24 evaluable patients responded. An earlier study had demonstrated objective tumor regression in 5 of 44 patients who received a drug with glucocorticoid and antiestrogenic properties, NSC-17256 [60]. In a Phase II study with this drug, 6 partial responses were documented in 111 patients with metastatic melanoma [61]. Seven studies with Tamoxifen (Nolvadex) have been carried out [62–68]. Some of these studies have reported response rate in the 15–35% range, mostly in cutaneous or soft tissue lesions [62, 63, 64], but others have failed to demonstrate any efficacy [65–68]. Anecdotal reports of tumor regression following hypophysectomy [69] have appeared.

Although overall results of hormonal manipulation appear to be discourag-

Table 1. Clinical trials with hormonal agents in metastatic melanoma

Therapeutic agents	Number of patients			References
	Total (evaluable)	Responders PR	CR	
Diethylstilbestrol (5 mg tid)	35	2	0	2
Estramustine phosphate (15 mg/m^2/day)	26	0	1	50
Medroxyprogesterone^{19} acetate (400–600 mg/day)	24	2	0	51
NSC-17256 (200–600 mg/day)	44	5	0	52
NSC 17256 (200–600 mg/day)	111	6	0	53
Tamoxifen (10 mg bid)	8	2	1	54
Tamoxifen (20–40 mg/day)	26	3	1	55
Tamoxifen (20 mg bid)	17	1	2	56
Tamoxifen (40 mg/day)	25	0	0	57
Tamoxifen (10 mg/bid)	23	0	0	60
Tamoxifen (100 mg/m^2/day)	13	1	0	58
Tamoxifen (100 mg/m^2/day)	17	0	0	59

ing, there exists a small group of patients with metastatic melanoma who respond favorably to hormonal therapy. Identification of this subset of patients should be the major objective of future studies.

10. Conclusions and Summary

Abundant laboratory and clinical data suggest hormone responsiveness of some experimental animal melanomas and even some human melanoma cell lines *in vitro*. Results of endocrine manipulation in humans, whether by virtue of the use of estrogens, antiestrogens, hypophysectomy (MSH ablation), or androgens, have been largely disappointing. The heterogeneity of tumor cells may explain some of these failures, but it is essential that we study the factors responsible for growth inhibition in those melanoma lines which may be hormone sensitive. The relationship between glucocorticoid and other hormone receptors is a crucial link in this type of study. The possibility that expression of glucocorticoid receptors may be enhanced is currently under active study [70]. Additional molecular mechanisms which mediate these kinds of hormone sensitivity are crucial if we are to improve the therapy of this highly chemotherapy-resistant tumor.

Future directions of research might include (1) further work in receptors and their physiology in melanoma, (2) identification of the clinical features of a subset of hormonally responsive melanoma patients, (3) possible use of differentiation or other agents to promote expression of hormone receptors and hormone sensitivity of melanoma cells, and (4) the use of hormones in combination with cytotoxic chemotherapy and the pharmacologic advantage to both agents.

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7. Psychosocial Factors Associated with Prognostic Indicators, Progression, Psychophysiology, and Tumor-host Response in Cutaneous Malignant Melanoma

Ralph J. DiClemente and Lydia Temoshok

1. Introduction

This chapter will present findings from a logically progressive series of studies investigating biopsychosocial aspects of cutaneous malignant melanoma conducted by an interdisciplinary group of researchers at the University of California, San Francisco (U.C.S.F.). Commencing in 1980, U.C.S.F. investigators have examined relationships between psychosocial factors and a variety of outcome measures. To facilitate a clear presentation of this body of work, each study will be briefly described including study objectives, methodology, and results. While some of these studies were originally summarized in a 1985 article [1], the present chapter describes additional studies and provides more extended discussion. During our discussion, we will integrate the findings across studies pointing out consistencies in research findings as well as reconciling any discrepancies. Finally, we will present a brief discussion of the implications of psychosocial research and these findings for future research and care of patients with cutaneous malignant melanoma.

1.1. Psychosocial factors associated with prognostic indicators

Cutaneous malignant melanoma, a tumor arising from the pigment-producing cells in the skin, presents an advantage as a model system for investigating the relationship between psychosocial factors and prognostic indicators in that there are precise and reliable staging systems for defining extent of disease. For the past decade, the prognosis for cutaneous malignant melanoma has been estimated from a combination of clinical and histological factors. The most important of these factors is tumor invasion, measured by the anatomic level of dermal invasion [2], or more reliably, by the maximal vertical thickness of the tumor [3]. These histopathologic indices appear to be good estimators of disease progression and outcome: the five-year survival rate of patients decreases progressively with either increasing level of dermal invasion or thickness of the primary lesion [4].

The psychosocial factors employed in the present series of investigations were suggested by prediction and longitudinal studies of psychosocial factors

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and cancer that included some measure of medical status as a dependent variable [5–9], as well as by our own preliminary observations and hypotheses.

1.1.1. Methods Patients are referred from throughout northern California to the malignant melanoma clinics at the University of California, San Francisco and Children's Hospital in San Francisco. All patients over 18 years of age with Clinical Stage I or II cutaneous malignant melanoma who were seen between March 31, and October 30, 1980 were asked to participate in the study. Over 90% of the patients asked agreed to participate in the study. Of the 59 subjects, 49 were diagnosed as Clinical Stage I, and ten were diagnosed as Clinical Stage II (regional lymph node involvement). Subjects were all Caucasian and ranged in age from 18 to 72 years. Sex distribution was 55% male and 45% female.

A single pathologist rated two histopathologic indices: Clark's level of invasion (II–V) and Breslow's thickness measurement in vertical millimeters (ocular micrometer from stratum granulosum to the deepest tumor) by microscopic examination of paraffin-embedded sections of the biopsied primary lesion.

1.1.2. Psychosocial assessment A one hour structured interview, conducted by a clinical psychologist, was recorded on videotape and later independently rated according to a precise coding manual (available upon request from Dr. Temoshok). Independent *a priori* theoretical aggregation and factor analysis of variables from the interview yielded thirteen scales: (1) Catastrophic Reaction, in which melanoma has disturbed most areas of functioning and provoked dire thoughts; (2) Coping by Avoidance, particularly of emotional discussions; (3) Coping by Changing, e.g., attitudes or relationships as a result of having melanoma; (4) Coping by Denial, by not thinking or talking about melanoma; (5) Coping by Optimism, by being hopeful about the future; (6) Coping with Strength, by trying hard to be positive, strong, and stable; (7) Emotionally Expressive, expressing a high degree of emotion when first told of the diagnosis of melanoma; (8) Faith, placing faith in external authorities, i.e., God or physicians; (9) Minimization, minimizing the seriousness of melanoma; (10) Nonverbal Type C, nonverbally characterized as emotionally constricted, accepting, appeasing, etc., rather than rated as having more 'Type A' characteristics; (11) Stressful Changes, or life stress during past five years; (12) Type A, reporting being more easily agitated, turns aggression outward, high-pressured, easily angered, and (13) Type C, reporting that one tries to please others, avoid conflicts, avoid expressive negative feelings. Each scale derived from the interview demonstrated satisfactory internal reliability, ranging from 0.43 to 0.96. Of the thirteen scales, 11 had reliability coefficients greater than 0.60, and five were greater than 0.80.

Standardized self-report instruments were also administered to subjects. These instruments were selected because of their previous use with cancer patients or because they tapped particular psychological dimensions which were

of hypothesized theoretical importance. These included: the Marlowe-Crowne (M-C) Social Desirability Scale [10], Beck's Depression Inventory [11], the Psychological Distress Scale from the MMPI [12], the short form of the Taylor Manifest Anxiety Scale [13], the McNair profile of Mood States (POMS) [14], and a new Character Style Inventory developed by one of the authors [15].

1.1.3. Results The main findings may be summarized as follow: the psychosocial variables most strongly correlated with tumor thickness in millimeters were delay in seeking medical attention for suspicious lesions and five scales derived from the interview material, which were positively and significantly correlated: faith in God and/or doctors, less understanding of treatment for melanoma, being relatively more 'Type C' in expression than 'Type A' on 17 semantic differential scales rated by the coders, less previous knowledge of melanoma, as well as two specific styles from the Character Style Inventory [15], Narcissistic Character Style and Histrionic Character Style (both significant negative correlations). Although tumor thickness was used more than Clark's Level of Invasion as a dependent variable, because it is presumed to be a superior measure [16], correlations of these same psychosocial variables with level were very similar.

Tumor thickness was considered as a dependent variable in a hierarchical multiple regression analysis in which biological variables (age, complexion/eye color) were entered first, followed by situational/behavioral variables (knowledge, understanding, and delay). Psychosocial variables ('Type C' and 'faith') were entered last. Delay emerged as the most significant variable predicting tumor thickness.

Semi-partial correlation analyses between psychosocial variables and tumor thickness demonstrated that the associations between the psychosocial variables and tumor thickness are not diminished when potentially confounding demographic variables (age and occupational status) are controlled. However, when the sample was dichotomized into subjects less than age 55, or 55 years and over, all but three variables (histrionic and narcissistic styles, and less previous knowledge of melanoma) that were significantly associated with tumor thickness and levels, were even more strongly and significantly correlated with prognostic indicators in the elder age group.

1.2. A quasi-replication

In order to highlight and elaborate the psychosocial variables suggested by the previous study with prognostic indicators, additional subjects were studied using a modified interview protocol and self-report battery. Psychosocial constructs describing emotional and control-coping reactions, in particular, were operationalized.

1.2.1. Methods Sixty subjects were recruited between November 20, 1980 and June 20, 1981 from the malignant melanoma clinic at U.C.S.F. A major difference

in the structured interview was the addition of a series of questions (e.g., 'Describe for me a time recently when you were angry ...') about how the subject reacted emotionally, behaviorally, physically, and cognitively to situations involving anger, sadness, happiness, or fear. Responses to these questions, which were recorded on videotape, were later rated on seven-point scales by independent coders on the following dimensions: (1) verbal articulateness in describing emotion, (2) nonverbal, paralingual expressiveness, (3) differentiated affective expressiveness, (4) elaboration of bodily sensations, and (5) cognitive expression in content of description. Interviewers rarely had time to inquire about more that two emotions; these were most always anger and sadness.

The same self-report measures were used as in the previous study, with the additions of Bryne's Repression-Sensitization Scale [17] and Barron's Ego Strength Scale [18], and the deletion of the Character Style Inventory [15].

1.2.2. Results Several findings from the previous study were replicated: (1) the significant positive association between delay in seeking medical attention for suspicious lesions and Clark's Level of Invasion, (2) the significant positive correlation between less previous knowledge of melanoma and level, and (3) the significant positive relationship of less understanding of treatment to tumor thickness.

In terms of these new variables, those significantly negatively associated with tumor thickness included interview-coded scales assessing (1) differentiated affective expression, (2) elaboration of bodily sensations regarding sadness, (3) verbal articulateness of sadness, (4) minimizing the seriousness of melanoma, (5) verbal articulateness of anger, and, as a tendency, (6) the self-report scale of Ego Strength.

1.3. Factors related to patient delay in seeking medical attention

The next study [19] focused on both clinical and psychosocial factors associated with patient delay in seeking medical attention for a suspicious lesion. Given the evidence from the two studies reported above, that length of patient delay is significantly associated with increasingly unfavorable prognostic indicators in malignant melanoma, it becomes more critical to understand this behavior and to identify factors related to delay.

1.3.1. Methods The total number of subjects was 106, which included the entire first subject series and the first 47 of the 60 subjects from the second series.

This study focused on a subset of psychosocial variables derived from the structured interview: (1) length of time patient delayed, if at all, seeking medical attention for a suspicious lesion, (2) whether the melanoma was detected by the patient (or family member) or 'coincidentally diagnosed' by a physician during a visit for an unrelated problem, and (3) the degree to which patients minimized the seriousness of melanoma, in general, and of their own condition, in particular.

The same histopathologic measures were used as previously described. Additional tumor characteristics, which were derived from the U.C.S.F. Melanoma Clinic computerized data base files [20], included site of lesion and type (superficial spreading melanoma, nodular melanoma, lentigo malignant melanoma, or not classifiable).

1.3.2. Results and discussion The longest mean delay (six months) in seeking medical attention for a suspicious lesion was associated with lesions of the upper and lower back. The difference between patients with back lesions compared with all other sites combined was statistically significant. This finding suggests the need for better public awareness of the possibility of malignant lesions in less visually accessible places.

Considerably more nodular melanomas were self-detected than were found during visits to physicians for unrelated problems. Because nodular melanoma is the most malignant variety of cutaneous melanoma, this finding suggests the need for more attention by physicians to the possibility of nodular melanoma.

Patients who had little or no previous knowledge of melanoma had significantly thicker lesions, on average, than patients with some or a great deal of knowledge. Because patients with less previous knowledge delayed an average of 1.4 months longer than those with more knowledge, it is probable that patient delay mediated the knowledge-tumor thickness relationship. Similarly, patients with less understanding of treatment had significantly thicker lesions and delayed significantly longer before seeking treatment. The implications of these results for public health education are obvious.

Patients who minimized substantially the seriousness of melanoma, in general, and the seriousness of their own condition had *shorter* periods of delay than those who minimized little or not at all. A possible explanation for this is that minimizing may reduce fear and anxiety about the disease or its treatment and thus facilitate more prompt seeking of medical attention. This finding, coupled with that of the relationship between knowledge and delay, implies that public health education that enhances understanding of the disease and its treatment without making dire health warnings may have significant impact upon early detection of cutaneous malignant melanoma [21].

1.4. Repressive coping reactions in patients with melanoma vs cardiovascular disease

This study [22] attempted to demonstrate that certain psychosocial factors previously found to be associated with unfavorable prognosis in melanoma and other cancer patients [5, 6] would be associated with a particular pattern of psychophysiological response. It was also hypothesized, based on the notion of a bipolar Type A-Type C dimension [23–25], that the psychosocial and psychophysiological responses of melanoma patients would be significantly different from those of a comparison disease contrast group — patients with cardiovascular disease.

1.4.1. Methods Subjects were 20 malignant melanoma patients, with no current evidence of disease, seen for follow-up examinations at the U.C.S.F. Malignant Melanoma Clinic; 20 patients also seen for follow-ups (at least 4 months postdiagnosis) at the U.C.S.F. Outpatient Cardiology Clinic; and 20 disease-free controls who were friends or family members of patients seen in the same outpatient medical clinics building at U.C.S.F. The three groups were matched by gender, age, and ethnic origin. In addition, the two disease groups were selected to be as equivalent as possible in terms of disease severity. The groups did not differ significantly for the number of patients assigned by their physicians to low, intermediate, or high risk categories on the basis of likelihood of death from disease over the next five years.

The experimental procedure operationalized the construct of 'repressive coping reaction' as the discrepancy between relatively *high* physiological arousal-electrodermal activity measured by a dermograph, in conjunction with relatively *low* self-reports of perturbation in response to 50 anxiety provoking statements. Each statement was projected as a slide for five seconds while the subject's skin conductance reaction was monitored. After each statement, the subject marked 0–10 on a scale for how much the statement 'bothered me.'

The following self-report measures, which have been used in other relevant studies [8, 26] to assess concepts akin to 'repressiveness,' were also administered: the Bryne Repression-Sensitization Scale (R-S) [17], the short form of the Taylor Manifest Anxiety Scale (MAS) [27], the Marlowe-Crowne Social Desirability Scale (M-C) [10], and a difficulty in Adjustment Scale (DADS), which was an adaptation of one item from the Recent Life Changes Questionnaire [28].

1.4.2. Results As predicted, the melanoma group exhibited significantly more repressive coping behavior in the experimental procedure than both the cardiovascular disease and the control groups. The melanoma group scored in the 'least sensitized' direction, as hypothesized, on three of the four self-report measures (the R-S, MAS, and DADS): The melanoma group also scored on the more 'repressed' or differences on these three measures between the melanoma and cardiovascular disease groups were statistically significant.

There were no significant differences across risk category subgroups for either disease group on repressive coping scores derived from the experimental procedure, nor on any self-report measure. High predicted intercorrelations among all the various measures of 'repressiveness' employed in this study (except M-C) provide evidence for convergent validity, and suggest that the study tapped more general *response styles.*

1.5. Relationship of psychological variables to tumor-host response

A number of recent studies have investigated relationships among stress, psychological, and immunologic variables [29–34]. There are, however, only a

few such reports, of which we are aware, focusing on cancer patients [35, 22]. The present study [36] was undertaken to explore whether psychological variables found to be associated with a clinical variable, tumor thickness [23], might also be linked with tumor and/or host response factors.

1.5.1. Methods Subjects included the 59 patients from the first series and the 60 patients from the second series, described above, at the U.C.S.F. Malignant Melanoma Clinic. The same psychosocial variables and histopathologic microstage features included in the first two studies outlined above were used for the analyses. Additional tumor and host factors recorded in the U.C.S.F. Melanoma Clinic computerized data base files [20] were included. These files were built upon the pathology data base forms completed by the pathologist on each patient prior to clinical and/or psychological examination (i.e., without knowledge of age, sex, or location of tumor). From examination of paraffin-embedded sections of the primary lesions using the light microscope, the following tumor and host factors were recorded: (1) mitotic rate (number of mitotic figures/mm^3), which is a function of tumor growth, (2) lymphocytes at the base of the deepest invasion (rated few, moderate, or many), (3) macrophages in the host response (rated few, moderate, or many), and (4) plasma cells (rated few, moderate, or many). The computerized data base was also used to obtain follow-up information about patients' subsequent medical status and stage of disease.

1.5.2. Results In terms of clinical variables, tumor thickness was positively, significantly correlated with mitotic rate, and negatively, significantly correlated with lymphocytic infiltration. These two factors (in addition to tumor thickness) also significantly discriminated patients who, by three-year follow-up, had died or had disease progression (higher mitotic rate, fewer lymphocytes), from those with no evidence of disease (the reverse pattern). More advanced initial stage, greater Clark's Level of Invasion, and greater tumor thickness, also significantly discriminated the two outcome groups in the expected directions.

Considering psychosocial variables, it should be noted that intercorrelations among emotional expression variables derived from the interview were, in general, *negatively* correlated with self-report emotion variables from POMS. In terms of mitotic rate, all correlations with emotional expression were negative, and six out of eight were strong and significant. The findings were reversed for lymphocytes: correlations with emotional expression were *positive.*

1.6. Psychosocial variables discriminating patients with disease progression from matched controls

This study [37] is based on 1.5- to 3-year follow-up reports on subjects from both series. By follow-up, a total of 20 of 119 patients had died or had severe disease progression (visceral spread).

1.6.1. Methods Each of the 20 patients in the disease progression groups was matched with a control subject who had no evidence of disease at follow-up. Matching was done on the basis of five attributes demonstrated to be predictors of survival in malignant melanoma [20]: sex, age, histologic type, anatomic location of primary tumor, tumor thickness, and level of invasion. T tests indicated that the matching was successful; there were no significant differences between the two outcome groups on any of these attributes. Because of the small *N* involved, only psychosocial variables *in common* between series one and two were used for these analyses. There were complete data for 11 subjects in each group.

1.6.2. Results Out of ten conservative nonparametric tests (Mann-Whitney U), five were statistically significant and three more approached significance. Compared to controls, the unfavorable outcome group had *higher* scores on six emotion-related scales, Tension-Anxiety, Fatigue-Inertia, Depression-Dejection, Anger-Hostility — all from POMS, as well as the Taylor MAS and MMPI Distress Scale. The unfavorable outcome group had *lower* scores on the POMS Vigor-Activity scale.

1.7. Psychological adjustment to malignant melanoma as a predictor of disease progression

We conducted another long-term follow-up study [38] in order to replicate the findings of Greer and his colleagues [6, 39, 40] on the relationship between psychological adjustment and disease progression. In earlier work with breast cancer patients, these investigators found that two particular psychological adjustment styles, namely, 'stoic acceptance' and 'helplessness/hopelessness,' were associated with greater likelihood of disease recurrence at both five- and ten-year follow-up. This finding has withstood the longest test of time in the psychological literature. However, as the sample consisted only of women with Stage I or Stage II breast cancer, we attempted to replicate these findings with both men and women diagnosed with cutaneous malignant melanoma. In addition, we developed a quantitative method, which we term the 'profile method,' for coding and scoring patients' adjustment responses, which could be more easily scored and amenable to parametric analysis.

1.7.1. Methods The study sample consisted of 117 patients with a pathology-confirmed diagnosis of cutaneous malignant melanoma seen at the University of California, San Francisco and Children's Hospital, San Francisco, Malignant Melanoma Clinics between 1979 and 1981. All patients were seen within one month of biopsy. Patients had been referred to the two clinics for confirmatory diagnosis and treatment recommendations. Most patients were aware at the time that they had melanoma, but few knew exactly the severity of the disease or its prognosis. Patients whose initial consult was for a disease recurrence were excluded from the patient population.

Patients ranged in age from 15 to 86, with a mean age of 45 years. Forty-eight percent fell within the age range of 30 to 49 years. Sex distribution was 54% male and 46% female. The demographic characteristics of the study population are comparable to the clinics' patient population [20] as well as to melanoma patients in general [41]. Eighty-six percent of the patients were diagnosed with Clinical Stage I disease while 11% and 3% were diagnosed with Clinical Stage II and III disease, respectively. Twenty-five died or had disease progression. Of this group, the mean time to death or time to follow-up which identified disease progression was 18 months. Patients with no evidence of disease progression had a mean follow-up of 29 months. Patients were treated with a variety of therapeutic approaches which included surgery, radiation therapy, chemotherapy, immunotherapy, and various combinations of these treatments. Description of the psychosocial interview and assessment of tumor pathology were described previously [23].

The predictor variables were the psychological adjustment reactions reported by patients during the initial clinic interview. Two categorization procedures were used: the Greer et al. [6] procedure which assigns patients to one of four mutually exclusive adjustment categories (fighting spirit, stoic acceptance, denial, hopelessness/helplessness) and a second procedure which derived a quantitative measure for each of the four categories, thus yielding a profile of adjustment reaction scores.

All patients seen at the two malignant melanoma clinics who participated in the psychological study of melanoma were followed periodically. Clinical status, i.e., disease progression, death, or no evidence of disease, was assessed for each patient over the course of the follow-up period.

1.7.2 Results A contingency table analysis was conducted in order to evaluate separately the relationship between the four mutually exclusive adjustment reaction categories, as determined by the procedure of Greer et al. [5], and follow-up clinical status for men and women. Of the 53 women included in this analysis, seven had disease progression and 46 had no evidence of disease. Of the women categorized as stoic ($n = 17$), five had disease progression. Stated another way, of all women who relapsed, then, 71% were categorized as stoic ($p = .05$). None of the other psychological adjustment categories made a significant contribution to the overall chi-square. A similar analysis for men only showed no significant association between the psychological adjustment categories and clinical status.

For both men and women, a stepwise logistic regression analysis was conducted, which included the primary histopathologic and epidemiologic determinants of disease progression (clinical stage, age of the patient, location of primary tumor, and tumor thickness) and the profile of psychological adjustment scores. For the men only, a two-factor risk model, which included a biological characteristic of the tumor (tumor thickness) and a psychological adjustment response (feelings of helplessness/hopelessness), emerged as significant ($p = 0.03$). The psychological and the biological risk fac-

tors were independently significant at the $p = 0.06$ and $p = .001$ level, respectively.

2. Discussion

A number of studies have been described relating psychosocial factors to cutaneous malignant melanoma, ranging from factors affecting prognostic indicators, delay in seeking medical attention, tumor-host response, and styles of coping and their relationship to disease progression. Any overarching summarization would only serve to obfuscate rather than clarify the relationship between psychosocial factors and cutaneous malignant melanoma. There are, however, common threads which can be identified within each of the research areas. These commonalities are also similar to findings from related studies, which suggests that biopsychosocial cancer investigators are indeed tapping constructs which do exert an influence on patients' medical status. Therefore, noting our own caveat, we will present a brief discussion of the findings from related studies, each addressing a particular aspect of cutaneous malignant melanoma.

The initial U.C.S.F. studies addressed the issue of whether psychosocial factors were related to prognostic indicators in cutaneous malignant melanoma, particularly tumor thickness in millimeters. One study [23] showed that age demonstrated strong interactions with hypothesized psychosocial variables. This finding is consistent with the notion, also advanced by other investigators [42], that environmental factors and age-related deterioration of the immune system functioning may play a larger role relative to psychosocial factors in older patients, while psychosocial factors may play a more predominant role in younger patients. This study also found that coping styles associated with thicker, more invasive lesions included being relatively nonself-involved and being more Type C (e.g., passive, appeasing, helpless) than Type A. These characteristics are consistent with the notion of a Type C constellation of attitudes, cognitive and emotional proclivities, verbal and nonverbal expressive patterns, specific coping strategies, and more general character styles that describe persons for whom psychosocial factors play a role in the development and/or progression of their malignancies. Replication of these findings in our second study suggests that we have indeed identified a pattern of coping which may be associated with greater tumor invasiveness.

In a separate study [22], it was demonstrated that the psychosocial factors found to be associated with less favorable tumor indicators (a 'repressive' coping style) is also associated with a *psychophysiological* response pattern characterized by relatively high physiological arousal — electrodermal activity measured by a dermograph, in conjunction with relatively low self-report of perturbation in response to 50 anxiety-provoking statements capable of discriminating between two disease groups — patients with cardiovascular disease and cutaneous malignant melanoma.

There are at least two routes by which psychosocial factors could, hypothetically, influence cancer prognosis. First, the association between particular dimensions of Type C coping style and unfavorable prognostic indicators could be behaviorally mediated. That is, the Type C style may be a more structural personality factor underlying the specific behavior of delay in seeking medical care for symptoms of melanoma. An alternative explanation is that certain types of coping responses may be associated with particular biological responses that may exacerbate tumor growth and/or weaken the body's resistance to the malignant process.

Two studies address this issue. First, in one study using a matching design to control for biological sources of variability between patients who had disease progression and those who did not during the 1.5 to 3 years after initial diagnosis, seemingly conflicting findings emerge [37]. That is, compared to the control patients (those without a disease progression), the index patients (unfavorable outcome group) had higher scores on six emotional-related scales. This finding does not mesh with another study [23] which suggested that less emotionally expressive patients exhibiting characteristics associated with our hypothesized Type C coping style would have more advanced disease and consequently a greater probability of disease progression (recurrence).

In trying to reconcile these results, Temoshok et al. [37] speculate that a high degree of consciously perceived stress, subjectively experienced as anxiety, distress, and/or dysphoric emotion, contributes significantly to melanoma progression over and above that of clinical melanoma prognostic risk factors. It is possible that coping with this stress and its felt consequences by expressing the emotion will buffer these otherwise negative effects. Thus, it was proposed that negative effects on melanoma progression of consciously experienced stress and dysphoric emotions, as well as the hypothesized buffering effects of coping through expressing emotion, are mediated by cellular immune factors. Therefore, to the extent that the course of disease is influenced by the host's immune response (43), these psychosocial factors will have an indirect, but significant effect upon disease progression.

More to the point, perhaps, are the results of the three-year follow-up in which we developed and tested a new coding procedure for assessing psychological adjustment to the diagnosis of malignant melanoma [38]. We hypothesized that the styles of stoicism and helplessness/hopelessness would be associated with greater likelihood of disease progression (recurrence) at follow-up. Both these features are to some extent represented in the Type C coping style, although, stoicism perhaps captures the meaning of Type C more fully than the adjustment style of helplessness/hopelessness.

The findings of this study confirm earlier results reported by Greer and his colleagues [6, 39, 40]. Each study used a semi-structured interview to elicit information which was later coded to identify the patient's psychological adjustment responses. The psychological adjustment categories used in the present study are also an extension and more detailed version of those employed in the work of Greer et al. [6]. The criterion variable in both studies was disease

progression (any form of disease recurrence or no evidence of disease). Of course, Greer and his associates investigated only women with breast cancer, while we chose to study both men and women with cutaneous malignant melanoma. Stratifying our patient sample by gender, however, permitted an approximation of the contingency table analysis by examining the relationship between psychological adjustment and disease progression for women only.

The results suggest that a psychological factor, adjustment to having cutaneous malignant melanoma, may be a significant predictor of disease progression. Patients who had responses characteristic of stoicism (among women) and feelings of helplessness/hopelessness (among men) were more likely to have had disease progression. It should be emphasized, however, that although a psychological factor had a statistically significant influence on the course of disease, the major prognostic determinant remains the biology of the tumor. Based on the logistic regression analysis, the biological prognostic risk factor, tumor thickness, made a relatively greater contribution to explaining the variance in disease progression than did psychological adjustment.

3. Clinical implications

In summary, our investigations have pointed to particular coping responses, conceptualized as Type C coping style, as being associated with poorer prognosis and disease outcome. Biological factors remain of primary prognostic importance, however. By and large, though, biological and epidemiological prognostic risk factors are not mutable, whereas psychological risk factors may be subject to modification. Thus, one implication to be drawn from the findings is that patients who are identified as possessing psychological adjustment responses associated with unfavorable outcomes may be counseled or taught behavioral strategies which maximize their probability of remaining disease-free. Of course, before behavioral intervention can be realistically considered, a prospective study designed along the lines of a clinical trial would be necessary to establish the efficacy of any intervention approach.

In conclusion, while the finding that psychological adjustment may influence the probability of disease progression in patients with cutaneous malignant melanoma is provocative, further studies are necessary using larger patient samples and defining in greater detail the relationships among psychosocial and biological variables, before more definitive suggestions for the development and implementation of intervention studies can be proposed.

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8. Central nervous system metastases in malignant melanoma

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1. Introduction

Of all the potential complications of malignant melanoma, none is more devastating or life threatening than the development of central nervous system (CNS) metastases. Once this occurs most patients are dead within a few months. While treatment may improve symptoms, it does little to prolong life in the majority of patients. Indeed, no effective methods of prevention or treatment have been devised to date, and these are urgently needed if we are to look forward to long-term survival and cure of such patients.

Melanoma has a very clear predilection for spread to the CNS. It is one of the most common neoplasms to metastasize to the brain exceeded only by carcinoma of the lung and breast [1, 2]. This may reflect the neuroendocrine origin of melanoma cells which, when they find themselves in the CNS, recognize a compatible environment for continued growth. The probability of any newly diagnosed patient eventually developing CNS metastases is probably on the order of 20–30% if one assumes that 30–40% of all new patients diagnosed with cutaneous melanoma will develop metastatic disease, and, of these, 75% will eventually be found to have CNS metastases. Such a figure gives some idea of the magnitude of the problem. Although CNS metastases may be the sole manifestation of spread of melanoma, they are usually found in association with disease in other organs. Two recent large reviews have discussed this in detail (3, 4). In Amer's series [3] from Wayne State University, of 56 patients with CNS metastases, only 16 had this as the sole manifestation of spread of their melanoma. The opposite side of this question, namely, how many patients who have metastatic melanoma in other organs will be found to have CNS metastases at autopsy, has been documented at approximately 75% by these same authors. The incidence of CNS involvement in metastatic ocular melanoma has not been fully documented but is probably even higher than the figures noted for patients with cutaneous primaries.

2. Clinical manifestations of brain metastases

Table 1 shows the clinical characteristics of patients with cutaneous melanoma most likely to develop CNS metastases. They are males with primary lesions

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Table 1. Clinical characteristics of patients with cutaneous malignant melanoma most likely to develop CNS metastases

Clinical characteristics		Approximate percent of patients
Sex	Male	60%
Primary site	Above the waist in approximate order of frequency:	
	Trunk	40%
	Upper extremity	25%
	Head and neck	15%
	Lower extremity	10%
	Unknown primary	10%
Presence of other metastases	Lung	75%
	Liver	75%

above the waist, who have known metastases in other anatomic areas, particularly the lungs and liver. Amer and his colleagues reviewed the records of 122 patients with metastatic malignant melanoma seen at Wayne State University over a 12-year period between 1964 and 1976 [3]. Forty-six percent of these patients were found to have central nervous system metastases, which were diagnosed clinically, and another 29% were found to have clinically unsuspected CNS involvement at autopsy. They noted a predominance of males among the patients who developed this complication. In their series, 61% of the patients with CNS metastases were males and 39% were females for a male:female ratio of 1.56:1. Madajewicz and his colleagues [4] from Roswell Park Memorial Institute reviewed 700 patients with metastatic malignant melanoma seen at that institution between 1972 and 1978. One hundred twenty-five were found to have brain metastases. Like Amer, they found that the majority of patients were males with a male:female ratio of 1.9:1. We reported a similar male:female ratio of 1.5:1 in reviewing the records of patients at the University of Colorado with brain metastases from melanoma [5].

In all of these series, patients most likely to develop central nervous system metastases, in addition to being males, usually had primary lesions on the trunk, upper extremity, or head and neck. Forty-three of Amer's 56 patients (75%) had primary lesions above the waist, and 72% of Madajewicz's patients had primaries in the same area. We noted similar findings in the patients reviewed here, and found that only 10.3% of our patients developing CNS metastases had primary lesions on the lower extremities. It is well known that males are more likely to develop truncal and head and neck primary melanomas than females [6], and the difference in the male:female ratio may simply reflect the difference in where primary lesions occur. Why there is a propensity of truncal lesions to metastasize to the brain remains unknown. In a recent review of patients developing CNS metastases here, however, we have shown that the distribution of the metastases suggest that spread to the central nervous system in melanoma may be by routes other than the classic intra-arterial route, namely through Batson's intra-vertebral plexus [6, 7], and that this may explain the

Table 2. Common clinical manifestations of cerebral metastases in malignant melanoma

Signs and symptoms	Approximate per cent of patients
Headache	70%
Impaired mentation	60%
Motor deficit including cranial nerve palsies	50%
Seizures	25%
Cerebellar dysfunction	15%

differences noted. This data, however, needs to be expanded and confirmed, but will be discussed later in this section.

Long intervals between the diagnosis of a primary malignant melanoma and the subsequent development of metastases have been described for almost all sites of metastatic disease. The interval between the diagnosis of a primary and the development of CNS metastases averages about three years [3]. Intervals of up to 11 years have been recorded, but are relatively unusual with cutaneous primaries. It is also interesting to note that in Amer's study the interval between the diagnosis of a primary lesion and subsequent development of brain metastases was longest in patients with lower extremity lesions as compared with all other groups, suggesting a longer transient time, or perhaps a reflection of the fact that patients with lower extremity lesions are more likely to be female.

Table 2 shows the signs and symptoms most likely to occur in patients developing brain metastases from malignant melanoma. The most common complaints are headaches, which occur in about 70% of the patients, with or without other neurologic complaints or findings. This is followed by motor loss and impaired mentation occurring in 50–60%, convulsions, cerebellar dysfunction, and cranial nerve palsies. The neurologic findings may vary widely depending upon the location of the metastases and the extent of involvement at the time of diagnosis. Not infrequently, the neurologic examination will be entirely normal, but the presence of severe and persistent headache strongly suggests the diagnosis. In all such patients, a careful neurologic examination should be undertaken, followed by a computerized axial tomogram (CT scan) of the brain. In our experience, 75% of the patients will have some neurologic symptoms at the time of diagnosis, but of these, only one-half will have specific neurologic abnormalities on routine examination. In our series of 44 patients, 11 patients were asymptomatic at the time of diagnosis and were found to have brain involvement only following routine brain scans for staging and evaluation. Our recommendations for initial and subsequent evaluation of patients for CNS metastases are shown in Table 3.

The most important feature for early diagnosis of central nervous system metastases is a high index of suspicion. Patients with a profile as described above — namely males with primary lesions above the waist, presenting with headache or other neurologic complaints — in particular should be evaluated

Table 3. Suggested evaluation of patient suspected of having intracranial metastases from malignant melanoma

High index of suspicion in all patients, but particularly those with characteristics noted in Table 1		
Immediate evaluation of any patient presenting with signs and/or symptoms noted in Table 2		
Careful Neurologic examination		
CT or MRI brain scans in all patients		
Metastases found	No metastases found	
Dexamethasone therapy	Electroencephalogram	
Consider surgery	Abnormal	Normal
Radiation therapy	Repeat scan in two weeks	If signs or symptoms persist repeat scan in four weeks

immediately for the presence of central nervous system metastases. Indeed, patients who are at high risk should perhaps have routine scans at periodic intervals after the diagnosis to detect and treat early disease. The mainstay of diagnosis at the present time is CT scanning of the brain. This should be undertaken as soon as a suspicion of this complication is present. It has not been determined whether magnetic resonance imaging (MRI) is as effective as CT scanning in detecting early lesions. The sensitivity of both of these techniques for this particular tumor remains to be determined, but probably is in the range of 80–90% for lesions which are greater than 2 cm in diameter. Considerably less sensitive is nuclear medicine brain scanning. In Amer's series, in patients with proven brain metastases, nuclear medicine scanning was able to detect only about 68%. Indeed, these authors felt that electroencephalography was more sensitive than nuclear medicine brain scanning, with all 40 patients with clinically apparent brain involvement in their series having an abnormal tracing. This has not been widely used, however, largely because of the development of CT scanning. Amer and his colleagues also comment on the use of lumbar puncture as a useful diagnostic technique, particularly for patients with leptomeningeal metastases. In their series, eight of nine patients with this complication had some evidence of this on lumbar puncture. Lumbar puncture as a routine means of making the diagnosis of metastatic malignant melanoma involving the CNS is not, however, recommended because of the frequent finding of markedly increased intracranial pressure, edema around the metastatic lesions on CT scan, and the possibility of herniation. This should be strictly reserved for patients in whom leptomeningeal disease is suspected and the diagnosis cannot be made by other means.

3. Sites of metastases

The finding noted previously — that most patients who develop central nervous system metastases are males with truncal, head and neck, and upper extremity primaries — led us to the question of whether the spread to the brain might have

Table 4. Number and percent of brain metastases by site in 28 patients with malignant melanoma with a total of 122 metastases

Major division	Subdivision	Number of metastases	% of total metastases
Cerebellum	Superficial cerebellum	8	6.6
	Cerebellar white matter	1	0.8
	vermis	2	1.6
Brainstem		2	1.6
Supratentorial area			
	Central sylvian	—	—
	Lentiform	5	4.1
	Caudate	2	1.6
	Thalamus	—	—
	Juxtaventricular	—	—
	Intraventricular	—	—
Suprasylvian area			
	Frontal white matter	5	4.1
	Parietal white matter	5	4.1
	Occipital white matter	6	5.0
	Temporal white matter	—	
	Frontal gyral	20	16.4
	Parietal gyral	32	26.2
	Occipital gyral	14	11.5
	Temporal gyral	12	9.8
Interhemispheric		—	—
Subarachnoid		7	5.7
Bone		1	0.8
	Total	122	100%

occurred via routes other than the classic intra-arterial route described for other neoplasms [8]. We subsequently reviewed the records of 43 patients with metastatic brain metastases from malignant melanoma seen at the University of Colorado Health Sciences Center [6]. The most common site of metastases was the suprasylvian area which accounted for 77% of the total. Of these, 83% were in the gyral area with a predominance of these in the parietal regions. This distribution is shown in Table 4. Previous authors, in particular Kindt [8], noted a definite predilection for cancers of other types to metastasize along the posterior aspect of the Sylvian fissure in the area of the junction of the temporal, parietal, and occipital lobes corresponding to the blood supply from the middle cerebral artery. Our finding of a predominantly superficial parietal distribution with a low frequency of white matter or grey matter junction metastases suggested to us that the spread may have been along Batson's vertebral venous plexus [7]. Batson's original work consisted of defining a vertebral venous plexus which he postulated might be at the route of spread of prostatic cancer to the vertebral bodies. To demonstrate this, he injected the dorsal vein of the penis in monkeys with radio-opaque paint and observed the spread under varying conditions of increasing intra-abdominal pressure. In these studies, dyes were seen at progressively higher locations along the spinal column in veins and

sinuses, eventually reaching venous areas within the cranial vault. Batson speculated that with daily activities, such as coughing or straining, which result in increased intra-abdominal pressure, spread could occur along this venous plexus. Further work to confirm and/or refute both his findings and ours needs to be done. Taken together with the finding of CNS metastases being more common with truncal primary melanomas, the question should be raised as to whether new means of diagnosis and/or prophylaxis might be considered.

4. Treatment

Table 5 shows the expected response rates and survival after various forms of treatment. Most patients will be found to have multiple areas of metastases at the time of diagnosis. For the occasional and uncommon patient with a single metastastic lesion found on scan, surgery should indeed be considered. We and other authors have shown that a combination of surgical removal followed by radiation therapy gives the longest survivorship in patients with this complication (3, 4, 5). It has not been determined, however, whether this apparently prolonged survival is simply the result of the fact that these patients have fewer lesions at the time of diagnosis.

Almost all the patients will be found to have marked edema on scans after the diagnosis of metastatic malignant melanoma is made. The initial mainstay of treatment is corticosteroid therapy, particularly with dexamethasone. We recommend starting with a dosage of about 20 mg per day divided into four daily doses. This usually leads to prompt relief of headache and sometimes the mental confusion through relief of the edema around the metastatic foci. Frequently, however, 20 mg per day is not adequate to obtain complete relief and the dosage may be increased rapidly to as high as 200–300 mg of dexamethasone per day. Indeed, some authors have advocated starting higher than 20 mg per day, but we find this adequate in most patients.

Following the institution of dexamethasone therapy, consideration should be given to surgery in all patients found to have metastatic lesions. The decision as to whether the patient is an operative candidate will be based upon whether there are multiple lesions, their location, and the presence of other life-

Table 5. Expected results of various methods of treatment in patients with malignant melanoma and CNS metastases

Primary form of treatment	Percent expected to respond	Duration of response (months)	Mean survival (months)
Surgery plus radiation therapy	100%	5–20	10–20
Radiation therapy	36–60%	3–4	4–5
Chemotherapy	less than 10%	2–4	2–4
Corticosteroid therapy	50%	1	1
No therapy		1	1

threatening metastatic disease. Patients who should be considered surgical candidates are those who have little or no evidence of life-threatening disease elsewhere, single or at most two lesions amenable to surgery without significant neurologic damage, and the understanding that, at surgery, multiple other lesions may be found. Although there are a few reports of patients with a single lesion who have survived for years, and we have experienced this in at least one patient, this is uncommon. Our recommendation is that because most patients eventually develop new lesions or relapse after surgical treatment only, this be followed with whole brain radiation. The median survival of patients treated with a combination of surgery and radiation therapy is approximately ten months in both Amer's series and that reported from this institution. As indicated above, however, it cannot be determined whether this represents the fact that these patients are, in general, in better condition and have fewer other visceral metastases, or whether it represents a real difference in the effect of the treatment.

For all other patients, the mainstay of treatment after dexamethasone has been started is radiation therapy. Carella et al [9] have reviewed the experience of the Radiation Therapy Oncology Group in the treatment of 60 patients with cerebral metastases from malignant melanoma. They used a variety of dosing schedules varying from 1,000 rads in a single fraction to 4,000 rads given over four weeks and found little difference in the probability, promptness, or duration of benefit and lumped all of these patients together for analysis. They reported symptomatic improvement in 76% of the patients treated with radiation therapy with 31% completely improved. In their series, of the four most frequently associated problems, headache cleared in 73%, motor loss improved in 61%, impaired mentation improved in 62%, and convulsions ceased or improved in 83%. The median survival for these patients was about 12 weeks with brain metastases as the cause of the death in 34, or slightly greater than half of the patients. In the remainder of the patients, all except one had died before the end of one year. Similar results have been reported by others (11, 12). It is unclear, however, what the contribution of corticosteroids was to the radiation therapy. There has been no randomized comparative trial of dexamethasone alone versus dexamethasone plus radiation therapy. Even after the radiation therapy is completed, most patients remain on corticosteroid therapy until their death, because of continued symptoms when the drug is withdrawn. Further, there are no comments in the literature regarding the incidence of actual sterilization brain metastases following radiation therapy in large series of patients, and this has not been reviewed in the University of Colorado series.

There have been numerous attempts in the past to try to treat brain metastases, both in melanoma and other neoplastic disorders, with chemotherapy without significant success. The hope that some of the lipid soluble agents such as BCNU (1, 3-bis (2-chloroethyl)-1-nitrosourea) and CCNU 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea) would cross the blood-brain barrier and be of significant benefit has not been borne out. We reported a few years

ago a study in which we treated patients with advanced metastatic malignant melanoma with escalating high doses of a combination of melphalan and BCNU combined with autologous bone marrow transplantation [14]. The inclusion of the nitrosourea in this combination was done in the hope that we could prevent the development of brain metastases. This turned out not to be the case, and even at the high doses of BCNU, we saw no apparent benefit in decreasing the incidence of intracerebral metastases. Indeed, the majority of patients in this series subsequently died of this complication. No effective chemotherapeutic regimen to treat or prevent this complication has been devised. The use of chemotherapy for the treatment of brain metastases in malignant melanoma cannot be recommended, with the exception of dexamethasone, except in experimental settings.

In all series that have been reported to date, the mean and median survivals of patients developing brain metastases from malignant melanoma is less than one year, usually in the order of two to six months. About half these patients die of complications of the central nervous system metastases and the other half as a result of progressive disease in other organs. As indicated above, no effective form of therapy has as yet been devised for the treatment of this most dreaded complication of this ever-increasing disorder. Surgery, radiation therapy, and corticosteroids all appear to have some limited benefit, particularly in the relief of neurologic signs and symptoms which may make the eventual death of the patient easier. They are, however, ineffective, as presently used, in prolonging survival in the majority of patients.

Prophylactic cranial radiation has been used in the leukemias and in a few other neoplastic disorders. In all instances in which it has been used, serious long-term neurologic complications have apparently arisen even when administered to adults. Thus, this form of therapy would not appear to be beneficial in this disorder.

5. Summary

Central nervous system metastases remain one of the most common and dreaded complications of malignant melanoma. Twenty to 30% of all new patients will develop CNS metastases, and most of these will die in a few months after this occurs, with or without treatment. Patients most likely to develop CNS metastases are males with a primary melanoma above the waist who have metastatic lesions in other areas, particularly the lungs and liver. Headache, impaired mentation, motor deficits, and seizures are the most common presenting complaints. Diagnosis is best confirmed by CT or MRI brain scans. The most common sites of metastases are in the suprasylvian area, predominantly in the frontal and parietal gyral areas. The mainstays of treatment are corticosteroids and radiation therapy, but all patients should be evaluated as possible surgical candidates. Even with such therapy, most patients will die of this or other complications of malignant melanoma in one to six months.

Further understanding of the methods of spread of malignant melanoma to the CNS, prevention of this complication, and more effective means of treatment are urgently needed if prolonged survival and cure in this disease is to be obtained.

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III

Therapy

9. Interferon Trials in the Management of Malignant Melanoma and Other Neoplasms: An Overview

E.T. Creagan

1. Introduction

Interferons comprise a family of glycoproteins which are endogenously produced by cells in response to viral infections, double-stranded RNA, mitogens, or small molecular weight agents. Issacs and Lindeman initially coined the term 'interferon' to describe a macromolecular particle which had the properties of an interfering agent to viral infectivity in the chick chorio-allantoic membrane model [1]. Since their pioneering discovery, there has been keen interest in the biological characteristics of these unique substances.

There are three major classifications of interferon: α, β, and γ. α-interferon is predominantly derived from viral-stimulated leukocytes, β-interferon is mainly produced by fibroblasts, and γ-interferon is produced by T-lymphocytes following exposure to specific antigens or mitogens (Table 1). Approximately 15 years ago an enormous amount of blood products was required to obtain α-interferon, and this precluded any meaningful attempt at clinical trials. The α-interferon obtained from virally-stimulated buffy coat leukocytes used in most early European and American trials was only approximately 1% pure. However, advances in recombinant DNA technology have provided the mechanics for producing substantial quantities of interferon suitable for clinical investigations. This technique involves the insertion of the leukocyte interferon gene into *E. coli* bacteria followed by additional purification using a specific

Table 1. Human interferon nomenclature

Species	Current		Former Abbreviation
	Natural	Recombinant	
Interferon alpha	IFN-α(Le) IFN-α(Ly)	rIFN-α	Le(leukocyte), Ly(lymphoblastoid), Type I, pH 2, stable, classical
Interferon beta	IFN-β	rIFN-β	F(fibroblast), Fi, Type I, pH 2, stable
Interferon gamma	IFN-γ	rIFN-γ	II F(immune), Type II, pH 2 labile, antigen-induced, mitogen-induced

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monoclonal antibody to leukocyte A interferon. The extracted material is > 95% pure.

α-interferon consists of 166 amino acid sequences with an additional 20 amino acid moieties contiguous with the amino terminal end. It now appears, from endonuclease analysis that several species of α-interferons might exist.

β-interferon has been typically produced by polynucleotide stimulation of foreskin fibroblasts. Approximately 30% of the molecule is similar to α-interferon in amino acid sequence (Figure 1) [2]. However, unlike α-interferon, which has at least 12–18 subtypes, there now appears to be probably two subspecies of β-interferon.

γ-interferon, immune interferon, is produced by T-lymphocytes in response to specific antigens or mitogen stimulation. The molecule is relatively unstable in an acid medium (pH 2), and this has precluded large-scale production of quantities needed for clinical trials. Other obstacles towards clarifying the molecule included the following: different T-cell subsets may respond to different stimuli, some cells may produce mixtures of interferon- and interferon-γ, and the molecule may be heterogeneous. However, a burgeoning of technical advances in recombinant methodology have been refined to the point where adequate quantities are now available for clinical investigation.

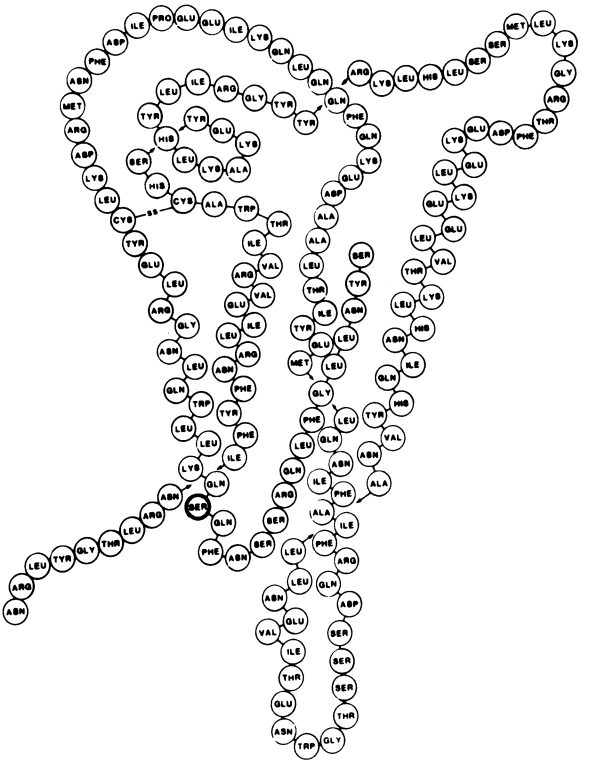


Figure 1. Amino acid sequence of an interferon molecule[31]. (Reproduced with permission.)

Despite apparent differences in structural, physicochemical, and cellular origins, all these classes of interferons demonstrate three common characteristics: immunomodulation, anti-viral activity, and anti-proliferative features.

2. Mechanism of Action

2.1. Antiviral

Although the precise intracellular mechanisms of interferon activity are not completely defined, it does appear that the antiviral effects of the molecules are mediated through the induction of enzymatic pathways [3]. Interferon therapy enhances the activity of a protein kinase. This molecule mediates complex pathways phosphorylating cellular factors essential in protein synthesis, thereby impeding the translation of viral and cellular messenger RNAs.

Interferon also increases the activity of endoribonuclease which inhibits the transcription and translation of both RNA and DNA viruses.

The 2′, 5′-oligodenylates are conversion products of adenosine phosphate in the presence of oligodenylate synthetase which is induced by interferon exposure [4]. The end result is an inhibition of cell growth by cleaving single-stranded RNA.

Another effect of interferon results in an ersatz glycoprotein envelope of viral particles. This results when interferon inhibits the conversion of selected aspects of uridine and dolichyl phosphate metabolism [5].

2.2. Antineoplastic properties

Antiproliferative activities are indigenous to virtually all species of interferons. Cells from diverse histogenesis including embryonic tissue, fibroblasts, and lymphoid cells demonstrate growth inhibition in the presence of interferon. All phases of the mitotic cycle are elongated, but the most pronounced effect appears to be restricted to cells which are not actively proliferating. Although the mechanics of growth inhibition are undoubtedly multifaceted, this phenomenon seems to involve the following: inhibition of cytokinesis [6], extension of the intermitotic phase [7], and depression of DNA synthesis [8].

It also appears that the antiproliferative effects of interferons are most evident during the actual duration of treatment [9]. A more durable inhibition of tumor development is achieved by prolonged exposure to interferon rather than by the usage of an intermittent schedule. In addition, these phenomena may explain the reversible myelosuppression following discontinuation of interferon therapy.

In vitro studies indicate that interferons are directly antiproliferative in experimental tumor models. For example, the addition of lymphoblastoid interferon to human tumors grown in the immunodeficient nude mouse results in unequivocal tumor inhibition [10]. This observation is obviously independent

of any immunoregulatory property of human interferons in this experimental model.

2.3. Immunoregulatory properties

Interferons have definite immunomodulatory characteristics clearly distinct from their antiproliferative and antiviral features (Table 2) [11]. However, the complexity of these inter-relationships currently defies any unifying concept. Moreover, selected aspects of humoral immunity may be depressed or augmented, depending upon the relationship of the interferon usage to the timing of the antigenic challenge [12].

Interferons also have well-documented effects on selected immunologic parameters [13–17]. These molecules augment cell-specific T-cell cytotoxic and natural killer cell activities [13]. These may also occur following the usage of interferon inducers such as BCG and Poly I-C. Interferon increases the natural killer activity by transforming natural killer cells and precursors to natural killer effector cells. Interferons also have a well-documented role in the activation of macrophages. Moreover, serum interferon titers induced by Newcastle disease virus directly correlate with enhanced phagocytic activity in murine peritoneal macrophages [14]. However, the clinical significance of these experimental observations is obscure. For example, a recent study assessed natural killer activity among patients receiving α-interferon for multiple myeloma [18]. Although natural killer activity increased in the macrophages of patients following the first injection of interferon, there was no apparent correlation between the response of the tumor and the interferon-induced enhancement of natural killer activity *in vitro* or interferon-induced enhancement of natural killer activity *in vivo*.

3. Interferon receptors

Prior to the biological expression of interferon activity, these glycoproteins bind to specific high-affinity receptors on the surface of both normal and transformed cells. There appears to be a common binding site for α- and β-interferons. In the experimental model utilizing the Daudi lymphoblastoid cells, pretreatment with β-interferon substantially decreases the subsequent binding of ^{125}I-labelled α-interferon [19]. This, in turn, abrogates the antiproliferative activity of α-interferon. On the other hand, there appears to be a distinct receptor class for

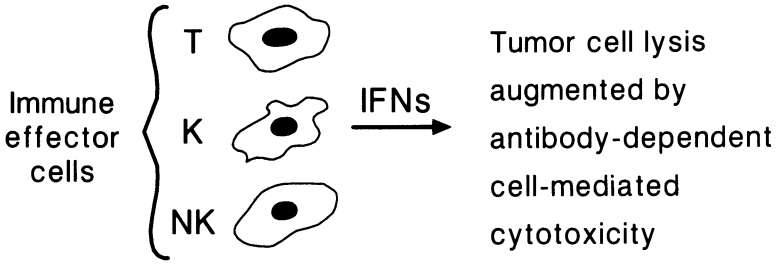
Table 2. Immunmodulatory enhancement by interferons

Natural killer (NK) lymphocyte cytotoxicity[13]
Mononuclear phagocytosis[14]
IgE-mediated histamine release[15]
Fibroblast-generated prostaglandin E production[16]
Antibody-dependent cell-mediated cytotoxicity[17]

γ-interferon. In experimental studies, α and β, but not γ, compete for ^{125}I-labelled α-binding sites. The binding of radio-labeled γ-interferon to human cell lines was not blocked by α. Moreover, γ did not inhibit the binding of labeled interferon α/β when aggregates of α and β as well as separated α and β species did inhibit the binding.

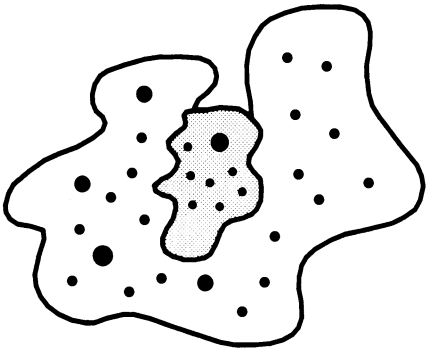
The concept of divergent receptor sites for α/β and γ interferons has been reinforced by studies of synergistic potentiation. Antiviral and antiproliferative effects of combinations of interferon-α/β and interferon-γ appear to be fivefold to twentyfold more active compared with results expected from simply additive effects.

Following the interaction of interferon with an appropriate receptor, a cascade of intracellular events unfolds which is schematically designated in Figure 2. The receptor complex rapidly induces an array of intracellular



A. In the presence of interferons, immunocompetent peripheral blood mononuclear cells may be recruited to form activated cytolytic cells. Tumor lysis appears to be manifested through antibody-dependent cell-mediated cytotoxicity[17].

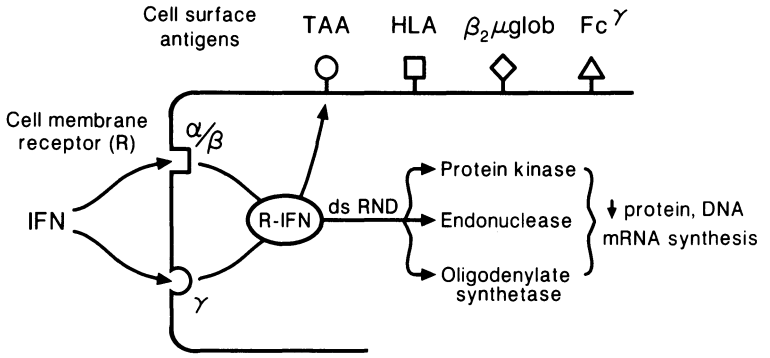
TUMOR CELL PHAGOCYTOSIS



B. Interferons enhance phagocytic activity of macrophages with morphologic and cytochemical evidence of activation[14].

Figure 2. Possible mechanisms of antiproliferative characteristics of interferons (IFNs) include cytotoxic and immunomodulatory interactions.

Figure 2 continue



C. Following the interaction of a single interferon molecule with a single interferon cell surface receptor, an array of enzymes are induced in the presence of double-stranded RNA (dsRNA). This process results in a decrease of protein, DNA, and mRNA synthesis. In addition, interferons impact upon cell membrane physicochemical discriminants resulting in the development of tumor-associated (TAA) and histocompatibility antigens (HLA) and the expression of beta$_2$-microglobulin and Fc gamma receptors[32]. Conceptually, host cellular mechanisms recognize cell surface antigens as 'foreign' and impair neoplastic cellular replication by cytotoxic and phagocytic mechanisms.

proteins which have antiviral, antiproliferative, and immunomodulatory properties. The technique of transmission electron microscopy has provided a fascinating glimpse into the fate of the interferon-receptor complex. When colloidal gold was coupled to *E. coli*-derived human α-interferon, the complex was visualized on the cell surface. An invagination of the cell membrane formed a coated vesicle (receptosomes) within the cytoplasm [20]. The interferon undoubtedly becomes degraded while the receptor is possibly 'recycled' by again being located on the cell surface.

Several lines of inquiry indicate an important relationship between interferon binding and biological expression. The antiviral and antiproliferative characteristics of interferon are directly correlated with binding. In addition, cells which bind to interferon respond to the molecule whereas cells which are resistant to the biological effects of interferon are the same cells which do not bind interferon specifically.

4. Interferon in the clonogenic assay

The human tumor cloning system for the human tumor stem cell assay (HTSCA) as popularized by Hamburger and Salmon now provides a unique probe to further expand fundamental knowledge of the interferon systems. This technique has been extensively utilized in the screening of chemotherapeutic agents and seems to be of most usefulness in screening out agents which have a high probability of being ineffective in the clinical setting. This can be reasonbly predicted with approximately 90% accuracy.

In one trial, investigators at the University of Arizona studied recombinant human leukocyte interferon (IFN-αA and IFN-αD) against 273 and 71 tumors, respectively [21]. For the A-subspecies there was substantial inhibition of cell growth in 38.1% of tumors tested, while 16% of tumors were inhibited by interferon-D. For interferon-A, the most marked inhibition occurred in the following neoplasms: malignant melanoma (51.7%), lung cancer (50%), myeloma (33.4%), and ovarian cancer (33.9%). Interferon-D appeared to be slightly less cytotoxic ($p < 0.01$) than interferon-A, in that only 8% of tumors demonstrated marked inhibition from the same dosage range of interferon.

A most interesting spin-off of this study was the correlation between interferon and chemotherapeutic drug sensitivity. Neoplasms which were sensitive to interferon (> 50% decrease in tumor colony forming units) had an enhanced propensity for cytotoxic sensitivity compared with tumors which were insensitive to interferon. For example, in one testing series of 101 tumors which were sensitive to interferon-A, 36% were also sensitive to cytotoxic drugs. However, among 117 neoplasms which were insensitive to interferon-A (4 of 11), only 10% (1 of 10) were sensitive to chemotherapy. The interferon-D produced similar results. These observations suggest that both interferons and cytotoxic agents have characteristics of antiproliferative sensitivity and resistance which may be expressed through similar cellular mechanisms.

These intriguing experimental investigations may be of value in targeting patients who would most likely derive benefit from interferons. Unfortunately, there has been little broad experience in correlating the clonogenic assay with clinical reality. In one report, a survey of the clonogenic assay in biopsies from 62 patients demonstrated a dose-dependent cytotoxic effect in only five patients [22]. The investigators utilized human leukocyte interferon. One patient with small cell lymphosarcoma cell leukemia was of some interest. This 50-year-old man demonstrated a progressive lymphadenopathy despite two standard polychemotherapeutic regimens. On the basis of an 85% decrease in colony forming units with interferon, the patient received an escalating dosage starting with 3×10^6 units/day. The nodal disease decreased to less than half its former size for six weeks. It is clearly hazardous to extrapolate from this isolated situation to broad patient groups. Nevertheless, the clonogenic assay, at least in this case, was of predictive value. It is presently unclear if a clonogenic assay will be consistently of predictive value in the clinical management of patients with disseminated disease.

Despite somewhat clouded clinical implications, the clonogenic assay may refine our understanding of interferon's antiproliferative future. In one report of six human cell lines, no apparent cytotoxic effects were manifested from a variety of interferon concentrations utilized by exposing the cell to one hour of interferon. However, from the highest concentration of interferon continuously exposed to each cell line, four of five model systems were clearly inhibited by interferon [23]. These observations have been extended to the clinical setting in that most investigators now favor a continuous program or an intensive treatment at periodic intervals rather than an intermittent schedule.

Additional refinements using the human tumor cell lines *in vitro* have

enormously contributed to the therapeutic potential of interferons. British investigators studies the impact of α and γ interferons on human lung cancer cells of squamous, adeno, large, and small cell types [24]. They documented differential cytotoxicity in that the large cell lines and the small cell lines (POC) were the most sensitive. Yet, the NCI-H6S small cell line was relatively resistant. In two cell lines (COR-L23 and POC) interferon-γ was consistently more effective than interferon-α. Daily injections of interferon-γ against cell lines COR-L23 and POC, when given in nude mice, achieved no overt inhibition of tumor growth. The authors appropriately point out that there are an endless number of imponderables in trying to correlate *in vitro*, *in vivo*, and clinical observations. Although antitumor activity was selectively documented, it is not presently clear how this data can be effectively applied in the clinical setting. It is also unclear why neoplasms, which have been sensitive to interferon, would cease to be effective. *In vitro* studies with human melanoma cell lines exposed to both crude and bacterial-cloned human leukocyte interferon indicated that prior interferon therapy may select out clones of cells which are increasingly resistant to rechallenge with additional doses of interferon [25].

A particularly interesting application of the clonogenic assay involved assessments of therapeutic synergism of IFN-β (ser) and interferon-γ [26]. Among six human melanomas, three were sensitive to both interferons, two were resistant to interferon-γ, and one was resistant to both species. However, five of six tumors were sensitive to combinations of interferon-β (ser) and interferon-γ. In one tumor, sensitivity to the combination was manifested at doses which were 10–20% lower than those required when the interferons were individually tested. In another melanoma model, 95% growth inhibition occurred from concomitant usage of interferon-β (ser) plus interferon-γ. In contrast, from single agent trials, interferon-β (ser) and interferon-γ achieved 80–90%, and only 23%, growth inhibition, respectively. These types of investigations hold promise for expanding the therapeutic potential of interferons in the management of advanced neoplasia.

5. Pharmacokinetics

Interferons can be administered by the intravenous, intramuscular, or subcutaneous routes. In the initial study with recombinant interferon, 16 patients with an array of disseminated neoplasms, chronic myelogenous leukemia, or multiple myeloma received escalating intramuscular doses ranging from 3 to 198 $\times$ 10^6 units each 72–96 hours [27]. Mean maximum serum concentrations of interferon were determined by both an enzyme-linked immunoassay using two monoclonal antibodies to interferon-A and by a modified bioassay utilizing MDBK cells as targets. Overall, the mean maximum serum concentration proportionately increased with increasing doses. Regardless of the dose, the half-life ranged from six to eight hours. In another study, the peak serum levels following intramuscular injection occurred at four to six hours [28]. The recombinant preparation was not detected in the urine as measured by the enzyme immunoassay following doses of 3 to 72 $\times$ 10^6 units.

Pharmacokinetic comparison was made between the recombinant interferon and a partially pure leukocyte interferon (IFN-C) preparation [27]. At the lower range of doses tested there was no difference in the mean serum concentration of interferon tested, but at a dose of 9×10^6 units the mean serum level of interferon-C was significantly greater than the value from interferon-rA. The clinical relevance of this finding is presently unclear. Antibodies to IFN-rA were detected in 3 of 16 patients. Although there was no immediate or overt sequelae to these findings, the impact of antibody formation on objective regressions and toxicity patterns must await further evaluation and ongoing surveillance. Neutralization by antibody of interferons has been documented, and this could have some clinical relevance [28]. For example, in a trial of recombinant derived interferon-alpha in metastatic renal cell cancer, 20 of 53 patients (38%) developed neutralizing antibodies to rIFN-αA. Seven of 12 responding patients (58%) developed antibodies and each of the seven developed recurrent disease coincident with antibody formation. In addition, the median duration of remission among antibody-positive and antibody-negative patients was two and ten months, respectively. These differences were statistically significant ($p < 0.009$). These data suggest that antibodies may interfere with the antineoplastic effects of rIFN-αA.

6. Clinical studies

Although clinical evaluations with interferons commenced in the early 1970s, the most meaningful experience with these preparations was launched in 1983 with the advent of recombinant technology which spurred the production of large quantities of interferons.

From Phase I trials an array of clinical laboratory sequelae have been consistently reported. Most patients developed a flu-type syndrome characterized by weakness, myalgias, chills, and fever (38°–40°). In general, these symptoms have been less significant with successive treatments. Leukopenia in the range of 2,000–4,000 cells/mm^3 is common as well as elevation of hepatic transaminase. Occasionally these developments have necessitated dose modification but these changes were usually transient and of little clinical impact.

The initial Phase II trials of interferon utilized limited quantities of impure interferon-α obtained by Sendai virus infestation of buffy-coated leukocytes obtained by Doctor Cantell and colleagues through the Finnish Red Cross. These substances consisted of a variety of subtypes of interferon-α whose precise proportion was unclear. In one of the larger initial studies published in 1980, 38 patients with either advanced breast cancer, multiple myeloma, or malignant lymphoma received an induction program of 3 to 9×10^6 antiviral units per day [30]. Nineteen patients demonstrated evidence of objective regression. The toxicities were typical of those anticipated for previous trials: low-grade fever, fatigue, anorexia, and partial alopecia. Myelosuppression occurred in most patients (lowest median leukocyte count, 2,500/mm^3). This trial was followed by a surfeit of publications addressing the role of interferons

in a diverse spectrum of lymphoproliferative, hematologic, and solid neoplasms. The results of three well-summarized reviews [31–33] and selected trials are listed in Table 3 [34–69]. As might have been predicted by previous surveys of the chemotherapy literature, there is a striking panorama of response rates and types of regressions. This undoubtedly reflects the influence of different types of interferons, the unique dosages and schedules of administration, and the impact of the yet unrecognized prognostic clinical and immunological discriminants among patient populations. Despite these obvious and well-acknowledged limitations, several trends have emerged from the literature. Renal adenocarcinoma and malignant melanoma have been the most intensely studies neoplasms. In renal adenocarcinoma, an accurate response rate appears to be approximately 25%, and this has been obtained from an array of doses and schedules. The median response duration has been in excess of four months, with regressions typically occurring within three months from the commencement of therapy, and predominantly affecting pulmonary metastases. Not surprisingly, a better performance score and a longer disease-free interval between diagnosis and metastases are positive harbingers of response. In recently completed randomized trials comparing low (1×10^6 units/day, days 1–28) versus high dose (10×10^6 units/day, days 1–28), human leukocyte α-interferon, six of the seven responses occurred in patients who had received the higher dosage and three of the seven were major regressions (two partial responses and one complete response) [43]. The response duration has been 4, 15, and 28 months in the patients with major regressions. Five of the seven regressions were observed in patients with pulmonary metastases. The briefest response duration, four months, occurred in a patient who had received the lower dose program. Interestingly, among nine patients receiving the low dose schedule who then received the higher dose program, one of four manifested an objective response. Conversely, there were no regressions among the five patients who continued on the low dose program. There did not appear to be any compelling relationship between antitumor response and toxicities.

Malignant melanoma is the other solid neoplasm which has been the focus of interferon investigation. Melanoma, like renal cell carcinoma, is virtually bereft of any systemic therapy which even remotely approaches consistent effectiveness. Moreover, like renal cell cancer, the immunologic implications of malignant melanoma certainly have justified interferon trials. Three reviews in the literature which encompass both human leukocyte and recombinant interferon programs confirm objective response rates of approximately 10–20% [31–33]. Most of the melanoma regressions have been partial, transient, usually limited to soft tissue or nodal disease, and have not dramatically impacted upon survival.

Among other neoplasms, the most sanguine benefit from α-interferon has occurred with hematologic malignant disease, most notably hairy cell [65] and chronic myelogenous leukemia [63], and cutaneous T-cell lymphoma [69]. The actual benefit from this preparation in acute leukemia [62], chronic lymphocytic leukemia [64], and multiple myeloma [66] is more problematic. Interferons have

Table 3. Composite response rates in selected interferon trials by tumor type*

Solid neoplasms	IFN species†	Evaluable patients	Response rates, % CR	PR
Malignant melanoma	HuIFN-α(Le)[34]	44	0	2
	HuIFN-α(Ly)[35]	15	0	6
	HuIFN-α(Ly)[36]	8	0	0
	rIFN-α2b[33]	23	0	8
	rIFN-αA[37–40]	96	4	18
	rIFN-α[41]	29	0	3
	rIFN-α2[42]	63	6	6
Genitourinary				
Renal adenocarcinoma	HuIFN-α(Le)[43]	30	3	6
	rIFN-αA[29]	56	2	19
	rIFN-αA	19	0	10
Bladder	HuIFN-α(Le)‡,[44]	8	100	0
Prostate	HuIFN-α	10	10	20
Bronchogenic				
Non-small cell	HuIFN-α(Le)	52	0	2
Small cell	HuIFN-α(Le)[45]	15	0	0
	HuIFN-α(Ly)[46]	10	0	0
Gastrointestinal				
Colorectal	HuIFN-α(Le)[47, 48]	43	0	0
	rIFN-αA;-α2[49]	46	0	4§
Carcinoid	HuIFN-α(Le)[50]	9	0	6
Hepatocellular	rIFN-γ[51]	7	0	0
Gynecologic				
Ovarian	rIFN-α2[52]	11	36	9
	rIFN-αA[53]	15	0	0
	HuIFN-Ly[54]	28	7	10
Cervical	HuIFN-Le[55]	15	20	NA
Head and neck				
Squamous/basal cell	HuIFN-Le‡,[56]	30	33	33
Lymphoepithelioma	rIFN-αA[57]	20	0	45
Central nervous system	HuIFN-β[58]	20	5	35
	HuIFN-αA(Ly)	3	0	33
	rIFan-αA	9	0	22
	IFN‡,[59]	13	0	15
Osteosarcoma	HuIFN-Le[60]	11	0	0
Breast	HuIFN-α(Le,Ly)[32]	56	0	21
	rIFN-αA[32]	47	0	29
	(rDNA)HuIFN-α2[61]	14	0	0
	HuIFN-α(Le)[32]	23	0	22
Hematologic Neoplasms				
Leukemia				
Myelogenous				
Acute	HuIFN-αN(Ly)[62]	14	0	14
Chronic	HuIFN-α(Le)[63]	7	71	0
Chronic lymphocytic	rIFN-αA[64]	18	0	11
Hairy cell	HuIFN-α(Le)[65]	7	43	57
Myeloma	HuIFN-α(Le)[66]	97	3	15
Lymphoma	HuIFN-α(Le)[32]	28	11	28
	rIFN-α-2,-A[32]	61	3	46
	rIFN-αA[67]	37	24	11
Nodular	HuIFN-α[68]	18	16	0
Cutaneous T cell	rIFN-αA[69]	20	45	0

*Adapted from 31-33.

† Leukocyte (Le) or lymphoblastoid-derived (Ly)

‡ Topical therapy

§ Decrease in tumor markers without objective regressions.

Table 4. Pretreatment characteristics of 31 patients treated with rIFN-αA

	Total	Better risk	Poor risk
Number of evaluable patients	31	21	10
Median age (yr)	56	52	58.5
Number of males/females	23/8	16/5	7/3
Prior chemotherapy (yes/no)	9/22	0/21	9/1
Number of patients with ECOG PS* (0/1/2, 3)	12/14/5	11/10/0	1/4/5
Dominant metastatic site Visceral/nonvisceral	19/12	13/8	6/4

*Eastern Cooperative Oncology Group performance score: 0 (fully active) to 4 (totally disabled). rIFN = αA: recombinant leukocyte A interferon.

Table 5. Results of treatment with 50 × 10^6 units/m^2 rIFN-αA and site of metastasis*

Risk category	Visceral-dominant	Nonvisceral	All patients
Better†	2/13	3/8	5/21
Poor‡	1/6	1/4	2/10
All patients	3/19	4/12	7/31

*Values: Number of objective regressions/number of evaluable patients.
† Eastern Cooperative Oncology Group performance score: 0, 1, and no prior chemotherapy.
‡ Eastern Cooperative Oncology Group performance score: 2, 3, or prior chemotherapy.
rIFN-αA: recombinant leukocyte A interferon.

been of reported benefit in head and neck cancer, cervical cancer, bladder cancer, and Karposi's sarcoma. However, further clinical trials are obviously necessary to define the optimum patient population, dosage, and schedule for these neoplasms. To date, there have been few, if any, really promising results among patients with nonsmall-cell bronchogenic carcinoma. Similarly, most gastrointestinal neoplasms have been relatively refractory to the use of interferon with the possible exception of carcinoid tumor. In one report, six of nine patients demonstrated biochemical evidence of a reduction in tumor markers. Unfortunately, five of the six quickly relapsed following discontinuation of interferon, and none of the patients manifested any objective decrease in tumor measurements [50].

Our experience at the Mayo Clinic has been reasonably consistent with the results of other investigators [37–40]. In our initial Phase II trial [37], 31 patients with disseminated malignant melanoma received intramuscular recombinant leukocyte A interferon (rIFN-αaA), 50 × 10^6 units/m^2 three times weekly for a planned treatment duration of three months (Table 4). Seven objective regressions (23%), which ranged in duration from 3 to 11.2+ months, were observed (Table 5). Forty-two percent of 12 patients who were fully active (Eastern Cooperative Oncology Group [ECOG] performance score, 0) responded compared to 11% of 19 patients with impairment of performance status (ECOG, 1–3). Of particular note is that prior chemotherapy did not influence response rate. For all patients the median time to progression, and of survival, was two months and six months, respectively (Figure 3). Four patients

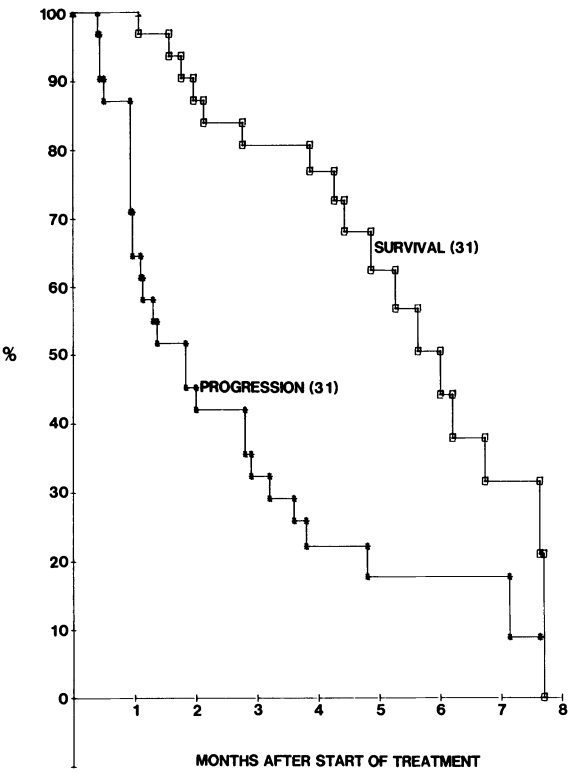


Figure 3. Survival and time to progression after start of rIFN-αA.

had partial regressions in soft tissue (3, 4.6 months), pulmonary (7 months), and prostatic lesions (three months). The latter was biopsy-proven and assessed by serial computerized tomography (CT) scans. Three had complete regressions of soft tissue disease (two patients, 6.4 and 10+ months each), and liver involvement (11.2+ months). The major toxicities were moderate to severe fatigue (87%), anorexia (58%), and confusion (23%) (Table 6). Performance score deterioated in 84% of patients during the time they were receiving rIFN-αA. Among the 13 patients whose tumors did not progress for at least 12 weeks, seven required dose reductions or termination of treatment due to toxicities. Hematologic and hepatic toxicity was transient and of little clinical significance. The study indicated that rIFN-αA had some antitumor activity accompanied by difficult side effects in patients with disseminated malignant melanoma.

In light of substantial clinical toxicities, we launched a subsequent Phase II trial in disseminated malignant melanoma utilizing a dose of 12×10^6 U/m^2, three times weekly among 30 patients [38] (Table 7). We selected this dose because it was clinically tolerable from our prior experience and there were no firm data which convincingly indicated an 'optimum dose' of interferon in malignant melanoma. We observed three objective partial regressions (20%) among the 15 better-risk patients (performance score 0, 1, and no prior

Table 6. Clinical toxicity of rIFN-αA from 50 × 10^6 units/m^2 three times weekly

	Percent (N = 31)
Moderate to severe	
Chills, fever	100
Myalgias	100
Fatigue	87
Anorexia	58
Confusion	23
Median maximum weight loss	5.6 kg
(N = 28) (range)	0.9–23.6 kg

rIFN-αA: recombinant leukocyte A interferon

Table 7. Pretreatment characteristics of 30 patients treated with rIFN-αA

Characteristics	Total	Better risk	Poor risk
Number of evaluable patients	30	15	15
Median age in years	53	53	47
Number of men/women	17/13	9/6	8/7
Prior chemotherapy (yes/no)	13/17	0/15	13/2
Number of patients with ECOG PS (0/1/2, 3)	12/15/3	6/9/0	6/6/3
Dominant metastatic site (visceral/nonvisceral	22/8	12/3	10/5

Note: ECOG score: 0 (fully active) to 4 (totally disabled).

chemotherapy) with times to disease progression of 1.9, 9.6, and 12.9+ months. There were also three regressions (one complete and two partial) among the 15 poor-risk patients (performance score 2, 3, or prior chemotherapy) with progression times of 3, 3.2, and 9.6+ months (Table 8). For all patients, the median survival time was 4.2 months (Figure 2). One-half of the patients were observed to have progressive disease within one month of commencing treatment. Responding metastatic lesions were limited to soft tissue, although one patient also had a partial response of a lung nodule. The most substantial toxicities were moderate-to-severe myalgias (27%), nausea (33%), anorexia (47%), and fatigue (50%) (Table 9). Among the 22 patients with weight loss, the median was 2.3 kg (range, 0.6 to 8.4 kg). Hematologic and hepatic toxicity was transient and of little clinical significance. This study indicated that rIFN-αA in the dose and schedule that we used was clinically tolerable and had antitumor activity in malignant melanoma. The response rate was similar to results observed in our previous study of a higher dose regimen.

It was becoming obvious from our two trials and the experience of other centers that interferon *per se* would be an unlikely candidate to offer a major therapeutic advance against malignant melanoma. Therefore, it was with keen interest that we followed the studies from European investigators reporting synergistic efficacy from the combination of cimetidine and interferon. The use of IFN-rA with an immunomodulatory agent is an appealing concept. Cimetidine, a histamine H_2 receptor antagonist and an inhibitor of suppressor T-cell function, may have a role in the management of patients with dissemi-

Table 8. Results of treatment with 12 × 10^6 U/m^2 rIFN-αA and site of metastasis

Risk category	Visceral-dominant	Nonvisceral	All patients
Better*	1/12	2/3	3/15
Poor†	0/10	3/5	3/15

Note: The data represent number of objective regressions/number of evaluable patients.

*ECOG PS 0, 1, and no prior chemotherapy.
† ECOG PS 2, 3, or prior chemotherapy.

Table 9. Clinical toxicity of rIFN-αA from 12 × 10^6 U/m^2 three times weekly

Moderate-to-severe	Percent (n = 30)
Fever	80
Myalgias	27
Nausea	33
Fatigue	50
Anorexia	47
Confusion	3
Median maximum weight loss (n = 29)	2.1 kg
(range)	0–8.4 kg

nated melanoma. An intriguing study described two patients with malignant melanoma who had received intratumoral inoculation of α-interferon without objective regression [70]. Cimetidine was then orally administered with intratumoral interferon. Both patients had complete regression of the injected lesions, and one individual demonstrated an objective response of multiple metastases not directly inoculated with interferon. Subsequent observations confirmed that intramuscular leukocyte interferon complemented by oral cimetidine was capable of inducing regressions of distant melanoma metastases [71]. In a more recent study, 15 evaluable patients received percutaneously injected α-interferon and oral cimetidine, 300 mg four times a day [72]. There were five objective regressions, and one was complete. Three regressions were partial and one was minor.

An expanded and updated analysis described a trial of human leukocyte interferon plus cimetidine among 20 patients with disseminated melanoma [73]. There were no objective regressions from interferon alone. Yet, subsequent treatment with interferon plus cimetidine resulted in six objective regressions, five of which involved only skin metastases. There was no apparent hematologic toxicity.

Thirty-five eligible patients with disseminated malignant melanoma in our study received intramuscular recombinant leukocyte interferon (IFN-rA), 50 × 10^6 U/m^2 three times weekly (TIW) for an intended duration of 12 weeks concomitant with daily oral cimetidine, 1200 mg/d in four divided doses [39] (Table 10). For all study participants, the median survival time was six months. Among 21 good-risk patients (performance score [PS] 0, 1, and no prior chemotherapy), we observed seven partial regressions (33%). Six patients had

Table 10. Pretreatment characteristics of 35 patients treated with IFN-rA plus cimetidine

	Total	Better risk	Poor risk
Number of evaluable patients	35	21	14
Median age in years	57	56	59.5
Number of males/females	20/15	12/9	8/6
Prior chemotherapy: yes/no	10/25	0/21	10/4
Number of patients with ECOG PS*: 0/1/2, 3	10/18/17	9/12/0	1/6/7
Dominant metastatic site: visceral/nonvisceral	23/12	14/7	9/5

*ECOG score: 0 (fully active) to 4 (totally disabled).

stability of disease (29%), seven had immediate disease progression, and one discontinued treatment after two doses without tumor evaluation due to side effects (Table 11). Times to disease progression of five patients with regressions of soft-tissue disease were 2.1, 3.3, 3.5, 3.7, and 4.3 months. Two patients had partial regressions of lung nodules for 2.0 and 3.8 months. We observed one regression among 14 poor-risk patients (PS 2, 3, or prior chemotherapy). A 46-year-old woman with prior treatment had a partial regression of soft-tissue disease for 4.1 months. Four poor-risk patients achieved disease stability, and nine progressed immediately. Leukopenia (WBC count $< 4{,}100/\mu L$) affected 21 (66%) of 32 patients with WBC count data. The median count was $3{,}100/\mu L$; range, 1,300 to $8{,}400/\mu L$. We detected two cases of mild thrombocytopenia (100,000 and $120{,}000/\mu L$) (Table 12). Other noteworthy toxicities included moderate-to-severe nausea (34%), anorexia (63%), and fatigue (80%). All patients experienced myalgias. Twenty patients had dosage decreases during the first cycle, and 14 of the 16 patients remaining on study after the first cycle required dosage reductions. The overall response rate is similar to our prior studies with IFN-rA as a single agent using TIW doses of 50×10^6 U/m^2 and 12×10^6 U/m^2 among 31 and 30 patients, respectively.

Although IFNs as single agents may have limited application in the treatment of human malignant disease, an emerging volume of data, both experimental and clinical, portends a potentially useful role for IFNs in combination with other antiproliferative modalities.

7. Interferons with other antiproliferative modalities

7.1. Cytotoxic programs

IFL-rA is an especially attractive agent to supplment chemotherapeutic regimens. IFL-rA has documented antitumor activity and has relatively little hematologic or gastrointestinal toxicity, the typical sequelae from most cytotoxic programs. The agent also appears to be synergistic with selected antitumor agents. The addition of interferon produced by Newcastle disease virus significantly enhances mean survival ($p < 0.05$) when combined with methotrexate (MTX) or MTX-t mercaptopurine (6-MP). There was no detected

Table 11. Results of treatment with 50 × 10^6 U/m^2 IFN-rA plus cimetidine and site of metastasis*

Risk category	Visceral-dominant	Nonvisceral	All patients
Better†	2/14	5/7	7/21
Poor‡	0/9	1/4	1/14

*Values = Number of objective regressions/Number of evaluable patients.
† ECOG PS 0, 1 and no prior chemotherapy.
‡ ECOG PS 2, 3 and prior chemotherapy.

Table 12. Clinical toxicity of IFN-rA from 50 × 10^6 U/m^2 three times weekly plus cimetidine

Moderate-to-severe	
Fever (≥ 38.3°C; n = 28)	89%
Fatigue (n = 35)	80%
Anorexia (n = 35)	63%
Nausea (n = 35)	34%
Confusion(n = 35)	11%
Median maximum weight loss (n = 30)	3.65 kg
(range)	0 to 19.5 kg

synergism between interferon and doxorubicin, 6-MP, cytosine arabinoside, or cyclophosphamide. The tumor model was L1210 murine leukemia in BDF/1 mice [74]. In another trial, the combination of interferon and cyclophosphamide prolonged the survival of leukemia AKR mice [75]. Of particular importance is a study in which the combination of interferon and BCNU produced therapeutic synergism and some cures in murine leukemia [76].

We have extended these observations by assessing synergistic antiproliferative activity of human interferon in combination with a variety of cytostatic and cytocidal drugs. Three human tumor cell lines, A498 (renal cell carcinoma), A101D (melanoma), and A375 (melanoma) are used in our laboratories (77, 78) for screening purposes because they differ up to 10,000 fold in their sensitivity to inhibition of colony formation by human interferon. For a variety of drugs, synergism has been detected against some, but not all lines. For example, human leukocyte recombinant A interferon (Ro 22-8181, Hoffman-La Roche Inc, Nutley, NJ), in combination with BCNU, had greater than additive (synergistic) antiproliferative activity against the two melanoma cell lines but not against the renal cell line. These data are summarized in Table 13. For these studies, interferon and BCNU were added at the same time so that the exposure to the agents was continuous. However, we and others have demonstrated that the half-life of BCNU at 37° is approximately 10 minutes, so that the actual time of exposure of cells to BCNU is relatively short. On the other hand, in additional studies (JS Kovach, unpublished data) we have demonstrated that against each of the cell lines the effects of interferon are dose- and time-dependent, with maximal inhibition occurring after approximately 120 hours of exposure.

The data in Table 13 demonstrate that interferon, in combination with BCNU, has greater than additive activity against the two melanoma cell lines, A375 and A101D, but not against the renal carcinoma cell line, A498. This can best be appreciated by comparing the ratio of actual percent survival to the expected survival based on the highest concentrations of interferon for each cell line. Thus, for A375, concentrations of interferon/ml produced synergistic activity with BCNU at concentrations of BCNU of 0.01/μg/ml and greater. For line A101D, synergy is present at almost all concentrations of interferon; however, there is no synergy with interferon at 3 units/ml and BCNU at 0.001 μg/ml.

We have completed a pilot study assessing the toxicity patterns of the combination of IFL-RA plus BCNU among 18 patients with a spectrum of advanced malignant disease (Table 14) [79]. The initial 12 patients received a thrice weekly intramuscular regimen of IFL-RA, 12 $\times$ 10^6 U/m^2 concomitant with BCNU at escalating doses of 50 mg/m^2 to 150 mg/m^2 administered intravenously on a monthly basis. At the highest dosage level, common toxicities are myelosuppression, thrombocytopenia, and manageable constitutional symptoms of fatigue and anorexia (Table 15). Experimental data have indicated that continuous, long-term exposure of cell lines to interferon may have greater cytotoxic and antiproliferative characteristics compared with short-term exposure [23]. In addition, the antiproliferative impact of interferon

Table 13. Inhibition of colony formation by human tumor cell lines caused by interferon and BCNU[79]

		Colony survival — percent of control [expected survival]		
	BCNU concentration	interferon concentrations (Units/ml)		
Cell line	(μg/ml)	300	3000	30,000
A375 (melanoma)	0.001	91 ± 8 [95]	92 ± 1 [91]	92 ± 8 [88]
	0.005	100 ± 9 [95]	88 ± 7 [91]	84 ± 9 [88]
	0.01	85 ± 2 [95]	64 ± 7 [91]	49 ± 3 [88]
	0.025	17 ± 1 [34]	6 ± 2 [32]	0 ± 0 [31]
	0.05	0 ± 0 [0]	0 ± 0 [0]	0 ± 0 [0]
		3	30	300
A101D (melanoma)	0.001	88 ± 11 [84]	67 ± 6 [75]	29 ± 8 [55]
	0.005	57 ± 2 [65]	45 ± 3 [58]	14 ± 4 [42]
	0.01	7 ± 1 [13]	10 ± 2 [12]	2 ± 1 [8]
	0.025	0 ± 0 [0]	0 ± 0 [0]	0 ± 0 [0]
	0.05	0 ± 0 [0]	0 ± 0 [0]	0 ± 0 [0]
		3	30	300
A498 (renal carcinoma)	0.001	93 ± 11 [90]	67 ± 5 [66]	32 ± 8 [29]
	0.005	80 ± 9 [79]	56 ± 9 [58]	20 ± 6 [25]
	0.01	70 ± 10 [50]	30 ± 4 [36]	13 ± 2 [16]
	0.025	3 ± 1 [5]	3 ± 1 [4]	0 ± 0 [2]
	0.05	0 ± 0 [0]	0 ± 0 [0]	0 ± 0 [0]

Table 14. Patient characteristics

Case	Age	Sex	Neoplasm	ECOG PS*	Indicator lesion	Prior therapy†
1. JM	62	F	Breast ACA‡	1	CXR	XRT
2. EO	70	F	Malignant melanoma	1	Soft tissue	XRT
3. RG	59	F	Breast ACA	0	CXR	H; C
4. HG	62	M	Renal ACA	0	Abd CT scan	H
5. MS	53	F	Malignant melanoma	2	Soft tissue	C; H; XRT
6. EH	56	F	Breast ACA	0	CXR	C; H
7. EO	60	F	Renal ACA	0	CXR	H; XRT
8. DB	57	M	Malignant melanoma	1	CXR	C
9. RR	60	M	Renal ACA	1	Abd CT scan	—
10. HT	59	M	Renal ACA	0	CXR	—
11. RH	43	M	Seminoma	2	CXR	C; XRT
12. KJ	69	M	Malignant fibrous histiocytoma	2	CXR	C; XRT
13. RL	62	M	Renal ACA	0	CXR	—
14. TK	39	F	Renal ACA	0	Soft tissue	—
15. JK	65	M	Gastric ACA	2	Liver	C; XRT
16. VO	65	M	Renal ACA	2	CXR	H; C
17. FL	66	F	Renal ACA	1	CXR	—
18. BC	56	F	Renal ACA	2	CXR	—

*ECOG PS: Eastern Cooperative Oncology Group Performance Score: 0 (fully active) to 4 (totally disabled).

‡ACA = adenocarcinoma

†XRT = radiation therapy; H = hormonal therapy; C = chemotherapy

Table 15. Toxicities observed by treatment dosage with BCNU and IFN-rA

	IFN-rA (12 × 10^6U/m^2)			
	Thrice weekly BCNU dosage (mg/m^2 q4wks)			Days 1–3 each month
	50 (N = 3)	100 (N = 3)	150 (N = 6)	150 (N = 6)*
Constitutional				
Deterioration in PS				
1 grade	—	—	—	2
≥ 2 grades	3	1	1	—
Weight loss (kg)	4.4; 4.6; 8.5	1.6; 5.8; 7.9	0.9; 3.3; 3.5; 6.6; 7.0	1.2; 2.1; 4.6
Moderate-to-severe				
Fatigue	2	3	3	1
Anorexia	2	2	3	—
Myalgias	2	1	—	—
Nausea, vomiting	—	—	3	2
Hematologic (cells × 10^3/mm^3)				
Leukocytes	2.6; 2.6; 3.8	1.6; 2.3; 3.6	1.6; 2.1; 2.5; 2.7; 3.0; 4.4	1.7; 2.7; 3.1; 3.2; 3.4
Platelets	109, 120, 188	56, 118, 136	29, 39, 80, 90, 118, 125	54, 60, 139, 172, 224

*One patient died within the month of commencing treatment.

dissipates within 24–48 hours once cells are no longer exposed to the agent. In light of these findings, we expanded our initial pilot trial to include six additional patients who received IFL-RA, 12 × 10^6 U/m^2, administered intramuscularly on three consecutive days each month in conjunction with a monthly intravenous dose of BCNU, 150 mg/m^2. With the aforementioned regimen, dose-limiting toxicities were similar to that achieved with the intermittent schedule of interferon.

We initially utilized the thrice weekly IFL-rA regimen because we had considerable experience with it from prior Phase II trials [37–40]. However, the optimum schedule for concomitant IFL-rA and cytotoxic agents remains unclear. As previously noted [23], it now appears that the antiproliferative characteristics of IFL-rA are enhanced when there is an extensive duration of cellular exposure to the agent. A more prolonged inhibition of tumor growth results from continued treatment *in vivo*. Phase II trials of the IFL-rA and BCNU combination may be more efficacious utilizing IFL-rA on a daily times three schedule with monthly BCNU.

Concomitant IFN therapy with a cytotoxic agent is conceptually appealing. IFNs have a direct antiproliferative effect on cells of most histologies and this seems to reflect the presence of IFN-specific receptors [19, 20]. In addition, IFNs have a profound immunomodulatory impact by enhancing the cytotoxicity of T-lymphocytes and natural killer cells (NK) [13] and augmenting the phagocytosis of tumor cells by macrophages [14]. The antitumor activity, relative absence of substantial hematologic and gastrointestinal toxicity, and possible synergism in animal tumor models suggest that IFN may be useful to complement other antiproliferative regimens (Table 16). Nevertheless, there are complex interactions which demand careful clinical monitoring. For example, rat fibroblast IFN inhibited the antitumor effects of cyclophosphamide when

Table 16. Enhanced antiproliferative activity among combinations of biological response modifiers

Regimen	Tumor systems*	Results
IFN-γ plus TNF-α	HTCFA: 8226 — myeloma; non-small cell lung cancer[88]	In 8226 — myeloma, single agent Rx reduced survival to 35–40% of controls *vs* 4% survival from IFN-γ plus TNF-α.
INF-γ plus IFN-αA or IFN-β_1	Hs294T†[90]	Enhanced antiproliferation only from IFN-γ plus IFN-αA or IFN-β_1.
IFN-γ plus IFN-β (ser)	HTCFA: Human melanoma cells[26]	5 of 6 cell lines manifested synergistic antiproliferative response to the combination program.

*HTCFA: Human tumor colony forming assay.
† Human melanoma cell line.

both agents were given concomitantly [80]. In another experimental system, recombinant IFN abrogated the efficacy of high dose cyclophosphamide in hamsters with TBD 932 lymphosarcoma [81]. However, additive or synergistic effects occurred at lower IFN doses. Similarly, synergistic antitumor efficacy was convincingly demonstrated from the combination of concomitant human lymphoblastoid IFN with either cyclophosphamide or adriamycin [82]. The same authors had previously reported that human lymphoblastoid interferon substantially increased the antitumor activity of suboptimal doses of cyclophosphamide and adriamycin, using the model of a human breast tumor xenograft growing in nude mice [83].

Doxorubicin is a cytotoxic anthracycline antibiotic with a broad spectrum of antineoplastic activity. Objective regressions have been confirmed in diverse solid tumors and in hematologic malignant disease. Noteworthy toxicities consist of myelosuppression, nausea, vomiting, alopecia, and vesication with possible tissue necrosis following extravasation of the agent. Congestive heart failure and cardiomyopathy may occur particularly in patients whose total dose exceeds 550 mg/m^2. The currently recommended single agent intravenous dose is 60–75 mg/m^2 administered at three week intervals [84].

British investigators have recently published intriguing studies involving a human breast tumor xenograft growing in nude mice [82]. A combination of human lymphoblastoid interferon with either cyclophosphamide or doxorubicin caused regression, and in some cases, total disappearance of tumor at doses of drug and interferon that, when employed alone, were capable only of inhibiting tumor growth. The tumor masses in the interferon and doxirubicin alone groups were significantly larger than in the combined groups. In addition, mice who received interferon-doxorubicin had a substantially enhanced survival compared with animals who received either agent separately. A particularly important observation was the absence of any bone marrow toxicities as measured by peripheral blood leukocytes among mice who received combination treatment.

Interferon plus cytotoxic agents in human tumor cell cultures has provided potential therapeutic alternatives for two groups of neoplasms which are traditionally refractory to systemic strategies: adenocarcinoma of bronchogenic [85] and salivary gland origin [86]. In xenografts, human α lymphoblastoid interferon significantly potentiated the activity of cisplatin, and Japanese investigators have clearly documented synergistic antiproliferative properties from the combination of 5-fluorouracil plus human leukocyte interferon. It appears that the combination approach is selectively effective against neoplastic cells of human salivary gland adenocarcinoma, but does not influence nonneoplastic tissue.

The mechanisms of enhanced antineoplastic characteristics from combinations of IFNs and chemotherapy have been addressed in the nude mouse experimental model utilizing a human tumor xenograft [82]. In elegant analyses the authors were able to dissociate the effects of human lymphoblastoid interferon [HuIFN-α(Ly)] on drug metabolizing enzymes in the liver from direct

interferon-chemotherapy relationships on the tumor. HuIFN-α(Ly) had no effect on mouse hepatic enzyme systems, whereas the murine IFN caused significant changes in isozymic forms of the enzymes. However, the addition of mouse IFN to HuIFN-α(Ly)/cyclophosphamide or adriamycin complexes did not impact upon cytotoxicity. Therefore, it appears that the malignant cell is the pivotal site for IFN-chemotherapy-metabolizing enzymes.

7.2. Biological response modifiers

In addition to cytotoxic-IFN regimens, another area with intriguing clinical application involves attempts to augment antineoplastic properties of IFNs by biochemical and immunological modulation (Table 17). Polyamines, including putriscine, spermidine, and spermine, stabilize DNA and inhibit its enzymatic degradation. α-difluromethylornithine (DFMO) is an inhibitor of ornithine decarboxylase, which thus depletes polyamines, rendering cells more susceptible to cytotoxic interventions. We have now documented that recombinant leukocyte A human interferon and human lymphoblastoid interferon with DFMO have markedly synergistic antiproliferative activity against human tumor cells [77]. Similarly, the combination of IFN and DFMO with doxorubicin have greater antiproliferative activity than expected on the basis of additive effects based on the activity of doxorubicin alone and the synergistic activity of IFN plus DFMO. As expected, the addition of putriscine to the cell cultures abrogated the synergistic interactions of IFN and DFMO but not the activity of IFN itself. Our studies have also indicated that neither aspirin nor indomethacin had any substantial negative impact upon the antiproliferative activity of human IFN or the synergistic properties of the IFN-DFMO complex. These observations also have clinical applicability in that it might be possible to ameliorate the constitutional toxicities of IFN by the use of nonsteroidal anti-inflammatory agents. It is becoming increasingly apparent that developments and techniques in molecular biology, coupled with advances in cloning methodology, may have relatively immediate applicability in the clinical setting.

In addition to the synergistic enhancement of IFNs by both cytotoxic agents and biochemical modulators, another area of interest focuses on the possible beneficial interactions between combinations of IFNs and tumor necrosis factor. In the mid-1970s sera from endotoxin-treated rodents that had been previously sensitized with M bovis strain BCG effected extensive hemorrhagic necrosis of murine neoplasms without untoward sequelae on the host [87]. This protein also demonstrates discriminatory cytotoxic and cytocidal characteristics against several transformed cell lines, but does not affect normal cell cultures. Initial clinical investigations with TNF indicate dose-limiting toxicities of renal abnormalities, clotting disturbances, and hypotension. A provocative experimental study has documented that combinations of IFN-γ plus TNF is therapeutically synergistic *in vitro* in soft agar clonogenic assays against human tumor cell lines. In one experimental model, either TNF or IFN-γ reduced cell

Table 17. Interactions between interferons (IFN) and selected antiproliferative agents in experimental studies

type of IFN	Agents	Interactions	Tumor model
rIFN-α_2	doxorubicin[23]	synergism	human tumor cell lines: cervical carcinoma (CASXI); malignant melanoma (SKMEL-28)
MuIFN*	methotrexate (MTX);[74] MTX + 6-mercaptopurine	synergism	L-1210 murine leukemia in BDF/1 mice
MuIFN*	cyclophosphamide[75]	synergism	lymphoma in AKR mice
MuIFN*	1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)[76]	synergism	LSTRA murine leukemia in CDF, mice
rIFN-αA; HuIFN(Ly)†	α-difluoromethyl-ornithine (DFMO)[77]	synergism	human cell lines: malignant melanoma (A375); renal cell carcinoma (A498)
R-IFN‡	cyclophosphamide(C)[80]	R-IFN + C were inferior to C alone	LS175 rodent liposarcoma in female BN rats
rIFN-αA	cyclophosphamide[81]	synergism detected at C, 50 mgm/hamster and rIFN-αA, 10^5 IU/hamster but no synergism with C, 2.5 mg/hamster	TBD932 hamster lymphosarcoma cell line; human breast cancer xenograft in nude mice.
HuIFN-α(Ly)†	cyclophosphamide; Adriamycin[83]	Using rIFN-αA, 10^5 IU/hamster, combination regimens significantly reduced tumor size ($p < 0.001$) *vs* controls	human breast cancer xenograft
HuIFN-α(Ly)†	cis-platinum[85]	synergism	human tumor non-small cell adenocarcinoma xenografts in CBA mice
HuIFN-α	5-fluorouracil[86]	synergism	human salivary gland adenocarcinoma cell line HSG

*Murine interferon
† Human lymphoblastoid interferon
‡ Sendai viral-produced rat interferon

survival to 35–40% of control. On the other hand, the combination of TNF (α) and IFN-γ reduced survival to only 4% of control [88].

The differences in the antiproliferative mechanisms of IFN-γ and TNF offer potentially interesting clinical opportunities which may be therapeutically exploitable. IFN-γ, the immune interferon, has an array of directly cytotoxic as well as immunomodulatory characteristics. On the other hand, it appears that TNF disrupts cellular replication by a temperature-dependent, non-cell cycle dependent process after a lag period of 10–12 hours. This event appears to be

consistent with a catalytic phenomenon characteristic of an enzymatic reaction. This event is probably preceded by TNF binding to some type of cell surface receptor [89].

In addition to the reported synergy from combinations of IFN-γ and TNF, additional investigations have documented provocative relationships from concomitant assessments of all three classes of IFNs: α, β, and γ [90]. Using the human melanoma cell line Hs294T, the relative potency of each subspecies of IFN was compared. Although IFN-β was the most potent in inhibiting cell growth, substantial antiproliferative characteristics were also evident from IFN-αA and IFN-γ. Dose-response curves were then calculated from IFNs alone (α, β, γ); from combinations of increasing concentrations of IFN-γ with fixed amounts of IFN-αA or IFN-β; and from combinations of increasing concentrations of IFN-αA or IFN-β with fixed amounts of IFN-γ. The exposure of the human melanoma cell lines to IFN-γ, in combination with either IFN-αA or IFN-β, clearly potentiated IFN-related biological characteristics. There were no consistent antiproliferative results from the combinations of IFN-αA and IFN-β, that is, combinations which did not include IFN-γ. These observations are consistent with another experimental study which documented enhanced efficacy from IFN-γ and IFN-β (ser) in human melanoma cell lines [26]. Since these IFN preparations were at least 95% pure, the observed antiproliferative properties were reflective of the IFN molecule *per se*.

In addition to the increasingly complex relationship between the IFN molecules, cell surface receptors, and biological activities, there are now evolving studies which indicate a marked temperature dependency to the antiproliferative properties of IFNs [91]. Using the murine B-16 melanoma model and murine IFN-α, -β, and -γ, studies show that the antiproliferative features of all three species were augmented by cellular incubation of the cells at elevated temperatures. However, the elevated temperatures had more dramatic effects on the antiproliferative activity of MuIFN-γ (tenfold enhancement) than in MuIFN-α or MuIFNγ (2.9 and 3.4 fold enhancement, respectively). In addition, the specific augmentation of MuIFN-γ's antiproliferative activity with the increased temperature occurred with increasing concentrations of the murine γ-IFN, but not with increasing concentrations of the other IFN species. These experimental studies obviously need clinical confirmation and may have important therapeutic implications in the management of advanced human malignant melanoma.

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10. The treatment of Malignant Melanoma by Fast Neutrons

Peter R. Blake

1. Introduction

Malignant melanoma is posing an increasing problem in the temperate zones as the incidence is rising and the population presenting with the disease is becoming younger [1]. Conventional treatment has involved wide excision of the lesion with skin grafting which has produced high local control rates for small superficial tumors. However, it is not a satisfactory form of treatment for recurrent or deeply infiltrating tumors or for lesions in sites where wide excision would cause disfigurement or morbidity [2]. For tumors such as these and for widely disseminated disease, alternative forms of therapy have been sought for many years. Radiotherapy, chemotherapy, and immunotherapy have all been studied in clinical trials both alone and as adjuvants to surgery.

Several researchers in the fields of surgery and histopathology have shown that depth of invasion through the skin and maximum tumor thickness are related to local recurrence, metatasis, and prognosis of malignant melanoma. Clark [3] showed that in patients with primary disease only (Stage I) survival decreased with increasing depth of invasion through the papillary and reticular dermis into the subcutaneous tissues. Similarly Breslow [4] found that patients with lesions less than 0.76 mm thick had a 100% five-year survival, which dropped to approximately 75% for lesions up to 2.25 mm thick and 25% for lesions thicker than this. This was only true for patients with Stage I disease, as for patients with involved local nodes (Stage II), it was the number of nodes involved that correlated with prognosis [5].

Recently the results of excision have been related to local recurrence and survival in terms of the histological stage of the primary tumor [6]. It is probable that the survival of patients with very thin lesions (< 0.76 mm) or very thick lesions (> 3 mm) is unaltered by performing wide local excision, with margins of 5 cm peripheral to the lesion and down to deep fascia, rather than simple excision with margins of 0.5–2 cm [2]. It is rare for very thin lesions to recur and metastases are uncommon. Very thick lesions, on the other hand, are frequently accompanied by nodal or distant metastases, and it is these, not local disease, that limit prognosis [7]. Wide excision is, therefore, unnecessary as local recurrence is not likely to occur in the short lifespan of the patient. However, a

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large proportion of melanomas fall into the 0.76–3 mm thick group, and in these patients survival is related to local control.

Many patients find the scars of wide excision or of block dissection disfiguring and distressing. This is particularly so when the scars are depressed or are on the face [8]. There are also sites, particularly on the face and perineum, where wide excision cannot be achieved without some morbidity. These factors, together with the necessity for treatment of widespread disease, make it important to study methods of treatment other than surgery.

Malignant melanoma has, for many years, been regarded as a 'radioresistant' tumor [9, 10]. Yet the reports that formed the basis of this belief often lacked histological diagnoses, staging, or comparison with similar surgical series [11]. In addition, the radiation energy, total dose delivered, dose per fraction, and overall treatment time were not standard and were not quoted in all reports. Having been labeled 'radioresistant', referral of patients with melanoma for radiotherapy has been almost entirely restricted to those with disease recurrent after primary surgery or with metastatic disease. These patients have, in comparison with patients undergoing primary surgery, fared badly because of the more aggressive nature of their disease.

However, a few reports have supported the use of both interstitial and external beam radiotherapy for the treatment of nodular melanoma. Ellis [12] reviewed his own work with radium needles and concluded that, although melanoma had varying sensitivity, responses were seen after x-ray doses of 5,500–6,000 Roentgen (6,500–7,000 cGy) delivered over seven to ten days. Hellriegel [13] achieved 62% five-year survival and 42% ten-year survival using 'high' local doses delivered by contact radiotherapy, although his series included some pigmented tumors that were not biopsied. In a Canadian series [14], 121 patients with nodular melanomas were treated by local excision followed by radiotherapy and were compared with a group undergoing wide excision only. After 5,000 cGy given in ten fractions over a two-week period, a 41% five-year disease-free survival was achieved, similar to that of the comparative series treated by wide local excision.

In studying treatment with large fractions, Habermalz and Fisher [15] analyzed the response of 144 skin and subcutaneous masses treated with fractions ranging from 200 to 800 cGy. They found no response in lesions treated with 500 cGy or less and saw responses in 28/31 lesions treated with fractions of 600 cGy or more. The response of lymph node metastases was less markedly dependent on dose per fraction, but the trend of more complete responses being seen after treatment with large fractions was still present. In a review of the relationship of the response of melanoma to total dose, the number of fractions and fraction size, Hornsey [16] found that twice as many tumors responded to fractions of 400–800 cGy than to fractions of 200–300 cGy. Overgaard [17] confirmed the finding that large fractions of 500 or 900 cGy produced a higher response rate than conventionally sized fractions, but was not able to determine which was superior. Khan and Ross [18], however, did not find a significant difference between response to small or large fractions.

These reports have stimulated radiobiological study of melanoma xenografts grown in laboratory animals and of established cell lines [19]. The results of these studies suggest that melanoma cells may differ from other cells in their radiobiological properties. In particular, it appears that melanoma cells are able to accumulate or repair large amounts of sublethal radiation damage [20] and that a large percentage of melanoma cells *in situ* may be hypoxic and relatively radioresistant [21].

Fast neutrons differ from conventional x-rays in producing very little sublethal damage and in being less dependent on tissue oxygen concentrations for their lethal effect. These properties of fast neutrons make them theoretically attractive for the treatment of malignant melanoma.

2. Factors affecting tumor response

The response of a tumor to a dose of radiation depends on a number of factors, which include

(1) the type and energy of the radiation,
(2) the sensitivity of the cells to the radiation, and
(3) environmental conditions influencing either the sensitivity of the cells or their ability to repair radiation induced damage.

3.1. Radiation type and energy

All ionizing radiations cause damage by the deposition of energy within the cells. This deposition produces free radicals which interact with molecules in the strands of DNA within the nucleus of the cell [22]. Ionization damage of DNA can either be repaired, leading to no lasting effects, or 'fixed' by the attachment to the ionized molecule of an electronaffinic compound, commonly oxygen. This fixed damage can lead to misreadings of the DNA strand at replication and subsequent biological effects which may be lethal to that cell or progeny.

Different types of radiation deposit energy in different patterns. Megavoltage x-rays deposit energy sparsely relative to particle radiation and cause ionizations indirectly by the production of 'recoil' electrons. As x-ray energy increases, the penetration of the beam into matter improves, but the interactions between the beam and the matter become less frequent. Consequently, the energy deposited per unit length of track falls. This deposition of energy is known as the Linear Energy Transfer (LET) of the radiation.

Particles that have a large mass — move slowly and ionize directly by virtue of being charged — produce ionizations in dense, short columns and have a high LET. Alpha particles and protons are of this type. Neutrons, while being relatively heavy, are not charged, but, at the energies used for therapy, produce ionizations indirectly by the production of 'knock-on' protons. The LET of fast neutrons is approximately 40 times that of ^{60}Co γ-rays. The result of this is that many more ionizations are produced in a cell nucleus by neutrons than are produced by x- or γ-rays. Nuclei are, to some extent, able to repair a limited

amount of ionization damage, but this ability fails rapidly as the number of ionizations produced within the nucleus increases. As neutrons produce a high density of ionizations, the damage is less easily repaired than the sparse ionization damage produced by x- or γ-rays. As less of the damage produced can be repaired, neutrons could be considered to be more effective in producing damage for a given dose. This leads to the concept of the relative biological effectiveness (RBE) of neutrons. This is the ratio of the dose to produce a given effect in the test system to that of 250 kVp x-rays [22]. The RBE is dependent on the total dose of radiation, the end-point, the dose rate, field size, and the number of treatment fractions. Thus, it is important to appreciate that RBE is not a constant factor but depends on the treatment regimen and the tissue under study. In the clinical studies using neutrons, RBE values of between two and five have been found for the effects on normal tissues.

The concept of RBE can be applied to any tissue response that is dose related. This includes the death of clonogenic cells irradiated *in vitro*, growth delay of tumors irradiated *in vivo*, and the production of normal tissue effects.

3.2 Sensitivity of cells

Apart from differences in the response of body tissues to different types of radiation, there is considerable variation in the degree of response between different tissues. This difference is also seen in experimental animal tumors and cells grown *in vitro*.

Both human and animal tumor cells have been used in studies of cellular radiation sensitivity. The cells have either been grown in liquid culture or have been disaggregated from tumors grown in laboratory animals. The basis of the method is that cell suspensions containing a known number of clonogenic cells are irradiated with graded doses and the surviving fraction estimated by culture of the clonogenic cells.

If the $\log^{10}$ of the fraction of clonogenic cells surviving x-ray treatment is plotted against a linear plot of dose, a curve is obtained which is similar in shape for many cell types, whatever the method of assay. There is an initial part to the plot in which the steepness of the curve increases with increasing dose. After a point, which varies from one cell type to another, the plot continues as a straight line. Both the size of the curved portion and the gradient of the straight portion are characteristic of that particular cell type irradiated under those particular conditions. The curved part of the plot is known as the shoulder and the exponential part as the straight portion of the dose-response curve. The presence of the shoulder on the clonogenic cell survival curve implies that radiation damage can either be accumulated or repaired without affecting the viability or reproductive capacity of the cell. This damage is, therefore, called 'sublethal' damage (SLD).

After x-irradiation, cells show variation in their ability to repair SLD. It appears that some tumor cells, including melanoma cells, can demonstrate considerable amounts of SLD repair [19]. However, neutrons, because of the

high LET, produce very little SLD, and almost all the damage produced is lethal whatever the cell type. This has been demonstrated both in cell culture [23] and in tissues *in vivo* [24].

3.3 The oxygen effect

In 1952 Read [25] published an account of the dependence on oxygen of the response of the bean sprout root growth to irradiation with alpha particles. The following year Gray et al. [26] published a wider review of the studies on oxygen, and two years later, in conjunction with Thomlinson [27], he published a report on the histological structure of bronchial carcinomas, the presence of hypoxic cells within these, and the implications that this had for radiotherapy. These studies had shown that, in the absence of oxygen, the dose of x-rays needed to produce a given effect was approximately three times that needed when oxygen was present. The ratio of these doses was called the oxygen enhancement ratio (OER). Whereas for single doses of x-rays the OER was 3, that of neutrons was in the region of 1.5 [28]. Neutrons could, therefore, be potentially a more effective form of treatment than x-rays for tumors with large hypoxic cell populations.

Guichard et al. [29] have reported that melanoma may have a large hypoxic cell population and that in a xenograft established from a melanoma, hypoxic cells comprised 80% of the total. However, it is uncertain whether this is an important advantage of neutrons in fractionated therapy. In clinical x-ray treatment regimens, single fraction therapy is rare, and most courses of radical radiotherapy comprise 15–30 fractions. Van Putten and Kallman [30] found that the proportion of hypoxic cells in a tumour after four fractions of radiation was the same as before the treatment started. For this to be so, hypoxic cells must become oxygenated between fractions; otherwise, the proportion of hypoxic cells in the tumor would increase as the more sensitive oxygenated cells were killed at each exposure. This process is known as re-oxygenation and the mechanism is poorly understood. While it is apparent that the demand for oxygen in the tumor will fall as sensitive cells die, allowing oxygen to diffuse further into previously hypoxic areas, cells die at the next mitosis, which is later than the time at which re-oxygenation appears to be complete.

The effect of re-oxygenation in therapy is to improve the effectiveness of x-rays and reduce the ratio of the values of the OER for fractionated courses of x-rays and neutrons.

4. Early biological and clinical results

Biological studies of fast neutrons started remarkably soon after their discovery in 1932. These showed that neutrons produced similar acute effects on biological systems as did x- and γ-rays. However, although these effects were qualitatively similar, they were not quantitatively the same. In addition, the effects were not

the same for all living tissues. This raised the prospect that this type of radiation, different from x- and γ-rays, could possibly have more lethal effects on tumors than on normal tissues.

To investigate this, Stone [31] began treating 'incurable' patients using the cyclotron at Berkley, California. The tumors treated were advanced and often large. Many had undergone previous surgery or radiotherapy, and infection was common and often untreatable. The neutron treatments were not standardized in that a variety of doses, field sizes, and fractionation regimens were used. A further problem was that many courses of treatment did not proceed as planned due to machine breakdown or unavailability. Although some cures were achieved in this group, the high rate of late complications was sufficient for Stone to say that 'neutron therapy as administered by us has resulted in such bad late sequelae in proportion to the few good results that it should not be continued.'

The treatment schedules used by Stone were based on the experience of matching acute skin reactions produced by neutron therapy with those of x-ray treatment. Subsequent analysis of these schedules has shown many of his doses to have been the equivalent of higher x-ray doses than would have been prescribed in normal practice [32]. This retrospective review indicated that those fields in which ulceration occurred had received doses equivalent to 6,100–11,000 cGy of x-ray therapy. It is understandable that this difficulty in determining tissue tolerance arose, as there was no knowledge of how regimens could be made biologically equivalent other than by matching skin reactions. Sheline was able to complete his review because knowledge of the factors which affect the relative biological effectiveness of neutrons increased considerably in the period after Stone's work [33, 34].

5. Recent clinical results with fast neutrons

Neutron therapy of malignant disease has been studied in several centers in Europe and North America since 1970. The difficulty in producing neutrons of sufficient energy and in sufficient quantity for clinical use has meant that many trials, especially in North America, have been centered on machines provided for scientific research, often some distance from hospital departments. In addition, many machines were not designed initially to have a clinical facility, and so there have been local problems as well as geographical ones. Neutron beams in planes or positions inconvenient for therapy limit the treatment techniques which can be used. Many of these problems have been overcome with great ingenuity. However, treatment technique, dose, fractionation regimen, and overall treatment time have varied from one center to another and comparison of results with other neutron trials or with patients treated with x- or γ-rays has been seldom possible.

Some centers found that the logistic problems of treating patients with neutrons was so great that a proportion of the course of treatment was given

using γ-irradiation. Neutron therapy was then given as part of a mixed modality schedule to give a boost to the tumor locally.

The largest number of patients treated with neutrons alone have been at the Medical Research Council Cyclotron Unit at Hammersmith Hospital, London [35]. Predominantly, patients have had late stage disease or have had previous surgery or radiotherapy and subsequent recurrence. Patients have, for the most part, been prescribed a standard dose and fractionation schedule, whatever the histological type of tumor. Variations in treatment have been necessitated by machine failure and the cutting short or replanning of treatments to spare sensitive normal tissues.

Neutron therapy at Hammersmith has been characterized by a high rate of response and subsequent local control for most histological types of tumor. This has been particularly noteworthy in tumors regarded as resistant to x-ray therapy. These include tumors of the salivary glands, malignant melanoma, and tumors of the air-sinuses.

Much of the neutron therapy in North America has been with mixed modality treatment. Results of this type of treatment have been conflicting. In some trials an advantage has been found [36], while others have not confirmed this finding [37]. This may be due to adverse features of the machines used in different centers or may indicate that improved results are only achieved with neutrons used as a single modality. One group [37], who reported no advantage in mixed modality therapy, has reported an improved survival in patients with advanced squamous carcinoma of the head and neck treated with fast neutrons alone [38]. The Fast Neutron Clinic at Hammersmith Hospital has reported its experience of the treatment of malignant melanoma with neutrons as a single modality [39].

6. Experience of the MRC fast neutron clinic in treating melanoma

6.1. Patients

The results of treatment of malignant melanoma with fast neutrons were analyzed in November 1983 and included all those patients referred with a diagnosis of malignant melanoma prior to this. It had not been the policy at Hammersmith to use neutron therapy as an adjuvant to surgery, and it had been intended that only those patients with measurable deposits of melanoma would be treated. Of 144 tumors in 79 patients, only one was completely excised and did not fulfill this criterion. Of the remaining 78 patients with 143 tumors, three died before treatment was completed, leaving for analysis 75 patients with 140 tumors. Some patients had more than one tumor treated. Of these, three tumors did not receive the prescribed treatment. The analysis is of the remaining 137 tumors in 75 patients.

Many patients had several tumors treated either concurrently or consecutively. A single tumor was treated in 48 patients, two tumors in 13 patients,

three in four patients, four in five patients, five tumors in one patient, six in three patients, and one patient had eight separate tumors treated. All these irradiated areas were observed for at least one month from the commencement of treatment.

6.2. Treatment techniques

All the patients were treated on the 16 MeV cyclotron at the MRC Cyclotron Unit, Hammersmith Hospital. The beam was produced by the action of deuterons, accelerated in the cyclotron, on a beryllium target. The emergent neutron beam passed through a collimator into the neutron treatment room. The beam was fixed in a horizontal plane 150 cm above the floor. Patients were positioned so that the beam was perpendicular to the tumor. This was achieved by a couch and a chair capable of movement in the vertical and horizontal planes and also capable of rotation.

Prior to 1978, collimation of the neutron beam was achieved using shaped wooden collimators impregnated with boron salts. These provided only a limited range of field sizes and were heavy and unwieldy to use. After 1978 the beam was fitted with the first fully adjustable collimator made of steel alloy and polythene. This allowed any rectangular field to be produced from 4 × 4 cm to 20 × 20 cm. Shielding of corners and other irregular field shapes could be produced by the use of rubber or wax inserts held in the collimator with magnets.

The dose rate from the cyclotron was dependent on the tuning of the radiofrequency field across the magnets. When working optimally, the dose rate at the treatment distance of 135 cm from the target was approximately 30 neutron cGy per minute.

6.3. Treatment volume

Superficial tumor nodules on flat areas of the body, such as the trunk, thigh, or upper arm, were treated with direct fields sufficient in size to cover the tumor and a suitable margin. The margin given to the tumor was usually 2–3 cm but, on occasions, this had to be reduced to spare sensitive organs or previously treated areas. Deeply infiltrating lesions or those on the head and neck were treated with two or three beams positioned to converge on the tumor while giving as little radiation as possible to the normal tissues.

6.4. Dose

The prescribed dose to the tumor was, with a few exceptions, 1,560 cGy in twelve fractions given three times per week over 26 days. When it was necessary to use fractionation schedules other than the standard twelve-fraction regimen, the modified Ellis formula [34] was used. The most frequently used alternative schedule was 1,395 cGy in six fractions over 12 days. The range of the prescribed (model tumor) doses, expressed as NSD, is represented in Figure 1.

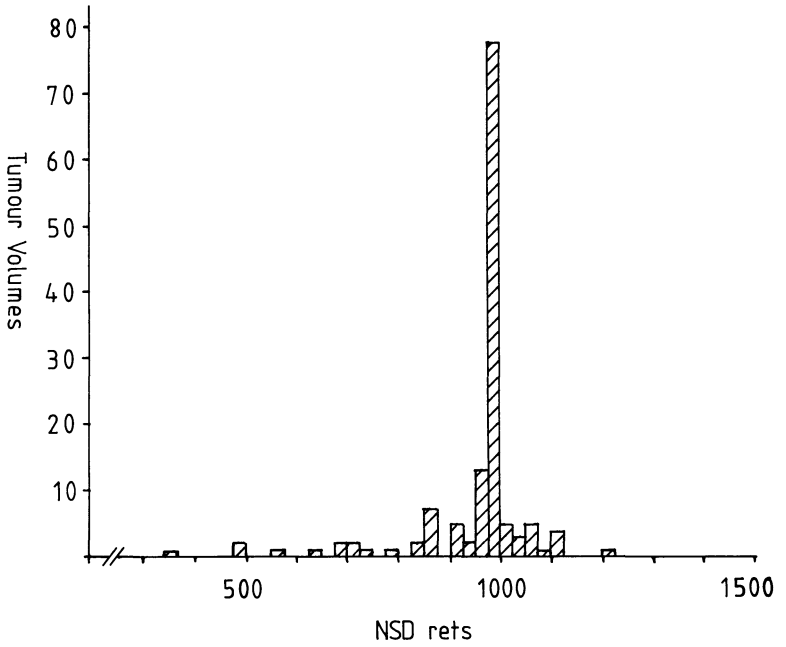

Figure 1. The range of prescribed (modal) tumor doses expressed as nominal standard dose (NSD).

Conversion of treatment regimens varying in total dose, number of fractions, and overall treatment time to the single term of 'nominal standard dose' allows comparison of regimens. Many tumors were described as superficial, and no plan was drawn for these when treated with a single radiation field. The tumor was all-encompassed in the high-dose region of the distribution and received greater than 90% of the prescribed dose. For some tumors the exact depth of invasion of the tumor was known, and for these the calculation of minimum tumor dose could be made. This calculation could also be made for those tumors where plans were drawn, either because more than one field was used or in order to calculate the dose to normal tissues that had to be spared, such as spinal cord.

6.5. Measurement of response and patient follow-up

Patients were seen at biweekly intervals immediately after treatment, unless severe skin reactions required more frequent attendance for dressings. After four or six weeks patients were seen monthly for three months and then at three-month intervals. All the patients were assessed by a doctor at each visit, and any palpable tumor masses were measured.

Histological evidence of complete regression was not sought, as early experience had shown that there was a risk of causing necrosis by performing a biopsy in the neutron treated area. Many residual masses showed no evidence of regrowth over the follow-up period, and it is likely that they represented fibrous

tissue and not viable tumor. However, residual masses were recorded as partial regressions.

Complications of treatment were assessed at follow-up visits and fell broadly into the categories of fibrosis or necrosis. Local recurrence and the appearance of metastases were also recorded.

6.6. Clinical analyses

The aim of analyzing the data was to determine the dose-response relationship for melanoma treated with fast neutrons. In addition, the incidence of normal tissue complications was to be related to the dose received by these tissues. The assumptions made were:

(1) That the degree of regression obtained was related to the prescribed (modal) dose, as the great majority of tumor cells received a dose within a few percent of this. Cells receiving less than this dose would be in a minority and would not be expected to influence the overall response of a tumor in the short term.
(2) Cells that did receive less than the prescribed dose, although being small in number, could provide a nidus for later recurrence, and therefore recurrence would be related to minimum tumor dose.
(3) Normal tissue complications would be related to the maximum normal tissue dose.
(4) Field size could be related to both recurrence and to complications, as there would be greater inhomogeneity in dose in a larger volume.

In a published article reporting the results of treatment of melanoma with fast neutrons [39] only those sites observed for at least three months after treatment were analyzed. However, for the purposes of this present analysis, all sites in which treatment was completed are included.

6.7. Clinical results

The median age of the patients was 60 years and the range was 20–86 years. The median survival after treatment was 12 months with deaths occurring from 1 to 30 months after treatment. The longest survival was 152 months (Figure 2).

In the majority of cases death occurred at hospitals other than Hammersmith Hospital or at home. Consequently, post-mortem examinations were not performed as a routine. However, many of the patients were known to have widespread metastatic disease and death was attributed to this. Metastases beyond the treated area were present in 11 of 75 patients (15%) when first seen. Of the remaining 64 patients 15 (23%) developed metastases within three months of starting treatment. At the time of the analysis all these patients with early metastases were dead. Of the remaining 49 patients 36 had died. Thirty-one of these had metastases at the time of death. Twelve of the 13 patients remaining alive were free of metastases. Of the whole group of 75 patients only

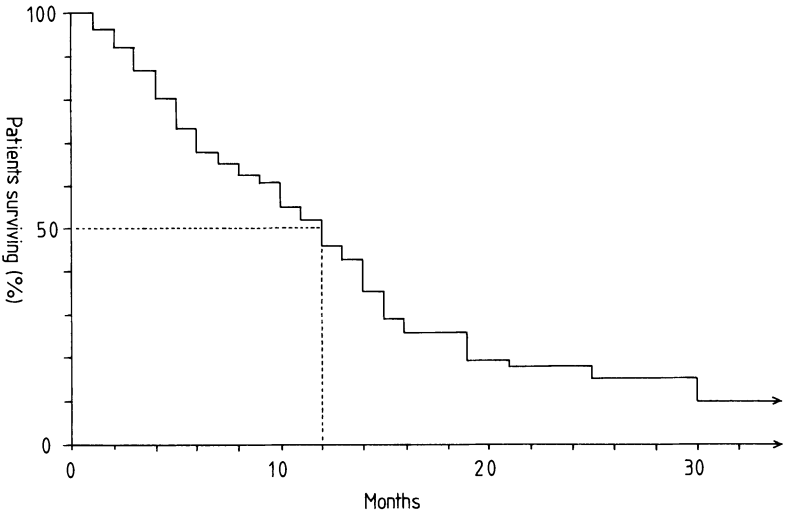

Figure 2. Patient survival following neutron therapy.

17 (23%) had not been shown to have evidence of metastatic disease outside the treated area at the time of the analysis.

The median period between diagnosis of the lesion to be treated and further distant metastasis was 12 months and between the start of neutron therapy and metastasis was six months.

6.8. Disease-free period

The median period for which the treated areas could be observed was six months and the range was 1-152 months, although 132 of 137 (96%) were observed for 30 months or less.

The disease-free period of each treated area is shown in Figure 3. Recurrence was seen in only 10 of 137 sites at a median interval of 6.5 months from the beginning of treatment.

The median observation period for sites showing complete regression of tumor was 11 months, while for those undergoing partial regression it was three months. This difference is significant ($p < 0.01$).

6.9. Complete regression related to dose

Complete regression of tumor was achieved in 80 of 137 sites (58%). Of the remaining 57 sites (42%), all but four (3%) underwent partial regression, with an estimated reduction in tumor volume of at least 50%. The remaining four tumors did not diminish in size after treatment but showed no sign of regrowth during the remainder of the patients' lives. In further analyses, these static tumors are included with those undergoing partial regression.

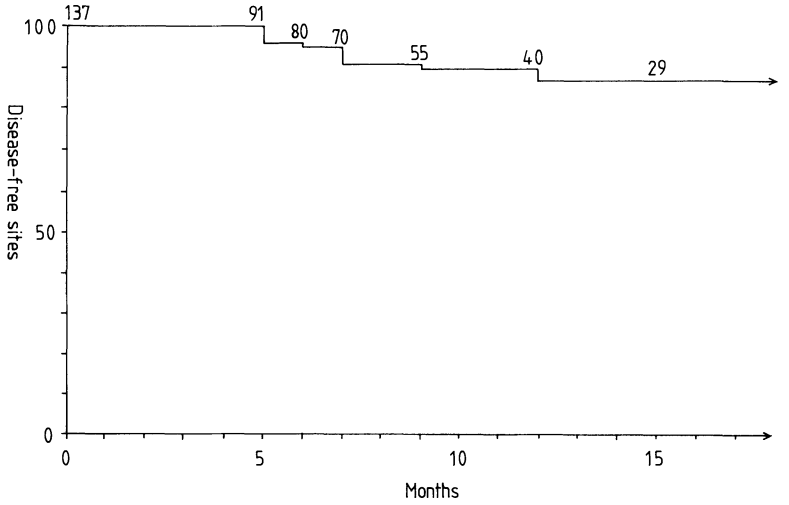

Figure 3. Sites remaining disease-free following neutron therapy. Numbers indicate those sites at risk of recurrence.

In considering all treated sites, whatever the subsequent survival, the median of the prescribed doses delivered to those undergoing complete regression had an NSD value of 988 rets and was the same as that received by sites showing only partial regression (Figure 4). However, the range of doses around the median was greater for those sites undergoing partial regression than for those showing complete regression (Table 1).

The median field size of sites undergoing complete regression was 138 cm^2, while that of sites showing only partial regression was 144 cm^2.

6.10. Recurrence

Ten of 137 (7%) treated sites showed evidence of recurrent tumor. The median minimum tumor dose for sites recurring was 975 rets, which was not significantly different from that of 988 rets for nonrecurring sites.

The median period between the commencement of treatment and recurrence was 6.5 months and ranged from 5 to 12 months (Figure 3).

The median area of the fields in which the tumors recurred was 155 cm^2 (range 80–546 cm^2), which was larger, though not significantly so, than that of 106 cm^2 (range 20–903 cm^2) for fields in which recurrence did not occur.

Six recurrences arose in sites that had only undergone partial regression, which was 11% of this group. Four further recurrences arose in sites where tumor had undergone complete regression (5%). None of the four static tumors regrew in the observation period.

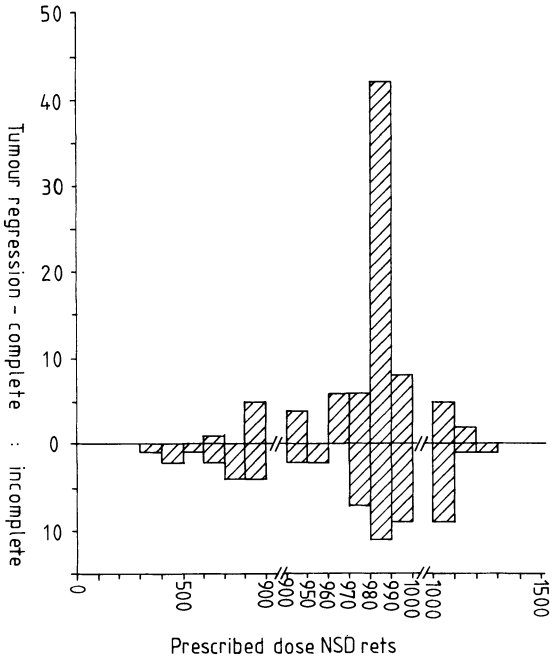

Figure 4. Complete or incomplete regression related to the prescribed (modal) tumor dose expressed as nominal standard dose (NSD).

Table 1. Regression related to dose and field size

Regression:	Complete	Incomplete
Number	80 (58%)	57 (42%)
Median dose	988 rets	988 rets
Dose range	676–1,188 rets	374–1,201 rets
Median ± 10%	89%	70%
Median ± 1%	66%	39%
Field size	138 cm^2	144 cm^2

6.11. Complications

In assessing the complication rate, those sites in which recurrence occurred have been excluded from the analysis. Recurrent tumor can be mistaken for fibrosis and can also lead to tissue necrosis and ulceration.

A total of 21 sites (15%) developed either severe fibrosis (8 sites), or necrosis that would not heal with conservative measures (11 sites). Both fibrosis and necrosis occurred in two sites.

The median period between treatment and severe fibrosis was 14 months (range 7–96 months) and for necrosis, 15 months (range 6–50 months). The median period of observation of sites that did not develop complications was

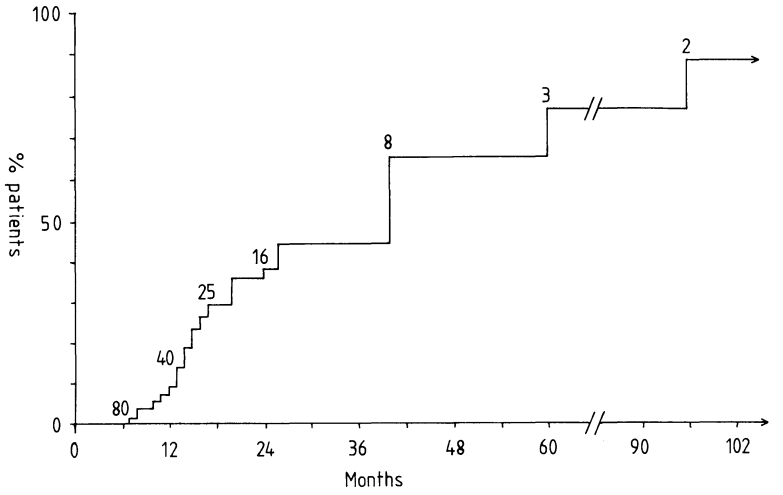

Figure 5. Actuarial complication rate (Kaplan-Meier). Numbers indicate sites that have undergone complete regression and remain at risk.

only six months. Consequently, the actuarial complication rate is considerably higher than the overall complication rate (Figure 5).

The median maximum tissue dose received by sites in which severe fibrosis developed was 1,100 rets (range 960–1,200 rets), whereas for sites in which necrosis occurred it was 989 rets. The median maximum tissue dose of the uncomplicated sites was 988 rets, so there was no difference between the dose received by uncomplicated sites and those that developed necrosis. However, the difference between the doses of uncomplicated sites and those developing severe fibrosis approached significance with $0.1 > p > 0.05$.

The median field area for uncomplicated treatments was 106 cm^2 (20–900 cm^2). For sites developing fibrosis, the median area was 210 cm^2, which is significantly greater at $0.1 > p > 0.05$. Necrosis occurred in sites where the median field area was 357 cm^2. This is highly significant in being greater than the uncomplicated field area with $p < 0.01$.

Sites that developed fibrosis showed no particular anatomical distribution. However, sites developing necrosis were in areas of diminished blood supply. Nine of these 13 sites were in the lower limb, and only one site not in the lower limb had not had previous treatment with either surgery or x-ray treatment, both of which adversely affect the vasculature. Trauma within the treated area commonly preceded the development of necrosis.

7. Discussion

7.1. Effect of survival on the assessment of response

In this group of patients with advanced and recurrent malignant melanoma the short survival after treatment, due to the high incidence of widespread

metastatic disease, had a considerable influence on response rate, local tumor control, and recurrence. Metastases beyond the treated area were recorded in 77% of patients and were the most common cause of death resulting in a median survival of only 12 months. This high incidence of metastatic disease is no different from that expected after local treatment of similarly advanced disease by surgery.

The median observation period of treated sites was six months and provided little time for the assessment of response, complications, or the regrowth of tumor. It is noticeable that there was a significant difference between the median observation period of those sites that underwent complete regression (11 months) and those that did not (3 months). Longer follow-up may have recorded a higher incidence of complete regression. Similarly, as the median period to the development of recurrence was 6.5 months, longer follow-up may also have revealed more recurrences.

The development of severe fibrosis and of necrosis occurred at median times of 14 and 15 months, respectively. Only 40% of treated sites were at risk at this time. Therefore, the true risk of developing a complication in a disease-free site with long survival is considerably higher than the overall complication rate (Figure 5).

7.2. Effect of tumor burden and field size

Over the 15-year period that malignant disease was treated in the Neutron Clinic at the MRC Cyclotron Unit, great attention was paid to measuring tumor masses before, during, and after treatment. Unfortunately, in the case of melanoma, the tumors were frequently multiple and measurements were often of the whole mass of tumor nodules rather than of individual deposits. This was particularly so for lymph node metastases which were seldom solitary. In retrospectively analyzing the tumor measurements there is the added problem of accurate measurements usually having been obtainable in two dimensions only. If it were known that the measured mass was a solitary nodule, then a rough assumption could be made that the tumor was approximately spherical and the third dimension calculated as the mean of the two superficial dimensions. However, for a group of nodules, no such assumption of shape can be made, and it is impossible to calculate even an approximate tumor volume. This is in contrast to tumors of the oral cavity which were commonly solitary and could be measured in three dimensions [40]. It is difficult, therefore, to calculate the regression rate of tumors or to analyze the achievement of complete or partial regression or of recurrence in terms of measured tumor volume. However, field size bears a relationship to tumor volume and can be measured with a reasonable degree of accuracy retrospectively from treatment specification and plans.

Complete regression of tumor appears not to be related to field size, while recurrence is related to field size but not significantly so. The median size of field used for those tumors which recurred was 50% larger than that used for those

that did not. Assuming field size to relate to tumor size, then it appears that recurrence becomes more likely in large tumors in which the distribution of dose of neutrons was poorer than in small tumors.

It is not surprising that complications were related to field size as this is the case in conventional radiotherapy. This was particularly so for those fields developing necrosis which were three times the median size of the uncomplicated fields. It is likely that the larger tumors, requiring larger fields, caused more normal tissue damage than did smaller tumors. However, it was also the case that fibrosis in the treated area caused shrinkage of the skin to approximately 50% of the treatment field size. In the case of very large fields, the degree of shrinkage was less because surrounding, untreated skin could not be drawn the distances proportional to the area undergoing fibrosis. The skin at the center of large fields became more devascularized than that at the center of small fields. This fibrotic, devascularized tissue was less able to heal after trauma or infection than smaller areas. This same situation arose in sites where the blood supply of the skin was already deficient before treatment, as in the lower leg.

7.3. The effect of dose on response of tumor and normal tissues

In order to study the relationship between dose and response, all the treatment schedules were reduced to an NSD value [34]. There was no difference in the prescribed (modal tumor) dose received by tumors undergoing complete or partial regression. Equally, there was no significant difference in the minimum dose received by tumors that recurred and those that did not. The incidence of severe fibrosis within the treated area was clearly related to maximum normal tissue dose, but that of necrosis was not. This latter problem was usually precipitated by trauma.

8. Conclusion

The results of treatment of malignant melanoma in these patients indicate that fast neutrons are an effective form of treatment for localized malignant melanoma. Response rates are higher than for conventional x-ray treatment and are maintained for longer periods, usually the remainder of the patient's life.

The radiobiological basis for this improved response must be related to the inability of melanoma cells to repair sublethal damage produced by neutron irradiation. This is apparent in the lack of a shoulder on the melanoma cell survival curve following neutron irradiation *in vitro*, relative to the large shoulder on the cell survival curve following x-irradiation. It is unlikely that the difference in oxygen enhancement ratio (OER) between the two radiation modalities is an important advantage of neutrons in fractionated therapy.

While the tumor response rate is high, the actuarial complication rate is also high. It has been the deleterious effects of neutrons on normal tissues that has

been a particular criticism of this form of radiation treatment [41]. As there is almost no skin sparing with neutrons of this energy, late fibrosis and telangiectasia are marked. The changes in the subcutaneous tissues lead to restriction of movement by fibrosis in those sites overlying joints, and trauma to the skin in treated areas frequently leads to deep-seated infection and necrosis. It is the low energy of the neutron beam, producing a poor penetration and delivering the maximum dose to the skin, that has been responsible for this high complication rate [42]. However, there exists the opinion that the effect of neutrons on those tissues which determine the late reaction is greater than would be expected from the degree of acute reaction. The doses used clinically have been determined by the acute tolerance of tissues, especially skin, and the formulae used to adjust these doses for changes in fractionation are also based on the observations of acute skin reactions. It may be that the cause for the high complication rate is related as much to this disparity between acute and late reactions as it is to the poor technical qualities of the treatment beams. However, it is clear that the incidence of fibrosis and necrosis is related to field size, and these complications could be reduced by using new high-energy cyclotrons.

As in all areas of cancer medicine the wish is to see patients at an earlier stage of their disease. The high incidence of metastatic disease and short survival following treatment of localized disease is extremely disappointing and limits the analysis that can be made of neutron therapy in the treatment of malignant melanoma. In addition, it appears that the recurrence rate, although low, would be further reduced if the tumors that were treated were smaller. Both these observations have encouraged clinicians to propose that fast neutrons are used for the treatment of less advanced melanoma. None of the patients treated in this series had disease less than 3 mm thick, and so all fell into the poor prognosis group. With new high-energy cyclotrons becoming available, a trial treatment of biopsy and wide excision versus biopsy and fast neutron therapy could assess not only the achievement of local control but also the morbidity of the two methods of treatment.

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Subject Index