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The Role of the Vitronectin Receptor $\alpha_v\beta_3$, in Melanoma Invasion and Metastasis

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Doctor of Philosophy

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Preface

The following excerpt is reprinted from the 'Guidelines for Thesis Preparation' of the Faculty of Graduate Studies and Research of McGill University to inform the reader of Faculty regulations:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

*If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.*

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement

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In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.

In accordance with the above guidelines, I have decided to include the following published works as part of the body of the thesis:

Nip, J., Shibata, H., Loskutoff, D. J., Cheresh, D. A., and Brodt, P. Human melanoma cells derived from lymphatic metastases use integrin $\alpha_v\beta_3$ to adhere to lymph node vitronectin. J.Clin.Invest. *90:* 1406-13, 1992.

Nip, J., Rabbani, S. A., Shibata, H., and Brodt, P. Coordinated expression of the vitronectin receptor and the urokinase-type plasminogen activator receptor in metastatic melanoma cells. J.Clin.Invest. *95:*, 1995.

These two papers are reproduced from **The Journal of Clinical Investigation** by copyright permission of The American Society for Clinical Investigation.

I am responsible for all of the experimental work and analysis carried out in these two aforementioned papers with the exception of flow cytometric analyses. All of the work was performed in the laboratory of Dr. Pnina Brodt (Department of Surgery, Division of Surgical Research, McGill University) with some of the experiments being done in Dr. Shafaat A. Rabbani's laboratory (Calcium Research Laboratory, Royal Victoria Hospital, Montreal) for the latter paper.

As well, some of the work described in the papers on which I am a co-author has been included as follows:

Brodt, P., Fallavollita, L., Sawka, R. J., Shibata, P., **Nip, J.**, Kim, U., and Shibata, H. Analysis of tumor cell adhesion to frozen lymph node sections - a correlate of lymphatic metastasis in breast carcinoma models of human and rat origin. Breast Cancer Research and Treatment. *17:* 109-120, 1990.

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Tawil, N., Gowri, V., Djoneidi, M., **Nip, J.**, Carbonetto, S., and Brodt, P. Integrin $\alpha 3\beta 1$ promotes adhesion of metastatic breast carcinoma cells to

lymph node stromal cells and fibronectin. 1996. International Journal of Cancer. In press.

学海无涯
为勤是岸

九五 [illegible] 书

Learning is like being in the middle of a vast ocean, only through hard work can one reach the shore.

-A Chinese proverb

Calligraphy by Mr. Chung Tong Ng

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Original Contributions to the Body of Knowledge

- The development of novel animal models of human melanoma and breast carcinoma metastasis to the lymph nodes.

- Melanoma metastasis correlates with integrin $\alpha_v\beta_3$-mediated adhesion to lymph node vitronectin.

- Human breast carcinoma metastasis correlates with integrin $\alpha_3\beta_1$-mediated adhesion to lymph node fibronectin.

- α_v mRNA and cell surface $\alpha_v\beta_3$ levels in human melanoma cells correlates with urokinase receptor mRNA expression.

- This is the first study which demonstrates a transcriptional link between α_v and uPAR as unique α_v antisense phosphorothioate oligonucleotides inhibit the expression of α_v mRNA and protein and specifically reduces uPAR mRNA expression and ligation of the $\alpha_v\beta_3$ integrin with immobilized antibodies causes a specific increase in uPAR mRNA expression.

Abbreviations

Collagen	coll
Extracellular matrix	ECM
Fibrinogen	Fgn
Fibronectin	FN
Laminin	LN
MeWo LNI 6I	6I
Monoclonal antibody	MAb
Nanometers	n m
Osteopontin	OP
Thrombospondin	Tsp
Urokinase	uPA
Urokinase-type plasminogen activator receptor	uPAR
Vitronectin	VN
Vitronectin receptor	VNR
von Willebrand's Factor	vWF

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Abstract

The incidence of melanoma worldwide has been increasing steadily in recent years with an associated rise in mortality rates. If detected early, melanoma lesions can be surgically removed with wide excisional margins resulting in high cure rates. However, as the melanoma invades the deep layers of the skin at the primary site and subsequently metastasizes to the regional and distant nodes, the prognosis for the patient becomes progressively worse. As the melanocytic lesion progresses from a benign nevus to a highly malignant metastatic melanoma, it undergoes changes in protein expression which may be relevant to melanoma development. Among the proteins found to be upregulated during melanoma progression are the vitronectin adhesion receptor $\alpha_v\beta_3$ and the receptor for the proteolytic enzyme urokinase plasminogen activator (uPAR). The present work describes our results with two new animal models of human melanoma which were developed to study the cellular and molecular mechanisms involved in lymphatic metastasis of this disease. Each model consisted of two cell lines with different metastatic abilities. Using these models, a correlation was found between the adhesion of the melanoma cells to cryostat sections of human lymph nodes and their metastatic ability in nude mice. When compared to the parent cells, the lymph-node metastasizing melanoma cells were found to

express increased levels of the cell surface vitronectin receptor (VNR) $\alpha_v\beta_3$ which was utilized to mediate the increased adhesion to lymph node. In addition to the VNR, the metastatic cells also expressed increased levels of the uPAR. To study the relationship between expression of these two receptors, α_v expression was suppressed with α_v antisense phosphorothioate oligonucleotides. In the antisense-treated cells a 9-fold reduction in α_v mRNA was seen with a corresponding 2.8-fold decrease in adhesion to vitronectin. This led to a specific 2-fold reduction in uPAR mRNA levels. Conversely, when the vitronectin receptor was ligated by specific immobilized monoclonal antibodies, uPAR mRNA expression was specifically increased and this increase was reflected in a functional increase in invasiveness of the cells as measured with the Matrigel invasion assay. This increased invasion could subsequently be inhibited by a MAb directed to the uPAR as well as the plasmin-specific inhibitor ε-amino caproic acid. Together these results suggest that there is a regulatory link between the expression of the vitronectin receptor $\alpha_v\beta_3$ and the uPAR in metastatic melanoma cells. This may provide the cells with a mechanism for coordinating adhesion, matrix degradation, and motility thereby facilitating the development of metastasis.

In a parallel study, a model of human breast carcinoma was used to study the role of cell adhesion in breast carcinoma metastasis. A

correlation was observed between cell adhesion to frozen sections of human lymph nodes and metastasis *in vivo*. However, unlike the melanoma cells, the metastatic breast carcinoma cells were found to utilize receptors $\alpha_3\beta_1$ and $\alpha_5\beta_1$ to adhere to lymph node fibronectin suggesting that different adhesion receptors may be involved in lymph node metastasis of different malignancies.

Sommaire

La fréquence du mélanome a augmenté fermement mondiallement dans les dernières années avec une montée associée avec la mortalité. Si le mélanome est détecté dès son début, lésions peuvent être remuer chirurgiquement avec de larges marges excisées et on obtient un haut taux de cure. Cependant, comme le mélanome envahit dans la profondeur de la peau au site primaire, et subséquemment se métastase aux noeuds régionaux et distants, le pronostic pour le malade devient progressivement bien pire. Comme la lésion du mélanocitique progresse d'un nevus bénin à mélanome du métastatique malin élevé, il subit des changements dans expression de la protéine qui peut être pertinent au développement du mélanome. Les protéines élevées trouvées dans la progression du mélanome sont le récepteur adhésif vitronectin, $\alpha_v\beta_3$, et le récepteur pour les enzymes proteolitique urokinase plasminogène activateur, uPAR. Dans cette thèse, je décris deux nouveaux modèles animal du mélanome humain pour étudier les mécanismes cellulaires et moléculaires impliqués dans les métastases lymphatiques de cette maladie. Chaque modèle consisté de deux lignes cellulaires dont la capacité métastatique est différente. Avec ce modèle on a demontré que l'adhésion des cellules du mélanome humain aux sections des noeuds cryostates de la lymphe humaine et la capacité métastatique des cellules mesurée avec les

souris nues était en correlation. En outre, les cellules du mélanome métastasisentes au noeud de la lymphe expriment des hauts niveaux du récepteur vitronectine à la surface de la cellule en comparaison avec les cellules parentales. En outre, ces cellules métastasisentes du mélanomes expriment un taux élevé du uPAR. En plus, la modulation de $\alpha_v\beta_3$ avec α_v antisense phosphorothioate oligonucleotides ou la ligature du $\alpha_v\beta_3$ avec des anticorps monoclonals spécifiques a eu pour résultat des modifications semblables dans l'expression du uPAR. Comme bien l'inhibition de l'expression de α_v mRNA et la protéine par l'antisense oligomers a eu pour résultat une réduction dans les niveaux du mRNA du uPAR. Par contre, quand le récepteur du vitronectine était modifié par une ligature spécifique des anticorps immobilisés, l'expression du mRNA de l'uPAR peut-être présentée comme une augmentation utilitaire dans dans l'envalissement des cellules à travers la Matrigel.

Ensembles ces résultats suggèrent qu'il se trouve un lien entre l'expression du récepteur vitronectine et l'uPAR dans les cellules métastatiques du mélanome. En plus ces observations fournissent un mécanisme en quoi les cellules organisent l'adhésion, la déchéance de la matrice, et la motilité qui ensemble facilitent le développement du mélanome métastatique.

J'ai aussi utilisé un modèle semblable pour étudier la tendance métastatique du carcinome mammaire humain et il se trouve une

corrélation entre l'adhésion aux sections cryostats des noeuds de la lymphes humaines et la métastase en vivo. Comme bien, les cellules du carcinome métastatique utilisent le récepteur de l'adhésion $\alpha_3\beta_1$ de la fibronectine pour adhérer aux noeuds de la lymphe. Ceci suggère qu'un autre récepteur est impliqué dans le système du cancer mammaire.

General Overview and Objectives of this study

The purpose of this study was to gain a better understanding of the cellular and molecular mechanisms involved in lymphatic metastasis of human malignancies. This information can ultimately lead to the development of more effective treatment modalities based on a better understanding of the biology of the metastatic process. The specific objectives of this study were as follows:

1. to develop experimental models of for the study of human melanoma and breast carcinoma metastasis to the lymph nodes;
2. to identify the adhesion receptors involved in lymphatic metastasis of these tumors; and
3. using the melanoma model, to elucidate the consequences of tumor cell adhesion to lymph node ECM in the process of metastasis.

In the first four chapters of this thesis, I present introductory material relevant to this study. In the first chapter, I discuss clinical aspects of melanoma namely current theories on the etiology, development and progression of the disease, and the current trends in melanoma treatment. The significance of lymph node metastasis is also discussed. Concepts of metastasis in general and lymphatic metastasis in particular are discussed

in chapter two and the importance of cell adhesion and proteolysis are highlighted. A brief description of the anatomy of normal lymph nodes is included. In the third chapter, the adhesion receptors and their role in invasion and metastasis are discussed. This chapter was divided into three sections dealing with families of adhesion molecules. The structure, expression, and role of these receptors in normal physiological processes are discussed. This is followed by a description of their role in tumor invasion and metastasis in general, and in melanoma metastasis in particularly. In the latter part of this chapter, the discussion focuses on the melanoma progression marker $\alpha_v\beta_3$ and its role in melanoma metastasis. In chapter four, the families of proteolytic enzymes known to be involved in invasion are discussed with emphasis on the urokinase plasminogen activation system. In the fifth chapter, the results of my experimental work on the human melanoma system are presented in the form of two published papers. I provide an overview of our findings in the beginning of the chapter. The next chapter contains the results of published and unpublished work on the role of cell-ECM adhesion in human breast carcinoma metastasis. The last chapter presents a summary of our findings and a discussion of their importance to melanoma metastasis.

Chapter I

Cutaneous Malignant Melanoma- a Clinical Perspective

1.1 Introduction

The worldwide incidence of cutaneous melanoma and related mortality have been rising steadily in the past few years (1) the latter increasing at approximately 5% annually (2). Early detection of melanoma is important as the lesion can be easily removed by a wide surgical excision resulting in a high cure rate (3). The poor prognosis of melanoma patients is directly related to the depth of invasion of the primary tumor and the involvement of regional and distant lymph nodes at the time of diagnosis (4). However, once melanoma metastasizes, the methods of treatment available today do not reliably affect the course of the disease.

1.2 History

Melanoma, like other cancers, has afflicted man since prehistoric times. One of the earliest examples of melanoma was found in a group of 2400 year-old pre-Colombian Incan mummies (5). Rounded melanotic masses were evident on the skin of these mummies, which also presented with diffuse metastases to the bones of the skull and extremities (5). The first reliable description of the disease was given by Hippocrates around 500 B.C. followed much later by accounts from physicians in the 1600 and

1700s. The French physician Laennec, inventor of the stethescope, was the first to describe melanoma as a disease and to use the term 'melanosis' (6). In England, Norris (6) was the first physician to extensively document and study melanoma. He described the epidemiology as well as clinical management of the disease. The designation of the the word "melanoma" for these pigmented tumors was given by Carswell in 1838 (7). Handley later described the early concepts of lymphatic metastasis of melanoma in his study of "lymphatic permeation by melanoma" (8). His ideas were later used by Pringle in 1908 (9), to advocate radical *en bloc* excision of the primary lesions, regional lymph nodes, and surrounding tissue for the surgical treatment of melanoma.

1.3 Epidemiology

In most populations, melanoma is a rare disease, representing less than 1% of all malignancies (10). The disease primarily afflicts Caucasians (11), although it is also found in Asians and blacks. While the incidence of this disease has increased in the general population in the past 20-30 years, the increase is most alarming in Caucasian populations of North America. The increase in incidence for the white population of North America is between 30-50% every 5 years (10) and the age adjusted annual incidence in

the U.S. is now approximately 13 per 100,000 and as high as about 22.7 per 100,000 in some states. The highest incidence of melanoma has been recorded in Australia with 17 per 100,000. Although the incidence has been increasing rapidly, no similar rise in related mortality has been seen. The five-year survival rate has in fact increased from 49% in 1950 to the present rate of approximately 81% (12). This increase in survival is probably due to improved early diagnosis and the efficacy of new treatments for early stage melanoma.

1.4 Etiology

The factors leading to the onset of melanoma are not known but numerous studies have identified several risk factors for the disease. One major environmental factor is exposure to sunlight (13-15), especially ultraviolet B radiation associated with solar rays (1). This type of solar radiation (λ 280-320nm) may penetrate the skin to reach the melanocytes found at the epidermal-dermal junction. Although ultraviolet A (λ 320-400nm) and C (λ 200-280nm) rays can also penetrate the dermis and epidermis respectively to varying degrees, they are usually not associated with the development of melanoma (16). Increased risk has been associated with intermittent acute exposure to sunlight and exposure in

childhood and adolescence causing severe sunburn (11). Despite its importance as a risk factor for melanoma development, exposure to sunlight does not explain the etiology of all melanomas. In one type of melanoma, acral lentiginous melanoma (17), the affected areas of skin, usually the palms and soles, receive comparably little exposure. As well, melanomas usually don't occur in areas most exposed to the sun's radiation such as the face (1).

Genetic factors may also contribute to disease development. The possession of a fair complexion, blond or red hair, increased number of nevi or freckles, or a family history of melanoma are some known genetic factors (18). Individuals having one or more of these factors may have a 2-148 fold increased risk for melanoma development (18). In addition, non-random chromosomal abnormalities have been identified in melanoma cells. They have been localized to chromosomes 1p (19), 6p (20), 6q (21), 7p, 7q (22), 9p (23), and 10q (24). Recent studies have focused on a region on chromosome 9p21 (23, 25) which was shown to be frequently rearranged or deleted in metastatic melanomas suggesting that this locus may contain a melanoma susceptibility gene. In fact, this region contains the MTS1 (Multiple Tumor Suppressor 1) gene, which encodes for a tumor suppressor protein, p16, which inhibits one of the kinases involved in cell division - Cdk-4 (26). Alterations in tumor suppressor

genes including the Rb, NF1, p53, and nm23 (27) genes may also contribute to the development and progression of melanoma (25).

1.5 Clinical Presentation

The superficial presentation of benign or malignant pigmented lesions may be deceptively similar. Visual examination employing the so-called "ABCD" rule has helped clinicians determine which lesions may be potentially malignant (1). Simply, the rule is a mnemonic for changes in the benign lesions which may indicate malignancy. The malignant pigmented lesion will show Asymmetry, Border irregularities, Color variations, and Diameters exceeding 6mm. In females, melanoma usually occurs on the lower extremities and in males it is commonly found on the trunk (28). However, any skin surface can be affected. Once a lesion is suspected of being malignant, a full-thickness biopsy (1) is performed. Histological examination of the tissue will determine the type of treatment to be followed.

Based on growth patterns and histopathological findings, there are several types of primary melanomas: superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, acral lentiginous melanoma, and mucosal lentiginous melanoma (1, 3, 17, 29). The first three types make up the majority (80-85%) of melanomas diagnosed (3), with superficial spreading melanoma being the most common at 70% of all melanomas (29). In most melanoma types, the disease progresses

through the radial and then the vertical growth phases however, nodular melanoma which is more aggressive characteristically lacks a distinct radial growth phase.

1.6 Precursor lesions of melanoma and progression to metastatic disease

The development and progression of melanoma from the neural crest-derived pigmented cells-the melanocytes, to metastatic melanoma follows discrete steps which can be clinically and histologically defined (30-32). Melanocytes may become transformed into nevus cells (30). Although these cells produce melanin, they, unlike melanocytes, do not transfer the pigment to the surrounding keratinocytes (16). A collection of nevus cells forms the common acquired and congenital nevus. These lesions are usually benign but may change to the classic precursor lesion, the dysplastic nevus (1, 33). Histological evidence of atypical architecture and cytology distinguish this lesion from the congenital nevus (34). The dysplastic nevus, may progress to the first malignant stage of melanoma namely, the radial growth phase. Growth of the malignant cells at this stage occurs in all directions but the overall effect is radial (horizontal) enlargement of the lesion. Growth at this stage is not normally associated

with metastatic potential (11). The next stage in the progression is the development of a vertical growth phase primary melanoma which is characterized by net growth in the direction perpendicular to the radial growth phase, that is, invasive growth through the layers of the skin, as well as the competency for metastasis (11). The last and most "deadly" stage is metastatic melanoma. The first sites of metastasis are usually the regional and distant lymph nodes followed by hematogenous dissemination. The preferred distant sites of metastases are the brain, liver, and lungs, however any organ may be the target in this highly malignant disease (35). As the melanoma progresses, the tumor cells acquire a number of properties relevant to the metastatic potential. These may include deletions in tumor suppressor genes, such as p53 and nm23 (25, 27), the acquisition of growth factor independence, through production of autocrine factors or the loss of susceptibility to negative growth regulation (36, 37), and the acquisition of the invasive phenotype which may be reflected in upregulation of molecules involved in adhesion, proteolysis, and/or migration - events crucial for invasion (38). The upregulated expression of cell surface molecules such as the integrin vitronectin receptor $\alpha_v\beta_3$ and the receptor for the urokinase plasminogen activator (uPAR) (39-41) is also associated with melanoma progression. A variety of adhesion receptors of the integrin and Ig superfamilies (42, 43)

and the melanoma-associated ganglioside GD_2 (44) have also been identified as progression markers for melanoma.

1.7 Microstaging and clinical staging

The depth and extent of invasion of the primary lesion are prognostic criteria essential for assignment of proper treatment. **(Fig. 1)** One method of microstaging developed by Clark (29) has shown that the level of invasion of the primary melanoma into the different layers of the skin is a valuable predictor of regional lymph node metastases as well as patient survival (45). A simpler and more accurate microstaging technique (46), which gave similar predictions of survival and prognosis, was Breslow's tumor thickness method (47), which involves measurement of the vertical thickness of the primary lesion.

Once microstaging of the melanoma is performed, clinical staging of the disease can follow. This may take into account the microstage of the primary tumor, the presence of regional lymph node metastases and the presence of distant metastases. Two accepted systems for clinical staging have been used. The original three-stage system (48) categorizes the melanoma into Stage I - localized melanoma, Stage II - regional

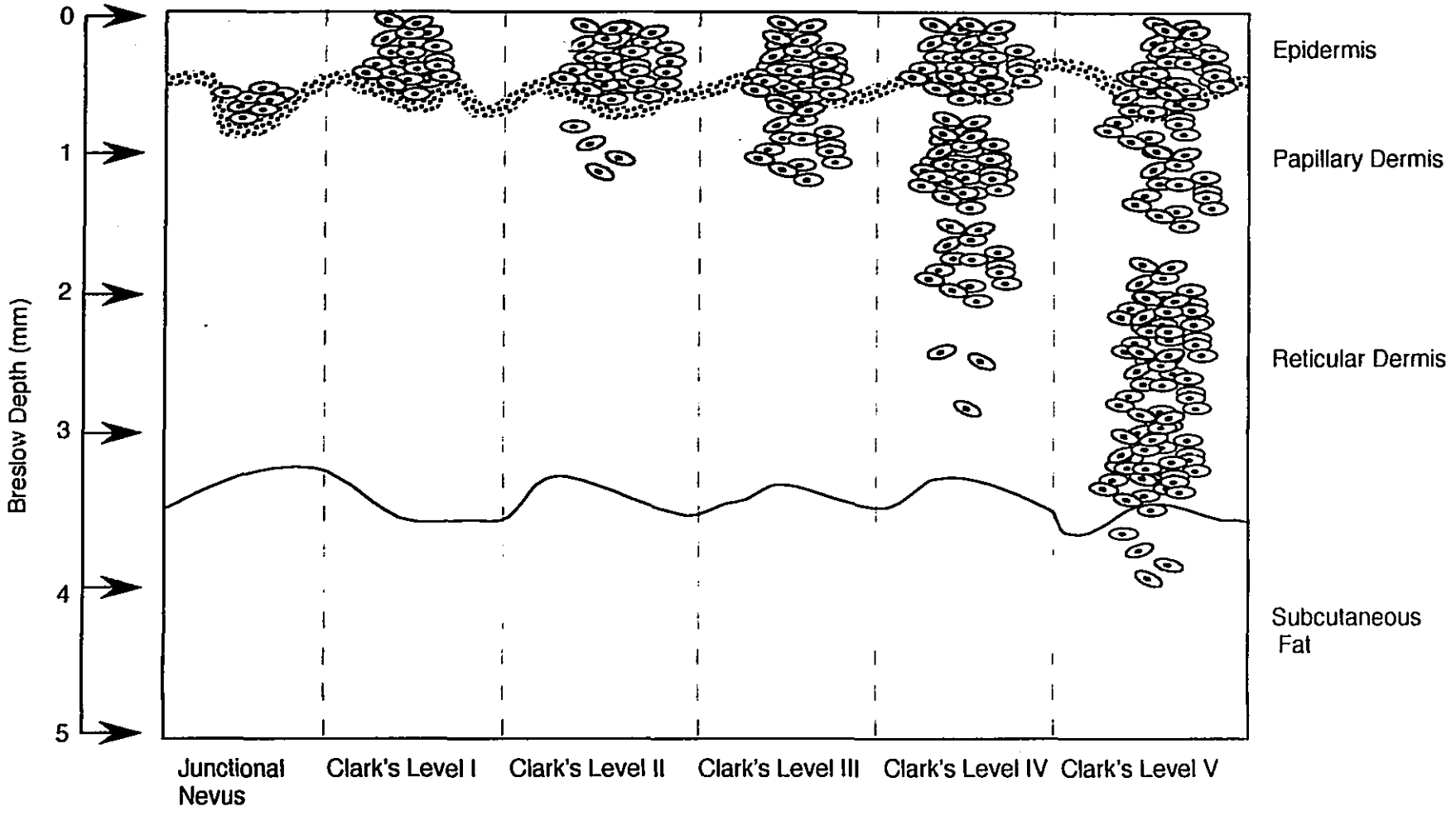

Fig. 1 Microstaging of melanoma*

A schematic diagram of the skin showing the various layers and the extent of melanoma invasion. The classical Clark's level of microstaging is represented along the bottom axis and the newer Breslow's method is shown along the left border. The oblong objects represent nevus or melanoma cells at various stages of melanoma progression.

*Modified from Yohn *et al*

metastases, and Stage III - distant metastases. A newer and more reliable method of staging, which also incorporates microstaging data, is the four-stage system proposed by the American Joint Committee on Cancer (AJCC) (49). **(Table I)**

1.8 Treatment

Resection of the melanoma with wide local excision (50) is the preferred treatment for Stage I disease. Treatment of patients who have regional and/or distant lymph node metastases (Stage II and III) involves the excision of the primary tumor as well as therapeutic lymph node dissection (1). To prevent distant metastases after surgical resection, stage II and III patients require adjuvant therapies, which may be in the form of chemotherapy, immunotherapy, hyperthermic isolation limb perfusion therapy, radiotherapy or a combination of the above (11). Although many of these treatment modalities have produced high response rates in the patients, none have significantly increased patient survival (3). In the management of Stage IV metastatic melanoma, the treatment options are limited and the disease is considered incurable. Autopsies revealed that melanomas may metastasize to all organ systems and visceral sites of the body (51). As the progression of the disease may be very rapid, with a

I. Traditional three-stage system

Stage	Criteria
I (thickness in mm)	Skin
≤ 0.75	
0.76 - 1.49	
1.50 - 2.49	
2.50 - 3.99	
2.50 - 3.99	
≥ 4.00	
II	Nodal metastases
III	Distant metastases

II. American Joint Committee on Cancer Staging System

TNM	Classification
pT1	≤ 0.75 mm
pT2	> 0.75 mm to 1.5 mm
pT3	> 1.5 mm to 4 mm
pT4	> 4.0 mm/satellites
N1	Regional ≤ 3 cm
N2	Regional > 3 cm and/or in-transit metastasis

Clinical Stage	Grouping		
Stage I	pT1	N0	M0
	pT2	N0	M0
Stage II	pT3	N0	M0
Stage III	pT4	N0	M0
	Any pT	N1, N2	M0
Stage IV	Any pT	Any N	M1

pT =primary tumor; N = node; M = metastasis

Modified from Koh et al and Sherman et al

Table I. Methods of Clinical Staging of Melanoma

median survival time of 4-6 months, treatment plans are largely palliative and may take the form of the therapies mentioned above (3). Apart from surgical resection of metastases, immunotherapy may prolong patient survival. The underlying mechanism of the various immunotherapeutic treatments is to invoke a host immune response which will help to fight the disease. These novel therapies include melanoma cell membrane vaccines (52), monoclonal antibodies directed to specific melanoma cell surface antigens, such as GD_2 and GD_3 (53), biological response modifiers such as interferons α and γ (54), adoptive immunotherapy (55), which involves administration of patient-derived lymphokine (IL-2)-activated killer cells (LAK), and tumor-infiltrating lymphocytes (TIL) (56). Chemotherapeutic alkylating agents used for palliation are dacarbazine, nitrosourea, and cisplatin with the first two compounds producing better response rates in the patient (4). Dacarbazine is the only single-agent chemotherapeutic drug that causes reproducible partial remission of metastatic melanoma in up to 20% of patients. The yew tree alkaloid, taxol, which binds to tubulin and promotes the formation of microtubules, causes mitotic arrest of the tumor cell by preventing microtubule depolymerization. This new drug has been shown to be as effective as dacarbazine with respect to patient response rates (57). Palliative radiotherapy (3) has also been somewhat effective in melanoma patients

with brain and bone metastases. Although some of these treatment modalities may increase the response of the melanoma patient with distant metastases, the improvement is not long-lasting and usually does not improve overall patient survival.

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Chapter II

Metastasis-an overview

2.1 Introduction

Since the development of regional lymph node metastases followed by distant nodal and systemic metastases is the major cause of death among melanoma patients, a better understanding of the basic mechanisms underlying the metastatic process is necessary in order to develop better prognostic and therapeutic protocols. Most of the experimental research in metastasis has focused on hematogenous or blood-borne dissemination of tumors with the bulk of information pertaining to it. However, in the case of melanoma the major determinant of poor prognosis in patients is the involvement of regional and distant lymph nodes (1). Metastasis to these sites has been shown to dictate patient survival. As this process is crucial to our understanding of malignant melanoma, this chapter will focus on the basic and current concepts of lymphatic metastasis.

2.2 General concepts on the process of metastasis

2.2.1 Tumor Heterogeneity

Originally it was believed that tumors were homogeneous in nature and that tumor cells did not vary from one generation to another. This

misconception may have been due to the type of tumor models used in many studies. For example, the use of acute lymphoblastic leukemia models to study cancer may have been misleading as these types of tumors consist of cells which may indeed be quite homogeneous in biological and physical properties (2). Similar to other solid tumors, primary melanomas are known to consist of subpopulations of cancer cells which are heterogeneous with respect to metastatic ability (3). Early experiments by Fidler and Kripke (3) using a murine melanoma model of experimental metastasis showed that different clones isolated from the parental primary melanoma exhibited highly divergent metastatic abilities. The phenomenon of tumor cell heterogeneity has also been shown with human colon and renal carcinoma cell lines using nude mice models (4). In terms of clinical management of metastases, tumor heterogeneity can present many problems. For example, heterogeneous tumor cell populations may respond differently to chemotherapy, radiotherapy, and thermotherapy (2). In fact, in one classical study, a breast adenocarcinoma cell line and three subpopulations derived from it were injected subcutaneously into syngeneic mice and subsequently treated with chemotherapeutic agents. The mice were monitored for primary tumor growth and metastases formation and it was found that the various subpopulations responded quite differently to the drugs (5) with responses

ranging from tumor regression to increased metastasis. Similarly, these cells had divergent drug sensitivities when tested *in vitro* suggesting that tumor cell heterogeneity may make development of effective therapies difficult. Likewise, in a human colon adenocarcinoma model, tumor cell heterogeneity resulted in variability in responses of the various subpopulations to x-rays (6, 7) and hyperthermia (8) *in vivo*. Other studies have demonstrated that although some of the cells in a heterogeneous primary tumor can give rise to metastases, the majority of cells (>99.99%) are destroyed by mechanisms such as shear forces and the host immune surveillance system (NK cells and macrophages) suggesting that metastasis is a highly selective process (9).

2.2.2 Organ specificity of metastasis

Experimental evidence and clinical obseravtions suggest that tumor cells metastasize in an organ selective manner (10). Our present understanding of organ specificity of metastasis is based on the classical theories of Paget (11) and Ewing (12). According to Paget's "seed and soil" hypothesis, site-specific metastasis was believed to be a consequence of a tumor cell, the 'seed', coming into contact with a favourable tissue microenvironment, 'the soil' leading to growth and metastases formation.

In contrast, Ewing's mechanical theory postulated that tumor cells colonize a particular organ due to the anatomical location of the primary tumor and to local hemodynamic factors, namely, the metastatic cells are carried from the primary site by the blood or lymph and become trapped in the first organ encountered, resulting in a metastasis. The former theory may explain thyroid metastasis of clear cell carcinoma of the kidney (13) whereas the latter may be the dominant factor in liver metastasis of colorectal carcinoma (14). However it appears that a combination of both mechanisms is likely responsible for the propensity of certain cancers to metastasize to specific secondary organs. Numerous organ-specific factors have been identified in support of Paget's theory. They include specific growth factors, hormones, adhesion receptors or the protein composition of the extracellular matrix (ECM) (15-17) which may all contribute to the establishment of a supportive microenvironment for tumor cells in specific organs.

2.2.3 Steps of metastasis and the importance of host-tumor cell interactions

Metastasis is a relatively inefficient process (18, 19). The tumor cells which escape from the primary tumor must be capable of completing a

Figure 1

The steps of cancer metastasis. A schematic representation of the key steps involved in tumor cell metastasis. Tumor cells may detach and escape from the primary tumor site and begin to invade the local stroma (I). Next the cells adhere to and migrate through the subendothelial matrix as well as the endothelial cells of the blood or lymph vessels (II) and are carried by the blood or lymph (III) to the site of tumor cell arrest. At this point, the cancer cells must extravasate by adhesion to the endothelium and diapedesis between the endothelial cells (IV). Finally, the tumor cells will invade local parenchymal tissue and form secondary deposits (V). The shaded cells represent cancer cells.

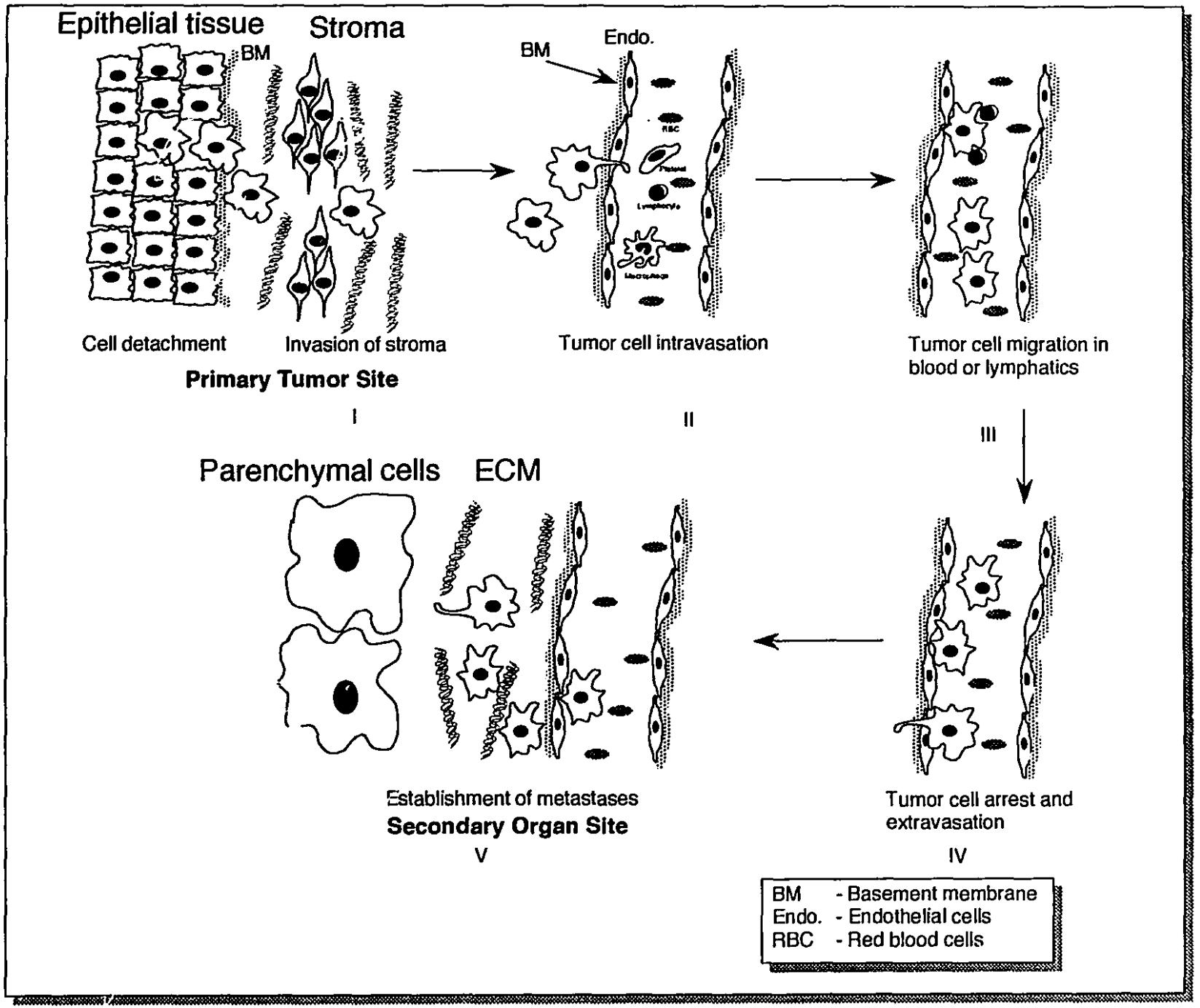

Fig. 1 The Steps of Cancer Metastasis

series of sequential, non-random steps in order to establish distant metastases (9, 20). Whether it is blood- or lymph-borne, some of the underlying cellular and molecular mechanisms are similar. In hematogenous dissemination, the melanoma cell must detach from the primary tumor, invade the local stroma, intravasate and and survive in the blood vessels, arrest and extravasate in secondary organ site, and finally proliferate to form a metastasis (Fig. 1). Each of these steps requires various host cell/tissue and tumor cell interactions such as cell adhesion and proteolysis. In addition, tumor cells which metastasize to regional nodes (see below) may leave the lymph node through efferent lymphatic vessels and may be carried via the thoracic duct to the left brachiocephalic vein, thus allowing entry into the systemic circulation. Anastomoses between the efferent lymph node vessels and the venous circulation, the lymphatico-venous channels, may also contribute to entry into the vasculature, thereby allowing formation of hematogenous metastasis.

One of the major determinants of metastases formation is the ability of tumor cells to adhere to specific host cells and their ECM including (15, 17) the microvascular endothelial, stromal and parenchymal cells. These adhesion events are required for tumor cell intravasation, extravasation, and target organ colonization. As well, in the vasculature or lymphatics, adhesion may protect the tumor cell from host immune cell lysis (NK

cells) (21), and allow transport of tumor cells in lymph and blood by formation of homotypic (22) or heterotypic (adhesion with lymphocytes or platelets) emboli (23, 24). Subsequent to adhesion, a host of cellular functions important for metastatic competency may be elicited. These include cell migration and the release of degradative enzymes, such as metalloproteinases, serine proteases, glycosidases, and cathepsins which are released by the tumor cells or by host cells and lead to the dissolution of the ECM (25, 26). Migration or locomotion of the tumor cells may be enhanced by autocrine motility factors and host chemotactic factors (27). Since adhesion, as well as proteolysis, are central to the metastatic cascade, a more detailed account of these processes will be given in subsequent sections.

2.3 Lymphatic metastasis

After the melanoma cells detach from the primary tumor and invade the local stroma, they may disseminate lymphatically. According to the studies of Carr (28), lymphatic metastasis can be divided into a series of steps starting with entry and transport of tumor cells in the lymphatic vessels followed by settling and growth in the regional lymph nodes, and finally metastasis to other nodes. Before a closer examination of the steps

involved in lymphatic metastasis can be undertaken, it is necessary to describe the anatomy and ultrastructure of the normal human lymph node.

2.3.1 The Anatomy of the lymph node

Forming the major part of the lymphatic system, lymph nodes are bean-shaped lymphoid organs ranging in size from 2 to 20 mm along their longest diameter (29). The nodes, which are usually found in clusters or chains, can be divided into two groups depending on their location in the tissues, the superficial lymph nodes - located in the subcutaneous connective tissue and the deep lymph nodes - usually found beneath the muscle layers. Under normal physiological conditions, the lymph nodes are involved in antigen processing, lymphopoiesis, and lymph filtration (30).

The framework of the node consists of the capsule, composed of layers of fibroblasts and their deposited collagen along with smooth muscle and nerve cells, the trabeculae, an internal continuation of the fibrous capsule whose fiber bundles are directed towards the center of the node, and the reticular cells and their deposited reticulin fibers, which form a network that supports the lymphoid cells of the entire node (29, 31). Forming the bulk of the ECM of the node, the reticulin fibers are composed of a central

core of bundles of type III collagen fibrils surrounded by a discontinous mesh of ECM proteins such as laminin, type IV collagen, heparin sulphate proteoglycans, entactin (32), and possibly, fibronectin and vitronectin (33, 34).

Apart from the supporting structures of the node, there are also important functional entities. One such entity is the lymphatic sinus which can de divided into the marginal (subcapsular), cortical, and medullary sinuses (31). These sinuses, which are interconnected, allow the passage of lymph through the node. The sinuses are lined with reticuloendothelial cells and macrophages with their lumens containing a variety of different lymphocytes (35) and criss-crossing reticular fibers.

The cortical, paracortical, and medullary areas are the three main areas of the lymph node parenchyma based on morphology and function (36). Containing the lymphocytic follicles and their associated germinal centers, the cortical area is the main area for B-cell humoral immunity in the node. The germinal centers contain lymphoid cells (large lymphocytes and blast cells) showing different degrees of maturation, as well as reticular cells and macrophages. Antigenic stimulation of the node causes these germinal centers to enlarge reflecting an increase in B cell mitosis. As it is the main site for the cellular immune response (37), the paracortical area of the lymph node is made up largely of T-cells with some macrophages

and interdigitating reticular cells. This area also contains the postcapillary venules, called high endothelial venules, which are involved in targeting of circulating lymphocytes to the lymph nodes (38). The medullary area is structurally arranged into cords which contain mature plasma cells and other lymphocytes. This region is enlarged by plasma cells during an immune response.

After the melanoma cells invade the lymphatic vessels, they are carried by the lymph to the lymph nodes where they enter via the afferent lymphatic vessels. The cells may then be retained in the marginal or subcapsular sinus where they may adhere to sinusoidal cells and possibly lymph node ECM found on the reticular fibers. Migration of the tumor cells to other parts of the node via the interconnected lymphatic sinuses may lead to invasion and destruction of the nodal parenchyma (28). In an attempt to destroy the tumor cells, a host immune response may be elicited in the nodes. This response involves both B- and T-cells responses residing in the cortical and paracortical areas of the node, respectively, as well as a macrophage-mediated response - sinus histiocytosis. Despite this host response, tumor cells generally proceed to destroy the node and may disseminate to distant nodes and organs (39).

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Chapter III
The Role of Cell Adhesion in Melanoma Metastasis

3.1 Introduction

Changes in adhesive events, in the form of increased or decreased cell adhesion to host cells or extracellular matrix are critical to the metastatic process. In order to escape from the primary tumor, tumor cells must lose their adhesion to adjacent cells, an interaction mediated by receptors such as the cadherins (1, 2). Conversely, tumor cell arrest in the target organ site and establishment of metastases appear to require increased adhesion, which may be mediated by adhesion molecules of the integrin (3-5), immunoglobulin (6) or selectin (7) families. Indeed, deletions in tumor suppressor genes encoding either an adhesion receptor (the NCAM-like molecule, DCC) or receptor-linked protein (APC, a catenin-binding molecule) have been implicated in the process of colorectal carcinoma progression. Since the progression of melanoma is associated with an increased expression of many types of adhesion receptors including receptors MUC18 (8) and ICAM-1 (9) of the Ig superfamily and the integrins (10) $\alpha_2\beta_1$ (11), $\alpha_3\beta_1$ (12), $\alpha_4\beta_1$ (13), and in particular $\alpha_v\beta_3$ (13-15), a more extensive review of these families of adhesion receptors is provided below.

3.2 Families of Adhesion molecules

To date, four major families of adhesion receptors have been identified based on sequence homologies and structural similarities. These include the cadherins, Ig supergene family, selectins, and integrins (16-18).

3.2.1 Cadherins

The cadherins mediate calcium-dependent intercellular adhesion. Each molecule consists of cytoplasmic, transmembrane, and extracellular domains (1, 2), the latter of which contains four homologous repeats (17). As homotypic adhesion molecules, cadherins mediate cell binding through interactions with identical cadherins found on the partner cells. They are present in adherens junctions (zonula adherens) where they are required for organization of the cytoskeleton (2) through interactions with the catenins (α, β, and γ), cytoplasmic proteins which control cadherin function (19).

The members in the cadherin family may, according to Takeichi (2), be subdivided into two main groups, those with an extracellular domain containing four cadherin repeats and those with more than four repeats. The classical cadherins are E-cadherin, first detected in epithelial tissues, P-cadherin, found in the placenta, and N-cadherin, present in neural tissues. In normal cells, these molecules are involved in maintaining epithelial tissue integrity (5). In tumor cells, their expression is frequently decreased and their levels were shown to be inversely correlated with the invasive capacity of the cells (20). As well, antibodies directed to E-cadherin were able to increase the invasiveness of a normally non-invasive canine kidney cell line (21), suggesting that loss of E-cadherin mediated adhesion

may contribute to invasion. With respect to metastasis, in some cancers such as human breast cancer (22), the incidence of regional and distant metastases was found to correlate with reduced E-cadherin expression. However, this may be tumor specific as expression of E-cadherin in human stomach cancers resulted in increased metastasis (23),.

Under physiological conditions, E- and P-cadherins are required to maintain skin tissue integrity (24). In melanomas, expression of these cadherins may be lost and this may contribute to metastasis. A recent report by Tang *et al* (24) demonstrated that melanocytes expressed both of these cadherins and could adhere to keratinocyte monolayers *in vitro* whereas several melanoma cell lines as well as transformed melanocytes tended to lack mainly E-cadherin and to a lesser degree P-cadherin and thus resulted in loss of adhesion to the keratinocytes. As keratinocytes are situated in close proximity to melanocytes (melanoma cells) *in vivo*, loss of tight adhesion by the latter cells as a result of loss of cadherin expression may lead to the development of the metastatic potential.

3.2.2 Immunoglobulin (Ig) Supergene Family

Members of this family include adhesion receptors as well as other molecules such as the receptors for the growth factors PDGF and CSF-1, and immune system proteins such as the T cell receptor and the MHC

antigens (25). The adhesion molecules of this family can mediate homotypic as well as heterotypic cell-cell adhesion. In the former group are molecules such as N-CAM, L-1 (10, 25), which are involved in regulation of neural tissue morphogenesis, CEA (carcinoembryonic antigen) (26), and PECAM-1 (27, 28), which has been implicated in leukocyte motility and migration across vascular vessels. The latter group includes proteins such as ICAM-1, ICAM-2, ICAM-3, VCAM-1 (INCAM-110), and LFA-3, which play a role in lymphocyte-endothelial cell interactions (29).

Several members of the Ig family have been implicated in melanoma progression and metastasis. The expression of the N-CAM-like cell surface glycoprotein, MUC-18, was shown to be increased in cryostat sections of metastatic melanoma lesions as compared to sections of benign nevi (30). The expression of MUC-18 was found to correlate with vertical thickness of the primary lesion as well as with poor prognosis and development of metastasis (31). Another Ig family member whose expression is upregulated in metastatic lesions is ICAM-1 (32). Along with its counter receptor, the leukocyte integrin LFA-1 ($\alpha_L\beta_2$), ICAM-1 (as well as ICAM-2 and ICAM-3) (33) normally plays a regulatory role in the host immune response by bringing together in close proximity antigen-presenting cells and T lymphocytes (34). Close contact between the ICAM-expressing antigen-presenting cells and LFA-1 on the T cells is needed for proper

antigen presentation and subsequent immune response. In fact, antibodies to ICAM-1 were shown to block lysis of melanoma cells by TIL (tumor-infiltrating lymphocytes), LAK (lymphokine-activated killer), and NK cells (35) suggesting that it was involved in melanoma cell recognition by the killer cells. In addition to elevated expression in metastatic melanoma lesions, ICAM-1 is also present in the serum of melanoma patients and is a marker for progression of the disease (36-38). Stage II and III melanoma patients with high levels of circulating ICAM-1 showed lower survival (37, 38).

The Ig family molecule VCAM-1 (also called INCAM-110), normally expressed on cytokine-activated vascular endothelium, binds to its counter receptors VLA-4 ($\alpha_4\beta_1$) (39) and possibly $\alpha_4\beta_7$ (40, 41). VCAM-1 and its counter receptor mediate lymphocyte-endothelial cell interaction required for leukocyte transmigration during inflammation. Recent studies have shown that the expression of VCAM-1 on tumor-infiltrating vascular endothelial cells may be decreased as compared to endothelial cells distant to the melanoma (42). It was postulated that down-regulation of VCAM-1 may protect highly vascularized melanoma lesions from destruction by host cytotoxic lymphocytes as the lymphocytes cannot extravasate into the lesion in the absence of VCAM-1 expression (42). The ligand for VCAM-1, VLA-4 ($\alpha_4\beta_1$), is also expressed on melanoma cells (14, 43). The interaction between endothelial VCAM-1 and melanoma VLA-4 may be important for

metastasis formation. A recent report by Garofalo *et al* (44) demonstrated that experimental lung metastases of VLA-4+ A375M melanoma cells could be enhanced by pre-injections of Il-1, which was shown to induce VCAM-1 expression on lung endothelial cells. Together these results suggest that tumor cell interaction with the endothelium can regulate the metastatic process.

3.2.3 Selectins

The selectins constitute another family of adhesion receptors (7, 16, 45). These cell surface glycoproteins mediate Ca^{2+}-dependent leukocyte-endothelial and leukocyte-platelet cell adhesion. The three main members of the family were identified originally on different cells such as endothelial, leukocyte, and platelets and were designated E-, L-, and P-selectin, respectively. Sharing structural similarities such as an N-terminal calcium-type lectin domain, an EGF-(epidermal growth factor) like domain, complement binding protein-related modules, and a transmembrane domain, the selectins play a role in leukocyte transmigration to inflammatory sites and in lymphocyte recirculation and homing (29) The lectin and EGF-like domains are required adhesion to the major selectin ligands, sialyl Lewis x, sialyl Lewis a, and related blood-group carbohydrate antigens (45), as antibodies directed to these

domains were found to inhibit selectin-mediated adhesion (46, 47). Through the identification of biological ligands for the selectins (48-50), it has been shown that the interaction of the selectins with the protein components of the ligands may also contribute to adhesion (50).

Selectins, especially E- and P-selectin, have also been implicated in metastasis of various tumors such as colorectal (51), breast (52), skin squamous cell (53), and pancreatic (54) carcinomas. Tumor cells which express the appropriate carbohydrate ligands for host endothelial cells selectins are believed to adhere to the endothelial cell during intravasation and extravasation of the tumor cell through vascular or lymphatic vessels (55). In fact, colorectal carcinoma cells expressing high levels of sialyl Lewis x antigens were shown to adhere *in vitro* to E-selectin expressed on activated endothelial cells and this adhesion correlated with the metastatic potential of the cells (56). In another report (53), investigators working on cutaneous basal and squamous cell carcinomas found that the ligands for the selectins were present in squamous but not in basal cell carcinomas as measured by immunohistochemical analysis suggesting that the absence of appropriate ligands on basal cell carcinoma may be responsible for their reduced metastatic potential.

In melanoma tissue, positive staining for E- and P-selectin on intratumoral endothelial vessels was found to be inversely correlated with disease-free interval and survival time (57). Another lectin-like molecule

involved in melanoma is the 90 kDa lung-specific endothelial cell adhesion molecule-1 (Lu-ECAM-1) (58). This molecule selectively binds highly lung metastatic murine B16-F10 melanoma cells as pre-injection of a MAb directed to Lu-ECAM-1 prior to B16-F10 cell injection resulted in a significant reduction in lung metastases whereas a similar reduction was not seen for another lung-metastasizing cell line (59). Further studies revealed that the staining pattern of lungs for Lu-ECAM-1 correlated with the distribution of B16-F10 lung metastases (60). These results suggest that metastasis is a selective process and that tumor-endothelial cell interactions play a crucial role in this process.

3.2.4 Integrins

Another important event in melanoma metastasis is adhesion of the tumor cells to the stromal, parenchymal, or subendothelial extracellular matrix at the primary and metastatic sites. This adhesion to the ECM leads to subsequent tumor cell invasion of the tissues and is mediated mainly by a family of adhesion molecules known as the integrins (3, 5, 61-64). The integrins are transmembrane glycoproteins which consist of non-covalently linked α and β subunits and serve as major mediators of cell-substratum and cell-cell adhesion. In addition to their involvement in tumor invasion and metastasis, integrins are also involved in various

physiological processes such as embryonic development and morphogenesis (65-68), inflammation (69), wound healing (70, 71), and blood coagulation (70, 72). To date, 15 α and 9 β subunits have been identified which combine to form at least 21 different integrin heterodimers (Table I). Each of these integrins can bind to one or more extracellular matrix proteins through specific peptide sequences (73, 74) such as the Arg-Gly-Asp peptide on fibronectin or they may mediate cell-cell adhesion as is the case for $\alpha_4\beta_1$ (39), and $\alpha_L\beta_2$, (29), which recognize their counter receptors VCAM-1 and the ICAM's, respectively. In addition to the RGD sequence found in several matrix proteins such as fibronectin and vitronectin, other peptide sequences have been identified as integrin-binding peptides. These include the peptides KGAGDV, EILDV, and DGEA expressed on fibrinogen, fibronectin, and collagen, respectively (3).

Originally, the integrin family was divided into three subfamilies based on their β subunit (61), the VLA (very late antigen) (β_1), the leucam (β_2), and the cytoadhesin (β_3) subfamilies. However, the discovery of new β subunits and the fact that α subunits can associate with β subunits from more than one family render this traditional classification system somewhat dated.

Generally, both subunits of the integrins consist of a substantial

extracellular domain, a single transmembrane domain, and a short cytoplasmic region. The exception to this general structure is the β_4 subunit which contains a very large cytoplasmic domain (75, 76) consisting of over 1000 amino acids which is approximately 20-25 times larger than other β subunits. The extracellular domain of the α subunit contains the divalent cation binding sites and that of the β subunit is characterized by a region containing highly conserved cysteines. The N-terminal domains of both of these subunits combine to form the ligand-binding region. The cytoplasmic domain of the β subunit is required for interaction with the cytoskeletal proteins (77, 78). Ultrastructural studies on the fibronectin receptor, revealed that the three-dimensional structure of an integrin consists of a globular head formed by the N-terminal regions of both the subunits (79) which measures about 80x120Å. This region also contains the ligand- and divalent cation-binding domains. Connected to this head are two tails of about 180-200Å in length.

Human cancer cells often display an integrin expression profile which is altered in comparison to normal cells. The tumor cells may express increased or decreased levels of specific integrin molecules or new integrins not generally expressed on the benign tissue. The patterns of expression cannot however be generalized and appears to be integrin and tumor-type and stage specific (4). In the case of breast (80) and colon carcinomas (81) for example, the expression of several integrin subunits

β_1, β_4 and α_2, α_6, β_1, β_4, respectively, was found to be decreased in comparison to the respective normal tissue. On the other hand, certain tumors such as the highly malignant cerebral glioblastoma (82) show increased expression of specific integrins such as the vitronectin receptor $\alpha_v\beta_3$ when compared to less maligant tumors and normal brain cortical and cerebral tissue. Melanoma tumors and cell lines also show changes in expression of integrins including upregulation of $\alpha_2\beta_1$ (83), $\alpha_3\beta_1$ (12), $\alpha_4\beta_1$ (13), and $\alpha_v\beta_3$ (14, 15). The upregulation of the $\alpha_v\beta_3$ appears to be of particular significance. This receptor was identified as a marker of melanoma progression and its expression is greatly increased in vertical growth phase primary and metastatic melanoma lesions (14).

In the following sections, a more detailed description of the $\alpha_v\beta_3$ integrin will be presented with an emphasis on its role in melanoma metastasis.

3.2.4.1 Structure and Biosynthesis of the $\alpha_v\beta_3$ integrin

Full length cDNA for α_v (84, 85) and β_3 (86, 87) chains have been cloned and the amino acid sequences determined. In addition, the promoter region of the α_v gene has been cloned and sequenced (88). The α_v subunit does not contain the 200 amino acid I ('inserted/interactive') domain (89)

characteristic of some α subunits but belongs to a subgroup of α subunits which is cleaved into heavy and light chains bridged by disulphide bonds. The α_v subunit shows sequence homology with other α chains especially α_5 with which it shares a 60% homology (90). The β_3 subunit is also homologous to the other β subunits with the overall homology among these subunits of about 40% (90).

The early work by Cheresh *et al* (91, 92) showed that the synthesis, processing. and assembly of the $\alpha_v\beta_3$ receptor as analyzed using human melanoma M21 cells occurs as follows: Initially, the α_v and β_3 chains are translated and transported to the endoplasmic reticulum, where they associate to form heterodimers. These heterodimers are functional with respect to adhesion to immobilized RGD peptide columns, an ability acquired about 7 minutes, post-synthesis. They also react with MAb LM609 which recognizes an epitope found on both the α and β chains (91). The next phase, at 60 minutes, post-synthesis, is rapid transport of the $\alpha_v\beta_3$ complex to the Golgi apparatus for further processing, which involves trimming of the high-mannose oligosaccharides and addition of sialic acid to the chains. The final step is transport of the receptor complex to the cell membrane.

3.2.4.2 Ligands of the $\alpha_v\beta_3$ integrin

α	β	Ligand
α1	β1	coll, LN
α2		coll, LN, echo virus
α3		coll, epiligrin, FN, LN
α4		FN, invasin, VCAM
α5		FN, denatured coll
α6		LN
α7		LN
α8		FN, VN, tenascin
α9		?
αv		VN
αL	β2	ICAMs
αM		iC3b, Factor X, Fgn, ICAMs
αX		Fgn, iC3b
αv	β3	VN, FN, Fgn, Tsp, vWF, OP, denatured coll, tenascin, disintegrins, HIV Tat
αIIb		VN, FN, Fgn, Tsp, vWF, coll, denatured coll, disintegrin
α6	β4	LN
αv	β5	VN, HIV Tat protein
αv	β6	FN, tenascin
α4	β7	FN, VCAM
αE	β7	E-cadherin
αv	β8	?

Table I. Integrins and their ligands

other integrins such as $\alpha_{IIb}\beta_3$, can be modified by the type of divalent cation present. For example, Mn^{2+} but not Ca^{2+} was able to promote adhesion of M21 melanoma cells to fibrinogen (99). In another study, the $\alpha_v\beta_3$ receptor isolated from human placenta was able to bind to the RGD peptide in fibronectin in the presence of Mn^{2+}, but not in the presence of Ca^{2+} or Mg^{2+} (100). This phenomenon of divalent cation regulation of the integrin-ligand specificity is not unique to the vitronectin receptor and has been demonstrated with the fibronectin receptor $\alpha_5\beta_1$ (101) and with the platelet integrin $\alpha_{IIb}\beta_3$ (99).

Vitronectin, the ligand first identified for $\alpha_v\beta_3$ (102), is a 75 kDa, liver-derived glycoprotein originally isolated from human serum using glass beads (103), and known also as serum spreading factor (104) or complement S-protein. It is present in the plasma at concentrations of 200-400μg/mL and can exist in a two-chain form (65 and 10kDa) (105). In addition to its soluble form found in plasma, vitronectin can also be ECM-associated. It was identified with the extracellular matrix of fibroblast cell lines (104) and found in tumors such as advanced glioblastoma biopsies (82) and late-stage melanoma (15). It is also found in the elastic fibers of the papillary and reticular dermis (106), in loose connective tissue, blood vessel walls, and the reticulin stroma of lymphatic tissue (107). The latter location is of particular relevance to our study (107-109) as the lymph

nodes are a major site for melanoma metastasis. Because infiltration of the lymph nodes by tumor cells was found to be accompanied by increased production of fibronectin and vitronectin (109), the local microenvironment in the node could become increasingly more favourable for metastatic melanoma cells which express high levels of $\alpha_v\beta_3$.

In addition to cell adhesion, vitronectin is also involved in many diverse processes such as fibrinolysis, thrombosis, and control of the complement system (105, 110). In fibrinolysis, vitronectin binds to and stabilizes type I plasminogen-activator inhibitor (PAI-1), a component of the urokinase proteolytic system, maintaining it in its active form (111). Vitronectin also prevents thrombin from heparin-dependent inactivation by anti-thrombin III (105) and can bind to the C5b-7 complex preventing it from adhesion to cells thereby protecting them from complement-mediated lysis (112).

3.2.4.1.3 Expression, function, and regulation of $\alpha_v\beta_3$ and the consequences of $\alpha_v\beta_3$-mediated ligation to vitronectin

Under physiological conditions, the expression of integrin $\alpha_v\beta_3$ is restricted. The receptor is expressed on vascular endothelial cells (93), on chorionic villi cells of the placenta (113), on smooth muscle cells (114), on

lymphocytes such as B, T, and NK cells (115-117), on bone marrow-derived mast cells (118), and on macrophages (119) and other cells of the macrophage/monocyte lineage such as osteoclasts (120).

The $\alpha_v\beta_3$ receptor has been implicated in the migration on vitronectin of several different cell types including endothelial cells (121), macrophages (119), neural crest cells (122), smooth muscle cells (123) and malignant cells such as lung and pancreatic carcinoma (124) and melanoma cells (15). Vitronectin receptor-mediated migration on vitronectin is an important event in physiological processes such as angiogenesis (125), wound healing (126), and embryonic development (122). Vascular cells expressing the receptor migrate to form new blood vessels, a process required for metastases formation. Tumor cells including malignant melanoma cells produce angiogenic factors such as bFGF which trigger this process (127). As well, keratinocytes involved in re-epithelialization of skin wounds, a process requiring extensive cell migration, show increased expression of the $\alpha_v\beta_3$ receptor (126). During embryonic development, neural crest cells must migrate through various extracellular matrices to their final target site where they can begin differentiation (128). *In vitro*, the neural crest cells required $\alpha_v\beta_3$ for migration on vitronectin implying a role for this receptor in development (122).

The $\alpha_v\beta_3$ receptor also plays a role in pathological conditions

requiring cell migration. For example, the receptor has been implicated in migration of smooth muscle cells into the neointima, an event believed to be important in atherosclerosis (123). As tumor cell metastasis requires highly motile and migratory cells, cells possessing the vitronectin receptor may have an advantage in terms of metastatic ability. In a study of human pancreatic carcinoma cells, a failure to express this receptor resulted in the inability of the tumor cells to spread or migrate on vitronectin (124). When the cells were then stably transfected with a full length β_3 cDNA, resulting in functional cell surface expression of $\alpha_v\beta_3$, the cells gained migratory competence on vitronectin.

In addition to its role in adhesion and migration, $\alpha_v\beta_3$ has been implicated in the regulation of tumorigenesis. Using the M21-L human melanoma cell model mentioned above, Felding-Habermann *et al* showed that these cells, which lacked cell surface $\alpha_v\beta_3$, could not adhere to vitronectin *in vitro* and were compromised in their ability to form tumors *in vivo* in nude mice (129). However, M21-L cells regained their adhesive and tumorigenic phenotype when stably transfected with an α_v cDNA (129).

The receptor has also been implicated in cell rescue from programmed cell death - apoptosis (130), the physiological process responsible for maintaining normal cell turnover (131). In a recent study,

Montgomery *et al* have shown that when embedded in 3-D dermal collagen gels (the major ECM protein of the dermis), melanoma cells required ligation of the $\alpha_v\beta_3$ receptors in order to escape apoptosis and form colonies. Similarly, using the chick chorioallantoic membrane model, vascular cells undergoing angiogenesis were shown to require ligation of the $\alpha_v\beta_3$ receptor for growth, differentiation, and the inhibition of apoptosis (127). One of the key steps in apoptosis is the removal of the apoptotic bodies by phagocytic macrophages. In fact, murine bone marrow-derived or human monocyte-derived macrophages use the $\alpha_v\beta_3$ receptor to recognize and remove the apoptotic bodies (132).

Recently, antibody-mediated crosslinking of the $\alpha_v\beta_3$ receptor on melanoma cells was shown to lead to increased invasiveness in the reconstituted basement membrane Matrigel, and this was attributed to upregulated expression of the type IV collagenase gene (133). In summary, the vitronectin receptor has been implicated in many activities ranging from adhesion and migration to tumorigenesis and invasion. Melanoma cells which express this receptor are therefore likely to have a survival advantage and an increased ability to complete the inter-cellular and cell-ECM interactions required for metastases formation.

The synthesis and expression of the $\alpha_v\beta_3$ receptor can be regulated by a variety of cytokines (119, 134), growth factors (135, 136), phorbol esters

(85), and the steroid hormone, vitamin D-3 (137). These modulators of vitronectin receptor expression usually act on the transcription or translation of either the α_v or β_3 subunits resulting in increased cell surface expression of the complex. Cytokines known to increase the expression of the vitronectin receptor on murine bone marrow-derived macrophages include IL-4, IL-6, and the inflammatory cytokines TNFα and GM-CSF (granulocyte-macrophage colony-stimulating factor) (119, 134). All of these cytokines exert their effect on vitronectin receptor synthesis by upregulating expression of the β_3 mRNA with IL-4 being the most potent activator (134). Human microvascular endothelial cells incubated with IFN-γ showed decreased cell surface $\alpha_v\beta_3$ levels, presumably due to a decreased expression of β_3 (135). In rabbit vascular smooth muscle cells, TGF-ß1 and PDGF-BB induced expression of $\alpha_v\beta_3$, again through the increased expression of β_3 mRNA (136). The stimulation seen with PDGF-BB was more rapid and transient than that seen with TGF-ß1, lasting only 24 hours, whereas the latter was more lasting. In the human endothelial cell model, bFGF was able to increase β_3 mRNA synthesis, resulting in increased cell surface expression of the receptor. However, co-incubation of these cells with IFN-γ and bFGF resulted in no increases in receptor expression suggesting that the inhibitory effect of the inflammatory cytokine cannot be overcome by bFGF stimulation (135). In

contrast to the above modulators, 1α,25-Dihydroxyvitamin D3 (137) and the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (85) stimulate $\alpha_v\beta_3$ expression by increasing α_v mRNA levels. In fact, stimulation of avian bone-marrow-derived osteoclast precursor cells with vitamin D resulted in increased α_v mRNA through increased rates of transcription (137). Together these findings show that in cells expressing $\alpha_v\beta_3$ receptor, levels may be regulated by autocrine growth factors or as well as by factors produced by neighbouring host cells (paracrine regulation).

Some of the consequences of $\alpha_v\beta_3$-mediated ligation to vitronectin have been described above , these are increased growth, differentiation, invasion, migration, and survival (rescue from apoptosis). Many of these processes are probably mediated through intracellular signals resulting from receptor crosslinking. For example, migration of endothelial cells on immobilized vitronectin was mediated by $\alpha_v\beta_3$ and this event resulted in increases in second messengers such as cytosolic free calcium $(Ca^{2+})_i$ and intracellular pH (124) suggesting that adhesion to this ECM protein triggered causes intracellular signaling events leading to cell migration. Like other integrins, the vitronectin receptor has a relatively short cytoplasmic domain with no known kinase or phosphatase activity characteristic of the more traditional signal transducing receptors (growth factors). The possible transducers of integrin-dependent message are likely

to be integrin associated proteins such as the focal adhesion kinase ($pp125^{FAK}$) (138). Focal adhesion kinase (FAK) is a cytoplasmic protein tyrosine kinase localized to focal contacts, where integrins and cytoskeletal elements are clustered. Binding of $\alpha_v\beta_3$ to vitronectin results in localization of the receptor to focal contacts where it associates with several integrin-associated proteins including the 50kDa, CD47 blood group antigen-related protein involved in the regulation of intracellular Ca^{2+} (139), the 190 kDa protein which is tyrosine-phosphorylated in response to PDGF (140), and the insulin and insulin-like growth factor receptor substrate-1 (IRS-1), an insulin signalling intermediate involved in binding Grb2 and PI-3 kinase, in response to insulin (141). The latter two integrin-associated proteins point to a possible integration of integrin and growth factor signaling pathways. In addition, the vitronectin receptor-mediated signaling events may also involve activation of protein kinase C. For example, human umbilical vein endothelial cells require $\alpha_v\beta_3$ for cord (lumen-less capillary-like structures) formation and this process is mediated by protein kinase C (PKC) as Calphostin C and staurosporine, inhibitors of PKC, could inhibit cord formation and the PKC activator phorbol 12-myristate 13-acetate (PMA) caused lengthening of the cords (142).

When viewed collectively, these findings present evidence that expression of the $\alpha_v\beta_3$ vitronectin receptor by melanoma cells may provide

them with a central mechanism for the promotion of adhesion, migration, invasion and growth, processes which are all required for metastasis to regional lymph nodes and distant organs. Other receptors may act in concert with $\alpha_v\beta_3$ to enhance the metastatic potential of the melanoma cells.

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Chapter IV

Proteolysis and the Urokinase System in Melanoma Metastasis

4.1 Introduction

In the process of invasion and metastasis, proteolysis is required for degradation of ECM proteins (1, 2) and cell migration through organ stroma and parenchyma (3, 4). This process of ECM proteolysis is controlled by various degradative enzymes including matrix metalloproteinases such as type IV collagenases (5, 6) and serine proteinases such as the plasminogen activators, tPA and uPA (7, 8). An overview of the various classes of proteolytic enzymes relevant to cancer metastasis in general and melanoma dissemination in particular, is presented with a closer examination of the urokinase plasminogen activation system which was the subject of the present study.

4.2 Proteinases and invasion

A key step in invasion, in both physiological processes such as angiogenesis, embryogenesis, inflammation, and tissue repair, and in pathological conditions such as tumor invasion and metastasis, is the release by invading cells of proteolytic enzymes which degrade the complex ECM. Structurally, the ECM consists primarily of collagens which are triple helical proteins (9) and make up the bulk of the proteins in mammals. Of the more than dozen characterized collagens, the most

common types are types I, II, and III interstitial collagens found in connective tissue. The collagen fibrils assemble into the collagen fibers which organize into different structural lattices depending on the type of tissue (9). In addition to collagen, the extracellular matrix also contains glycoproteins such as fibronectin, laminin, and entactin, and proteoglycans which vary depending on the tissue and include heparan sulphate, chondroitin sulphate, dermatan sulphate, and keratan sulphate proteoglycans (10). These interact with the collagen fibers to form the three-dimensional structure of the ECM. The glycoprotein vitronectin has been localized to the ECM of loose connective tissue and the elastic fibers of the papillary and reticular dermis (11).

In addition to connective tissue, invading tumor cells must also transverse basement membranes. This specialized ECM also known as the basal lamina is found at the interface between epithelial and connective tissues and between the epithelium and vascular endothelial cells. The basement membrane is composed largely of type IV collagen, which unlike the interstitial collagens, forms end to end dimers instead of fibrils. In the basal lamina, these dimers connect to form a polygon-like structure as seen both *in vitro* and *in vivo* (12). Electron microscopic analyses have shown this 50-100 nm membrane (9) to consist of three layers (4):

1. the lamina lucida, which is in contact with the cells of the epithelial layer and is composed mainly of laminin;

2. the lamina densa, made up of type IV collagen with some proteoglycans; and

3. the lamina reticularis, which consists of interstitial collagens, fibronectin, and proteoglycans.

The ECM structures normally form efficient barriers to cell migration. Under physiological or pathological conditions requiring invasion however, the invading cells secrete or bring about the secretion of proteases which may cause dissolution of these barriers. Among these proteases are the metalloproteinases, the cysteine proteinases, the serine proteinases, and the aspartic proteinases (13).

4.2.1. The Metalloproteinases

The metalloproteinases, also known as matrix metalloproteinases (MMP), are a family of matrix degrading proteinases which share several characteristics. They are usually secreted as inactive zymogens and require activation by other enzymes for function and they depend on a Zn^{2+} atom in the active site for the enzymatic activity. Members of this family all share sequence homology and have been divided into three main groups: interstitial collagenases, type IV collagenases, and stromelysins (4).

Collagen types I, II, III, VII, and X are cleaved by the interstitial collagenase (MMP-1). Melanoma cells can produce this collagenase *in*

vitro and it is thought to contribute to degradation of interstitial collagen *in vivo* (14).

The stromelysins (15), which consist of stromelysin (MMP-3), stromelysin-2 (MMP-10), and matrilysin (MMP-7) and more recently stromelysin-3 (ST3) (16), form another subgroup of MMPs. They are involved in degradation of collagens type I, IV, V, VII, IX, of ECM glycoproteins such as fibronectin and laminin, and of proteoglycans. Numerous studies have implicated the stromelysins in progression and metastasis of various tumors (15) including breast carcinomas (17) and squamous cell carcinomas of the head and neck (18, 19). In situ hybridization studies on breast carcinomas revealed that ST3 mRNA levels were higher in invasive ductal and lobular carcinomas than in early stage *in situ* tumors (17). In this study, a significant correlation was found between ST3 levels and metastatic disease. In another study, expression of ST3 mRNA was analyzed in numerous breast tumors and only the highly malignant tumors were found to express the message for this enzyme. Expression in this study correlated with the presence of lymph node metastasis (20). However, in this and other studies (21), expression of ST3 mRNA was associated with the stromal elements, particularly fibroblastic cells, surrounding the malignant breast tumor suggesting that this enzyme plays a role in stromal epithelial interaction. In head and neck squamous cell carcinoma, both stromelysin (19) and stromelysin-3 (18) were

implicated in invasiveness and progression. Again the latter study, demonstrated that ST3 mRNA was associated with stromal cells surrounding the invasive tumor cells (18).

The type IV collagenases include the 72-kDa and 92-kDa gelatinases, MMP-2 and MMP-9, respectively. These enzymes degrade type IV collagen, as well as type V, VII, IX collagens, gelatins, and fibronectins (4). The gelatinases characteristically cleave type IV collagen at a triple-helical domain which is ¼ the molecule length from the NH_2-terminus, resulting in 2 fragments of a ¼ and ¾ length (22). This is in contrast to interstitial collagenase, which cleaves the interstitial collagens at the COOH-terminal end, again generating ¼ and ¾ fragments. The stromelysins may also cleave type IV collagen but the cleavage site is at a different site than that of type IV collagenases producing less well-defined fragments.

Type IV collagenases have been implicated in the invasion and metastasis of different tumors ranging from breast carcinomas to melanomas (6). In colon adenocarcinomas, the expression of MMP-2 as measured by immunohistochemical analysis of tumor samples, was elevated in primary adenocarcinomas as compared to adjacent normal colonic mucosa and this expression correlated with the stage of the disease as determined by Duke's classification level (23). Tissue staining of invasive ductal and lobular breast carcinomas also revealed similar

increases in expression of MMP-2 when compared to normal duct epithelium (24). In other tumors such as two squamous cell carcinoma cell lines, expression of MMP-2 as well as MMP-9 was demonstrated by gelatin-substrate-zymography (25). In the A2058 human melanoma cell line, the expression of activated 72-kDa type IV collagenase could be inhibited by a synthetic peptide derived from a region on the inactive proenzyme. This peptide contained a crucial cysteine residue required to maintain the latent form of the enzyme. Inhibition of MMP-2 in this way resulted in a suppression of melanoma cell invasion through reconstituted basement membrane (Matrigel), (26) suggesting that this enzyme was required for invasion *in vitro* and possibly for metastasis *in vivo*. In addition, ligation of the $\alpha_v\beta_3$ vitronectin receptor on melanoma cells has been shown to cause an upregulation of the 72-kDa type IV collagenase gene and an increase in invasiveness through Matrigel (27) providing a mechanism to explain the observed increased invasiveness in melanoma cells which express high levels of vitronectin receptor. Along similar lines, a study using A2058 human melanoma cells revealed that MMP-2 not only mediates ECM proteolysis but can also alter cell adhesion and spreading (28). In this study, the activity of MMP-2 in the melanoma cells was altered by overexpression or suppression of the specific inhibitor, TIMP-2. Suppression of inhibitor expression by an anti-sense RNA to TIMP-2 reduced inhibitor levels and resulted in decreased adhesion of the

melanoma cells to fibronectin, vitronectin, and gelatin coated plates as well as to uncoated plates. Conversely, an increase in adhesion was seen in cells overexpressing TIMP-2. Together these results suggest that proteolysis by MMP-2 can decrease tumor cell adhesion thereby promoting migration and invasion.

4.2.2. Cysteine and Aspartate proteinases (Cathepsins)

The cysteine proteinases consist mainly of cathepsins B, H, L, and S whereas the aspartate proteinases include cathepsins D and E (4, 13). The former peptidases have at their active site a cysteine residue whereas the latter have two aspartic acid groups. With the exception of cathepsin E, all the cathepsins are lysosomal enzymes with broad substrate specificities which may include proteoglycans, glycoproteins, and collagens. These enzymes have been found in a variety of tumors and the evidence suggests that they play a role in invasion and metastasis. For example, melanomas may produce one or more of several cathepsins including cathepsins B, D, H, and L (29, 30). Despite the lysosomal localization of the majority of cathepsins, studies have shown that in murine melanoma cells, cathepsin B activity in the plasma membrane fractions of these cells is increased and this is associated with increased metastatic potential (31). In another study, cathepsin B-like enzyme activity was shown to be higher

in melanomas as compared to pigmented nevi (32). This study also demonstrated that patients with high activity of this enzyme had the shortest disease-free interval suggesting that the enzyme may be an important prognostic predictor in human melanoma. Immunohistochemical studies revealed that in addition to cathepsin B, cathepsins D, H, and L levels were also higher in metastatic melanomas compared to primary melanomas and nevi (29, 33) suggesting that expression of these enzymes may be correlated with melanoma progression and metastasis.

4.2.3. Serine proteinases

These endopeptidases which have at their active site serine, histidine, and aspartic acid residues catalyze the cleavage of a broad spectrum of substrates. In fact, many components of the coagulation, complement, and fibrinolytic systems are serine proteases (13). Of particular importance in melanoma invasion and metastasis is the urokinase plasminogen activator. This serine proteinase and its receptor were recently identified as markers of progression in melanoma as their appearance and increased expression *in situ* were found to signal the conversion of benign melanocytic nevi to invasive and malignant lesions (34, 35).

4.2.3.1. Urokinase and its receptor - expression and function

The urokinase plasminogen activator (uPA) and its receptor (uPAR) constitute an important component of the cell migration apparatus. They play a role in diverse physiological and pathological processes mediating cellular migration and homing (36). For example, on vascular endothelial cells, the expression of uPA and its receptor are increased on migrating cells in wounded endothelial monolayers suggesting that these proteins may be necessary in physiological processes such as angiogenesis in wound healing (37). Similarly, these proteins have been implicated in embryogenesis and inflammation (36). Urokinase and/or its receptor have also been implicated in invasion and metastasis of several different tumor types including human carcinomas of the breast (38), colon (39, 40), pancreas (41), prostate (42), and skin (squamous cell carcinoma) (43). Evidence for involvement of uPA and its receptor in tumor invasion and metastasis has come mainly from immunohistochemical analysis or *in situ* hybridization. However, more direct methods have also been used such as genetic manipulation of uPA or uPAR gene expression. For example, in the squamous cell carcinoma study, stable transfection with a plasmid vector expressing 300 bp antisense DNA for uPAR resulted in a reduction in uPAR mRNA and a decrease in invasion as measured using

chick chorioallantoic membranes. In SV40-transformed human fibroblasts, the uPAR mRNA and protein expression was inhibited by a uPAR antisense oligomer and this resulted in decreased invasiveness of the cells through Matrigel coated filters (44). Overexpression of uPA in a rat prostate cancer cell line transfected with a plasmid expressing a full length rat uPA cDNA resulted in both accelerated and more widespread development of skeletal metastasis in recipient rats (42). Along different lines of investigation, in a study using ovarian cancer tumor extracts, the levels of uPA strongly correlated with lymphatic metastasis (45). Moreover, patients with low levels of uPA in the tumor extracts were found to have a better prognosis than those with higher levels.

The upregulated expression of these proteins in metastatic melanoma has been well documented (34, 35). In one of these studies, immunohistochemistry, *in situ* hybridization, and histological zymography were used to show that uPA expression and activity were present in atypical nevi and melanomas but not in benign melanocytes (34). Using similar techniques, another group showed that uPA as well as uPAR expression and activity were increased in late-stage primary and metastatic melanoma as compared to benign nevi and early stage primary melanoma (35). It should also be noted that while the urokinase system can act in an autocrine fashion with all the essential components synthesized by the same cell, cooperativity between different cells, in a

paracrine manner, where uPAR-expressing cells bind and utilize uPA secreted by other cells has also been demonstrated. For example, in studies on colon adenocarcinomas, tumor cells which expressed high levels of uPAR were found to bind uPA synthesized by neighbouring stromal cells or tumor-infiltrating macrophages (40, 46).

Urokinase converts the enzymatically inactive glycoprotein plasminogen, found in plasma and extracellular fluids to plasmin - a broad-spectrum serine protease which can degrade circulating and tissue proteins and can also contribute to the proteolytic activity of other enzymes such as metalloproteinases, by catalyzing their conversion from latent zymogens to active enzymes (4). Urokinase consists of an NH_2-terminal growth factor domain, a kringle domain, and a COOH-terminal region containing the active site (7). The enzyme is synthesized and secreted in an inactive single-chain precursor form, the pro-uPA (47). In this form, uPA binds with high affinity through its amino-terminal domain, to a 55-60 kDa membrane receptor (uPAR) and is then converted to a two-chain active enzyme by plasmin and possibly other proteases, such as trypsin and kallikrein (48).

The human urokinase receptor is a highly glycosylated 335 amino acid polypeptide. The molecule has no transmembrane domain and is anchored to the cell membrane by glycosylphosphatidylinositol (GPI) (49). The primary structure of the receptor consists of three repeated consensus

sequences named domains I, II, and III. Cross-linking studies using uPA and uPAR (50) demonstrated that domain I is the uPA binding region as antibodies directed to this region prevented uPA binding (51). It is interesting to note that uPA, found in murine mammary and lung carcinoma cells, may be involved in the cleavage of its own receptor thereby specifically releasing the ligand binding domain of the receptor (Domain I) while leaving the other two domains (II and III) attached to the cell membrane (52). This finding is relevant as levels of the remaining receptor can be measured and correlated with uPA activity in the tumor cells. This self-cleavage phenomenon was first observed in U937 - a leukemic monocytic cell line (53).

Plasminogen, the uPA substrate, also binds to a cell surface receptor and its conversion to plasmin occurs at the cell surface. In its bound form plasminogen is protected from the action of the circulating inhibitor α_2-antiplasmin. A third component of this cell surface proteolytic system are the PA inhibitors PAI-1, PAI-2, protease nexin 1 and the less well characterized PAI-3 (54, 55). Of these inhibitors, PAI-1 is known to be an extracellular matrix protein associated with vitronectin (56).

Several inhibitors of various components of the plasminogen activation system have been shown to inhibit the invasiveness or metastatic ability of tumor cells. These include suramin, a polysulfonated

naphthylurea compound which was shown to modulate the expression of uPA and PAI-2 in a human metastatic renal cell carcinoma cell line transplanted into nude mice (57). This drug increased the production of PAI-2 and decreased that of urokinase resulting in inhibition of lung and liver metastases of this tumor. Another group of inhibitors which alter the function of uPA are the synthetic urokinase inhibitors. Recently, the substituted carboxamidines, B428 and B623 (58) were shown to be competitive inhibitors of human urokinase Fibrosarcoma cells treated with these inhibitors showed an inhibition of cell surface uPA-mediated degradation of fibronectin suggesting that these compounds may be useful for prevention of invasion. Urinary trypsin inhibitor and a synthetic peptide derived from it, could both inhibit plasmin and reduced the invasion of HOC-1 human ovarian cancer cells through Matrigel-coated filters. (59) Recent work by this group (60) has shown that a hybrid molecule consisting of the amino terminal fragment (ATF) of urokinase (which binds to uPAR), and the urinary trypsin inhibitor, could inhibit tumor cell invasion through Matrigel. Specific targeting of the urinary plasmin inhibitor to the uPAR probably localized this inhibitor to areas of active proteolysis thereby increasing their efficiency. In another study (61) a hybrid molecule consisting of human uPA and a ribosome-blocking plant-derived protein, saporin was synthesized. Cytotoxicity assays revealed that this molecule was effectively targeted to cells expressing the

uPAR. These studies show that the urokinase plasminogen activation system is crucial for invasion *in vitro* and metastasis *in vivo* and could also serve as a target for synthetic compounds with tumor-inhibitory activities.

4.2.3.2. Regulation of expression of urokinase and its receptor

The synthesis of uPA and its receptor are tightly regulated (62). Similarly to the vitronectin receptor expression, many factors have been implicated in the control of these proteins. These include growth factors, inflammatory cytokines, hormones, and phorbol esters (63, 64). For example, bFGF has been consistently shown to stimulate production of uPA in endothelial cells in an autocrine (65) or paracrine fashion (66). Recent studies have shown that this upregulation of uPA production in bovine aortic endothelial cells was the result of an increased rate of uPA gene transcription (67). The study also demonstrated that the presence of bFGF was required during the delay period between increased uPA gene transcription and uPA protein synthesis because a mutated bFGF protein increased uPA mRNA levels but not uPA protein synthesis. This growth factor could also increase the expression of cell surface receptors for urokinase on vascular endothelial cells (68). Another growth factor which regulates uPA and uPAR expression is TGFß1. The effects of this factor are

varied depending on the cell system used. In vascular endothelial cells, TGFß1 (69) caused inhibition of uPA synthesis and did affect the expression of uPAR (68). Similar findings were reported with 8387 fibrosarcoma cells (70). However, in human skin fibroblasts TGFß1 increased the production of uPA (71) with a resulting increase in enzymatic activity which could be inhibited by an anti-uPA antibody. Likewise, in a human lung carcinoma cell line A549 and in a murine macrophage cell line RAW264.7, (72, 73), TGFß1 increased uPA mRNA levels and protein synthesis. This was determined by casein substrate zymography and immunoprecipitation in the first study and in the second study by a unique method for quantitating uPA activity in which macrophages stimulated with TGFß1 were incubated on a bovine endothelial cell-deposited ECM containing bound, ^{125}I-labelled bFGF and the liberated radioactivity was measured and visualized by SDS-PAGE and autoradiography. It is of interest to note that in endothelial cells, plasminogen activity may be regulated by both bFGF and TGFß1 (74). That is, bFGF in these cells upregulates the levels of uPA which in turn, through plasmin generation, can activate (or liberate from the ECM) latent TGFß1. TGFß1 then downregulates uPA levels, possibly through increased synthesis of an inhibitor to uPA, PAI-1, thereby providing a negative feedback mechanism for control of uPA activity in these cells (74). In addition to TGFß1, urokinase via plasmin can also convert the precursors

of other growth factors and scatter factor, HGF (75, 76), to their biologically active forms. These factors, in addition to providing a growth or migratory stimulus for tumor or host cells, can also modulate the expression of various components of the urokinase system. Other growth factors known to effect cell surface expression of uPAR are EGF (77) and vascular endothelial growth factor (VEGF) (78), as found with HeLa cells and vascular endothelial cells, respectively.

In addition, growth factors, inflammatory cytokines such as IFNγ and TNFα (64, 79) have been shown to alter uPA and uPAR expression. In the U937 mononuclear phagocyte cell line, IFNγ increased cell surface levels of uPA and its receptor whereas TNFα increased uPAR mRNA levels without affecting uPA expression (79). In T-cells derived from normal donors, cytokines such as IL-2, IL-4, and IL-7 upregulated the expression of uPAR (80). It is possible that these cytokines also play a role in regulating urokinase and its receptor on melanoma cells.

Steroid hormones such as the glucocorticoid, dexamethasone, also affect uPA function, usually by suppression of uPA activity (8). For example, in MAT 13762 mammary adenocarcinoma cells, treatment with this compound led to a reduction in uPA mRNA caused by decreased stability of the mRNA transcripts (81). On the other hand, estrogens were found to increase uPA activity in human mammary carcinomas (82).

Urokinase (83, 84) and urokinase receptor mRNA levels have been

shown to significantly increase in response to phorbol 12-myristate 13-acetate (PMA) implicating PKC in the regulation of uPAR synthesis (85). Interestingly, PKC has recently been implicated in post-adhesion, integrin-mediated signalling and its inhibition was shown to block cell spreading and the phosphorylation of the focal adhesion kinase, pp125^{FAK} (86). In a recent report, $\alpha_v\beta_3$-mediated endothelial cell migration and tube formation which depend on uPA activity were shown to require PKC activation (87). Moreover, melanoma cell adhesion to vascular endothelium and metastases formation could be blocked by PKC inhibitors (88). These findings suggest that post-adhesion, integrin-mediated signalling and uPA-mediated proteolysis both involve PKC activation.

4.2.3.3. Focal contacts - sites of linkage between adhesion and proteolysis

uPAR and uPA have been localized to cell-cell and cell-substratum (focal) contact sites and plasminogen has been shown to bind to ECM proteins (36). Thus all the components of this proteolytic apparatus are concentrated at focal contact sites where they can coordinate cell adhesion and migration and may tilt the balance in favor of one cellular function or the other. PAI-1 can stabilize vitronectin-mediated cellular adhesion (89) thereby acting in cooperation with vitronectin receptors such as $\alpha_v\beta_3$.

Vitronectin and other ECM proteins may then be degraded by plasmin resulting in cell detachment and migration (62). Moreover, receptor bound uPA is not internalized nor processed. However, once it binds to PAI-1 via its carboxyl-terminal catalytic domain, the complex is internalized via the low-density lipoprotein (LDL) receptor-related protein (LRP), also known as the α_2 macroglobulin receptor. LRP consists of two noncovalently-linked subunits, an extracellular 515 kDa subunit including the ligand binding region, and an 85 kDa subunit consisting of extracellular, transmembrane, and cytoplasmic domains, the latter of which contains the endocytosis signal sequence, NPXY (90-92). Following PAI-1 binding, the uPAR:uPA:PAI forms a complex with the 515 kDa chain of LRP. Subsequently, the signal sequence of the 85 kDa LRP subunit allows endocytosis of the entire complex, with the uPA:PAI-1 being targeted to lysosomes for eventual degradation and the uPAR and LRP being recycled and reexpressed at new focal adhesion sites on the cell surface (90). Binding of uPA to vitronectin-associated PAI-1 can therefore trigger a process of uPAR repositioning, thereby maximizing its utility in conjunction with cell migration, reattachments and the formation of new focal adhesion contacts. In addition to this functional link between cell adhesion to vitronectin and uPA-mediated proteolysis, recent reports based on studies using an embryonic kidney epithelial cell line stably transfected with a full-length uPAR cDNA isolated from macrophages,

also suggest that the uPAR receptor can act directly as a vitronectin receptor mediating VN recognition through its primary structure domains II and III (93).

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Chapter V

The Role of the $\alpha_v\beta_3$ Vitronectin Receptor and the Urokinase-type Plasminogen Activator in Melanoma Metastasis

5.1 Overview

The findings of my studies on the role of the $\alpha_v\beta_3$ receptor in lymphatic metastasis of human melanoma cells are presented in this chapter in the form of two published papers and additional unpublished results. I was responsible for all of the experimental work described in these papers with the exception of flow cytometric analyses, which was carried out by Mr. Kenneth McDonald at the Royal Victoria Hospital in Montreal.

In the first paper (Nip, J., Shibata, H., Loskutoff, D. J., Cheresh, D. A., and Brodt, P. Human melanoma cells derived from lymphatic metastases utilize integrin $\alpha_v\beta_3$ to adhere to lymph node vitronectin. J.Clin.Invest. *90:* 1406-13, 1992), the two animal models developed in an attempt to study the role of adhesion in lymphatic metastasis of human melanoma are described. They consist of the MeWo model (see Fig. 1) which was developed by intradermal inoculation of MeWo, an established human melanoma cell line, into nude mice. Metastasis of these cells to the lymph nodes usually occurred within 9 weeks following injection. Lymph nodes were removed at that time and lymph node-metastasizing cells were isolated. These cells were cloned *in vitro* and a single clone MeWo LNI 6I was selected for further study. A similar approach was used to develop a second melanoma model which was derived from an inguinal lymph

node metastasis of a melanoma patient. This model consists of the MIM/8 parental cells and MIM/8 LNI lymph-node metastasizing cells which were obtained in a manner similar to MeWo LNI 6I.

Using these two models, a good correlation was found between cell adhesion to cryostat sections of lymph node and the metastatic potential *in vivo* in nude mice. Subsequently, vitronectin (VN) was identified as the ECM protein on the lymph node sections recognized by the metastatic cells and $\alpha_v\beta_3$ was found to mediate the adhesion. Flow cytometric analysis confirmed that the expression of this receptor on the metastatic cells was significantly and specifically increased as compared to the parental cells.

In the second paper (Nip, J., Rabbani, S. A., Shibata, H., and Brodt, P. Coordinated expression of the vitronectin receptor and the urokinase-type plasminogen activator receptor in metastatic melanoma cells. J.Clin.Invest. *95:*, 1995), further evidence based on Northern blot analysis and cell surface immunoprecipitation is presented which confirms the initial observations of increased expression of the vitronectin receptor in the lymph-node metastasizing cells. In these cells, an increase was also found in the expression of the urokinase receptor. Furthermore, when vitronectin receptor expression in the metastatic melanoma cells was suppressed by α_v antisense phosphorothioate oligonucleotides causing a substratum-specific decrease in adhesion to VN, a reduction was also

found in the levels of uPAR mRNA. Antisense oligonucleotides can block protein synthesis through several different mechanisms including:

1. physical inhibition of protein translation by ribosomes;
2. interference with cytoplasmic translocation of mRNA; and
3. formation of RNA-RNA duplexes, resulting in activation of cellular RNase L. All of which can lead to decreased mRNA and protein levels. In our system, the method used was the uptake of oligonucleotide by simple endocytosis. The use of cationic lipids such as lipofectin, did not enhance the uptake of the oligonucleotides. Conversely, ligation of the vitronectin receptor by crosslinking antibodies caused a specific increase in uPAR mRNA expression. Together, these findings suggest that uPAR transcription in the metastatic melanoma cells is regulated by α_v expression and function.

In the last part of the chapter, recent unpublished findings are described showing that ligation of the α_v-containing integrins on the metastatic melanoma cells resulted in specific uPAR-mediated increase in invasiveness through Matrigel-coated filters confirming that ligation of α_v integrins indeed increased uPA-mediated proteolysis and invasiveness.

Coordinated Expression of the Vitronectin Receptor and the Urokinase-type Plasminogen Activator Receptor in Metastatic Melanoma Cells

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Abstract

Integrin $\alpha_v\beta_3$ is a marker of progression in malignant melanoma. Previously we reported that human melanoma cells derived from regional lymph node metastases had increased $\alpha_v\beta_3$-mediated adhesion to lymph node vitronectin. In the present study, the expression and function of $\alpha_v\beta_3$ were further investigated with emphasis on the functional relationship between $\alpha_v\beta_3$ and the urokinase-type plasminogen activator system of proteolysis. We found that metastases-derived melanoma MeWo LNI 6I (6I) and MIM/8 LNI cells had a markedly increased expression of α_v mRNA transcripts relative to the parent lines which was reflected in significantly elevated levels of the $\alpha_v\beta_3$ heterodimers on the cell surface. These cells also expressed elevated levels of urokinase plasminogen activator receptor (uPAR) mRNA and had higher levels of surface bound urokinase plasminogen activator as detected by immunolabeling. To determine whether the expression of uPAR and α_v were linked, α_v synthesis in the metastatic melanoma cells was suppressed using α_v antisense phosphorothioate oligonucleotides. This resulted in a marked decrease in detectable α_v mRNA and protein and a corresponding substratum-specific reduction in cell adhesion to vitronectin. When uPAR expression in these cells was subsequently analyzed, we found a reduction of ~ 50% in uPAR mRNA levels. On the other hand, ligation of the $\alpha_v\beta_3$ receptor on the melanoma cells by immobilized antibody resulted in a twofold increase in uPAR mRNA. The results suggest that the expression of uPAR in metastatic melanoma cells is linked to the expression and function of the vitronectin receptor. (*J. Clin. Invest.* 1995. 95:2096–2103.) Key words: metastasis • melanoma • integrin • vitronectin receptor • urokinase plasminogen activator receptor.

Introduction

Malignant melanoma is becoming increasingly prevalent worldwide (1) and its incidence is growing more rapidly than any other form of cancer (2). Metastasis to the draining regional and distant lymph nodes precedes wide-spread dissemination of malignant cells and is normally associated with poor prognosis (3). Little is presently known about the molecular mechanisms involved in melanoma dissemination to regional nodes.

Tumor cell metastasis to secondary sites is a complex, multistep process involving cell detachment from the primary site, migration through lymph (or blood) vessels, cell adhesion to cellular and extracellular matrix (ECM)[1] elements of the invaded organ, proteolytic degradation of matrix proteins, and cell proliferation (4). Integrins, a family of cell adhesion receptors consisting of α/β heterodimers, mediate cell–ECM as well as cell–cell interactions and are known to regulate cell migration and growth (5). Expression of the vitronectin receptor integrin $\alpha_v\beta_3$ has been linked to malignant progression of melanoma (6) but its function in the process of invasion and metastasis are not presently clear. Recent studies have suggested that clustering of $\alpha_v\beta_3$ by antibody or ligand can trigger increased expression of the metalloproteinase type IV collagenase thereby promoting invasion (7). Other studies suggested that $\alpha_v\beta_3$ plays a role in regulation of melanoma cell growth in vitro and in vivo (8). We recently reported (9) that human melanoma cells derived from regional lymph nodes had an increased $\alpha_v\beta_3$-mediated adhesion to lymph node vitronectin.

In addition to the metalloproteinases, the serine protease urokinase plasminogen activator (uPA) and its membrane-linked receptor (uPAR) have also been repeatedly implicated in the metastatic process. Recently we have shown that increased skeletal metastasis of prostate cancer cells is directly related to uPA overproduction (10). The evidence is particularly strong for involvement of the uPA/uPAR system in melanoma invasion and dissemination (11, 12). This activity is regulated by plasminogen activator inhibitors 1 and 2. Binding of plasminogen activator inhibitor-1 to receptor-linked uPA has been shown to trigger internalization of the complex and subsequent processing and reexpression of the receptor at a second site providing a mechanism for receptor mobility as the cells migrate through degraded ECM (13). As the binding of uPA to its receptor occurs at focal adhesion contacts, and plasminogen activator inhibitor-1 is associated with ECM vitronectin and can stabilize vitronectin-mediated adhesion, it was of interest to investigate the relationship between $\alpha_v\beta_3$ and the urokinase system in melanoma cells selected in vivo for increased metastasis to regional nodes. The present results show that uPAR transcription in malignant melanoma cells is linked to α_v expression.

Methods

Animals and tumor lines. Female nu/nu (CD-1)Br mice, 4–6 wk old (Charles River Canada; St-Constant, Canada) were used for propagation

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1. *Abbreviations used in this paper:* ECM, extracellular matrix; GFD, growth factor–like domain; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor.

of the human melanoma cell lines and for isolation of the metastatic variants. The mice were housed in Microisolator cages (Lab Products Inc., Maywood, NJ) and handled in biohazard cabinets. They remained disease free for up to 1 yr.

Cell line MIM was established from an inguinal lymph node metastasis of a male melanoma patient as we described previously (9). A pigmented clone-MIM/8 was derived by serial dilution cloning of MIM cells. Subsequent intradermal injection of 5×10^6 MIM/8 cells into the right lateral flanks of nude mice gave rise to palpable primary tumors at 3 wk and regional lymph node metastases at 12 wk, after inoculation. The lymph node–invading melanoma cells were isolated by mincing the lymph nodes and mechanically dispersing the cells through an 85-μm nylon filter (Nitex; B&SH Thompson, Town of Mont Royal, Canada). Cells were washed and seeded onto 25-cm^2 tissue culture flasks (Falcon Labware; Fisher Scientific Co., Montreal, Canada) containing RPMI-1640 supplemented with 5% FCS, 1% gentamycin sulfate, 1% penicillin-streptomycin, and 2 mM glutamine (all reagents were from Gibco Laboratories, Burlington, Canada) (RPMI-1640-FCS). Nonadherent cells and cellular debris were removed 24 h later and the adherent melanoma cells designated MIM/8 LNI, were maintained in culture until a confluent monolayer was obtained. The tumorigenicity and metastatic properties of the melanoma cells were determined after intradermal injection of 5×10^6 cells into nude mice. Of five animals inoculated with MIM/8 cells, only two developed regional lymph node metastases whereas four/four animals bearing MIM/8 LNI tumors developed regional lymph node metastases.

The origin and metastatic properties of cell line MeWo LNI 6I (6I) were described in detail previously (9).

All cell lines were maintained as monolayer cultures in RPMI-1640-FCS. Cell lines were subcultured twice weekly using $Ca2^+$ and $Mg2^+$-free PBS containing 0.02% EDTA to disperse the cells.

Antibodies. mAb W6/32 directed to human HLA class I which recognizes an antigenic determinant common to products of the HLA-A, B, and C loci was obtained from Cedarlane Laboratories (Hornby, Canada). Normal mouse immunoglobulin was from Bio-Can Scientific (Mississauga, Canada), rabbit anti–mouse IgG was from Dakopatts A/S (Glostrup, Denmark), peroxidase-conjugated goat anti–rabbit IgG from Sigma Chemical Co. (St. Louis, MO), and peroxidase-conjugated rabbit anti–mouse IgG from Dimension Laboratories (Mississauga, Canada). The following antibodies were kind gifts; mAb's LM142 and LM609 to the α_v subunit, and $\alpha_v\beta_3$ complex (14), respectively, were from Dr. D. Cheresh (Scripps Research Clinic, LaJolla, CA), mAb P3G8 to α_v (15) and mAb A3-IIF5 to α_3 (16) from Dr. M. Hemler (Dana-Farber Cancer Institute, Boston, MA), mAb AP-3, directed to the β_3 integrin subunit, from Dr. P. J. Newman (Blood Research Institute, Milwaukee, WI), and rabbit antibody to uPA, which recognizes the growth-factor–like domain (GFD) (17), from Dr. Andrew Mazar (Abbott Laboratories, Chicago, IL).

cDNA probes and oligomers. α_v cDNA (18) was obtained from Gibco Laboratories. The mouse 72-kD type IV collagenase cDNA was a kind gift from Dr. Ann F. Chambers (London Regional Cancer Centre, London, Ontario, Canada). An antisense oligonucleotide corresponding to the first 15 amino acids of the uPA receptor (19) was synthesized by Sheldon Biotechnology (McGill University, Montreal, Canada). uPA cDNA (pHUK-1) was obtained from the American Type Culture Collection (Rockville, MD). Antisense phosphorothioate oligonucleotides directed to the α_v subunit of human vitronectin receptor were chosen on the basis of the BLAST alignment program (20) to have no homology with other known human DNA sequences including integrin α subunits such as α_5 (6). They include AS2 (5′-TCAGCATCAATATCTTGT-3′), complementary to bases 563 to 580 of the human vitronectin receptor sequence; AS3 (5′-AAGCCATCGCCGAAGTGC-3′), complementary to bases 31 to 48 of the human sequence; and AS4 (5′-GACTGT-CCACGTCTAGGT-3′), complementary to bases 136 to 153. As a control, a sense sequence S1 (5′-GCACTTCGGCGATGGCTT-3′), corresponding to AS3 was used. The oligonucleotides were synthesized on a DNA/RNA synthesizer (392; Applied Biosystems, Foster City, CA) and purified three times using ethanol precipitation (2.5 vol of ethanol and 1/4 vol of 10 M ammonium acetate).

Immunoprecipitation. Tumor cell-surface proteins were ^{125}I-labeled using the lactose peroxidase method (21). Equal numbers of cells of each cell line were labeled. The cells were washed repeatedly and lysed for 15 min on ice with a 0.5% NP-40 buffer containing 1 mM PMSF, 1 mg/ml leupeptin, and 1 mg/ml pepstatin A (all from Sigma Chemical Co.). The lysate was cleared by centrifugation at 10,000 g for 15 min. To aliquots containing equal numbers of cpm from each tumor lysate (volumes adjusted to 500 μl with lysis buffer), 1–5 μg of the appropriate mAb were added for a 2-h incubation at 4°C and this was followed by incubation overnight at 4°C with 100 μl of rabbit anti–mouse IgG complexed to protein-A CL-4B Sepharose beads (Pharmacia LKB Biotechnology, Uppsala, Sweden). The beads were washed repeatedly with 0.1% NP-40, resuspended in SDS sample buffer, and boiled for 15 min. The eluted proteins were electrophoresed on 7.5% SDS-polyacrylamide gels under nonreducing conditions. For radiolabeled proteins, gels were dried and exposed to x-ray film (X-OMAT AR; Eastman Kodak Co., Rochester, NY) for 7–14 d. The relative intensity of the integrin bands on the autoradiographs was analyzed by laser densitometry using an Ultroscan XL Enhanced Laser Densitometer (LKB Instruments Inc., Bromma, Sweden).

RNA isolation and Northern blot analysis. Total RNA was isolated using the acid guanidium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (22). Poly (A)$^+$RNA was isolated using an mRNA purification kit obtained from Stratagene Inc. (La Jolla, CA). The RNA (30 μg of total RNA or 5 μg of poly (A)$^+$RNA) was separated by electrophoresis through a 1.1% agarose gel containing 2 M formaldehyde. The RNA was transferred to a nylon membrane (Hybond N; Amersham International, Oakville, Canada) by capillary action and hybridized with cDNA probes radiolabeled by random primer extension with [α-^{32}P]dCTP (23) or with oligomers which were end-labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase (24) for 48 h at 42°C. The membranes were washed twice for 30 min at room temperature with 1 × SSC containing 0.1% SDS and twice for 30 min at 55°C with 0.1 × SSC containing 0.1% SDS. The blots were exposed for 2–7 d at −70°C. As a control for RNA loading, the blots were subsequently probed with a ^{32}P-labeled, 800-bp BamHI restriction fragment of rat cyclophilin cDNA (25). The relative amounts of the mRNA transcripts were analyzed by laser densitometry using an Ultroscan XL Enhanced Laser Densitometer and normalized relative to the internal cyclophilin controls.

Reverse transcriptase-PCR. 1 μg of total RNA extracted as described was used for the reverse transcription reaction. Subsequent amplification of the resultant first strand cDNA in a GeneAmp PCR System 9600 (Perkin-Elmer Cetus Instruments, Emeryville, CA) thermal cycler was performed using established procedures (26). 30 cycles of amplification were performed each consisting of denaturation at 94°C for 2 min, reannealing at 55°C for 1 min, and extension at 72°C for 1 min. The PCR product was analyzed on a 1% Tris-borate/EDTA agarose gel. Based on the known number of basepairs between the specific sense and antisense primers used for the amplification, the size of the human uPA DNA product was predicted to be 750 bp. For adequate detection of uPA DNA, a second amplification of 5 μl of the first reaction product was necessary.

Indirect immunocytochemistry. Tumor cells were cultured overnight at 37°C on eight-chamber slides (Lab-Tek; Nunc, Inc., Naperville, IL). The cells were fixed with 2% formaldehyde/PBS at room temperature for 30 min and permeabilized with 100% acetone for 2 min at −20°C. After rehydration with PBS, rabbit anti–human uPA (anti-GFD) antibody was added at a dilution of 1:250 for 45 min at room temperature followed by a peroxidase-conjugated goat anti–rabbit IgG diluted 1:100. The slides were developed with 100 μl/chamber of a 0.1-M Tris-HCl (pH 7.6) solution containing 400 μg/ml 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, CA) and 0.03% H_2O_2 (Fisher Scientific Co., Montreal, Canada). The chamber slides were dried, mounted with Immuno-Fluore (ICN Biomedicals Inc., Costa Mesa., CA), and visualized by light microscopy.

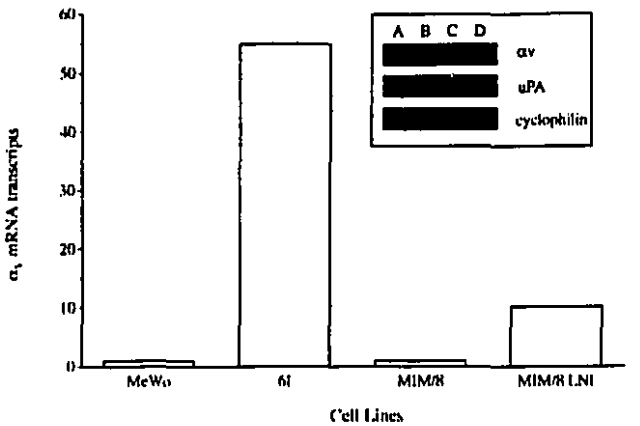

Figure 1. Northern blot analysis of α_v mRNA transcripts in melanoma sublines. Poly (A)$^+$ RNA derived from MeWo, 6I, MIM/8, and MIM/8 LNI cells (*inset*, lanes *A*, *B*, *C*, and *D*, respectively) were size-fractionated on 1.1% formaldehyde-agarose gels after loading of 5 μg of mRNA onto each lane. The blots were probed sequentially with ^{32}P-labeled α_v and uPA cDNA. Laser densitometry was used to quantitate the levels of α_v mRNA, relative to control cyclophilin mRNA. Results of this analysis are shown in the bar graph and are expressed as the ratios of α_v to cyclophilin mRNA in the metastatic cells relative to the parental cells, which were assigned an arbitrary value of 1.0.

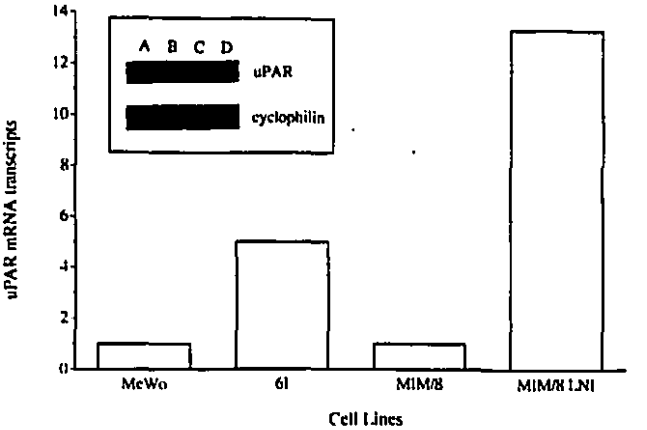

Figure 3. Northern blot analysis of uPAR mRNA transcripts. Total RNA derived from MeWo (*A*), 6I (*B*), MIM/8 (*C*), and MIM/8 LNI (*D*) cells (*inset*) was size-fractionated by electrophoresis on 1.1% formaldehyde-agarose gels. 30 μg of RNA were loaded in each lane. The blots were probed with a ^{32}P-labeled uPAR antisense oligomer. Laser densitometry was used to quantitate the intensity of the bands relative to control bands of cyclophilin mRNA. Results of this analysis are shown in the bar graph and are expressed as the ratios of uPAR to cyclophilin mRNA in the metastatic cells relative to the parental cells, for which the uPAR:cyclophilin ratio was assigned a value of 1.0.

Cell treatment with oligonucleotides. Melanoma cells were plated in 6- or 96-well plates (Falcon Labware; Fisher Scientific Co.). To each well 100 (96 wells) or 700 (6 wells) μl of the indicated concentrations of oligonucleotides diluted in RPMI-1640-FCS medium was added every 24 h for a period of 3 d. Cells were analyzed on day 4.

Detection of cell surface α_v by ELISA. Oligonucleotide-treated cells plated in the 96-well plates were analyzed by ELISA (16). After removal of the oligonucleotide-containing medium, the cells were washed and incubated with PBS containing 1% BSA for 30 min. Anti-α_v mAb LM142 was used at a dilution of 1:500 followed by an alkaline phosphatase-conjugated rabbit anti-mouse IgG diluted 1:1,000. ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) was used as a substrate and the colorimetric reaction measured with a microplate reader (3550; Bio-Rad Laboratories) at a wavelength of 415 nm.

Cell adhesion assays. Microtiter plates were coated with 5 μg/ml of human vitronectin (Gibco Laboratories), human fibronectin, or mouse laminin, (both from Collaborative Research Inc., Bedford, MA). Cell adhesion was measured as we previously described using 4×10^4, ^{51}Cr-labeled tumor cells/well and a 30 min incubation at 37°C (9).

mAb-mediated ligation of α_v complexes. One million MeWo LNI 6I cells were seeded in 6-well culture plates precoated with 10 μg/ml of mAb P3G8 for a 24-h incubation. RNA was extracted and uPAR mRNA analyzed as described above. As controls, wells precoated with mAb A3-1IF5 to human α_3 or mAb W6/32 to MHC class I antigens were used.

Statistics. The Student's *t* test was used to analyze differences in cell adhesion to ECM proteins.

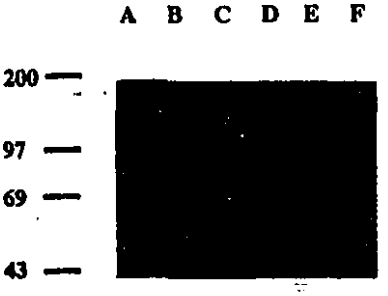

Figure 2. Analysis of cell surface $\alpha_v\beta_3$ by immunoprecipitation. ^{125}I-radiolabeled proteins were precipitated from NP-40 extracts of 6I (lane *A*), MeWo (lane *B*), MIM/8 LNI (lanes *C* and *E*), and MIM/8 (lanes *D* and *F*) cells with mAbs LM142 (lanes *A–D*), or AP-3 (lanes *E* and *F*). The immunoprecipitated proteins were separated by electrophoresis on 7.5% polyacrylamide gels under nonreducing conditions and the protein bands visualized by autoradiography of the dried gels. The positions of prestained molecular weight standards are indicated by the bars.

Results

To measure $\alpha_v\beta_3$ expression in the parental and lymph node metastases-derived cells, Northern blot analysis and immunoprecipitation with monoclonal antibodies to α_v (mAb LM142) and β_3 (mAb AP-3) were used. The results were in accord with our previous observations based on flow cytometry (9). They show that the expression of α_v mRNA in the metastatic cells was significantly increased as compared to the parent lines. When normalized against control cyclophilin mRNA levels and analyzed by densitometry, MIM/8 LNI and 6I cells were found to express 10- and 55-fold more α_v mRNA than MIM/8 and MeWo cells, respectively, (Fig. 1). This was reflected in in-

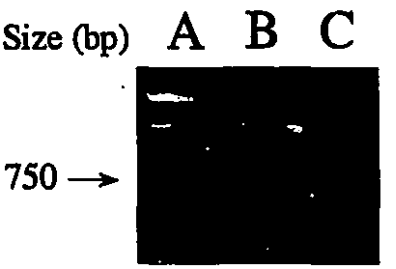

Figure 4. Detection of uPA message by RT-PCR. 1 μg of total cellular RNA derived from MeWo (*B*) and 6I (*C*) cells was reverse transcribed and the subsequent cDNA was amplified using specific uPA primers. The cDNA product was electrophoresed on a 1% TBE-agarose gel. The expected size of the PCR product was 745 bp. Size standards are shown in lane *A*.

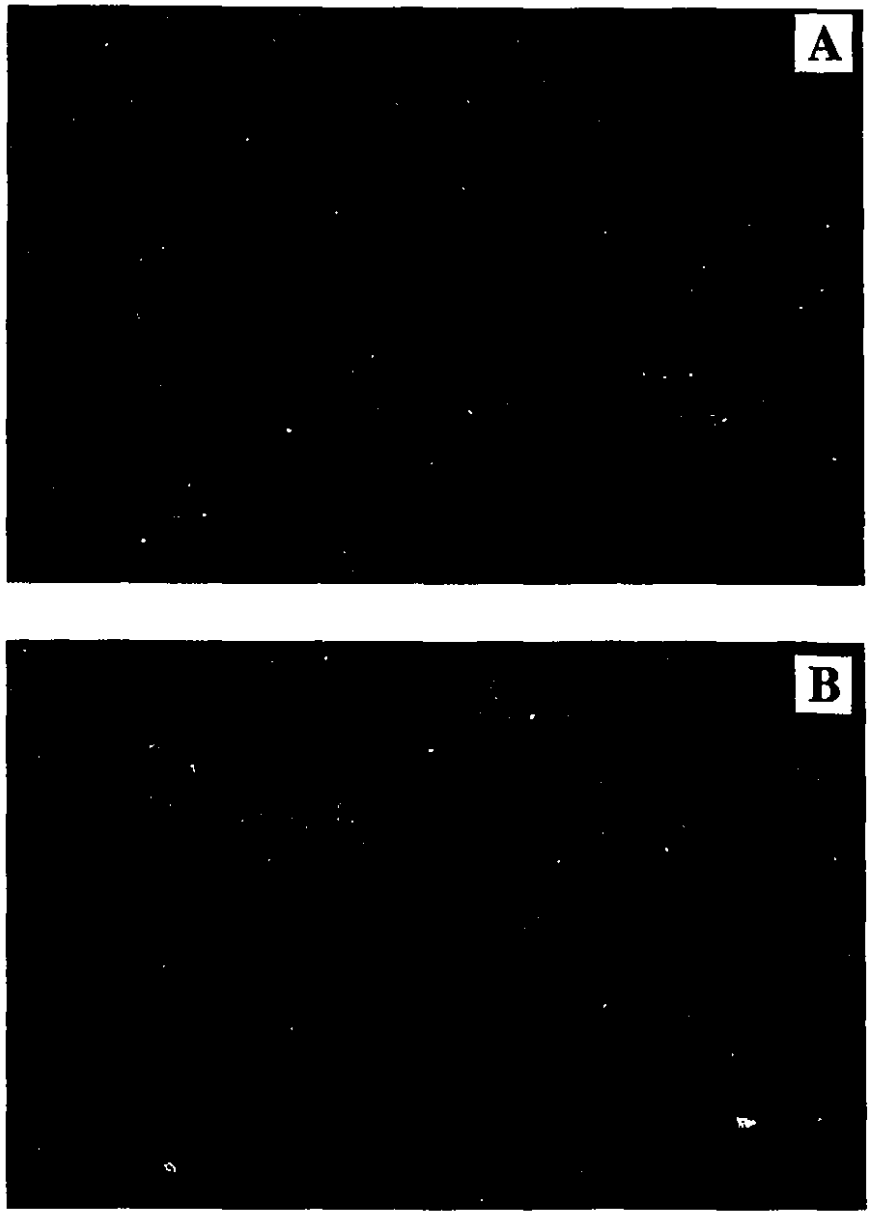

Figure 5. Immunocytochemical analysis of cell-surface-associated uPA. Melanoma cells were cultured on 8-well chamber slides and incubated first with a polyclonal antiserum to uPA (anti-GFD) and then with a peroxidase-conjugated goat antiserum to rabbit IgG. Photomicrographs of representative areas from MIM/8 (*A*) and MIM/8 LNI (*B*) cultures are shown. ×400.

creased cell surface expression of both α_v and β_3 as shown in Fig. 2. mAb LM142 and mAb AP-3 each immunoprecipitated two bands with molecular weights corresponding to α_v and β_3. Densitometric analysis revealed increases of 5- and 90-fold, respectively, in immunoprecipitable α_v on 6I and MIM/8 LNI cells and a 47-fold increase in immunoprecipitable β_3 on MIM/8 LNI cells, as compared to the respective parent lines.

Urokinase has been strongly implicated in melanoma metastasis (11, 12, 27) and ECM vitronectin in its regulation (13). It was therefore of interest to determine whether expression of urokinase and its receptor were also modulated in the metastatic melanoma cells. When mRNA transcripts for uPAR and uPA were measured, we found that the patterns of uPAR expression on the metastatic sublines paralleled those seen for α_v mRNA, namely the expression was significantly increased for 6I (X5) and MIM/8 LNI (X13) cells as compared to the respective parent lines (Fig. 3). No uPA mRNA could be detected by the Northern blot assay when as much as 5 μg poly(A)$^+$ RNA of each cell type were analyzed (Fig. 1), although under similar conditions, uPA transcripts were detectable in RNA extracted from a uPA-rich human prostate carcinoma cell line, PC-3 (data not shown). When analyzed by the reverse transcriptase-PCR method, urokinase cDNA was detectable in all melanoma cell lines suggesting that low levels of the enzyme were expressed (Fig. 4).

When a rabbit anti-uPA antibody (anti-GFD) was subsequently used to immunolabel the cells, we found that significantly higher levels of uPA could be detected on the metastatic cells than on the parental cells (Fig. 5), suggesting that the former could more efficiently bind the enzyme on the cell surface.

To address the question of a possible coordination at the transcriptional level in expression of the vitronectin and urokinase receptors, two approaches were used. First, expression of α_v in 6I cells was suppressed by treatment with α_v antisense oligonucleotides. This treatment had no apparent effect on cell

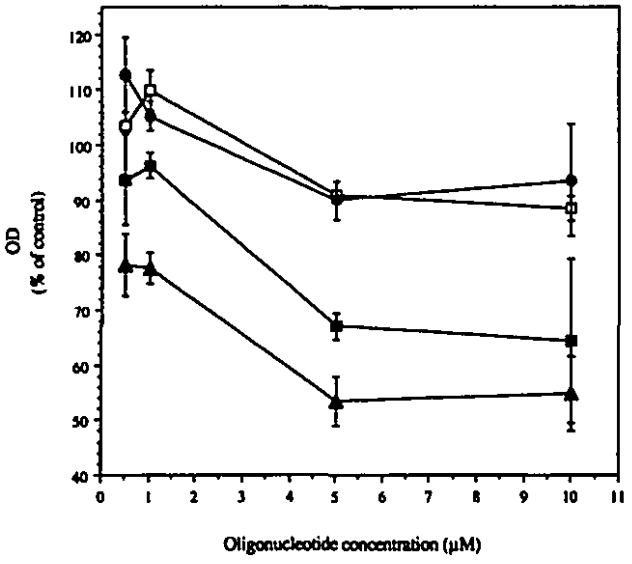

Figure 6. Inhibition of α_v synthesis by antisense oligonucleotides. 6l cells were treated with antisense oligonucleotides, AS2 (●), AS3 (■), and AS4 (▲); or with the sense oligonucleotide, S1 (□). After treatment, cell surface expression of α_v was measured with mAb 142 using an ELISA. Results shown are expressed as absorbance at 415 nm relative to control untreated 6l cells and represent means ±SD of triplicate wells.

morphology or the integrity of the monolayer as confirmed by phase contrast microscopy and did not reduce cell viability as assessed by trypan blue exclusion dye. A reduction in cell surface $\alpha_v\beta_3$ expression was confirmed by ELISA (Fig. 6) and was reflected in a marked and substratum-specific decrease in cell adhesion to vitronectin (up to 70% inhibition; $P = 0.009$) in the antisense-treated cells (Table I). When α_v expression was analyzed, a nine-fold decrease in α_v mRNA expression was seen as assessed by Northern blot analysis (Fig. 7 *A*). In these cells, a significant decrease was also seen in uPAR mRNA. In a total of three experiments performed, uPAR mRNA transcripts were decreased 1.78–2.5 (mean 2.2)-fold relative to control cells. A representative experiment is shown in Fig. 7 *B*. In all the experiments, treatment with 10–20 μM sense oligonucleotides failed to reduce uPAR mRNA and in fact caused a slight increase (up to 21%) in some of the experiments. The reduction in uPAR mRNA was specific, as no decrease was seen in the mRNA levels of another related proteinase expressed by these melanoma cells, namely, MMP-2 (Fig. 7 *C*).

As a second approach and to determine whether uPAR expression was linked to vitronectin receptor function, we investigated the effect on uPAR mRNA expression of antibody-mediated ligation of the vitronectin receptor. Melanoma 6l cells were plated on culture dishes precoated with mAb P3G8 and their RNA extracted and analyzed. mAb W6/32 directed to an MHC determinant and mAb A3-IIF5 directed to integrin subunit α_3, which is also expressed by 6l cells (unpublished data), were used as controls. Results of a representative experiment shown in Fig. 8, demonstrate an antibody-specific 2.4-fold increase in uPAR message in cells attached to P3G8-coated plates (Fig. 8, lane *C*). In three experiments performed, the increases observed in uPAR mRNA ranged from 1.54- to 2.48-fold in cells plated on P3G8-coated dishes (mean stimulation was 1.97±0.47). On

Table I. Reduced Adhesion to Vitronectin in Melanoma Cells Treated with α_v Antisense Oligonucleotides

	Adhesion (percentage of control)	
	Oligonucleotide	
ECM protein	AS3	S1
Vitronectin	42.7±9.4	118.8±14.*
Laminin	145.6±11.1	
Fibronectin	139.5±5	
None	189±20	

6l cells were treated with 25 μm of the oligonucleotides for 3 d and then dispersed and used to measure adhesion to microtiter plates precoated with 5 μg/ml of the ECM proteins. Adhesion of untreated cells was measured at the same time and used as control to calculate relative adhesion levels. Results shown are means±SD of triplicate samples. Adhesion of AS3-treated cells to vitronectin was significantly reduced ($P = 0.023$) relative to untreated cells, whereas it was unchanged for laminin ($P = 0.187$), fibronectin ($P = 0.124$), and increased for uncoated plates ($P = 0.0152$).

the other hand, cells plated on mAb A3-IIF5 showed no increase in uPAR mRNA with levels ranging from 0.76 to 1.06 of controls while in cells plated on mAb W6/32 the levels were slightly reduced and ranged from 0.79 to 0.94 of controls. Similar findings (2.2-fold specific stimulation of uPAR) were obtained with MeWo cells in which the $\alpha_v\beta_3$ receptor was ligated with mAb LM609 under the same conditions (not shown).

Discussion

The evidence for the involvement of both $\alpha_v\beta_3$ and uPAR in melanoma invasion and metastasis is compelling (6, 7, 11, 12, 27). Recent findings also suggest that the two receptors are functionally linked (13, 28). In the present study, the possibility of a regulatory link in the expression of these receptors was investigated.

We found that human melanoma cells selected for increased metastasis to regional lymph nodes expressed higher levels of α_v mRNA transcripts and increased levels of cell surface α_v and β_3 as compared to the parent cell lines. This confirms and extends our previous finding that metastatic melanoma cells have an increased $\alpha_v\beta_3$-mediated adhesion to lymph node vitronectin (9). In addition, our results show that the highly metastatic cells expressed significantly more uPAR mRNA transcripts than the parental cells. These cells provide therefore a unique experimental model for analysis of the functional relevance of $\alpha_v\beta_3$ to melanoma metastasis on one hand and the link between expression and synthesis of these two receptors on the other. In this context, it should be noted that the increases observed in expression of the $\alpha_v\beta_3$ complex in the two melanoma models were not proportional to the increases in α_v mRNA transcripts (Fig. 1). This may be due to differences either in the levels of the β_3 subunit available for complex formation or in posttranscriptional processing of the α_v and β_3 subunits in these cells.

While the expression of uPAR transcripts was increased in the metastatic cells, expression of uPA mRNA was relatively low. However, higher levels of cell surface uPA could be de-

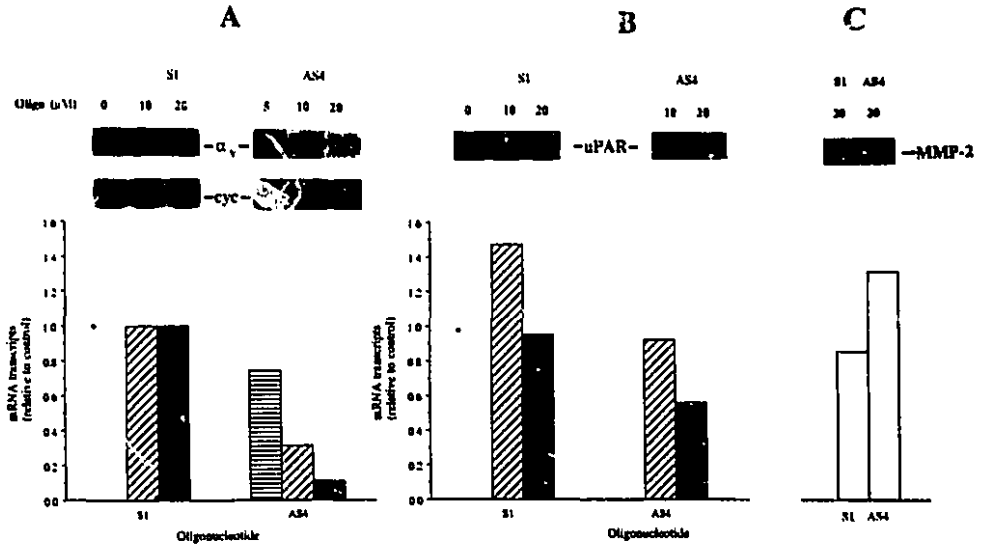

Figure 7. Northern blot analysis of α_v and uPAR mRNA transcripts in cells treated with antisense oligonucleotides. 15 μg of total RNA extracted from cells treated with oligonucleotides AS4 or S1, were electrophoresed on a 1.1% agarose gel, blotted to nylon membranes, and probed as described in Methods (*top*, panels *A*, *B*, and *C*). Results of the densitometric analysis are shown (*bottom*, panels *A*, *B*, and *C*). All values have been normalized relative to the respective untreated cells which were assigned a value of 1.0 (indicated by *).

tected on these cells by immunoperoxidase labeling suggesting that they could more efficiently immobilize uPA from the extracellular environment (13). In a recent study of colon adenocarcinoma tissue, high levels of uPAR were observed in association with the malignant cells while uPA was detected only on the adjacent stromal cells (29, 30). Other findings also suggest that the uPA/uPAR system can function either in an autocrine fashion or it may involve the cooperation of several different cell types, each contributing a different component of the system (13, 29, 30). It is conceivable that in the present model, the invasive and metastatic melanoma cells could utilize either endogenous and/or exogenous uPA produced and secreted into the microenvironment of the lymph node by stromal or monocytic cells (31).

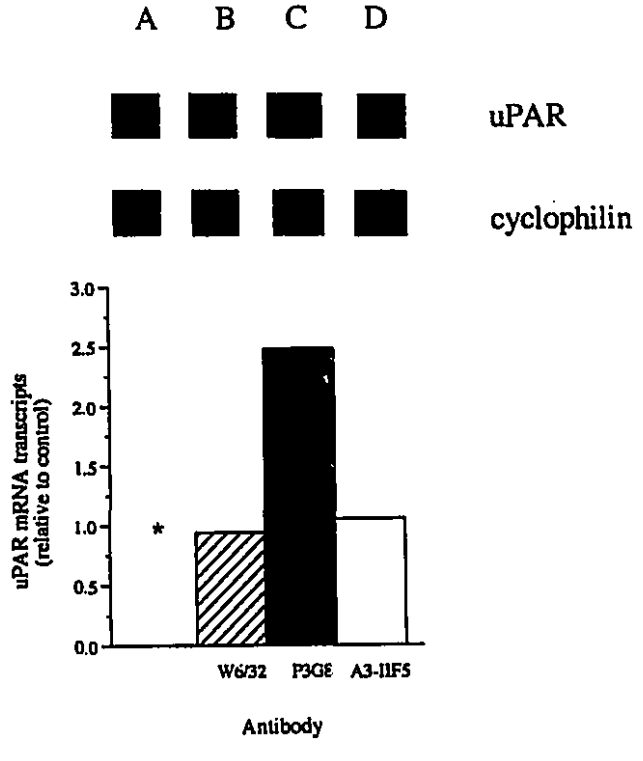

Figure 8. Increased uPAR mRNA expression in response to ligation with an mAb to α_v. RNA was extracted from 6l melanoma cells grown overnight on uncoated (*inset*, lane *A*), mAb W6/32- (*B*), mAb P3G8- (*C*), or mAb A3-IIF5- (*D*), coated wells. Northern blot analysis and laser densitometry were performed as described in the legend to Fig. 3. The results of the densitometry are shown in the bar graph. The values have been normalized relative to the respective untreated cells which were assigned a value of 1.0 (indicated by *). Results shown in the inset are from a representative experiment of three performed.

In an attempt to determine whether the increased expression of uPAR in the metastatic cells was linked to the elevated expression of $\alpha_v\beta_3$, two approaches were used. First, antisense phosphorothioate oligonucleotides corresponding to unique sequences in the α_v subunit were used to suppress synthesis of the vitronectin receptor. Our results show that treatment with antisense oligonucleotides significantly reduced cell surface expression of the α_v integrin complex and cell adhesion to vitronectin. It also resulted in a concomitant specific reduction in uPAR message. This suggests that uPAR synthesis in these melanoma cells is regulated by α_v levels.

Several mechanisms may be invoked in interpreting these results. It is conceivable that uPAR synthesis is transcriptionally regulated through a feedback mechanism involving cellular α_v or the α_v-containing integrin complexes. This could provide a mechanism for coordinating cell adhesion, matrix degradation, and motility since $\alpha_v\beta_3$ mediates adhesion to matrix vitronectin, and the uPAR/uPA complex is involved in localized matrix degradation at the cell–substratum interphase during cell migration. Alternatively, ligand binding by $\alpha_v\beta_3$ may trigger a signaling cascade leading to upregulation of uPAR synthesis. This may be mediated through upregulation of autocrine growth factors which are produced by melanoma cells and can modulate uPA and uPAR expression (32, 33) such as basic fibroblast growth factor (34) and TGF-β (35). The latter mechanism is supported by our finding that mAb-mediated ligation of the vitronectin receptor caused a specific increase in cellular levels of uPAR mRNA. In cells whose α_v production has been suppressed by treatment with antisense α_v oligonucleotides, the decline in uPAR expression may be a consequence of the re-

duced adhesion of these cells to vitronectin present in the culture medium or deposited into the ECM by the melanoma cell. As these melanoma cells also express a receptor for fibronectin ($\alpha_5\beta_1$, results not shown) it is not entirely surprising that despite the reduction in α_v levels, no immediate changes were observed in the adhesiveness or viability of the antisense-treated cells.

While our results are consistent with a role for $\alpha_v\beta_3$ in regulation of uPAR expression, the participation of other α_v integrins such as $\alpha_v\beta_5$ and $\alpha_v\beta_1$ cannot be entirely ruled out. It should be noted however, that FACS® analyses with mAb IA9 to subunit β_3 (36), (a gift from Dr. M. Hemler, Dana-Farber Cancer Institute, Boston, MA) failed to show increases in β_3 expression on the metastatic cells relative to parental lines. MIM/8 LN1 cells showed in fact a slight reduction (63% positive cells; mean intensity of fluorescence 588) in cell-surface β_3 as compared to the parent MIM/8 cells (76% positive cells, mean intensity of fluorescence 620) (results not shown). We have also previously shown that cell surface levels of β_1 were not altered in the metastatic cells (9). These findings suggest that the other α_v complexes if involved, may play more minor roles.

Antibody- or ligand-mediated cross-linking of integrins has been shown to trigger signal transduction mechanisms leading to protein tyrosine phosphorylation (37), changes in calcium influx (38), gene transcription (39), and altered cell growth. Several lines of evidence suggest that $\alpha_v\beta_3$ is also involved in signal transduction mechanisms. Thus, $\alpha_v\beta_3$ was implicated in the regulation of processes such as cell migration (40), differentiation, angiogenesis (41), and tumorigenicity (8). Cross-linking of $\alpha_v\beta_3$ was shown to increase melanoma cell invasiveness (7) and several $\alpha_v\beta_3$-associated proteins have been described, at least one of which, a 190-kD protein, is phosphorylated in the presence of PDGF (42). As several of these processes such as cell migration and invasion also involve uPA-mediated proteolysis (43), it is conceivable that some of the effects attributed to $\alpha_v\beta_3$ were actually mediated through upregulation and cell-surface localization of the uPAR/uPA complex. In a recent series of reports (32, 44, 45) it has been shown that uPA and uPAR expression in cultured endothelial cells is regulated by basic fibroblast growth factor and that uPA in turn can catalyze the proteolytic conversion of the precursor of TGFβ into its biologically active form. As TGFβ may enhance melanoma cell proliferation (46), the induction of uPAR synthesis in response to $\alpha_v\beta_3$ ligand binding may also provide a mechanism for control of melanoma cell growth. In the regional lymph node, stromal cell–derived or autocrine basic fibroblast growth factor (47) may initiate this cascade when tumor cells adhere to stromal vitronectin (9) resulting in increased melanoma cell invasion and migration.

The transcriptional regulation of uPAR synthesis is poorly understood (13). Yet the evidence is strong that this receptor is of major importance to the invasion and metastasis of several malignancies, including melanoma (11, 12, 27). A better understanding of the regulatory role that integrin $\alpha_v\beta_3$-mediated adhesion plays in uPAR synthesis and function will shed light on the mechanisms regulating this receptor and can potentially provide a basis for the development of specific biological reagents designed to inhibit the aggressive behavior of malignant melanoma.

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Human Melanoma Cells Derived from Lymphatic Metastases Use Integrin $\alpha_v\beta_3$ to Adhere to Lymph Node Vitronectin

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Abstract

Human melanoma is a highly metastatic cancer and the regional lymph nodes are generally the first site of metastasis. Adhesion to cryostat sections of human lymph nodes was therefore studied using two human melanoma models established from lymph node metastases, namely, MeWo cell lines of diverse metastatic potentials and a highly metastatic cell line of recent origin designated MIM/8. We found a good correlation between the metastatic potentials of the melanoma cells as measured in nude mice and their ability to adhere to cryostat sections of human lymph nodes. When adhesion to immobilized extracellular matrix proteins was measured, a significant increase in adhesion, which correlated with increased metastasis, was seen mainly on vitronectin and to a lesser extent on fibronectin. The adhesion to vitronectin and to the frozen sections were specifically blocked by an RGD-containing peptide, mAb 661 to vitronectin and mAb LM609 to integrin $\alpha_v\beta_3$. FACS® analysis revealed a significant and specific increase in cell surface expression of $\alpha_v\beta_3$ on the metastatic cells as compared to the parent line. Together these results suggest that the adhesion of melanoma cells to lymph node vitronectin via the $\alpha_v\beta_3$ receptor plays a role in the process of lymphatic dissemination. (*J. Clin. Invest.* 1992. 90:1406–1413.) Key words: lymphatic metastasis • adhesion • vitronectin receptor

Introduction

The regional draining nodes are the first site of metastasis for the majority of primary human neoplasms, including malignant melanoma. In melanoma patients, the appearance of regional and distant lymph node metastases is a major determinant of poor prognosis (1). Despite the importance of this process to the prognosis and treatment of human malignancies the underlying mechanisms remain virtually unexplored.

Cellular adhesion is now known to play a major role in the process of cancer dissemination. Tumor cell adhesion to various host cells such as lymphocytes, endothelial cells, or platelets (2–4) as well as to extracellular matrix proteins has been shown to regulate invasion and proliferation and promote tumor metastasis (for review see references 5 and 6). Integrins, a family of cell adhesion proteins that mediate cell–cell and cell–matrix interactions, some through recognition of the Arg-Gly-Asp peptide, have been implicated in the process of hematogeneous dissemination, as they mediate tumor cell attachment to vascular endothelial cells and to subendothelial matrix proteins such as laminin and fibronectin (5, 7). The involvement of integrins in the process of lymphatic metastasis has not, however, been investigated.

Previously, we have shown that tumor cell potential to metastasize lymphatically correlated well with tumor cell adhesion to frozen sections of lymph nodes in several carcinoma models of human, rat, and mouse origin (8–10). Here we extend these findings to two human melanoma models and show that adhesion of the melanoma cells to the frozen sections is $\alpha_v\beta_3$ mediated and that vitronectin is the primary adhesion ligand. The increased expression of $\alpha_v\beta_3$ on melanoma cells may therefore promote their potential to adhere in and metastasize to the lymph nodes.

Methods

Animals. 4–6-wk-old female nu/nu (CD-1)Br mice obtained from Charles River Canada (St. Constant, Quebec, Canada) were used to propagate the human melanoma line and to isolate lymph node–metastasizing cells. The mice were housed in Micro-isolator cages obtained from Lab Products Inc. (Maywood, NJ) and handled in a biological containment cabinet. Under these conditions, they remained disease free for ≥ 12 mon.

Tumor lines. The human melanoma cell line MeWo, which was originally established from a lymph node metastasis of a malignant melanoma patient (11), was obtained courtesy of Dr. R. S. Kerbel (Mt. Sinai Hospital, Toronto, Ontario, Canada). The cells were maintained as monolayer cultures in RPMI 1640 supplemented with 10% FCS, 1% gentamycin sulfate, 1% penicillin–streptomycin, and 2 mM glutamine (all reagents from Gibco Laboratories, Burlington, Ontario, Canada) (RPMI–FCS). Monolayers were dispersed twice weekly with Ca^{2+}- and Mg^{2+}-free PBS containing 0.02% EDTA and the cells were reseeded at a dilution of 1:4. All tumor cell cultures were incubated at 37°C in a 5% CO_2 incubator.

Selection and cloning of lymph node–metastasizing MeWo LNI 61 cells. Regional (inguinal, axillary, or brachial) lymph node metastases were detectable in some of the nude mice within 9 wk after the intradermal injection of 5×10^6 tumor cells when the primary tumor had reached a mean tumor diameter of ~ 1.0 cm. The animals were killed when the primary tumor was > 2.5 cm in diameter or when moribund. Pulmonary metastases were occasionally observed. The regional draining lymph nodes were aseptically removed and mechanically dispersed through an 85-μm filter (Nitex; B&SH Thompson, Town of Mount Royal, Canada). The cells were centrifuged and then seeded onto 25-cm^2 tissue culture flasks (Falcon; Fisher Scientific Co., Montreal, Canada) containing RPMI 1640 with 10% FCS. On the following day, the monolayers were washed with RPMI 1640 to remove nonadherent cells (mainly lymphocytes) and debris. This procedure was routinely

Portions of this work have appeared in abstract form (1990. *Clin. & Exp. Metastasis* 8[Suppl. 1]:25–26).

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carried out to confirm the presence of melanoma cells in the nodes. After one such procedure, confluent monolayers of lymph node–derived melanoma cells (designated MeWo LNI) were obtained. The monolayer was subsequently dispersed and the cells replated at low density leading to the isolation of individual clones. One of these clones, MeWo LNI 6I (6I),[1] was selected for further study as it had a high level of adhesion to frozen lymph node sections (see below). This clone was used for the studies described. For some of the experiments, tumor cells were also isolated from the primary MeWo tumor by enzymatic digestion with a trypsin/EDTA solution supplemented with DNAse (12). The dispersed tumor cells were passed through an 85-μm Nitex mesh, the filtrate spun down, and the cells plated in culture dishes. These cells were designated MeWo(v) to distinguish them from the MeWo cell line maintained in vitro only. MeWo(v) cells were cultured for ≤ 1 wk before use in the experiments.

Melanoma line MIM/8. A cell line designated MIM was established from an inguinal lymph node metastasis of a 66-yr-old male melanoma patient obtained recently through the pathology laboratory of the Royal Victoria Hospital, Montreal, Canada. The node was minced and the cells passed through an 85-μm Nitex mesh membrane as described above for MeWo LNI 6I. A single-cell suspension was obtained and plated onto a culture dish precoated with 10 μg/ml type I collagen. The melanoma cells were grown to confluence and then cloned by the limiting dilution method. A pigmented clone, MIM/8, was selected for further study since it was found to be highly tumorigenic and metastatic, giving rise to tumors in 4/5 intradermally inoculated nude mice and to regional node metastases in 3/4 tumor-bearing mice. FACS® analysis (Becton Dickinson Co., Mountain View, CA) with mAb LM609 revealed that 95% of MIM/8 cells were $\alpha_v\beta_3$ positive.

Lymph node metastasis assay. Nude mice were inoculated intradermally in the right lateral flank with 5×10^6 melanoma cells. The mice were monitored twice weekly and the size of the primary tumor measured. A local tumor was observed ~ 6 wk after inoculation. Positive regional axillary or brachial lymph nodes were palpable ~ 3 wk after the appearance of the local tumors when the mean diameter measured ~ 1.0 cm.

Anchorage-independent growth assay. To measure anchorage-independent growth of the melanoma cells, a modification of the standard soft agar cloning assay was used (13–15). Briefly, tumor cells were mixed with a solution of either 0.6 or 1.2% agar (Difco Laboratories Inc., Detroit, MI), added to an equal volume of a 2× concentrated complete RPMI–FCS medium, and plated at a concentration of 10^3 cells/well into six-well plates (Falcon; Fisher Scientific Co.) that were prelayered with a 1.2% agar solution. The overlay was allowed to solidify and covered with 1 ml RPMI–FCS. The medium was replenished on alternate days for 12 d, at which time the tumor colonies were fixed with 1% glutaraldehyde and stained with Coomassie Blue R-250 (16). The number and diameter of the colonies were determined using a microscope (Diaphot-TMD Inverted Nikon Canada) equipped with an ocular square millimeter grid.

Cell adhesion to cryostat sections of human lymph nodes. Human lymph nodes were obtained from the Surgical Pathology Laboratory of the Royal Victoria Hospital (Montreal, Canada). The nodes were removed from patients presenting with cervical node enlargement and were snap frozen in liquid N_2. They were routinely analyzed by electron microscopy and immunohistochemistry for evidence of malignancy and used when the pathology findings were negative. Cell adhesion to the cryostat sections was measured as described in detail elsewhere (8). In all the experiments 5-μm cryostat sections were used with 5×10^5 ^{51}Cr-labeled tumor cells suspended in a volume of 40 μl.

Cell adhesion to extracellular matrix proteins. Microtiter plates were coated with human fibronectin, mouse laminin, type IV collagen (all from Collaborative Research Inc., Bedford, MA), or human vitronectin (Sigma Chemical Co., St. Louis, MO). The proteins were added to the wells at concentrations ranging from 1 to 50 μg/ml and the wells allowed to dry in a laminar flow hood overnight. Before the adhesion assay, nonspecific protein-binding sites were blocked by the addition to each well of 0.05 ml of a 1% BSA solution in PBS for 1 h. The cell-adhesion assay was essentially that described elsewhere (17), with some modifications. Briefly, tumor cells (1×10^7 cells in 75-cm^2 flask) were metabolically labeled by incubation overnight with 250 μCi of ^{35}S-methionine (Tran ^{35}S-Label™, ICN Biomedicals Inc., Costa Mesa, CA). The following day, the cells were washed, added to the protein-coated wells, and incubated at 37°C for up to 60 min. The unbound tumor cells were aspirated and the wells were washed three times with PBS. Cells were lysed with 1 N NaOH and the radioactivity in the lysate measured in a liquid scintillation counter. Adhesion to BSA-coated wells was negligible (< 1%).

Antisera and peptides. The origin and specificity of mAb LM609 directed to the $\alpha_v\beta_3$ receptor were described in detail elsewhere (17). mAb P4C10 directed to a functional epitope on the β_1 integrin subunit (18) was a kind gift from Dr. E. A. Wayner (Cytel Co., La Jolla, CA). mAb W6/32 to human HLA class I, which recognizes an antigenic determinant common to products of the HLA-A, B, and C loci, was obtained from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada). mAb 661 to vitronectin was produced and characterized by Drs. D. J. Loskutoff and D. Cheresh. Balb/C mice were immunized with purified human vitronectin and the hybridomas initially tested by ELISA on vitronectin-coated wells. The supernatants of those hybridomas that were positive in the initial screening were subsequently analyzed for the ability to block the adhesion of human melanoma line M21 to vitronectin-coated dishes (17) and mAb 661 was found to be highly inhibitory. In initial tests this antibody blocked the adhesion of 6I cells to vitronectin but not to fibronectin (see Results, Fig. 5). mAb 3E3, which is directed to the cell attachment fragment of human fibronectin, was obtained from Boehringer Mannheim Canada (Laval, Quebec, Canada). Normal mouse immunoglobulin was obtained from Bio Can Scientific (Mississauga, Ontario, Canada). Rabbit anti-mouse IgG was obtained from Dakopatts A/S (Glostrup, Denmark). Peptides, Gly-Arg-Gly-Asp-Ser-Pro-Lys and Gly-Arg-Gly-Glu-Ser-Pro, were obtained from Hukabel Scientific (Longueuil, Quebec, Canada) and Peninsula Laboratories Inc. (Belmont, CA), respectively.

Inhibition assays. Tumor cells were incubated with the antibodies for 45 min at 4°C. Excess antibody was washed off before addition of the cells to lymph node sections or to vitronectin. Inhibition by peptides was measured after incubation of the cells with various peptide concentrations for 15 min at room temperature. Cells were added to the frozen sections or to vitronectin-coated wells without prior washing. When the effects of mAb 3E3 and 661 on cell adhesion were tested they were added to the frozen sections or to vitronectin-coated wells together with the radiolabeled cells.

Indirect immunofluorescence assay. Tumor cells were incubated for 2 h at 37°C on eight-chamber slides (Lab-Tek; Nunc, Inc., Naperville, IL) that had been precoated with 10 μg vitronectin. The cells were fixed with 1% glutaraldehyde/PBS and permeabilized with 0.4% Triton X-100 in PBS containing 50 mM glycine HCl. The chambers were washed with RPMI and mAb LM609 added at a dilution of 1:25 for 1 h at 4°C, followed by a second incubation with FITC-conjugated goat anti-mouse IgG (Bio/Can Scientific) at a dilution of 1:100. The slides were dried and mounted with a glycine–glycerol solution (19).

Flow cytometry. For flow cytometric analysis, 1×10^6 tumor cells were incubated for 1 h on ice first with 65 μg/ml of the mAb and then with FITC-conjugated goat anti-mouse IgG (150 μg/ml). After washing, the cells were resuspended in 1 ml of RPMI 1640 and analyzed using a cell analyzer (FACSTAR; Becton Dickinson, Mountain View, CA) equipped with an argon-ion (15 MW) laser at 488 nm with a 530/30 DF filter. The program used to analyze the data was Consort 30™ Version E 12/86 (Becton Dickinson). Control cells were incubated with the second antibody only. For each cell line ~ 5,000 cells were analyzed.

1. *Abbreviations used in this paper:* ECM, extracellular matrix; MIF, mean intensity of fluorescence; RGD, Arg-Gly-Asp; 6I, line MeWo LNI6I.

Statistics. Student's *t* test was used to analyze adhesion data and Fisher's Exact test was used to analyze results of in vivo studies (20).

Results

Metastatic MeWo cells were isolated from the regional lymph nodes of tumor-bearing nude mice, cultured in vitro for ~ 2 wk and their adhesion to cryostat sections of human lymph nodes measured and compared with that of the parental MeWo line. The results shown in Fig. 1 are consistent with previous findings we obtained using a breast carcinoma model (9) and demonstrate that the lymph node metastases-derived tumor cells were significantly more adherent to lymph node cryostat sections than the parent line. The metastatic cells were subsequently cloned and clone 6I was selected for further study as it was found to be highly adherent to lymph node cryostat sections (Fig. 1). When the tumorigenic and metastatic properties of clone 6I were subsequently analyzed by inoculation of the tumor cells intradermally in nude mice, we found that tumor-bearing animals inoculated with 6I cells had a significantly higher incidence of lymph node metastases than animals inoculated with the MeWo cells, whereas the incidence and rates of growth of the primary tumors were not markedly different (Table I). Furthermore, 6I cells had a significantly higher cloning efficiency in semisolid agar (Table II), a property previously shown to correlate with the metastatic potential of melanoma cells (13).

The adhesion of MeWo and 6I cells to extracellular matrix proteins that were previously identified in human lymph nodes (21, 22), namely, fibronectin, laminin, type IV collagen, and vitronectin, was subsequently investigated using matrix protein-coated dishes. As shown in Fig. 2, a significant difference in adhesion between MeWo and 6I cells at all time intervals tested was seen only on vitronectin-coated plates. Attachment of 6I cells to other matrix proteins was lower than that to vitronectin and differences between the adhesion of the two cell lines, where seen, were of a considerably lower magnitude. Adhesion to BSA was < 1%.

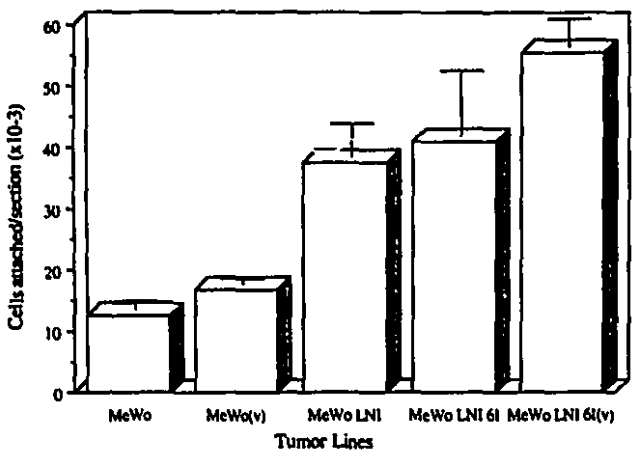

Figure 1. Adhesion of MeWo cells to cryostat sections of human lymph nodes. Sublines that have been passaged in vivo and readapted to culture in vitro are denoted with a (v). Results are means and SD of quadruplicate samples. The proportion of the 6I and MeWo LNI cells that adhered to the sections was significantly higher than that of MeWo cells ($P < 0.007$ and 0.039, respectively). There was no significant difference between the adhesion of MeWo LNI and 6I cells ($P > 0.1$).

Table I. Incidence of Primary Tumors and Lymph Node Metastases in Animals Inoculated with Tumor Lines MeWo and MeWo LNI 6I Cells

Cells injected	Incidence of primary tumors	Incidence of regional lymph node metastases
MeWo	14/19*	2/14‡
MeWo LNI 6I	10/10	6/10

Nude mice were injected intradermally in the right lateral region with 5×10^6 cells. Tumors developed within 5–6 wk and lymph node metastases observed when the mean diameter of the tumors measured 1.0 cm. * $P = 0.134$. ‡ $P = 0.032$ (Fisher's Exact Test).

That this differential adhesion to vitronectin was not an artifact of the prolonged in vitro culture of MeWo cells was established by reinoculation of the melanoma cells into nude mice to obtain-MeWo(v). These tumor cells were maintained in culture for only 1 wk to deplete the host cell infiltrate and their adhesion to cryostat sections was then measured. Results shown in Fig. 1 demonstrate that there was no significant difference ($P > 0.12$) between the adhesion of MeWo or 6I cells propagated in vivo and those maintained in vitro for an extended period of time, confirming that the increased adhesion of 6I cells was not due to the in vitro–in vivo selection process per se. Similar results were obtained when the adhesion of these lines to vitronectin-coated dishes was measured (data not shown).

To investigate the role of vitronectin in the adhesion of line 6I cells to lymph node sections, the tumor cells were treated before the adhesion assay with antibody LM609 directed to integrin $\alpha_v\beta_3$. Results shown in Fig. 3, *A* and *B* demonstrate a dose-dependent inhibition by the antibody of attachment to the sections and to vitronectin-coated dishes. Maximal inhibition ranged between 70 and 80% of control levels. A mAb directed to the functional domain of the β_1 subunit (P4C10) also inhibited adhesion to the sections with maximal inhibition ranging between 40 and 45% whereas a mAb directed to an HLA class I determinant (W6/32) had no measurable effect on the adhesion (Fig. 3 *C*).

Table II. Cloning Efficiency of MeWo Cells in Semisolid Agar

	Cloning efficiency*		Colony size‡	
Cell line	0.3%§	0.6%	0.3%	0.6%
			μm	
MeWo	0.27±0.047	0.15±0.05	19.5±2.6	53.3±14.0
MeWo LNI 6I	0.73±0.28	0.37±0.13	113.4±13.3	110.0±46.9

The cloning method is described in Methods. The results are means and SD of triplicate samples. The difference in cloning efficiency and colony size between MeWo and 6I cells was significant ($P < 0.05$) in both agar concentrations. * Cloning efficiency was calculated by counting the number of colonies 12 d after plating 10^3 tumor cells. ‡ The diameters of all the colonies were measured using a Nikon inverted microscope equipped with an ocular grid. § Final concentration of agar in the overlay.

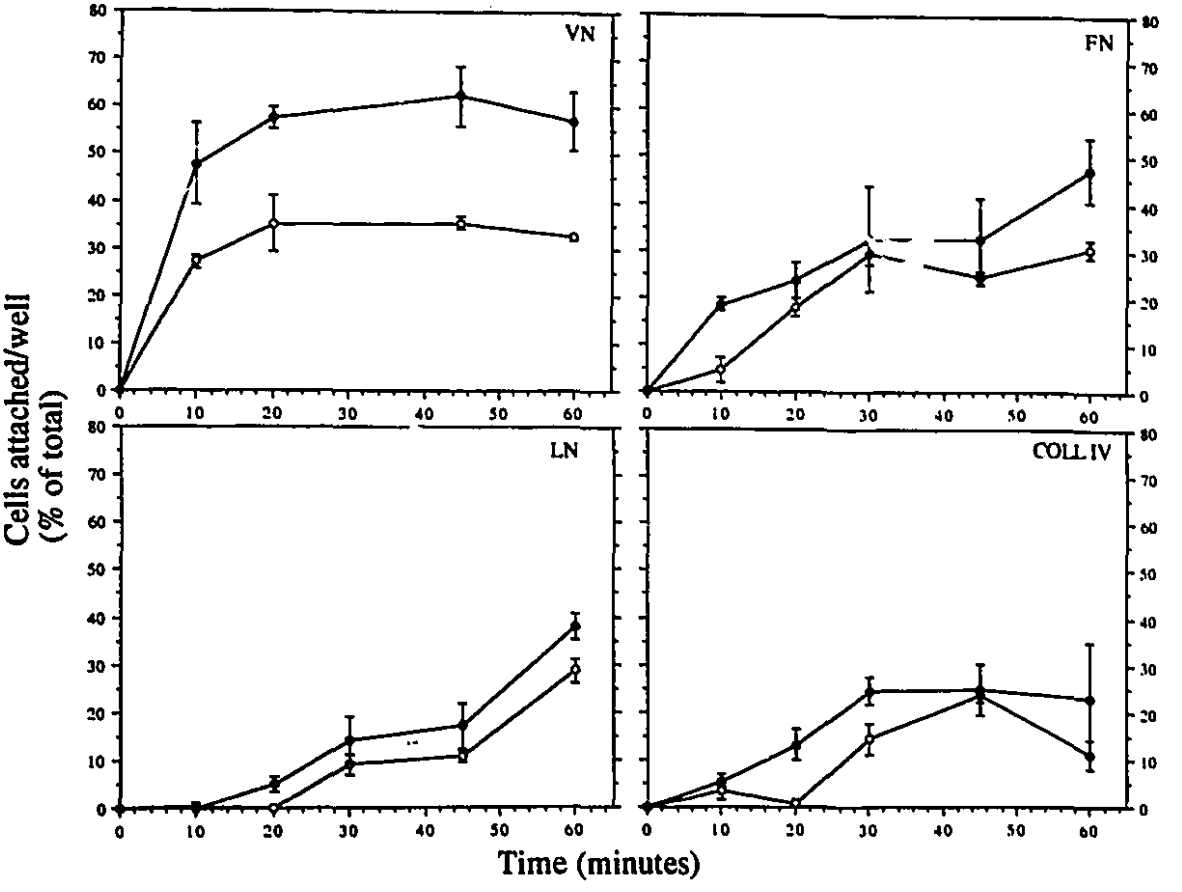

Figure 2. Adhesion of MeWo and 6I cells to ECM protein–coated plates. For coating, 50 μl of a 10 μg/ml solution of the proteins were added to each well of a microtiter plate. Results shown are means and SD of triplicate samples. The background adhesion to BSA-coated wells (< 1%) was subtracted. 6I (●) cells were significantly more adherent to vitronectin than MeWo cells (○) at all time intervals ($P < 0.04$ at 10 and 20 min and $P < 0.001$ at 40 and 60 min). Differences in attachment to fibronectin and laminin were significant only at 60 min ($P < 0.04$ and < 0.01, respectively) and to type IV collagen at 20 and 30 min ($P < 0.05$).

Consistent with the role of vitronectin as the ligand on the lymph node frozen sections was also our finding that an RGD-containing peptide significantly and specifically blocked the adhesion of 6I cells to the sections (shown in Fig. 4). Furthermore, a mAb to human vitronectin (mAb 661), which blocked the binding of 6I cells to vitronectin-coated dishes (Fig. 5 *B*) but not to fibronectin-coated dishes (Fig. 5 *C*), also inhibited the adhesion of these cells to the cryostat sections by 80% (Fig. 5 *A*). In contrast a mAb, directed to the cell attachment fragment of human fibronectin (mAb 3E3), inhibited adhesion to the sections by only 19%.

Cell surface expression of $\alpha_v\beta_3$ on the MeWo cells was subsequently measured by flow cytometric analysis using indirect immunofluorescence staining. As shown in Table III and Fig. 6, the proportion of positively labeled cells was significantly higher in the 6I subline relative to MeWo. This was also reflected in an increase in the mean intensity of fluorescence (MIF) (from 133.2 for MeWo to 163.2 for 6I). When the MIF measured with MeWo cells was used as an arbitrary lower limit (Fig. 6 *A*, *solid vertical bar*), to estimate the proportion of "brightly" fluorescing cells in the two cell lines, as many as 40% of 6I cells but only 8.1% of MeWo cells ($P < 0.001$) were found to be brightly stained. These results were in contrast to fluorescence profiles obtained with antibodies P4C10 and W6/32, where no increase in the proportion of labeled cells or in MIF for 6I cells was observed (Fig. 6 *B* and *C* and Table III). Fluorescein-labeled 6I cells cultured on vitronectin-coated dishes are shown in Fig. 7. The cells were virtually indistinguishable morphologically from MeWo cells.

To determine whether our findings with the MeWo line were relevant to other melanomas, in particular melanoma cells of more recent origin, a second melanoma model recently developed from a human lymph node metastasis was used. A pigmented clonal subline, designated MIM/8, was obtained and found to be highly tumorigenic and metastatic, giving rise to primary tumors in four of five intradermally inoculated nude mice and to regional lymph node metastases in three of four tumor-bearing mice.

When adhesion of MIM/8 cells to cryostat sections of human lymph nodes was measured, it was found to be as high as that of the 6I cells (i.e., 5–6 × 10^4 cells attached/section). Results of inhibition studies with the mAb are shown in Table IV. Similar to the results seen with 6I cells, the adhesion to the sections could be significantly inhibited by mAb LM609, whereas mAb P4C10 and mAb W6/32 did not significantly reduce binding. A significant inhibition of binding was also seen when either mAb 661 or mAb 3E3 were added to the sections together with the MIM/8 cells.

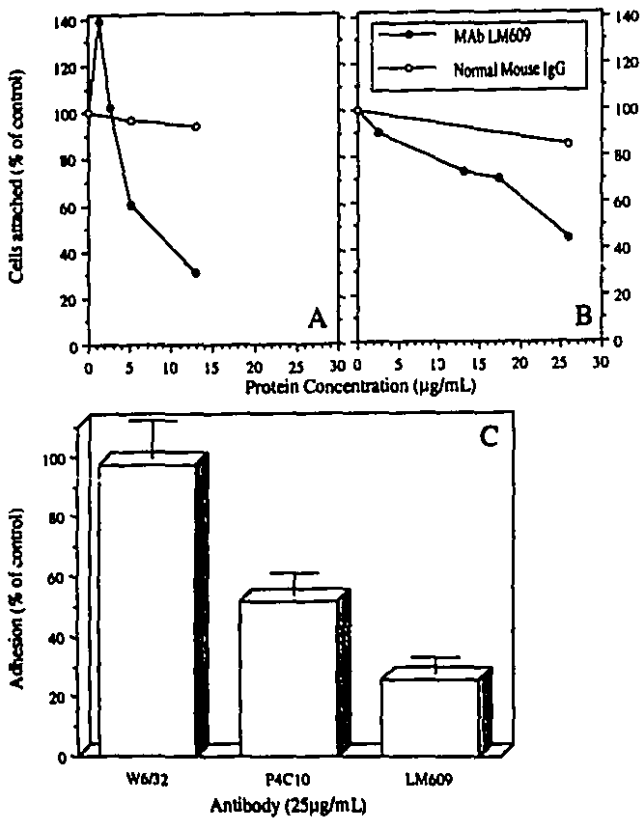

Figure 3. mAb LM609 blocks the adhesion of 61 cells to the lymph node sections. Cells were pretreated with the antibody and washed three times before their addition to the frozen sections (*A*) or to vitronectin (*B*). The results shown are the means and SD of quadruplicate samples. Adhesion to BSA-coated wells (< 1%) was subtracted. The effects of mAb P4C10 (directed to the β_1 subunit of integrins) and mAb W6/32 (directed to HLA class I determinant) on adhesion to the sections are compared with the effect of LM609 in *C*. There was no significant difference between the adhesion of untreated cells and cells treated with mAb W6/32.

Discussion

Our previous work has shown that tumor cell adhesion to frozen lymph node sections was an in vitro correlate of tumor cell

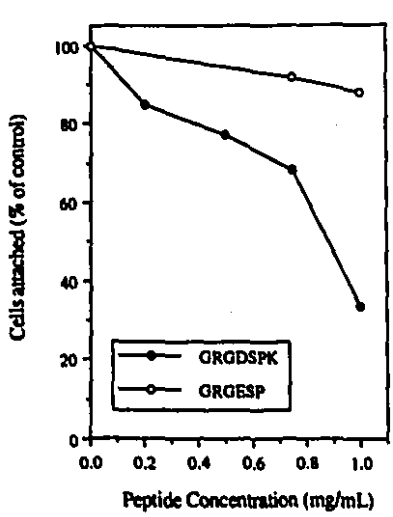

Figure 4. An RGD-containing peptide blocks the adhesion of 61 cells to lymph node sections. The cells were incubated with the peptide for 15 min before addition to the sections. The results are means and SD of quadruplicate samples.

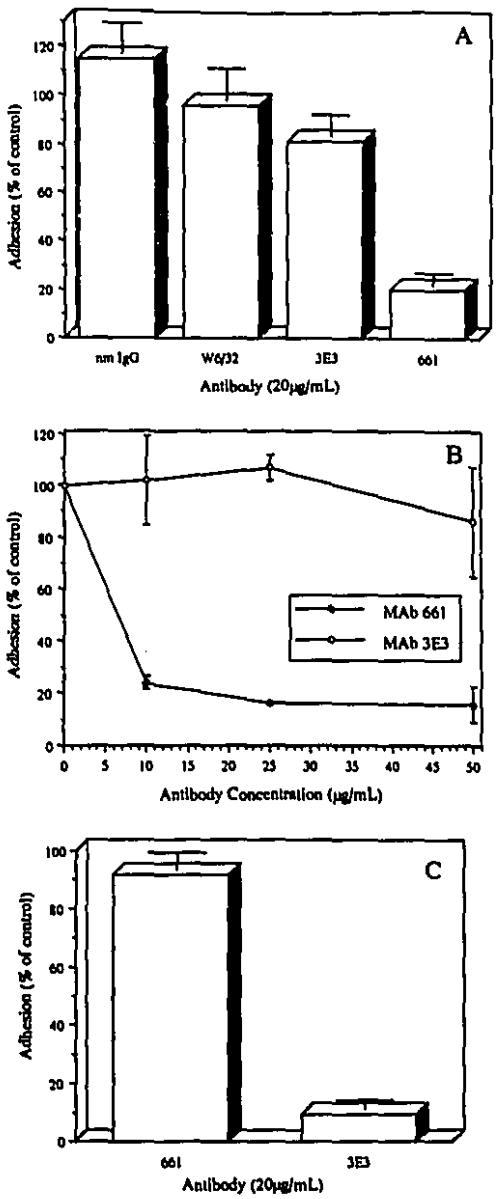

Figure 5. mAb 661 to human vitronectin blocks adhesion of 61 cells to frozen sections. Shown are the effects of mAb 661 on adhesion of 61 cells to frozen lymph node sections (*A*), vitronectin-coated dishes (*B*), and fibronectin-coated dishes (*C*). Cells were added to the different substrata together with the antibody. The results shown are the means and SD of triplicate samples. Adhesion to the sections in the presence of mAb 661 was significantly lower ($P < 0.02$) than that of any of the control groups.

potential for lymphatic metastasis in several animal and human carcinoma models (8–10). The present study extends these findings and establishes the relevance of this adhesion system to human melanoma. Our findings with the MeWo and MIM/8 lines are particularly significant as both lines were established from lymph node metastases and the major site of metastasis for these cells in nude mice after intradermal inoculation is the regional lymph node (11). The increased attachment to frozen sections seen with melanoma cells that originated from a lymph node metastasis in a nude mouse and similar findings obtained with a human breast carcinoma sub-

Table III. Flow Cytometric Analysis of mAb-stained Melanoma Cells

Antibody	Cell surface determinant	Cell type analyzed	Positive cells
			%
LM609	$\alpha_v\beta_3$	MeWo	62.6
		MeWo LNI 61	95.9
P4C10	β_1	MeWo	99.9
		MeWo LNI 61	99.8
W6/32	HLA class I	MeWo	100
		MeWo LNI 61	100

Cells were labeled using an indirect immunofluorescence assay and analyzed using a FACSTAR Cell Analyser. Control levels were determined by treating the cells with the second antibody only. Maximal staining under control conditions was used as a lower limit for gating positive cells.

line (10) suggest that cellular mechanisms that mediate lymphatic dissemination in this animal model are relevant to the clinical process. It should be noted in this context that there was no significant difference between the adhesion of MeWo and MeWo LNI 61 cells to cryostat sections of human kidneys (adhesion levels were 2.2 and 2.6%, respectively), suggesting that the adhesion ligand on the lymph node sections may be expressed in an organ-selective manner.

Our results show that binding of 61 cells to the sections was RGD dependent and mediated in large part by the receptor $\alpha_v\beta_3$. Adhesion studies on extracellular matrix-coated dishes confirmed that this metastatic subline was significantly more adherent to vitronectin than the parental line MeWo whereas the differences in attachment to collagen IV, fibronectin, and laminin were more minor. This increased adhesion was not the result of the in vivo selection process per se as no differences were found in either the levels of adhesion or in the expression of $\alpha_v\beta_3$ (as assessed by FACS; results not shown) between MeWo cells maintained in culture or derived from a solid tumor growing in vivo. Moreover, in recently published studies by Albelda et al. (23), it has been shown that adaptation of melanoma biopsy-derived cells to growth in vitro augmented rather than reduced their expression of $\alpha_v\beta_3$. It appears therefore that melanoma cells metastatic to the lymph node represent a subpopulation of tumor cells with increased adhesiveness to the lymph node extracellular matrix (ECM).

The ability of mAb LM609 to block the adhesion of 61 and MIM/8 cells to frozen sections confirmed that it was mediated by integrin $\alpha_v\beta_3$. The differences in the levels of inhibition by mAb LM609 seen on the frozen sections (Fig. 3 *A*) and on vitronectin-coated dishes (Fig. 3 *B*) suggest that although $\alpha_v\beta_3$ may be the main receptor mediating adhesion to vitronectin on the frozen sections, other receptors, possibly $\alpha_v\beta_5$, may play a role in the adhesion of the 61 cells to vitronectin-coated dishes.

Although receptor $\alpha_v\beta_3$ is also known to mediate attachment to fibrinogen and von Willebrand factor (17), these proteins are unlikely to play a role in the adhesion of the melanoma cells to the lymph node sections. In a recent study, fibrinogen could not be detected on lymph node cryostat sections whereas von Willebrand factor was found to be localized in the blood vessels (D. A. Cheresh, unpublished observations). Tumor cell attachment, on the other hand, has been localized to the lymph node stroma and the subcapsular sinus (10). Furthermore, we found that mAb 661 to human vitronectin, which inhibited binding of 61 cells to vitronectin but not fibronectin-coated dishes, also blocked adhesion of these cells to the frozen sections. A second mAb (3E3) directed to the cell-binding domain of fibronectin had only a minor inhibitory effect on the adhesion of 61 cells but could significantly reduce adhesion of MIM/8 cells, suggesting that for some melanoma cells $\alpha_v\beta_3$ may mediate adhesion to multiple ECM ligands in the node.

Flow cytometric analysis using indirect immunofluorescence labeling revealed that a significantly larger proportion of 61 cells compared with MeWo cells labeled intensely with antibody LM609. This increased expression was specific to $\alpha_v\beta_3$

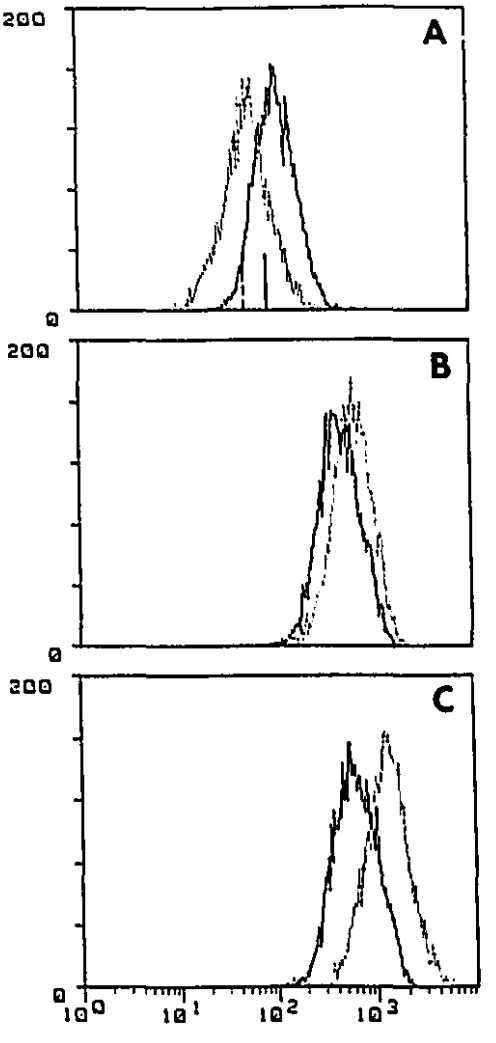

Figure 6. Flow cytometric analysis of immunofluorescence-labeled MeWo and 61 cells. An indirect staining method was used to label the cells with mAb LM609 (*A*), P4C10 (*B*), and W6/32 (*C*). Control cells that were incubated only with the second antibody were used for gating. The dotted vertical bar represents the maximum staining of control cells. The solid vertical bar in *A* denotes the mean intensity of fluorescence measured for MeWo cells. This was used as an arbitrary lower limit to determine the proportion of cells in the two cell lines for which cell surface expression of $\alpha_v\beta_3$ exceeded this level. Each profile is based on the analysis of ~ 5,000 cells. The ordinate in each graph represents the number of cells and the abscissa shows the relative intensity of fluorescence.

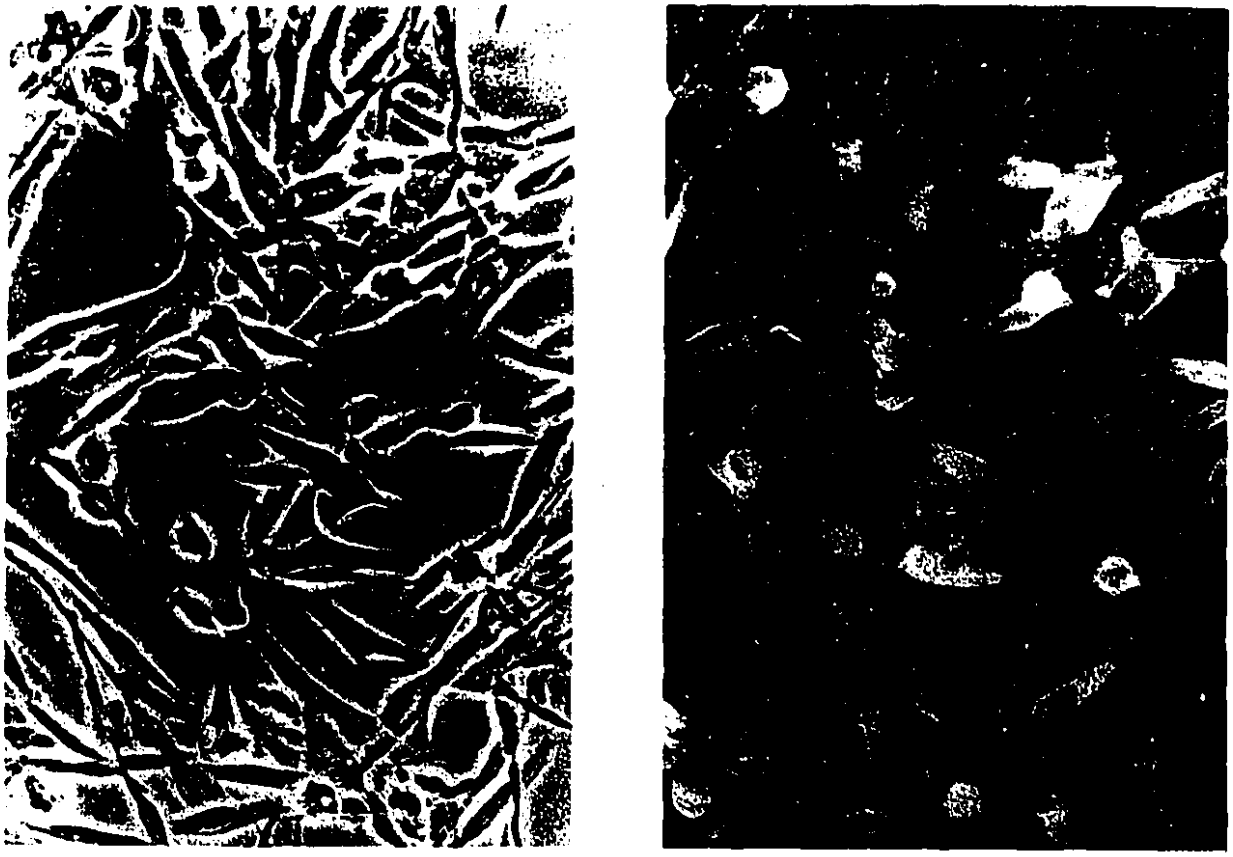

Figure 7. Immunofluorescence-labeled 6I cells. Cells were cultured on vitronectin-coated chamber slides and labeled with mAb LM609 and an FITC-conjugated goat anti-mouse IgG antibody. Positively stained cells are seen in *B*. The same microscopic field viewed by phase contrast microscopy is shown in *A*. ×400.

and did not reflect a general upregulation of integrins or differentiation antigens as neither β_1 nor MHC expression were increased in these cells. Together our results suggest that melanoma cells expressing high levels of $\alpha_v\beta_3$ may have a selective advantage with respect to metastases formation in the lymph node. As adhesion to vitronectin in the present tumor model is rapid (with maximal binding seen at 20 min) and since differences between adhesion of the two cell lines were apparent as early as 10 min after incubation with the ligand, it is likely that these "high expressor" cells also represent the major subpopulation mediating attachment in the in vitro assays.

Table IV. The Effect of mAb Treatment on the Adhesion of MIM/8 Cells to Frozen Sections of Human Lymph Nodes

mAb used	Adhesion
	% of control
661	51±10
3E3	50±11
LM609	62±8
P4C10	88±11
W6/32	97±20
mmIgG	137±23

Treatment with mAb was as described in the legends to Figs. 3 and 5. Antibody concentrations used were 20–25 μg/mL. Shown are means ±SD of two to three experiments. Mean adhesion of control (nontreated) cells was 5.3×10^4 cells/section (10% of total).

Our results with antibody P4C10 suggest that β_1 integrins also play a role in the adhesion of 6I cells to the frozen sections. However the failure to demonstrate a consistent difference in the attachment of MeWo and 6I cells to fibronectin, laminin, and type IV collagen and the results of the FACS analysis showing a decrease in the intensity of labeling with P4C10 in 6I cells compared with MeWo cells (Fig. 6 *B*) when taken together argue that the preferential attachment to vitronectin through $\alpha_v\beta_3$ plays a more decisive role in this tumor model. It is possible that adhesion through β_1-containing complexes represents a secondary event that may be triggered by $\alpha_v\beta_3$-mediated attachment and may play a stabilizing role.

Although changes in integrin-mediated adhesion of tumor cells to the extracellular matrix have been shown to regulate the growth and hematogenous dissemination of cancer cells (5), this report provides the first indication that an integrin receptor molecule may play an important role in lymphatic metastasis. In this context our findings are consistent with two recent reports that implicated the $\alpha_v\beta_3$ receptor in the malignant progression of human melanoma. Using a histochemical analysis to measure the expression of various integrin molecules on frozen sections derived from melanoma biopsies, Albelda et al. (23) have recently shown that a significant increase in the cell surface expression of this receptor in situ is associated with progression of the disease from benign nevus to vertical growth

phase–malignant melanoma. Marshall et al. (24), using immunohistochemistry and immunoprecipitation with an anti-VnR–specific mAb, have shown that $\alpha_v\beta_3$ expression is significantly augmented in human melanoma cells compared with foreskin-derived normal melanocytes.

The functional significance of cell adhesion to vitronectin in the process of lymph node metastasis requires further investigation. Important in this respect are recent reports by Kramer et al. (21) and by Reilly and Nash (22), the first demonstrating that basement membrane components such as fibronectin, laminin, and type IV collagen are associated with the reticular fibers found throughout the lymph node parenchyma, including the lymphatic sinuses, and the second showing a codistribution of vitronectin and fibronectin to the reticulin stroma of human lymph nodes (reference 22; see also references 25 and 26). We have confirmed these findings and found that these ECM proteins can be localized to the reticular fibers and germinal centers on lymph node cryostat sections (unpublished observation). As light-microscopy analysis revealed that some of the cells adhering to the frozen sections can be found in the marginal sinus (8, 10), it is possible that vitronectin and fibronectin provide a site of anchorage for cancer cells infiltrating the node and reaching the marginal sinus via the afferent lymphatic channels. The observation by Reilly and Nash (22) that synthesis of vitronectin and fibronectin in the node is upregulated after invasion by the tumor cells suggests that the local environment in the node may become increasingly favorable to cancer cells that express high levels of the VnR.

Our finding that 61 cells had a significantly increased metastatic potential in vivo compared with MeWo cells is consistent with the notion that $\alpha_v\beta_3$ plays a role in the increased metastatic potential of these tumor cell in vivo. The increased cloning efficiency of 61 cells in semisolid media suggests that, in addition, changes in other, growth-related properties of these cells (possibly indirectly related to $\alpha_v\beta_3$ expression) also contribute to their enhanced metastatic potential.

Boukerche et al. (27) have shown that the local growth of a human melanoma in nude mice could be inhibited by pretreatment of the cells with a mAb reactive against the platelet glycoprotein $\alpha_{IIb}\beta_3$. This glycoprotein complex is known to mediate platelet aggregation and shares the β_3 subunit with the $\alpha_v\beta_3$. Taken together with these observations our results strongly suggest that reagents such as mAb and peptides that can interfere with melanoma adhesion may provide potent therapeutic tools in the management of malignant melanoma during the early stages of dissemination.

Acknowledgments

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Chapter V
Increased Urokinase mediated Invasiveness Following Ligation of α_v Integrins in Metastatic Melanoma Cells

5.4

5.4.1. Introduction

Previously, we have shown that uPAR expression in lymph node-metastasizing MeWo LNI 6I melanoma cells was linked to the expression and function of the vitronectin receptor. In that study, ligation of α_v integrins by MAb resulted in a specific increase in uPAR mRNA. The aim of the present study, was to further explore the effect of α_v ligation by using a functional assay to measure cell-mediated proteolysis and invasion.

5.4.2. Materials and Methods

Tumor lines. The origin and metastatic properties of cell line MeWo LNI 6I (6I) were described in detail previously (1). The cells were maintained as monolayer cultures in RPMI-FCS and passaged twice weekly using Ca^{2+} and Mg^{2+}-free PBS containing 0.02% EDTA to disperse the cells.

Antibodies. MAb's LM142 and LM609 (2) directed to the α_v subunit and the $\alpha_v\beta_3$ complex respectively, were generous gifts from Dr. D. Cheresh

(Scripps Clinic, LaJolla, CA), MAb A3-IIF5 to integrin subunit α_3 (3) was a generous gift from Dr. M. Hemler (Dana Farber Cancer Institute, Boston, MA), MAb W6/32 directed to human HLA class I which recognizes an antigenic determinant common to products of the HLA-A, B, and C loci was obtained from Cedarlane Laboratories (Hornby, Ontario, Canada), and MAb 3936 which recognizes the human uPAR in its ligand-bound or unbound forms was from American Diagnostica Inc, (Greenwich, CT).

Proteinase Inhibitors. The plasmin inhibitor, ε-aminocaproic acid, was obtained from Sigma Chemical Co. (St.Louis, MO) and the synthetic metalloproteinase inhibitor U24522 (4) was from Dr. G. Dipasquale (Stuart Pharmaceuticals, Wilmington, DE).

MAb-mediated Ligation of the vitronectin receptor. One million MeWo LNI 61 cells were incubated for 24 hours at 37°C in 6 well culture plates precoated with 10μg/mL of MAb's LM142, LM609, A3-IIF5 or W6/32. The cells were then dispersed and used in the Matrigel invasion assay as follows. Matrigel (Collaborative Research, Bedford, MA.) was diluted with cold distilled water to a final concentration of 0.23mg/ml and 60 μl added to 8 μm polycarbonate filters. The filters were dried overnight, reconstituted with serum free-RPMI and placed in 24 well plates. To each filter $5x10^4$ cells in 100 μl RPMI containing 0.2% BSA were added with or

without the indicated concentrations of antibodies or inhibitors. Human fibronectin (5μg/ml, Gibco BRL) was used as a chemoattractant in the lower chambers. The plates were incubated for 48 hours at 37°C in a 5% CO_2, water-saturated atmosphere. The cells on the upper surface of the filter were then removed with a cotton swab and the filters fixed in 0.1% glutaraldehyde for 20 minutes and stained with 0.2% crystal violet for 30 minutes. For each filter 20 random fields were counted using an inverted microscope (Inverted Diaphot-TMD, Nikon, Canada) at 100X magnification. Duplicate samples were analyzed for each assay condition (see schematic representation Fig. 1).

5.4.3. Results and Summary

Results shown in Fig. 2 demonstrate that ligation of the vitronectin receptor $\alpha_v\beta_3$ by immobilized antibodies resulted in an increase of up to 2-fold in invasiveness through Matrigel as compared to control, untreated cells (Fig. 2 and 3). MAb LM609 directed to the $\alpha_v\beta_3$ complex as well as MAb LM142, directed to the α_v subunit both caused a significant increase in invasiveness of these cells whereas a control antibody to MHC Class I antigens, MAb W6/32, did not affect invasiveness. This increase in invasiveness was completely abrogated by MAb 3936 directed to the uPAR but not by control antibody MAb W6/32 (Fig. 3). A similar reduction in invasion was seen when a plasmin-specific inhibitor, ε-aminocaproic acid

was used, whereas a metalloproteinase-specific inhibitor U24522 had a partial effect (36% inhibition) (Fig. 4).

Previously, others (5) have shown that the ligation of the vitronectin receptor resulted in increased invasiveness of melanoma cells through Matrigel which was attributed to an increase in the expression of the metalloproteinase MMP-2. In our study, a similar increase in melanoma cell invasiveness was observed however our results suggest that this increase was due mainly to increased uPAR-uPA function as a MAb to uPAR and a uPA inhibitor completely abolished the increase in invasiveness and also reduced the base level of invasion. While MMP-2 may also play a role in invasiveness in our model it does not appear to be the major mediator affected by α_v ligation as a specific MMP inhibitor had a more minor effect on the invasion. It is possible that in fact, the upregulation of uPAR, which leads to increased production of cell surface plasmin, also plays an indirect role in MMP-2 mediated proteolysis as plasmin can convert pro-MMP-2 to its active form and thereby regulate MMP-2 activity (6). Taken together these results provide the first evidence that ligation of the vitronectin receptor on metastatic melanoma cells enhances invasiveness through upregulation of uPAR mRNA expression and a resulting increase in uPAR-mediated function.

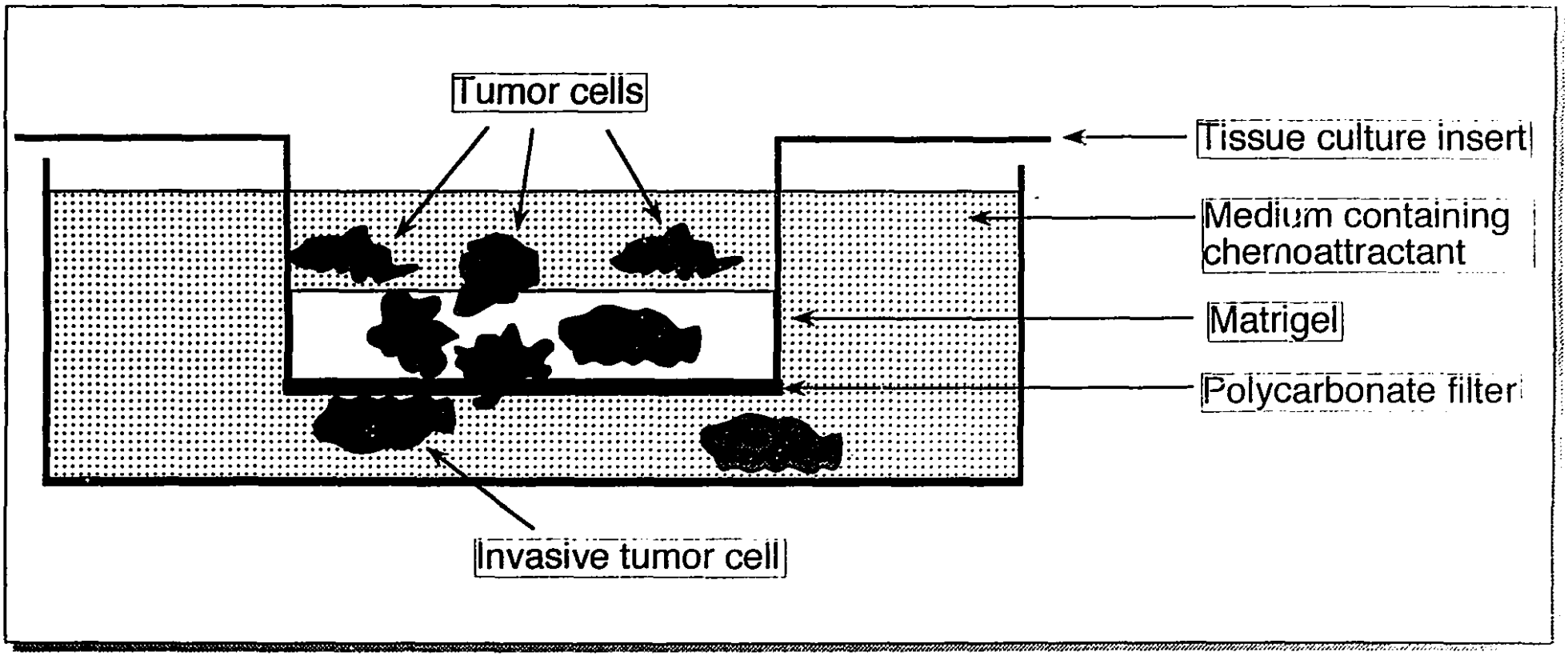


Figure 1. Matrigel Invasion Assay

Schematic representation of invasion of tumor cells through a Matrigel barrier.

Figure 2. Increased invasiveness of 6l cells in response to ligation with MAb to the vitronectin receptor.

6l melanoma cells grown overnight on MAb W6/32-, MAb LM609-, or MAb LM142 coated wells were dispersed and placed on Matrigel-coated wells. Control cells were grown on uncoated wells. After a 48 hour incubation at 37°C, the cells invading to the underside of the filter were fixed, stained, and quantitated by counting 20 random fields using an inverted microscope. The results shown are expressed as invasion relative to untreated cells. Shown are means±SD of duplicate experiments. The total number of cells counted on control filters was 932±15.6. The invasion levels for LM609- and LM142-treated cells were significantly increased ($p<0.036$) when compared to untreated cells. W6/32-treated cells showed no significant increase in invasion ($p=0.87$).

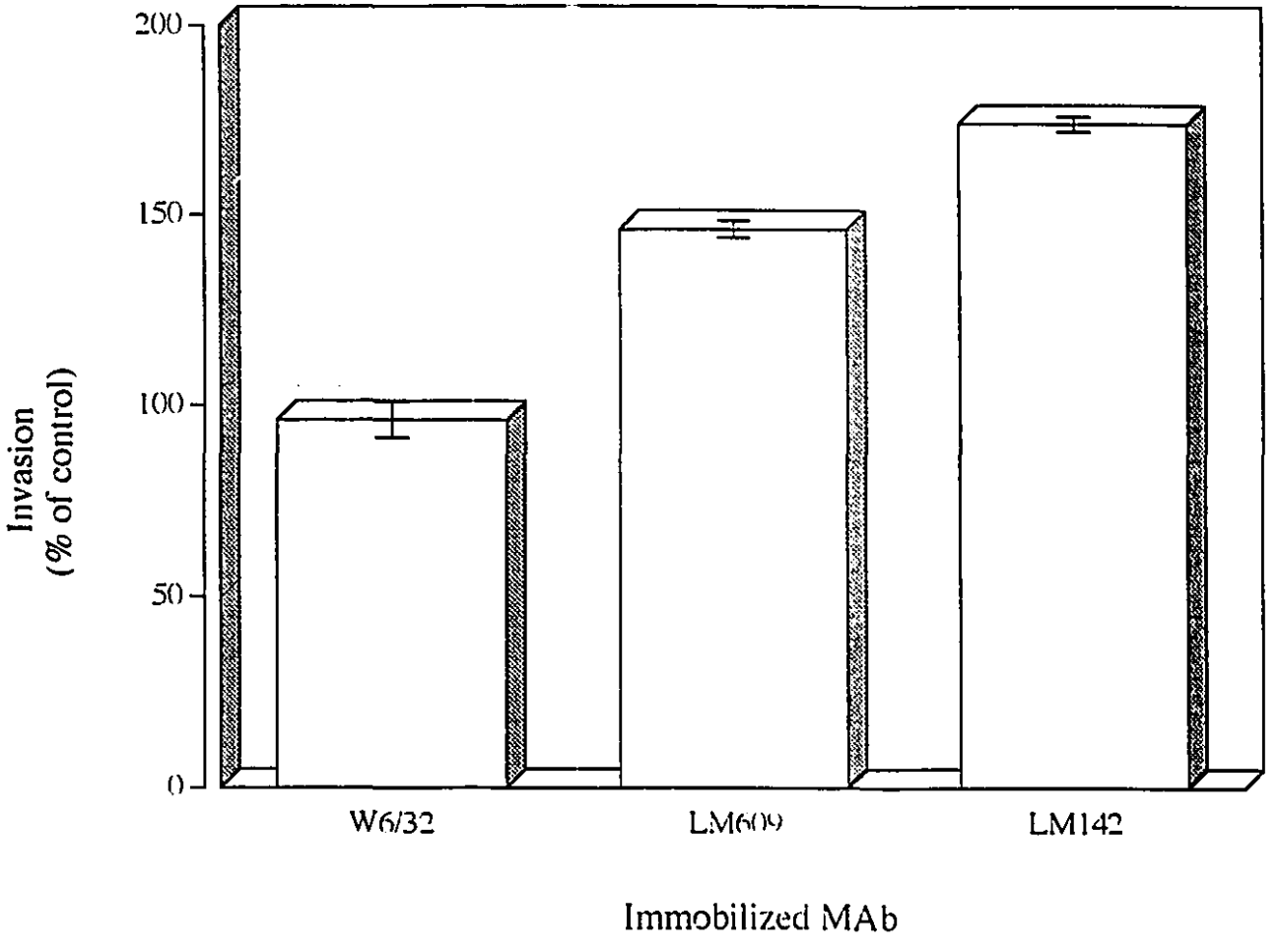

200
150
100
50
0
Invasion
(% of control)
W6/32
LM609
LM142
Immobilized MAb

Figure 3. MAb 3936 abrogates the incremental increase in 6l cell invasion due to ligation with anti-α_V MAb.

The invasion of 6l melanoma cells grown overnight on MAb LM142-coated (+) or uncoated (-) wells was measured using a Matrigel assay as described in the legend to Fig. 1. Cells were added together with 40μg/mL MAb 3936 or MAb W6/32. The results are expressed as invasion relative to control cells grown on uncoated wells. Shown are means±SD of duplicate experiments. When compared to untreated, LM142-stimulated cells, MAb 3936 treatment significantly reduced the level of invasion of the cells ($p<0.001$) whereas MAb W6/32 did not alter the invasion significantly ($p=0.64$).

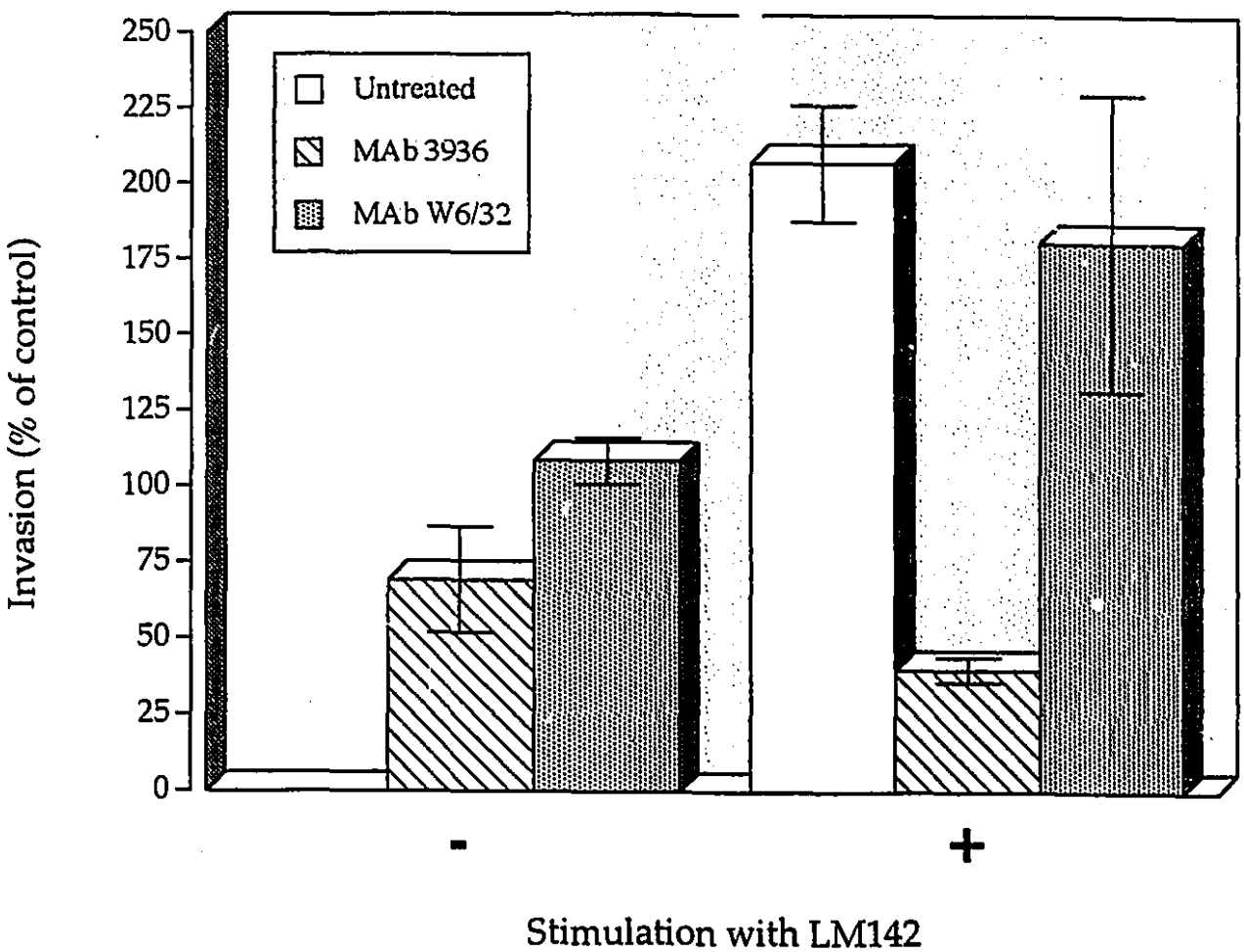
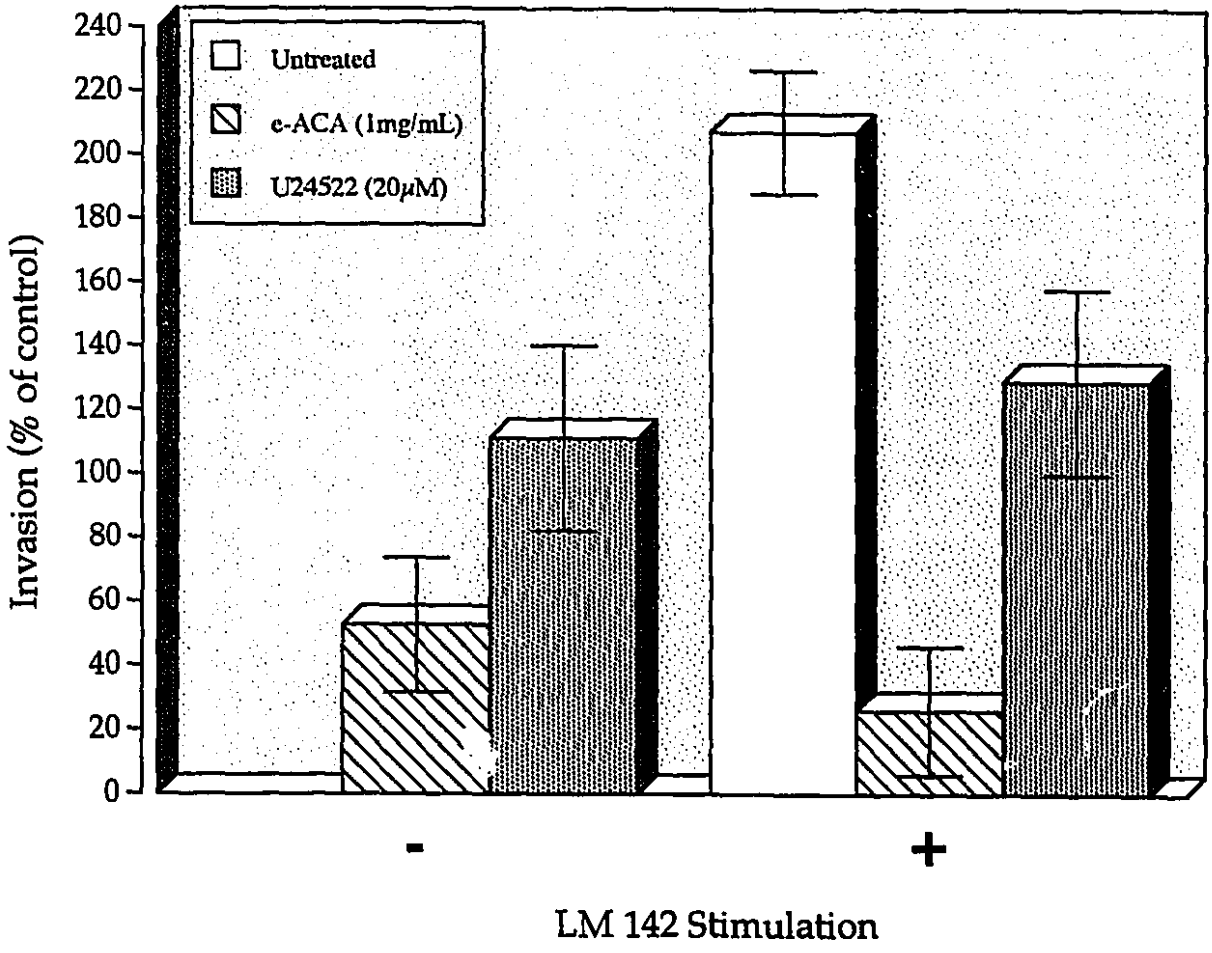

Invasion (% of control)
250
225
200
175
150
125
100
75
50
25
0
Untreated
MAb 3936
MAb W6/32
-
+
Stimulation with LM142

Figure 4. A plasmin inhibitor blocks the incremental increase in 6l invasion due to ligation with anti- α_V MAb.

The invasion of [illegible] melanoma cells grown overnight on MAb LM142-coated (+) or uncoated (-) wells was measured in a Matrigel assay as described in the legend to Fig. 1 alone, with 1mg/mL e-aminocaproic acid or 20μM U24522. The results are expressed as invasion relative to cells grown overnight on uncoated wells. Shown are means±SD of duplicate experiments. The levels of inhibition of invasion caused by e-aminocaproic acid ($p<0.001$) and U24522 ($p<0.003$) on LM142-stimulated cells were significant when compared to untreated LM142-stimulated cells.


Untreated
ε-ACA (1mg/mL)
U24522 (20μM)
Invasion (% of control)
240
220
200
180
160
140
120
100
80
60
40
20
0
-
+
LM 142 Stimulation

5.4.4. References for ligation studies

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Chapter VI

Integrin $\alpha_3\beta_1$ Mediates Adhesion of Human Breast Carcinoma Cells to the Lymph Node Stroma

6.1 Breast Cancer Studies

6.1.1. Introduction

In this chapter, studies on the role of $\alpha_3\beta_1$ in human breast cancer cell metastasis to the lymph nodes are presented in the form of a published paper and unpublished results. The paper is entitled "Analysis of tumor cell adhesion to frozen lymph node sections - a correlate of lymphatic metastasis in breast carcinoma models of human and rat origin" published in Breast Cancer Research and Treatment *17*: 109-120, 1990. This paper demonstrated that there was a positive correlation between the adhesion of the rat or human breast cancer cells to the respective lymph nodes and their metastatic ability *in vivo*. The increase in adhesion to lymph node cryostat sections was specific, as adhesion to rat liver or brain was not altered. For both human and rat models, an RGD-containing peptide, as well as a polyclonal antiserum to β_1 integrins, blocked adhesion to the lymph node sections. In this study, I was responsible for the selection of the lymph node-metastasizing Hs578T cell lines as well as adhesion assays with these cells using frozen sections of human lymph nodes. Additional studies using several antibodies to integrins and ECM proteins subsequently demonstrated that cell adhesion to the sections was mediated by fibronectin and the cell surface adhesion receptor mediating the adhesion was identified as the integrin $\alpha_3\beta_1$ (in press).

6.1.2. Materials and Methods

Animals and Tumor lines. Four to six week old female nu/nu (CD-1)Br mice (Charles River Canada; St-Constant, Quebec) were used for propagation of the human breast carcinoma cell lines and for isolation of lymph node-metastasizing cells. The mice were housed in Micro-isolator cages (Lab Products Inc.; Maywood, NJ) and handled in biohazard cabinets. Human breast carcinoma line Hs578T (1), was obtained from the American Tissue Type Collection. Hs578T was maintained as a monolayer culture in DMEM supplemented with 10% FCS, 1% gentamycin sulfate, 1% penicillin-streptomycin, and 2mM glutamine (DMEM-FCS) (all reagents were from Gibco Laboratories, Burlington, Ontario).

Selection of lymph node-metastasizing Hs578T cells. To obtain lymph node metastases of tumor Hs578T, 4×10^6 tumor cells were inoculated into the mammary fatpads of female nude mice. Tumors appeared approximately 2 wk later. The animals were sacrificed when mean tumor diameter was 3-3.5 cm or when they appeared moribund. The inguinal or the axillary and branchial nodes draining the tumor site were removed, minced, and cultured in RPMI-FCS. Tumor cells were harvested about 2 weeks later (LNI cells) and were reinjected into nude mice for selection of the next generation of metastatic (LNII) cells. This procedure was repeated

seven times.

Antibodies. MAb 661 (2) to the cell adhesion fragment of vitronectin was obtained from Dr. D. Loskutoff (Scripps Research Institute, LaJolla, CA), MAb 3E3 directed to the cell attachment fragment of human fibronectin was obtained from Boehringer Mannheim Canada (Laval, Quebec, Canada), and normal mouse immunoglobulin was obtained from Bio-can Scientific (Mississauga, Ontario). Rabbit antiserum to the β_1 subunit of integrin was a gift from Dr. R.O. Hynes (MIT, Boston MA)

Lymph node adhesion assays. The adhesion of breast carcinoma cells to cryostat sections of human lymph nodes was measured using 5μm sections and a 45 min incubation at 4°C as previously described (2). In some experiments blocking antibodies to integrins were incubated with the tumor cells for 45 minutes at 4°C prior to the adhesion assays. MAb to matrix proteins were added together with the tumor cells. Where appropriate, normal rabbit IgG was used as a control.

6.1.3. Results and Summary

To examine the relationship between cell adhesion and lymphatic metastasis in breast cancer, an experimental model was developed using

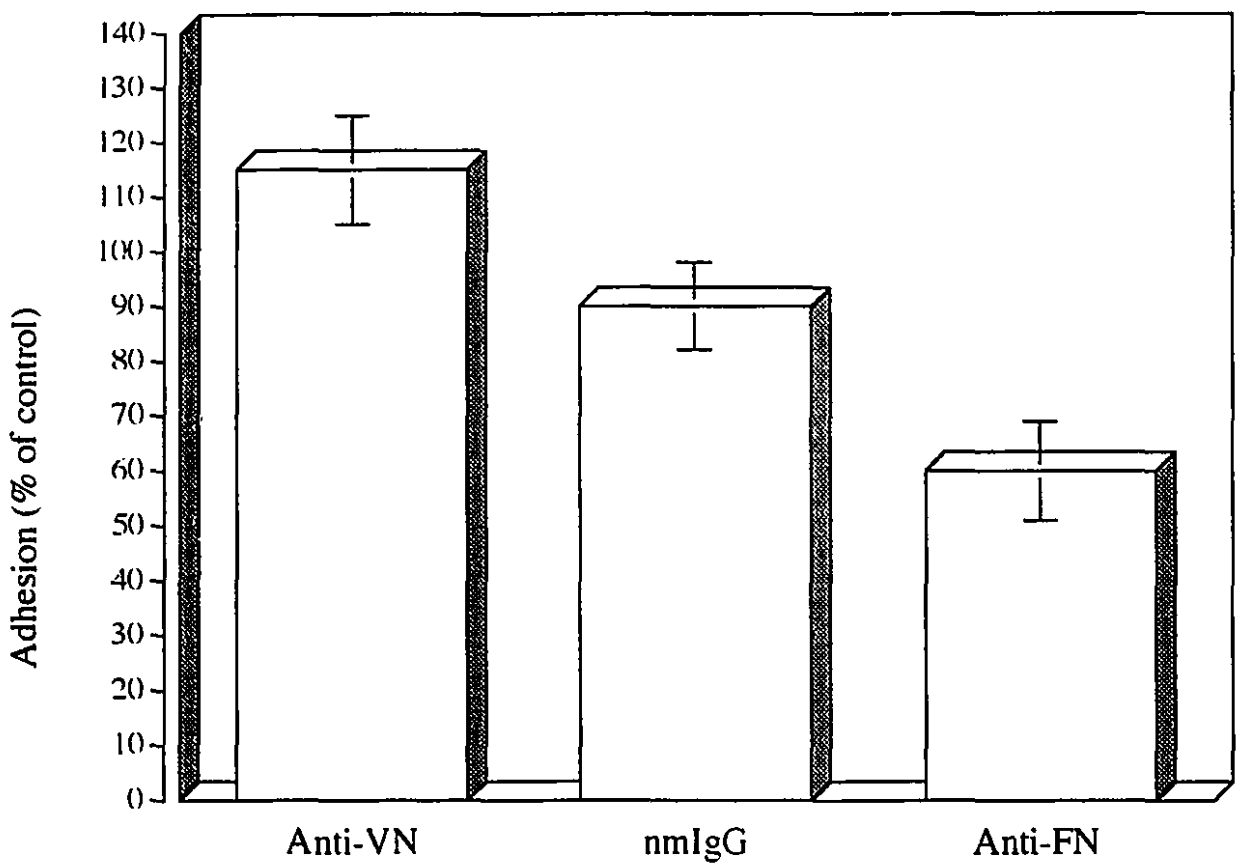
Hs578T breast carcinoma cells. These cells were injected into the mammary fatpads of female nude mice giving rise to local tumors as well as regional lymph node metastases. The adhesion of the parental Hs578T cells and the metastatic cells isolated from the regional lymph nodes was then tested using cryostat sections of human lymph nodes. The results shown in Figure 6 of the attached paper indicate that there was a significant increase in the adhesion of the metastases-derived cells to the cryostat sections as compared to adhesion of the parent cells. Serial propagation of the metastatic cells resulted in further increases in the adhesion (see Fig. 6). Other experiments revealed that an RGD-containing peptide could block carcinoma cell adhesion to the lymph node sections suggesting that an integrin receptor may have been involved. To further explore this possibility, a polyclonal antiserum directed to the β_1 integrin subunit was used. This antibody inhibited the adhesion of the breast carcinoma cells by 75% while control preimmune serum had no effect (see Fig. 9 in attached paper) suggesting that a β_1 integrin was involved. In a subsequent experiment, a metastatic variant Hs578T LNVII selected through seven lymph node metastases to mammary fatpad implantations was used. MAb 3E3 directed to the cell attachment fragment of fibronectin blocked adhesion of these cells to the lymph node sections but a MAb directed to the cell binding domain of vitronectin, previously shown to block human melanoma cell adhesion to vitronectin in these sections (2),

and a murine preimmune serum had no inhibitory effect (see Fig. 1). Others in Dr. Brodt's laboratory have subsequently isolated rat lymph node stromal cells which were shown by immunohistochemical staining to express cell surface- and ECM-associated fibronectin. Adhesion and spreading assays using these stromal cells have subsequently shown that the major receptor involved in human breast carcinoma cell adhesion and spreading on lymph node stromal fibronectin was integrin $\alpha_3\beta_1$ (paper submitted for publication).

The $\alpha_3\beta_1$ receptor, whose ligands include laminin and collagen (3) as well as fibronectin, has been implicated in the invasion and metastasis of several tumors including melanoma (4), basal cell carcinoma (5), and small cell lung cancer (6). Despite reports of downregulation of the receptor in some tumors (7, 8), immunohistochemical studies involving melanoma (4) and basal cell carcinoma demonstrated that its expression is increased with progression of the disease in the former and upregulated as compared to normal epidermal basal cells (5) in the latter. In one immunohistochemical study (9), analysis of a large bank of different tumors revealed that this receptor was expressed in the majority of primary and metastatic solid tumors tested. Additionally, metastases of some tumors, including breast and renal carcinomas, and melanomas expressed higher levels of the $\alpha_3\beta_1$ receptor than the respective primary tumor, implicating the receptor in tumor progression. It is conceivable

Figure 1. Inhibition of human breast carcinoma cell adhesion to lymph node cryostat sections by an anti-FN antibody.

To 5μm lymph node cryostat sections 5x10 ^{5} 51 Cr-labelled cells were added in the presence of antibodies to fibronectin, vitronection or control IgG for 45 min at 4°C. The sections were rinsed to remove the non-adherent cells, the adherent cells lysed with 1N NaOH and the radioactivity associated with the lysates determined in a γ counter. Shown are the results of experiments performed with human Hs578T-LNVII tumor cells on cryostat sectons of human peripheral lymph nodes. Anti-FN and anti-VN antibodies were used at concentrations of 25μg/ml. As a control, preimmune IgG was used at the same concentration. The results shown are means±SD of duplicate experiments.


Adhesion (% of control)
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
Anti-VN
nmIgG
Anti-FN

therefore that in breast carcinoma, $\alpha_3\beta_1$ is functionally relevant to disease progression by mediating epithelial-stromal interaction in the draining lymph nodes which promote tumor survival and growth.

Breast Cancer Research and Treatment 17: 109–120. 1990.

Report

Tumor cell adhesion to frozen lymph node sections – a correlate of lymphatic metastasis in breast carcinoma models of human and rat origin

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Key words: lymphatic metastasis, adhesion, integrims

Summary

The role of tumor cell adhesion in lymphatic metastasis of breast cancer was investigated *in vitro* using a rat mammary carcinoma model of four cell lines with different metastatic phenotypes, two human breast cancer cell lines, and cryostast sections of normal rat or human lymph nodes, respectively. A positive correlation was found between the adhesion levels obtained with three metastatic rat mammary cell lines (TMT-081 > MT-100M & TMT-50) and a non-metastatic line MT-W9B, the latter being 3–4 fold less adhesive to the lymph node sections than the metastatic tumors. This selective adhesion was specific, as it was not found with cryostat sections of rat liver and brain. Enzyme assays indicated that cell surface glycoproteins bearing terminal β-galactoside residues were involved in the adhesion of the rat tumors.

Adhesion of the human breast carcinoma cells Hs578T to sections of human lymph nodes was significantly higher than that of the normal breast epithelial cell line Hs578Bst, and comparable to adhesion of a second breast carcinoma line, MCF-7. Moreover, Hs578T cells isolated from regional lymph nodes of tumor-bearing nude mice were significantly more adhesive to human lymph node sections than the parental line.

Adhesion of both human and rat tumors could be partially blocked by the addition of the synthetic peptide GRGDSPK and by antibodies directed to the β1 chain of integrin, suggesting that an integrin receptor may played a role in the adhesion. The results suggest that tumor cell adhesion to cryostat sections of lymph nodes is a correlate of the malignant phenotype in mammary tumors of diverse origins, and could be used to delineate the adhesion factors mediating lymphatic metastasis.

Introduction

Among the numerous cellular properties now known to regulate the metastatic phenotype of tumor cells, the importance of cellular adhesion as a mediator of cellular invasion, motility, and growth has been well documented [for reviews see 1, 2]. Adhesive interactions have been demonstrated between metastasizing tumor cells and various components of the host microenvironment including the extracellular matrix [3–5], endothelial cells [6, 7], and parenchymal cells [8, 9] of invaded organs.

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Studies in several laboratories using experimental tumor models have shown that the metastatic process can be successfully arrested by reagents which inhibit tumor adhesion [10–13].

Human carcinomas, primary breast cancer in particular, frequently disseminate via lymphatic channels and may reach the circulation through lymphovenous connections in the nodes [14, 15]. Despite the clinical importance of lymphatic dissemination, the host-tumor interactions which underlie this process have received little attention, and the role of cell-cell and cell-matrix adhesion is therefore virtually unknown.

In previous studies we have shown that a lymph node metastasizing subline of the murine Lewis lung carcinoma (line H-59) was significantly more adhesive to cryostat sections of lymph nodes than a second subline (M-27) which disseminates only hematogenously [16]. Subsequent studies employing a monoclonal antibody specific for tumor H-59 (MoAb 12/50) identified a 37 kDa cell membrane protein as a possible mediator of tumor cell adhesion in this model [17].

In the present study, the relevance of this *in vitro* system to the metastatic properties of breast cancer cells was assessed using a rat mammary carcinoma model and two cell lines derived from primary human breast carcinomas. In both models, a good correlation was found between tumor cell ability to bind to the sections and the malignant phenotype. Subsequent studies suggested that integrins play a role in this attachment.

Materials and methods

Animals

Wistar/Furth (WF/HSD) female rats were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN, USA). They were used between the ages of 7 and 26 wk for maintenance of the mammary carcinoma lines and for assessment of the metastatic phenotype. Nu/rnu (CD-1) BR female mice were used to maintain the human tumor lines and for the selection of lymph node metastasizing tumor cells. They were obtained from Charles River Canada (St-Constant Quebec) and maintained in Micro-isolator cages obtained from Lab Products Inc. (Maywood, New Jersey). All procedures requiring handling of nude mice were carried out in a biological containment cabinet.

Tumor lines

Rat mammary carcinoma lines TMT-081, TMT-50,

Table 1. Tumorigenicity and metastatic properties of the tumor lines[a]

	Tumorigenicity	Incidence of lymph node metastases		Lung metastases
		Regional	Distant[b]	
MT-W9B	+	0/15	–	0
TMT-50	+	9/18	4/18	0
MT-100M	+	10/19	5/19	0
TMT-081	+	30/30	26/30	4/30[c]
Hs578Bst	–	–	–	–
Hs578T	+	8/10	–	0
MCF-7	+	0/5[d]		–[d]

[a] Results are based on complete autopsies and histological analysis of lymph nodes and lungs. Tumorigenicity and metastasis were assessed in 15–30 tumor-inoculated rats and (for the human cell lines) in 5–10 nude mice. Rat tumors were inoculated into the mammary fatpad using minced tumor tissue or 2×10^6 dispersed tumor cells. Human cells were inoculated into the mammary fatpad of nude mice using 2 (Hs578T) or 5 (Hs578Bst, MCF-7) million cells. Tumors appeared in all animals injected with tumorigenic cell lines. No animals inoculated with Hs578Bst cells developed tumors.

[b] The axillary and brachial nodes contralateral to the draining nodes were also involved.

[c] The number of metastases in positive lungs ranged between 5 and 10.

[d] Metastasis of tumor MCF-7 to lymph node and lungs of nude mice has been observed by other investigators [23].

MT-100, and *MT-W9B* were induced in W/Fu female rats by a protocol of combined chemical carcinogenesis with 3-MCA (3-methylcholanthrene) and immunosuppression. Their origin and malignant properties have been described in detail previously [18, 19]. A metastatic variant of MT-100 developed spontaneously in our laboratory and was used in all the experiments. It was redesignated MT-100M. The metastatic properties of the tumor lines as observed during the course of the present study are summarized in Table 1. The tumor lines were maintained *in vivo* by implantation of minced tumor tissue into the rat mammary fatpads adjacent to the inguinal or axillary nodes. Tumors were excised when they reached 3 cm in diameter, then minced and implanted into new rats. Autopsies were always performed at the time of tumor excision and the regional and distant nodes, the lungs, and the visceral organs were examined for macroscopic metastases and analyzed by histology. Cultures were established from tumor cells derived from the solid tumors by digestion with trypsin [20]. The dispersed cells were plated on ECM (extra cellular matrix) coated plates which were prepared as described by Vlodavsky *et al.* [21]. *BAE*, a bovine aortic endothelial cell line (obtained courtesy of Dr. B. Zetter, Dept. of Surgery, Harvard Medical School), was used as the source of ECM. The tumor cells were passaged once weekly (ECM plates were used only for the first three passages) and maintained in RPMI-FCS (RPMI containing 10% FCS, 0.01 M HEPES, and 2×10^{-3} M glutamine). To disperse the cell monolayer, cells were incubated for 10 min at 37° C with a solution of PBS-EDTA (Ca++ and Mg−+ free phosphate buffered saline containing 0.2% EDTA).

Breast carcinoma line *Hs578T* and the non-malignant cell line *Hs578Bst*, which was originally derived from the periphery of the carcinoma and characterized as myoepithelial in origin [22], were obtained from the ATCC. Hs578T was maintained as a monolayer culture in DMEM-FCS. Hs578Bst cells were maintained in DMEM-FCS containing also 1% non essential amino acids (Gibco, Burlington, Ontario, Canada), 1 mM oxalacetic acid, 0.2 units/ml insulin (Sigma), 0.5 mM sodium pyruvate, and 30 ng/ml EGF (epidermal growth factor, obtained from Collaborative Research, Mass.). These cells grew *in vitro* for up to 4 passages, at which time they normally became senescent. Line MCF-7 [23], obtained through the courtesy of Dr. B. Brenner (Lady Davis Research Institute, Montreal), was maintained by weekly passage in RPMI-FCS.

Selection of lymph node-metastasizing Hs578T cells

To obtain lymph node metastases of tumor Hs578T, 4×10^6 tumor cells were inoculated into the mammary fatpads of female nude mice. Tumors appeared approximately 2 wk later. The animals were sacrificed when mean tumor diameter was 3–3.5 cm or when they appeared moribund. The nodes draining the tumor site (the inguinal or the axillary and brachial nodes) were removed, minced, and cultured in RPMI-FCS. When tumor cells appeared in the culture (LN1 cells), they were reinjected into nude mice for selection of the next generation of metastatic (LN2) cells. This procedure was repeated 3 times.

Adhesion assay

Rat lymph nodes and other organs were obtained from normal W/Fu or Wistar rats. Human lymph nodes were obtained from the Surgical Pathology Laboratory of the Royal Victoria Hospital. The nodes removed from patients presenting with cervical node enlargement were snap frozen in liquid N_2 and analyzed by electron microscopy, by immunohistochemistry, and by marker analysis for evidence of malignancy. They were used in the adhesion assays only if the pathology findings were negative. Adhesion to cryostat sections was measured essentially as we described previously [16]. Briefly, ^{51}Cr-labelled cells (5×10^5 in 0.05 ml RPMI-FCS) were layered onto 5–7 μm cryostat sections for a 45 min incubation at 4° C. Unattached cells were removed by washing, and the sections were fixed in 1% glutaraldehyde in PBS before the radioactivity associated with the sections was measured in a γ counter. Treatment of the tumor cells with tunicamycin and various enzymes was carried out as described in detail elsewhere [16, 17].

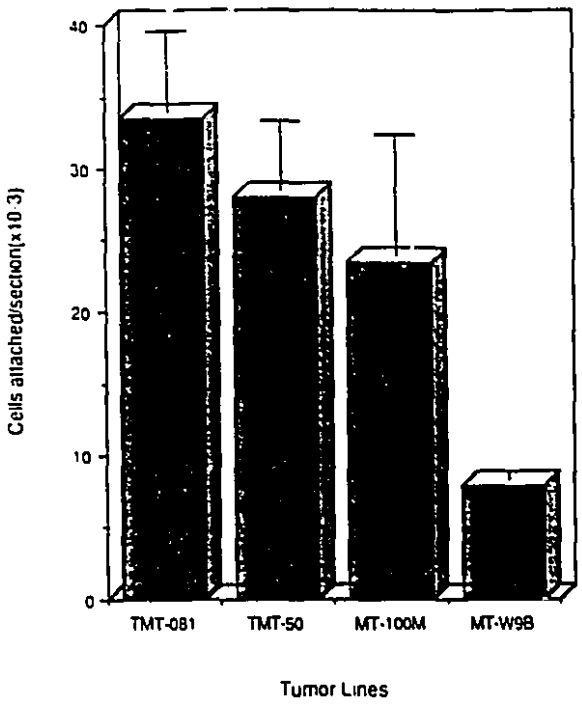

Fig. 1. Adhesion of rat mammary carcinoma lines to frozen sections of rat lymph nodes. To each section 5×10^5 ^{51}Cr-labelled cells were added. Results represent means and S.D. of 3–6 experiments. Adhesion of line MT-W9B was significantly lower ($P < 0.01$) than that of any of the other cell lines. Adhesion of tumor MT-100M was significantly lower ($P < 0.05$) than that of TMT-081.

Antisera and peptides

MoAb/LM609 reactive with the human vitronectin receptor $\alpha_v\beta_3$ [24] was a kind gift of Dr. D. Cheresh (Scripps Clinic, La Jolla, CA). Rabbit antiserum to the β_1 subunit of integrin [25] was a kind gift of Dr. R.O. Hynes (MIT, Boston, MA). Tumor cells treated with the antibodies at the specified concentrations were washed 4 times prior to the adhesion assay to remove unbound antibody. Peptide GRGDSPK [26] was obtained from Hukabel Scientific (Longueuil, Quebec, Canada) and peptide GRGESP from Penninsula (Belmont, CA). Cells were preincubated with the peptides at the specified concentrations for 15 min at room temperature and then added to the frozen sections without washing.

Statistics

The Student's t-test was used for all the statistical analyses.

Results

The tumorigenic and metastatic properties of the various cell lines used in this study are summarized in Table 1. The data, based on complete autopsies and confirmed by histology, was compiled over a period of two years and is based on analysis of 15–30 rats for each tumor type. The data on the human breast cancer cell lines Hs578T and MCF-7 is based on autopsy findings in 5–10 tumor-bearing female nude mice.

Adhesion assays were performed using the rat tumors and frozen sections of rat lymph nodes, spleen, liver, and brain. Results are shown in Fig. 1 and Fig. 2. A significant difference in the adhesion of the different cell lines was seen only with lymph node and spleen sections, and the level of adhesion correlated well with the metastatic phenotypes of the tumor lines. Thus, the adhesion of tumor TMT-081 was higher than that of tumors TMT-50 and MT-100M, and all three metastatic cell lines were significantly more adherent than the non-metastatic line MT-W9B (Fig. 1). Adhesion to brain sections was low for all tumor lines tested. Adhesion to liver sections was high (relative to lymph nodes) but did not vary significantly among the different cell lines and did not correlate with the metastatic phenotypes of the tumor cells (Fig. 2 and Table 1).

To determine the role of plasma membrane glycoconjugates in the adhesion to lymph node sections, tumors TMT-081 and MT-W9B were pretreated with tunicamycin, exoglycosidases, or pronase prior to the adhesion assay. Results of a representative experiment are shown in Fig. 3. None of these treatments resulted in an increase in the adhesion of tumor MT-W9B. Treatment of TMT-081 with β-galactosidase or neuraminidase alone failed to modify the adhesion while treatment with both enzymes, with tunicamycin, or with pronase all resulted in a significant reduction in adhesion.

Similar analyses were carried out with the human

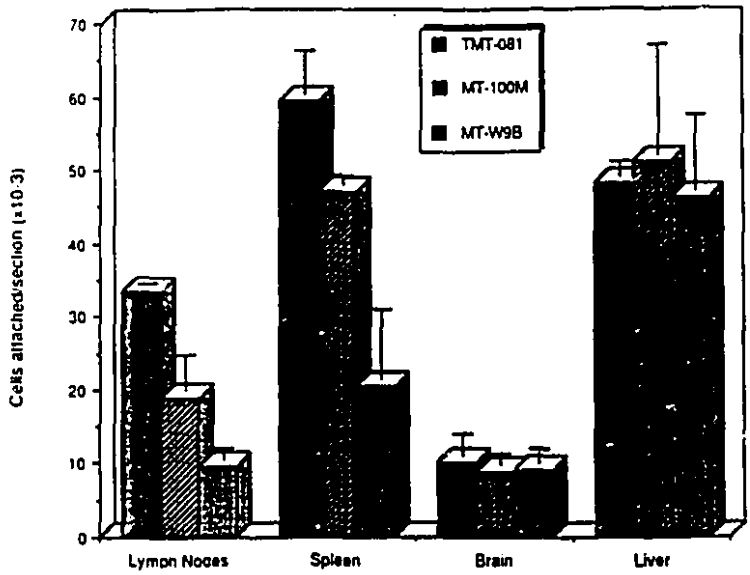

Fig. 2. Adhesion of rat cell lines to frozen sections of different organs. Results are means and S.D., of quadruplicate samples. The adhesion of tumor TMT-081 to lymph node and spleen sections was significantly higher than that of tumors MT-100M ($P < 0.005$ and < 0.025 respectively) and MT-W9B ($P < 0.005$ and < 0.01 respectively). Adhesion of MT-100M to these sections was also significantly higher than that of MT-W9B ($P < 0.001$).

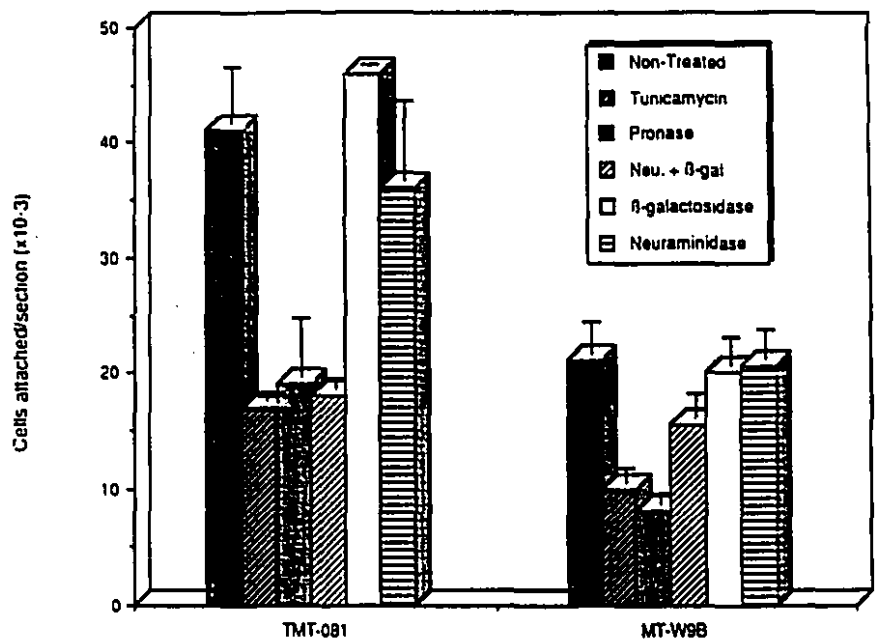

Fig. 3. The effects of tunicamycin and enzyme treatment on the adhesion of rat tumor cells to cryostat sections of rat lymph nodes. Enzyme treatment was carried out as described in the Materials and methods section and in greater detail previously [16]. To each section 5×10^5 untreated or treated cells were added. The results represent means and S.D. of triplicate samples. For both tumor lines there was a significant decrease in adhesion following treatment with tunicamycin or pronase ($P < 0.005$ for TMT-081 and < 0.025 for MT-W9B). The reduction in adhesion following treatment with neuraminidase combined with β-galactosidase was also significant ($P < 0.025$).

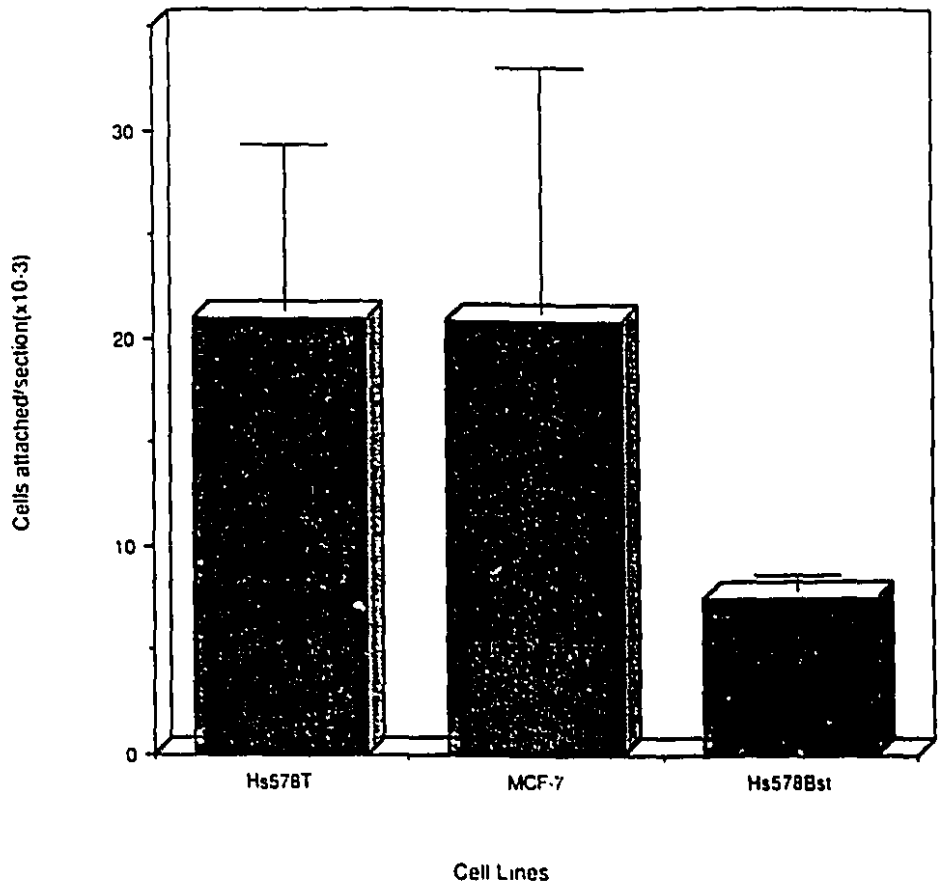

Fig. 4. Adhesion of human cell lines to frozen sections of human lymph nodes. Experiments were performed as detailed in the legend to Fig. 1. Results are expressed as means and S.D. of 3–4 experiments. In each experiment lymph nodes derived from different donors were used as a source of frozen sections. Adhesion of carcinoma line Hs578T was significantly higher than that of line Hs578Bst ($P < 0.025$).

breast cancer lines Hs578T and MCF-7 and the control epithelial line Hs578Bst. As shown in Fig. 4, the adhesion of line Hs578T was significantly higher than that of the normal epithelial cell line and comparable to the adhesion of line MCF-7. Treatment of tumor Hs578T cells with tunicamycin and pronase reduced adhesion significantly, while treatment with neuraminidase and β-galactosidase separately or in combination had no effect. None of the enzyme treatments increased the adhesion of line Hs578Bst (Fig. 5).

In order to determine whether the level of adhesion *in vitro* of line Hs578T correlated with metastatic potential *in vivo*, tumor cells were inoculated into the mammary fatpads of female nude mice. Regional lymph nodes were removed from the tumor bearing mice and the infiltrating tumor cells were harvested and expanded in culture. The adhesion of these cells to frozen sections of human lymph nodes was then compared to that of the parental cell line. Results shown in Fig. 6 demonstrate that tumor cells isolated from lymph node metastases in nude mice were significantly more adherent to the sections than the parental cell line, and that continuous selection of lymph node metastases resulted in further increase in the adhesion.

To test whether attachment to extracellular matrix proteins played a role in the adhesion, the ability of the Arg-Gly-Asp containing hepatpeptide GRGDSPK to block adhesion of the tumors to the lymph node sections was tested. Results shown in Fig. 7 and Fig. 8 demonstrate that the heptapeptide inhibited the attachment of tumors Hs578T and TMT-081 (respectively) significantly better

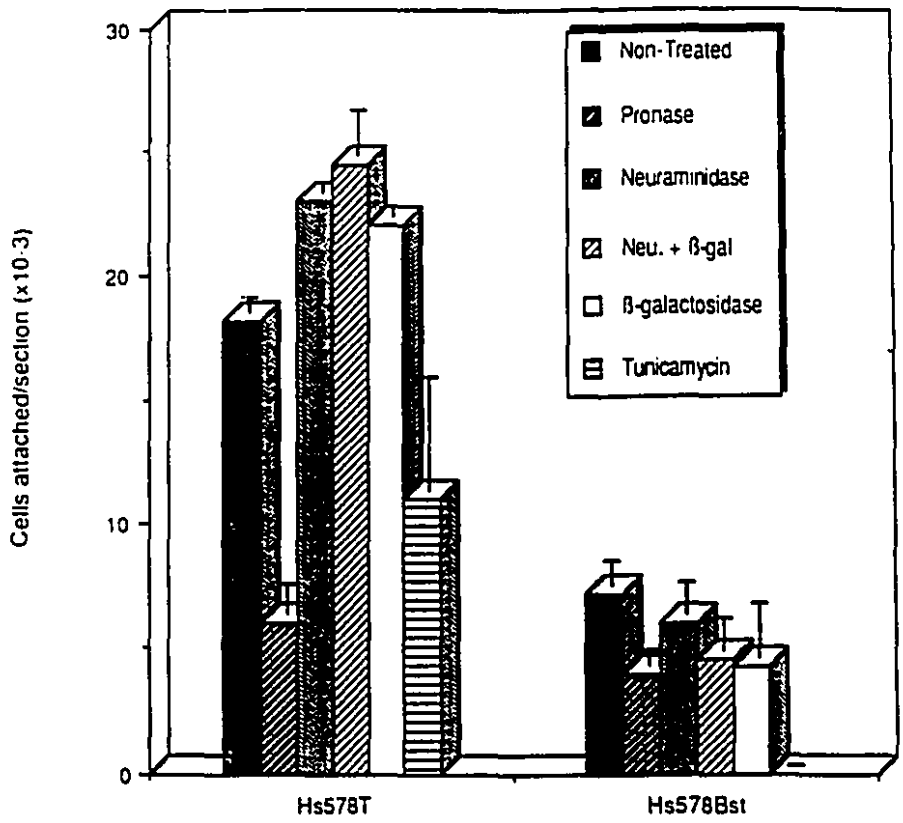

Fig. 5. The effects of tunicamycin and enzyme treatment on the adhesion of the human cell lines to frozen sections of human lymph nodes. The experiments were performed as described in the Materials and methods section. The reduction in adhesion of line Hs578T was significant following treatment with pronase ($P < 0.0025$) and tunicamycin ($P < 0.005$) only. The reduction in adhesion of Hs578Bst following treatment with pronase, β-galactosidase, or neuraminase and β-galactosidase was also significant ($P < 0.05$). (–) denotes experiment not done.

than the peptide GRGESP containing the sequence Arg-Gly-Glu. The inhibition of adhesion by the peptide was not complete, reaching 48% (TMT-081) and 77% (Hs578T) of control levels at a concentration of 0.75 mM. In comparison, inhibition by the control peptide was 6% and 33% respectively.

The role of integrin receptors in the adhesion to frozen sections was further assessed by pretreating tumors TMT-081 and Hs578T prior to adhesion with a polyclonal antibody directed to the β_1 subunit of integrin and with a monoclonal antibody (LM609) directed against the $\alpha_v\beta_3$ receptor for vitronectin [24]. The role of the receptors for fibronectin and vitronectin was of particular interest, as the synthesis of these matrix proteins is significantly augmented in lymph nodes infiltrated by metastasizing tumor cells [27]. We found that the antibody to β_1 reduced adhesion of both tumors (Fig. 9). The reduction in adhesion was, however, considerably greater with tumor Hs578T (>75% vs 30% for TMT-081). MoAb LM609 had no significant effect on the adhesion of either of the tumor lines (not shown).

Discussion

This investigation was prompted by our earlier finding that the potential of tumor cells to metastasize to regional lymph nodes correlated with adhesion to frozen lymph node sections in a murine lung carcinoma model [16]. The objective of the present experiments was to establish whether this

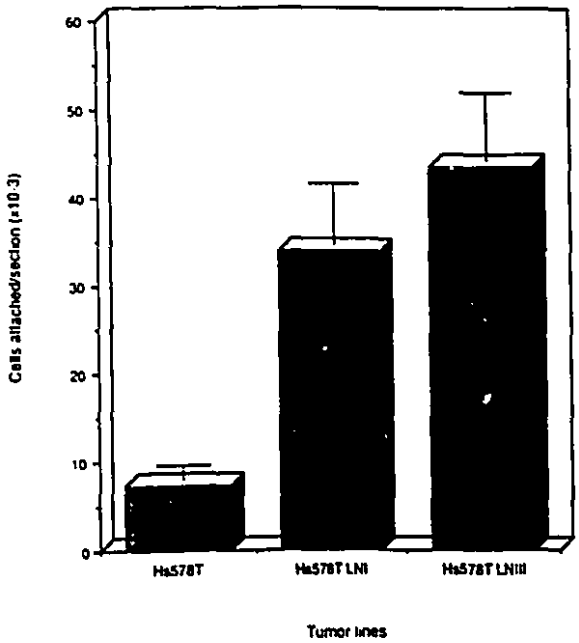

Fig. 6. Increased adhesion of Hs578T cells following selection of metastatic tumor cells from regional lymph nodes of nude mice. Hs578T cells were inoculated into the mammary fatpads of female nude mice and lymph node metastases were harvested and cultured as described in Materials and methods giving rise to line LNI. Line LNIII was derived following three consecutive cycles of isolation and reinjection of lymph node metastasizing cells. Results are means and S.D. of quadruplicate samples. Adhesion of lines LNI and LNIII cells was significantly higher than that of the parental line ($P < 0.005$). P value for LNIII vs LNI is < 0.1.

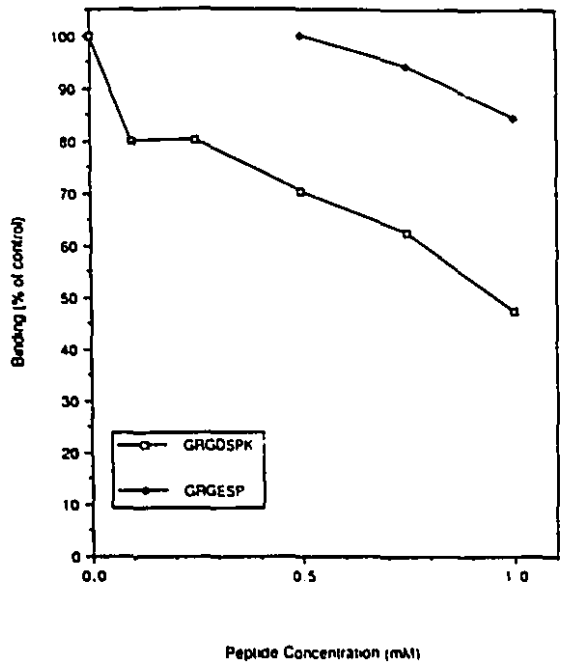

Fig. 7. Effect of GRGDSPK and GRGESP on adhesion of tumor TMT-081. Tumor cells (10^7/ml) were incubated with the peptide for 15 min. at 4°C and then added to the sections (5×10^5 cells in 0.05 ml) without washing. Experiments were carried out in quadruplicates and each point is the average of 2 experiments. The S.D. within experiments ranged between +–10% of the mean adhesion values.

correlation was also relevant to other tumor models including human carcinomas. Breast cancer models were selected for study because lymphatic metastasis is a common but little understood event in the pathology of this disease [5, 28, 29].

We found that tumor cell attachment to cryostat sections of lymph nodes and spleen correlated well with the ability of the rat tumors to metastasize lymphatically, while the attachment of a human breast carcinoma line was significantly higher than that of non-malignant breast epithelial cells. Furthermore, adhesion of the human carcinoma cells appeared to correlate with potential for lymphatic metastasis *in vivo* in immunodeprived nude mice. The results also demonstrated that, in both human and rat tumor models, cell surface glycoproteins were involved in the attachment. As the attachment of the rat but not of the human tumor cells was sensitive to treatment with neuraminidase and β-galactosidase, it appears that different carbohydrate residues are involved in the adhesion of these tumors.

Changes in cell surface carbohydrates have been linked to cell adhesion and to the metastatic phenotype in various other tumor models [30–32]. Of particular interest in this context is a recent report by Buckley and Carlsen [33] demonstrating a positive correlation between the potential of rat mammary carcinoma cells to metastasize to the regional lymph nodes and their ability to bind the lectin soybean agglutinin. In this model terminal galactose and N-acetylgalactosamine were implicated in the metastatic phenotype of the tumor cells. Our results with the rat model are similar to previous findings with the murine tumor line H-59, where β-galactoside and sialic acid residues were implicated in attachment to mouse lymph node sections

[16]. However, the adhesion molecules in the two tumor models appear to be antigenically distinct, since a MoAb (12/50) which was found to block the adhesion of the murine tumor cells to the sections failed to inhibit attachment of the rat tumor TMT-081 [17].

Our blocking experiments with synthetic peptides and with antibodies directed to the β_1 subunit of integrin suggest that receptors for extracellular matrix proteins and in particular for fibronectin are involved in the adhesion to the lymph node sections. As the inhibition of TMT-081 and Hs578T adhesion in the presence of the RGD-containing peptide did not exceed 50–75% of control, it is probable that other adhesion factors may also be involved [34]. In recent related studies, we found that tumor TMT-081 was more adherent to fibronectin than the other rat tumor cell lines, while there was no difference in the adhesion to collagen type I, collagen type IV, laminin and vitronectin [35]. In addition, light microscopy analysis confirmed that tumor TMT-081 cells attached to regions of the frozen sections which labelled positively with antibodies to fibronectin (manuscript in preparation). Furthermore, tumor line Hs578T (LN1) adhered significantly better to fibronectin and collagen type IV than the parental line Hs578T (unpublished observation). Together these results suggest that preferential adhesion to the frozen sections could be related to an increased attachment of the tumors to matrix proteins, fibronectin in particular.

While increased attachment to basement membrane (BM) fibronectin and laminin has been linked repeatedly to tumor cell potential to metastasize hematogeneously [36], few investigations have been reported on the role of matrix attachment in lymphatic metastasis. Relevant in this respect are two recent studies by Kramer *et al.* [37] and Reilley and Nash [27], the former showing that the reticular fibers of the node consist of collagen type III fibers surrounded by the BM proteins fibronectin, laminin, and collagen type IV, and the latter demonstrating an increased synthesis of fibronectin and vitronectin in lymph nodes infiltrated by carcinoma cells. These studies suggest that matrix proteins are likely to play a role in tumor disseminating to the lymph nodes, possibly by serving as sites of 'anchorage' for the disseminated tumor cells.

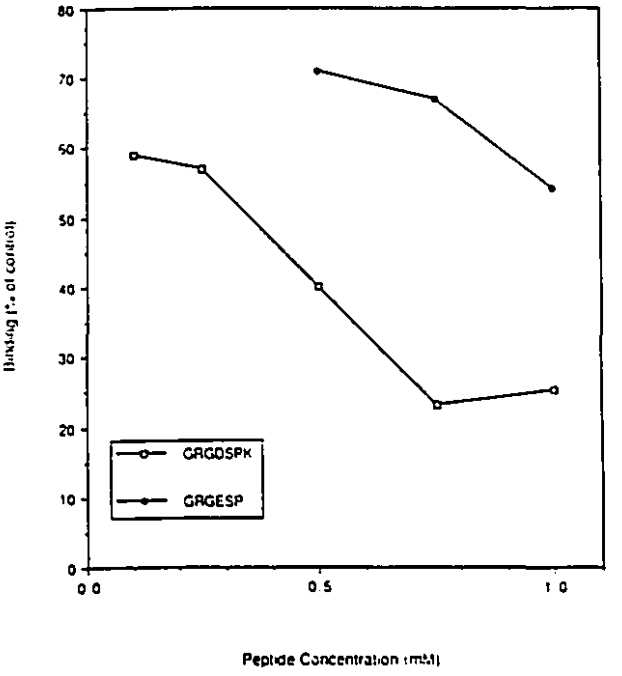

Fig. 8. Effect of GRGDSPK and GRGESP on adhesion of Hs578T. The experimental procedure was as described in the legend to Fig. 7. Each point is the average of 2 experiments. The S.D. within experiments ranged between 5 and 15% of the mean adhesion values.

It should be pointed out that tumor cell adhesion is only one in a chain of events which constitutes the 'metastatic cascade' [38]. Access to the organ microvasculature from the primary site (mechanical factors), invasion, and proliferation in the microenvironment of the secondary organs are some of the other limiting factors which influence the potential of the tumor cells to form metastases [2, 39]. This is demonstrated well by our findings that the rat tumor cell lines which exhibited higher adhesion to liver than to lymph node sections *in vitro* (Fig. 2) failed to metastasize to the liver *in vivo*.

Cell adhesion to frozen sections *in vitro* has previously been used to study the role of specific cell-cell interactions in lymphocyte traffic and homing [40] and in the organ specific patterns of metastasis of several tumor types [41, 43]. Lymphocytes and lymphoma cells have been shown to adhere preferentially *in vitro* to high endothelial venules of pe-

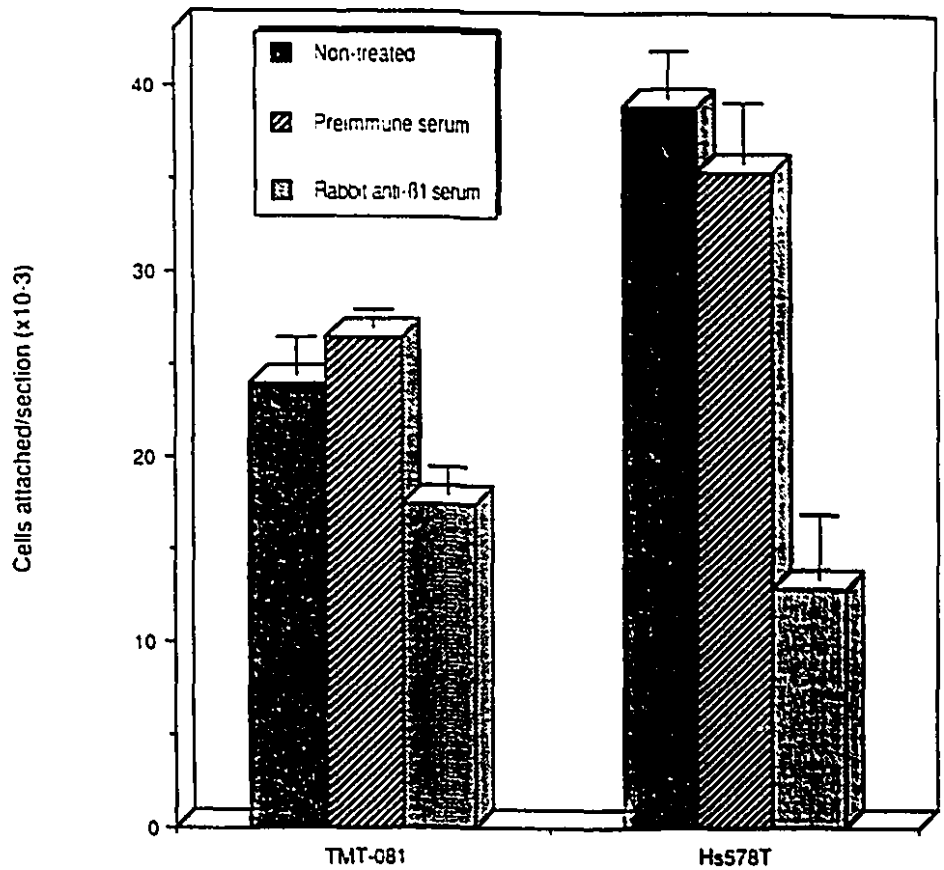

Fig. 9. Effect of rabbit antibodies to the β_1 chain of integrin on adhesion. Cells (4×10^5/ml) were incubated for 1 hr. at 4°C with the antibody or with preimmune rabbit serum diluted 1 : 25 in RPMI. Shown are means and S.D. of quadruplicate samples. The reduction in adhesion following treatment with the antibody was significant for tumor Hs578T ($P < 0.005$) and for tumor TMT-081 ($P < 0.01$). The serum dilution of 1 : 25 was selected on the basis of preliminary experiments showing maximal *specific* inhibition at this concentration.

ripheral nodes or Peyer's patches, corresponding to their preferential arrest in these nodes *in vivo* [40, 43]. Those studies eventually led to the identification of the lymphocyte 'homing receptors', among them one integrin molecule, which direct lymphocyte traffic [44, 45]. Studies with tumor cells have demonstrated a correlation between tumor cell potential to metastasize to liver and lung and their adhesion to frozen sections of these organs [41, 42], but the site of tumor attachment on these sections has not been identified. Other studies demonstrated that the endothelial cells [6, 7] and the extracellular matrix proteins [46] of different organs express unique adhesion properties, suggesting that they may provide the basis for the specificity of tumor cell attachment *in vitro* and 'homing' *in vivo*.

Although the adhesion ligands on the cryostat sections in our experimental models remain to be positively identified, our results taken together suggest that the tumor cells adhere to the lymph node ECM, possibly to ECM components exposed or deposited in the sinusoidal spaces, in particular fibronectin. This adhesion may in turn be required for tumor cell proliferation in response to local growth factors, as has been demonstrated in other models of organ specific metastasis [47]. Our findings that preferential adhesion to lymph node sections is a correlate of lymphatic metastasis in carcinoma models of diverse origins strongly suggest that this *in vitro* model provides a useful system for

analysis of adhesion factors relevant to lymphatic metastasis *in vivo*. Moreover, it could potentially be used as a clinical tool to aid in the staging and prognosis of breast carcinoma.

Acknowledgements

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Chapter VII

Discussion

7.1 Discussion

The evidence described demonstrates that the $\alpha_v\beta_3$ vitronectin receptor is involved in melanoma metastasis to the lymph nodes while for breast carcinoma integrin $\alpha_3\beta_1$ may play a metastasis facilitating role. The results described provide the first indication that the vitronectin receptor plays a role in regulating cellular invasion through a transcriptional link with the uPAR/uPA system of proteolysis. This could provide the cells with a central mechanism for coordinating cellular adhesion, matrix degradation, and migration since $\alpha_v\beta_3$ mediates cell adhesion to matrix vitronectin, and the uPAR/uPA complex is involved in localized pericellular matrix degradation at the cell-substratum interphase during cell migration.

Several mechanisms may account for the increase in uPAR expression and function seen following ligation of the vitronectin receptor. Antibody- or ligand-mediated crosslinking of integrins have been shown to trigger signal transduction mechanisms leading to protein tyrosine phosphorylation (1, 2), changes in calcium influx (3), gene transcription (4), and altered cell growth. Recently, the signaling pathways of integrins, including $\alpha_v\beta_3$ have been more closely examined. The molecule thought to play a central role in integrin-mediated signal transduction is pp^{125FAK}. This molecule has been shown to have binding

sites for proteins involved in the Ras and Src signaling pathways (5) further supporting a direct link between integrin-mediated and Ras signaling pathways. A recently identified integrin-linked protein kinase (ILK) was coimmunoprecipitated with β_1 and phosphorylate in the cytoplasmic domain suggesting that this molecule can also mediate integrin intracellular signal transduction possibly independently of FAK (6). Another FAK-independent tyrosine kinase Syk, was shown to be involved in post-adhesion signaling by monocytes (7). It is thought that Syk is involved in nuclear translocation of the transcription factor NF-κB. Interestingly, it has been shown that an antisense oligonucleotide directed to the p65 (RelA) subunit of NF-κB inhibits $\alpha_v\beta_5$-mediated PKC-dependent cell migration on vitronectin (8, 9). As well, studies on the fibronectin receptors $\alpha_5\beta_1$ and $\alpha_4\beta_1$ which recognize different regions on the fibronectin molecule, revealed differences in their effects on MMP (stromelysin-1 and 92-kD gelatinase) gene expression (10) upon ligand-binding suggesting that they mediate distinct ligand-specific signal transduction mechanisms. It is possible that in the melanoma cells, cross-linking of α_v integrins triggers a signaling cascade leading to the upregulated expression of uPAR possibly through the activation of PKC - a regulator of uPAR synthesis (please also see discussion in Chapter IV). Alternatively, it is conceivable that $\alpha_v\beta_3$-mediated ligand-binding brings

about increased synthesis of autocrine factors such as bFGF and TGFß (11,12) which were implicated in the regulation of uPA and uPAR synthesis (13-18).

In light of our findings, it is also conceivable that some of the effects previously attributed to $\alpha_v\beta_3$ function such as its role in cellular migration, invasion, and angiogenesis (19-21) were in fact mediated through upregulation and cell surface localization of the uPAR/uPA complex (22). In a recent series of reports, (13, 23, 24) it has been shown that uPA and uPAR expression in cultured endothelial cells is regulated by bFGF and that uPA in turn can catalyze the proteolytic conversion of the precursor of TGFß into its biologically active form. The induction of uPAR synthesis in response to $\alpha_v\beta_3$ ligand-binding may also provide a mechanism for control of melanoma cell growth as activation of TGFß may provide a signal for melanoma cell proliferation (25). Furthermore, plasmin was shown to catalyze the hydrolytic cleavage of the matrix-associated motility factor pro-HGF (scatter factor) to its active form (26, 27) and melanoma cells were found to express the tyrosine kinase receptor for HGF/SF, oncogene c-met (28), and respond to exogenous HGF synergistically with bFGF to promote motility and growth (29).

As mentioned previously, involvement of the regional and distant lymph nodes is a major determinant of poor prognosis in melanoma patients. As our studies revealed, metastatic melanoma cells isolated from

regional lymph nodes express increased levels of both $\alpha_v\beta_3$ and uPAR. This suggests that upregulated expression of these receptors may give the melanoma cells a selective growth advantage in the microenvironment of the node. When taken together with other observations, our results are consistent with the following postulated model for early events in the course of lymph node invasion and colonization (see Fig. 1 and 2):

Melanoma cells from the primary tumor may infiltrate the node through the afferent lymphatic vessels and enter the subcapsular (marginal) sinus, which is lined by reticuloendothelial cells enmeshed in reticular fibers traversing the sinusoidal lumen (30). These fibers, which are composed of a central core of type III collagen fibrils surrounded by ECM proteins such as laminin, type IV collagen (31) and possibly, fibronectin and vitronectin (32, 33) may initially provide a scaffolding for melanoma cell attachment and migration, two functions dependent on integrins. As nodal infiltration by tumor cells is accompanied by increased production of fibronectin and vitronectin (33), the local microenvironment could become increasingly more favourable for melanoma cells which express high levels of $\alpha_v\beta_3$. Breast carcinoma cells expressing increased levels of the $\alpha_3\beta_1$ receptor for fibronectin, collagen, and laminin would also benefit from this fibronectin-rich microenvironment. Others have shown using intravital microscopy

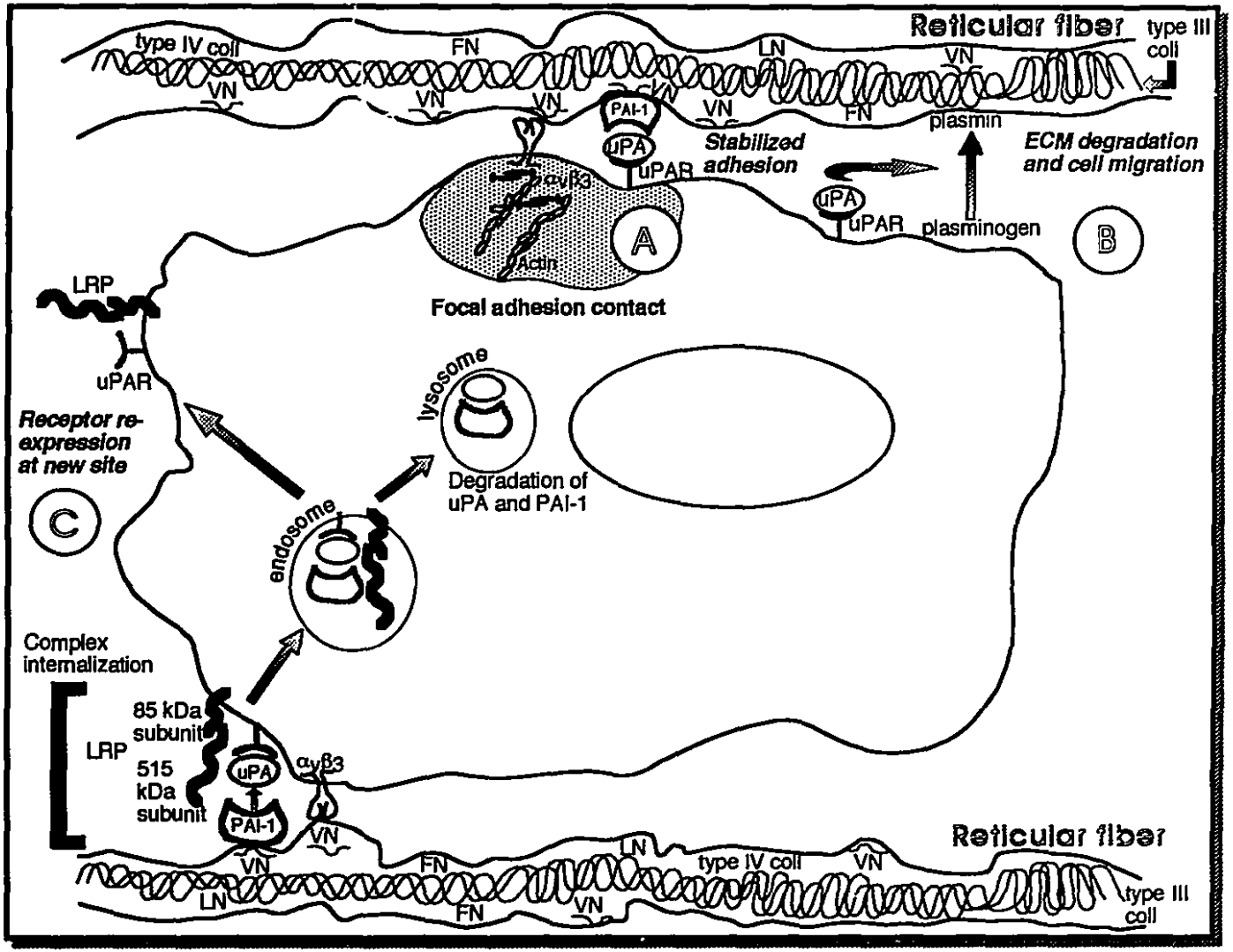

Figure 1. The uPAR:uPA system of proteolysis, the expression and processing of its components and their functional association with $\alpha_v\beta_3$-mediated adhesion to the ECM shown in the context of the lymph node sinus.

Schematic diagram of the known functional links between vitronectin receptor-mediated adhesion and the urokinase plasminogen activation system. Shown is a diagram of a melanoma cell within the confines of lymph node marginal sinus. This picture incorporates studies published by other authors and described in references 35, 36, and 47-49.

(IVVM) that mechanical arrest is involved in the early stages of metastasis formation and that specific adhesion may not be the predominant homing mechanism (34). These findings were observed in studies on the microvasculature of mouse liver and muscle cells during blood-borne metastasis. However, similar studies have not been done using the regional lymph nodes. Binding of the vitronectin receptor to local ECM vitronectin may trigger increased synthesis and clustering of the uPA receptors at focal adhesion contacts (35, 36), resulting in more efficient pericellular matrix degradation and cellular migration (see Fig. 2). It is conceivable that the increased proteolysis and migration we observed may have been due to more rapid turnover of the uPAR-uPA-PAI-1 complex formed via the vitronectin-bound PAI-1. Increased PAI-VN binding may have resulted in less vitronectin available for the VNR if the VNR-VN and PAI-VN binding are competitive. Likewise, α_v antisense treatment of the melanoma cells leading to decreased vitronectin receptor expression may have increased the availability of PAI-1 binding sites on vitronectin and this increase in PAI-1 sites could have resulted in reduced uPA-mediated proteolysis. This mechanism is unlikely however, since the binding sites on vitronectin for VNR and PAI-1 are distinct. The binding sites for PAI-1 and the vitronectin receptor have been localized to the somatomedin B and RGD domains of vitronectin, respectively (37). As well, the α_v antisense treatment resulted in a specific decrease in uPAR

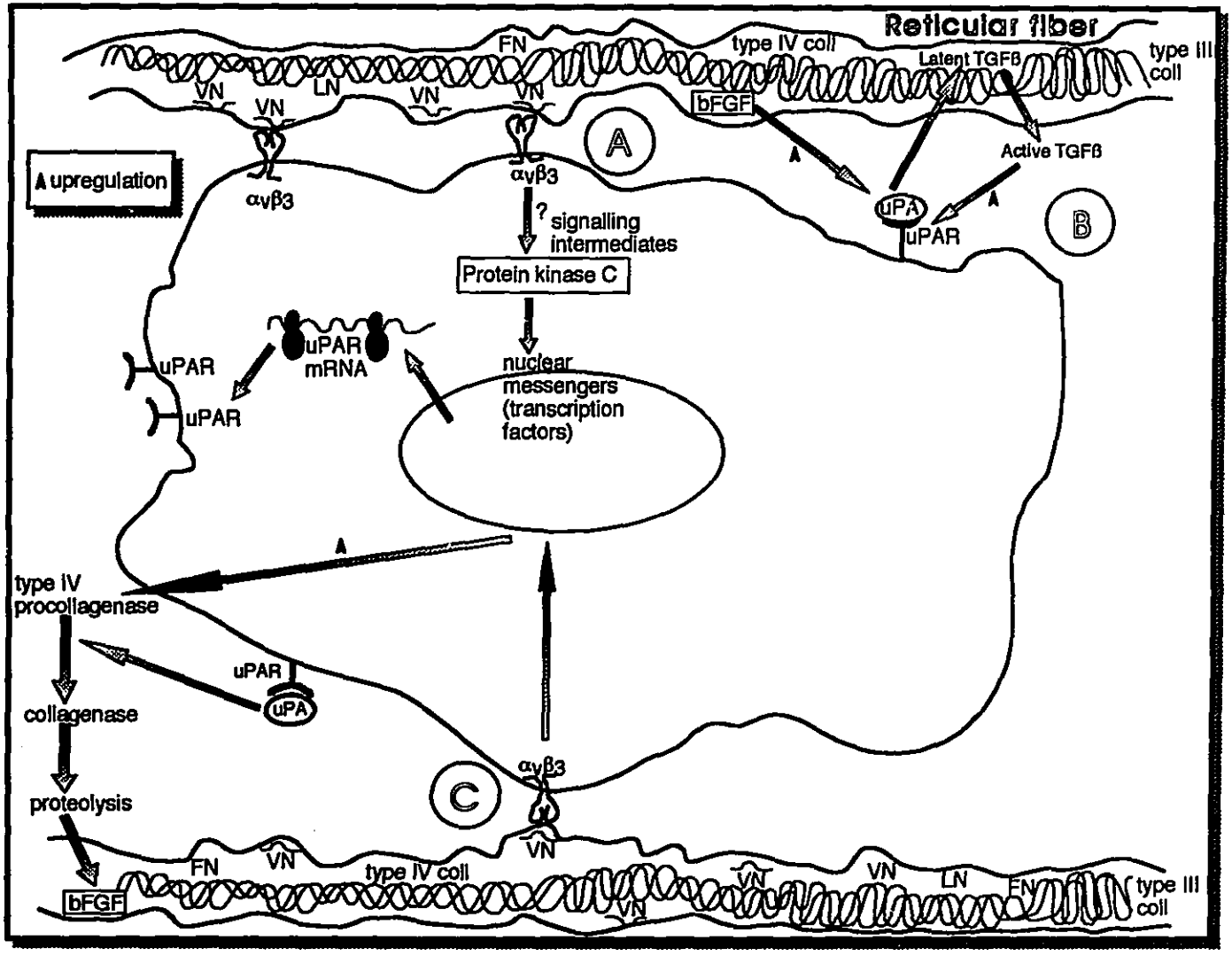

Figure 2. Postulated mechanisms for the observed regulatory link between $\alpha_v\beta_3$ and uPAR in the lymph node microenvironment.

Diagram of the regulatory link between $\alpha_v\beta_3$ and uPAR in the lymph node. Postulated mechanisms for such a link are as follows: 2A: Upregulation of uPAR synthesis through $\alpha_v\beta_3$-induced activation of protein kinase C, 2B: upregulated expression of uPAR-modulating growth factors or their receptors in response to melanoma cell attachment to ECM and 2C: increased expression of type IV collagenase in response to ligand binding by $\alpha_v\beta_3$, leads to release of ECM-bound uPAR-modulating growth factors. This picture incorporates studies published by other authors and described in references 13, 14, 20, 23, and 24.

mRNA suggesting that there is a true transcriptional link and not merely a functional association between $\alpha_v\beta_3$-mediated adhesion to vitronectin and the uPA proteolytic system. As uPAR-bound uPA can activate pro-MMP-2, this is also in accord with reports that MMP-2 activity in melanoma cells may promote cellular migration and invasion by reducing cellular adhesive (integrin-mediated) contacts (38). Increased proteolysis, possibly also involving upregulation of type IV collagenase production (20) may result in the release and/or activation of matrix-bound growth factors such as bFGF, TGFß, and HGF/SF, which may in turn increase melanoma cell proliferation and motility. Receptor $\alpha_v\beta_3$-mediated ligand binding may also result in a net increase in melanoma cell growth through its more direct role as a negative regulator of apoptosis (21). Thus the cumulative effect of increased $\alpha_v\beta_3$ expression is to enhance the ability of the melanoma cells to survive, proliferate, and eventually invade the lymphatic parenchyma.

Alterations in normal cellular expression of integrins may contribute to the increased propensity of melanoma and breast carcinoma cells for lymphatic metastasis. In the epidermis, it has been shown that melanocytes express cell surface α_3, α_6, α_v, and β_1 (although in culture, α_5 and β_3 expression are also upregulated) (39). Migration of the melanocytes was shown to be mediated by $\alpha_v\beta_3$ as well as $\alpha_5\beta_1$ suggesting that

expression of the vitronectin receptor indeed makes the cells more motile. Upregulation of the vitronectin receptor in melanoma progression would explain the increased motility and invasiveness of the tumor cells. The integrin subunits expressed in normal breast tissue *in situ* include α_1, α_2, α_3, α_6, α_v, β_1, and β_4 (40). In infiltrating ductal carcinomas, immunocytochemistry demonstrated that the expression of α_v was increased whereas the other subunits were generally decreased. However, in mucinous breast carcinoma, cell surface expression of α_2, α_3, α_6, α_v, and β_4 was upregulated. Similarly to melanoma, breast carcinomas whether infiltrating or mucinous usually metastasize first to regional lymph nodes (41). In our system, the finding that the adhesion of lymph node-metastasizing Hs578T LN cells to frozen sections of human lymph nodes correlated with metastatic potential is in accord with our findings with the melanoma model. However, in the breast cancer model, the integrin molecule involved was identified as the $\alpha_3\beta_1$ receptor. This integrin has been implicated in the metastasis of a variety of solid tumors including breast carcinoma. In one study, the $\alpha_3\beta_1$ receptor was present in 82% of metastatic tumors examined immunohistochemically and was associated with a ligand present at the tumor epithelial-stromal border (42). In our study, $\alpha_3\beta_1$-mediated adhesion of the breast carcinoma cells to lymphatic stromal cell fibronectin may be important for lymphatic

metastasis. Fibronectin adhesion was through the $\alpha_3\beta_1$ integrin despite the significant levels of the major fibronectin receptor $\alpha_5\beta_1$, expressed on these cells. Others have shown that the ability of $\alpha_3\beta_1$ to mediate adhesion in the presence of other integrins is poor (43). Our findings may be unique to our breast carcinoma system, but demonstrates that $\alpha_3\beta_1$ may be the major contributor to FN adhesion even in the presence of the major fibronectin receptor. Since fibronectin is a major component of the reticular fibers of the lymph nodes, carcinoma cells expressing high levels of the $\alpha_3\beta_1$ receptor may adhere to these fibers and establish colonies in the node. While these integrin-ECM interactions are probably not unique to the lymph node and may also be required for colonization of other, more distant organs, it is at the early stage of the disease, before widespread dissemination occurs, that therapeutic intervention aimed at disrupting these interactions is likely to be most effective.

Disruption of adhesive interactions between integrins and their ligands could prove to be useful in preventing metastatic disease. For the vitronectin receptor $\alpha_v\beta_3$, synthetic integrin ligand analogues such as cyclic RGD peptides as well as neutralizing anti-integrin MAb's have been used in experimental *in vivo* models to cause regression of various tumors including melanoma (21). In our study, the α_v antisense phosphorothioate oligonucleotides have been used to block α_v synthesis

and functions and these may also prove to be a useful therapeutic tool. In our studies on MAb-mediated integrin crosslinking, we showed that uPAR mRNA synthesis was increased in response to the crosslinking of the VNR and that this caused an increase in invasiveness through Matrigel. The mechanism of this increase in uPAR mRNA production and cell invasion is not known but we postulate that it may be linked to activation of the PKC signaling pathways. If this is confirmed, the use of appropriate PKC inhibitors may provide alternative means for disturbing signal transduction mechanisms downstream of the VNR activation event. Together these approaches for inhibition of cell adhesion or the resultant signaling mechanisms could prove useful in the clinical management of disseminated cancers probably as adjuvants to conventional surgical techniques.

Future research would focus on elucidating the signaling pathways linking uPAR transcription to receptor $\alpha_v\beta_3$. Additionally, the roles, if any, of other vitronectin receptors such as $\alpha_v\beta_5$ and $\alpha_v\beta_1$, or other integrin heterodimers in regulating uPAR synthesis and function should be examined to determine if our findings with $\alpha_v\beta_3$ are unique to this integrin. Recent reports suggest that the signaling pathways of growth factor receptors such as the insulin (or IGF-1) receptor (44) and PDGF receptor (45) may be linked to $\alpha_v\beta_3$-mediated signaling. The clarification of the relationship between growth factors and $\alpha_v\beta_3$ signaling in our system

specifically, further examination of the expression of various growth factors in response to $\alpha_v\beta_3$ ligation and how they affect $\alpha_v\beta_3$ signaling pathways may be of interest. Finally, the consequences of long-term α_v suppression in melanoma cells and its effect on growth and development of metastatic disease in laboratory animals remain to be studied. In addition to long-term suppression, the development of α_v-deficient transgenic mice could be highly informative. In this respect it is of interest to note recent observations made with transgenic mice bearing a null mutation in the vitronectin gene (46). These mice were normal in terms of development and subsequent fertility with the only measurable change being a loss of PAI-1 binding activity of the murine plasma, as well as the lack of vitronectin protein. This finding may not be totally surprising as there may be some redundancy in function of the integrin adhesion systems during development and fibronectin receptors, such as $\alpha_5\beta_1$, may replace the vitronectin adhesion system in these mice.

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