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by

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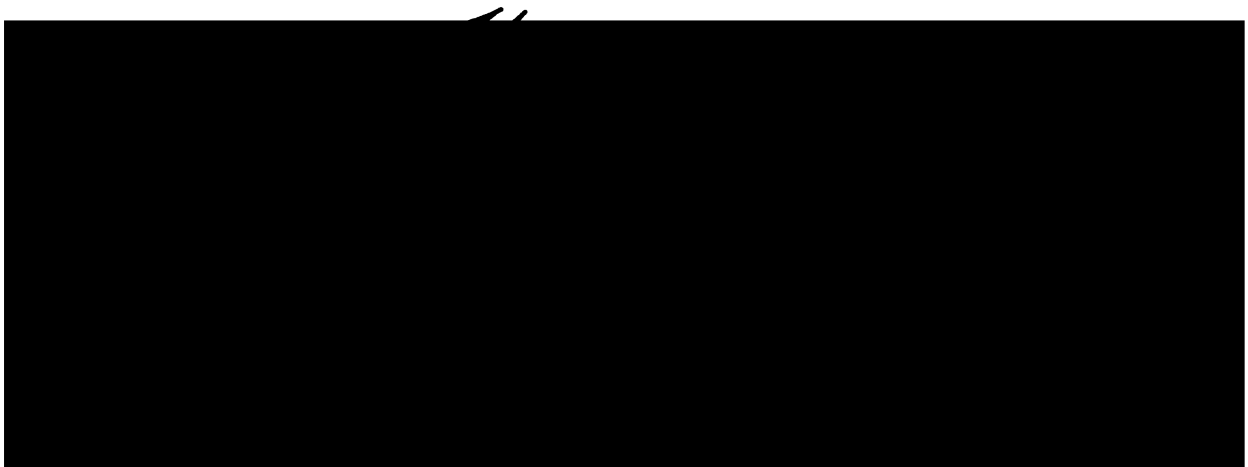

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III. Metastatic Melanoma Cells Interact with the Reticular Fibers of the Lymph Node. Berston, E.D., Ramos, D.M., and Kramer, R.H. *Melanoma Research* **4,** 21-27 (1994)

IV. Expression of High and Low Affinity Laminin-Binding Integrins is Coordinately Regulated During Melanoma Tumor Progression. Ramos, D.M., Waleh, N., Vu, M.P., Ziober, B.L., Yao, C.C., Chen, Y.Q., and Kramer, R.H. *Journal of Biological Chemistry* (submitted).

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ABSTRACT

DIFFERENTIAL EXPRESSION OF LAMININ-BINDING INTEGRINS IN METASTATIC TUMOR CELLS

by

DANIEL M. RAMOS

Tumor cell-substrate interactions are important in the development of metastases. These interactions are mediated by a variety of cell-surface receptors including the integrins. Laminin, a major component of basement membranes, has been shown to enhance metastatic potential. We tested the hypothesis that differential expression of laminin-binding integrins has functional consequences for tumor cell behavior.

A highly invasive fibrosarcoma cell line, (HT1080) expresses several putative laminin receptors: α2-, α3-, and α6β1. However, only the α6β1 integrin complex was functional in binding to laminin. In addition, HT1080 invasion through complex matrices, can be blocked by anti-α6 antibodies.

We also determined that the highly metastatic B16-BL6 murine melanoma cell line uses both the α6β1 and α1β1 integrin complexes to adhere to laminin, with α6 binding with higher affinity than α1. We also demonstrate that α1β1 is a receptor for collagen type IV.

Using the moderately metastatic B16F1 cell line we derived metastatic variants which were extremely efficient at targeting para-aortic lymph nodes. The metastatic variants were more efficient than the parental cells in adhering to cryostat sections of

lymph nodes and this adhesion could be substantially inhibited using anti-integrin antibodies.

In an additional study, we determined that the metastatic K1735 M2 melanoma adhere to and migrate on laminin using the $\alpha 6\beta 1$ complex. In contrast the nonmetastatic K1735C23 cells are nonmigratory on laminin, using the $\alpha 7\beta 1$ complex for adhesion. We also demonstrate that $\alpha 6\beta 1$ is a low affinity laminin receptor while $\alpha 7\beta 1$ binds laminin with high affinity.

The results from the four separate studies demonstrate that tumor cell-ECM interactions are mediated by a variety of integrin receptors, and that differential expression of laminin-binding integrins by tumor cells influences the metastatic phenotype. We suggest that expression of $\alpha 6\beta 1$ promotes the metastatic phenotype, while $\alpha 7\beta 1$ negatively influences metastasis.

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CHAPTER ONE

Introduction

Metastasis, the spread of cells from the primary tumor to distant sites and their subsequent growth at that site, is the most devastating aspect of cancer. Despite significant improvements in early diagnosis, surgical techniques and therapies, most deaths from cancer are due to metastasis (Fidler 1990). Metastasis, a highly selective process, is influenced by both host factors and intrinsic properties of the metastatic tumor cells. Cell-substrate adhesion occurs early in the invasive process when tumor cells interact with the basement membrane and later with the underlying connective tissue (Liotta 1986). Adhesion is implicated in all further steps of metastasis. Participants in this interaction are components of the extracellular matrix (ECM) such as collagens, fibronectin, laminin elastin, proteoglycans and others as well as cell surface receptors such as the integrins and laminin receptors. Liotta (1986) and collaborators have tried to simplify this by proposing a three step hypothesis of invasion. 1) Attachment of the cells to the ECM; 2) Local and pericellular degradation of the ECM by proteolysis; 3) Tumor cell locomotion into the region of the matrix modified by proteolysis. Progressive invasion of ECM may take place by the cyclic repetition of these three steps. The attached cell then secretes hydrolytic enzymes (i.e. plasminogen activator, collagenases) which can locally degrade the matrix. Displacement of tumor cells is an essential physical aspect of invasion and metastasis. This displacement can be explained by active translocation alternating between adhesion to and deadhesion from the ECM (Nicolson 1989).

During local invasion tumor cells must penetrate the basement membrane that surrounds blood vessels. During blood borne metastasis, tumor cells must survive the transport in the circulation, adhere to small blood vessels at distant capillary beds and once again invade the blood vessel wall (Killon and Fidler 1990). This series of steps also requires multiple interactions with the extracellular matrix using specific cell surface receptors.

It is well documented that malignant tumors are not composed of a homogenous population of tumor cells, but rather a heterogenous cell population with regards to surface antigens, growth potential, pigment production, and metastatic potential (Fidler 1978). This suggests that metastasis favors the survival and growth of a small subpopulation of cells that pre-exist with the primary tumor.
Very few (<.1%) tumor cells survive this multistep process suggesting that metastasis is quite selective (Fidler *et al.*, 1978). A successful metastasis depends on the interaction of metastatic cells with different organ environments (Fidler 1990).

Evidence that malignant tumors contain subpopulations of cells with differing metastatic potentials was shown by Fidler and Kripke (1977). Studying the B16 melanoma cell lines, they found that tumor cell populations of both high and low metastatic potential exist within a single parental tumor. Earlier work by Nowell (1976), demonstrated that tumor cell variants arising within developing tumors are subjected to host selection pressures and give rise to new sublines with increased metastatic potential. Price et al (1986) also provide evidence that melanoma metastasis is dependent on pre-existent metastatic cells within the primary tumor.

Three metastatic and three nonmetastatic clones of the K1735 murine melanoma were evaluated using experimental metastasis assays (Price *et al.*, 1986). There was no significant difference in the gross survival of metastatic and nonmetastatic cells in lungs and other organs within one hour post inoculation. However very few metastatic and no nonmetastatic cells survived to invade and grow at distant organ sites suggesting that metastasis is a very selective process and that most metastatic cells probably die from random events (i.e. host immune system). Further evidence that metastasis is highly selective is provided by Nicolson, who proposes that tumor cells in the primary mass must first diversify in order to metastasize (1988). As tumor cells become more diverse some will acquire the correct phenotypic characteristics to make them dominant and subsequently metastatic (Nicolson, 1988). Kerbel proposes that growth preference by highly metastatic clones within the primary tumor eventually allow them to dominate (1990). Although growth preference or dominance is not all that is required for tumor cells to acquire metastatic competence, it is a major trait of the metastatic cancer cell and is necessary for expression of metastatic ability.

Tumor cells and the extracellular matrix

Attachment of cancer cells to the ECM components is an important event occurring many times during invasion and metastasis. In order to move through the ECM a tumor cell must adhere to and migrate upon matrix molecules. The cell must express specific surface adhesion receptors for components of the matrix. Appropriate attachment sites within the matrix molecules must be available to the cell and the cell must be able to break its adhesive contacts as it migrates (Starkey 1993). The ECM

is a complex network composed of an array of very large multi-domain molecules linked together by covalent and noncovalent bonds into an insoluble component. The ECM is composed of a variety of polysaccharides and proteins secreted by the cells. Vascular basement membranes contain type IV collagen, laminin, fibronectin, vitronectin, heparan sulfate proteoglycan, entactin, thrombospondin and von Willebrand factor. The interstitial matrix contains fibrillar collagens (type I, III, V and others) fibronectin, hyaluronic acid and fibrillar associated proteoglycans (decorin). Many of these ligands contain a unique cell binding site represented by the amino acid sequence Arg-Gly-Asp (RGD). This sequence is present in the cell binding domain of fibronectin, and in other adhesion ligands such as vitronectin, fibrinogen, collagens, entactin, thrombospondin, and laminin. While the RGD site has been shown to be of great importance in cell attachment, many of these proteins contain additional domains which function as adhesion sites for integrin and non-integrin receptors. The ECM acts as a supporting scaffold which isolates tissue compartments, mediates cell attachment, and influences tissue architecture (Liotta 1986). A characteristic feature of extracellular matrix proteins in general, and of basement membrane components is that they are capable of multiple interactions, both with cells and other extracellular matrix components. In addition, the ECM helps regulate cell function. It can act a positive as well as a negative regulator of functional differentiation depending on the cell type and genes studied (Jones *et al.* 1993). The ECM can also interact with a variety of growth factors (Tsuboi *et al.*,1990). There is some evidence that the binding of growth factors to the ECM is critical for their biological activity (Flaumenhaft and

Rifkin 1990). Growth factors bound to the ECM often have altered potency, increased stability, and are concentrated in the vicinity of the cell. This suggests that not only can the ECM act as a structural element but also is capable of transducing signals via concentration of growth factors. Adhesion, the first step in the metastatic cascade has been the most extensively studied. It is apparent that tumor cell adhesion involves interactions between specific cell surface receptors and individual components of the ECM. The basement membrane is a specialized ECM which underlies all epithelia, surrounds muscle, peripheral nerves, and fat cells. During metastasis, tumor cells must cross endothelial basement membranes when entering and exiting the circulatory system. Thus, tumor cell interaction with the basement membrane is quite important. Laminin a major glycoprotein found in basement membranes plays an important role in the interaction of tumor cells of the basement membrane.

LAMININ

Laminin, the major noncollagenous glycoprotein component of basement membranes, has been implicated in several biological processes including cell adhesion, migration, differentiation, cell polarization, neurite outgrowth, and enhancing metastatic potential (reviewed in Timpl 1989). It also binds to other components in the matrix (i.e. type IV collagen, heparan sulfate proteoglycan and entactin) and to itself (Kleinman and Weeks 1989). The multifunctional properties of this molecule may relate to its large size and multidomain structure.

Laminin can best be thought of as a family of related proteins. The best studied form of laminin is derived from the murine EHS tumor and consists of a 400 k

A chain, a 210 k B1 chain, and a 200 k B chain. This form of laminin (A-chain) is ubiquitous in basement membranes. It is a cross shaped molecule with a large multilobulated globular domain at the base of the cross and smaller globular domains on each arm (Beck *et al.*, 1990). Several other laminin isoforms have been identified and include merosin, S-laminin, S-merosin, kallinin/epiligrin, K-laminin (reviewed in Tryggvason 1993). These isoforms usually contain a heavy chain (A, M, or K) and two light (B1, B, and S). The different laminin isoforms appear to be tissue specific and are expressed in a developmentally regulated pattern.

It is well established that laminin has multiple functional domains. A major adhesion and migration promoting region in the classical A chain laminin has been located in the long arm near the globular end (E8 fragment). Several integrin receptors have been shown to bind to this region of laminin, including $\alpha 3\beta 1$ (Gehlsen *et al.*, 1988, 1990), $\alpha 6\beta 1$ (Aumailley *et al.*, 1990; Hall *et al.*, 1990); and $\alpha 7\beta 1$ (Kramer *et al.*, 1991). Another major cell attachment site is present in the short arms of laminin that comprise a cross structure and seem to be the major binding site for the $\alpha 1\beta 1$ integrin (Kramer *et al.*, 1991; Goodman *et al.*, 1991). In the same inner cross region, there is a cryptic RGD sequence located in the A chain of EHS laminin that becomes exposed after pepsin digestion which promotes cell adhesion through interactions with the $\alpha v\beta 3$ receptor (Aumailley *et al.*, 1990; Sonnenberg *et al.*, 1991). Tumor cells express a wide variety of cell surface components capable of interacting with laminin including lectins, galactosyltransferase, 67 k family of receptors, cranin, dystroglycan and the integrins (Smalheiser and Schwartz 1987; Graf *et al.*, 1987;

Runyan *et al.*, 1988). The integrins have been identified as playing a major role in tumor cell adhesion to the extracellular matrix (Dedhar and Saulnier 1990).

Integrin Family of Adhesion Receptors

All integrins are αβ heterodimers. The α subunits range in size between 120 and 180 k and each is noncovalently associated with a β subunit. Most integrins are expressed on a variety of cells, and most cells express several integrins. At this time there are 8 known β subunits and 14 known α subunits (Reviewed in Hynes 1992). These include 8 known complexes that can serve as receptors for fibronectin, collagens, laminin, and vitronectin. Many α subunits can associate with only a single β subunit. However, several α subunits (α4, α6, and αv) can associate with more than one β subunit; with αv being quite promiscuous in this respect (Hynes 1992). αv has been shown to bind multiple β subunits including β1 (Bodary and McLean,1990; Vogel *et al.*, 1990), β3 (Cheresh and Spiro, 1987), β5 (Cheresh *et al*, 1989), β6 (Sheppard *et al.*, 1990) and β8 (Moyle *et al.*, 1991).

While the αvβ5 and the αvβ1 integrins appear to be restricted in their ligand binding specificity, the αvβ3 heterodimer interacts with multiple ligands in an RGD dependent manner (Cheresh and Spiro 1987; Charo *et al.*, 1987). Integrin cytoplasmic domains are believed to interact with cytoskeletal proteins and possibly other cytoplasmic components. Evidence for cytoskeletal connections come from a variety of sources. These include microscopic evidence for co-localization of integrin and cytoskeletal structures (Burridge and Faith 1989), as well as biochemical evidence for interactions of integrin or integrin cytoplasmic domain peptides with the

cytoskeletal proteins talin (Horwitz *et al.*, 1985) and α actinin (Otey *et al.*, 1990). Deletion of all or part of the β1 cytoplasmic domain, the portion of the β subunit which interacts with the cytoskeleton, interferes with association into focal contacts (Solowska 1989, Hagashi 1990). Although there have been no reports of cytoskeletal proteins binding to the α subunit cytoplasmic domains, recent studies suggest this possibility (Chan *et al.*, 1992; Tawil *et al.*, 1993; Briesewitz *et al.*, 1993). α subunits may play a role in regulating the recruitment of integrin receptors to focal contacts (Briesewitz *et al.*, 1993). For example α5β1 is found in focal contacts while α3β1 is not, although both interact with fibronectin (Elices *et al,* 1991). αvβ3 localizes to focal contacts whereas αvβ5 does not although both interact with vitronectin (Cheresh *et al.*, 1993). Additionally, α subunit cytoplasmic domains have been implicated in preferential association of laminin-binding integrins with either focal contacts or podosomes (Tawil *et al.*, 1993). These examples imply that different integrin α subunits may be important in mediating different cellular responses to a common ligand. Recently, a study using chimeric integrin α subunits demonstrates that the wild type α2 and α2\α5 cytoplasmic chimera promotes contraction of collagen gels, while the α2/α4 cytoplasmic chimera promotes cell migration on laminin substrates, while the adhesion to either substrate is not changed (Chan *et al* 1992). The α subunit cytoplasmic domain may interact with different cytoplasmic proteins than the β subunit and this interaction may modulate specific aspects of integrin function subsequent to adhesion (i.e. ability to promote migration or contraction).

Distinct but homologous β subunits termed β1-β8 have been identified, each of

which is capable of associating individually with one or more α subunits (Reviewed in Hynes, 1987, 1992; Hemler, 1990). These include eight known complexes that can serve as receptors for fibronectin, collagen, laminin, and vitronectin. Integrins with the β1 subunit are found on a variety of cell types and mediate adhesion to a variety of extracellular matrix components. There is some redundancy in β1 ligand binding. For example α1,α2,α3-,α6-, and α7β1 all bind laminin, although recently we demonstrate the α6- and α7β1 integrins have very different laminin-binding affinities (Ramos *et al.*, submitted). Since receptors with the same β subunit but distinct α subunits differ in their binding properties, ligand specificity has been attributed mainly to the α subunit.

Integrins with the β2 subunit are leukocyte-specific and mediate many adhesive interactions in these cells (Reviewed in Arnaout, 1990). The β2 subfamily of integrins also known as CD18 antigens consist of three leukocyte adhesion receptors: LFA-1, MAC-1, and GP150,95 (Reviewed in Albelda and Buck 1990). They share a common β subunit and are restricted to blood cells. LFA-1 is found in most white blood cells. The ligand for LFA-1 is ICAM-1 a member of the immunoglobulin family (reviewed in Albelda and Buck). MAC-1 found on macrophages and monocytes and some lymphocytes and has a wide range off ligands (ICAM-1, iC3B, Factor X, fibrinogen). It is believed MAC-1 can participate in complement binding, phagocytosis, and cell-cell adhesion. Relatively little is known about gp150,95 other than it binds to iC3b. Integrins with the β3 subunit include the platelet GPIIb-β3 complex and the vitronectin receptor αvβ3 (Phillips *et al.*,1988). GPIIb-β3 is restricted to platelets and

other cells of the megakaryocytic lineage, while αVβ3 is expressed in several cell types, including melanoma cells. The αvβ3 complex is able to interact with multiple ligands found within the extracellular matrix (VN, Fn, LM, fibrinogen, Von Willebrand's Factor, thrombospondin, osteopontin, and osteonectin). Both GPIIb-β3 and αvβ3 bind to RGD fragments and interaction between these receptors and their target ligands can be blocked by RGD containing peptides (Smith *et al.*, 1990). The β4 subunit is restricted to epithelial cells and associates with α6. The β4 subunit is unusual in that it has an extremely long cytoplasmic domain compared to other integrin β subunits. This complex has recently been identified in hemidesmosomes and its ligand is epiligrin (Sonnenberg *et al.*, 1991). There is some evidence that the α6β4 binds to laminin (Mercurio *et al.*, 1993). β7 (βp) a lymphoid integrin subunit has been shown to associate with α4. The α4βp complex mediates lymphocyte adhesion to Peyer's Patch HEVs (Holzman and Weissman, 1989). More recently by homology PCR, Erle *et. al.*, (1991) identified yet another β subunit (β8).

The discovery of alternatively spliced integrin subunits has added yet another level of complexity to the integrin family. In mammals several subunits have alternatively spliced cytoplasmic domains. These include β1 (Altrude *et al.*, 1990; Ruoslhati *et al.*, 1989) β3 (van Kuppevelt *et al.*, 1989; Ruoslhati *et al.*, 1989), β4 (Suzuki and Naitoh, 1990; Hogervorst *et al.*, 1990; Tamura *et al.*, 1990), α3 (Tamura *et al.*, 1991) α6 (Hogervorst *et al.*, 1991; Cooper *et al.*, 1991) and α7 (Ziober *et al.*, 1993; Quaranta *et al.*, 1993). In addition alternative splicing has been identified in the extracellular domain of at least α subunits. These include α6, α7 (Ziober *et al.*,

1993), the drosophila PS2 (Hirano *et al.*, 1991), and the IIb subunit (1990). Alternate splicing may be one way to provide unique signals to the cytoskeleton (i.e. motility, gene expression).

Integrins and Tumor Cells

A critical aspect of invasive and metastatic behavior involves adhesive interactions of tumor cells with other cells and with the extracellular matrix. Such interactions occur as tumor cells migrate locally from the primary tumor mass or as circulating tumor cells adhere to the basement membrane during initial steps in metastasis. It seems clear that many of the adhesive interactions of tumor cells are mediated by the integrin family of cell surface receptors. However the role of integrins in the pathophysiology of various tumor types is likely to be quite complex and is only beginning to be explored and understood. Data from in vivo and in vitro comparisons of normal and malignant cells suggest that there is a shift in integrin expression that accompanies malignant transformation (Albelda *et al.*, 1990). Squamous cell and basal cell carcinomas show a decrease in β1 integrin expression compared to normal epithelial cells (Peltonen *et al.*,1989). Plantefaber and Hynes (1989) demonstrate that transformation of rodent cells (rat 1, NRK, and NiL8) with Rous sarcoma virus results in reduced levels of α5β1. Klein *et al.*, (1991), showed that α2 is upregulated in some invasive melanoma cells. Nontumorigenic human osteogenic sarcoma (HOS) cells transformed with N-methyl-N'-nitrosoguanidine (MNNG) were shown to become highly tumorigenic and in addition MNNG-HOS cells show significant increases in α6β1, α2β1, and α1β1 receptors (Dedhar and Saulnier

1990). Levels of α5β1 and α3β1 are unaltered while αvβ3 is drastically reduced in the transformed cells. In nonmetastatic K1735 melanoma cells levels of α7β1 are upregulated with a concurrent decrease in α6β1 expression (Ramos *et al.*, submitted). Conversely, the highly metastatic murine melanoma cells express high levels of the α6β1 integrin while expressing almost no levels of α7β1.

It seems obvious that adhesion receptors such as integrins are important in tumor cell motility during invasive events and in the arrest of circulating tumor cells as part of the metastatic process. However, it is also quite apparent that no single integrin is responsible for the metastatic phenotype. Recently this picture has gained an additional degree of complexity as it has become clear that integrins can participate in signal transduction as well as in adhesive events.

Integrin Signaling

Recent work indicates that integrins can function as signaling receptors (Hynes 1992; Schwartz 1992; O' Toole *et al.*, 1994; Filardo and Cheresh 1994). Integrin mediated adhesion of cells to ECM proteins has been shown to activate a number of intracellular signaling events, including activation of the Na/H antiporter (Schwartz *et al.*, 1989) which causes an elevation in intracellular pH. This rise in intracellular pH is a consequence of adhesion to fibronectin thought to be due to integrin clustering. Work by Werb *et al.* (1989) demonstrates that monoclonal antibodies to the α5β1 fibronectin receptor induces expression of genes which encode secreted matrix-degrading enzymes stromelysin and collagenase. More recent studies demonstrate that antibody clustering of integrins can alter cellular patterns of tyrosine phosphorylation

of a 120 kD protein. Recently this protein has been cloned and sequenced and has been identified as a novel tyrosine kinase. Based on its localization in focal adhesion contacts this protein has been termed pp125 focal adhesion kinase or FAK. The potential importance of FAK in signaling has been further supported by observations that FAK is also activated in some cells via G protein coupled neuropeptide receptors. FAK seems to be at the convergence of pathways mediated by integrins, G protein receptors and src-family oncogenes. These are examples of events on the outside of the cell which influence downstream intracellular events (outside-inside signalling). Recent work by O'Toole *et al.*, (1994) demonstrates that cell type specific signals that modulate integrin affinity are transmitted through the cytoplasmic domains of both the α and β subunits. They also demonstrate that certain deletions in the conserved GFFKR sequence within the α subunit cytoplasmic domain reset the default state to "high affinity". Furthermore, the conserved GFFKR motif of the α subunit cytoplasmic domain maintains the default low affinity of the extracellular domain. In addition Filardo and Cheresh (1994) identified a region in the αv cytoplasmic domain that impacts the ligand binding and conformation of the $\alpha v\beta 3$ complex. This appears to be yet another way for integrins to transmit signals (inside-outside). Several integrins are expressed on the cell surface in an inactive state and require activation with a variety of agonists to acquire the capability to mediate adhesion to their ligands (Dustin and Springer 1991); Shaw *et al.*, 1990). Examples of such activation dependent integrins include $\beta 2$ integrins (Dustin and Springer 1991), the IIb$\beta 3$ integrin (Ginsberg *et al.*, 1991) and the $\alpha 6\beta 1$ integrin on macrophages (Shaw *et al.*, 1990). It

is clear that kinase activation plays an important part of integrin signaling (Shaw *et al.*, 1990). Although the details of signaling pathways have not been elucidated, it appears that they may induce a conformational change in the extracellular domain which may facilitate ligand binding (Frelinger *et al.*, 1991; Neugebauer and Reichardt 1991). The cytoplasmic domains of the activation dependent integrins are likely targets of intracellular signaling pathways. For example, the cytoplasmic domain of β3 (Hillery *et al.*, 1991) and α6 (Shaw *et al.*, 1990) integrin subunits are phosphorylated on serine residues in response to the appropriate agonists. It is becoming more apparent that integrins are more than just proteins whose functions are restricted to adhesion; rather integrins can also mediate signal transduction processes which may regulate gene expression and cell growth.

Melanoma

Melanoma is a tumor which has a high propensity for metastasis. Melanoma is caused by malignant transformation of normal melanocytes. Initially, melanomas grow superficially, in what has been termed the radial growth phase. The cells in this phase have little tendency to metastasize. Eventually melanoma enters a vertical growth phase which penetrates the dermis. The vertical thickness of the primary tumor is a measure of melanoma progression and is directly correlated with the potential to metastasize (Breslow 1970, Sober *et al.*, 1983). During the progression from melanocyte to malignant melanoma, there is a change in the repertoire of cell surface markers. There is an apparent increase in GD2/GD3 gangliosides in the metastatic cell type compared with the nonmetastatic phenotype (Cheresh and Klier 1986). Since

gangliosides have been implicated in attachment of tumor cells to a variety of substrates, the increased ganglioside levels seen in metastatic cells may be related to the invasiveness of the cells (Cheresh and Klier 1986). Another phenotypic difference between melanocytes and melanoma cells is the expression of I-CAM-1, a cell adhesion molecule which mediates adhesion through LFA-1 a member of the β2 integrin family. I-CAM 1 expression increases with increasing tumor thickness. I-CAM-1 is not detectable on normal melanocytes and is only sporadically detectable on proliferative benign nevi (Reithmuller, 1989). The expression of I-CAM-1 may contribute to the metastatic capability of melanoma cells through several mechanisms. I-CAM-1 has been shown to bind directly to LFA-1, found on the surface of hematopoietic cells (Johnson *et al.*, 1989). Through this interaction melanoma cells could establish cell contacts with leukocytes present in tumor infiltrate. This could lead to an enhancement of tumor cell adhesion to migrating and invading leukocytes, enabling individual tumor cells to dissociate from the primary tumor (Johnson *et al.*,1989; Johnson, 1988).

Integrins and Melanoma Progression

It is well documented that integrin profiles differ between melanocytes and melanoma; as well as between non invasive and invasive melanoma. It has been shown (Klein *et al.*, 1991) that the melanoma progression marker A.1.43 is identical to α2β1 (a previously described collagen receptor); and that this integrin complex is expressed on melanoma cells and not on normal melanocyte cultures. More recently, Natali *et al.*, (1993) demonstrated that the promiscuous α3β1 is upregulated in

metastatic melanoma compared with primary and benign lesions. We demonstrate high level expression of the laminin-binding integrin, α6β1, in highly metastatic K1735 murine melanoma cells and not in the non-metastatic cells. In the nonmetastatic K1735 murine melanoma cells we observe high levels of the α7β1 with little or no levels of α6β1 expression (Ramos *et al.*, submitted).

Albelda *et al.* (1990) demonstrate that a change occurs in the integrin profile of melanoma cell's *in vivo* when compared to its normal counterpart, the melanocyte. Using a panel of monoclonal antibodies against specific integrin subunits they defined the distribution of integrin receptors on cells in culture derived from normal and malignant melanocytes, and in tissue sections from benign to increasingly malignant melanocytic lesions (Albelda *et al.*, 1990). Differences were noted in integrin expression in tissue sections containing metastatic and vertical growth phase melanomas when compared to radial growth phase lesions and nevi. The most notable change was the expression of the β3 subunit which was restricted only to cells within vertical growth phase and metastatic melanomas. McGregor *et al.*, (1989) also identified the presence of a IIb-β3 like glycoprotein on human metastatic melanoma cells but absent from normal melanocytes. Tumor progression in malignant melanoma is associated with modulation of a number of integrins and this shift in integrin expression may reflect an important aspect of the melanoma cell's capacity to adhere to an migrate through multiple ECM substrates.

OUTLINE OF PRESENT STUDY

In order to better understand tumor cell interactions with the extracellular matrix, several different approaches were used. Biochemical characterization of ECM receptors as well as functional assays to determine ligand binding and interaction were key methodologies in this study. In the first part of the study, the HT1080 human fibrosarcoma cell line was used initially to examine tumor cell-matrix interactions. Immunoprecipitation was used to identify cell surface expression of integrin complexes. Affinity chromatography was performed to elucidate which of the expressed integrins play a role in adhesion to laminin. We next used adhesion and invasion assays to evaluate the HT1080 interaction with both purified ECM proteins and complex matrices (reconstituted basement membrane or RBM). Subsequently experiments were performed in the presence of function perturbing antibodies to determine the role that specific integrins play in the adhesive and invasive process. We convincingly demonstrate that although HT1080 cells express a variety of ECM binding integrins, $\alpha 6\beta 1$ appears to mediate invasion through complex matrices.
We next used the highly characterized B16 murine melanoma system to investigate malignant melanoma cell interactions with specific ECM molecules, laminin and collagen IV. These molecules were investigated because of their presence in basement membranes, barriers which melanoma cells must traverse during metastasis. Affinity chromatography and adhesion assays were used to characterize interactions of specific integrins expressed on these melanoma cells with laminin and collagen IV. We demonstrated that $\alpha 6\beta 1$ is the predominant laminin-binding integrin in the B16 cells. We also show that $\alpha 1\beta 1$ binds weakly to laminin and appears to be the major collagen

IV receptor found in these cells.

In the third part of the study, we generated a variety of metastatic variants of the B16 cells, including an amelanotic variant, which has increased propensities to metastasize to the regional lymph nodes in C57BL/6 syngeneic mice. We evaluated morphology and ability to adhere to a variety of ECM proteins including ECM isolated from human amnion and murine lymph node.

Finally we extended our study of tumor cell interactions with the ECM to include the K1735 melanoma cell system. The K1735 murine melanoma cell lines are highly characterized and consist of established clones of both high and low metastatic potential. Using the K1735 murine melanoma cells we identified the novel laminin-binding integrin $\alpha 7\beta 1$ which had been originally identified on human melanoma cells (Kramer *et al.*, 1989). To understand different interactions that high and low metastatic cells may have with the ECM, we characterized integrin expression on both high and low metastatic variants of the cell lines. Laminin-affinity chromatography, Western, Northern blots were used to identify laminin-binding in the K1735 cell lines. In addition, adhesive and migratory properties to ECM proteins were also evaluated. We demonstrate that metastatic K1735 cells use $\alpha 6\beta 1$ to interact with laminin and these cells are quite motile on laminin substrates. Affinity chromatography suggests that this interaction with laminin is quite weak. Conversely, the non-metastatic K1735 cells use primarily the $\alpha 7\beta 1$ complex to interact with laminin and these cells do not migrate on laminin substrates. We also generated a highly metastatic revertant of the K1735C23 cell lines and determined the expression of laminin-binding integrins was

identical to those expressed in the highly metastatic K1735M2 cells. These revertants expressed high levels of α1 and α6 with a coordinate decrease in the expression of α7.

MATERIALS AND METHODS

Cell Culture. The parental B16-F1, B16-BL6 and K1735 murine melanoma cell lines, whose origin and properties have been described (Fidler, 1973; Fidler and Kripke and Fidler 1977), were obtained from Dr. I. J. Fidler (MD Anderson Cancer Center, Houston, TX). Human HT1080 cells were originally isolated from a metastasis to the mediastinum and were obtained from the American Type Culture Collection. The cells were routinely cultured in the presence of 8% CO_2 in Falcon T-150 tissue culture flasks containing Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 50 μg/ml gentamicin added. Cells were passaged at preconfluence after a brief treatment with trypsin-EDTA. For ^{125}I-cell surface and ^{35}S-metabolic labeling, adhesion and migration assays, all cell lines were harvested with 2 mM EDTA. For experiments where the selected variants were compared with the parental cell line, the B16-F1 cell line was first passaged as a subcutaneous tumor in C57BL/6 mice and then readapted to culture and used for experiments from passage 5-10. For derivation of highly metastatic variants of the K1735 cell lines, the cells were injected into the footpad of C3H/HeN mice. After appropriate incubation period the animals were sacrificed and the metastatic lung colonies were harvested and adapted to tissue culture and used for experiments from passage 5-10.

Cell-Surface Labeling. Cell-surface proteins were radiolabeled with ^{125}I by the lactoperoxidase-glucose oxidase method, as previously described (Kramer *et al.*,

1988). Preconfluent cells were removed from dishes with 2 mM EDTA, 0.05% BSA in PBS, washed three times with cold 50 mM Tris-HCl, 20 mM glucose, 150 mM NaCl, 1 mM $MnSO_4$, and then resuspended at a final concentration of 1 x 10^6 cells/ml in the same buffer. In some experiments $MgCl_2$ replaced $MnSO_4$ as the divalent cation. Iodination was initiated by adding carrier-free $Na^{125}I$ (Amersham, Arlington Hts., IL), glucose oxidase (Sigma), and lactoperoxidase (Calbiochem) at final concentrations of 250 mCi/ml, 200 mU/ml, and 200 μg/ml, respectively. The suspension was gently mixed by rotating the tube at 5 rpm on ice for 20 min. The reaction was terminated by adding a large excess volume of the buffer (without glucose), and the cells were recovered by centrifugation.

Affinity Chromatography. Purified laminin, fibronectin, type I or IV collagen was coupled to CNBr-activated Sepharose. Surface-radiolabeled cells were solubilized in 200 mM β-octylglucoside, 50 mM Tris-HCl (pH 7.4), 1 mM $MnSO_4$ (or 1 mM $MgCl_2$), and 1 mM phenylmethylsulfonyl fluoride at 4°C. The cell extract was centrifuged to remove the insoluble cellular components and loaded onto the columns (0.7 x 5 cm). The unbound material was removed with 5 column volumes of running buffer (50 mM octylglucoside, 50 mM Tris-HCl, 1 mM $MnSO_4$, or $MgCl_2$ pH 7.4). Next the column was washed with 5 column volumes of 0.2 M NaCl in the running buffer. Bound material was eluted with 10 mM EDTA in divalent cation-free running buffer, followed by 1 M NaCl in running buffer. In some experiments elution of the column was attempted with 10 mM Ca^{2+} or 10 mM Mg^{2+} in running buffer. Column washes and elution fractions were collected in 1 ml volumes. Elution profiles were

analyzed by SDS-PAGE according to Laemmli, and the ^{125}I-labeled proteins were detected by autoradiography of the dried gels. Samples were reduced with 1% β-mercaptoethanol and heated at 100°C for 3 min. Nonreduced samples were treated the same except that 10 mM N-ethylmaleimide was substituted for β-mercaptoethanol. Two-dimensional gel electrophoresis was performed according to the procedure of Phillips *et al.* (1988). Prestained protein standards (Biorad) were used as M_r markers and included BSA, 67,000; phosphorylase b, 94,000; β-galactosidase, 116,000; and myosin, 200,000.

Immunoprecipitation. Column fractions or whole cell extracts were immunoprecipitated with polyclonal or monoclonal antibodies as previously described (Kramer *et al.*, 1988). Samples were incubated with antibodies at 4°C for 1 h. Species-specific anti-IgG-Sepharose was added and incubated for an additional hour at 4°C to recover the immune complexes. The unbound material was removed by extensive washing with 0.1% NP-40, 10 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.4 (TNC buffer). This was followed by two washes, first with 0.1% SDS in TNC buffer, then with 1 M NaCl in TNC buffer. The recovered immunoprecipitates were solubilized by heating at 100°C for 3 min and analyzed by SDS-PAGE followed by autoradiography.

Antibodies. Mouse monoclonal antibody (GoH3) to the α6 subunit of the human α6β1 receptor was kindly provided by Dr. A. Sonnenberg (The Netherlands Cancer Institute). Mouse monoclonal antibodies against α2β1 (P1H5), α3β1 (P1B5), and α5β1 (P1D6) were a generous gift of Dr. William Carter (University of Washington, Seattle). Rat monoclonal antibodies against the human fibronectin

receptor, α5β1, (B1E5) and the human β1 subunit (AIIB2) were a gift of Dr. Caroline Damsky (University of California, San Francisco). Anti-hamster β1 (originally named anti-ECM_R/anti-GP140), a polyclonal antibody prepared from BHK cells which reacts with the β1 subunit, was a gift from Dr. Caroline Damsky. Affinity-purified rabbit polyclonal antibodies prepared against the human placental fibronectin receptor, which react with the β1 and the α5 subunits were a gift of Dr. Erkki Ruoslahti (La Jolla Cancer Foundation). Polyclonal anti-mouse fibronectin receptor was a gift of Dr. V.P. Patel . Rabbit polyclonal antibodies to mouse laminin were from Dr. Hynda Kleinman (NIH). Mouse monoclonal antibody to vinculin, rhodamine-conjugated goat anti-rabbit IgG, and fluorescein-isothiocyanate-conjugated goat anti-mouse IgG were obtained from Boehringer-Mannheim (Indianapolis, IN). Species-specific antibodies conjugated to Sepharose were obtained from Sigma Chemical Co (St. Louis, MO).

Adhesion Assay. Cell adhesion to various ECM proteins was assessed as previously described (Kramer *et al.*, 1989). Laminin and type IV collagen were isolated from EHS tumor and fibronectin was isolated from human plasma (Kramer *et al.*, 1989); type I and type III collagen were obtained respectively, from Collagen Corp., Palo Alto, CA and Sigma Chemical Co., St. Louis, MO. Unmodified polystyrene 96-well flat-bottom microtiter plates (Serocluster, Costar, Cambridge, MA) were precoated with purified extracellular matrix proteins or BSA as a control in PBS at various concentrations for 1 hr at 37°C in a humidified chamber. In experiments with RBM, a thin layer of the liquid was spread across each well of a 96 well plate, allowed to dry in a laminar flow hood for 2 h, then rinsed three times with DMEM.

Wells were rinsed three times with PBS, and nonspecific adherence was blocked with 1 mg/ml BSA in PBS for 1 hr. Preconfluent cell cultures were labeled with 1 μCi/ml of 5-[^{125}I]IdUrd (ICN) for 24 hr in DME supplemented with 10% FBS. Cells were then resuspended in cold DME with 0.1% BSA and 1 X 10^4 cells was added to each well. The assay was initiated by floating the plate on a 37°C water bath in an 8% CO_2 chamber and incubated for 1 hr. The number of adherent cells was then determined.

Immunofluorescence. The distribution of β1-containing complexes that may act as adhesion receptors in the B16 cells was analyzed by immunofluorescence microscopy. Glass coverslips were coated for 24 h at 4°C with 50 μg/ml of type IV collagen, laminin or fibronectin in PBS. A cell suspension of 1 x 10^4 cells was added to the coverslips. After 4 h incubation at 37°C, the cells were processed as described (Ramos *et al.*, 1989). Cells were fixed and permeabilized by extraction with 0.5% Triton X-100 in PBS, and nonspecific staining was blocked with normal goat serum. The samples were incubated with polyclonal antibodies against the human fibronectin receptor, which react with the β1 subunit, and monoclonal antibody to vinculin. Next, samples were incubated with the appropriate secondary antibody coupled either with fluorescein isothiocyanate or rhodamine. The coverslips were mounted with Fluoromount (Fisher) and viewed on a Nikon microscope equipped with epilumine-scence optics. In some experiments we examined tumor cell interactions with basement membrane molecules contained within the ECM of lymph node or human amnion tissue. Tumor cells were seeded onto extracted lymph node or human amnion

matrices. After a predetermined time, tissue sections were stained with an appropriate dilution of primary rabbit polyclonal anti-laminin, anti-fibronectin or anti-type IV collagen (Kramer *et al.*, 1988; Ramos *et al.*, 1989). After removal of the primary antibody, secondary antibody conjugated either to fluorescein or rhodamine was added and the tissue was rinsed and mounted with Fluoromount.

Invasion Assay. A suspension of HT1080 cells and liquid RBM was prepared at a concentration of $2x10^5$ cells/ml. 5 µl of the suspension was placed in each well of a 48-well dish (Costar). The dish was then immediately inverted and incubated at 37°C for 30 min to allow for polymerization. 300 µl of DMEM alone or DMEM plus antibodies (GoH3 and AIIB2) was added to the cell-RBM suspension. The assays were monitored visually using phase-contrast optics at various time intervals. Cells migrating through the matrix and reaching the matrix-plastic interface were determined.

RESULTS

Invasion of Basement Membrane Matrices by Fibrosarcoma Cells Involves Laminin-Binding Integrin Receptors.

Identification of Cell-Surface Proteins

To identify laminin-binding proteins on HT1080 cells, we subjected ^{125}I-surface-labeled cells to laminin affinity chromatography followed by SDS-PAGE. The detergent extract of the cells contained several radiolabeled bands, ranging from $\underline{M}_r$ 30,000 to 200,000 (chapt 2). After the extract was applied to a laminin-Sepharose

affinity column, elution of material with 0.2 M NaCl in running buffer released several minor bands and two major bands at M_r 110,000 and 140,000 nonreduced. 10 mM EDTA eluted large amounts of the same two radiolabeled bands at M_r 110,000 and 140,000. Immune precipitation of EDTA-eluted fractions and whole-cell extract (chapt 2) with anti-α6 antibodies recovered the α6β1 complex of M_r 140,000 (α6) and 110,000 (β1). After exhaustive immunoprecipitation with anti-α6 antibody (GoH3), we did not detect any significant amounts of other integrin complexes in the EDTA-eluted fractions (not shown). We also subjected extracts of HT1080 cells to immunoprecipitation with antibodies to α1β1 (Ts2/7) and to αvβ3 (LM 609); however, α1β1 was not detectable and αvβ3 was present in only minor amounts (data not shown). We observed, by sequential immunoprecipitation of whole-cell extract with integrin-specific antibodies, that HT1080 cells express the α2β1, α3β1, and α5β1 complexes as well as α6β1. By immunoprecipitation we found that the α6β1 complex is expressed in relatively lesser amounts than the α2β1 and α3β1 complexes.

HT1080 Cell Interaction with the ECM

The α2β1 complex is a collagen receptor that appears to function also as a laminin receptor in endothelial cells (Languino *et al.*, 1989). Blocking antibody to the α2β1 complex (P1H5) was able to inhibit HT1080 cell adhesion to collagen types I and IV but not to laminin or fibronectin (chapt 2). Therefore α2β1 does not serve as a laminin receptor in the HT1080 cells. Antibody against the α5β1 receptor (P1D6) effectively blocked attachment to fibronectin but not to laminin or collagens. Monoclonal antibody ,B1E5, against the α5β1 was also effective in blocking HT1080

cell attachment to fibronectin substrates (not shown), but had no effect on attachment to laminin.

The affinity chromatography and immunoprecipitation results suggested that α6β1 may be important in HT1080 cell attachment to laminin. Monoclonal antibodies to α6β1 (GoH3) completely blocked HT1080 cell adhesion to immobilized laminin (chapt 2), but had no detectable effect on their attachment to fibronectin-coated substrates (not shown). We next looked at the effect of several monoclonal antibodies on HT1080 cell attachment to a reconstituted basement membrane (RBM). In the presence of a 1:30 dilution of GoH3, initial cell attachment was inhibited by almost 80%. However, antibody to α5β1 (B1E5) only slightly decreased cell attachment. Anti-β1 (AIIB2) antibody completely blocked attachment. While the initial phase of cell adhesion to the matrices was inhibited by GoH3, longer incubation (> 3 hr) allowed the cells to overcome the antibody and the cells were able to attach (not shown).

We previously reported (Kramer *et al.*, 1986) that when seeded onto reconstituted basement membrane, HT1080 cells spread out, migrate, and invade, leaving channels etched in the surface of this matrix. In contrast to the fibrosarcoma cells, in the same study human skin fibroblasts attached but spread poorly on this matrix. We used scanning electron microscopy to observe this behavior more closely. We saw that the cells actually burrowed into this matrix, dissolving it and leaving behind an empty channel.

We next studied the effect of integrin specific antibodies on the spreading of

HT1080 cells on RBM. After 5 hr, cells in medium alone had spread extensively on the matrix and were beginning to form cell-cell networks; some channels were present. In the presence of anti-α6 antibody (GoH3), most cells had attached but only limited spreading was evident. Similarly, when seeded in the presence of antibodies against the β1 subunit (AIIB2), the cells attached but failed to spread. However, in the presence of antibodies against the fibronectin receptor (B1E5), the cells were able to attach, spread out and migrate on the matrix.

Finally, we evaluated the effect of individual monoclonal antibodies on HT1080 cell invasion of the RBM. The assay consisted of suspending the cells in liquid RBM and placing a small droplet of this suspension in tissue culture wells. After the matrix had polymerized, the samples were incubated for increasing times and the number of cells that had migrated to the edge of the droplet was determined. Initial studies using [^{125}I]IdUrd-labeled cells indicated that virtually all cells were trapped in the matrix and could not be released by trypsin-EDTA treatment. However, with time, cells eventually penetrated the matrix and could be progressively recovered by the trypsin-EDTA treatment. This correlated with the out-migration of cells from the matrix that were scored visually. After 40 hr of incubation, 5.6% of the input cells could be recovered by extraction with trypsin/EDTA. Furthermore, anti-β1 antibody reduced the releasable radioactivity to 1.6%. Microscopic examination of the cells in the matrix droplet revealed that cells emerged from the matrix layer and then migrated on its surface, eventually concentrating at the matrix-plastic interface. Significant numbers of cells had migrated out of the matrix droplets by 15 hr and

could be easily counted; this movement continued with time. The effect of anti-α6 (GoH3) and anti-β1 antibody on this invasion process was examined. Both antibodies convincingly blocked the out-migration of cells, even after 24 hr of incubation. Neither antibody appeared to be cytotoxic, since removal of the antibody reversed the inhibitory effect (not shown). Antibody to the fibronectin receptor (B1E5) had no effect on RBM invasion (not shown).

Identification of Integrin Receptors for Laminin and Type IV Collagen in Metastatic B16 Melanoma Cells

The B16-BL6 melanoma cells were seeded onto substrates coated with fibronectin, laminin, collagen types I, IV, and V, gelatin and BSA to establish adhesion profiles. Cells adhered equally well to laminin and fibronectin, but gelatin and BSA were poor substrates for adhesion (chapt. 3). At the lower coating concentrations of collagens, cells attached best to type IV collagen and least to collagen types I and V. At higher coating concentrations (100 μg/ml), significant cell adhesion to collagens type I and V was observed.

We used anti-β1-integrin antibodies to determine to what extent integrins were involved in B16 cell adhesion to laminin, type IV collagen, and fibronectin-coated substrates. Immunofluorescence staining with antibodies to the β1 chain revealed focal contacts on B16 cells adhering to all three ligands. These adhesion contacts were typically concentrated at the outer margin of the cell. Counterstaining with monoclonal antibodies against vinculin demonstrated co-localization with the β1

integrin on laminin, type IV collagen and fibronectin substrates, confirming that the condensations represent focal adhesion plaques. Attempts at staining focal adhesion plaques with GoH3 antibody ($\alpha 6\beta 1$) were unsuccessful, possibly because the receptor/ligand binding site was inaccessible to the antibody.

Identification of B16 Cell-Surface Laminin and Collagen Binding Proteins

Laminin- and collagen-binding proteins on the surface of the B16 cells were identified by affinity chromatography followed by SDS-PAGE. A detergent extract of ^{125}I-labeled cells contained a number of radiolabeled bands, ranging from M_r 30,000 to 250,000. The extract was applied to a column of laminin-Sepharose. After washing of the column, elution with 0.2 M NaCl in starting buffer released several minor bands as well as a set of two major bands at M_r 120,000 and 140,000 (nonreduced) (chapt 3). Application of EDTA eluted large amounts of the same two radioactive bands at M_r 120,000 and 140,000 (chapt 3). Neither GRGDSP peptides (not shown) nor YIGSR-NH_2 peptides could specifically elute this laminin-binding complex. Immunoprecipitation of EDTA-eluted fractions with either anti-hamster $\beta 1$ or anti-mouse $\beta 1$ antibodies recovered the same two laminin-binding proteins. The reactivity of these two surface polypeptides with anti-$\beta 1$ antibody implies that they form an α-$\beta 1$ complex related to the integrin superfamily of receptors.

A minor band (M_r 180,000) was sometimes recovered from laminin columns depending on the conditions of elution. It appeared that under extensive washing, the M_r 180,000 band has a much lower affinity for laminin; when the column washing phase of the chromatography was shortened, the M_r 180,000 band

was consistently detected. This M_r 180,000 could be immunoprecipitated with anti-β1 antibody but not with anti-α6 antibody, and appears to be identical to the type IV collagen receptor complex (see below).

Since the electrophoretic properties of the major laminin-binding complex were similar to these of α6β1, we subjected the EDTA-eluted material to immunoprecipitation with anti-α6 antibody. The GoH3 monoclonal antibody specifically immunoprecipitated the complex from the column fractions as well as from the whole-cell extract. The YIGSR peptide which has been implicated in mediating B16 adhesion to laminin (Humphries *et al.*, 1987), was ineffective in eluting α6β1 (chapt 3) from laminin columns.

The finding that α6β1 appears to be a major laminin-binding complex on the B16 cells led us to test whether GoH3 monoclonal antibody would block the attachment of cells to laminin substrates. Anti-α6 substantially inhibited B16-BL6 cell adhesion to immobilized laminin. Significant inhibition was obtained with dilutions of 1:30 or less. In contrast, GoH3 antibody produced no detectable effect on the attachment of cells to either type IV collagen- or fibronectin-coated substrates. This result is in line with the observations of Sonnenberg *et al,* (1989) that α6β1 mediated the adhesion of human platelets to laminin.

On columns of type IV collagen-Sepharose, EDTA eluted two bands, an M_r 120,000 protein complexed with an M_r 180,000 protein (chapt 3). When reduced with β-mercaptoethanol, the M_r 120,000 band migrated at 130,000 while the M_r 180,000 remained essentially unchanged. Immunoprecipitation of specifically bound

and EDTA-eluted material from type IV collagen-Sepharose columns with anti-hamster β1 co-precipitated the M_r 120,000 (β1) and 180,000 (α) complex. An apparently identical receptor was recovered from type I collagen-Sepharose columns, although it bound very poorly to this ligand.

Metastatic Melanoma Cells Interact with the Reticular Fibers of the Lymph Node

Selection of B16 variant cell lines.

In initial studies, when parental B16-F1 cells were injected into the footpad of C57BL/6 syngeneic mice, all animals developed primary tumors. While most B16-F1-injected mice showed tumor involvement of the popliteal nodes, usually only a small fraction of the mice exhibited gross metastases to the para-aortic lymph nodes. Through a series of sequential selections, cells from the few para-aortic metastases were adapted to culture, expanded, and reinjected into the footpads of additional mice. Again, the few tumor-positive para-aortic lymph node metastases were obtained and were expanded in culture. This process was repeated and in as few as four sequential selections, we obtained a melanotic variant cell line (B16-PA4M) that exhibited a modest increase in its capacity to metastasize to popliteal, inguinal and para-aortic lymph nodes. Also during the fourth round of selection, we obtained an amelanotic variant (B16-PA4A) from a para-aortic metastasis in a single mouse that had an increased capacity to metastasize to regional nodes. In this mouse, metastases to the popliteal, inguinal, and para-aortic nodes were progressively amelanotic, while the primary tumor remained intensely melanotic. In contrast to the results with the parent

B16-F1 cells, almost all of the mice injected with B16-PA4A and B16-PA4M variant cells developed popliteal metastases, and a majority developed distant para-aortic metastases. After three more selections, the B16-PA4M cell line produced another melanotic variant (B16-PA7) with a similar metastatic profile (not shown). Comparison of the growth rates of subcutaneously injected parental and variant cells did not show any substantial differences, suggesting that the increased metastasis is not due simply to an increased proliferation.

In another set of experiments, mice were injected with either the B16-F1 parental or the amelanotic B16-PA4A variant cells. After two weeks, the leg containing the primary tumor was amputated to decrease tumor burden; the animals were followed for up to eight weeks. Interestingly, under these conditions of an extended tumor growth period, the B16-F1 cells frequently produced increased colonization of the para-aortic nodes. Whereas in the earlier selection studies, the footpad-injected B16-F1 cells rarely metastasized to distant nodes, after the extended incubation most of the mice showed tumor involvement in these sites. Gross examination of the B16-F1 lymphatic tumors revealed that in a few mice, there was decreased pigmentation of tumor involved nodes with increased tumor cell dissemination, resulting in predominantly amelanotic para-aortic nodes as in the original PA4A cell line (chapt 4). Histological examination of para-aortic nodes from these animals revealed that the node itself was often heterogeneous with respect to cell phenotype as evidenced by the presence of amelanotic tumor colonies interspersed with smaller melanotic deposits within the same node. The B16-PA4A cell line

remained amelanotic throughout the eight-week incubation period and produced grossly tumor-involved popliteal, inguinal, para-aortic and, frequently, mesenteric and axial lymph nodes (not shown).

Morphology and motility rates of selected cell lines.

The variant PA4M and PA4A cell lines appeared distinct from the parental cell line, not only in metastatic efficiency, but also in their unusual morphology: the B16-F1 parental cells, although pleomorphic, generally formed tight, epithelioid clusters, with many cell-cell contacts. In contrast, the variant cells (B16-PA4M or -PA4A) appeared more dispersed and displayed pronounced pseudopodial projections resembling dendritic processes. (chapt 4).

We next evaluated the motility of the B16 parental and variant cell lines. Monolayer wound assays were performed using the B16-F1, B16-PA4A, and B16-PA4M cells seeded on tissue culture plastic. By 8 hr, both variant cell lines had filled in nearly half of the wound area, and completely by 16 hr, while the parental B16-F1 barely entered the wound.

B16 cell adhesion to extracellular matrix.

To determine the relative adhesive properties of variant tumor cell subpopulations, we compared the ability of the parental and variant cells to adhere to the extracellular matrix proteins fibronectin, laminin, and collagen types I, III, and IV. All three cell lines tested adhered well to the basement-membrane-associated proteins fibronectin, laminin, and type IV collagen. Interestingly, the parental B16-F1 cell line showed a somewhat greater attachment to most substrates tested than did the variant

B16-PA4M and B16-PA4A lines. Tumor cell attachment to interstitial associated collagen types I and III, was minimal under these conditions for all cell lines; adhesion to BSA and gelatin was also negligible.

We next compared the ability of parental and variant cell lines to adhere to preparations of lymph node extracellular matrices. The variant B16-PA4A cells were more efficient than the parental cells in attaching to these matrices. Furthermore, the parental and the PA4A variant cells showed significantly greater adherence to exposed ECM prepared from cryostat sections of lymph node tissue than to unextracted tissue. The PA4A cells adhered with greater efficiency than the parental cells to the unextracted lymph node. To determine the relative adhesion potential of tumor cells for lymph node ECM, we also performed adhesion assays on interstitial dermis connective tissue. B16-F1 and B16-PA4A cells both exhibited relatively poor attachment to cryostat sections of collagen type I- and III-rich mouse dermis.

We previously established that the lymph node reticular fibers are composed of a core of types I and III collagen decorated with basement membrane macromolecules, including laminin and type IV collagen (Kramer *et al.*, 1988). Immunofluorescence staining for reticular fiber associated laminin demonstrated a good correspondence between attachment of tumor cells and location of the fibers within the lymph node. In unextracted node, where adhesion was less efficient, cell attachment was also concentrated at matrix fibers (not shown).

We next assessed cell attachment to cryostat sections of human amnion which is known to contain both an epithelial basement membrane and an underlying

interstitial stroma (containing collagen types I, III, V) (Amenta *et al.*, 1986). After removal of the amniotic cells, the tissue was cut in cross-section to provide access to both the basement membrane and the stromal matrix. Immunofluorescent staining of sections of amnion with antibodies to laminin confirmed the preferential binding of B16 tumor cells with the basement membrane.

Taken together, these results indicate that tumor cells adhere predominantly to the basement-membrane-containing reticular fibers and not to collagen types I and III, which compose the core of these fibers. Since the B16 cell lines appeared to adhere preferentially to the lymph node reticular fibers, we examined the distribution of the laminin-rich fibers in established nodal metastases. Immunofluorescence microscopy of para-aortic nodes infiltrated with B16-PA4M cells revealed that the melanotic tumor cells were arranged in close apposition with the reticular fibers.

To determine the mechanism by which B16 cells adhere to lymph node ECM, we evaluated the potential role of integrin adhesion receptors in mediating this adhesion, using a goat anti-β1 integrin anti-serum (GP-140) (Knudsen *et al.*, 1981). The metastatic B16-PA4A cells pre-incubated in the presence of increasing concentrations of β1 antibodies were effectively inhibited from attaching to lymph node extracellular matrix . A 1:100 dilution of the antibody was about 90% effective in inhibiting adhesion to lymph node. Control, non-immune goat serum had no significant effect on adhesion to the ECM preparations at all concentrations tested.

Expression of High and Low Affinity Laminin-Binding Integrins is Coordinately Regulated During Melanoma Tumor Progression

Adhesion of melanoma cell variants to laminin and the E8 fragment

In standard adhesion assays we found that both high (M2) and low (C23)metastatic cells adhered well to laminin, although adhesion efficiency was usually slightly better for the M2 cell line (Chapt 5). Similarly, both the M2 and C23 cells adhered well to the E8 fragment of laminin that represents a portion of the long arm of laminin. In the case of the M2 cells, adhesion to the E8 fragment, but not adhesion to intact laminin was significantly reduced in the presence of the blocking monoclonal antibody (GoH3) to the $\alpha 6\beta 1$ receptor; this antibody produced no inhibitory effect on the adhesion of the C23 cells to laminin or the E8 fragment. For both cell lines, a rabbit anti-$\beta 1$ antibody was effective in inhibiting attachment to laminin or the E8 fragment. Finally, anti-E8 antibody significantly inhibited the adhesion of C23 cells and to a lesser extent of M2 to intact laminin and the E8 fragment.

Migration of variant cells on laminin substrates.

Although highly and poorly metastatic variant cell lines can adhere with nearly the same efficiency to laminin, their migration on this ligand is dramatically different. Using a newly developed migration assay (Clyman *et al.,* 1992), we found that over a wide range of laminin coating concentrations, the highly metastatic M2 cells rapidly migrated on laminin while the poorly metastatic C23 cells barely moved (chapt 5). For both cells lines, optimal migration occurred at laminin coating concentrations of 10-30 μg/ml. In contrast, the relative migration efficiencies on fibronectin substrates were reversed, with the M2 cells migrating at reduced rates compared to the C23 cells; this

indicates that the poor migration of C23 cells on laminin is not due to a general defect in their locomotory activity.

We next evaluated the contribution of the α6β1 integrin in mediating cell migration on laminin. Specific blocking antibody to the α6 subunit (GoH3), significantly inhibited laminin-mediated motility in the M2 melanoma cells by over 50%. In addition, migration could be completely inhibited by anti-β1 antibody (not shown). More recent results demonstrate that antibodies to the E8 fragment reduce M2 migration on intact laminin approximately 50%. This indicates that both cell lines use E8 to adhere to laminin and that the E8 fragment of laminin mediates M2 migration. These results suggest that while α6β1 is important, additional β1 integrins (α1) also contribute to locomotion of M2 cells on laminin.

Identification of laminin-binding integrins.

We analyzed the relative integrin expression profiles of the C23 (non-metastatic), and the M2 (highly metastatic) by immunoprecipitation of lysates prepared from surface ^{125}I-iodinated cells (chapt 5). Numerous differences in integrin profiles between the two cell types were noted, particularly in the laminin-binding integrin. Both cell types expressed detectable levels of α2, α3, and α5. Their was somewhat higher levels of α2 in the M2 cells while the C23 had slightly higher levels of α5. The highly metastatic M2 cell line had high levels of α1 and α6. In contrast, the C23 cells had a much more limited integrin pattern and expressed little or no detectable levels of α1 or α6. For the αv integrins, we found that the C23 had predominately the

αvβ5 while the M2 expressed primarily the αvβ3 (not shown).

We used immunoblotting of cell lysates to measure the relative level of the α7 subunit in the two cell types. The blots were probed with rabbit antibodies generated against the α7B cytoplasmic domain (Ziober *et al.*, 1993). The α7B appears to be the predominant form rather than α7A (data not shown). Equivalent load amounts of cellular protein were analyzed. In agreement with the laminin-Sepharose columns and immunoprecipitation studies, the M2 cells had much reduced amounts of the α7 receptor. α7 was detected as a single reactive band corresponding to 120 kD that comigrated with the β1 subunit. In C19 cells, however, a second band of 70 kD of variable intensity was also detected that was reactive with the anti-α7 antibody and appears to be a proteolytic cleavage product (Song *et al.*, 1992; Ziober *et al.*, 1993). We examined the expression of the integrin receptors that bind laminin in the M2 and C23 cells. To measure surface levels of the receptor that can bind laminin, cells were metabolically labeled with ^{35}S-methionine and ^{35}S-cysteine and the level of laminin-binding integrins was assessed by laminin-Sepharose chromatography. EDTA elution, under non-reducing conditions, demonstrated a broad band of 120 kD was eluted that when reduced separated into the β1 subunit at 140 kD and the α7 subunit at 100 kD. This was verified by immunoprecipitation with specific antibodies to the cytoplasmic domains of α7 and β1 subunits (not shown). Significant levels of α6 are not detected in the EDTA fractions (as assessed by immunoprecipitation) since this integrin is mostly recovered in the 200 mM wash (Kramer *et. al.*, 1990). Much higher levels of α7 integrin were consistently recovered from the C23 cells than from the M2 cell

lines. To verify that the retrieval of the α7β1 complex was complete, the detergent lysates were sequentially passed over two identical columns of laminin-Sepharose; the majority of the integrin bound to the first column (not shown). In additional assays, ^{125}I-labeled cell lysates were similarly processed for chromatography on laminin-Sepharose columns and again subjected to sequential elution. As before, analysis of the EDTA eluted fraction by SDS-PAGE indicated that substantially higher levels of the α7β1 complex were present on the poorly metastatic C23 cells compared to the M2 cells. Densitometric analysis of the autoradiograms from metabolically- or surface-labeled cells indicated that compared to the M2 cells, the C23 cells had 7-10 fold greater levels of the α7 subunit. Immunoprecipitation and flow cytometry demonstrated that the M2 cells displayed high levels of the α6 integrin while the level of this integrin on the C23 cells was at near background. Since the α6 subunit has been shown to exist as two alternatively spliced isoforms in the cytoplasmic domains, we next examined the cytoplasmic form of α6 in the K1735 melanoma variants. We found that by RT-PCR the highly metastatic variants predominantly expressed the α6A for along with some minor amount of the B form; the low metastatic variants, on the other hand, had a much lower level of α6 and expressed almost exclusively the α6B from (chapt 5). As shown in previous studies, RT-PCR can yield a heteroduplex of the A and B forms of α6 that exhibit an intermediate size on analysis in agarose gels. The existence of the 6A and 6B forms was confirmed by immunoblotting and immunoprecipitation with anti-peptide antibodies (not shown). .

Relative affinity of melanoma integrins for laminin.

The K1735 parental cell line, whose repertoire of integrins contain all known β1 laminin-binding integrins (α1, α2, α3, α6, α7), was used to study relative receptor affinity on laminin-Sepharose columns. Cells were surface labeled with ^{125}I, solubilized in running buffer containing 1 mM Mn^{2+}, and loaded on laminin-Sepharose columns; after washing with running buffer, the column was sequentially eluted with running buffer containing 10 mM Ca^{2+} instead of Mn^{2+}, and finally, running buffer containing EDTA but no divalent cation. The elution profiles of individual integrins was then assessed by immunoprecipitation and SDS-PAGE. Replacement of the Mn^{2+} with 10 mM Ca^{2+} eluted a mixture of β1 integrins that consisted predominately of α6 with some amounts of α1; subsequent fractions contained essentially all of the remaining α6 and α1 along with some small aunts of α7. The elution with EDTA recovered exclusively α7β1. When Mg^{2+} was used as the divalent cation, we similarly observed preferential elution of both α6 and α1 from the laminin-Sepharose column. Again, α7β1 was exclusively eluted with EDTA. When using a stepwise NaCl gradient to elute laminin-binding integrins from the laminin column, α6 was eluted from the column with 50 mM NaCl. In contrast, α7β1 is resistant to NaCl elutions even at concentrations of 1 M and is preferentially eluted with EDTA. Thus in contrast to α1 and α6, α7β1 binding to laminin is completely resistant to elution with NaCl and substantially resistant to elution with Ca^{2+} and Mg^{2+}. Immunoprecipitation with specific antibodies to α2 and α3 failed to detect significant amounts of these integrins in the eluted fractions from laminin columns.

Expression of the α6 and α7 mRNA in tumor cell lines.

The difference in the expression of α6 and α7 integrins in the low and highly metastatic cell lines may reflect regulation at the transcriptional level. Using probes specific for each α chain, we screened total RNA from a panel of K1735 melanoma cell lines with different metastatic potentials by Northern blot analysis. The α7 probe hybridized with a mRNA species of about 4.2 kb in positive cell lines (chapt 5). The α7 mRNA and corresponding protein levels were consistently high in cells of low metastatic potential (C10, C16, C19, and C23). In contrast, the level of mRNA for α7 was several-fold lower or not detectable in cell lines that were highly metastatic (C2, C26, M2, and M4 cell lines).

In contrast, in all K1735 cell lines α6 mRNA expression was consistently opposite from the expression of α7 transcripts. In α6 positive cell lines, a mRNA species of about 6 kb was detected. High expressors of α6 mRNA transcripts were highly metastatic (i.e., C2, C26, M2, and M4). Usually the poorly metastatic set of variant cell lines (i.e., C10, C16, C19, C23) had very low or undetectable levels of α6 message, although it should be noted that C10 sometimes had significant α6 message. The K1735 parental cell line had an intermediate but variable level of α6 signal; this was similar to that observed in Northern blots of the parental cell RNA when hybridized with probe for α7: a variable but usually high level of expression was noted.

By immunoblotting we found that α6A (but not α7B) is strongly expressed in the metastatic M2 cell line, but not in the non-metastatic C19 and C23 clones; most significantly, selection of a revertant clone (C23m) by spontaneous metastasis from the

CHAPTER TWO

Role of Laminin-Binding Integrin in the Invasion of Basement Membrane Matrices by Fibrosarcoma Cells

ABSTRACT

Laminin is a large glycoprotein that is found in basement membranes and promotes cell adhesion, migration, and differentiation. In human fibrosarcoma cells we detected the presence of an integrin complex, with a M_r of 140,000/120,000 under nonreducing conditions, that bound specifically to laminin-Sepharose columns. Immunoprecipitation with monoclonal antibodies characterized this complex as $\alpha 6\beta 1$. Attachment of the fibrosarcoma cells to laminin substrates was completely inhibited in the presence of anti-$\alpha 6\beta 1$ antibody, while attachment to fibronectin and type IV collagen was unaffected. When seeded onto a reconstituted basement membrane, the fibrosarcoma cells spread out, migrated, and invaded the matrix. In the presence of anti-$\beta 1$ or anti-$\alpha 6\beta 1$ antibodies, initial invasion through the matrix was inhibited. The results indicate that the HT1080 cells express the $\alpha 6\beta 1$ complex and that it mediates their attachment to laminin. Furthermore, this receptor appears to be important during initial attachment and subsequent invasion of basement-membrane-like matrices.

INTRODUCTION

Invading tumor cells must adhere to and traverse basement membranes and stroma during metastasis. This process involves interactions between the extracellular matrix and specific cell surface adhesion receptors. Adhesion and invasion are mediated by a variety of surface adhesion receptors, among which are the integrin heterodimers, a superfamily of cell-surface transmembrane glycoproteins involved in cell-cell and cell-substrate interactions. Integrin heterodimers are composed of an α and a β subunit. These have been classified according to the presence of one of at least five β subunits which are combined with one of several α subunits (Hynes, 1990). Many of the extracellular matrix receptors share a common β1 subunit complexed to one of several α chains (Hemler, 1990). A group of integrins involved in leukocyte cell-cell interactions, such as LFA-1, Mac-1, and p150,95, belong to the β2 subfamily. The platelet IIb/IIIa complex and the vitronectin receptor belong to another subgroup which contains the β3 subunit. The β4 subunit is restricted to epithelial cells and is complexed with the α6 subunit. The β5 subunit is found on epithelial cells and has affinity for fibronectin and vitronectin (Cheresh *et al.*, 1989); it may be related or identical to the recently identified βs subunit associated with the vitronectin receptor. Many of the β1 and β3 integrins appear to bind sites containing the amino acid sequence Arg-Gly-Asp (RGD). For all five subfamilies, the ligand specificity appears to be defined by the α chain and the individual β chain to which it is complexed.

Laminin is a ubiquitous basement membrane protein which mediates a wide

range of cellular activities and appears to have an important role in tumor invasion and metastasis (Liotta *et al.*, 1986). The molecule is composed of three chains that form a cross-shaped structure (Timpl, 1989). Several cellular receptors for laminin have been identified. Liotta and coworkers (Rao *et al.*, 1983) first identified a non-integrin laminin receptor with a M_r of 67,000. The M_r 67,000 receptor has now been detected on many cell types, and in tumor cells its presence appears to be positively correlated with metastatic potential (Liotta *et al.*, 1985). This glycoprotein apparently interacts with the YIGSR sequence in the B1 chain of laminin. Recently, however, Mecham *et al.* (1989) demonstrated that the M_r 67,000 receptor interacts with a hydrophobic sequence (VGVAPG) in elastin, as well the YIGSR sequence in the B1 chain of laminin. Another group (Smalheiser and Schwartz 1987) identified a protein of M_r 120,000 as the major laminin-binding protein on a number of different cell lines.

The first account of an integrin receptor for laminin was provided by Buck and Horwitz (1987). They observed that antibodies to the β1 integrin complex in avian cells can inhibit attachment to laminin. Ignatius and Reichardt (1988) identified a laminin-binding protein on neuronal cells which corresponds to α1β1. Ramos *et al.* (1990) also identified a complex of M_r 200,000/120,000 on B16-BL6 cells which strongly resembles α1β1 and binds to laminin-Sepharose affinity columns. Languino *et al.*, (1989) recently demonstrated that α2β1, known to be a collagen receptor (Wayner and Carter, 1987) is also a receptor for laminin. The α3β1 (Gehlsen *et al.*, 1988) and α6β1 (Ramos *et al.*, 1990; Sonnenberg *et al.*, 1987; Sonnenberg *et al.*, 1988) complexes have also been shown to be receptors for laminin. Lotz *et al.* (1990)

recently suggested that α6β4 may be a laminin receptor. More recently, a novel integrin receptor for laminin was identified in our laboratory which has a $\underline{M}_r$ of 100,000/130,000 under reducing conditions (Kramer *et al.*, 1989).

Wayner and Carter (1987) have shown that human HT1080 fibrosarcoma cells express integrin receptors for collagen and fibronectin (α2β1, and α5β1, respectively) as well as a promiscuous receptor for collagen, fibronectin, and laminin (α3β1). In the present study, using affinity chromatography and immunoprecipitation with monoclonal antibodies, we identified a β1 integrin complex on HT1080 cells which binds to laminin and appears to be α6β1. We previously demonstrated that when seeded onto reconstituted basement membrane, the HT1080 cells migrate on and degrade the matrix, leaving behind a vacant channel (Kramer *et al.*, 1986). In the present study, using monoclonal antibodies to the α6 subunit (GoH3), we demonstrate that α6β1 appears to be important in the initial attachment to and subsequent invasion of basement membrane matrices by HT1080 cells.

MATERIAL AND METHODS

Cell Culture. Human HT1080 cells were originally isolated from a metastasis to the mediastinum (Rasheed *et al.*, 1974). The cells were obtained from the American Type Culture Collection and were maintained as monolayer cultures in Dulbecco's Modified Eagle's medium (DMEM) containing 5% fetal bovine serum and gentamycin (50 μg/ml). Cells were passaged at preconfluency using 0.25% trypsin-2 mM EDTA-0.05% BSA in Ca^{2+}- and Mg^{2+}-free PBS. For adhesion and migration

assays, the HT1080 cells were harvested with 2 mM EDTA.

Antibodies. Mouse monoclonal antibody (GoH3) to the α6 subunit of the human α6β1 receptor was kindly provided by Dr. A. Sonnenberg (The Netherlands Cancer Institute). Mouse monoclonal antibodies against α2β1 (P1H5), α3β1 (P1B5), and α5β1 (P1D6) were a generous gift of Dr. William Carter (University of Washington, Seattle). Rat monoclonal antibodies against the human fibronectin receptor, α5β1, (B1E5) and the human β1 subunit (AIIB2) were a gift of Dr. Caroline Damsky (University of California, San Francisco). Rabbit polyclonal antibodies to mouse laminin were from Dr. Hynda Kleinman (NIH).

Cell-Surface Labeling. HT1080 cell-surface proteins were labeled with ^{125}I by the lactoperoxidase method as previously described (Kramer *et al.*, 1989). Preconfluent cell cultures were removed from dishes with 2 mM EDTA, 0.05% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Cells were washed three times with cold 50 mM Tris-HCl, 20 mM glucose, 150 mM NaCl, 1 mM $MnSO_4$, then suspended in the washing buffer. Iodination was initiated by adding carrier-free $Na^{125}I$, glucose oxidase, and lactoperoxidase at final concentrations of 1 mCi/ml, 200 mU/ml, and 200 μg/ml, respectively. The cell suspension was gently mixed by rotating the tube at 5 rpm on ice for 20 min. The reaction was terminated by adding large volumes of the buffer, without glucose, and the cells were recovered by centrifugation.

Affinity Chromatography. Laminin was purified according to the protocol of Kleinman *et al.* (1982) and coupled to CNBr-activated Sepharose. Surface-

radiolabeled cells were solubilized in 200 mM β-octylglucoside, 50 mM Tris-HCl, 1 mM $MnSO_4$, and 1 mM phenylmethylsulfonyl fluoride at 4°C. The cell extract was centrifuged to remove the insoluble cellular components, placed on the column, and allowed to remain there for 1 h. The unbound material was then washed off with 5 column volumes of running buffer (50 mM octylglucoside, 50 mM Tris-HCl, 1 mM $MnSO_4$, pH 7.4). Next, the column was washed with 5 column volumes of 0.2 M NaCl in the running buffer. Specifically bound material was eluted with 10 mM EDTA in divalent cation-free running buffer, followed by 5 column volumes of 1 M NaCl in running buffer. Column washes and elutions were in 1 ml volumes. Half the samples were reduced with 1% β-mercaptoethanol and heated at 100° for 3 min. Nonreduced samples were treated the same, except that 10 mM N-ethylmaleimide replaced the β-mercaptoethanol. Elution fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970), and the ^{125}I-labeled proteins were detected by autoradiography of the dried gels. Prestained protein standards (Biorad) were used as molecular weight markers (BSA, M_r 67,000; phosphorylase b, M_r 94,000; β-galactosidase, M_r 116,000; and myosin, M_r 200,000).

Immunoprecipitation. Elution fractions and whole cell extracts were immunoprecipitated with polyclonal and monoclonal antibodies as previously described (Kramer *et al.*,1989). Samples were incubated with antibodies at 4°C for 1 h. Species-specific anti-IgG beads were added for 1 h to recover the antigen-antibody complexes. Nonspecific material was removed from the beads by washing with 10

mM Tris-HCl, 0.1% Nonidet P-40, and 1 mM $CaCl_2$, pH 7.4 (TNC buffer). Two more washes followed, first with 1 M NaCl in TNC buffer, then with 0.1% SDS in TNC buffer. This was followed by one more wash with TNC buffer alone. The immunoprecipitates were solubilized by heating at 100°C for 3 min and demonstrated by SDS-PAGE followed by autoradiography.

Reconstituted Basement Membrane. A reconstituted basement membrane (RBM) containing type IV collagen, laminin, entactin, nidogen, and heparan sulfate proteoglycan, was prepared as previously described (Kramer *et al.*, 1986; Kleinman *et al.*, 1986). Alternatively, a commercial preparation (Matrigel, Collaborative Research Inc.) was also used, with similar results. The liquid RBM is layered onto a tissue-culture dish and incubated at 37°C for 30 min, forming a gel onto which cells can be seeded.

Adhesion Assay. Cell adhesion to purified protein substrates and to RBM was quantified using our previously described protocol (Kramer *et al.*, 1989). Laminin and type IV collagen were purified from the EHS tumor according to the protocol of Kleinman *et al.* (1982). Their purity was verified by immunoblotting and enzyme-linked immunosorbent assay using specific antibodies as in previous studies (Kramer *et al.*, 1989). Fibronectin was isolated from outdated human plasma as described by Ruoslahti *et al.* (1982). Bovine skin type I collagen (pepsin-treated, 95-98% purity) was obtained from Collagen Corporation (Palo Alto, CA). 96-well plates (Costar) were precoated with fibronectin, laminin, collagen (type I and IV) and BSA (as a control) at a range of concentrations in PBS for 1 h at 37°C. In experiments with

RBM, a thin layer of the liquid was spread across each well of a 96 well plate, allowed to dry in a laminar flow hood for 2 h, then rinsed three times with DMEM. Cells in culture dishes were radiolabeled for 14-20 h with 2 μCi/ml 5-[^{125}I]iodo-2'-deoxyuridine (IdUrd) (ICN, Irvine, CA). They were removed with 2 mM EDTA, centrifuged, washed, and suspended in serum-free DMEM plus 0.1% BSA at a final concentration of $1x10^5$ cells/ml. Cells in suspension were pre-incubated in the presence of either control culture medium or dilutions of antibodies. 50 μl of the cell suspension was then added to each well. The plate was incubated for 15 min at 0°C, then for 30 min at 37°C. After incubation the plates were rinsed with medium and the number of adherent cells was determined.

Migration Assays. Each well of a 24-well plate (Costar) was coated with liquid Matrigel, which was placed in the well and evenly spread across the bottom. The Matrigel was allowed to polymerize at 37°C for 30 min. HT1080 cells were adjusted to a density of $5x10^4$/ml and seeded onto the matrix in control culture medium or in culture medium containing antibody. The 24-well plates were incubated at 37°C and photographed at 2 h and 5 h after seeding with a Nikon Diaphot inverted microscope equipped with phase-contrast optics.

For viewing with the Cambridge S-150 electron microscope, untreated cells and matrix were fixed in a 2% solution of glutaraldehyde in PBS at room temperature for 20 min, then post-fixed with a 2% solution of OsO_4 for 20 min. The specimens were then sequentially dehydrated in ethanol. Critical point drying was carried out with CO_2, in a modified Bomar critical point dryer. Finally, the specimens were sputter-

coated with gold.

Invasion Assay. A suspension of HT1080 cells and liquid RBM was prepared at a concentration of $2x10^5$ cells/ml. 5 µl of the suspension was placed in each well of a 48-well dish (Costar). The dish was then immediately inverted and incubated at 37°C for 30 min to allow for polymerization. 300 µl of DMEM alone or DMEM plus antibodies (GoH3 and AIIB2) was added to the cell-RBM suspension. The assays were monitored visually using phase-contrast optics at various time intervals. Cells migrating through the matrix and reaching the matrix-plastic interface were determined.

RESULTS

To identify laminin-binding proteins on HT1080 cells, we subjected ^{125}I-surface-labeled cells to laminin affinity chromatography followed by SDS-PAGE. The detergent extract of the cells contained several radiolabeled bands, ranging from M_r 30,000 to 200,000 (Fig. 1, lanes 1 and 6). After the extract was applied to a laminin-Sepharose affinity column, elution of material with 0.2 M NaCl in running buffer released several minor bands and two major bands at M_r 110,000 and 140,000 nonreduced (Fig. 1, lanes 2 and 7). Application of 10 mM EDTA eluted large amounts of the same two radiolabeled bands at M_r 110,000 and 140,000 (Fig. 1, lanes 3-4 and 8-9). Immune precipitation of EDTA-eluted fractions (Fig. 1, lanes 5 and 10) and whole-cell extract (Fig. 2, lanes 4 and 8) with anti-α6 antibodies recovered the α6β1 complex of M_r 140,000 (α6) and 110,000 (β1). Under reducing conditions, the M_r140,000 band migrated at M_r 115,000 while the M_r 110,000 migrated at

approximately M_r 140,000. This shift in electrophoretic mobility for α6β1 has been previously documented (Ramos *et al.*, 1990; Sonnenberg *et al.*, 1988). We also subjected extracts of HT1080 cells to immunoprecipitation with antibodies to α1β1 (Ts2/7) and to αvβ3 (LM 609); however, α1β1 was not detectable and αvβ3 was present in onlt trace amounts (data not shown).

We observed, by sequential immunoprecipitation of whole-cell extract with integrin-specific antibodies, that HT1080 cells express the α2β1, α3β1, and α5β1 complexes as well as the α6β1 (Fig. 2). The immunoprecipitation results suggested that the α6β1 complex is expressed in relatively lesser amounts than the α2β1 and α3β1 complexes. The α2β1 complex is a collagen receptor that appears to function also as a laminin receptor in endothelial cells (Wayner and Carter, 1987). We therefore tried to determine whether this complex is also important in mediating HT1080 cell adhesion to laminin. Blocking antibody to the α2β1 complex (P1H5) was able to inhibit HT1080 cell adhesion to collagen types I and IV but not to laminin or fibronectin (Fig. 3). Antibody against the α5β1 receptor (P1D6) effectively blocked attachment to fibronectin but not to laminin or collagen (Fig. 3).

The affinity chromatography and immunoprecipitation results suggested that α6β1 may be important in HT1080 cell attachment to laminin. This was borne out by the observation that monoclonal antibodies (GoH3) to this receptor completely blocked HT1080 cell adhesion to immobilized laminin (Fig. 4), but had no detectable effect on their attachment to fibronectin-coated substrates (not shown). We next looked at the effect of several monoclonal antibodies on HT1080 cell attachment to RBM. In the

presence of a 1:30 dilution of GoH3, initial cell attachment was inhibited by almost 80% (Fig. 5). However, antibody to α5β1 (B1E5) only slightly decreased cell attachment. Anti-β1 (AIIB2) antibody completely blocked attachment. While the initial phase of cell adhesion to the matrices was inhibited by GoH3, longer incubation (> 3 h) allowed the cells to overcome the antibody and the cells were able to attach (not shown).

We previously reported that when seeded onto reconstituted basement membrane, HT1080 cells spread out, migrate, and invade, leaving channels etched in the surface of this matrix (Kramer *et al.*, 1986). We now used scanning electron microscopy to observe this behavior more closely. We saw that the cells actually burrowed into this matrix (Fig. 6), dissolving it and leaving behind an empty channel.

We next studied the effect of antibodies on the spreading of HT1080 cells on RBM. After 5 h, cells in medium alone had spread extensively on the matrix and were beginning to form cell-cell networks; some channels were present (Fig. 7A). In the presence of anti-α6 antibody (GoH3), most cells had attached but only limited spreading was evident (Fig. 7B). Similarly, when seeded in the presence of antibodies against the β1 subunit (AIIB2), the cells attached but failed to spread (Fig. 7D). However, in the presence of antibodies against the fibronectin receptor (B1E5) (Fig. 7C), the cells were able both to attach and to spread out and migrate on the matrix.

Finally we evaluated the effect of individual monoclonal antibodies on HT1080 cell invasion of the RBM. The assay consisted of suspending the cells in liquid RBM and placing a small droplet of this suspension in tissue culture wells. After the matrix

had polymerized, the samples were incubated for increasing times and the number of cells that had migrated to the edge of the droplet was determined. Early observations using ^{125}I-Iudr-labeled cells indicated that virtually all cells were trapped in the matrix and could not be released by trypsin-EDTA treatment. However, with time, cells eventually penetrated the matrix and could be progressively recovered by the trypsin-EDTA treatment (data not shown). Microscopic examination of the cells in the matrix droplet revealed that cells emerged from the matrix layer and then migrated on its surface, eventually concentrating at the matrix-plastic interface (Fig. 8). Significant numbers of cells had migrated out of the matrix droplets by 15 h (Fig. 8) and could be easily counted; this movement continued with time. The effect of anti-α6 (GoH3) and anti-β1 antibody on this invasion process was examined. Both antibodies convincingly blocked the out-migration of cells, even after 24 h of incubation (Fig. 9). Neither antibody appeared to be cytotoxic, since removal of the antibody reversed the inhibitory effect (not shown). Antibody to the fibronectin receptor (B1E5) had no effect on RBM invasion (not shown).

DISCUSSION

Recent observations have suggested that integrin-related receptors can mediate cell interactions with laminin (Ramos *et al.*, 1990; Wayner and Carter, 1987; Kramer et al., 1989). In this study we report that the α6β1 integrin receptor is the major laminin-binding protein on the surface of the HT1080 fibrosarcoma cell line. The ability of specific monoclonal antibodies (GoH3) to effectively inhibit the attachment

of HT1080 cells to laminin suggests that the α6β1 complex is critical in mediating initial attachment to laminin. Furthermore, we provide evidence that α6β1 is also important in the invasion of basement membrane matrices.

To determine the importance of α6β1 in mediating attachment to a complex matrix, we seeded the HT1080 cells onto reconstituted basement membrane (RBM). The cells were able to attach firmly and spread. Conversely, when the tumor cells were preincubated with GoH3 antibody and then seeded onto the RBM, adhesion was significantly inhibited (Fig. 5). This reduction in attachment was dramatic but not complete, indicating the presence of other receptors mediating adhesion to the basement membrane. In a 5-h cell spreading assay, the cells eventually attached in the presence of GoH3, but were not able to spread effectively (Fig. 8). Similarly, antibodies against the integrin β1 subunit prevented cell spreading while allowing a reduced number to attach. Given the variety of integrin as well as non-integrin receptors capable of interacting with basement membranes, these results are not unexpected. HT1080 cells express integrin receptors not only to laminin but to type IV collagen as well, a major component of the basement membrane. These results also suggests the presence of several receptor systems, one for attachment and one for spreading and migration, as has previously been suggested by Runyan *et al.* (1988). This is a reasonable possibility, since during blood-borne metastasis, tumor cells must adhere to a variety of extracellular matrix proteins once they extravasate the blood vessel.

Graf *et al.* (1987) showed that a synthetic peptide containing the YIGSR

sequence from the B1 chain of laminin is able to support HT1080 cell attachment. Furthermore, the YIGSR peptide inhibited HT1080 cell attachment to intact laminin. YIGSR is one of the suggested determinants for the M_r 67,000 laminin receptor, although recent studies suggest that a hydrophobic sequence in the B1 chain of laminin is a more effective binding site. While HT1080 cells may express the M_r 67,000 receptor, they apparently also use the α6β1 integrin complex to mediate adhesion to laminin.

Using integrin-specific antibodies, we observed that the HT1080 cells also express α2β1, α3β1, and α5β1, and that α2β1 and α3β1 are expressed in relatively greater amounts than α6β1. This correlates with the earlier work of Wayner and Carter (1987) who found that the HT1080 cells' expression of these subunits is also α2>α3>α5. α2β1, a known collagen receptor, has recently been shown to function as a laminin-binding protein in endothelial cells (Languino *et al.*, 1989). However, in our experiments, monoclonal antibodies to α2β1 were able to block HT1080 cell attachment to collagens type I and IV, yet were ineffective in preventing their attachment to laminin. This suggests that in this system the α2β1 complex may be important as a collagen receptor, but that additional receptors such as the α6β1 are also active in laminin adhesion.

Many groups have used various invasion assays to study tumor cells invasion. The two most common systems used are the amnion (Liotta *et al.*, 1980; Russo *et al.*, 1980) and the RBM, (Albini *et al.*, 1987; Hendrix *et al.*, 1987) usually studied in Boyden-type chambers. However each of these methods have certain disadvantages.

The major problems associated with use of the amnion assay are inconsistency in thickness and composition of the amnion, and difficulty in collecting intact, post-invasive tumor cells. Previous invasion assays utilizing dried RBM on filters in Boyden chambers are cumbersome and invasion is difficult to quantitate due to cells becoming trapped within the pores of the filter. In this study we have developed a modified invasion assay using RBM which is reproducible, rapid, and easy to measure. Invasion is measured by determining the number of cells invading through the matrix and their accumulation at the plastic-matrix interface. This provides a simple means of measuring invasion without the cumbersome problems encountered with amnion or matrix-Boyden chamber assays. In addition, this method permits continuous morphological analysis of the invasion process.

We have successfully used this modified invasion system to study the effect of various anti-integrin blocking antibodies on tumor cell invasion of basement membrane matrices. Our results indicate that the β1-class of integrin receptors appears to be essential for cell migration and invasion of these matrices. Basement membranes are composed of several macromolecules, including laminin, type IV collagen, entactin, and heparan sulfate proteoglycan. All of these molecules are capable of supporting cell attachment (Reviewed in Timpl, 1989), yet antibodies to the α6β1 complex significantly reduced cell attachment, spreading, and invasion of basement membrane matrices. Thus, laminin, a major adhesive component of the basement membrane, appears to play an important role in the invasion process, despite the presence of other adhesion molecules. The potential function of the other β1 complexes and β3

FIGURE LEGENDS:

Fig. 2.1. Identification of a laminin-binding integrin complex. ^{125}I-labeled HT1080 cells were solubilized in starting buffer and the extract (lane 1, nonreduced and lane 6, reduced) was applied to a laminin-Sepharose column as described under "Material and Methods". After a washing with running buffer, the column was eluted with 200 mM NaCl (lane 2, nonreduced and lane 7, reduced) followed by EDTA (lanes 3-4 nonreduced and lanes 8-9 reduced). The fraction in lane 3 was immunoprecipitated with anti-α6 monoclonal antibody (GoH3) (lane 5, nonreduced and lane 10, reduced).

Fig. 2.2. Immunoprecipitation of β1 integrin receptors. The detergent extract of ^{125}I-labeled whole cells was immunoprecipitated with monoclonal antibodies as described in Material and Methods. The immunoprecipitates were then analyzed by SDS-PAGE. Anti-α2β1 (P1H5) (lane 1, nonreduced; lane 5, reduced); anti-α3β1 (P1B5) (lane 2, nonreduced; lane 6, reduced); anti-α5β1 (B1E5) (lane 3, nonreduced; lane 7, reduced); anti-α6β1 (GoH3) (lane 4, nonreduced; lane 8, reduced).

Fig. 2.3. Effect of anti-integrin monoclonal antibodies on cell adhesion. Cells were radiolabeled with 5-[^{125}I]iodo-2'-deoxyuridine and processed for adhesion to laminin, fibronectin, and collagen types I and IV, in the presence of various concentrations of anti-α2β1 (P1H5) and anti-α5β1 (P1D6). Values are expressed relative to the maximum attachment at 15 min. for each protein substrate and represent the mean of triplicate wells; bars show SD.

Fig. 2.4. Antibodies to α6β1 block cell adhesion to laminin. Cells

radiolabeled with 5-[^{125}I]iodo-2'-deoxyuridine were processed for adhesion to immobilized laminin in the presence of various dilutions of GoH3 hybridoma culture supernatant (anti-α6β1), B1E5 (anti-α5β1), or AIIB2 (anti-β1), or in control culture medium. Values are the mean of triplicate wells; bars show SD.

Fig. 2.5. GoH3 inhibits cell attachment to reconstituted basement membrane (RBM). Liquid matrix was placed evenly across the bottom of a 96-well dish and allowed to air dry. Cells radiolabeled with 5-[^{125}I]-iodo-2'-deoxyuridine were processed for adhesion to the matrix in the presence of various dilutions of GoH3 (anti-α6β1), B1E5 (anti-α5β1, ascites), or AIIB2 (anti-β1, ascites), or in control medium. Values are mean of triplicate wells; bars show SD.

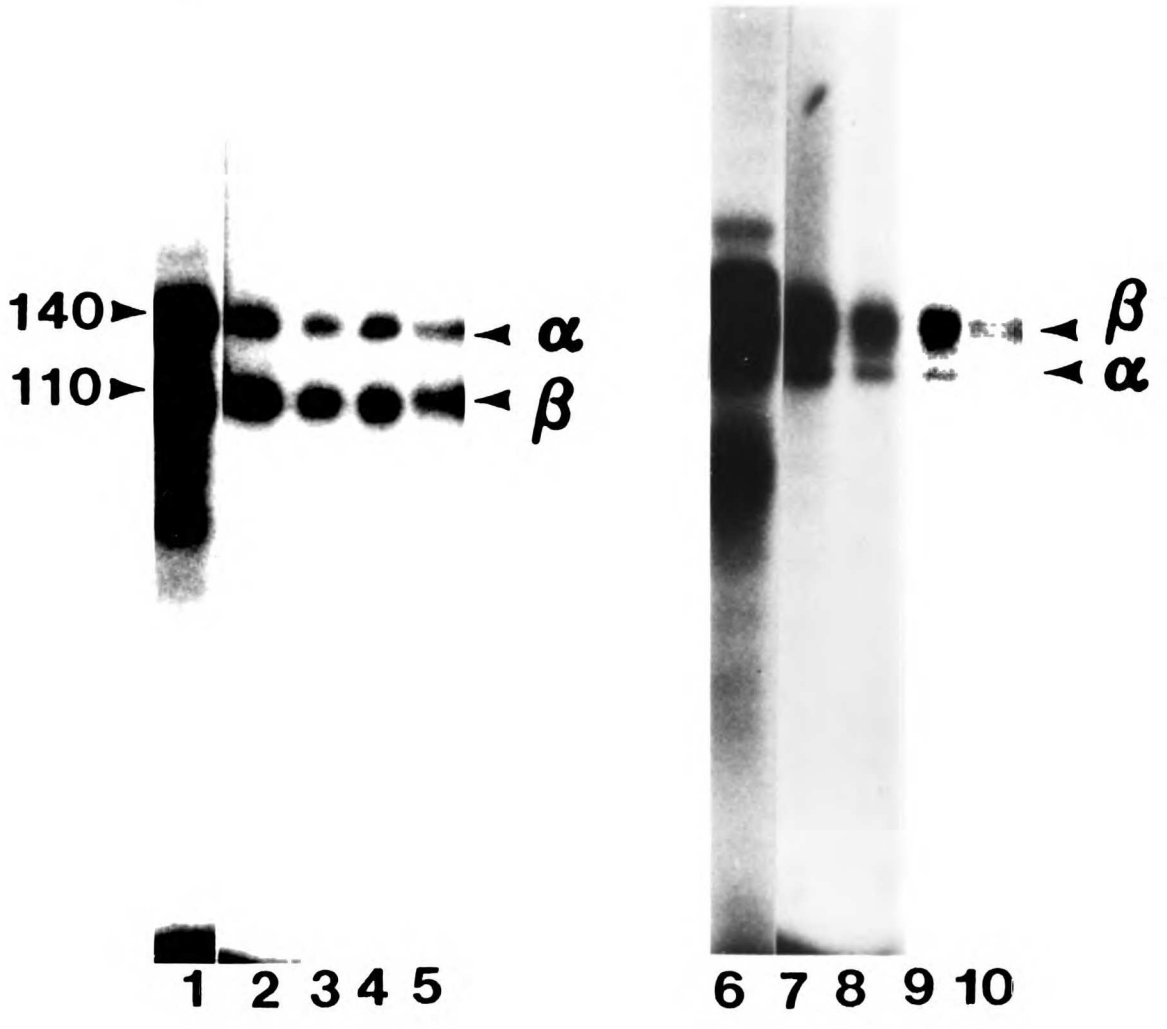
Fig. 2.6. Scanning electron micrograph of HT1080 cells invading RBM. HT1080 cells were seeded onto RBM and incubated for 12 h at 37°C. The specimens were fixed and dehydrated as described in "Material and Methods", then critical-point-dried, sputter-coated with gold, and viewed with a Cambridge S-150 electron microscope. Note that areas of dissolution of the matrix are evident and that cells have invaded the matrix (arrows).

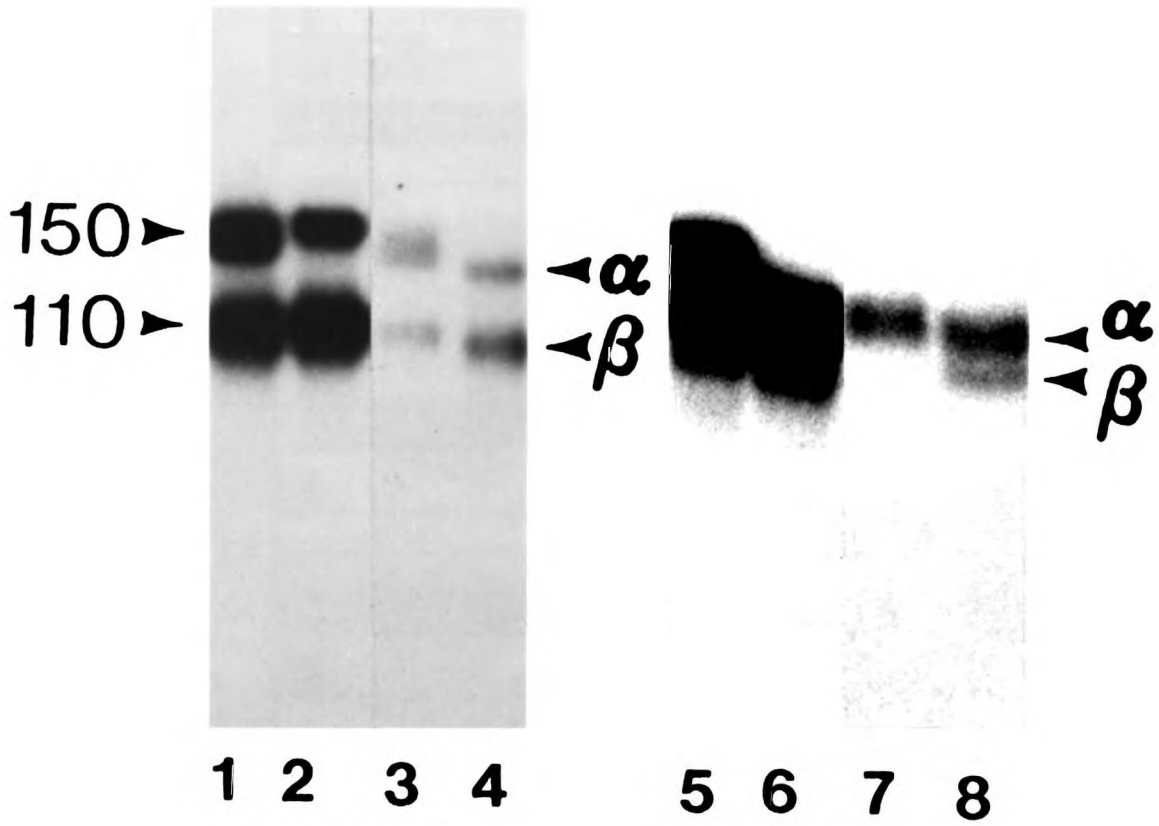
Fig. 2.7. Effect of monoclonal antibodies on HT1080 cell spreading on RBM. HT1080 cells were seeded onto the matrix in (A) control medium alone; (B) in the presence of anti-α6β1 (GoH3) (hybridoma culture supernatant, 1:30 dilution); (C) in the presence of anti-α5β1 (B1E5) (ascites, 1:500 dilution); or (D) in the presence of anti-β1 (AIIB2) (ascites, 1:500 dilution). Cells were incubated for 5 h at 37°C and photographed with phase-contrast optics.

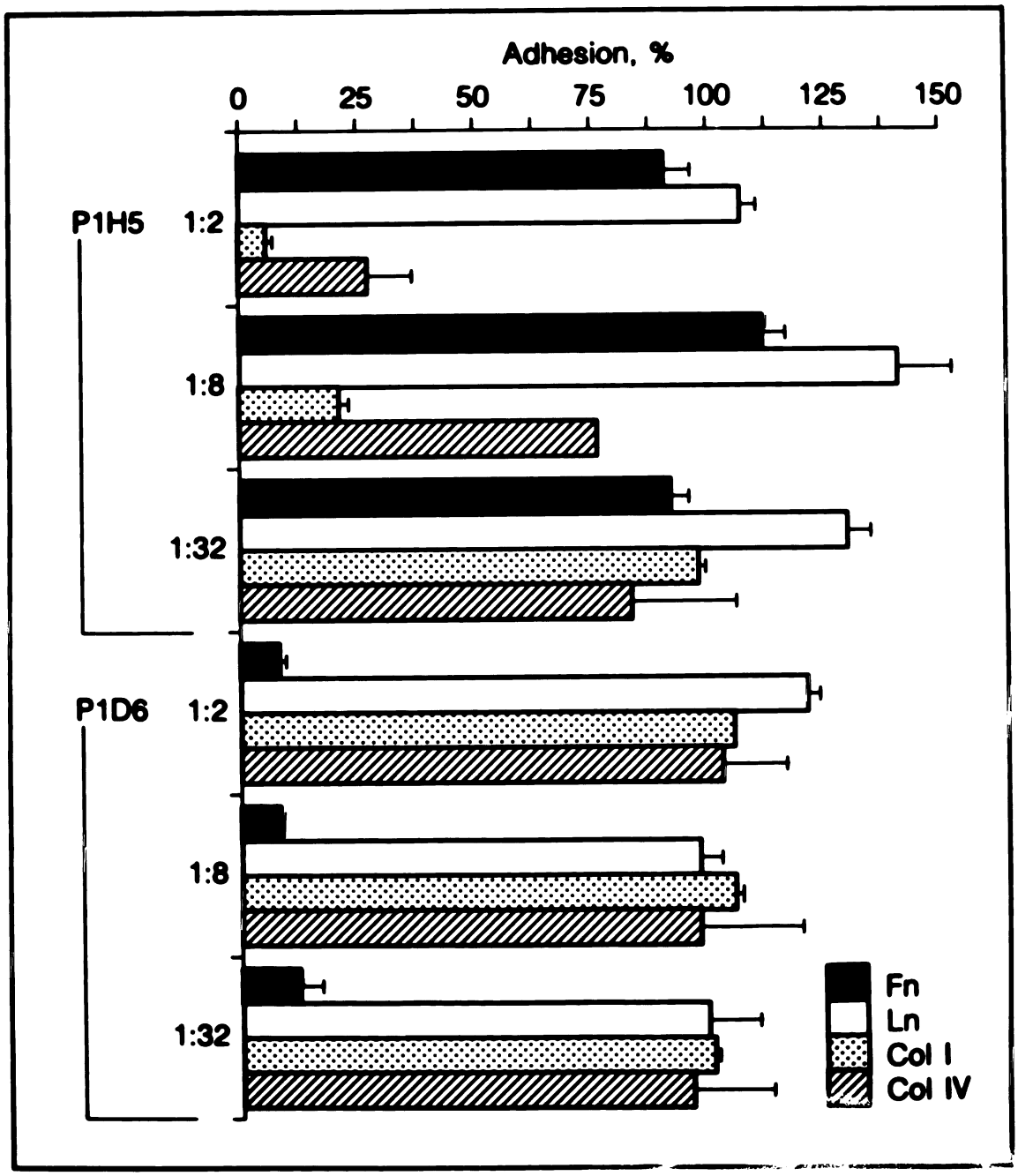
Fig. 2.8. Invasion of RBM by HT1080 Cells. Fibrosarcoma cells suspended in RBM invaded the matrix and reached the matrix-plastic interface (dashed line) at 15 h. (A) Cell invading the matrix (arrow indicates area of matrix dissolution. (B) Cell dissolving the RBM during invasion (arrows) and migrating to matrix-plastic interface. (C) Cell accumulating at the edge of the matrix after invasion.

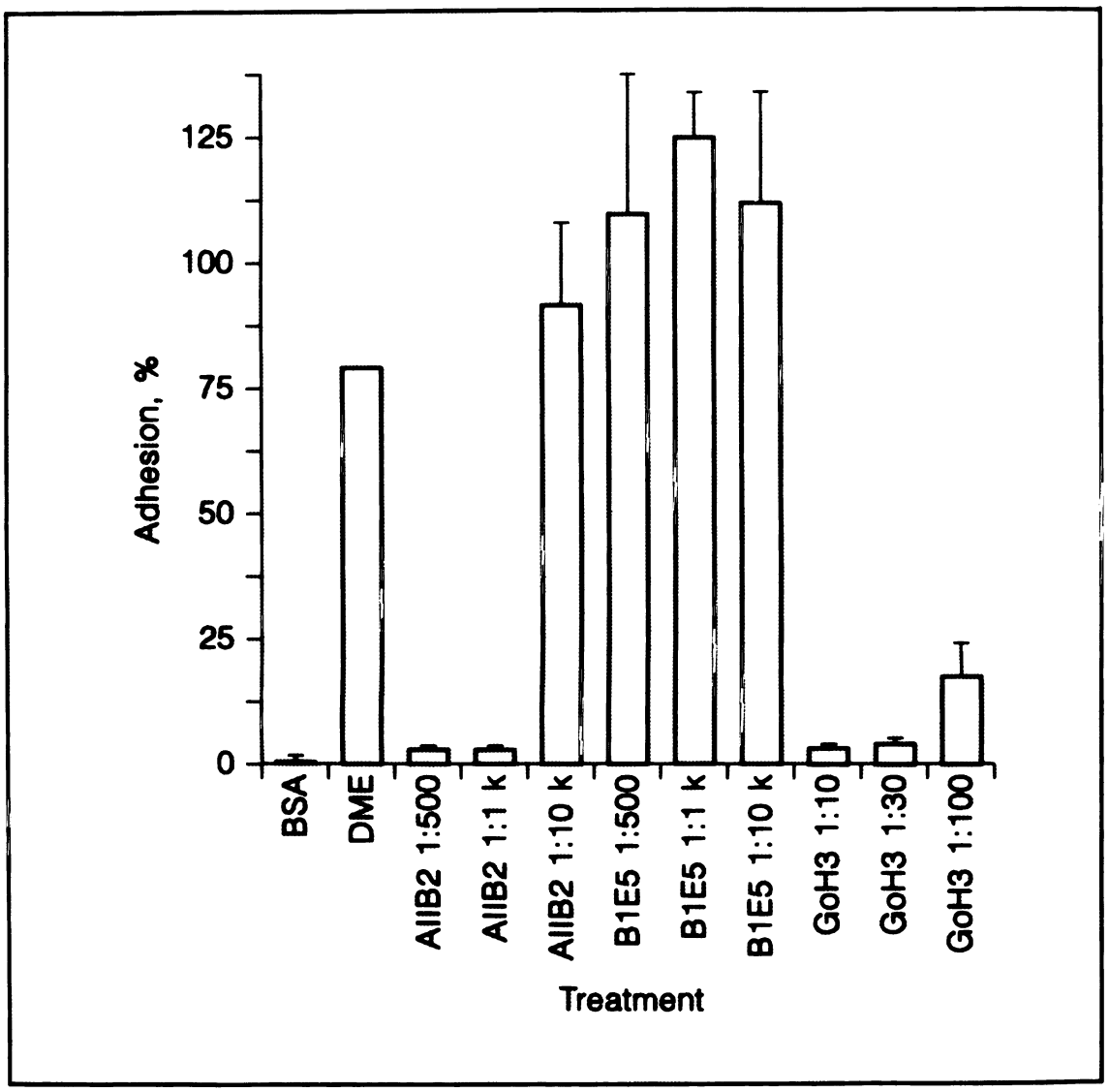
Fig. 2.9. Effect of monoclonal antibodies on HT1080 cell invasion of RBM. Control medium or medium with antibodies to $\alpha 6\beta 1$ or $\beta 1$ was placed in each well containing the HT1080 cells suspended in RBM. Cells that exited the matrix and had accumulated at the matrix-plastic interface were determined at the indicated time points. Values are means of triplicate wells; bars show SD.

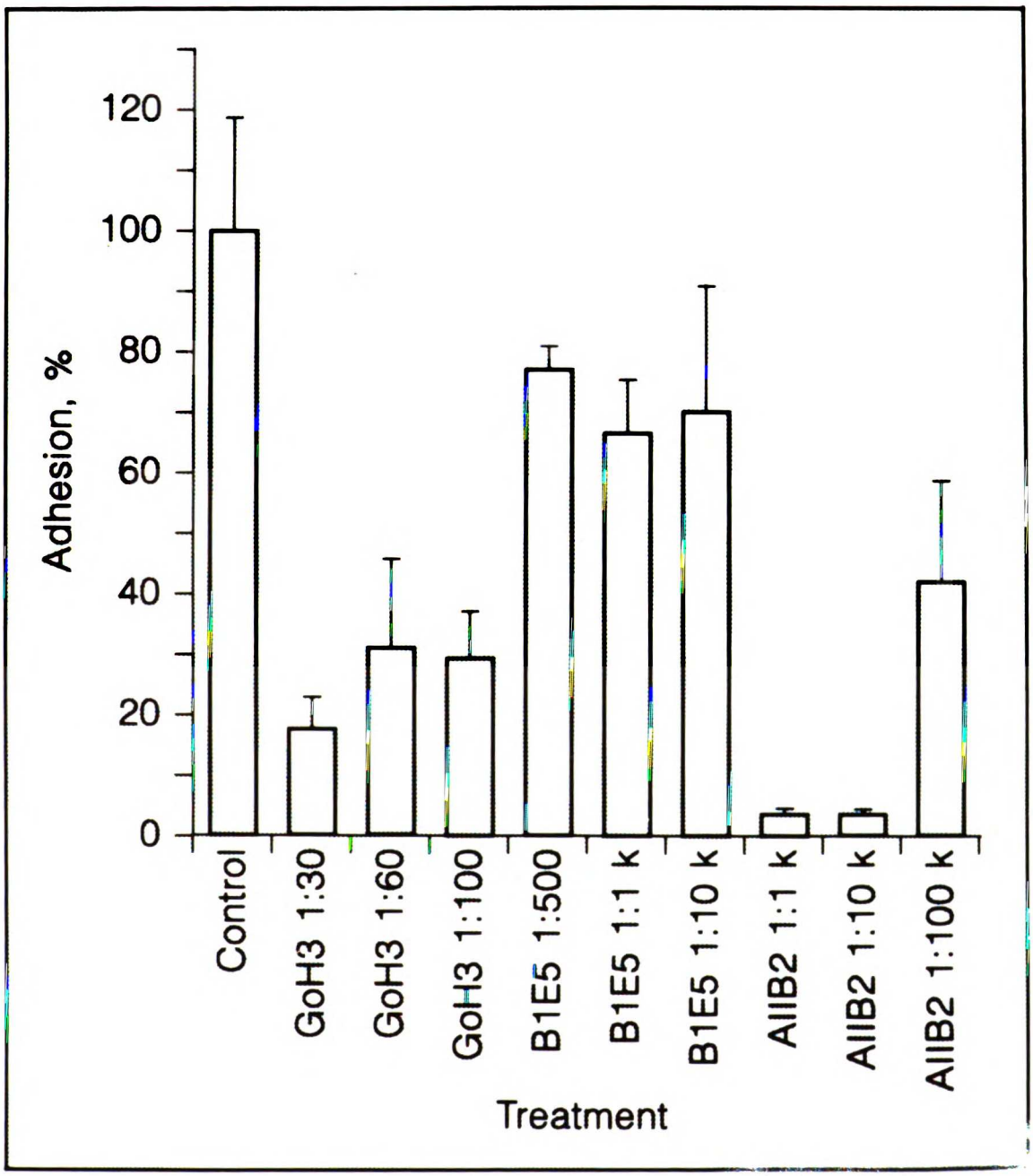
Fig.2.1

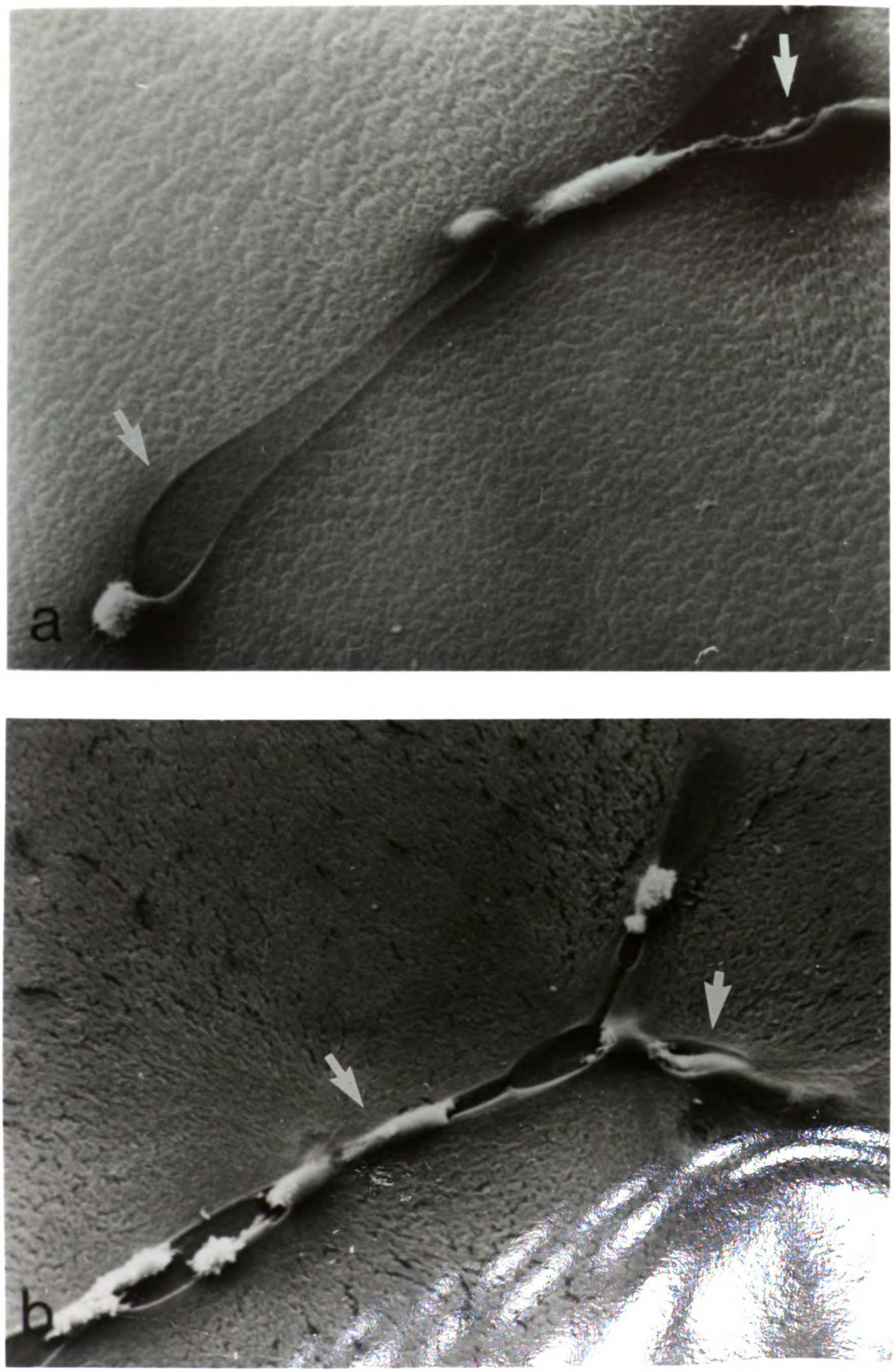



150
110
α
β
α
β
1 2 3 4 5 6 7 8


Adhesion, %
0
25
50
75
100
125
150
P1H5
1:2
1:8
1:32
P1D6
1:2
1:8
1:32
Fn
Ln
Col I
Col IV


Adhesion, %
125
100
75
50
25
0
BSA
DME
AIIB2 1:500
AIIB2 1:1 k
AIIB2 1:10 k
B1E5 1:500
B1E5 1:1 k
B1E5 1:10 k
GoH3 1:10
GoH3 1:30
GoH3 1:100
Treatment


Adhesion, %
120
100
80
60
40
20
0
Control
GoH3 1:30
GoH3 1:60
GoH3 1:100
B1E5 1:500
B1E5 1:1 k
B1E5 1:10 k
AIIB2 1:1 k
AIIB2 1:10 k
AIIB2 1:100 k
Treatment


a
b

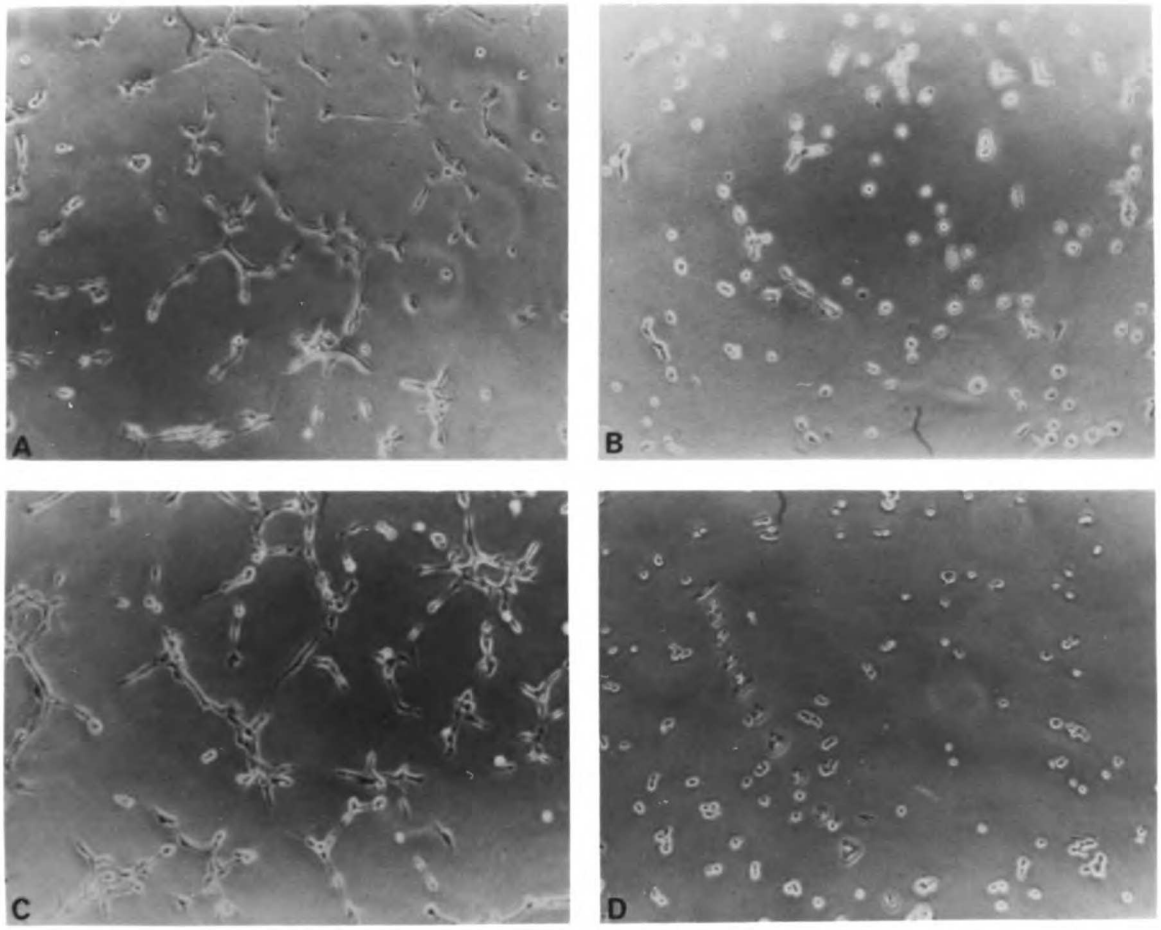
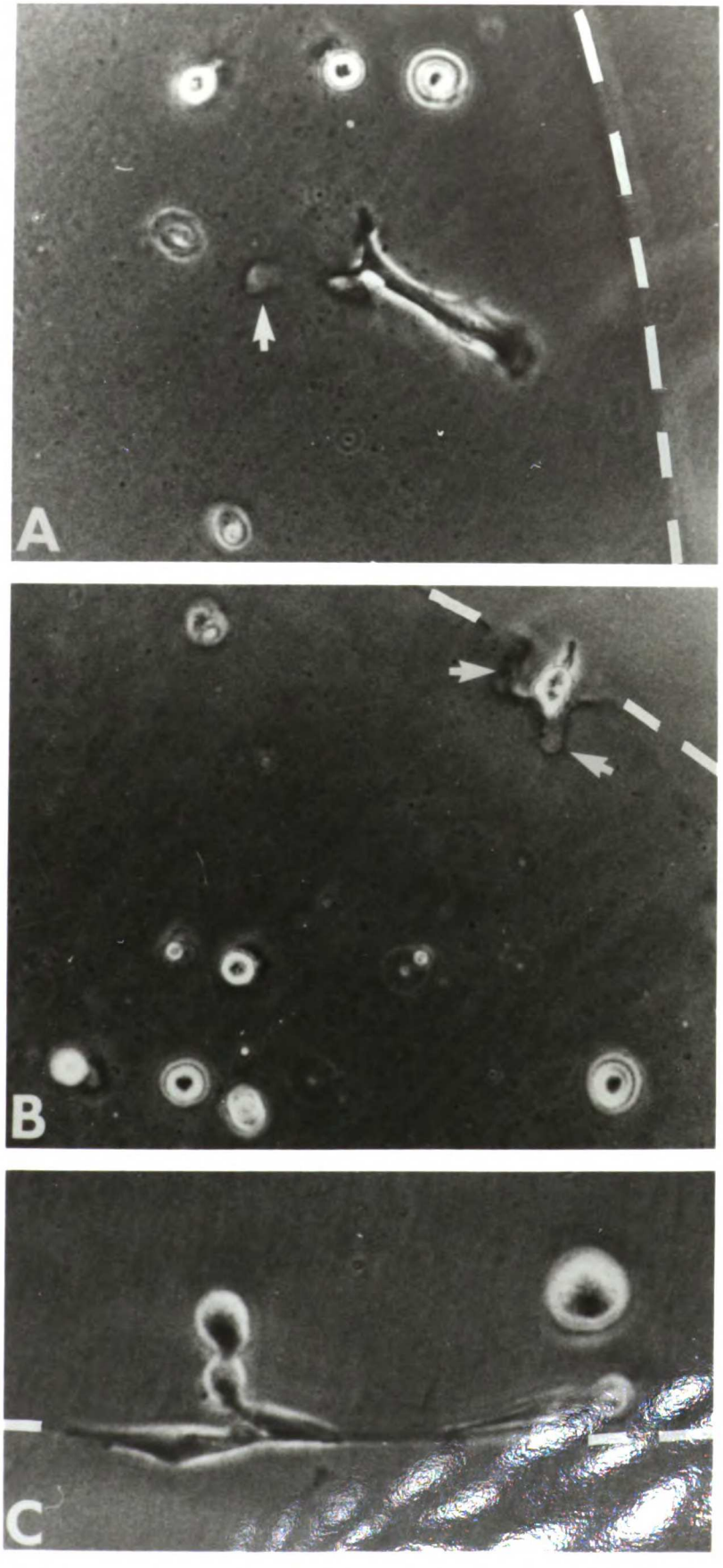
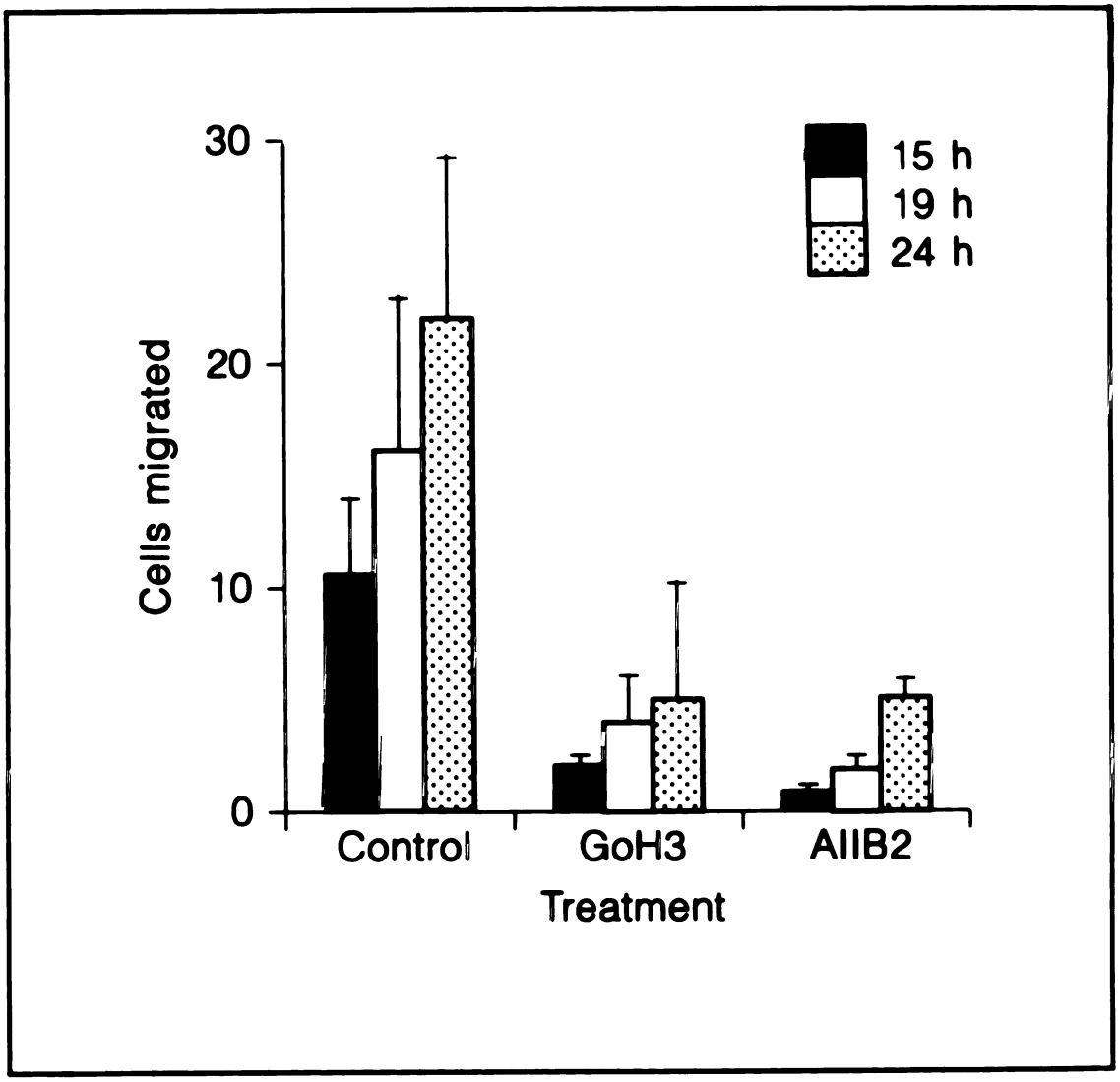

A
B
C
D

Fig.2.8




30
20
10
0
Cells migrated
15 h
19 h
24 h
Control
GoH3
AIIB2
Treatment

CHAPTER THREE

Identification of Integrin Receptors for Laminin and Type IV Collagen on Metastatic B16 Melanoma Cells

Footnotes

[3]The abbreviations used are: VLA, very late activation antigen; BSA, bovine serum albumin; PBS, phosphate buffered-saline; GRGDSP, Gly-Arg-Gly-ASP-Ser-Pro; YIGSR-NH_2, Tyr-Ile-Gly-Ser-Arg; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

ABSTRACT

As tumor cells invade surrounding tissue, they adhere to various extracellular matrix components. Previously we reported that B16-BL6 melanoma cell adhesion to both basement membrane and purified protein substrates was blocked by antibody to β1-integrin adhesion receptors (Kramer *et al.*, 1989). In the present study we found, using immunofluorescent staining, that β1-integrin complexes were co-localized with vinculin in focal adhesion plaques on laminin, type IV collagen and fibronectin substrates. To identify potential adhesion receptors on B16 cells, the cells were surface-labeled with ^{125}I, solubilized with detergent, and chromatographed on laminin-, type IV collagen-, and fibronectin-Sepharose columns. On laminin-Sepharose, an integrin heterodimer complex was eluted with EDTA that contained a β1 chain at M_r 120,000 and an α subunit at M_r 140,000 (nonreduced). This complex was specific for laminin and failed to bind to collagen- or fibronectin-Sepharose columns. Immunoprecipitation with specific monoclonal antibody identified this complex as α6β1 (VLA-6). Furthermore, monoclonal antibody to the α6β1 complex effectively blocked the attachment of B16-BL6 cells to laminin but did not affect adhesion to fibronectin or type IV collagen. We recovered a different integrin complex from type IV collagen-Sepharose columns that was composed of a β1 chain and an α chain of M_r 180,000 (nonreduced). This same complex also exhibited a weak affinity for laminin-affinity chromatography. The laminin-binding complex and the type IV collagen-binding complex were clearly distinct from the fibronectin-binding receptor and were not eluted by Arg-Gly-Asp-containing peptides. The results

INTRODUCTION

During invasion and metastasis, tumor cells must adhere to and penetrate the basement membrane and the interstitial stroma. This process clearly involves the specific interactions between tumor cell-surface adhesion receptors and components of the extracellular matrix. Previously we found that the highly metastatic murine B16-BL6 melanoma cell line is able to adhere to both complex basement membranes and to several of the individual proteins that compose the basement membrane: fibronectin, type IV collagen and laminin (Kramer *et al.*, 1989). In the same study we found that B16-BL6 cell adhesion to both basement membrane and purified protein substrates is blocked by anti-β1- integrin antibodies.

The integrins comprise a superfamily of transmembrane receptors that participate in cell-cell and cell-substrate interactions. Integrin receptors are heterodimeric proteins composed of an α and a β chain. Many of the extracellular matrix receptors (e.g., the family of VLA[3] antigens) are composed of a common β1 subunit complexed to one of several α chains. Integrins involved in cell-cell interactions, such as LFA-1, are part of the β2 subfamily. The vitronectin receptor and the platelet IIb/IIIa complex contain the β3 subunit. Some integrin complexes, such as the fibronectin receptor (α5β1) and the β3 receptors, bind to sites containing the amino acid sequence RGD.

Several potential laminin receptors have been identified, some of which are integrins. A non-integrin high-affinity receptor of $\underline{M}_r$ 67,000-72,000 has been detected on many different cell types, including B16 cells, and the expression of this

receptor has been correlated with metastatic potential (Liotta *et al.*, 1986). This receptor has been shown to interact with the YIGSR sequence in laminin. Several groups have identified another non-integrin glycoprotein of M_r 120,000 as the predominant laminin-binding protein in a number of cell lines (Smalheiser and Schwartz, 1987; Kleinman *et al.*, 1988). Buck and Horwitz (1987), provided the first evidence for an integrin receptor for laminin (CSAT). They demonstrated that the CSAT integrin complex (β1) in avian cells binds to laminin-affinity columns and can inhibit cell attachment to laminin. More recently, a laminin-binding β1 integrin with an α chain of M_r 150,000 was identified on a rat glioblastoma cell line (Gehlsen *et al.*, 1988). An apparently different laminin-binding integrin complex has been identified in a rat neuroblastoma cell line (Ignatius and Reichardt 1988). This complex is composed of a β1 chain and a M_r 200,000 α chain and may be related to VLA-1 (α1β1) in human cells. In addition, VLA-6 (α6β1) has been identified as the laminin receptor on platelets and other cell types (Sonnenberg *et al.*, 1987; 1988).

Several potential receptors for collagen have been identified. Dedhar *et al.* (1987) reported that a group of non-integrin surface proteins (M_r 30,000, 70,000, and 250,000) on human osteosarcoma cells appear to mediate adhesion to type I collagen. Carter and collaborators (1987, 1988) isolated a set of β1 integrin complexes on various human cells that are receptors for collagen and fibronectin or collagen alone. The collagen-specific receptor is identical to VLA-2 (Wayner *et al.*, 1988; Santoros *et al.*, 1988) and to the platelet Ia/IIa complex that mediates platelet adhesion to collagen (Ginsberg *et al.*, 1988). VLA-3 also appears to be involved in

adhesion to collagen, although this complex is able to interact with fibronectin and laminin as well (Wayner and Carter, 1987). Kramer and Marks (1989) recently demonstrated that VLA-1 ($\alpha 1\beta 1$) is a collagen receptor.

In the present study, using affinity chromatography, we have identified distinct $\beta 1$ integrin complexes on B16-BL6 cells that appear to be receptors for laminin, type IV collagen, and fibronectin. Immunofluorescence microscopy confirmed that $\beta 1$-containing complexes were concentrated in focal adhesion plaques in cells attached to laminin and type IV collagen. The present results are consistent with our previous observations that anti-$\beta 1$ antibody blocks B16-BL6 cell adhesion to laminin and type IV collagen, and suggest that $\beta 1$ integrin complexes participate in substrate adhesion.

MATERIALS AND METHODS

Cell Culture. The highly metastatic BL6 subline of the mouse B16 melanoma (Poste *et al.*, 1982) was obtained from E.G. and E. Mason Laboratories. The cells were routinely cultured in the presence of 8% CO_2 in Falcon T-150 tissue culture flasks containing Dulbecco's modified Eagle's medium with 5% fetal bovine serum and 50 μg/ml gentamicin added. Preconfluent cultures were dissociated from culture flasks with 2 mM EDTA, 0.05% BSA in Ca^{2+} and Mg^{2+}-free PBS.

Antibodies. Affinity-purified rabbit polyclonal antibodies prepared against the human placental fibronectin receptor, which react with the β1 and the α5 subunits (Pytela *et al.*, 1985) were a gift of Dr. Erkki Ruoslahti (La Jolla Cancer Foundation). Anti-hamster β1 (originally named anti-ECM_R/anti-GP140), a polyclonal antibody prepared from BHK cells which reacts with the β1 subunit, was a gift from Dr. Caroline Damsky (Knudsen *et al.*, 1981; Tomaselli *et al.*, 1987). Polyclonal anti-mouse fibronectin receptor was a gift of Dr. V.P. Patel (Patel and Lodish, 1987). Monoclonal antibody (GoH3) to human VLA-6 (α6β1) was kindly provided by Dr. A. Sonnenberg (Sonnenberg *et al.*, 1987; 1988). Mouse monoclonal antibody to vinculin, rhodamine-conjugated goat anti-rabbit IgG, and fluorescein-isothiocyanate-conjugated goat anti-mouse IgG were obtained from Boehringer-Mannheim (Indianapolis, IN). Species-specific antibodies conjugated to Sepharose were obtained from Sigma Chemical Co (St. Louis, MO).

Adhesion Assays. We measured cell adhesion using our previously described assay (Kramer *et al.*, 1989). Laminin and type IV collagen were purified

from the EHS tumor according to the protocol of Kleinman *et al.* (1982). Their purity was verified by immunoblotting and enzyme-linked immunosorbent assay, using specific antibodies as in previous studies (Kramer *et al.*, 1986). Fibronectin was isolated from human plasma and the $\underline{M}_r$ 120,000 cell-binding domain was purified from chymotrypsin digests as described (Ruoslahti *et al.*, 1982). Bovine skin type I collagen (pepsin-treated, 95-98% purity) was obtained from Collagen Corporation (Palo Alto, CA). Type V collagen was purchased from Calbiochem (La Jolla, CA). Gelatin from swine skin was purchased from Sigma. 96-well plates (Costar) were precoated with fibronectin, laminin, collagen types I, IV, and V, gelatin and BSA at a range of concentrations in PBS for 1 h at 37°C. Cells in culture dishes were radiolabeled for 16-20 h with 1 μCi/ml 5-[^{125}I]iodo-2'-deoxyuridine (ICN, Irvine, CA). Cells were removed with 2 mM EDTA, washed, and resuspended in serum-free Dulbecco's modified Eagle's medium plus 0.1% BSA at a final concentration of 1 x 10^5 cells/ml. 100 μl of the cell suspension was added to each well. After a 30 min incubation at 37°C, the plates were rinsed with medium, and the number of adherent cells was determined. In experiments in which monoclonal antibody was tested, cells were incubated in the presence of either control culture medium or dilutions (1:3-1:100) of GoH3 hybridoma culture supernatants. After incubation for 15 min at 0°C, the plate was warmed to 37°C and incubated a second time for 30 min, and the number of adherent cells was then determined as above. Controls included irrelevant rat monoclonal antibody, which always produced negligible inhibition of adhesion to laminin or other extracellular matrix protein substrates.

Immunofluorescence. The distribution of β1-containing complexes that may act as adhesion receptors in the B16-BL6 cells was analyzed by immunofluorescence microscopy. Glass coverslips were coated for 24 h at 4°C with 50 μg/ml of type IV collagen, laminin or fibronectin in PBS, then washed three times with cold PBS. Cells were suspended in serum-free culture medium containing 0.1% BSA (1×10^5 cells/ml). 100 μl of the cell suspension was overlaid onto the coverslips. After 4 h incubation at 37°C, the cells were processed as described (Kramer and Marks 1989). Cells were fixed by rapid exchange with 1% para-formaldehyde and 5% sucrose in PBS at 4°C. The monolayers were then permeabilized by extraction with 0.5% Triton X-100 in PBS for 15 min, and nonspecific staining was blocked by the addition of 10% normal goat serum. The samples were incubated for 1 h with polyclonal antibodies against the human fibronectin receptor, which react with the β1 subunit, and monoclonal antibody to vinculin. Next, samples were incubated for 1 h with goat anti-mouse IgG conjugated with fluorescein isothiocyanate (1:200) or goat anti-rabbit IgG conjugated with rhodamine (1:800). The coverslips were mounted with Fluoromount (Fisher) and viewed on a Nikon microscope equipped with epiluminescence optics.

Cell-Surface Labeling. B16-BL6 cell-surface proteins were radiolabeled with ^{125}I by the lactoperoxidase-glucose oxidase method, as previously described (Kramer *et al.,* 1989). Preconfluent cells were removed from dishes with 2 mM EDTA, 0.05% BSA in PBS, washed three times with cold 50 mM Tris-HCl, 20 mM glucose, 150 mM NaCl, 1 mM $MnSO_4$, and then resuspended at a final concentration

of 1 x 10^6 cells/ml in the same buffer. Iodination was initiated by adding carrier-free $Na^{125}I$ (Amersham, Arlington Hts., IL), glucose oxidase (Sigma), and lactoperoxidase (Calbiochem) at final concentrations of 250 mCi/ml, 200 mU/ml, and 200 μg/ml, respectively. The suspension was gently mixed by rotating the tube at 5 rpm on ice for 20 min. The reaction was terminated by adding a large excess volume of the buffer (without glucose), and the cells were recovered by centrifugation.

Affinity Chromatography. Purified laminin, fibronectin, or type I or IV collagen was coupled to CNBr-activated Sepharose. Surface-radiolabeled cells were solubilized in 200 mM β-octylglucoside, 50 mM Tris-HCl (pH 7.4), 1 mM $MnSO_4$, and 1 mM phenylmethylsulfonyl fluoride at 4°C. The extract was centrifuged to remove the insoluble cellular components and loaded onto columns (0.7 x 5 cm). The cell extract was incubated on the column for 45 min, after which the unbound material was removed with 5 column volumes of running buffer (50 mM octylglucoside, 50 mM Tris-HCl, 1 mM $MnSO_4$, pH 7.4). Next the column was washed with 5 column volumes of 0.2 M NaCl in the running buffer. Bound material was eluted with 10 mM EDTA in divalent cation-free running buffer, followed by 1 M NaCl in running buffer. When peptide elutions were attempted, one column volume of GRGDSP or YIGSR-NH_2, (1 mg/ml, Peninsula Laboratories) in running buffer, was loaded onto the column and incubated for 30 min prior to elution. Column washes and elution fractions were collected in 1 ml volumes. Elution profiles were analyzed by SDS-PAGE according to Laemmli (1970), and the ^{125}I-labeled proteins were detected by autoradiography of the dried gels. Samples were reduced with 1% β-mercaptoethanol

and heated at 100°C for 3 min. Nonreduced samples were treated the same except that 10 mM N-ethylmaleimide was substituted for β-mercaptoethanol. Two-dimensional gel electrophoresis was performed according to the procedure of Phillips *et al.* (1987). Prestained protein standards (Biorad) were used as M_r markers and included BSA, 67,000; phosphorylase b, 94,000; β-galactosidase, 116,000; and myosin, 200,000.

Immunoprecipitation. Column fractions or whole cell extracts were immunoprecipitated with polyclonal or monoclonal antibodies as previously described (Kramer *et al.*, 1989). Samples were incubated with antibodies at 4°C for 1 h. Species-specific anti-IgG-Sepharose was added and incubated for an additional hour at 4°C to recover the immune complexes. The unbound material was removed by extensive washing with 0.1% NP-40, 10 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.4 (TNC buffer). This was followed by two washes, first with 0.1% SDS in TNC buffer, then with 1 M NaCl in TNC buffer. Control immunoprecipitation performed with irrelevant antibodies always recovered negligible radioactivity. The recovered immunoprecipitates were solubilized by heating at 100°C for 3 min and analyzed by SDS-PAGE followed by autoradiography.

RESULTS

The B16-BL6 melanoma cells were seeded onto substrates coated with fibronectin, laminin, collagen types I, IV, and V, gelatin and BSA. Cells adhered equally well to laminin and fibronectin, but gelatin and BSA were poor substrates for

adhesion (Fig. 1). At the lower coating concentrations of collagens, cells attached best to type IV collagen and least to collagen types I and V. At higher coating concentrations (100 μg/ml), significant cell adhesion to collagens type I and V was observed.

We used anti-β1-integrin antibodies to determine if integrins were involved in B16 cell adhesion to laminin, type IV collagen, and fibronectin-coated substrates. Immunofluorescence with antibodies that react with the β1 chain revealed adhesion contacts on B16 cells adhering to coverslips coated with all three ligands (Fig. 2). It is expected that the anti-β1 antibodies would stain all α-β1 complexes present in the focal adhesion plaques. These adhesion contacts were typically concentrated at the outer margin of the cell. Counterstaining with monoclonal antibodies against vinculin demonstrated co-localization with the β1 integrin on laminin, type IV collagen and fibronectin substrates, confirming that the condensations represent focal adhesion plaques (Fig. 2). Similar results were obtained when B16 cells were preincubated with cycloheximide as described previously (Kramer *et al.*, 1989), indicating that the cells were not adhering to material secreted by the cells (data not shown). Attempts at staining focal adhesion plaques with GoH3 antibody (α6β1) were unsuccessful, possibly because the receptor/ligand binding site was inaccessible to the antibody.

Laminin- and collagen-binding proteins on the surface of the B16 cells were identified by affinity chromatography followed by SDS-PAGE. A detergent extract of ^{125}I-labeled cells contained a number of radiolabeled bands, ranging from M_r

30,000 to 250,000 (Fig. 3, lane 1). The extract was applied to a column of laminin-Sepharose. After washing of the column, elution with 0.2 M NaCl in starting buffer released several minor bands as well as a set of two major bands at M_r 120,000 and 140,000 (nonreduced) (Fig. 3, lanes 2-5). Application of EDTA eluted large amounts of the same two radioactive bands at M_r 120,000 and 140,000 (Fig. 3, lanes 6-8). Neither GRGDSP peptides (not shown) nor YIGSR-NH_2 peptides (Fig. 3, lane 16) could specifically elute this laminin-binding complex. Immunoprecipitation of EDTA-eluted fractions with either anti-hamster β1 or anti-mouse β1 antibodies recovered the same M_r 120,000 (β_1) (nonreduced) and 140,000 (α) laminin-binding proteins (Fig. 3, lanes 9-12). The reactivity of these two surface polypeptides with anti-β1 antibody implies that they form an α-β1 complex related to the integrin superfamily of receptors. Under reducing conditions (Fig. 3, lanes 10 and 12), the M_r 140,000 band migrated at M_r 115,000, while the M_r 120,000 band appeared to shift to approximately M_r 150,000. This inversion of the bands was confirmed by two-dimensional gel electrophoresis (Fig. 4).

A minor band (M_r 180,000) was sometimes recovered from laminin columns (Fig. 3, lane 14), depending on the conditions of elution. It appeared that under extensive washing, the M_r 180,000 band has a much lower affinity for laminin; when the column washing phase of the chromatography was shortened, the M_r 180,000 band was consistently detected. This M_r 180,000 could be immunoprecipitated with anti-β1 antibody but not with anti-α6 antibody, and appears to be identical to the type IV collagen receptor complex (see below).

Since the electrophoretic properties of the major laminin-binding complex were similar to these of VLA-6 ($\alpha 6\beta 1$) (Sonnenberg *et al.*, 1987), we subjected the EDTA-eluted material to immunoprecipitation with anti-$\alpha 6$ antibody. The GoH3 monoclonal antibody specifically immunoprecipitated the complex from the column fractions (Fig. 3, lane 13 and 14) as well as from the whole-cell extract (Fig. 3, lane 15). The YIGSR peptide which has been implicated in mediating B16 adhesion to laminin (Graf *et al.*, 1987; Iwamoto *et al.*, 1987), was ineffective in eluting VLA-6 (Fig. 3, lane 16) from laminin columns.

The finding that VLA-6 appears to be a major laminin-binding complex on the B16 cells led us to test whether GoH3 monoclonal antibody would block the attachment of cells to laminin substrates. Indeed, anti-VLA-6 substantially inhibited B16-BL6 cell adhesion to immobilized laminin; maximum inhibition approached 95% (Fig. 5). Significant inhibition was obtained with dilutions of 1:30 or less. In contrast, GoH3 antibody produced no detectable effect on the attachment of cells to either type IV collagen- or fibronectin-coated substrates (Fig. 6). This result is in line with the observations of Sonnenberg *et al.* (1988) that VLA-6 mediated the adhesion of human platelets to laminin.

On columns of type IV collagen-Sepharose, EDTA eluted two bands, an $\underline{M}_r$ 120,000 protein complexed with an $\underline{M}_r$ 180,000 protein (Fig. 7, lanes 5-8). When reduced with β-mercaptoethanol, the $\underline{M}_r$ 120,000 band migrated at 130,000 while the $\underline{M}_r$ 180,000 remained essentially unchanged (Fig. 7, lane 10). Immunoprecipitation of specifically bound and EDTA-eluted material from type IV collagen-Sepharose

columns with anti-hamster β1 co-precipitated the M_r 120,000 (β1) and 180,000 (α) complex (Fig. 7, lanes 9 and 10). An apparently identical receptor was recovered from type I collagen-Sepharose columns, although it bound very poorly to this ligand; negligible amounts of radiolabeled polypeptides were retrieved on denatured collagen (gelatin)-Sepharose columns (not shown).

We previously reported that B16-BL6 cells express a β1-integrin fibronectin receptor complex (Kramer *et al.*, 1989). When the electrophoretic migration patterns of the eluates from the fibronectin-, type IV collagen-, and laminin-Sepharose columns were compared (Fig. 8), it was clear that the α chains of all three complexes were distinct. On fibronectin-Sepharose columns, EDTA eluted a complex composed of a β1 chain and an α chain of M_r 160,000 (Fig 8, lane 1). When reduced with β-mercaptoethanol, both bands co-migrated as a broad band at an M_r of 130,000 (Fig. 8, lane 4). This contrasts with the collagen-binding complex, which has an α chain of M_r of 180,000 that remains essentially unchanged after reduction, and with the laminin-binding complex, with an α chain of M_r 140,000 that shifts to 115,000 after reduction.

DISCUSSION

We reported (Kramer *et al.*, 1989; Ramos *et al.*, 1989) that B16-BL6 cell adhesion to amnion basement membrane, and to various extracellular matrix proteins (fibronectin, laminin, and type IV collagen), was inhibited in the presence of anti-β1 antibody. Immunofluorescence staining showed that on all three substrates, β1-containing complexes were associated with vinculin-positive focal adhesion plaques. The results of the present study support our prediction that B16-BL6 cells express β1-containing integrin complexes that bind to laminin and collagen. The results also underscore the conclusion that, like other cells, tumor cells such as the B16 melanoma express a multiplicity of extracellular matrix adhesion receptors.

This is the first report that identifies integrin-like complexes that are specific for laminin and collagen in the well-studied B16 melanoma cell system. Neither receptor could be eluted with RGD peptides, indicating that unlike several other integrins, such as the fibronectin receptor, vitronectin receptor, and IIb/IIIa complex, the adhesion of these receptors to their substrates is not mediated through this sequence. Furthermore, YIGSR-NH_2 was also ineffective in eluting the laminin-binding complex (Fig. 3), suggesting that this receptor binds to a site on laminin that is distinct from that of the $\underline{M}_r$ 67,000 receptor. The B16 cells may express additional integrin receptors that bind laminin or type IV collagen. Unfortunately, specific antibodies that react with the α1, α2, or α3 subunits of the β1 family in rodent cells are not readily available.

The laminin-binding receptor complex migrated at $\underline{M}_r$ 140,000 (α) and

120,000 (β) nonreduced. When the samples were run under reducing conditions, the β shifted to an apparent M_r of 150,000 while the α shifted to 115,000. This reversal in electrophoretic mobility was confirmed by two-dimensional gel electrophoresis. The reversal in position of the α and β components is similar to that which occurs in the human laminin-binding integrin complex VLA-6 (Sonnenberg *et al.*, 1988). In fact, anti-VLA-6 monoclonal antibody (GoH3) immunoprecipitated the complex from the fractions recovered from laminin-Sepharose columns and from the whole-cell extract, indicating that this complex is composed of an α6 and a β1 subunit.

The ability of GoH3 monoclonal antibody to substantially block B16 adhesion to laminin substrates establishes that the VLA-6 complex is important in mediating the initial adhesion of cells to this substrate. However, the inhibition was not always complete, implying that other receptors, such as the M_r 67,000 receptor identified by Liotta and collaborators (1986), may also be important in adhesion to this substrate. The M_r 180,000/β_1 collagen-binding complex also appears to bind to laminin, but with low affinity; it may be related to the laminin-binding β1 complex identified on rat neuroblastoma cells by Ignatius and Reichardt (1988), which also had an α chain of M_r 180,000. This complex may be involved in mediating B16 adhesion to laminin. Other work indicates that B16 cells express a surface galactosyltransferase that binds laminin oligosaccharides and can promote cell spreading but is not essential for the initial phase of cell attachment (Runyan *et al.*, 1988).

The original observations of Liotta and collaborators have established the importance of the basement membrane protein laminin in metastatic colonization

(Liotta *et al.*, 1986). Preincubation of murine melanoma cells with intact laminin was found to enhance metastasis (Barsky *et al.*, 1984). In addition, the M_r 67,000 receptor for laminin is reportedly increased on highly metastatic cells (Liotta *et al.*, 1986). Recent studies using synthetic peptides indicate that the laminin-specific amino acid sequence YIGSR (Graf *et al.*, 1987) can elute this receptor from laminin-affinity columns. YIGSR can inhibit lung colonization by B16 tumor cells (Iwamoto *et al.*, 1987), confirming that this particular site in laminin acts as an important substrate during metastasis. On laminin affinity columns, YIGSR failed to elute material at M_r 67,000; presumably this is because this receptor is poorly labeled by the lactoperoxidase mediated ^{125}I-iodination procedure (Kramer *et al.*, 1989). The identification of the VLA-6 complex specific for laminin on these highly metastatic murine B16 cells, and the ability of antibody against this class of receptor to block cell adhesion to laminin, indicates clearly that there are multiple mechanisms for cell interactions with this ligand.

The type IV collagen-binding receptor migrated at M_r 180,000 (α) and 120,000 (β) nonreduced. Upon reduction the β subunit shifted to an apparent M_r of 150,000 while the α subunit remained unchanged. Using affinity chromatography on columns of type I collagen, we recovered a complex with the same α chain as the type IV collagen-binding complex, although it was recovered in minor amounts. Similarly, the receptor complex failed to bind to gelatin-Sepharose columns. This suggests that the collagen-binding complex preferentially binds to type IV collagen and interacts weakly with interstitial collagens. The lower affinity of this receptor for type I

collagen and denatured collagen could account for the poor binding of the B16 cells to these substrates in the adhesion assay (Fig. 1), in which the cells showed a preference for type IV collagen. The collagen-binding complex could not be eluted with GRGDSP, implying that other sequences are involved in this interaction.

The α subunit of the type IV collagen-binding complex is similar in size and electrophoretic behavior after reduction to the α1 subunit of the VLA-1 receptor complex found in humans. The fact that the VLA-1 receptor has also been shown to be a receptor for type IV collagen (Kramer and Marks,1989) suggests that it may be related to the collagen receptor identified in this study. In addition, like the collagen receptor we identified, VLA-1 on human melanoma cells also binds to laminin-Sepharose columns (Kramer *et al.*, unpublished results). Unfortunately, antibodies against the human α1β1 complex that are cross-reactive with the rodent antigen are not yet available; evidence that the M_r 180,000 subunit identified here is related to the human α1 subunit awaits either sequence data or availability of specific antibodies. We conclude that this M_r 180,000 β1 complex binds preferentially to type IV collagen but also exhibits some affinity for laminin. It therefore appears to be a promiscuous receptor, like VLA-3 (α3β1) identified in human cells (Wayner *et al.*, 1988).

Collagens are ubiquitous proteins found not only within the basement membrane but throughout the interstitium. Tumor cells must interact with these proteins during adhesion to and invasion of tissue extracellular matrix. However, type IV collagen is restricted to the basement membrane while collagen types I, III, V, and

VI are associated with the interstitium. Type V collagen, once believed to basement-membrane-specific, is now known to be widespread throughout most interstitial tissues (Fessler and Fessler 1987). During hematogenous colonization, tumor cell emboli must be able to adhere rapidly and firmly to the exposed basement membrane once they penetrate the microvascular endothelium (Liotta *et al.*, 1986). It is reasonable that they would therefore express multiple receptors that could bind strongly to basement-membrane-specific components, such as laminin and type IV collagen. On the other hand, interstitial tumor cells at the primary tumor site must be highly motile in order to reach neighboring vascular beds for subsequent dissemination. Once tumor emboli have arrested in a vascular lumen and penetrated the endothelium and underlying basement membrane, they must again invade the interstitium.

It is now known that stationary cells form stable focal adhesion contacts with the substrate while locomotory cells form weak and transient close contacts (Reviewed in Burridge, 1986). Similarly, integrin adhesion receptors (e.g., the fibronectin receptor) are concentrated in focal contacts when the cells are stationary, but are diffusely distributed in motile cells (Duband *et al.*, 1988). The expression of high-affinity receptors for one substrate (i.e., basement membrane) and low-affinity receptors for another substrate (interstitium) may provide malignant cells with a mechanism to attach firmly to one site yet migrate rapidly on another. On fibronectin, laminin and type IV collagen substrates (Fig. 2) and on amnion basement membrane (not shown), B16-BL6 cells frequently formed focal adhesion contacts. Further work is needed to determine the type of adhesion contacts that these cells form on interstitial collagen, but our current results indicate that this form of adhesion is of the weaker type.

FIGURE LEGENDS

Fig. 3.1. B16-BL6 cell adhesion to extracellular matrix proteins. Cells radiolabeled with 5-[^{125}I]iodo-2'-deoxyuridine were processed as described under "Materials and Methods." Cells were plated onto wells precoated with extracellular matrix proteins at the indicated concentration; BSA (1 mg/ml), gelatin (100 μg/ml), or fibronectin and laminin (both at 10 μg/ml). Cell attachment to laminin was taken as the maximum binding. Data is expressed as the mean of triplicate wells; bars show S.D.

Fig. 3.2. Localization of integrin β_1 complexes in focal adhesion plaques. B16-BL6 melanoma cells were allowed to adhere to laminin-coated (a,b), type IV collagen-coated (c,d) or fibronectin-coated (e,f) coverslips as described under "Materials and Methods." After samples were fixed and permeabilized, they were stained by double immunofluorescence with rabbit polyclonal anti-β1 (a,c,e) and monoclonal anti-vinculin (b,d,f). On both laminin and collagen substrates, β1 complexes (arrowheads) were concentrated in vinculin-positive focal adhesion plaques at the marginal edges of the cell. Bar, 30 μm.

Fig. 3.3. Identification of an integrin laminin receptor. ^{125}I-labeled B16-BL6 cells were solubilized in starting buffer and the extract (lane 1) was applied to a laminin-Sepharose column as described under "Materials and Methods." After a washing with running buffer, the column was eluted with 200 mM NaCl (lanes 2-5), followed by EDTA (lanes 6-8). Fraction in lane 6 was immunoprecipitated with either anti-mouse β1 antibody under nonreduced (lane 9) and reduced (lane 10) conditions, or with anti-hamster β1 antibody under nonreduced (lane 11) and reduced (lane 12) conditions. Laminin-binding material eluted with EDTA was immunoprecipitated with

rat monoclonal antibody GoH3 to α6β1(lane 13, nonreduced, lane 14 reduced). Whole cell extract was immunoprecipitated with monoclonal antibody GoH3 and recognized the α6β1 complex.(lane 15). Elution of laminin-Sepharose column with the YIGSR peptide failed to yield either the α6β1 complex or the M_r 67,000 receptor (lane 16). Using a modified protocol with limited washing of the laminin-Sepharose column, a high-molecular-weight α subunit (M_r ~180,000) was also recovered (lane 17).

Fig. 3.4. Two-dimensional SDS-PAGE analysis of the integrin laminin receptor. A sample of the EDTA-eluted laminin-binding complex was analyzed nonreduced in the first dimension followed by reduction and electrophoresis in the second dimension. The position of the α6 subunit (arrow) and β1 subunit confirms their reversal following reduction with β-mercaptoethanol.

Fig. 3.5. Anti-VLA-6 (α6β1) monoclonal antibody blocks B16-BL6 cell attachment to laminin. Cells radiolabeled with 5-[^{125}I]iodo-2'-deoxyuridine were processed for adhesion to laminin in the presence of various dilutions of hybridoma culture supernatant medium or control medium. GoH3, rat monoclonal antibody against α6β1. AIIB2, rat monoclonal antibody against human β1 subunit that is not reactive with the β1 of murine cells (1:3 dilution). Values are the mean of triplicate wells; bars show S.D.

Fig. 3.6. Anti-VLA-6 does not block cell adhesion to fibronectin or collagen. Cells were radiolabeled with 5-[^{125}I]iodo-2'-deoxyuridine were processed for adhesion to the indicated protein substrates in the presence of GoH3 hybridoma culture supernatant (1:4 dilution) or control culture medium or in culture medium. Values are the mean of triplicate wells; bars show S.D.

Fig. 3.7. Identification of an integrin type IV collagen receptor. B16-BL6

cells surface labeled with ^{125}I were extracted with starting buffer and the extract (lane 1) was applied to a type IV collagen-Sepharose column as described under "Materials and Methods." After a washing with starting buffer, the column was eluted with 200 mM NaCl (lanes 2-4). Next, the column was eluted with 10 mM EDTA (lanes 5-8). Fractions were analyzed by SDS-PAGE (nonreduced). EDTA eluted two discrete bands at M_r ~180,000 and 120,000. Immunoprecipitation of material in lane 6 with anti-β1 antibodies recovered the same components as seen under nonreduced (lane 9) and reduced (lane 10) conditions.

Fig. 3.8. Comparison of fibronectin-, type IV collagen-, and laminin-binding integrin complexes isolated by sequential affinity chromatography. B16-BL6 cells surface-labeled with ^{125}I were extracted with starting buffer and processed for chromatography on the cell binding domain of fibronectin- (lanes 1 and 4), type IV collagen- (lanes 2 and 5), and laminin-Sepharose columns (lanes 3 and 6). EDTA-eluted material from each column was compared by SDS-PAGE under nonreduced (lanes 1-3) and reduced (lanes 4-6) conditions.

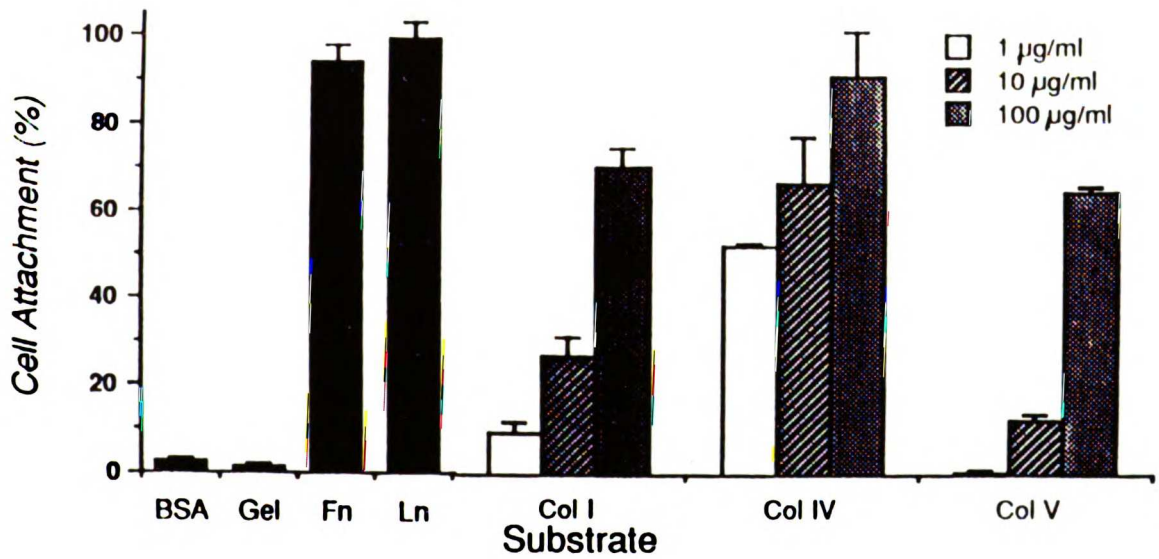

Cell Attachment (%)
100
80
60
40
20
0
BSA
Gel
Fn
Ln
Col I
Col IV
Col V
Substrate
1 µg/ml
10 µg/ml
100 µg/ml

Fig.3.2

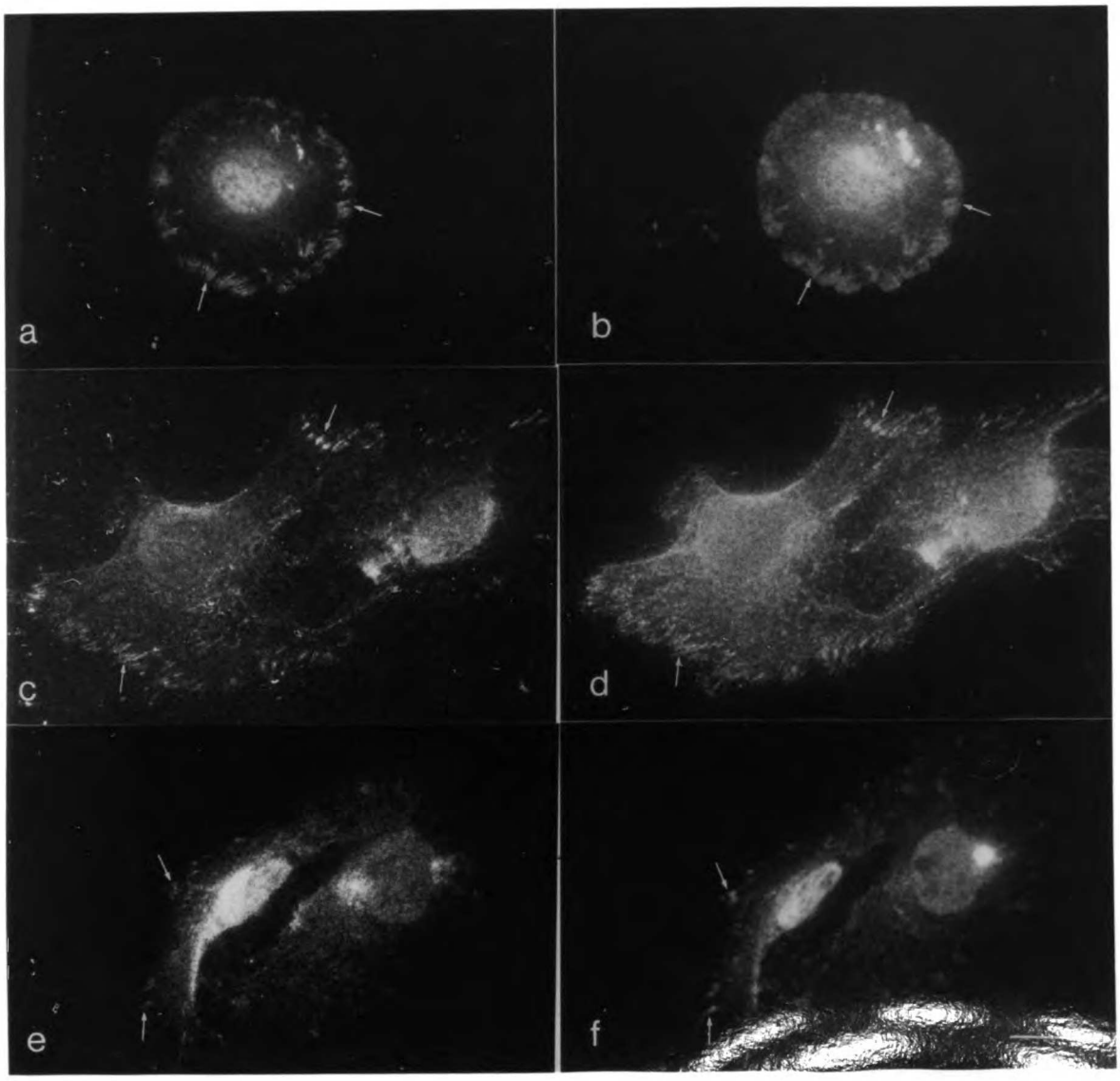
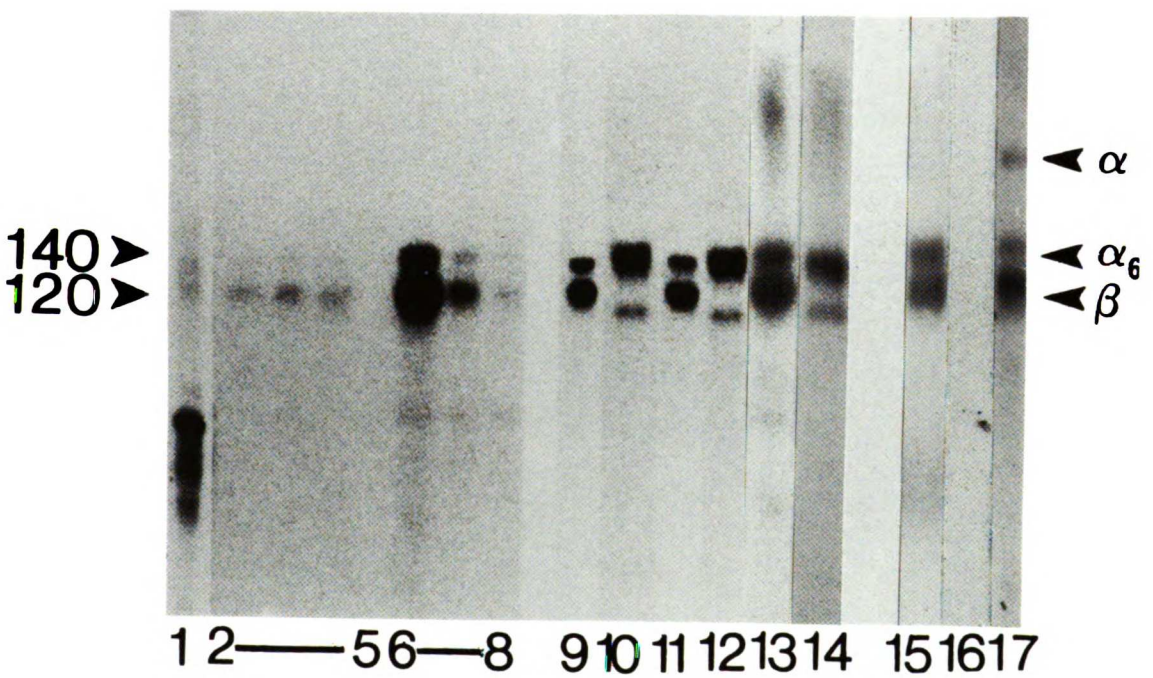


Fig.3.3



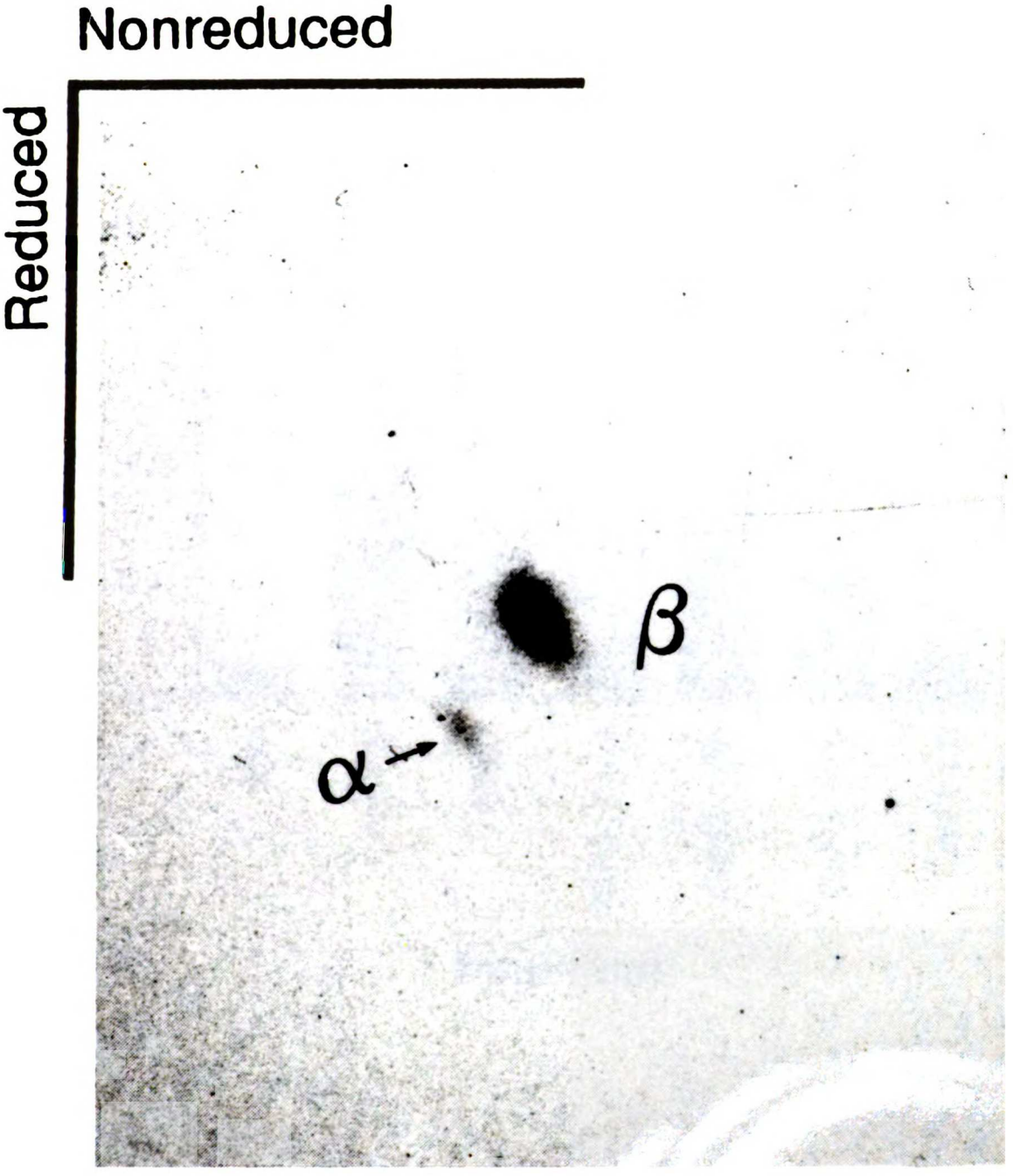

Nonreduced
Reduced
β
α

Fig.3.5

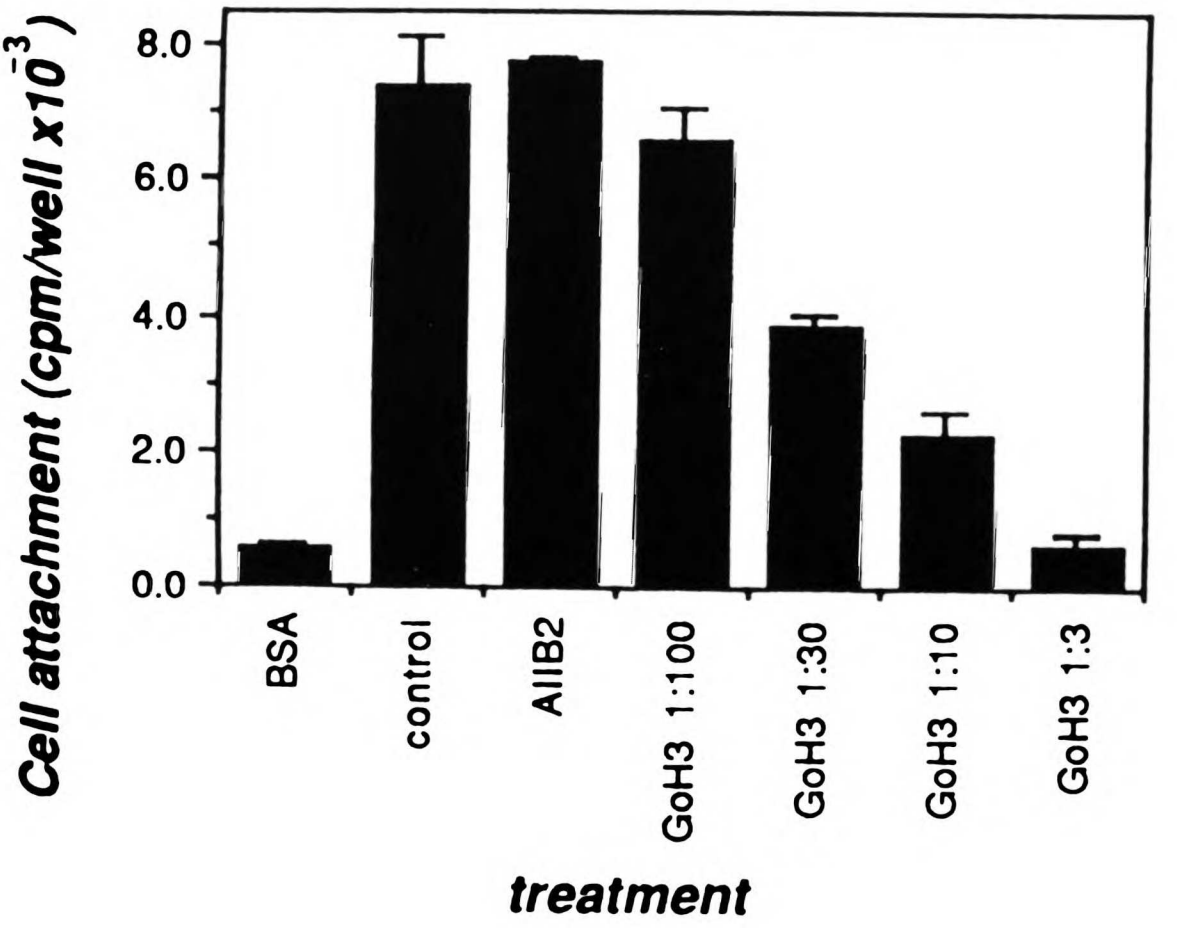
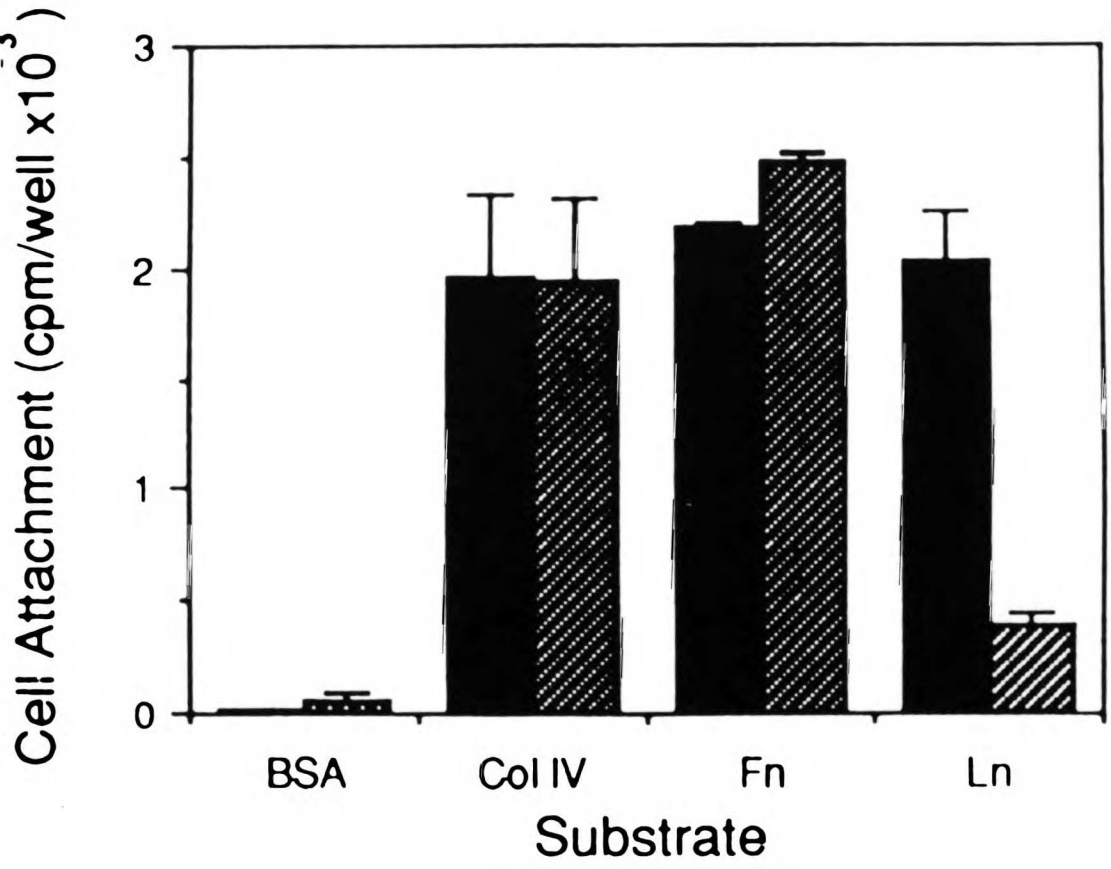
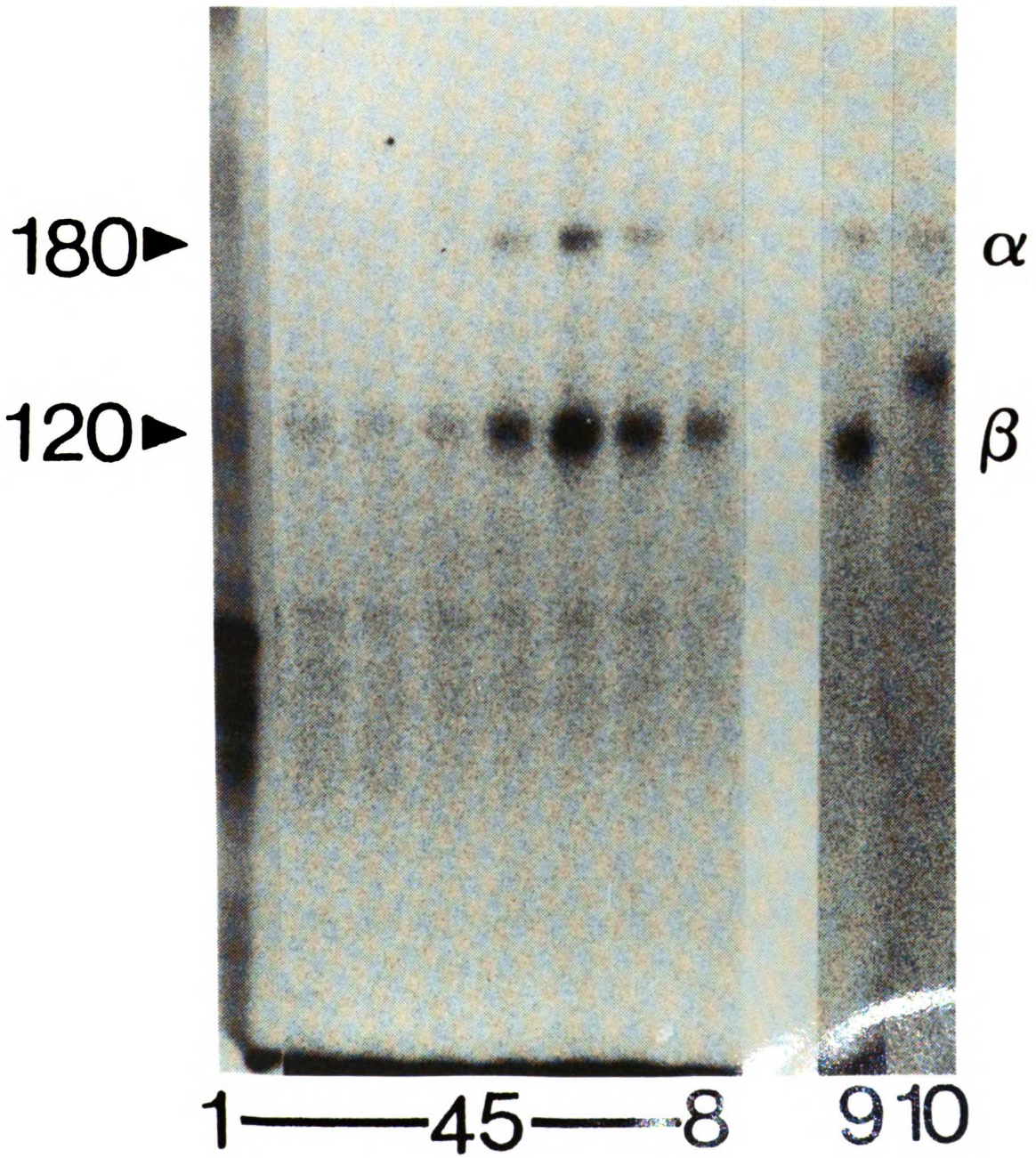
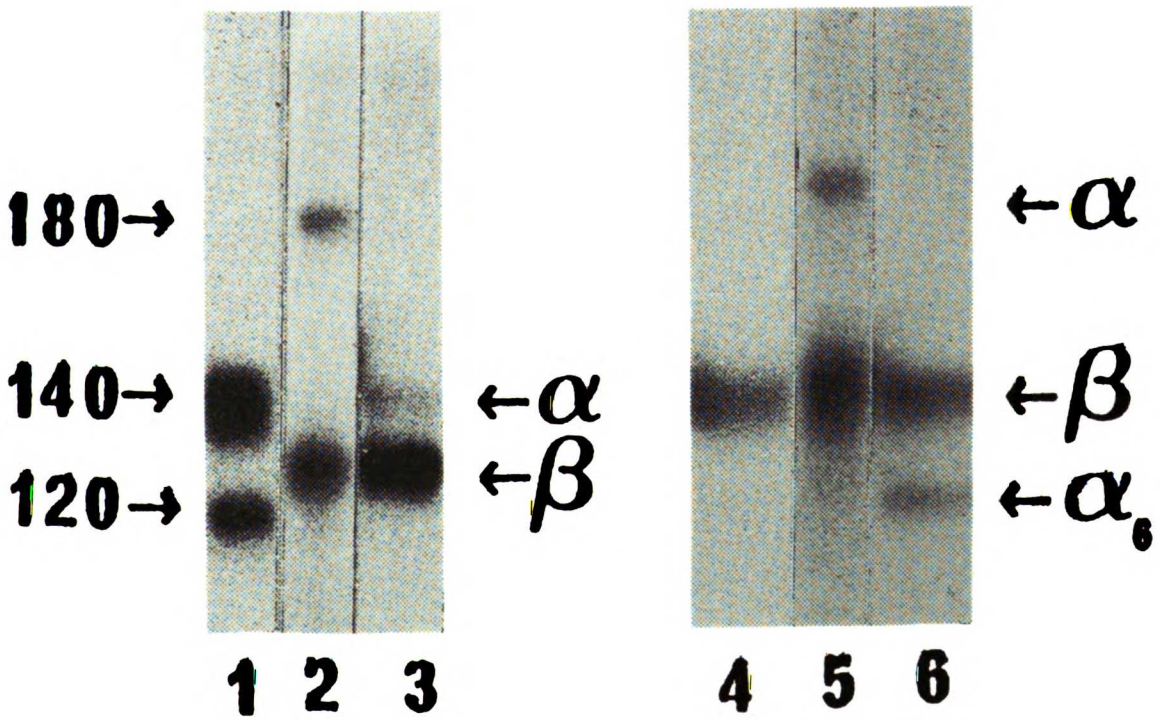

Cell attachment (cpm/well x10^{-3})
8.0
6.0
4.0
2.0
0.0
BSA
control
AIIB2
GoH3 1:100
GoH3 1:30
GoH3 1:10
GoH3 1:3
treatment

Fig.3.6


Cell Attachment (cpm/well x10^{-3})
3
2
1
0
BSA
Col IV
Fn
Ln
Substrate


180►
120►
α
β
1——45——8
9 10


180→
140→
120→
←α
←β
←α
←β
←α6
1 2 3
4 5 6

CHAPTER FOUR

Metastatic Melanoma Cells Interact with the Reticular Fibers of the Lymph Node

ABSTRACT

Murine B16 melanoma sublines have been selected in vivo for enhanced metastasis to lymph nodes. Successive selections from the footpad to para-aortic nodes yielded variant tumor cell lines, including an amelanotic line, with moderately increased potential for lymph node metastasis. The phenotype of the variant cells was distinct from that the parental cells. The lymph node-selected cells had extensive dendritic-like pseudopodial projections and were more motile than the parental cells. In addition, the variant cells were more efficient than the parental cells in attaching to and spreading on preparations of lymph node extracellular matrix. This matrix is composed of an array of reticular fibers containing a core of collagen type III decorated with a basement-membrane-like material rich in laminin and type IV collagen. In adhesion assays, the melanoma cells attached best to laminin, collagen, and fibronectin, and poorly to the interstitial matrix proteins collagen types I and III. This pattern of ligand preference was confirmed in adhesion assays to cryostat tissue sections of amnion where the tumor cells attached to the basement membrane aspect but not the interstitial stromal matrix. Experiments using specific antibodies establish that cell attachment to lymph node reticular fibers was mediated by the β_1 class of integrin receptor complexes. These results indicate that highly motile variant B16 sublines can be selected for distant lymphatic dissemination, and that interaction between invasive tumor cells and nodal reticular fibers may facilitate this metastatic process.

Abbreviations used: ECM, extracellular matrix; DME, Dulbecco's modified minimum essential medium; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; IdUrd, iodo-2'-deoxyuridine; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

INTRODUCTION

The most common mode of initial dissemination of human cancer is through the lymphatic system (Van de Velde *et al.*, 1977). Lymphatic metastasis occurs when malignant cells are released from the primary tumor into the lymphatic vessels and transported to regional lymph nodes. After arrest and growth in the nodes, tumor cells may detach and migrate further, to secondary nodes and then into the bloodstream. Successful blood-borne metastasis involves a series of highly selective processes whereby fewer and fewer tumor cells survive after each step. This may also be true of lymphatic metastasis, in that tumor cells capable of invading the lymphatics and surviving transport to regional nodes represent a small, select fraction of the original population.

During both lymphatic and blood-borne metastasis, malignant cells must interact with various host organ systems that consist of both cellular and extracellular components. The ECM, which can include basement membrane and interstitial stroma, must be traversed by tumor cells as they cross these tissue boundaries. Since the ECM serves as a mechanical barrier that must be penetrated at several stages during the metastatic process, studies of the mechanisms of tumor cell invasion have focused on tumor-ECM interactions. The initial attachment of tumor cells to the matrix involves cell surface receptors that bind specific ECM components such as collagens and matrix glycoproteins (Liotta, 1985, 1991). Various types of receptors may mediate these interactions; currently it is generally accepted that it is the integrin class of receptor complexes that together provide broad ligand specificity for promoting tumor cell adhesion to the diverse set of ECM proteins (Dedhar, 1990; Kramer *et al.*, 1991; Ruoslahti *et al.*,1991). The next step in invasion requires that the

ECM is hydrolyzed by specific proteinases, collagenases and glycosidases expressed on the tumor cell surface or released locally. Dissolution of the ECM is thought to be the rate-limiting step in the exit of tumor cells from the circulation to initiate metastatic colonies. Tumor cells may migrate into the digested region of the ECM under the influence of autocrine or chemotactic factors and attach to the newly exposed matrix. This repeated cycle of attachment, degradation and migration permits extensive tumor cell invasion into neighboring tissues.

Evidence that malignant tumors contain subpopulations of cells with differing metastatic abilities was obtained by Fidler and Kripke (1977). Studying the B16 melanoma, they found that cell populations of both high and low metastatic potential existed within the same parental tumor. This supported earlier work by Nowell (1976), who suggested that tumor cell variants arising within developing tumors are subjected to host selection pressures and give rise to new sublines with increased metastatic potential. There is now evidence that dominance by highly metastatic clones in the primary tumor can enhance eventual metastatic spread of the tumor (Kerbel, 1990).

We used a sequential in vivo selection protocol to obtain variant tumor cell lines from the murine B16-F1 melanoma cells with greater potential to form lymphatic metastases. By amplifying these highly metastatic subpopulations through repeated *in vivo* passaging in mice, we selected for variant cell lines that were more efficient in metastasizing to target lymph nodes. We characterized these site-specific variants with respect to morphology, motility, and adhesiveness in order to identify unique properties that may be important in lymphatic metastasis. A preliminary report describing these studies has been published (Berston *et al.*, 1988).

MATERIALS AND METHODS

Cell culture. The parental B16-F1 melanoma cell line, whose origin and properties have been described (Fidler, 1973), was obtained from Dr. I. J. Fidler (MD Anderson Cancer Center, Houston, TX). Parental B16-F1 and lymph-node-selected variant cell lines were grown as previously (Fidler, 1973). Cells were passaged at preconfluence after a brief treatment with trypsin-EDTA. For most experiments where the selected variants were compared with the parental cell line, the B16-F1 cell line was first passaged as a subcutaneous tumor in C57BL/6 mice and then readapted to culture and used for experiments from passage 5-10.

Selection of B16 lymph-node-derived variants. To select lymph-node-derived variants of the B16-F1 melanoma, we injected tumor cells into the footpads of 6-to 8-week-old female C57BL/6 mice as described (Fidler, 1978). B16 tumor cells were harvested from tissue-culture plates by brief treatment with a trypsin-EDTA solution and washed twice with PBS. After mice were anesthetized by an appropriate injection of sodium pentobarbital, 1 x 10^5 tumor cells in 25 µl of DME were injected into the footpad. At selected times after injection, animals were sacrificed by cervical dislocation and autopsied for the presence of tumors in the regional lymph nodes and other sites. Normally mice were sacrificed at four weeks. In some studies, the hind limb of footpad-injected C57BL/6 mice was amputated at the mid-femur when the primary tumor reached a mean diameter of approximately 3-5 mm (about 14 days). Individual nodes involved with tumor were measured with calipers. The presence of tumor in popliteal, inguinal, and para-aortic nodes was verified either by routine histology or by detection in a bioassay after culturing. Tissue sections were stained with hematoxylin and eosin or for melanin by the method of Becker.

In the initial selection, a single para-aortic lymph node metastases was removed from the mouse, and the node was gently teased apart into fragments, and the recovered cells were introduced into culture and expanded. The cultured cells were reinjected back into the footpads of fresh mice as described above, to obtain the next generation of cells that spread to the para-aortic lymph nodes.

To characterize potential differences in the growth rate of B16 variants, tumor cells were injected subcutaneously into 6- to-8-week-old female C57BL/6 mice as follows. B16 parental and variant cells were harvested from tissue-culture plates by brief treatment with trypsin-EDTA solution. Mice were injected subcutaneously in the hind quarter flank with 5 x 10^5 in 0.1 ml. Tumor growth was assessed at day 14 by determining the geometric mean tumor size.

Migration assay. B16 parental and lymph-node-derived cell lines were seeded at 4 X 10^5 cells/ml/well in DME containing 5% FBS on 12-well tissue-culture plates (Falcon, Lincoln Park, NJ). After 24 hr, monolayer cultures were wounded with a plastic pipette tip, and cell migration into the denuded area was observed at 0, 4, 8, and 16 hr. At these time points, monolayers were fixed with 1% paraformaldehyde in PBS, followed by 70% ethanol for 2 min, and stained for 10 min with 0.2% Coomassie blue in 10% acetic acid.

Adhesion assay. Cell adhesion to various ECM proteins was assessed as previously described (Kramer *et al.*, 1989). Unmodified polystyrene 96-well flat-bottom microtiter plates (Serocluster, Costar, Cambridge, MA) were precoated with purified extracellular matrix proteins. Laminin and type IV collagen were isolated from EHS tumor and fibronectin was isolated from human plasma (Kramer *et al.*, 1989); type I and type III collagen were obtained respectively, from Collagen Corp.,

Palo Alto, CA and Sigma Chemical Co., St. Louis, MO, or BSA as a control in PBS at various concentrations for 1 hr at 37°C in a humidified chamber. Wells were rinsed three times with PBS, and nonspecific adherence was blocked with 1 mg/ml BSA in PBS for 1 hr. Preconfluent cell cultures were labeled with 1 μCi/ml of 5-[^{125}I]IdUrd (ICN) for 24 hr in DME supplemented with 10% FBS. Cells were then resuspended in cold DME with 0.1% BSA and 1 X 10^4 cells was added to each well. The assay was initiated by floating the plate on a 37°C water bath in an 8% CO_2 chamber and incubated for 1 hr. The number of adherent cells was then determined.

Attachment of B16 cells to tissue sections. Mesenteric and axial lymph nodes were removed from normal C57BL/6 mice and rinsed in cold PBS. For cryostat sections, nodes or other tissue (mouse dermis or denuded human amnion [Liotta *et al.*, 1980]) were embedded in gum tragacanth (Sigma), and snap-frozen in liquid nitrogen. Glass slides were prepared by coating with a thin layer of 2% gelatin (Sigma) in distilled water and allowed to dry, and then incubated for 16 hr at 4°C in 4% formaldehyde in PBS, pH 7.2. After extensive washing with distilled water, the slides were allowed to air dry. Cryostat sections of tissue (8-12 μm thick) were placed on the gelatin-coated slides and were then either untreated or extracted with detergents to solubilize the cellular components and expose the extracellular matrix. Sections to be extracted were first treated with hypotonic buffer (10 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) for 5 min, then with a non-ionic detergent solution (0.5% NP-40 in 50 mM Tris-HCl) for another 5 min. Next, they were extracted for 5 min with 0.1% deoxycholate in the same hypotonic buffer. The extracted sections were washed extensively with PBS containing 0.1% BSA and then incubated with 10 μg/ml of DNase (Sigma) plus 1 mM phenylmethylsulfonyl fluoride in PBS containing 0.1%

BSA.

B16 parental and variant cells were radiolabeled with [^{125}I]IdUrd and brought up to a final concentration of 1 X 10^5 cells/ml in DME containing 0.1% BSA and 25 mM HEPES, pH 7.2. Cells (5 X 10^4) were added to wells of a sectional 24-well flat-bottom tissue culture plate (Cluster, Costar), and slides were placed over these wells, with tissue sections face down, and clamped onto the plate with a silicon rubber gasket. The assay was initiated by inverting the assembly and placing the plate on an orbital shaker at 90 rpm at 37°C for a range of time intervals (shear conditions). To terminate the assay, slides were extensively, but gently, washed with DME containing 0.1% BSA. The adherent cells were solubilized with 1 N NaOH/0.1% SDS for 1 hr at 37°C, and radioactivity was measured in a gamma counter.

We also explored the potential role of integrin receptors in mediating B16 cell adhesion to lymph node tissue by testing the effect of anti-integrin antibody. This antiserum, GP-140, a gift of Dr. Caroline Damsky (UCSF), reacts with β_1 integrins and was previously shown to inhibit B16-BL6 cell adhesion to immobilized fibronectin, laminin, and type IV collagen (Kramer *et al.*, 1989; Ramos *et al.*, 1990). We incubated [^{125}I]IdUrd-labeled B16 variant cells in control medium (DME medium containing 1 mg/ml BSA), medium containing normal goat serum or medium containing the GP-140 antiserum for 30 min at 4°C; cell suspensions were then placed over the cryostat sections of isolated lymph node ECM as above; the extent of adhesion was then assessed.

Immunofluorescent staining. We used immunostaining procedures to characterize specific interactions between tumor cells and basement membrane macromolecules contained within the ECM of lymph node or human amnion tissue.

Cryostat sections (10 μm thick) of normal lymph node tissue as well as denuded human amnion were prepared and extracted as described above to produce insoluble extracellular matrices onto which tumor cells were seeded. B16 parental and variant cells were allowed to adhere to tissue sections as described above for lymph node cryostat sections. After washing to remove non-adherent cells, the tissue was fixed with 1% paraformaldehyde for 10 min at 4°C. In certain experiments, cryostat sections of both normal and tumor-involved lymph node were prepared to determine the distribution of reticular fibers and tumor cells. For staining, tissue sections were then incubated for 30 min with 30% normal goat serum in PBS at 37°C to block nonspecific reactions, and stained for 1 hr with an appropriate dilutions of primary rabbit polyclonal anti-laminin, anti-fibronectin, or anti-type IV collagen (Kramer *et al.*, 1988). After removal of the primary antibody and three rinses with PBS plus 0.1% BSA, the secondary antibody (goat anti-rabbit IgG conjugated with either fluorescein or rhodamine) was added at a 1:500 dilution in PBS containing 0.1% BSA for 1 hr. Tissue was rinsed with PBS containing 0.1% BSA, mounted in Fluoromount (Fisher).

RESULTS

Selection of B16 variant cell lines. In initial studies, when parental B16-F1 cells were injected into the footpad of C57BL/6 syngeneic mice, all animals developed primary tumors. While most B16-F1-injected mice showed tumor involvement of the popliteal nodes, usually only a small fraction of the mice exhibited gross metastases to the para-aortic lymph nodes. In a series of sequential selections, cells from the few para-aortic metastases were adapted to culture, expanded, and reinjected into the footpads of additional mice. Again, the few tumor-positive para-aortic lymph node

metastases were obtained and were expanded in culture. This process was repeated and in as few as four sequential selections, we obtained a melanotic variant cell line (B16-PA4M) that exhibited a modest increase in its capacity to metastasize to popliteal, inguinal and para-aortic lymph nodes. Also during the fourth round of selection, we obtained an unusual amelanotic variant (B16-PA4A) from a para-aortic metastasis in a single mouse that had an increased capacity to metastasize to regional nodes. In this mouse, metastases to the popliteal, inguinal, and para-aortic nodes were progressively amelanotic, while the primary tumor remained intensely melanotic. Subsequent studies of the amelanotic cell line isolated from the para-aortic node revealed that it remained essentially amelanotic after culturing and when reinjected into new host mice, produced amelanotic tumors with high efficiency. In contrast to the results with the parent B16-F1 cells, almost all of the mice injected with B16-PA4A and B16-PA4M variant cells developed popliteal metastases, and a majority developed distant para-aortic metastases (Fig. 1). After three more selections, the B16-PA4M cell line produced another melanotic variant (B16-PA7) with a similar metastatic profile (not shown). Comparison of the growth rates of subcutaneously injected parental and variant cells did not show any substantial differences. Thus, when groups of six mice each were injected with equal numbers of tumor cells, at 14 days post-injection the average tumor volumes (mm^3 ± S.D.) were: B16-F1, 1580 ± 750; B16-PA4M, 1575 ± 300; B16-4A, 1930 ± 450.

In another set of experiments, mice were injected with B16-F1 parental or the amelanotic B16-PA4A variant cells and after two weeks, the leg containing the primary tumor was amputated; the animals were followed for up to eight weeks. Interestingly, under these conditions of an extended tumor growth period, the B16-F1

cells frequently produced increased colonization of the para-aortic nodes. Whereas in the earlier selection studies, the footpad-injected B16-F1 cells rarely metastasized to distant nodes, after the extended incubation most of the mice showed tumor involvement in these sites. Gross examination of the B16-F1 lymphatic tumors revealed that in a few mice, there was decreased pigmentation of tumor involved nodes with increased tumor cell dissemination, resulting in predominantly amelanotic para-aortic nodes as in the original PA4A cell line (Fig. 2). Histological examination of para-aortic nodes from these animals revealed that the node itself was often heterogeneous with respect to cell phenotype as evidenced by the presence of amelanotic tumor colonies interspersed with smaller melanotic deposits within the same node (Fig. 3). The B16-PA4D cell line remained amelanotic throughout the eight-week incubation period and produced grossly tumor-involved popliteal, inguinal, para-aortic and, frequently, mesenteric and axial lymph nodes (not shown).

Morphology and motility rates of selected cell lines. The variant PA4M and PA4A cell lines appeared distinct from the parental cell line, not only in metastatic efficiency, but also in their unusual morphology: the B16-F1 parental cells, although pleomorphic, generally formed tight, epithelioid clusters, with many cell-cell contacts. In contrast, the variant cells (B16-PA4M or -PA4A) appeared more dispersed and displayed pronounced pseudopodial projections resembling dendritic processes. (Fig. 4). We next evaluated the motility of the B16 parental and variant cell lines. Monolayers of B16-F1, B16-PA4A, and B16-PA4M cells seeded on tissue culture plastic were wounded with a plastic micropipette tip to form a denuded area about 1 mm wide, and the migratory capacity of cells at the wound edge was observed over time. By 8 hr, both variant cell lines had filled in nearly half of the wound area, and

completely by 16 hr (Fig. 5). This result contrasted with the lesser motility of the parental B16-F1 cells, which had barely entered the wound area after 16 hr (Fig. 5).

B16 cell adhesion to extracellular matrix. To determine the relative adhesive properties of variant tumor cell subpopulations, we compared the ability of the parental and variant cells to adhere to the extracellular matrix proteins fibronectin, laminin, and collagen types I, III, and IV. All three cell lines tested adhered well to the basement-membrane-associated proteins fibronectin, laminin, and type IV collagen (Fig. 6). Interestingly, the parental B16-F1 cell line showed a somewhat greater attachment to most substrates tested than did the variant B16-PA4M and B16-PA4A lines. Tumor cell attachment to interstitial associated collagen types I and III, was minimal under these conditions for all cell lines; adhesion to BSA and gelatin was also negligible. Similar adhesion profiles were obtained when the assays was performed under high shear conditions (not shown).

We next compared the ability of parental and variant cell lines to adhere to preparations of lymph node extracellular matrices. The variant B16-PA4A cells were more efficient than the parental cells in attaching to these matrices (Fig. 7). With increasing time of incubation, the greater the difference in the extent of adhesion between the two cell lines. Furthermore, the parental and the PA4A variant cells showed significantly greater adherence to exposed ECM prepared from cryostat sections of lymph node tissue than to unextracted tissue (Fig. 8). The PA4A cells adhered with greater efficiency than the parent cells to the unextracted lymph node. To determine the relative adhesion potential of tumor cells for lymph node ECM, we also performed adhesion assays on interstitial dermis connective tissue. B16-F1 and B16-PA4A cells both exhibited relatively poor attachment to cryostat sections of

collagen type I- and III-rich mouse dermis (Fig. 8).

We previously established that the lymph node reticular fibers are composed of a core of types I and III collagen decorated with basement membrane macromolecules, including laminin and type IV collagen (Kramer *et al.*, 1988). Immunofluorescence staining for reticular fiber associated laminin demonstrated a good correspondence between attachment of tumor cells and location of the fibers within the lymph node (Fig. 9). Scoring of adherent cells indicated that more than 75% were present directly over lymph node reticular fibers. In unextracted node, where adhesion was less efficient, cell attachment was also concentrated at matrix fibers (not shown). Melanoma cells adherent to the reticular fibers appeared to spread along the axis of the fibers (Fig. 9C,D).

We next assessed cell attachment to cryostat sections of human amnion which is known to contain both an epithelial basement membrane (containing type IV collagen, laminin, entactin/nidogen, and heparan sulfate proteoglycan) and an underlying interstitial stroma (containing collagen types I, III, V) (Amenta *et al.*, 1986). After removal of the amniotic cells, the tissue was cut in cross-section to provide access to both the basement membrane and the stromal matrix. Immunofluorescent staining of sections of amnion with antibodies to laminin confirmed the preferential binding of B16 tumor cells with the basement membrane aspect and not with the stromal regions containing interstitial collagens (Fig. 10).

Taken together, these results indicate that tumor cells adhere predominantly to the basement-membrane-containing reticular fibers and not to collagen types I and III, which compose the core of these fibers. Since the B16 cell lines appeared to adhere preferentially to the lymph node reticular fibers, we examined the distribution of the

laminin-rich fibers in established nodal metastases. Immunofluorescence microscopy of para-aortic nodes infiltrated with B16-PA4M cells revealed that the melanotic tumor cells were arranged in close apposition with the reticular fibers (Fig. 11A). The distribution and density of reticular fibers present in adjacent and apparently normal regions of the same lymph node were similar to that of the tumor-involved region of the node (Fig. 11B).

To determine the mechanism by which B16 cells adhere to lymph node ECM, we evaluated the potential role of integrin adhesion receptors in mediating this adhesion, using antiserum to integrin receptor complexes. A goat anti-β1 integrin anti-serum (GP-140) against rodent-derived β1-integrins (Knudsen *et al.*, 1981) was tested. The metastatic B16-PA4A cells pre-incubated in the presence of increasing concentrations of β1 antibodies were effectively inhibited from attaching to lymph node extracellular matrix (Fig. 12). Even a 1:500 dilution of the antibody produced more than 50% inhibition of tumor cell adhesion to nodal matrix. A 1:100 dilution of the antibody was about 90% effective in inhibiting adhesion to lymph node. Control, non-immune goat serum had no significant effect on adhesion to the ECM preparations at all concentrations tested (not shown).

DISCUSSION

The B16 mouse melanoma has been used extensively as a model to study preferential metastasis to organ-specific sites. From the B16 melanoma, tumor cell subpopulations have been selected that preferentially metastasize to lungs (Fidler, 1973), liver (Tao *et al.*, 1979), ovary (Brunson *et al.*,1979) and brain (Miner *et al.*, 1982; Schackert and Fidler, 1988) as well as other sites. In our studies, we selected

and characterized variant tumor cell subpopulations from the parental B16-F1 tumor that are more efficient in metastasizing to target lymph nodes. The cell lines obtained after just four in vivo passages exhibited an increased affinity for lymph node in vivo and in vitro. These findings paralleled work by various groups (Nicolson and Winkelhake, 1975; Netland *et al.*,1984; Schackert and Fidler, 1988; Miner *et al.*, 1982) in which tumor cells were found to bind preferentially to organ tissue, to which they also metastasized in vivo after appropriate selections.

The results indicate that within the B16 parental cell line there are unique subpopulations of cells that are efficient in forming lymph node metastases. The amelanotic cell line derived by para-aortic lymph node metastases appears to represent a clonal cell population. It now accepted that within the primary tumor cell population there are preexisting subsets of cells that have differing degrees of metastatic potential (Fidler, 1978). Although the B16 variants selected here appeared to disseminate to regional lymph nodes at a greater frequency than the parental cell line, the subcutaneous growth rate of these three cell lines did not differ significantly. Nicolson (1986) suggested that tissue-specific factors present in unique organ environments may stimulate or inhibit growth of tumor cells. This concept of organ-regulated tumor cell growth relates back to the original "seed and soil" hypothesis proposed by Paget (1889). Thus, it is possible that differences in proliferation of the B16 variants are only observed in the unique milieu provided by the lymph node. This would be consistent with the concept that highly metastatic subpopulations can be preferentially amplified during initial tumor growth at the primary site (Kerbel, 1990). This dominance in the case of the B16 cells appears to occur in the environment of the lymph node. For example, macrophages or other nodal cells may be stimulated to

produce a variety of growth factors during tumor cell invasion, and thus enhance tumor cell growth in this environment. Alternatively, the relatively high density of basement membrane components encasing the reticular fibers (Karttunen *et al.*, 1986;1989; Kramer *et al.*, 1988) may act to enhance tumor cell survival and growth at this site. In the present study it seems likely that a specific subclone of the melanoma cells, the amelanotic cells, frequently undergo rapid proliferation in the lymph node and may displace the majority of slower growing melanotic cells. Whether this is also occurring at the primary tumor site is not known.

Both the parental and selected cell lines adhered with good efficiency to the basement membrane-specific macromolecules laminin and type IV collagen as well as fibronectin. The presence of integrin receptors on B16 cells for fibronectin (Kramer *et al.*, 1989), laminin (Kramer *et al.*, 1989; Ramos *et al.*, 1990), and type IV collagen (Kramer *et al.*, 1989) has been documented. In contrast, attachment to collagen types I and III was minimal for all cell lines. This finding parallels our results from adhesion assays with B16 cells on dermis and human amnion, in which cell attachment to the interstitial stromal components of the tissue (rich in collagen types I, III, and V) was minimal. Conversely, we observed significant cell adhesion to basement-membrane-rich regions of the human amnion. This provides further evidence that basement membrane components on the outer surface of nodal reticular fibers (Kramer *et al.*, 1988) may aid in tumor cell arrest during lymphatic metastasis. It is possible that the organ-specific structure of the lymph node extracellular matrix is required for preferential attachment of the B16 variants. Since it has been established that molecules contained within an organ's extracellular matrix may influence tumor cell

attachment and growth properties (Nicolson *et al.*, 1985), it is possible that lymph node ECM contains unique adhesive ligands that play a role in organ-specific metastasis.

Preferential attachment of tumor cells to organ-specific components has been suggested experimentally by others (Fidler, 1978). Nicolson (1986) found that high-lung-colonizing B16-BL6 cells preferentially adhered to target organ suspensions of lung tissue rather than heart or ovary tissue. Furthermore, Nicolson *et al* (1975) obtained specific adhesion of organ-selected tumor cells to cell suspensions of target organs such as lung, liver and ovary. Brodt (1989) found that the ability of tumor cells to bind to cryostat sections of lymph node tissue in vitro correlated well with their ability to metastasize in vivo. Apparently, in this system tumor cells interact with the cellular components of the lymph node rather than the extracellular matrix. However, using a Lewis lung carcinoma cell line and two variants that differed in their ability to metastasize to lung and liver, Chung *et al.* (1988) found marked differences in their abilities to adhere to defined ECM macromolecules.

It is well established that cell-surface receptors for specific extracellular matrix adhesion macromolecules, such as collagens, fibronectin and laminin, are important during cell migration and invasion (Liotta, 1991). Numerous investigators have used specific antibodies to receptor molecules to establish the role of these molecules in cell adhesion to purified extracellular matrix proteins. We used this approach to evaluate the attachment of the B16 melanoma cell line to isolated nodal reticular fibers. That tumor cell attachment to lymph node ECM was abolished by anti-integrin antibodies implies that this type of receptor mediates this adhesion. In future studies it will be important to identify which specific integrin complexes (and their respective ligands)

FIGURE LEGENDS

Fig. 4.1. Relative metastatic potential of parental B16-F1 and variant B16-PA4M and B16-PA4A.D melanoma cell lines for regional lymph nodes. The melanotic B16-PA4M and amelanotic B16-PA4A cell lines were derived as described in Material and Methods after four sequential *in vivo* selections in which the primary tumor metastasized to para-aortic lymph nodes. Mice, 10 in each group, were injected with 1 X 10^5 cells of each cell line in the hind footpad. Four weeks later, the animals were autopsied and scored for the presence of primary tumor and nodal metastases at the indicated sites.

Fig. 4.2. Lymph node metastasis by parental B16-F1 cells. Example of a mouse with widespread dissemination of melanoma cells to lymph nodes after extended incubation time. Mice were injected with the parental B16-F1 cells in the hind footpad as described in Materials and Methods. At two weeks, when the primary tumor reached a mean diameter of approximately 3-5 mm, the hind limb was amputated. Mice were sacrificed eight weeks later and autopsied. This B16-F1-injected mouse shows tumor involvement in the popliteal (p), inguinal (i) and para-aortic (pa) nodes. Note the apparent heterogenous tumor cell population of melanotic (black arrows) and amelanotic (white arrows) tumor cells within the para-aortic node (inlay).

Fig. 4.3. Histology of tumor-involved lymph node from B16-F1 injected mice. Para-aortic nodes from several mice injected in the footpad with B16-F1 as described in Fig. 2 were processed for histological examination. (A) Becker's melanin staining of a para-aortic lymph node confirms populations of melanotic tumor cells that have

invaded the interior of the node. (B) At an adjacent region of the same para-aortic lymph node shown in (A), large areas of amelanotic tumor cells are present; this indicates that amelanotic tumor cells derived from a predominately melanotic primary tumor can co-exist within the node and apparently have been preferentially expanded in number. (C) Para-aortic lymph nodes from five mice were fixed, embedded in paraffin, and sectioned. The paraffin block (unstained) was then photographed by transmitted light under low magnification to show the cross-sectional regions of tumor-involved para-aortic lymph nodes. These nodes represent the spectrum of amelanotic to melanotic colonies observed within these nodes. Note the heterogenous and focal populations of melanotic and amelanotic tumor cells in individual nodes, while other nodes appear primarily amelanotic or melanotic.

Fig. 4.4. Phase contrast microscopy of (A) B16-F1 parental cells and (B) B16-PA4A (amelanotic) and (C) B16-PA4M (melanotic) lymph-node-selected cells. Cells were seeded on plastic culture dishes and incubated for 48 hrs. Note the compacted morphology of the parental cell line as compared with the highly dendritic-like processes (arrows) and dispersed morphology of the variant cell lines.

Fig. 4.5. Migration of B16 parental and variant cells. B16-F1 parental or the variant cells (PA4M and PA4A) cells were seeded on culture plates as described in Materials and Methods. After 12 hrs, strips of the confluent cell monolayers were denuded with a plastic tip (1 mm diameter). At selected time intervals (A = time zero, B = 4 hours, C = 8 hours, D = 16 hours), plates were fixed, stained, and photographed.

Fig. 4.6. Adhesion of B16 cell lines to extracellular matrix proteins. [^{125}I]IdUrd-labeled cells were added culture wells precoated with the indicated ECM

proteins, and after 1 hr of incubation (under non-shear conditions) the number of adherent cells was determined. Values are the mean of triplicate wells; error bars show standard deviation. Bovine serum albumin, BSA; gelatin, Gel; type I collagen, type I Col; type III collagen, type III Col; type IV collagen, type IV Col; laminin, LN; and fibronectin, FN. Similar results were obtained in five separate experiments. Solid bar, B16-F1, stippled bar, B16-PA4M, open bar, B16-PA4A.

Fig. 4.7. Adhesion of B16 parental and variant cells to lymph node extracellular matrix. Cryostat sections of mouse lymph node were extracted as described in Materials and Methods to selectively solubilize the cellular components and expose the extracellular matrix. Radiolabeled tumor cells were allowed to bind to the tissue selections for the indicated times under shear conditions and adhesion was assessed as described in Materials and Methods. Each value is the mean of triplicate sections; error bars show standard deviation. Similar results were obtained for eight separate experiments. Solid bar, B16-F1; stippled bar, B16-PA4A.

Fig. 4.8. Attachment of B16 parental and variant cells to tissue sections of dermis or lymph node. Cryostat sections were either untreated (dermis or node) or were extracted to expose the extracellular matrix (node ECM). [^{125}I]IdUrd-labeled cells were seeded onto the sections, incubated under shear conditions for 1 h, and the number of adherent cells was determined as described in Materials and Methods. Gelatin coated substrates were used as a control for tumor cell adhesion. Note the poor affinity of B16 cells to the collagen type I- and III-rich dermis. Values are mean of triplicate sections; error bars show standard deviation.

Fig. 4.9. Localization of adherent tumor cell to lymph node reticular fibers. Cryostat sections of lymph node were prepared and extracted to produce the insoluble

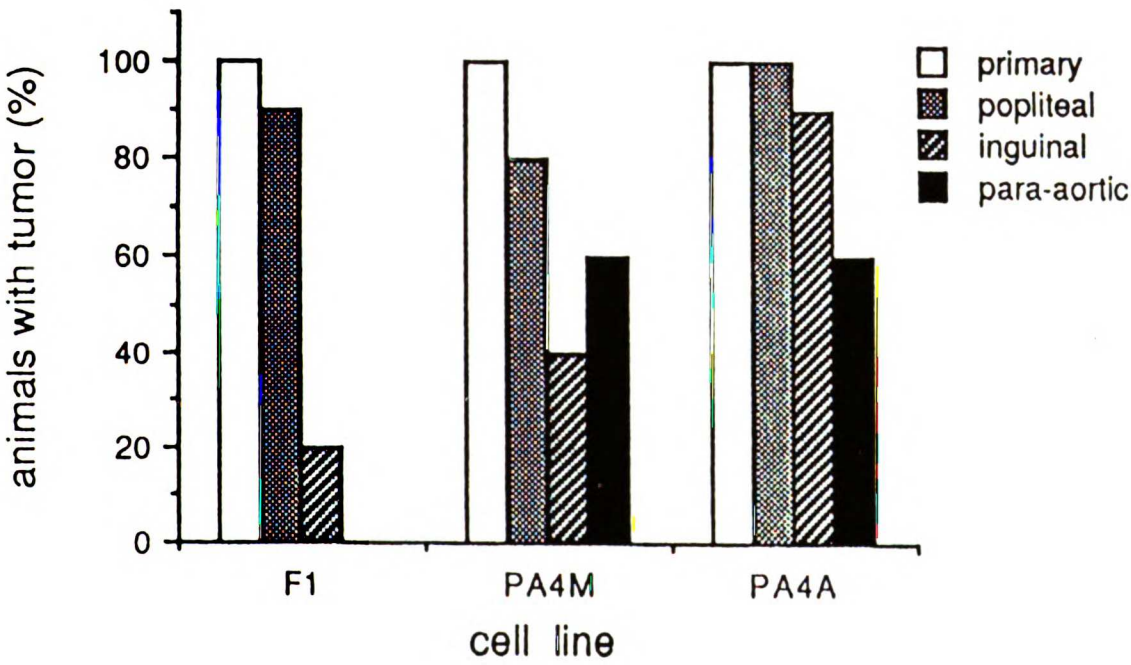
extracellular matrices on which B16-PA4A cells were seeded and incubated for 30 min. Immunofluorescent microscopy for type IV collagen (A) coupled with phase-contrast microscopy (B) reveals a good correspondence between the attachment of tumor cells (circles) and the co-localization of reticular fibers. Sections stained with Giemsa show extensive adhesion of tumor cells to the nodal reticular fibers at high (C) and low (D) magnification (reticular fibers are visible as dark linear arrays).

Fig. 4.10. Adhesion variant melanoma cells to amnion extracellular matrix. Human amnion was denuded of epithelial cells and cryostat sections perpendicular to the plane of the membrane sheet were then prepared. Tumor cells (B16-PA4A) were allowed to attach for 1 hour to the tissue sections (under shear conditions), and then fixed and stained. (A) Phase-contrast optics of the same region in (A) showing the location of adherent tumor cells (stars) at the basement-membrane zone of the amnion, but not at the adjacent stromal matrix; (B) Immunofluorescent staining of amnion sections with antibodies to laminin reveals basement-membrane-rich regions of the tissue; (C) A lower magnification by phase-contrast of the amnion with tumor cells (arrows) preferentially binding to the basement membrane zone of the amnion.

Fig. 4.11. Co-localization of reticular fibers with B16 tumor cells. Para-aortic nodes were removed from mice after footpad injection with B16-PA4M (at 4 weeks), sectioned, and stained with anti-laminin antibodies. More than half of the enlarged lymph node was infiltrated with the melanotic B16 tumor cells (A,B), which are visible in the phase-contrast micrograph (A) as large cells, usually containing numerous melanin granules. The distribution and density of the reticular fibers as detected by immunofluorescence staining with antibodies to laminin (B), was similar to that found in the remaining portion of lymph node lacking tumor cell deposits

(C,D), as seen by phase-contrast microscopy (C) and by staining with anti-laminin antibodies (D).

Fig. 4.12. Inhibition of B16 cell adhesion to lymph node extracellular matrix with anti-integrin antibody. Radiolabeled B16-PA4A cells were plated onto preparations of lymph node matrix in the presence of increasing concentrations show as dilution of anti serum of anti-β1 integrin antibodies as described in Materials and Methods. After incubation for 30 min (under shear conditions), the number of adherent cells were then determined. Values represent the mean of triplicate wells; error bars show standard deviation.


animals with tumor (%)
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60
40
20
0
F1
PA4M
PA4A
cell line
primary
popliteal
inguinal
para-aortic

Fig.4.2

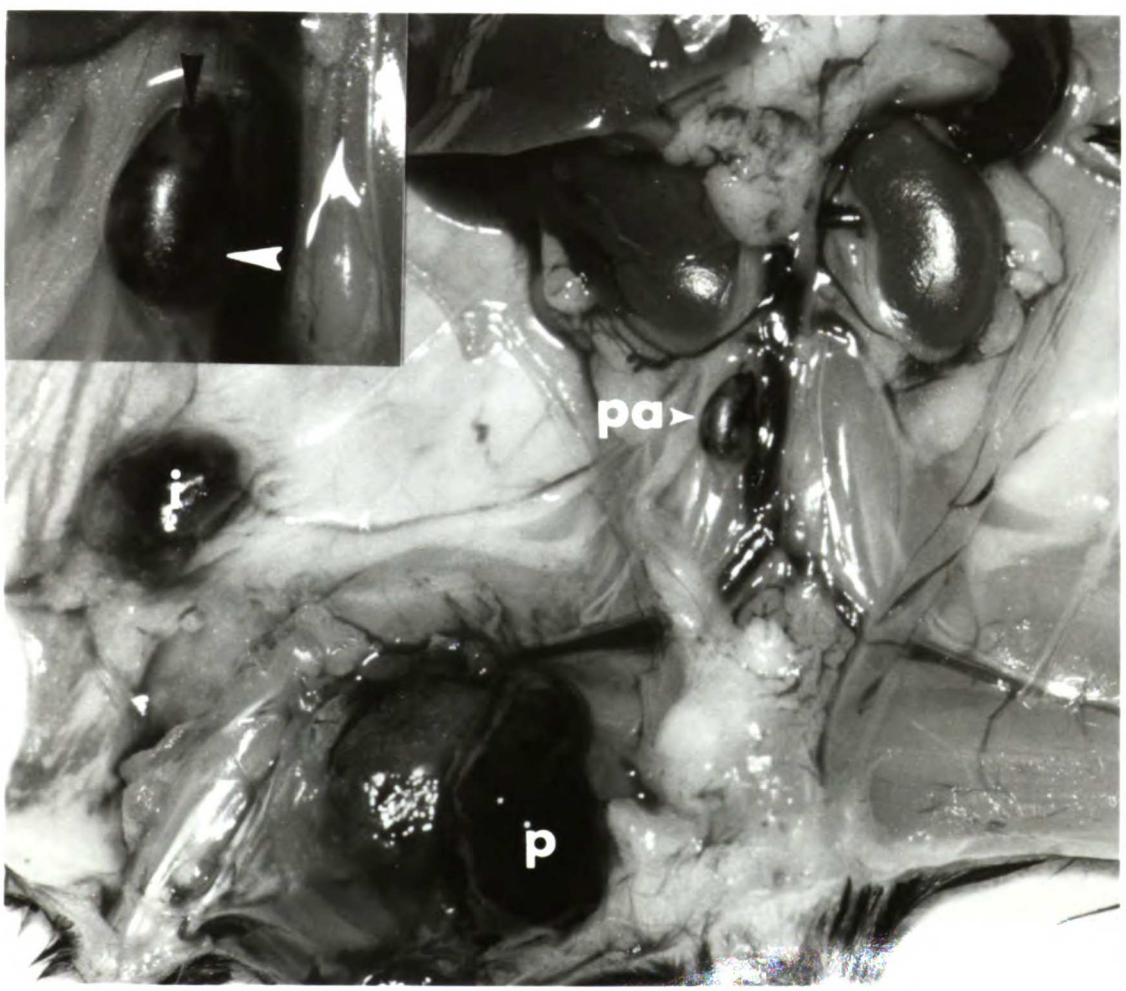


Fig.4 . 3

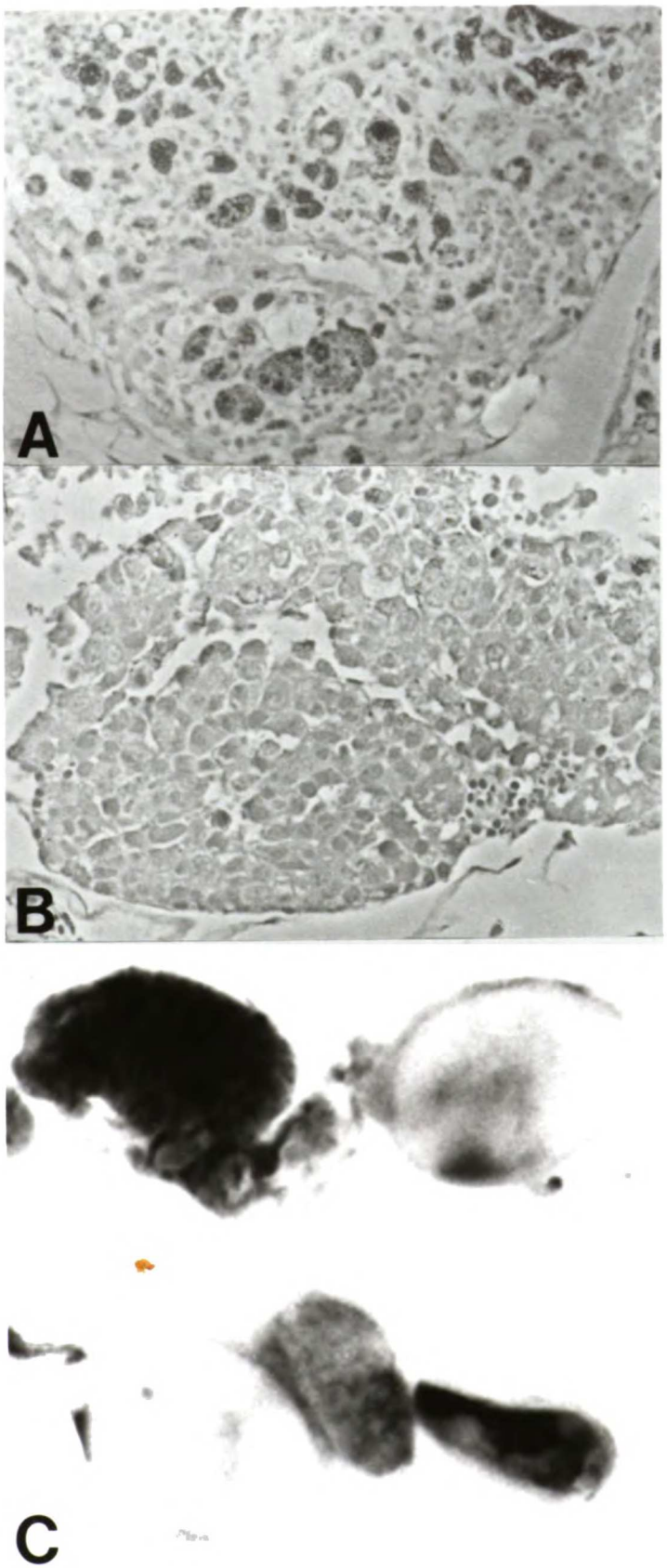
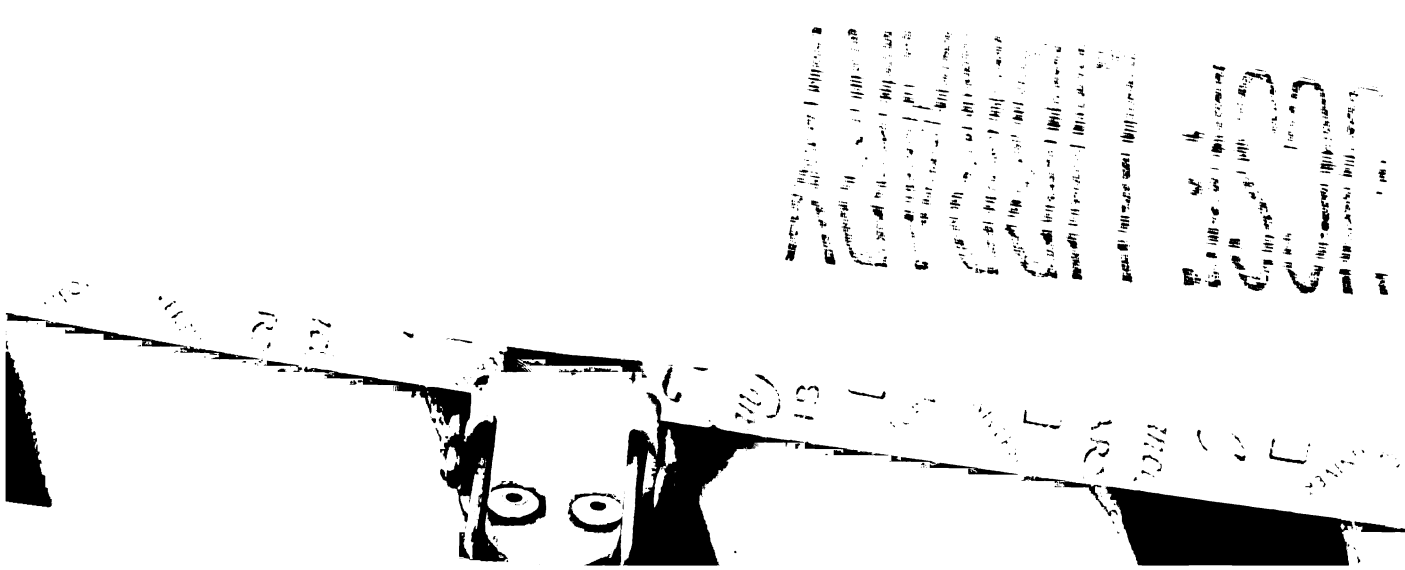


Fig.4 - 4



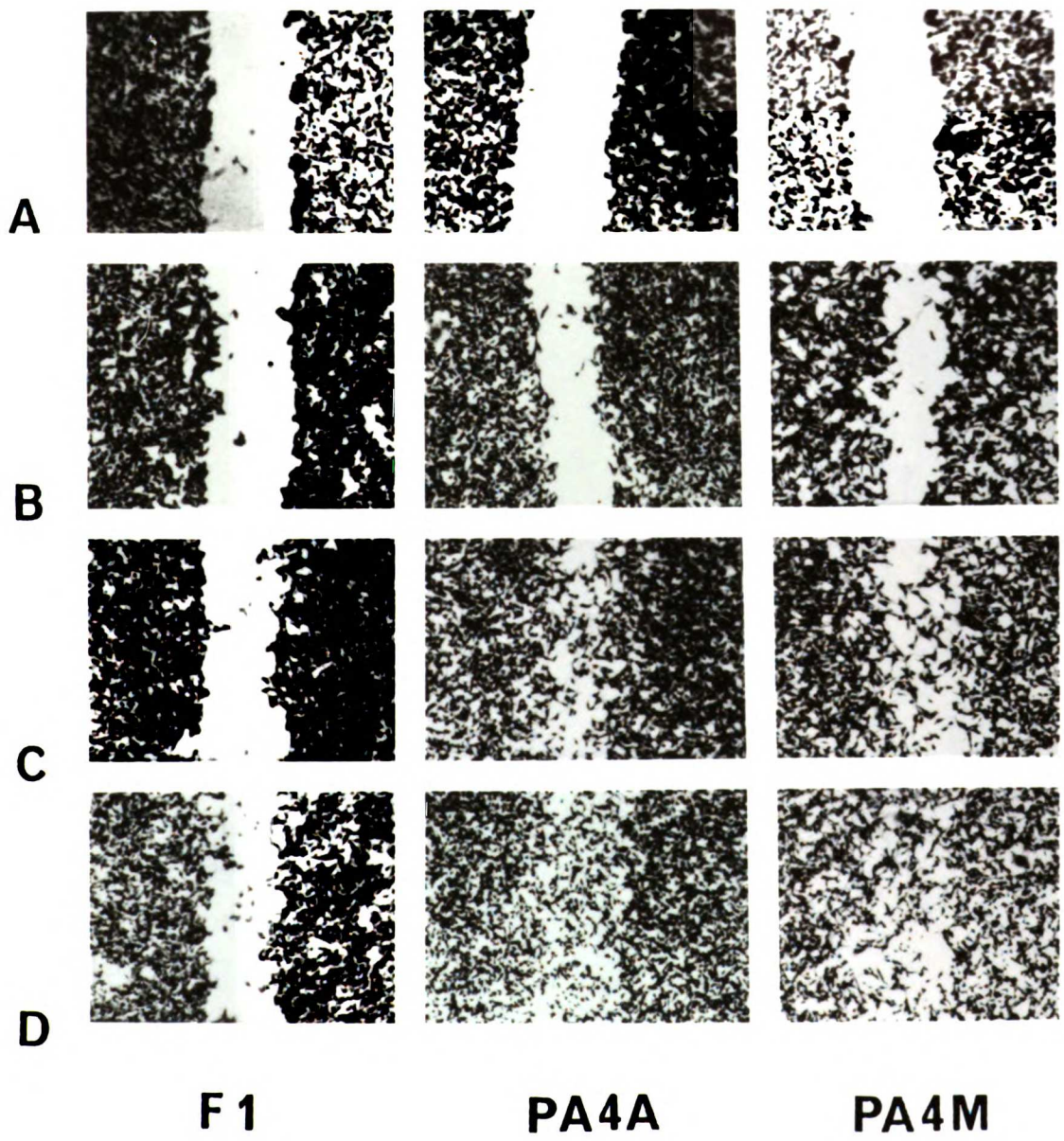
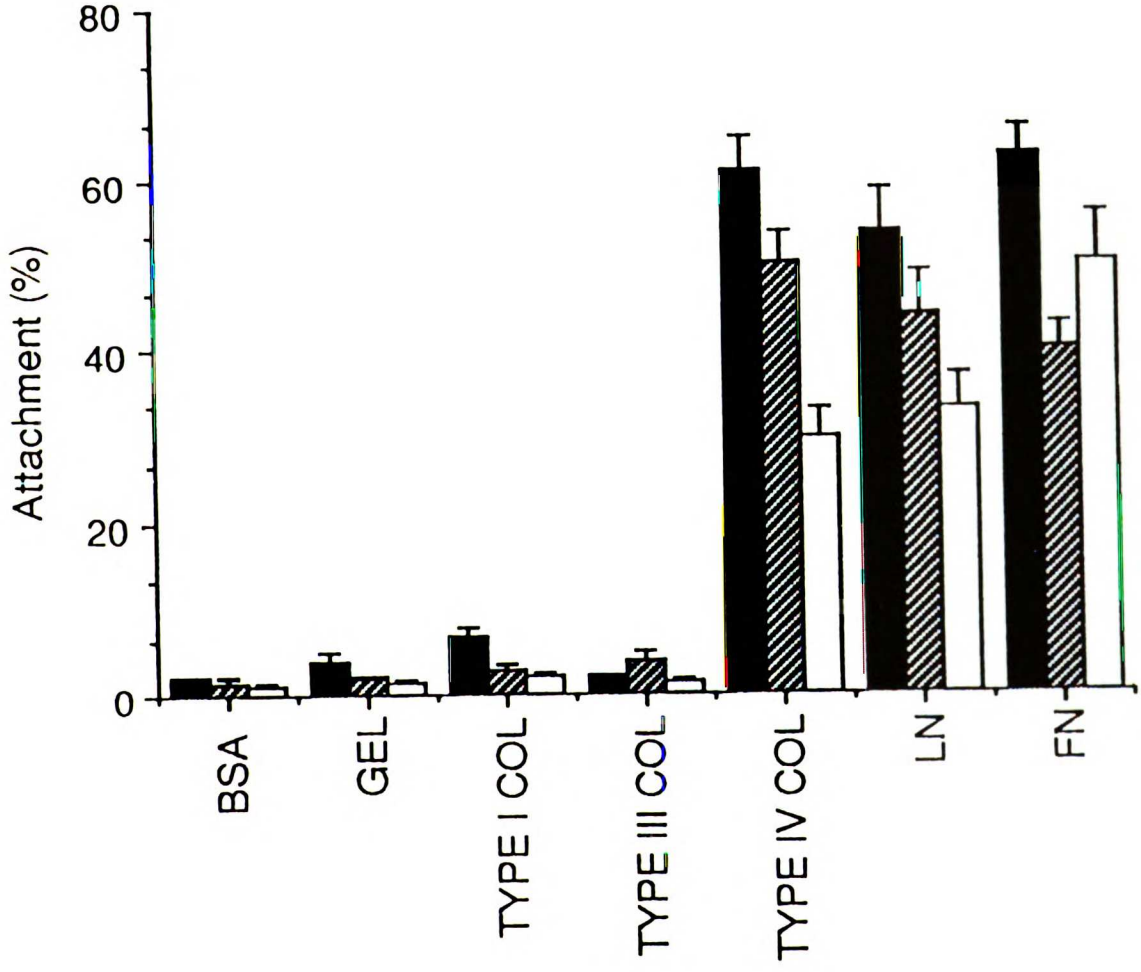
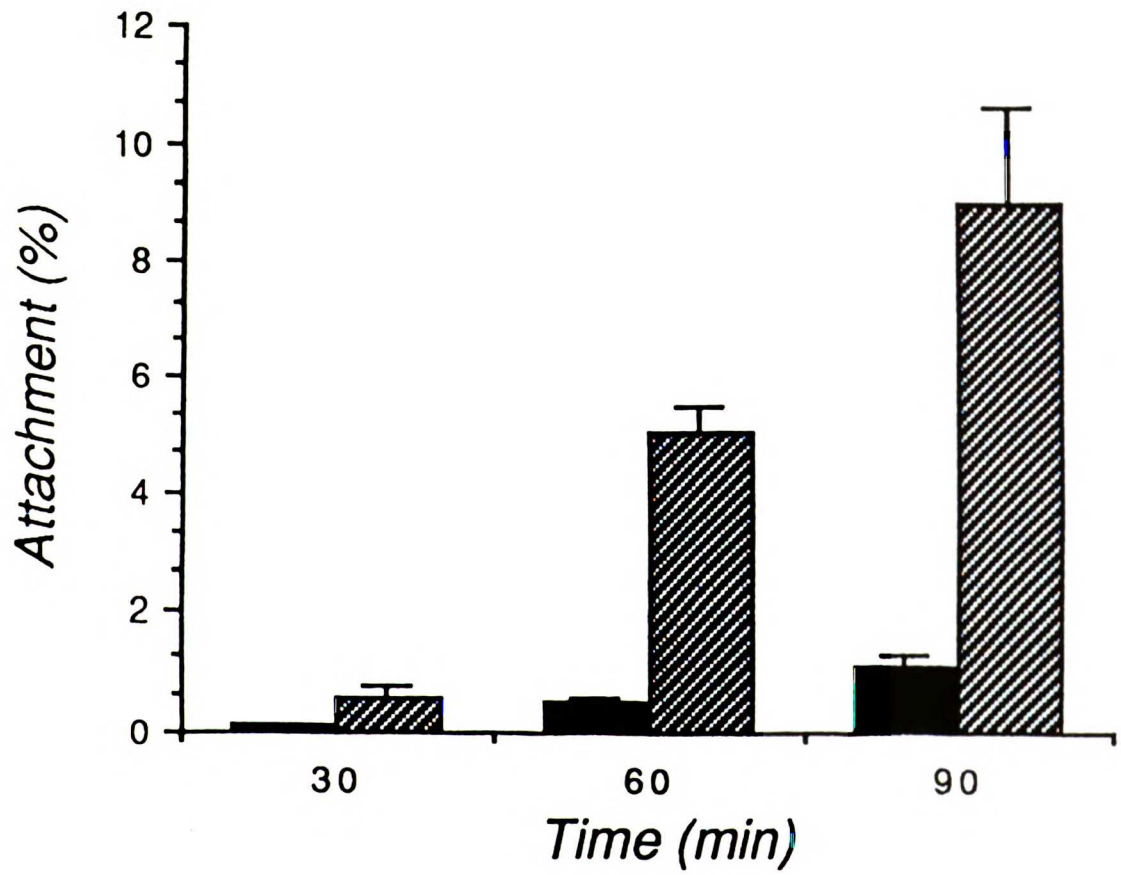

A
B
C
D
F1
PA4A
PA4M

Fig.4.6


80
60
40
20
0
Attachment (%)
BSA
GEL
TYPE I COL
TYPE III COL
TYPE IV COL
LN
FN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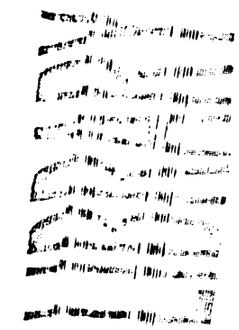
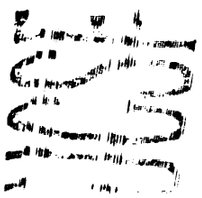
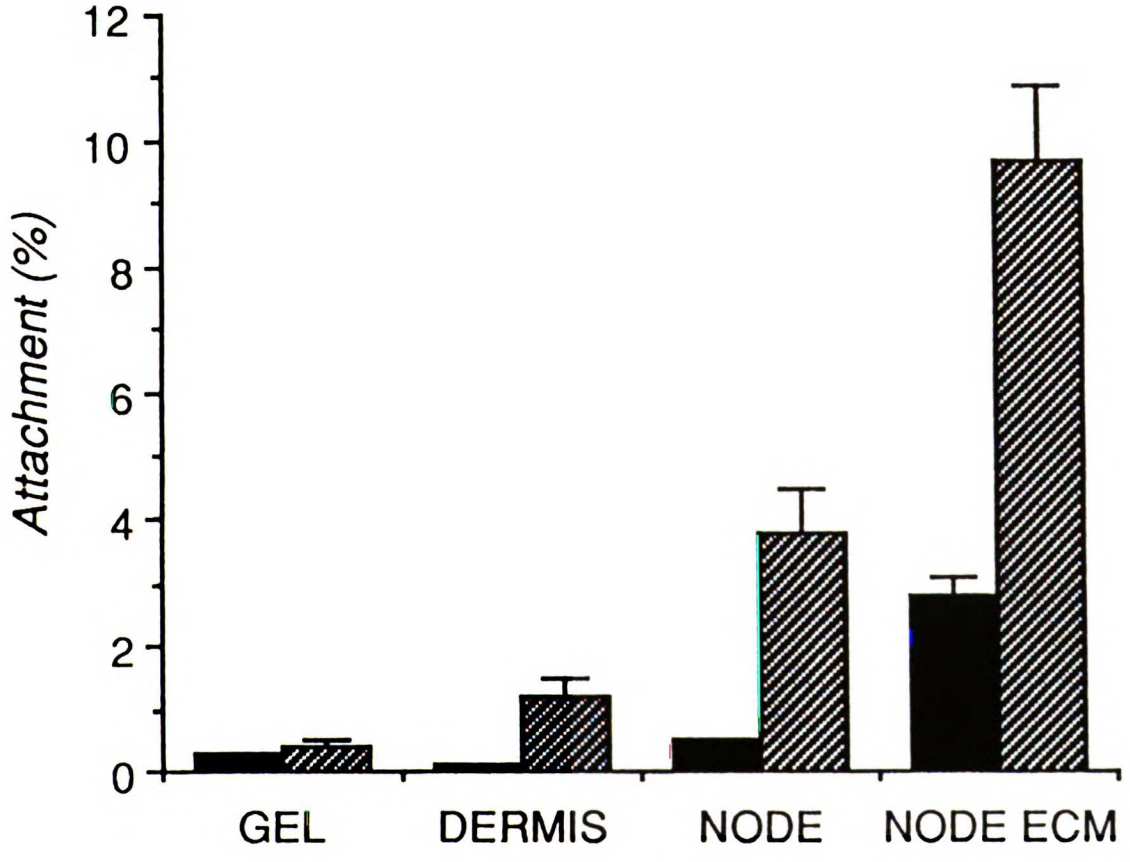
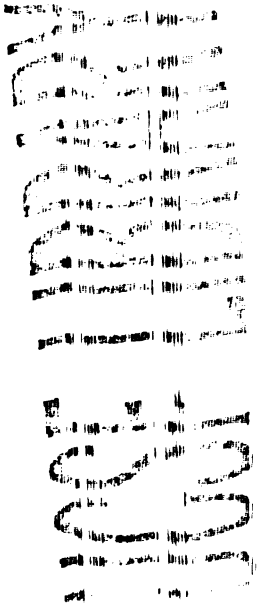
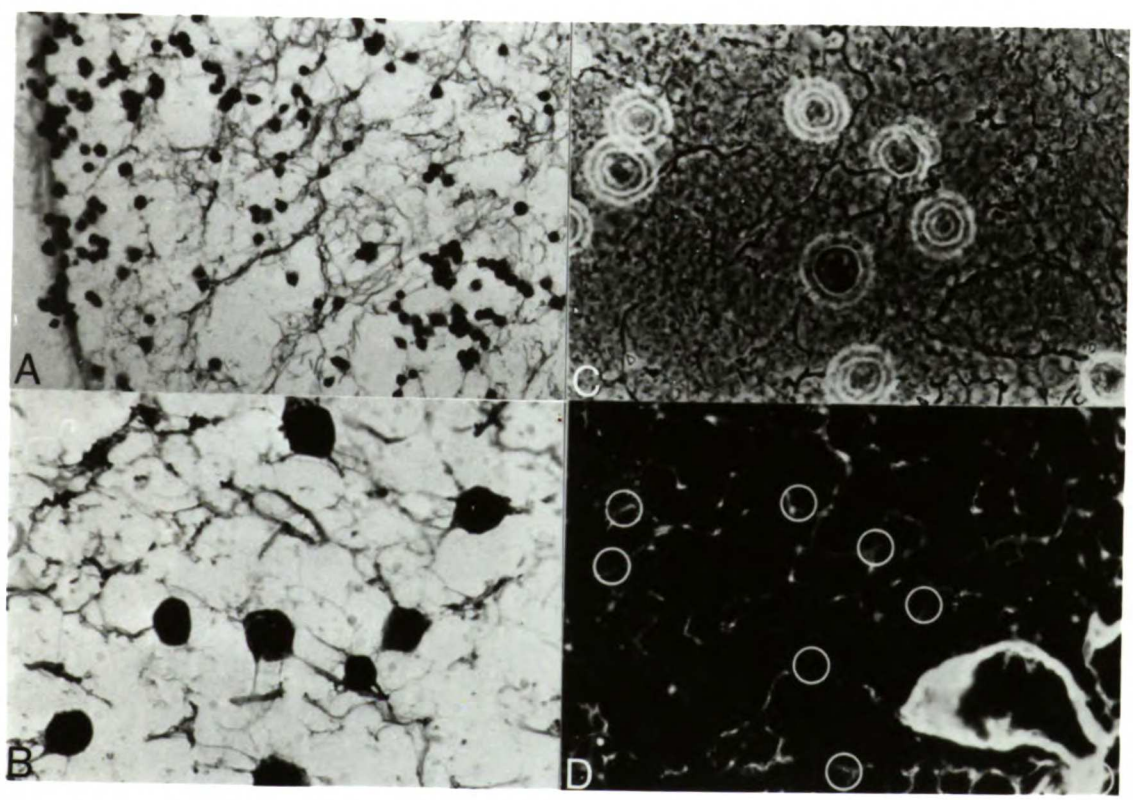
10
8
6
4
2
0
Attachment (%)
30
60
90
Time (min)

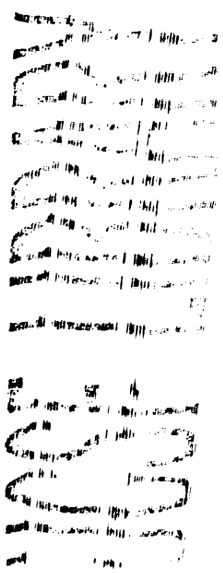


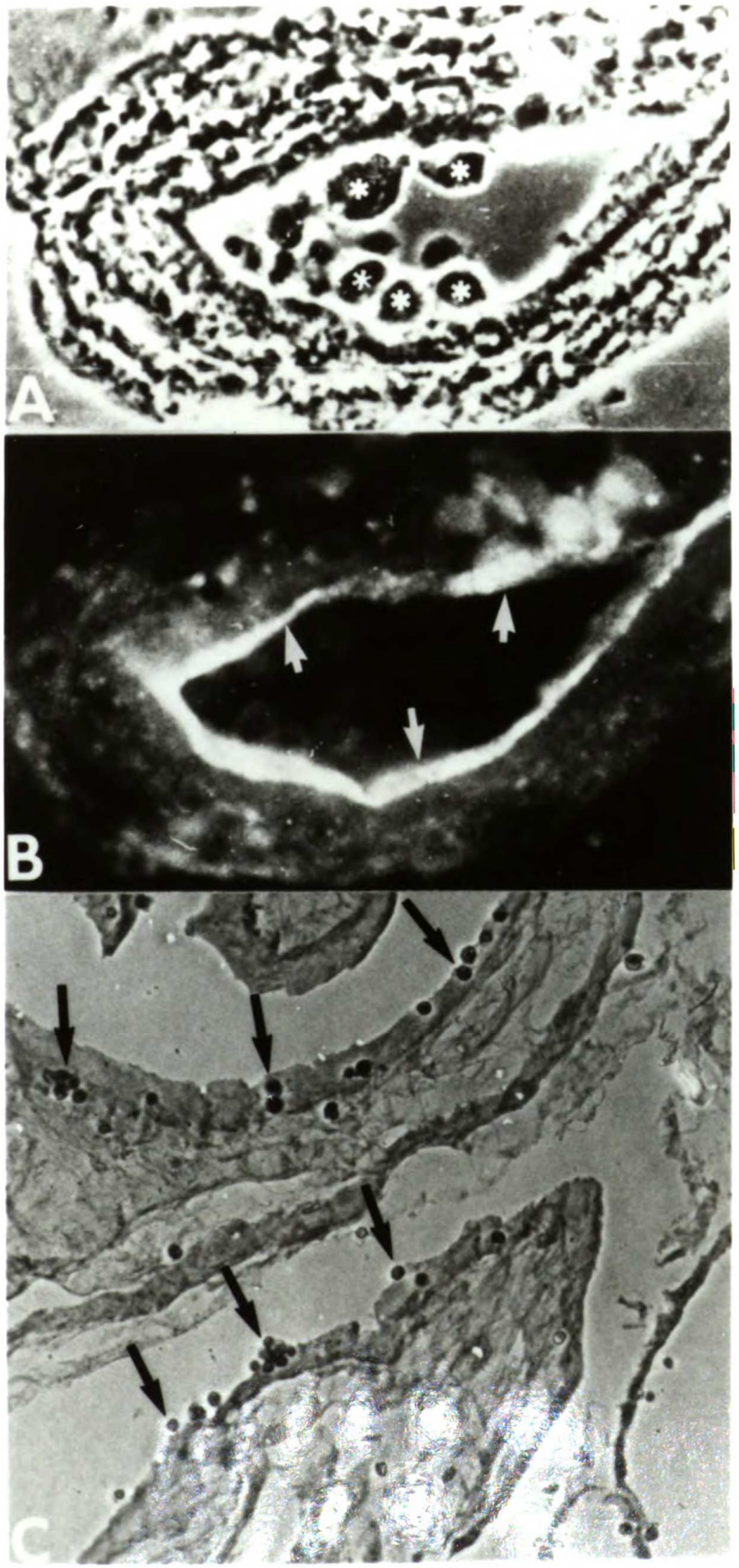


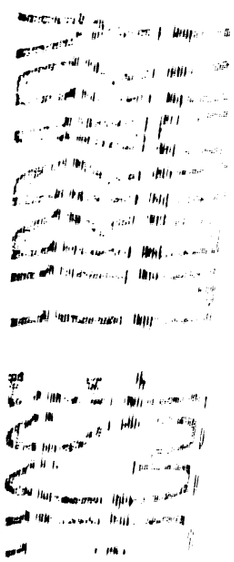

12
10
8
6
4
2
0
Attachment (%)
GEL
DERMIS
NODE
NODE ECM

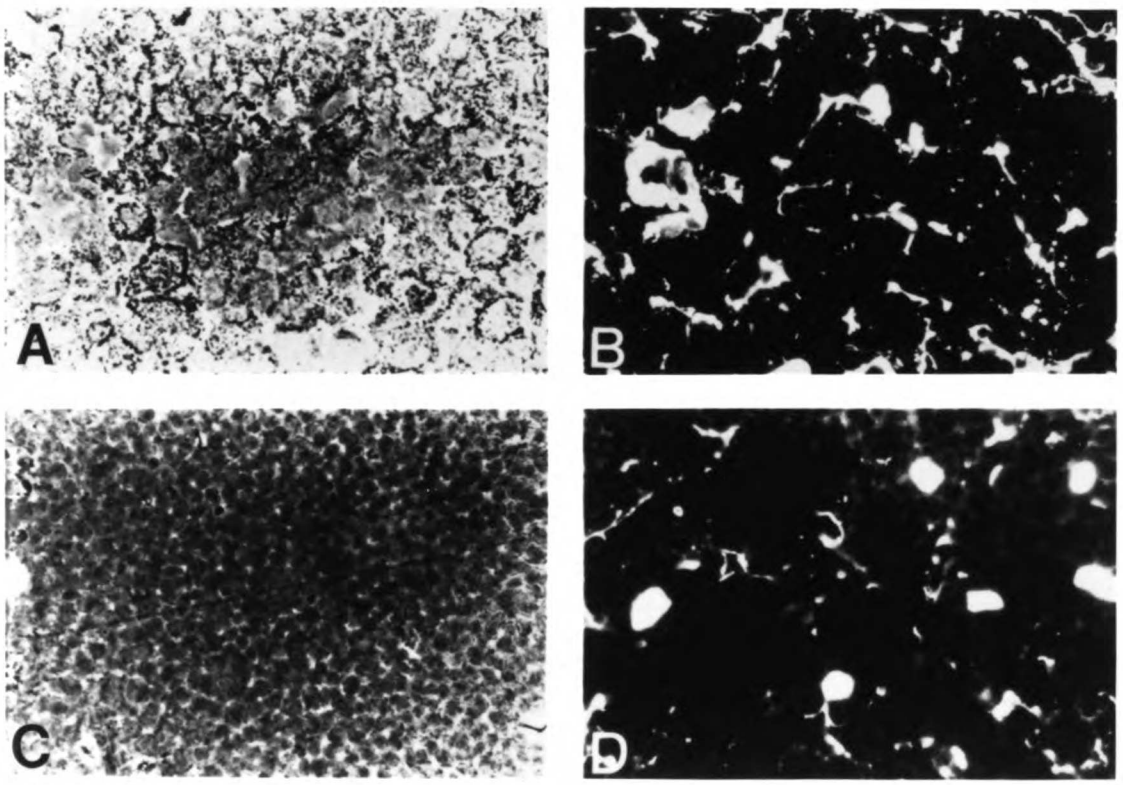


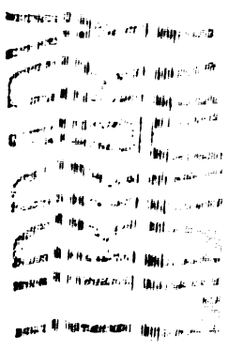

A
C
B
D

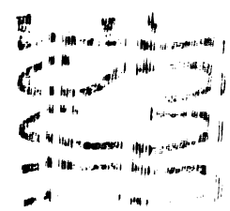


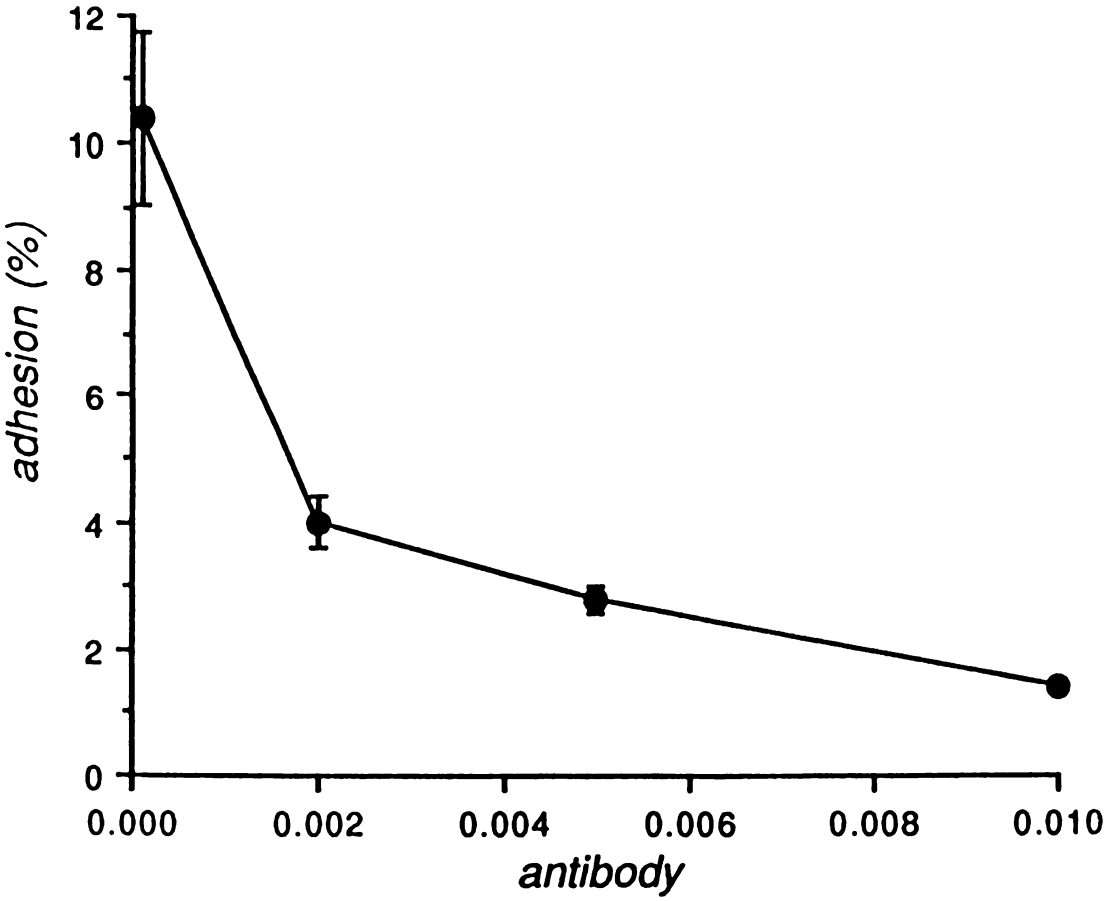

A
B
C




A
B
C
D






12
10
8
6
4
2
0
adhesion (%)
0.000
0.002
0.004
0.006
0.008
0.010
antibody

CHAPTER FIVE

Expression of High and Low Affinity Laminin-Binding Integrins Is Coordinately Regulated During Melanoma Tumor Progression

INTRODUCTION

Several integrins of the β1 series, namely α1, α2, α3, α6, and α7, are important receptors for laminin. The first laminin to be extensively characterized was isolated from rodent tumor cells and is composed of A (α), B1(β), and B2(γ) chains. This form of laminin is abundant in various tissues such as skin and blood vessels (Chung *et al.*, 1979; Timpl 1989; Marainkovich *et al.*, 1993). Several other laminin isoforms have been identified and include merosin, S-laminin, S-merosin, kalinin/epiligrin, and K-laminin (Marainkovich *et al.*, 1993; Engvall 1993; Lindblom *et al.*, 1994). Most laminin isoforms are adhesive and can induce a multitude of cellular responses, including migration, invasion, cell polarization, and neurite outgrowth, as well as influence proliferation and differentiation (Timpl 1989; Beck *et al.*, 1990; Mecham 1991). Furthermore, several of the laminin isoforms are tissue-specific and are expressed in a developmentally regulated pattern. The biological response to laminin appears to be cell-type-specific, due in part to the specific receptors that are expressed by individual cells.

Laminin contains multiple sites for cell attachment (Timpl 1989), which appears to be mediated by several β1 integrins (Mercurio and Shaw 1991; Kramer 1994). The long-arm fragment E8 of laminin, produced by elastase digestion, is the binding site for the α3β1, α6β1, and α7β1 integrins (Gehlsen *et al.*, 1988; Sonnenberg *et al.*, 1990; Kramer *et al.*, 1991). The α1β1 integrin appears to bind to the cross region of laminin represented by the P1 or similar fragments (Hall *et al.*, 1990; Kramer *et al.*, 1991). The α2β1 integrin also binds laminin (Elices and Hemler 1989; Languino *et al.*, 1989; Kramer *et al.*, 1990), although its specific binding site has not been defined. Several important nonintegrin receptors have also been identified and include the 32/67-kDa receptor,

dystrophin, and galactosyl transferase (Mecham 1991). The laminin-derived Tyr-Ile-Gly-Ser-Arg (YIGSR) peptide appears to bind to the 67-kDa laminin receptor (Graf *et al.*, 1987; Kleinman *et al.*, 1988).

The α7 subunit was first detected on certain melanoma cells and shown to be part of a unique αβ1 integrin complex that bound laminin, as it was biochemically distinct from all known α subunits (Kramer *et al.*, 1989b; Ziober *et al.*, 1993). The α7β1 receptor is also expressed on myoblasts and skeletal and cardiac muscle (von der Mark *et al.*, 1991; Kramer *et al.*, 1989b; Ziober *et al.*, 1993). While certain melanoma cell lines express this integrin, others appear to express little or none (Kramer *et al.*, 1991; Kramer *et al.*, 1989b; Kramer *et al.*, 1991b). The α7 subunit has greatest sequence similarity with the α6 subunit and somewhat less with α3 (Ziober *et al.*, 1993; Kramer *et al.*, 1991b). These three integrin subunits are similar not only in binding to the same ligand (laminin) at the same site (the E8 fragment) (Gehlsen *et al.*, 1988; Kramer *et al.*, 1991; von der Mark *et al.*, 1991), but also in being alternatively spliced in both the cytoplasmic (Kramer *et al.*, 1989b; Ziober *et al.*, 1993; Tamura *et al.*, 1991; Collo *et al.*, 1993) and extracellular domains (Ziober *et al.*, 1993).

The murine K1735 melanoma cell line, derived from a primary tumor, has been shown to be composed of a mixture of phenotypically different clones with metastatic heterogeneity (Fidler *et al.*, 1981) In this study we examined a series of clonal cell lines derived from the parental K1735 cells for their expression of laminin-binding integrins. While multiple laminin-binding integrins were present in the melanoma cells, only α1, α6 , and α7 could be demonstrated to bind this ligand actively. Of the group of laminin-binding integrins, α1 and α6 exhibited low affinity binding whereas α7 showed high

affinity. The data indicates that in the panel of K1735 melanoma cell variants there is a strict inverse correlation between the expression of the α7 subunit and the variant cell line's metastatic potential. Furthermore, the results suggest that rapid migration of melanoma cells on laminin substrates requires expression of low affinity integrins, such as α1 and α6, in the metastatic cells whereas the poor migration of the nonmetastatic cells reflects expression of high affinity α7 receptor.

EXPERIMENTAL PROCEDURES

Cell Culture. The mouse K1735 melanoma cell lines we studied, the highly metastatic C26, M2, and M4 lines and the poorly metastatic C10, C19, and C23 lines, were from Dr. I.J. Fidler (University of Texas M.D. Anderson Hospital and Tumor Institute), and their isolation and metastatic activity has been previously described (Fidler *et al.*, 1981; Aukerman *et al.*, 1986). All cell lines were routinely cultured in 10% fetal bovine serum in Dulbecco's minimal essential medium (H-16). Cells were routinely passaged in log growth phase by brief treatment with 0.25% trypsin/2 mM EDTA. To minimize phenotypic drift, cell lines were replenished after 10 passages or less from frozen stocks. For selection of potential metastatic variants of the cloned K1735-C23 melanoma cell line, we injected C23 cells into the footpads of 6-to 8-week-old female C3H/Hen mice. C23 tumor cells were harvested from tissue-culture plates by brief treatment with a trypsin-EDTA solution and washed twice with PBS. After mice were anesthetized by pentobarbital sodium, 5 x 10^4 tumor cells in 25 µl of DMEM[1] was injected into the footpad. After 4 weeks, animals were sacrificed by cervical dislocation and autopsied for the presence of tumors in the lungs and other sites. The presence of tumor in lung foci was verified by detection in a bioassay after culturing in vivo. The C23m cell line was derived from a single pulmonary metastasis by excision of the tumor, which was then gently teased apart into fragments, the cellular contents of which were introduced into culture and expanded.

Cell Adhesion Assay. Cell adhesion to immobilized ligands was measured as described (Kramer *et al.*, 1989). The ligands used were the laminin-entactin complex, the E8 laminin fragment (both from the mouse EHS tumor) and fibronectin (from human

plasma) and were purified as previously described (Kramer *et al.*, 1991; Kramer *et al.*, 1990). Bovine skin collagen type I was from Collagen Corporation (Palo Alto). Laminin fragments E1, E3, E4, or P1' were a gift from Dr. P. Yurchenco (Robert Wood Johnson Medical School, New Jersey). Briefly, wells of virgin polystyrene 96-well flat-bottom microtiter plates were coated with purified ligands at 37°C for 1 h. The wells were washed, and nonspecific binding was then blocked with 0.1% BSA in PBS for 15 min. Cells were detached from culture dishes by treatment with 2 mM EDTA for 10 min at 37°C, washed, and resuspended in serum-free medium with 0.1% BSA. The cells were seeded at 2 x 10^4 cells/well in 50 µl of medium (with or without antibodies) and then incubated at 37°C in a humidified 8% CO_2 incubator for 30 min. The assay was terminated by washing, and the number of adherent cells was then estimated by a microcolorimetric assay for hexosaminidase at 405 nm (Landegren 1984). The data are presented as a percentage of the total cells seeded that become bound.

Migration Assay. Cell migration on surfaces coated with extracellular matrix components was measured using our recently described assay (Clyman *et al.*, 1992). Briefly, sheets of virgin polystyrene were assembled in a 96-well dot blot apparatus (Schleicher and Schuell, Keene, NH); the wells were then coated with ligand for 1 h. After a washing, the sheet was washed and reassembled with a polished stainless steel screen with 0.9-mm-diameter perforations. Cells were seeded onto the exposed surface of the polystyrene sheet (1 x 10^5/ml, 50 µl/well). After 1 h incubation at 37°C to permit cell attachment, the screen was removed, the apparatus was reassembled, and cells were incubated for 8 h. Cells on the sheet were then processed by fixation with 0.5% formaldehyde and stained with hematoxylin. The area covered by the out-migrating cells was measured by computer-assisted image analysis (NIH Image); the data were expressed

as the mean and S.D. of at least 8 individual measurements in pixel units (x 10^3) and represent "relative migration".

Ligand-Affinity Chromatography. Laminin-entactin complex or laminin E8 fragment was conjugated to Sepharose CL-4B (Pharmacia-LKB Biotechnology Inc., Piscataway, NJ). Laminin-Sepharose columns were equilibrated with running buffer, consisting of 50 mM Tris-HCl, 50 mM octyl-β-D-glucopyranoside, 0.1 mM PMSF, and 1 mM $MnSO_4$ (or with substitution of a different divalent cation), pH 7.4 (Kramer *et al.*, 1989).

K1735 cells in log growth phase were detached from culture dishes with 2 mM EDTA, 0.05% BSA in PBS and surface-labeled with ^{125}I by the lactoperoxidase method. For metabolic labeling, preconfluent cultures were first incubated with methionine- and cysteine-free Iscove's medium for 1 h and then labeled for 18 h with 40 μCi/ml of [^{35}S]methionine and [^{35}S]cysteine (NEN Research Products, Wilmington, DE). Labeled cells were solubilized directly on the plates with TNC buffer (10 mM Tris-HCl, 0.5 mM $CaCl_2$, 0.5% Nonidet P-40, pH 7.4) containing 5 mM cold methionine and cysteine for 45 min at 4°C. To remove nuclei and debris, labeled cells were solubilized in running buffer containing a total of 100 mM octyl-β-D-glucopyranoside and centrifuged first at 700 x g for 10 min and then at 14,000 x g for 15 min. The resulting supernatant was applied to columns of approximately 1 ml packed laminin-Sepharose. Fractions (1 ml) were collected, subjected to immunoprecipitation with anti-integrin antibodies, and analyzed by SDS-PAGE in 7% polyacrylamide gels (Laemmli 1970). Reduction was performed with 5% fresh 2-mercaptoethanol followed by alkylation with N-ethylmaleimide. Molecular mass standards included prestained protein markers of 180, 116, 84, 58, and 48 kDa (Sigma Chemical Co., St. Louis, MO).

Rabbit antibodies were generated against the mouse α7B cytoplasmic sequence (CPELGPDGHPVSVTA) (Ziober *et al.*, 1993) after coupling to KLH carrier protein. Rat monoclonal antibody to α6, GoH3, was a gift of Dr. A. Sonnenberg, Netherlands Cancer Institute (Laemmli 1970). Rat monoclonal antibody to human β1, AIIB2, was a gift from Dr. C. Damsky. Rabbit antibody to the E8 fragment of EHS-laminin was a generous gift of Dr. Peter Yurchenco (Robert Wood Johnson Medical School, New Jersey). Goat anti-mouse IgG- and goat anti-rat IgG-Sepharose were from Sigma Chemical Co. (St. Louis, MO). Rabbit antibody against rodent β1 integrin was a gift from Dr. K. Rubin (Uppsala)(von der Mark *et al.*, 1991;). Rabbit polyclonal antibodies to α1, α2, α6A, and α6B were from Dr. L. Reichardt (UCSF), and those to α3 and α5 were from Dr. R. Hynes (MIT). Secondary antibodies for immunofluorescence staining and cytofluorometric analysis (goat anti-rat and anti-rabbit IgG conjugated with FITC) were from Jackson ImmunoResearch (West Grove, PA).

Cytofluorometric Analysis. Cultured cells were detached with 2 mM EDTA and washed with PBS containing 1% normal goat serum. The cell suspensions were then incubated with appropriate dilutions of anti-integrin antibody for 45 min at 4°C. After a washing, the cells were incubated with secondary FITC-labeled goat anti-rat IgG (Jackson ImmunoResearch) for 20 min. After a final washing, the cells were fixed in 0.1% formaldehyde for 10 min at 4°C and then analyzed with a flow cytometer (FACScan, Laboratory for Cell Analysis, UCSF). Cell analysis was gated on forward and size scatter intensities. The results were presented in single-parameter flow cytometric histograms.

RT-PCR Amplification. Total cellular RNA from each cell line (15-20 μg/ml) was used for cDNA synthesis. The reverse transcriptase reaction was carried out in a 30-μl volume at 42°C using the RNase H-preparation of the Molony murine leukemia virus

reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD). One fifth of the reverse transcriptase reaction mixture was used for PCR. The two-round PCR reaction was carried out in a 100-µl mixture containing 10 mM Tris-HCl, pH 9.0 at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 0.1% Triton X-100, 0.2 mM each of dATP, dCTP, dTTP and dGTP, 1 µM of each primer, and 2 units of AmpliTaq polymerase (Perkin/Elmer, Emeryville, CA). The primers used were based on the previously published sequences of the mouse α7 (Song *et al.*, 1992) and the human α6 subunits (Tamura *et al.*, 1991). The reaction mixture was heated to 94°C for 5 min; this was followed by 30 amplification cycles consisting of 45 s of denaturation and annealing at 94°C and 50°C respectively, and a 90 s elongation step at 72°C. The reaction mixture was maintained at 72°C for 10 min after completion of the last cycle. All PCR reactions were carried out in a Perkin/Elmer thermal cycler. All DNA sequencing was performed by the dideoxy-chain-termination method using the Sequenase version 2.0 system (United States Biochemical Corp., Cleveland, OH) and 2'-deoxyadenosine 5'-α[^{35}S]thiotriphosphate ([^{35}S]dATP[αS]) as the radioactive nucleotide. Sequences were obtained from both strands.

Immunoblotting. Cultured cells were removed from culture dishes by a brief treatment with 2 mM EDTA, 0.05% BSA in PBS. They were then solubilized with 100 mM octyl-β-D-glucopyranoside and centrifuged, first at 700 x g for 10 min and then again at 14,000 x g for 15 min. The protein concentration of the cell extract was determined by the Bradford Assay. Proteins were separated by SDS-PAGE (30 µg/well), then electrophoretically transferred to nitrocellulose and probed with specific antibodies to α6A or α7B, according to protocols that we have used previously (Kramer *et al.*, 1986). The ProtoBlot horseradish peroxidase (HRP) system of Promega was used for

detection of antigens. Briefly, the membrane was rinsed with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl (TBS) and soaked in the blocking solution consisting of TBS and 1% BSA. The primary antibody (α6A or α7B) was added at the appropriate concentration in a solution containing TBS and 0.05% Tween-20 (TBST). After 2 h incubation at room temperature, the unbound antibody was removed by washing the membrane in TBST three times for 5-10 min each. A 1:2,500 dilution of anti-IgG-HRP conjugate prepared with TBST was then incubated with the membrane for 30 min. After several washes in TBST solution, the membrane was placed in the HRP color development solution. When the color was developed to the desired intensity, the filter was rinsed with deionized water and photographed.

RNA Isolation and Northern Blot Analysis. Total RNA from cultured cells was isolated by the guanidinium-cesium chloride method as described (Cheng *et al.*, 1991). RNA samples (15 μg) were electrophoresed in 1% agarose gels containing 6% formaldehyde. After electrophoresis, the RNA was transferred to nylon membranes (Amersham Hybond N) by capillary blotting and fixed to the filter by exposure to UV light. The RNA was hybridized with the 700-bp PCR-amplified α7 cDNA fragment or mouse α6 cDNA fragment (from Dr. R. Pytela, UCSF) labeled with ^{32}P by the random priming method of Feinberg and Vogelstein (Feinberg and Vogelstein 1983). Hybridizations were carried out at 60°C in 50% formamide, 5x SSC, 5x Denhardt's solution, 0.1% SDS, and 0.3 mg/ml salmon sperm DNA. Filters were washed twice in 1x SSC, 0.1% SDS at room temperature for 15 min and once at 65°C in 0.1x SSC, 0.1% SDS for 1 h. Filters were then exposed to X-ray film at –80°C with intensifying screens.

RESULTS

Adhesion and Migration of Melanoma Cell Variants on Laminin and E8

In adhesion assays we found that both highly (M2) and poorly (C23) metastatic melanoma cells adhered well to laminin, although adhesion efficiency was usually slightly better for the M2 cell line (Fig. 1). Similarly, both the M2 and C23 cells adhered well to the E8 fragment, which represents a major portion of the long arm of laminin. In contrast, both cell lines attached poorly to additional laminin fragments, including E1, E3, E4, and P1' (not shown). In the case of the M2 cells, adhesion to the E8 fragment, but not to intact laminin, was significantly reduced in the presence of the blocking monoclonal antibody (GoH3) to the $\alpha 6\beta 1$ receptor; this antibody did not inhibit adhesion of the C23 cells to laminin or the E8 fragment. For both cell lines, a rabbit anti-$\beta 1$ blocking antibody (Gullberg *et al.*, 1989) was effective in inhibiting attachment to laminin and E8. Finally, anti-E8 antibody significantly inhibited attachment to intact laminin and E8 by C23 cells and, to a lesser extent for intact laminin, by M2 cells. These results indicated that integrins of the $\beta 1$ series are important for adhesion of both cell types to laminin. In particular, the $\alpha 6$ integrin significantly contributes to the attachment of M2 cells to the E8 fragment.

Although the M2 and C23 cell lines adhered with nearly the same efficiency to laminin, their migration on this ligand was substantially different. The highly metastatic M2 cells accelerated rapidly on laminin over a wide range of ligand-coating concentrations outpacing the poorly metastatic C23 cells (Fig. 2A). For both cell lines, optimal migration occurred at coating concentrations of 10-30 μg/ml; at higher concentrations migration was inhibited. The E8 fragment as ligand was also able to induce a differential locomotive response, with the M2 cells moving more rapidly on E8

than the C23 cells (Fig. 2B). On fibronectin substrates however, the relative migration efficiencies were reversed, with the M2 cells migrating more slowly than the C23 cells (Fig. 2B); this indicates that the poor migration of C23 cells on laminin is not due to a general defect in their locomotory activity. Antibody against the laminin E8 fragment significantly inhibited migration of M2 cells on laminin (Fig. 2D) and on the E8 fragment (not shown).

We also evaluated the role of the β1 class of integrins in mediating laminin-induced motility in the M2 cells. Polyclonal anti-β1 antibody produced a nearly complete inhibition of M2 cell locomotion over laminin substrates (Fig. 2C). The GoH3 blocking antibody to the α6 subunit inhibited laminin-mediated motility in the M2 cells by over 50% (Fig. 2D). This indicated that for the M2 cells, movement on laminin is dependent on α6β1 but that additional β1 integrins also participate. In contrast to the M2 cells, GoH3 had no effect on the migration of the C23 cells (not shown).

Identification of Laminin-Binding Integrins

We compared the integrin expression profiles of the C23 and the M2 cells by performing immunoprecipitation of lysates prepared from surface ^{125}I-iodinated cells (Fig. 3A). Numerous differences in integrin profiles between the two cell types were noted, particularly in the laminin-binding integrins. Overall, M2 cells had consistently higher levels of several integrin α chains than did C23 cells, which had a more limited α integrin pattern and expressed little or no detectable α1 or α6. Both cell types expressed moderate levels of α3. M2 cells had higher levels of α2 while C23 cells had higher levels of α5. That M2 cells, but not C23 cells, were sensitive to perturbation of adhesion to E8 by anti-α6 antibody (Fig. 1) suggested a difference in the cells' expression of the α6β1 receptor. This was confirmed by flow cytometry with monoclonal GoH3 antibody

to α6 (Fig. 3B) in which M2 cells displayed high levels of the α6 integrin while levels of α6 on the C23 cells were near background levels.

Since the α6 subunit has been shown to exist as two alternatively spliced isoforms in the cytoplasmic domains, we next examined the relative levels of the α6 isoforms in the K1735 melanoma variant cell lines. We found by RT-PCR that the highly metastatic variants (M2, M4, C26) predominantly expressed the α6A form, whereas the low metastatic variants (C19, C23) expressed roughly equal amounts of α6A and α6B, albeit at low levels (Fig. 3C). As shown in previous studies (Kramer *et al.*, 1991b), RT-PCR also yielded a heteroduplex of the A and B forms of α6 that exhibited an intermediate size on analysis in agarose gels. The relative abundance of α6A and α6B isoforms was confirmed by immunoblotting with anti-peptide antibodies (Fig. 5C and data not shown). By PCR, with specific primers, only the α7B form is detectable in C23 and M2 cell lines (unpublished observations).

We used affinity chromatography to identify functional high affinity laminin receptors in the K1735 melanoma variant cell lines and to estimate their relative level of expression. M2 and C23 cells were metabolically labeled and detergent extracts were processed for chromatography on laminin-Sepharose columns (Fig. 4A). After the column was washed with 0.2 M NaCl, bound receptors were eluted with EDTA and the fractions analyzed. In SDS-PAGE under nonreducing conditions, a single broad band of ≈120 kDa was eluted with EDTA that on reduction separated into the β1 subunit at 140 kDa and an α subunit at 100 kDa. This electrophoretic behavior in nonreducing and reducing gels is unique for α7β1 (Kramer *et al.*, 1991, von der Mark *et al.*, 1991, Kramer *et al.*, 1989b) and its identity was verified by immune precipitation with specific antibodies to the cytoplasmic domains of α7 and β1 subunits (not shown). Much higher levels of α7

integrin were consistently recovered from the C23 cells than from the M2 cell lines. To verify that the retrieval of the α7β1 complex was complete, the detergent lysates were sequentially passed over two identical columns of laminin-Sepharose; the majority of the integrin was bound to the first column (not shown). In additional assays, cells were surface ^{125}I-labeled and cell lysates similarly processed for chromatography on laminin-Sepharose columns and again subjected to sequential elution as above (Fig. 4B). As before, analysis of the EDTA-eluted fractions in reducing SDS-PAGE indicated that substantially higher levels of the α7β1 complex were present on the poorly metastatic C23 cells than on the M2 cells. This indicated that α7β1 is at the cell surface and is functional with respect to its binding to laminin. Densitometric analysis of the autoradiograms from metabolically or surface-labeled cells indicated that levels of the high affinity α7 subunit in the C23 cells were at least 7- to 10-fold higher than levels in the M2 cells (not shown). Significant amounts of α1, α2, α3, and α6 were not detected in the EDTA fractions (confirmed by immunoprecipitation); if present, these integrins are usually recovered in the 200 mM wash step that precedes the EDTA elution (see below).

Next, we used immunoblotting of lysates to more accurately measure the relative level of the α7 subunit in the M2, C23, and C19 cell lines. Equivalent load amounts of total cellular protein were analyzed and the blots were probed with rabbit antibodies generated against the α7B cytoplasmic domain (Kramer *et al.*, 1989b). RT-PCR indicated that for these cell lines α7B, rather than α7A, appears to be the predominant form (unpublished results). In agreement with the laminin-affinity chromatography (Fig. 4), the M2 cells had negligible amounts of the α7B receptor (Fig. 5). For C19 and C23 cells, α7B was detected as a major reactive band corresponding to 120 kDa that comigrated with the β1 subunit. However, a second band of ≈70 kDa of variable intensity was also

detected that was reactive with the anti-α7 antibody and appears to be a proteolytic cleavage product (Ziober *et al.*, 1993; Song *et al.*, 1992). Specific antibodies were also used to estimate the relative levels of α6A in the cell lysates (Fig. 5.). As expected, α6A was strongly expressed by the metastatic M2 cells, but not by the C19 or C23 cells.

We also analyzed the expression of α7 in spontaneous revertant subline that we had selected from the normally nonmetastatic C23 cells by injection into the footpads of syngeneic mice. One animal developed a small pulmonary metastatic deposit which was excised and introduced into culture. From this explant we derived the C23m cell line. In this revertant C23m cell line there was a high level of expression of α6B (and α1, not shown) and a reduction in the level of α7B (Fig.5). Control experiments with soluble peptide validated the specificity of the antibodies to the sequence in the cytoplasmic domain of α6A and α7B (not shown). These results show a consistent relationship between the loss of α7 and increase in expression of α6 and the acquisition of the metastatic phenotype.

Expression of α6 and α7 mRNA in Melanoma Cell Variants

The difference in the expression of α6 and α7 integrins in the poorly and highly metastatic melanoma cell lines may reflect regulation at the transcriptional level. Using probes specific for each α chain, we screened total RNA from a panel of K1735 melanoma cell lines with differing metastatic potential by Northern blot analysis at high stringency. The α6 probe hybridized with an mRNA species of about 6.0 kb in positive cell lines (Fig. 6A). The α6 mRNA levels were consistently high in cells of high metastatic potential (C26, M2, M4) but low or nondetectable in cell lines that were poorly metastatic (C10, C19, C23). In α7-positive cell lines, a mRNA species corresponding to α7 of about 4.2 kb was detected. α7 mRNA expression was consistently opposite from

that of α6 transcripts (Fig. 5B), being abundant in the poorly metastatic K1735 cell lines and poorly expressed, if at all, in the metastatic clones. By densitometric analysis, the relative level of α7 transcripts in the poorly metastatic cell lines was at least 10-fold that of the highly metastatic cell lines. The parental cell line from which the individual clonal variant cell lines were derived had an intermediate level of expression of α6 and α7 (unpublished results). From these results it appears that the difference in expression of α6 and α7 is due to regulation at the transcriptional level.

Analysis of the Relative Affinity of Melanoma Integrins for Laminin

To address possible functional differences between the different laminin receptors in the K1735 variant cell lines, we examined the relative affinity of melanoma integrins for laminin and E8. We used the K1735 parental cell line, which expresses a β1 integrin repertoire that reflects a composite of the integrin profiles found in the metastatic and nonmetastatic variants and is a mixture of all known laminin-binding integrins (α1,2,3,6,7). The parental cells were surface-labeled with ^{125}I and processed for chromatography on E8- or laminin-Sepharose columns; the bound mixture of integrins was subjected to selective elution with buffers containing different divalent cations (Fig. 7). The standard protocol for affinity chromatography was to prepare cell lysates in buffer containing octylglucoside detergent and 1 mM Mn^{2+}, and after a wash with running buffer, the column was sequentially eluted with 0.2 M NaCl to displace weakly associated receptors followed by 10 mM EDTA to elute the more strongly bound integrins. The elution pattern of individual integrins was then assessed by SDS-PAGE and identity of α subunits was verified by immunoprecipitation. The laminin-binding integrins of parental K1735 cells were chromatographed on columns of E8- or laminin-Sepharose following this protocol (Fig. 7A). A prominent integrin of 120 and 140 kDa was eluted

with 0.2 NaCl and corresponded to α6β1 (lanes 2-3); following reduction the α6 and β1 subunits showed an inverted position in the gel (lanes 9-10). Subsequent elution with EDTA yielded a single band at 120 kDa (lanes 5-7) identified as α7β1, which characteristically split into two bands at 140 and 95 kDa after reduction (lanes 12-13). The parental cells also expressed α1β1 but this integrin, as expected, did not bind to the E8 fragment, consistent with its proposed binding site at the cross region of laminin (Sonnenberg *et al.*, 1990; Hall *et al.*, 1990, Kramer *et al.*, 1991). However, α1 was recovered from columns of intact laminin (lanes 15 and 16).

Next, we evaluated the role of divalent cations in moderating integrin affinity for laminin. After the column was loaded with detergent lysate from surface ^{125}I-labeled parental cells and washed with Mn^{2+}-containing running buffer, it was eluted first with 200 mM NaCl (Fig. 7B, lanes 1-2), followed by buffer containing 10 mM Ca^{2+} instead of Mn^{2+} (lanes 3-6), and finally with 10 mM EDTA (lanes 7-9). The 200 mM NaCl eluted substantial amounts of α1 and α6, but no α7 was detected. Replacement of the Mn^{2+} with 10 mM Ca^{2+} displaced a mixture of β1 integrins that in the early fractions consisted predominately of α6 with some amounts of α1; subsequent fractions contained essentially all of the remaining α6 and α1 along with significant amounts of α7. Finally, elution with 10 mM EDTA recovered exclusively α7β1. Thus, in contrast to α1 and α6, α7 bound to laminin is completely resistant to elution with 200 mM NaCl buffer (Kramer *et al.*, 1991) and substantially resistant to elution with Ca^{2+}. When the affinity chromatography was repeated but with the Mn^{2+} replaced first with Mg^{2+}, then with Ca^{2+}, and finally with EDTA, a somewhat different pattern of elution emerged (Fig. 7C). α6 was effectively eluted by the Mg^{2+} while α1 was partially eluted and α7 was completely resistant to elution; again, switching the buffer to Ca^{2+} provoked the complete retrieval

of the residual α1 and partial recovery of α7; EDTA completely displaced the remaining α7.

The strength of K1735 integrin binding to laminin was also evaluated by examining their resistance to dissociation from laminin-Sepharose columns with a step salt gradient of 50 mM to 1M NaCl. Whereas α1β1 and α6β1 were effectively eluted early at 50-200 mM NaCl, α7β1 was resistant even to buffer containing 1 M NaCl and was recovered in the final wash with 10 mM EDTA (Fig. 7D). Again this pattern of elution reflects the high affinity of the α7 receptor for its ligand and contrasts with the behavior of α1 and α6. In other experiments using initial loading buffers containing Mg^{2+} alone or a mixture of Ca^{2+} and Mg^{2+}, α7 still efficiently bound to laminin-Sepharose and was not displaced by the 200 mM NaCl wash; α1 and α6 were readily eluted with the salt wash under these conditions; in the presence of Ca^{2+} as the only divalent cation, no significant amounts of any integrin were detected binding to laminin columns (unpublished data). Immunoprecipitation with specific antibodies to α2 and α3 failed to detect significant amounts of these integrins in the eluted fractions from laminin columns (not shown). Taken together, this differential elution pattern indicates a fundamental difference in how salt and divalent cations influence the interaction of this set of integrins with laminin.

DISCUSSION

We compared the expression of β1 integrin receptors for laminin by metastatic variants of the K1735 melanoma to analyze their role in adhesion and migration. Results obtained with several of the K1735 cell lines indicate that the highly metastatic cells show accelerated migration on laminin substrates while the nonmetastatic cells exhibit little motility on the same ligand. Earlier work had also indicated differences in the relative motility and invasiveness of melanoma variant cell lines (Albini *et al.*, 1987; Raz 1983; Albini *et al.*, 1989; McCarthy and Furcht 1984). Laminin induced a strong haptotactic response in the metastatic M2 cells, but not the C23 cells with a relatively narrow range of coating concentrations that promoted locomotion. A minimum density of laminin was needed for attachment and subsequent motility; however, at higher coating concentrations movement was stopped. These results are consistent with the work of others who have shown that migration is stimulated at an optimal ligand density but inhibited at higher ligand densities (McCarthy and Furcht 1984; Goodman *et al.*, 1991). We found that for the K1735 cells, much of the adhesion and motility-promoting activity of laminin appears to reside in the E8 fragment. Both metastatic (M2) and nonmetastatic (C23) clonal cell lines bound well to the E8 fragment and adhesion to intact laminin was substantially inhibited with antibodies to the E8 domain. In addition, migration of M2 cells on both laminin and E8 was inhibited by anti-E8 antibodies. Anti-E8 did not inhibit attachment of M2 cells, indicating that there is substantial interaction of these cells with other sites in laminin.

Integrin receptors of the β1 class mediated the attachment of both the C23 and the M2 cells to laminin and to the E8 fragment, as indicated by the blocking effect of β1 antibodies. In addition, the β1 integrins were crucial for migration of M2 cells on

laminin, since blocking antibodies ablated motility. The adhesive behavior of the C23 and M2 cells could be differentiated by their sensitivity to anti-α6 blocking antibody (GoH3). Whereas neither of the cell lines was inhibited by GoH3 from binding to intact laminin, this antibody significantly inhibited adhesion to the E8 fragment by M2 cells but not the C23 cells. The sensitivity of M2 cells to GoH3 is consistent their relatively high expression of α6 integrin, as determined by immune precipitation and FACS analysis. When parental K1735 cell lysates were applied to columns of E8- or laminin-Sepharose, α6 was retained on the columns. In contrast, α1 receptor bound to columns of laminin-Sepharose, but not to E8-Sepharose. This suggests that for the M2 cells the α1 receptor confers the resistance to blocking by the anti-α6 antibody that was seen in adhesion assays with intact laminin. However, M2 cell migration on laminin was significantly inhibited by GoH3 thereby implying that α6 plays a more prominent role in migration than it does during the initial adhesion to this ligand.

Under the conditions used, we did not observe significant binding of α2 or α3 to murine laminin or its E8 fragment in studies with the parental K1735 cells or the M2 and C23 variants, even though these receptors are expressed by both variant cell lines at significant levels. The lack of α3 binding to EHS-derived A-chain laminin has been observed previously in both rodent and human systems (Kramer *et al.,* 1991; Goodman *et al.*, 1991; Weitzman *et al.,* 1993; Delwel *et al.,* 1994) but contrasts with studies reporting that α3 binds laminin or that anti-α3 antibodies could block adhesion to laminin (Wayner and Carter 1987; Elices *et al.*, 1991). Subsequently, it was shown that α3 binds efficiently to kalinin (or epiligrin), a laminin isoform with a truncated A-like chain (Delwel *et al.,* 1994; Carter *et al.*, 1991). It is well established that α2 can function as both a collagen and a laminin receptor; however, α2 functionality is activation- and cell-

type-specific and can be modulated by various cellular mechanisms, including protein kinase C-dependent pathways, and externally by β1-activating antibody (Chan *et al.*, 1992; Chan and Hemler, 1992; Masumoto and Hemler, 1993; Hynes 1992). In the case of the K1735 cells, both α2 and α3 integrins appear to be inactive toward laminin or its E8 fragment. The fact that C23 and M2 cells adhered well to collagen (data not shown) suggests that α2 is functional for this ligand. Collectively, these results indicate that for the M2 cells, interaction with laminin is mediated by a subset of β1 integrins that includes α1 and α6.

In the case of the C23 cells, several lines of evidence indicate that the primary laminin receptor is α7β1. First, α7 is the most abundant laminin receptor identified in C23 cells and appears to account for over half of the β1 group of integrins on these cells (Ramos *et al.*, manuscript in preparation). Second, blocking antibodies to α6 had no effect on C23 cell adhesion to laminin or the E8 fragment whereas anti-β1 antibody completely inhibited adhesion to laminin and the E8 fragment. Third, α7 is the only integrin in C23 cells with functional activity as detected by ligand chromatography on laminin- or E8 fragment-Sepharose columns, even under highly permissive conditions (e.g., Mn^{+2} as the divalent cation and low ionic strength). Fourth, C23 cell adhesion to laminin was blocked by anti-E8 antibodies, consistent with the known binding site for α7 (Kramer *et al.*, 1991; von der Mark *et al.*, 1991). Fifth, α7 is prominently concentrated in vinculin- and talin-positive focal adhesion contacts in C23 cells adherent to laminin (unpublished observations). However, final confirmation of the role of α7 in adhesion awaits the availability of blocking antibody specific to α7.

Recent work has shown that many integrin receptors require activation before they can bind ligand (Hynes 1992; Ginsberg *et al.*, 1992). The mechanisms that modulate

receptor activity and specificity are not completely understood, but certain evidence indicates that receptor function is controlled post-translationally at different levels. In particular, integrin activity and ligand specificity appear to be under the control of the cellular microenvironment. Furthermore, post-ligand-binding events have been shown to be regulated by the α and β cytoplasmic domains (Ginsberg *et al.*, 1992). Numerous factors can alter receptor function, including divalent cations, lipids, status of the cytoplasmic domain, and the binding of certain anti-β1 antibodies (Ginsberg *et al.*, 1992). For example, as mentioned above, α2β1 can be inactive, or can bind collagen, or can bind both collagen and laminin; its cell-type-specific activity, referred to earlier, can be modified by phorbol esters and by activating antibodies to the β1 subunit. Finally, recent work by Sonnenberg and colleagues (Hogervorst *et al.*, 1993) indicates that the phosphorylation of the α6A cytoplasmic tail can downregulate α6Aβ1-mediated adhesion to laminin.

The type of divalent cation available to the integrin complex has a significant impact on the activation as well as affinity and specificity of the receptor for its ligand. In fact, integrins require divalent cations for their binding to ligands. There are numerous examples of modulation of integrin receptor activity by the specific divalent metal salts present in assays of cell adhesion and migration (Grzesiak *et al.*, 1992), ligand-affinity chromatography (Kramer *et al.*, 1990; Greziak *et al.*, 1992; Pytela *et al.*, 1987), or in solubilized receptor ligand-binding assays (Gailit and Ruoslhati 1988; Charo *et al.*, 1990). Divalent cations can induce multiple effects that include stimulating or inhibiting ligand binding or altering ligand specificity. The physiologically relevant divalent cation mix is Ca^{2+} and Mg^{2+}-rich with significant Mn^{+2} (Olinger 1989; Schramm 1986). However,

for several integrins studied so far, Ca^{2+} alone is not sufficient for activity and Mg^{2+} and/or Mn^{+2} is required (Pytela *et al.*, 1987; Kirchhofer *et al.*, 1990; Smith and Cheresh 1991; Smith *et al.*, 1994). In particular, Mn^{+2} has been shown to be a potent activator of integrin function (Masumto and Hemler, 1993; Dransfield *et al.*, 1992).

In our study, we found that α1, α6, and α7 integrins bound to laminin with different affinities and their binding could be differentiated by the type of available divalent cation and by the ionic strength of the elution buffer. With Mn^{+2} as the only divalent cation, most of α6 was eluted with 50 mM NaCl while a fraction of α1 remained bound and was eluted with 200 mM NaCl. However, α7 was resistant even to 1 M NaCl, reflecting its high affinity interaction with laminin. Under intermediate conditions of stringency for chromatography that included 200 mM NaCl and Mg^{2+} instead of Mn^{2+}, α6 was completely and α1 partially eluted whereas α7 remained bound to the laminin column. Substituting Ca^{2+} for Mn^{2+} or Mg^{2+} caused the displacement of α1 and α6 and eventually the elution of α7. This response to Ca^{2+} is similar to the behavior of several other integrins that display loss of ligand-binding function in the presence of high Ca^{2+} (Staatz *et al.*, 1989; Santoro 1986; Kirchhofer *et al.*, 1991). In other studies, Forsberg *et al.* (1990) reported that the rat α1 receptor on laminin columns was not detected in EDTA eluates when a Ca^{2+}/Mg^{2+} buffer was used but could be isolated when Mn^{2+} was substituted.

Although these initial results represent a crude approach for analysis of integrin affinity, they indicate fundamental differences in the interaction of each of these receptors for laminin. Furthermore, they suggest that α1 and α6 may be expected to exhibit low affinity binding with laminin under physiological ionic strength and concentrations of Ca^{2+}

and Mg^{2+}. In one study by Sonnenberg *et al.* (1991), binding of soluble laminin to α6β1 on *intact* platelets was detected in medium containing Mn^{2+} but not in Ca^{2+} or Mg^{2+} buffers. It has been predicted that during tissue injury, local concentrations of Ca^{2+} and Mg^{2+} may be altered and could thereby modulate integrin-mediated events such as adhesion and migration (Greziak *et al.*, 1992). In a recent study by Michishita *et al.* (1993), a structural basis for a relatively high affinity Mn^{2+}-binding site that could not be competed for by Ca^{2+} was identified in the αM integrin subunit that is homologous to the first divalent-cation site on other α integrins. This implies that small amounts of Mn^{+2} may be physiologically relevant in activating or modulating integrin function. In fact, as discussed above, several studies have shown that even low concentrations of Mn^{+2} (0.1 mM) can activate integrin function, even in the presence of Ca^{2+}. Levels of Mn^{2+} in tissue fluid probably vary but estimates range between 1-10 μM (Dransfield *et al.*, 1992). In the context of intact cells, the apparent low affinity of α1 and α6 may be important for cell migration. Furthermore, integrin receptors can condense into small aggregates or larger focal adhesion contacts with their associated array of cytoskeletal proteins and filaments. These structures clearly provide a complex machinery of the cell whereby receptors that are individually of low affinity are able to establish high affinity interactions that represent a summation of the integrin collective.

We suggest that one factor that may regulate the migratory response of melanoma cells' to laminin is the level of the α7 receptor. A major difference in integrin profiles between the highly and poorly metastatic cell types was their relative levels of expression of the high affinity α7 and the low affinity α1 and α6 integrins. It is likely that α6 and α7 may transduce different cytoplasmic signals when binding to their common ligand, the E8 domain of laminin. Alternatively, it may be the extracellular domain that confers

different activities on the two integrins, thereby producing distinct migratory phenotypes. Sequence analysis of the α6 and α7 subunit domains corresponding to the ligand-binding region shows high homology (>70%) (Ziober *et al.*, 1993; Song *et al.*, 1992). Yet on affinity columns, α7 exhibited a much higher affinity for laminin than did α6. This difference is presumably due to structural elements in the ectodomain. To further complicate this issue, recent results indicate that there are at least two candidate alternative splicing sites in α7: one at the ectodomain near the divalent cation-binding sites, and one at the cytoplasmic domain; α6 has homologous alternative splicing at these two sites. These new findings point to the existence of a molecularly heterogeneous population of α7 subunits that needs to be further defined.

The cytoplasmic domains of integrins interact with the cytoskeleton and potentially with other cytoplasmic proteins. It is through this interface with the cytoplasmic compartment that signals are transmitted to the cell. In addition, it is now apparent that functionality of the extracellular domain of the integrin is influenced by "inside-out signaling" (Ginsberg *et al.*, 1992). It is also generally believed that the α subunit cytoplasmic domain may regulate the interactions of the β subunit cytoplasmic tail with the cytoskeleton (Chan *et al.*, 1992). Thus, the existence of cytoplasmic domain splice variants could regulate the quality and strength of signal input from the extracellular space and vice versa. Hogervorst *et al.* (1993) recently demonstrated that α6A and α3A are phosphorylated on phosphoserine and weakly on phosphotyrosine. The B isoforms are not phosphorylated. They suggest that this difference in phosphorylation of the two isoforms may be an important functional regulatory mechanism. More recently, Sonnenberg and collaborators (Delwel *et al.*, 1994) showed that phosphorylation

of α6A inhibits ligand-binding activity. Again, because of the extensive similarity between α6 and α7, it is possible that regulation of α7 function at the cytoplasmic domain may involve its phosphorylation.

The development of metastases, a highly selective process, depends on the existence of tumor cell variant subpopulations; certain subsets of cells with the appropriate phenotype can survive the rigors of the metastatic cascade and successfully establish secondary tumor foci. It is likely that a successful phenotype includes a suitable array of integrin receptors that would directly determine the cell's adhesive and migratory activity. In the K1735 series of variant cell lines, the expression patterns of the α1β1 and α6β1 integrins were opposite from that of α7β1, in that the former group of integrins were significantly increased in the highly metastatic cell lines (e.g., M2) and reduced in the non-metastatic cell lines (e.g., C23). The results indicate that the acquisition of metastatic potential in these cells is associated with loss of α7 expression and a coordinate increase in low affinity α1 and α6 expression. Northern blot analysis of RNA prepared from a panel of the K1735 cell lines demonstrated a good correlation between the level expression of α6 or α7 protein level and the level of corresponding transcripts. The K1735 parental cell line expressed a mixture of laminin-binding integrins, including α1, α2, α6, and α7. The parental cell line, which was derived from the primary tumor, has been shown to be composed of a mixture of phenotypically different clones with metastatic heterogeneity (Fidler *et al.*, 1981). It is therefore not surprising that the integrin profile of the parental cell line represents a composite of the profiles of the metastatic and nonmetastatic cell types. The results suggest that during tumor progression, there is selection of cells with an invasive phenotype that includes an integrin repertoire enriched in low affinity laminin-binding integrins (i.e., α1 and α6) but lacking

significant levels of the high affinity α7 receptor.

A number of studies suggest that α6 may be upregulated in highly metastatic melanoma cells. Albelda *et al.* (1990) found by immunostaining that α6 is present not only in primary melanoma but also in vertical growth phase and metastatic lesions. Two recent studies (Etoh *et al.*, 1993; Vink *et al.*, 1993) showed that α6 as well as α2 were upregulated in cells derived from human metastatic melanoma but not in primary nonmetastatic cells. Similarly, Danen *et al.*(1993) reported that α2 and α6 were increased in a series of metastatic melanoma cell lines but not in primary nonmetastatic tumor cells. Analogous results were also found by Mortarini *et al.*(1991). In studies with the B16 murine melanoma cells, which have high levels of α6 (Ramos *et al.*, 1990), it was shown that antibodies to this receptor would abolish experimental pulmonary metastases and that the arrest and retention of tumor emboli in the lung were diminished with co-injection of anti-α6 antibody.

Figure Legends

Fig. 5.1. Adhesion of K1735 variant cell lines to laminin and E8. C23 (*A*) and M2 (*B*) cells were processed for adhesion to laminin (solid bars) and the E8 fragment (hatched bars) in the presence of the indicated dilutions of rat monoclonal antibody against the α6 integrin subunit (GoH3), polyclonal antibody against the β1 integrin, or polyclonal against the E8 fragment of laminin; the dilution of the hybridoma supernatant or concentration per ml of rabbit IgG is given. The number of adherent cells was determined as described in "Experimental Procedures." Values are the mean of triplicate wells; bars show S.D.

Fig. 5.2. Migration of K1735 melanoma variants over laminin substrates. (*A*) Relative migration of highly metastatic M2 cells (•) and nonmetastatic C23 cells (■) on laminin. Assay was performed as described in "Materials and Methods". (*B*) Comparison of M2 (solid bars) and C23 (hatched bars) cell migration on substrates coated with laminin, laminin E8, and fibronectin at 10, 5, and 20 μg/ml respectively. (*C*) Inhibitory effect of polyclonal anti-E8 and anti-β1 antibodies on M2 cell migration on laminin-coated substrates. (*D*) Inhibitory effect of monoclonal anti-α6 antibody (GoH3) on M2 cell migration on laminin. A control monoclonal antibody (AIIB2) is a negative control. For A-D, values are means of at least 8 individual measurements; bars show S.D.

Fig. 5.3. Identification of β1 integrins in K1735 melanoma variant cell lines. (*A*) Immunoprecipitation of β1 integrins. C23 and M2 cells were surface-labeled with ^{125}I and processed for immunoprecipitation with polyclonal antibodies to integrin α subunits (α1,

α2, α3, α5, α6, listed at the bottom of each lane). The immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions followed by autoradiography. C23 cells, left panel; M2 cells, right panel. The positions of the α and β1 chains are given.

(*B*) Flow cytometry measurement of α6 integrin expression. C23 and M2 cells were reacted with monoclonal antibody to the α6 subunit (GoH3), then stained with secondary FITC-labeled antibody and analyzed by FACS analysis. The profile marked "o" represents the background level of C23 cells with secondary antibody only. M2 cells had high levels of expression of α6 while C23 cells had almost no detectable signal.

(*C*) RT-PCR for α6A and α6B. RNA samples were processed using α6 primers directed to the extracellular domain near the transmembrane region and cytoplasmic domain near the end of the coding region for two 30 cycle rounds of amplification as described in "Experimental Procedures." The upper band (~510 bp) is derived from the α6A mRNA while the lower band (~385 bp) is generated from the α6B mRNA. Note that the metastatic (M2, M4, C26) have exclusively the A form of α6 while the nonmetastatic cells (C19, C23) have a mixture of both A and B. The intermediate band visible in the C19 and C23 cells is an artifact arising from the hybridization of the A and B forms.

Fig. 5.4. Detection of the α7β1 laminin receptor in C23 and M2 melanoma cells. C23 and M2 cells were metabolically ^{35}S-met/cys labeled (*A*) or surface ^{125}I-labeled (*B*) and the cell lysates were processed for laminin-Sepharose affinity chromatography. After washing with running buffer containing 300 mM NaCl, bound receptor was eluted with 10 mM EDTA. The samples in *A* and *B* were separated in SDS-PAGE under nonreducing conditions (left panel) and reducing conditions (right panel); *B* shows reduced samples

only. Lanes are numbered according to fractions from laminin-Sepharose columns after elution with 10 mM EDTA. The positions of α7 and β1 subunits are indicated. Note that the nonmetastatic C23 cells have much higher levels of α7 than the metastatic M2 cells.

Fig. 5.5. Immunoblot analysis of the α6 and α7 subunit in K1735 variant cell lines. The highly metastatic M2, and nonmetastatic C19 and C23 cells were compared with the C23m cells, derived from a single pulmonary metastases that formed after subcutaneous injection of C23 cells. Cell lysates were subjected to SDS-PAGE (nonreducing) at 50 μg protein/lane, transferred to membrane and probed with rabbit polyclonal antibodies to α6A and α7B cytoplasmic domains. Molecular mass is shown in kDa. A major breakdown product of α6A (at ~80 kDa) and of α7B (at ~70 kDa) appears to correspond to a proteolytic fragment produced by each.

Fig. 5.6. Comparison of α6 and α7 mRNA expression levels in a panel of K1735 variant cell lines. Detection of α6 and α7 mRNA transcripts in K1735 variant cell lines. Samples of total cell RNA (25 μg/lane) were separated in agarose gels, blotted onto membranes, and probed with ^{32}P-labeled α6 (*A*) or α7 (*B*) cDNA. The C10, C19, C23, cell lines are nonmetastatic while the C26, M2, M4 cell lines are highly metastatic. The α6 and α7 transcripts had the expected size of 6.0 and 4.1 kb, respectively.

Fig. 5.7. Analysis of the relative affinity of K1735 cell integrins for laminin. (A) Affinity chromatography of melanoma integrins on E8- and laminin-Sepharose columns. Parental K1735 cells, whose repertoire of integrins contains a mixture of all known β1 laminin-binding complexes, were surface-labeled with ^{125}I and processed for chromatography on

E8- (*lanes 1-14*) and laminin- (*lanes 15 and 16*) Sepharose in the presence of Mn^{2+}. Columns were loaded with cell lysates (*lanes 1 and 8*) and after washing with running buffer, the column was washed with 200 mM NaCl (*lanes 2-4 and 9-11*), and then eluted with 10 mM EDTA (*lanes 5-7 and 12-14, and 15-16*). Samples were processed for SDS-PAGE under nonreducing (*lanes 1-7 and 15*) and reducing (lanes 8-14) conditions. α6β1 was selectively eluted from the E8 column with 0.2 NaCl (*lanes 2-3, 9-10*), while α7β1 was resistant to NaCl elution but was eluted with EDTA (*lanes 5-7, 12-14*). α1β1 did not bind to E8 columns but a fraction of this receptor bound to intact laminin after washing with 200 mM NaCl and was subsequently eluted with EDTA (*lanes 15 and 16*); α7 bound to both E8 and laminin columns.

(*B*) Effect of Ca^{2+} on integrin binding to laminin. K1735 parental cells were ^{125}I-surface-labeled and processed for laminin-Sepharose chromatography. Cell lysates were prepared in the presence of 1 mM Mn^{2+} and applied to the column as in (*A*) above. The column was washed with running buffer containing 50 mM Tris-HCl (column fractions 1-6), then successively eluted with 200 mM NaCl (column fractions 7-11), with low salt buffer containing 10 mM Ca^{2+} instead of 1 mM Mn^{2+} (column fractions 12-16), and finally with 10 mM EDTA (column fractions 17-19). Samples of selected fractions were analyzed by SDS-PAGE; *lanes 1-2*, 200 mM NaCl fractions 7,8; *lanes 3-6*, Ca^{2+}-eluted fractions; *lanes 7-9*, EDTA eluted fractions 17-19. α1 and α6 were effectively eluted while α7 was only partially eluted with Ca^{2+}.

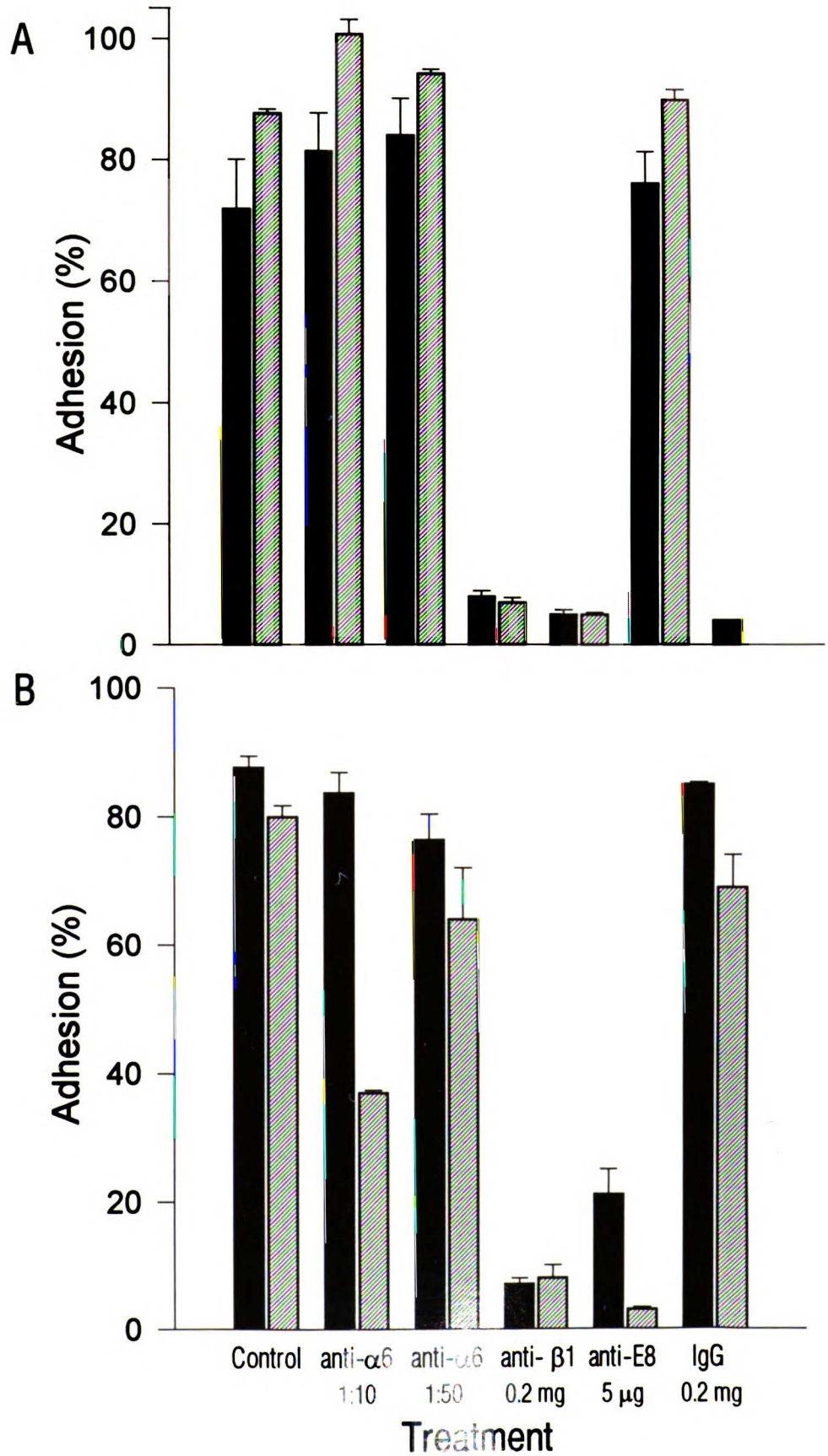
(*C*) Effect of Mg^{2+} on integrin binding to laminin. K1735 parental cells were ^{125}I-surface- labeled and processed for laminin-Sepharose chromatography. *Lane 1*, running buffer alone containing 50 mM Tris-HCl and mM Mg^{2+}. *Lanes 2-4*, running buffer

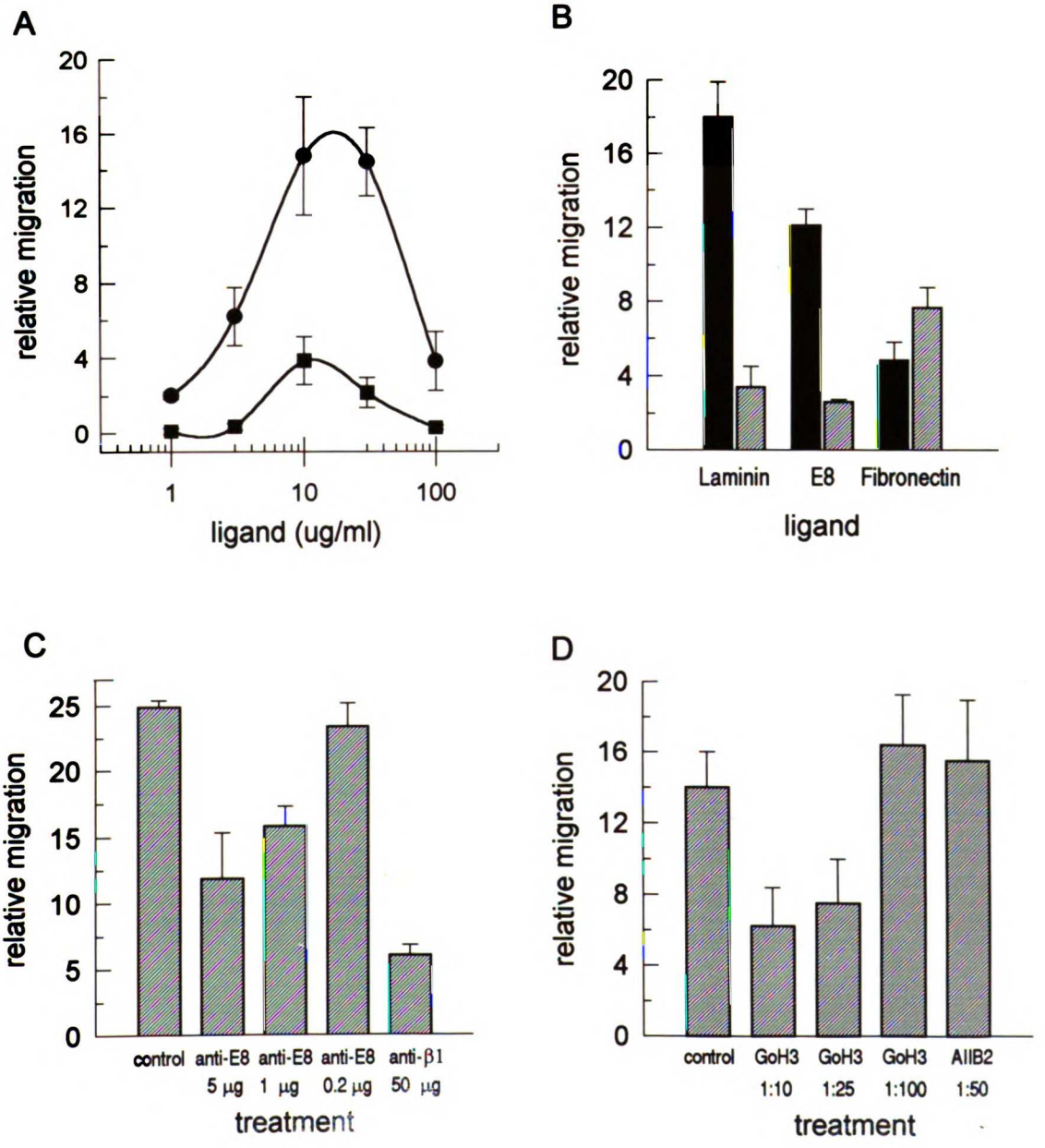
containing 10 mM Mg^{2+} instead of Mn^{2+}. *Lanes 5-7*, running buffer containing 10 mM Ca^{2+} instead of Mn^{2+}. *Lanes 8-10*, EDTA elution. α1 and α6 were eluted by Mg^{2+} while α7 remained bound to the column.

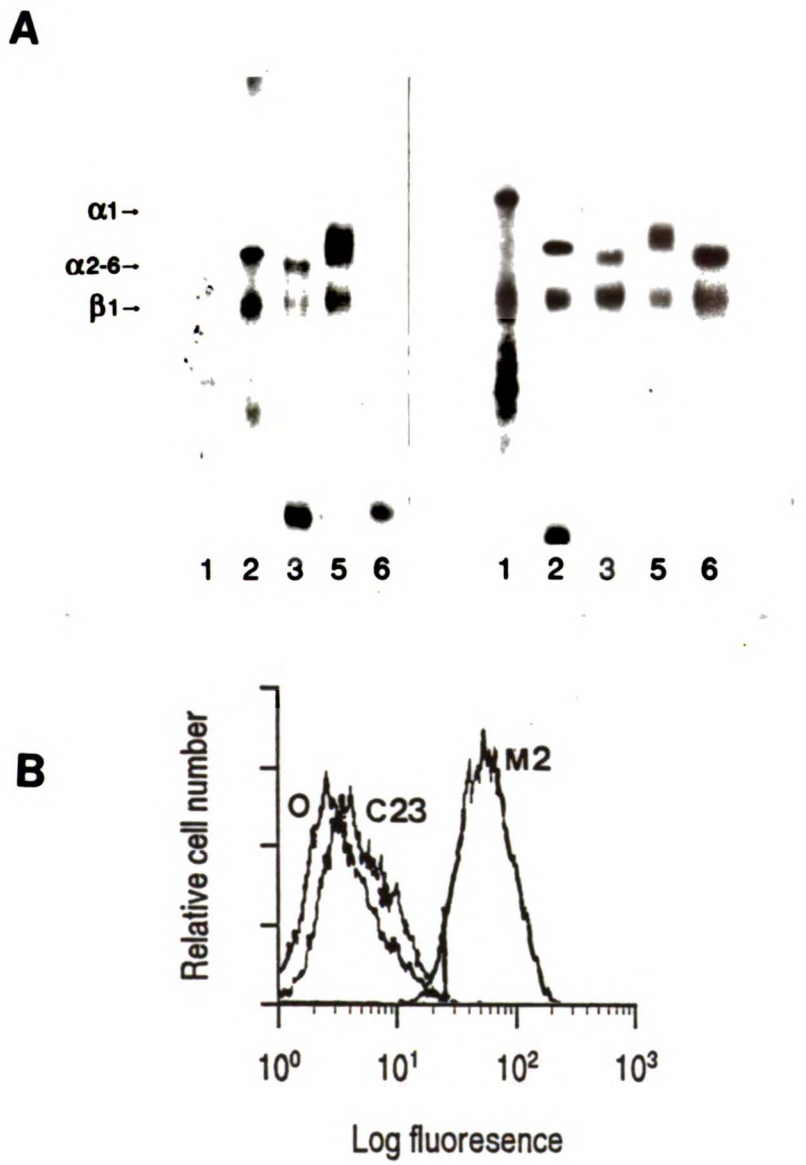
(*D*) Effect of NaCl on integrin binding to laminin. K1735 parental cells were ^{125}I-surface-labeled and the cell lysate processed for laminin-Sepharose chromatography in the presence of 1 mM Mn^{2+} as the divalent cation. After loading of the lysate, the column was first washed with low salt (50 mM Tris-HCl) running buffer *(lanes 1-2)* and then eluted with a step gradient of NaCl: 50 mM (*lanes 3-4*), 100 mM (*lanes 5-7*), 200 mM (*lanes 8-10*), 500 mM (*lanes 11-13*), 1 M (*lanes 14-15*), and 10 mM EDTA (*lanes 16-18*). α6 was eluted in the low salt buffer, while α1 was mostly eluted by the 100 mM NaCl buffer; α7 was resistant to 1 M NaCl and recovered with EDTA.

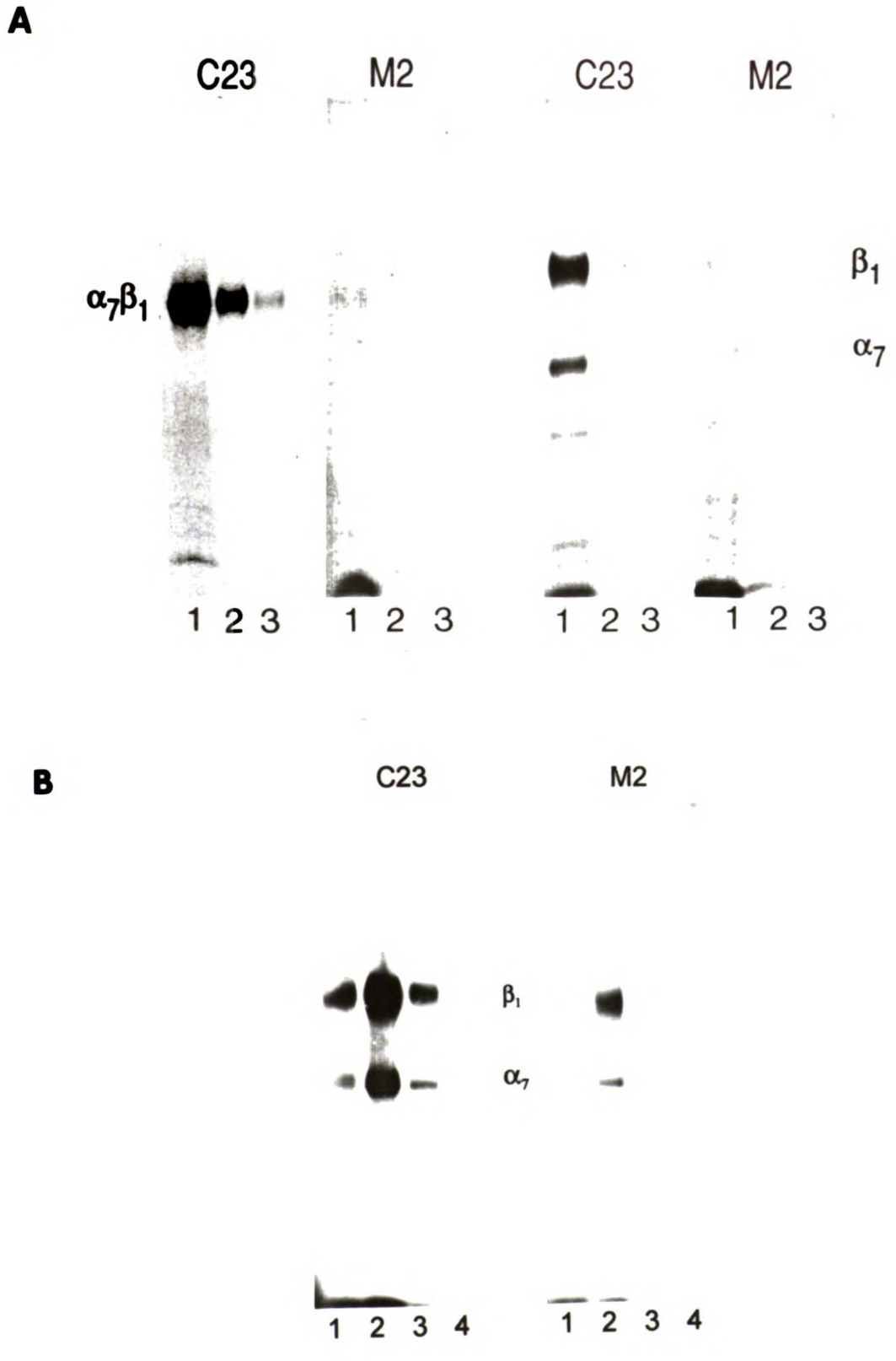
Footnote

[1] The abbreviations used are: DMEM, Dulbeccos's minimum essential medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; PCR, polymerase chain reaction.


A
B
Adhesion (%)
0
20
40
60
80
100
Control
anti-α6 1:10
anti-α6 1:50
anti- β1 0.2 mg
anti-E8 5 μg
IgG 0.2 mg
Treatment


A
relative migration
20
16
12
8
4
0
1
10
100
ligand (ug/ml)
B
relative migration
20
16
12
8
4
0
Laminin
E8
Fibronectin
ligand
C
relative migration
25
20
15
10
5
0
control
anti-E8 5 µg
anti-E8 1 µg
anti-E8 0.2 µg
anti-β1 50 µg
treatment
D
relative migration
20
16
12
8
4
0
control
GoH3 1:10
GoH3 1:25
GoH3 1:100
AIIB2 1:50
treatment


A
α1→
α2-6→
β1→
1 2 3 5 6 1 2 3 5 6
B
Relative cell number
O
C23
M2
10⁰ 10¹ 10² 10³
Log fluoresence
C
M2 M4 C19 C23 C26


A
C23
M2
C23
M2
$\alpha_7\beta_1$
β_1
α_7
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1 2 3
1 2 3
1 2 3
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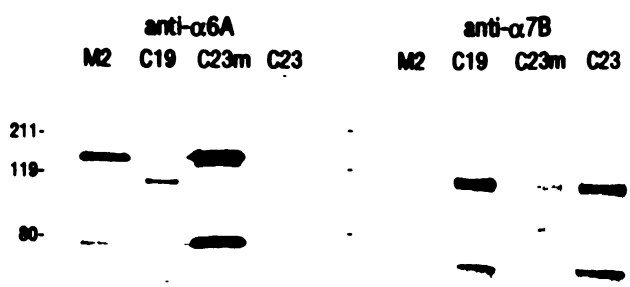
β_1
α_7
1 2 3 4
1 2 3 4


anti-α6A
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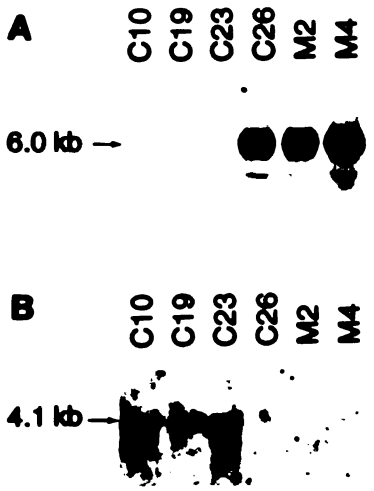
C19
C23m
C23
anti-α7B
M2
C19
C23m
C23
211-
119-
80-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C10
C19
C23
C26
M2
M4
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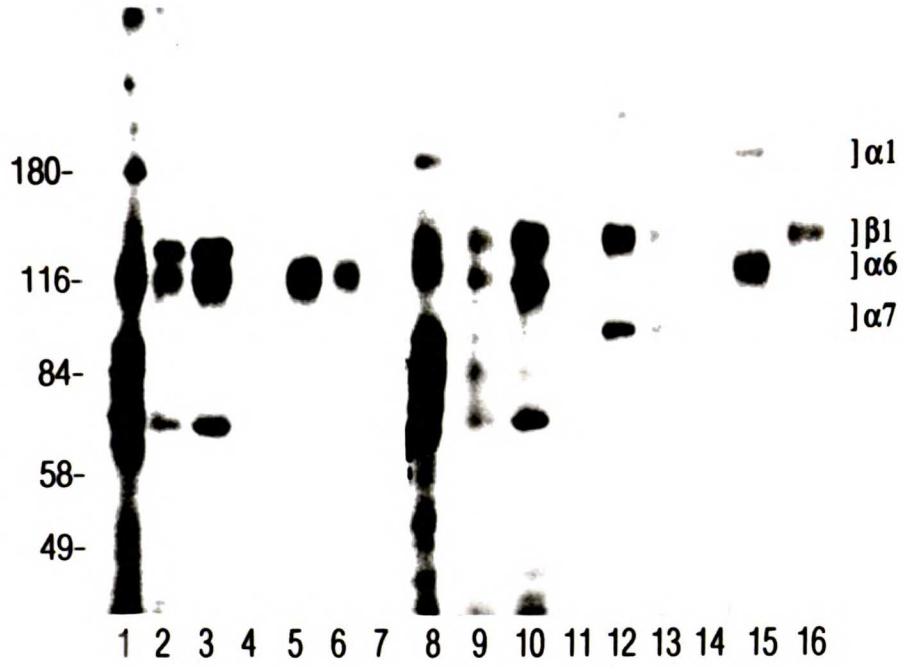
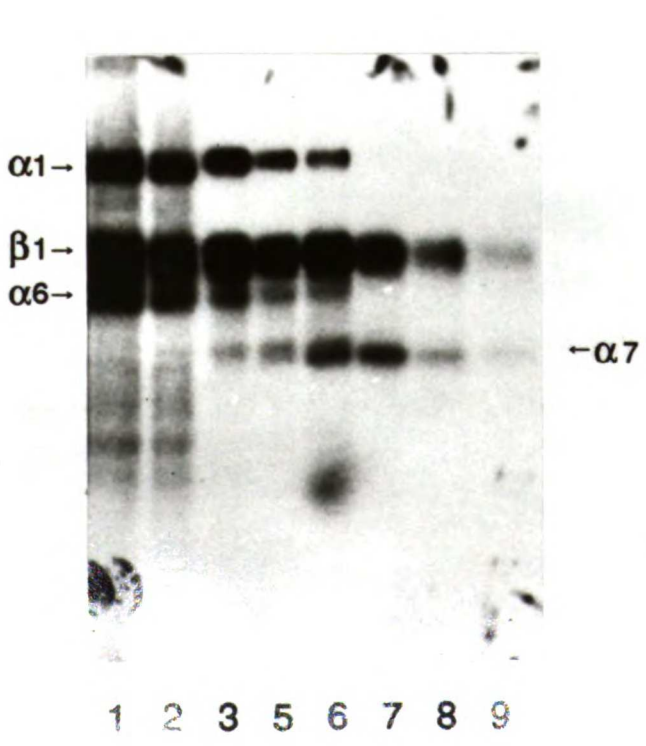
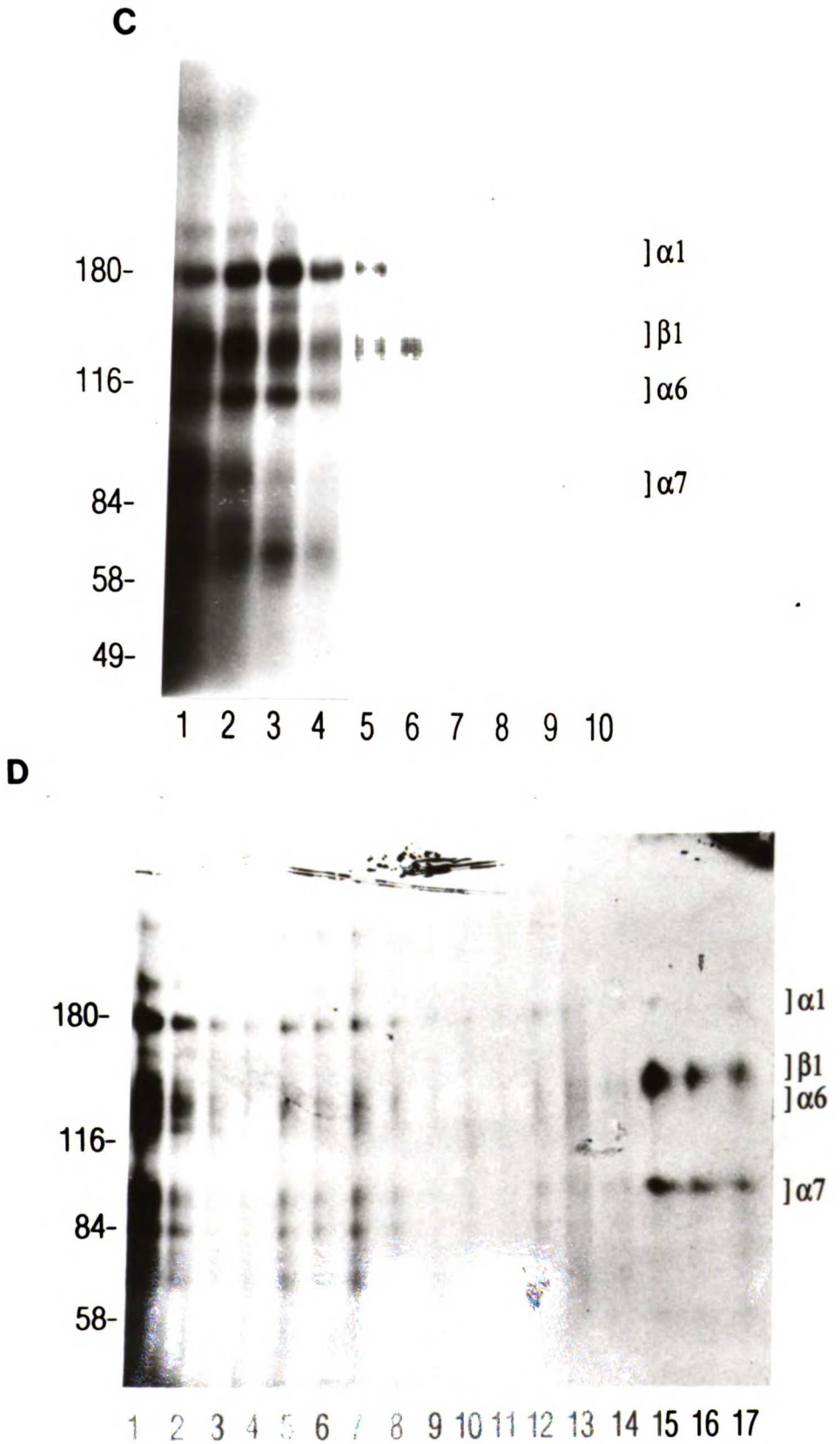
B
C10
C19
C23
C26
M2
M4
4.1 kb

A


180-
116-
84-
58-
49-
]α1
]β1
]α6
]α7
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

B


α1→
β1→
α6→
←α7
1 2 3 5 6 7 8 9


C
180-
116-
84-
58-
49-
]α1
]β1
]α6
]α7
1 2 3 4 5 6 7 8 9 10
D
180-
116-
84-
58-
]α1
]β1
]α6
]α7
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

CHAPTER SIX

DISCUSSION

The development of metastases, a highly selective process, depends on the existence of tumor cell variant populations within the primary tumor. Certain subsets of cells with the appropriate phenotype can survive the rigors of the metastatic cascade and successfully establish metastases. It is likely that a successful phenotype includes a suitable array of integrin receptors that could directly influence the adhesive properties of the cell. The availability of tumor cell lines with distinct metastatic profiles allowed us to study and compare β1 integrin expression and function in these cells. Recent observations suggest that integrin receptors mediate cell interactions with laminin (Ramos *et al.*, 1990; Hall *et al.*, 1990; Languino *et al.*, 1989). In our studies, a major emphasis was placed on the role of laminin-binding integrins in tumor cell adhesion, migration and invasion.

We determined that the HT1080 human fibrosarcoma cell line expresses multiple putative laminin receptors including α2-α3-and α6β1. The ability of specific monoclonal antibodies to α6 (GoH3) to effectively inhibit the attachment of HT1080 cells to laminin suggests that the α6β1 complex is critical in mediating initial attachment to this ligand. Antibodies to α2 inhibit adhesion to collagen types I and IV. The fact that α2 can bind laminin, or collagen in various cell types suggests that the biological response appears to be cell type specific. In this system α2 and α3 do not function as laminin receptors. A given integrin may manifest varying adhesive competence depending on its cellular environment, or even the state of differentiation of the cell (Adams and Watt 1990; Chan and Hemler 1993). Variation in function may be due to changes in ligand binding affinity as occurs with β3 (Bennet and Vilaire, 1989) or β1 (Faull *et al.*, 1993) integrins. Changes in adhesive function may also occur without changes in ligand-binding affinity.

To determine the importance of α6β1 in mediating attachment to a complex

matrix, we seeded HT1080 cells on reconstituted basement membrane (RBM). The cells were able to attach firmly and spread upon this matrix. When pre-incubated with GoH3, and then seeded on the RBM, adhesion was significantly inhibited. In a 5 h spreading assay, the cells eventually spread in the presence of GoH3 but were not able to spread effectively. This indicates the presence of other receptors mediating adhesion to basement membranes. Similarly, antibodies against β1 prevented cell spreading while allowing cell adhesion. HT1080 cells express integrin receptors not only to laminin but also to type IV collagen, a major component of the basement membrane. The results also support the presence of several receptor systems, one for attachment and one for spreading and migration as has previously been suggested by Runyan *et al.* (1988). Using integrin-specific antibodies we observed that HT1080 cells express α2β1, α3β1, α5β1, and that α2β1, and α3β1 are expressed in relatively greater amounts than α6β1. Languino *et al.* (1989) showed that α2β1, a known collagen receptor, is also a laminin receptor. However in our experiments antibodies against α2 were effective in blocking adhesion to collagen I and IV but were ineffective in blocking adhesion to laminin. This suggests that in this system α2β1 may be important as a collagen receptor but other receptors such as α6β1 are active in adhesion to laminin. We demonstrated that antibodies to α6β1 block invasion through RBM. Basement membranes (BM) are composed of several macromolecules, yet antibodies to α6β1 significantly reduced cell attachment spreading and invasion. Thus laminin, a major component of basement membranes appears to play an important role in the invasion process despite the presence of other adhesion molecules. Receptors for laminin have previously been implicated in tumor cell metastasis by Liotta and collaborators (Liotta *et al.*, 1986).

Since laminin appears to play a significant role in the metastatic cascade, we

examined the highly invasive B16-BL6 cell line for their expression of laminin-binding integrins. The results of this study supported our prediction that B16-BL6 cells express β1 containing integrin complexes that bind to laminin and collagen. This was the first report that identified integrin complexes specific for laminin and collagen in the B16 system. Using ligand affinity chromatography and immunoprecipitation with specific anti-α6 antibodies we demonstrated that α6β1 is also the major laminin-binding complex in these cells. The majority of the α6β1 complex is eluted from the laminin-Sepharose column with 0.2 M NaCl, with the remainder of this complex eluted with 10 mM EDTA.

The B16-BL6 cells express another laminin-binding integrin, the α1β1 complex. The affinity of α1β1 for laminin appears to be lower than α6β1, as we needed to shorten the washing phase, in order to consistently recover the α1β1 complex. Together these results suggest that both α6β1 and α1β1 bind to laminin with low efficiency, since neither complex is specifically eluted with EDTA. This low affinity interaction may enhance the highly metastatic phenotype by which tumor cells need to interact with a given substrate, but not to such an extent as to restrict motility. Using ligand affinity chromatography, we also demonstrated that B16-BL6 cells also use α1β1 to bind collagen IV. However, unlike its association with laminin, α1β1 interacts with collagen IV with higher affinity as the complex is only eluted from the type IV-Sepharose using EDTA. This suggests that the cellular "microenvironment" influences how a given receptor will interact with its ligand. For example, α1β1 binds laminin with extremely low affinity in B16 melanoma cells, yet in the same cell type mediates much higher affinity interactions with collagen IV.

The α6β1 and α1β1 integrins bind to different regions within the laminin molecule. α6β1 binds to the bottom of the long arm of the A chain (E8). α1 is believed

to bind to the center of the cross (P1). More recent evidence demonstrates that α1β1 in neural crest cells binds to the B1-A-B2 mid-portion (T'8) of the E8 domain (Lallier *et al.*, 1993). Although both α1β1 and α6β1 bind to laminin with relatively low affinity, it is attractive to suggest that upon binding to the different regions of the laminin molecule, conformational changes occur in the integrin molecule and these changes mediate an outside-inside signalling resulting in a variety of cytoplasmic events which may influence the metastatic phenotype.

We previously demonstrated that B16-BL6 murine melanoma adhesion to complex matrices and individual basement membrane proteins such as laminin and collagen type IV could be inhibited by antibodies to the β1 integrin subunit (Kramer *et al.*, 1989). In the present studies, we determined that α6β1 is important in mediating initial adhesion of B16-BL6 cells to laminin substrates as demonstrated by the sensitivity to GoH3 monoclonal antibody. However the inhibition was not always complete, suggesting that adhesion to laminin is mediated by contributions by both α1β1 and α6β1.

Although β1 could be localized to focal adhesions, we were unable to identify α6 in such structures in cells plated on laminin. Formation of focal contacts is associated with the stationary phenotype, therefore the inability to identify α6β1 focal adhesions on laminin is the B16 cells may be indicative of a more motile and hence migratory/metastatic phenotype.

The B16 murine melanoma has been highly characterized and used extensively as a model to study organ-specific metastasis. We continued to use this system to pursue an *in vivo* approach to understand metastasis. Other groups selected tumor cell populations from B16 melanoma which preferentially metastasize to various organs, such as the lungs, liver, ovary, brain and other sites (Fidler, 1973; Tao *et al.*, 1979; Brunson

et al., 1979; Miner *et al.*, 1982; Schackert and Fidler, 1988). We wanted to evaluate potential differences in adhesion and migration between poorly metastatic and highly metastatic B16 cells. Using the B16-F1 cell line, we selected and characterized tumor cell subpopulations from the parental population which are efficient in targeting para-aortic lymph nodes. One cell population was intensely melanotic (B16PA4M) while the other cell type was amelanotic (B16PA4A). Our results confirm the work of others which indicates that within the B16 parental cell line exist subpopulations of cells that are efficient in forming lymph node metastases (Miner *et al.*, 1982; Schackert and Fidler 1988; Nicolson and Winkelhake 1975).

The metastatic variants (B16-PA4A and B16-PA4M) were isolated and adapted to culture conditions. In a monolayer wound assay, tumor cells were grown to confluency on tissue culture plastic in the presence of 5% fetal bovine serum, and "wounded" with a plastic pipette tip. Cells were monitored at several different time points and relative migration was determined. In all wound assays, the metastatic variants (B16PA4A and B!6PA4M) were much more motile compared with the parental (B16F1) cell population. The enhanced migratory properties of the variants coincide with the enhanced metastatic potential compared with the poorly metastatic phenotype of the parental B16F1 cell line, suggesting that the metastatic properties of the tumor cell variants are retained *in vitro*. Although both the parental and variant cell lines adhered to laminin, fibronectin, collagens types I, III, and IV, the metastatic variants did not adhere as effectively. This is not surprising, since metastatic cells need to interact transiently with the ECM to maintain the motile and invasive phenotype.

Our laboratory previously demonstrated that reticular fibers of the lymph node are composed of a core of type I and III collagen decorated with the basement membrane

molecules laminin and type IV collagen (Kramer *et al.*, 1988). Immunofluorescence staining for reticular fibers laminin showed a good correspondence between attachment of tumor cells and location of fibers within the node. Laminin, is a known chemotactic ligand for some cell lines. One could speculate that these basement membrane-rich reticular fibers may attract and aid in tumor cell recruitment and lymph node arrest during metastasis.

Having generated tumor cells which efficiently targeted lymph nodes, we examined their adhesion to lymph node matrices. We showed previously that β1 integrins are involved in B16 melanoma adhesion to purified ECM proteins and complex matrices like human amnion basement membrane. We now show that B16 adhesion to isolated lymph node reticular fibers is mediated by integrins as demonstrated by total inhibition of adhesion to lymph node ECM in the presence of anti-β1 GP-140 (ECM_R), a polyclonal antibody which recognizes β1 and β3 integrins. Not only do integrins mediate adhesion to isolated matrix molecules and reconstituted basement membranes but also appear to be functionally active in adhesion to *in vivo* matrices found within human amnion and lymph node reticular fibers (Kramer *et. al.*, 1989; Berston *et. al.*, 1994). Brodt *et al.*, (1991) provide evidence that human melanoma cell interactions with lymph node sections are mediated by αvβ3 integrins. Our data, in conjunction with Brodt *et al.*, (1991) demonstrate that integrins mediate human and mouse melanoma adhesion to lymph node matrices.

Tumor cell interaction with laminin via integrins appear to be critical to achieve a successful metastasis. We have identified the α6β1 complex as the major laminin-binding integrin in both the highly invasive HT1080 fibrosarcoma cells and in the highly

metastatic B16 murine melanoma (Ramos *et. al.*, 1990; Ramos *et. al.*, 1991). Although the major laminin-binding integrin may be the α6β1 in the B16 cell line, we also demonstrate that the expression of even trace amounts of additional laminin receptors contribute to laminin interactions (i.e. α1β1).

Obviously there is more than one factor which contributes to the metastatic phenotype, however tumor cell interactions with laminin appear to be important in enhancing metastasis. We next examined the highly characterized K1735 murine melanoma cell lines which have been selected for both high and low metastatic potential. Results obtained with several of the K1735 cells indicate that the highly metastatic cells show accelerated migration on laminin substrates while the nonmetastatic cells show little motility on the same substrates. The β1 class of integrins mediate attachment of both the C23 and M2 cells to laminin and E8 fragment as indicated by the blocking effect of β1 antibodies. β1 was also crucial for migration of M2 cells on laminin since blocking antibodies ablated migration.

While GoH3 did not block adhesion to intact laminin in either the M2 or C23 cells, this antibody significantly inhibited adhesion to the E8 fragment by the M2 cells but not the C23. This sensitivity of M2 cells to GoH3 is consistent with their high expression of α6 as determined by immunoprecipitation and FACS. For the M2 cells, α6 bound to E8-Sepharose or intact laminin-Sepharose. In addition, α1 bound to intact laminin but failed to bind to E8 fragment, suggesting the α1 which is strongly expressed on M2 cells confers resistance to anti-α6 blocking antibody seen in adhesion assays with intact laminin. However, M2 migration on laminin could be significantly inhibited by GoH3 implying, α6 plays a more prominent role in migration than it does during initial adhesion to laminin. These results indicate that for the M2 cells, interaction with laminin

is mediated by a subset of β1 integrins that includes α1 and α6.

A number of studies suggest α6 maybe upregulated on highly metastatic melanoma. Albelda *et al.*, (1990) found that by immunostaining, α6 is present in not only primary melanoma but also in vertical growth phase and metastatic lesions. In recent studies (Etoh *et al.*, 1993; Vink *et al,* 1993) it has been shown that α6 as well as α2 were upregulated in cells derived from human metastatic melanoma but not in primary nonmetastatic cells. Similarly, Danen *et al.,* (1993) reported that in a series of metastatic melanoma cell lines that both α2 and α6 were also increased but not from primary non metastatic tumor cells. Analogous results were also found by Mortarini *et al.*, (1991).

Under the conditions used we did not observe significant binding of α2 or α3 to murine laminin or its E8 fragment, in studies with parental K1735 cells, M2 or C23 variants, even though these receptors are expressed by all three cell lines at significant levels. The lack of α3 binding to EHS-derived A-chain laminin has been observed previously in both rodent and human systems (Goodman *et al.*, 1991; Delwel *et al.*, 1994), but contrasts with studies reporting that α3 binds to laminin or anti-α3 antibodies could block adhesion to laminin (Wayner and Carter 1987; Elices *et al.*, 1991). It has subsequently been shown α3 binds efficiently to kalinin (Carter *et al.*, 1991). It is well established α2 can function as both a collagen and laminin receptor, however α2 functionality is activation- and cell type specific and can be modified by various cellular mechanisms, including PKC dependent pathways and externally by β1-activating antibodies (Masumoto and Hemler 1993). In the case of the K1735 cells, α2 and α3 appear to be inactive toward laminin and its E8 fragment.

In the case of the poorly metastatic C23 cells, several lines of evidence indicate

that the primary laminin receptor is α7β1. First α7β1 is the most abundant laminin receptor identified in the C23 and appears to account for more than half of β1 integrins on these cells (Ramos et al., in preparation). Second, blocking antibodies to α6 have no effect on C23 adhesion to laminin and the E8 fragment. Thirdly, α7 is the only integrin in C23 with functional activity as detected by ligand chromatography on laminin- or E8-Sepharose columns, even under highly permissive conditions (Mn^{2+} as the divalent cation, and low ionic strength). Fourth, cell adhesion to laminin is blocked with anti-E8 antibodies, consistent with the known binding site for α7 (Kramer et al., 1991). Fifth, α7 is prominently concentrated in vinculin and talin positive focal adhesion contacts in C23 cells adherent to laminin. Recent work has shown that many integrin receptors require activation before they can bind ligand (Hynes 1992, Ginsberg *et al.,* 1992). The mechanisms that modulate receptor activity and specificity are not completely understood but certain evidence suggests that receptor function is controlled post-translationally at different levels. In particular, integrin activity and specificity appears to be under the control of the cellular microenvironment.

Numerous factors can alter receptor function including divalent cations, lipids, status of the cytoplasmic domain, and binding of certain β1 activating antibodies. For example, as mentioned above α2β1 can be inactive, or bind collagen, or bind collagen and laminin. Its cell type specific behavior can be modified by phorbal esters and by activating antibodies to the β1 subunit.

The type of divalent cation available to the integrin complex has a significant impact on the affinity and specificity of the receptor for its ligand. Integrins require divalent cations for binding their ligands. Additionally, divalent cations induce multiple effects that include stimulating or inhibiting ligand binding or altering ligand specificity.

The physiological relevant divalent cation mix is Ca^{2+}-rich with significant Mg^{2+} and decreased concentration of Mn^{2+}. However, for several integrins in solubilized form Ca^{2+} alone is not sufficient for activity and Mg^{2+} or Mn^{2+} is required. In particular Mn^{2+} is a potent activator of integrin function. In our study we find that α1, α6, and α7 integrins bind to laminin with different affinities and their binding could be modulated by the type of divalent cation and by the ionic strength of the buffer. In the presence of Mn^{2+}, α6 was eluted between 50-100 mM NaCl and α1 was displaced in 50-100 mM NaCl. However α7 was resistant to even 1 M NaCl, reflecting its high affinity interaction with laminin. Intermediate stringency conditions of chromatography that included 200 mM NaCl and Mg^{2+} instead of Mn^{2+} caused immediate elution of α6 and partial elution of α1 whereas α7 remained bound to the laminin column. Substituting Ca^{2+} for Mn^{2+} or Mg^{2+} caused the immediate elution of α1 and α6 and eventually the elution of α7. This response to Ca^{2+} is similar to the behavior of several other integrins which lose ligand binding function in the presence of increased Ca^{2+} concentrations (Grzesiak *et al.*, 1992)

In other studies, Forsberg *et al.*, (1990) reported that the rat α1 receptor on laminin columns was not detected in EDTA eluates when Ca^{2+}/Mg^{2+} buffer was used but could be isolated when $Mn^{2+}$was substituted. These results indicate fundamental differences in the interaction of each of these receptors with laminin. Furthermore, this suggests that α1 and α6 may be expected to exhibit low affinity binding under physiological concentrations of Ca^{2+}, Mg^{2+}, and NaCl. In a recent study by Michishita *et al.* (1993), a structural basis for the relatively high affinity Mn^{2+} binding site that could not be competed by Ca^{2+} was identified in the αM integrin subunit that is homologous to the first divalent cation site on other α integrins. This implies that small amounts of Mn^{2+} may be physiologically relevant in modulating integrin function.

Several studies show that even low concentrations of Mn^{2+} (0.1 mM) can activate integrin function even in the presence of Ca^{2+}. In intact cells, integrin receptors condense into focal adhesion contacts with their associated cytoskeletal components. This complex provides a mechanism to allow receptors that are individually of low affinity but are able to establish high affinity interaction which represents a summation of integrins.

We suggest that one factor that may regulate melanoma response to laminin is the expression level of the α7 receptor. Since a major difference in the two cell types appears to be the expression of either the α6 or α7, it is possible that the two integrins may transduce different cytoplasmic signals when bound to their common ligand, the E8 domain of laminin. Alternatively it may be the extracellular domain that confers the different affinities between the two integrins. Sequence analysis of the α6 and α7 subunits corresponding to ligand binding regions show high homology. The different affinities seen on ligand columns may be due to structural differences in the extracellular domains or may reflect a mechanism related to divergent cytoplasmic domains of the 2 α chains. It is generally believed that the α cytoplasmic domain may regulate the β cytoplasmic tail interaction with the cytoskeleton. Thus the existence of cytodomain variants could regulate the quality and strength of signal input from the extracellular environment and vice versa.

In further support of our suggestion that α6β1 assists in promoting the metastatic phenotype. We generated a metastatic variant of the poorly metastatic K1735C23 cell line (K1735C23m). When we harvested the single metastatic colony and adapted it to cell culture we observed high expression of both the α6- and α1β1 complex with a coordinate down expression of the α7β1 complex. This suggests that metastatic tumor cells must downregulate α7β1 or upregulate α6β1 to go through the metastatic cascade.

The work presented here describes several different mechanisms by which tumor cells interact with laminin. In all four studies β1 integrins appear to be involved in the metastatic phenotype. In the metastatic cells evaluated, α6β1 appears to be the primary laminin receptor. This is true for the HT1080 fibrosarcoma cells as well as the B16 and the K1735 murine melanoma cell lines. Non-metastatic K1735 melanoma cells use the α7β1 as the primary laminin receptor and this complex appears to be associated with reduced motility and high affinity to laminin substrates. It appears that we have two different mechanisms by which tumor cells interact with laminin substrates and we suggest these differences influence metastatic potential and are related to alterations in motility and invasion of laminin-rich matrices. Additionally, Albini *et al.*, (1989) demonstrate that among the K1735 melanoma cells, clones with low and high metastatic potential can be discerned on the basis of interactions with laminin. The K1735 melanoma response to fibronectin is essentially the same for the two cell types (high and low metastatic potential). In contrast, the highly metastatic cell lines show enhanced attachment to, spreading on, and migration toward laminin. The highly metastatic cells had~2 fold more high affinity laminin receptors and in addition carry a class of low affinity laminin-binding sites which was lacking in low metastatic cells (Albini *et al.*, 1991).

We suggest that one factor that may regulate the migratory response of melanoma cells to laminin is the level of α6 or α7 receptor. It is likely that α6 and α7 may transduce different cytoplasmic signals when binding to their common ligand, the E8 domain of laminin. Alternatively it may be the extracellular domain that confers different activities on the two integrins, thereby producing distinct migratory phenotypes. On

laminin-affinity columns α7 exhibited a much higher affinity for laminin than did α6. These differences may be due to structural elements in the ectodomain. The cytoplasmic domain of integrins interact with the cytoskeleton and other cytoplasmic proteins. It is through this interface with the cytoplasmic compartment that signals are transmitted to the cell. It is now recognized that functionality of the extracellular domain is influenced by "inside-out" signalling (Ginsberg *et al.* 1992). The existence of cytoplasmic domain variants could regulate the quality and strength of signal input from the extracellular space and vice versa.

Hogervorst *et al.,* (1993) recently demonstrated that α6A and α3A are phosphorylated on serine and weakly on tyrosine. The B forms are not phosphorylated. This suggests that the difference in phosphorylation of the two isoforms may be an important functional regulatory mechanism. More recently, Sonnenberg and collaborators (Delwel *et al.*, 1994) showed that phosphorylation of α6A inhibits ligand binding. Because of the extensive homology between α6 and α7 it is possible that regulation of α6 and α7 at the cytoplasmic domain may involve phosphorylation.

The development of metastasis, a highly selective process, depends on the existence of tumor cell variant subpopulations with the appropriate phenotype that can survive the rigors of the metastatic cascade and establish secondary foci.

The evidence presented in these studies suggest that differential expression of laminin-binding integrins by tumor cells influences the metastatic phenotype. We propose that upregulation of α6 in metastatic cells positively contributes to the metastatic phenotype while expression of α7 negatively influences the metastatic phenotype.

One approach to investigate the role of specific integrins on migration and invasion *in vivo* is to explore the normal migratory processes in development. During

normal murine development, melanoblasts are highly motile and migrate from the neural crest, penetrate the basement membrane and enter the epidermal ectoderm where they become encapsulated into developing hair follicles. During this migration melanoblasts remodel and actively invade the ECM much in the same way tumor cells do. Using this system we can evaluate the role of integrins in the invasive process.

Tyrosinase is regarded as the key enzyme in pigment synthesis. Tyrosinase is expressed before or as soon as melanoblasts enter the follicle, long before melanin is produced and secreted into the growing hair that emerges after birth.

To test our concluding hypothesis that differential expression of laminin-binding integrins by tumor cells directly influences the invasive phenotype, I propose the following : A) Using transgenic mice, examine whether constitutive expression of α6 by melanocytes alters their normal behavior. I will design a recombinant gene comprised of the α6 gene under the control of the tyrosinase promoter (Tyr-α6). This construct will be microinjected into fertilized eggs and the embryo placed in C57BL/6 pseudo pregnant mice. Since this transgene is under the control of a tissue-specific promoter (tyrosinase), theoretically only pigmented cells (ie. melanocytes) should express this Tyr-α6 construct. Southern hybridization analysis of tail biopsies will be done to look for integration of our transgene. These experiments using α6 under the control of a tissue-specific promoter (TYR) will allow us to examine: 1) Whether a constitutive boost in α6 expression effects melanoblast migration. 2) Does early constitutive expression of α6 alter the normal melanocyte phenotype by inducing malignancy?

In contrast we suggest the expression of α7 inhibits the invasive phenotype. As previously mentioned melanocytes are highly migratory during development. To examine an earlier period of melanocyte development we can use another melanocyte specific

gene, Tyrosinase Related Protein-2 (TRP-2). TRP-2 is expressed by melanoblasts 6 days before expression of tyrosinase and 4 days prior to tyrosinase related protein-1 (TRP-1). To target an earlier period (10 dpc) in the melanocyte migratory process, we can use the TRP-2 promoter to construct a TRP-2/α7 transgene. This gene can then be microinjected into fertilized eggs and planted into pseudo pregnant mice. In theory only melanoblasts/melanocytes will express the transgene. Since we propose that α7 negatively influences the metastatic phenotype which includes active migration and invasion, we would expect the migratory properties of the melanocyte to be disrupted (probably somewhat diminished). In this way we can study events usually associated with metastasis (migration and invasion) in normal cells. We could then compare these experiments with the previously described transgenic experiments to: 1) Compare differences between migratory and invasive potential of melanocytes expressing α6 vs. α7. 2) In addition we would be able to determine if α6 is sufficient to promote conversion of normal melanocytes. The transgenic experiments are primarily designed to evaluate the role of α6 and α7 in normal migrating melanocytes. Although it is possible that expressing high levels of α6 in normal melanocytes may confer the metastatic phenotype, it is not guaranteed.

B) However there is a way to produce melanoma by introducing external factors known to induce tumors. Kripke (1979) has previously described a method by which external factors can produce melanoma. Kripke's group used UV light as an initiator and croton oil as a promoter. In our experiments I propose to use transgenic mice carrying TYR-α6 or TYR-α7. The mice will be given a previously described dose of UV (1 hour/day; 5 days/ week x 2 weeks). Each treatment has a total UV dose of $1x10^4$ J/M^2 over the wavelength range of 280-340 nM. Beginning 2 weeks after the last UV

treatment; 0.025 ml of Croton oil in acetone will be delivered to the scapular region of the mice twice a week. This protocol has been effective in producing melanoma. Larue *et al.*, (1992) demonstrated increased expression of melanin in murine melanocytes after exposure to UV treatment which indicates induction of tyrosinase; which in our system would lead to increased expression of either α6 or α7. Melanoma will be evaluated for both expression of α6 and α7 as well as metastatic potential.

(C) Yet another series of experiments will be done to transfect the highly metastatic K1735M2 cells with α7 cDNA. How will expression of α7 in these metastatic cells which do not normally express α7 alter their behavior? Normally the M2 cells are quite motile on laminin using the α6β1 complex and are highly efficient at forming lung colonies *in vivo*. In contrast, the poorly invasive C23 cells, which exclusively use the α7β1 complex to adhere to laminin do not migrate on this substrate and rarely form pulmonary metastases. We expect that the M2-α7 transfectants will have decreased motility on laminin and be less efficient at forming lung colonies compared to the mock transfected M2 cells. A total reversal of the metastatic phenotype is not expected but we do expect significant reduction in metastatic behavior. However, the M2 cells also express the "low affinity" laminin-binding complex α1β1. This complex participates in initial adhesion to laminin and we have seen its expression elevated in metastatic K1735 melanoma. It is yet to be determined what role this complex plays in the metastatic process. Future experiments will be directed to understanding what role α1β1 plays in invasion and metastasis.

The above experiments have been designed to give further insight into determining to what extent differential expression of laminin binding integrin influences the metastatic behavior of melanoma cells as well as the invasive phenotype of melanocytes which

mimic several steps in the metastatic cascade. At the same time these experiments will allow us to see if expression of a single laminin-binding integrin is sufficient to promote the metastatic phenotype. Although several other factors obviously play a role in the metastatic process, such as growth factors, ECM components, proteinases, the expression of integrins contributes to this process and deserves attention. We obviously will not fully dissect the metastatic process with these experiments, but we should have a better understanding of what effect the apparent switching of laminin-binding integrins plays in metastasis.

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