ANALYSIS OF INTEGRINS AND CELL ADHESION ON INVASIVE TUMOR CELL LINES USING AN IN VITRO INVASION ASSAY

by

Ronald Benoit Saulnier

B.Sc., Acadia University, 1988

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Pathology)

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

APRIL, 1991

© Ronald Benoit Saulnier, 1991

Department of _Pathology_

The University of British Columbia
Vancouver, Canada

Date _May 6, 1991_

ABSTRACT

Little is known about the mechanisms which cause tumor cells to become invasive. For this thesis an <u>in</u> <u>vitro</u> tumor cell invasion assay was developed and used to investigate the role of a family of cell surface receptors, called integrins, in the invasion of tumor cells across basement membranes. It was also used to isolate an invasive cell line in order to study some of its properties.

Two osteosarcoma cell lines, HOS and MNNG-HOS, with known <u>in</u> <u>vivo</u> metastatic potentials were assayed in the <u>in</u> <u>vitro</u> invasion assay. Invariably, the highly tumorigenic and metastatic MNNG-HOS cells demonstrated greater invasive ability than the non-tumorigenic HOS cells. The chemical transformation of HOS into tumorigenic MNNG-HOS cells resulted in an increase in the expression of $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_6\beta_1$ integrins which are laminin and collagen receptors. The expression of $\alpha_3\beta_1$ and $\alpha_5\beta_1$ were unchanged on MNNG-HOS cells and the expression of $\alpha v\beta3$ was strongly downregulated on the more invasive cells. The invasion of HOS and MNNG-HOS cells through matrigel could be significantly inhibited when anti-fibronectin receptor or anti-α_6 subunit antibodies were present in the invasion assay, demonstrating the important role of integrins in tumor cell invasion.

The <u>in</u> <u>vitro</u> invasion assay described in this thesis was used to isolate a more invasive cell line from the prostate carcinoma cell line, PC-3, and called IPC-3. The morphology of these cells was distinct from the parent population, showing a spherical morphology as opposed to the triangular or spindle shaped morphology of PC-3 cells. These cells were also several times more invasive than the PC-3 cells and proliferated at a faster rate than the parent PC-3 cells. IPC-3 cells gradually lost their invasive potential after several months in tissue culture but retained their morphology and the characteristic expression of integrins. Adhesion of PC-3 and IPC-3 cells to

purified extracellular matrix components revealed that IPC-3 attached well to laminin and to vitronectin. In adhesion kinetic experiments to purified extracellular matrix proteins, IPC-3 cells attached more quickly than PC-3 cells to laminin and vitronectin. Although the IPC-3 cells attached to the extracellular matrix proteins, fibronectin, vitronectin, laminin and collagen type IV, they were only able to spread on laminin and required several hours to do so. PC-3 cells also attached well to the extracellular matrix proteins but required only several minutes to spread on the matrix proteins including laminin. When plated on stock matrigel PC-3 cells organized themselves in tube-like structures while IPC-3 cells aggregated in clusters.

Analysis of the integrins on PC-3 and IPC-3 cells demonstrated that IPC-3 cells downregulated the expression of the $\alpha_1\beta_1$, $\alpha_2\beta_1$ and an almost completely downregulated the $\alpha_3\beta_1$ integrin while the expression of the fibronectin receptor, $\alpha_5\beta_1$, and the vitronectin receptor, $\alpha_v\beta_3$, were unchanged. The expression of $\alpha_6\beta_1$ in both PC-3 and IPC-3 cells was not prominent. However the $\alpha_6\beta_4$ receptor was present in large amounts and was upregulated in IPC-3 cells, particularly the 200 kDa subunit of β_4.

Immunofluorescence staining of PC-3 and IPC-3 cells demonstrated that PC-3 cells distributed their $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrin receptors mainly along the cell periphery and their $\alpha_v\beta_3$ receptor in focal adhesion plaques, while the invasive IPC-3 cells concentrated their integrin receptors in circular adhesion structures.

Although much remains to be learned about integrins, they have an instrumental role in the invasion of tumor cells across basement membranes during the metastatic cascade of malignant cells.

TABLE OF CONTENTS

Table of Contents

Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BSA   bovine serun albumin

CPM   counts per minute

DMEM   Dulbecco's Modified Eagle Medium

DPM   disintigrations per minute

EDTA   Ethylenediaminetetraacetic acid

FCS   fetal calf serum

MHC   major histocompatibility complex

PBS   phosphate buffer saline

PMSF   phenylmethylsulfonyl fluoride

RIPA   radioimmunoprecipitation assay

SDS-PAGE   sodium dodecyl sulfate polyacrylamide gel electrophoresis

TIMP   tissue inhibitor of metalloproteinases

amino acids

R   arginine

G   glycine

D   aspartic acid

Y   tyrosine

I   isoleucine

S   serine

L   leucine

E   glutamic acid

Acknowledgments

I would like to express my sincerest gratitude:

INTRODUCTION

Overview

The management and treatment of cancer patients is a growing concern with the increasing number of cancer patients being diagnosed each year. Many patients have benign neoplasms which are not life-threatening and can be treated with surgery. However, of greater importance are the individuals who have malignant tumors capable of metastasizing. Metastasis is defined as the spread of malignant tumor cells from their origin to a distant site by one of three possible routes:(i) the bloodstream, (ii) the lymphatic system or, (iii) across body cavities. It is these patients having malignant tumors which are of concern. For many of these patients the treatments currently available are not sufficient to eradicate the entire tumor. The few cells that survive treatment will reestablish new lesions and eventually lead to the patient's death (Fidler et al., 1978).

One of the more serious problems in treating cancer is that malignant tumors have often metastasized before they are clinically detectable. Often, at the time of diagnosis, a very large number of cells have established themselves at distant sites making surgery a futile attempt at removing the lesion. Chemotherapy and radiation therapy are sometimes beneficial but often make the patient very ill. More advanced and sophisticated methods for treating cancer are therefore required. In order to develop better methods, we must first understand more about the mechanisms involved in tumor cell invasion and metastasis and more about the properties of malignant cells.

PATHOGENESIS OF METASTASIS

Tumor cell metastasis is a very complex multi-step process. In order for metastatic lesions to be established, the tumor cell must successfully complete several steps during the metastatic cascade (Poste and Fidler, 1980).

A tumor arises from a single transformed cell which grows slowly as a small avascular lesion called an "in situ" carcinoma. Once the tumor has attained a sufficient size it becomes vascularized and grows much more rapidly giving rise to a large heterogenous population of cells, some of which have acquired the genetic and biochemical characteristics that enable them to become metastatic. The cells which have acquired these metastatic characteristics must first detach from their neighbouring cells and invade any surrounding matrix, then penetrate the basement membrane of the nearby blood or lymphatic vessels (Fidler and Hart, 1982). Once in the circulatory or lymphatic system, the tumor cells must evade the host immune defences such as the lymphocytes and macrophages. The invasive cells must cross the basement membrane of the blood vessel a second time and establish a colony in a new location (Fidler and Hart, 1982). If the tumor is incapable of completing any of these steps because it is lacking an important biochemical or phenotypic characteristic such as proteases, extracellular matrix receptors or growth factors, it will be eliminated. The metastatic process is a very inefficient process (Weiss, 1983). Few cells which leave the primary lesion actually survive long enough to establish metastases. Poste and Fidler, (1980) injected radiolabelled murine B16 melanoma cells into mice and measured the radioactivity from the cells having established metastases in the various organs. From their experiments they concluded that far less than 0.1% of the cells were able to colonize and form new lesions.

The invasion of tumor cells through the extracellular matrix , especially the basement membrane, is often the first step in the metastatic process. The basement membrane is considered

a significant barrier for the tumor cell and is therefore an important area to investigate.

Liotta et al., (1986) have described a 3 step mechanism for the invasion of tumor cells across a basement membrane (Fig. 1). The first step involves the attachment of tumor cells to the components of the basement membrane such as laminin or type IV collagen via cell surface receptors, many of which belong to a superfamily of adhesion receptors called integrins. The second step involves the release of proteolytic enzymes such as collagenases, stromelysin and plasminogen activator by the tumor cell which degrade the components of the basement membrane. The third and final step is the active migration of the tumor cell through the broken down matrix.

The cell surface receptors for the extracellular matrix play an important role in all three steps of the invasion process. Attachment of the tumor cell to the basement membrane, mediated by receptors for extracellular matrix molecules, is crucial for invasion. If attachment is inhibited with antibodies directed against cell surface receptors or peptides containing the cell binding domain, the cells are unable to invade (Kramer et al., 1989, De Luca et al., 1990, Dedhar and Saulnier, 1990).

There is now evidence that integrins may be involved in signal transduction from the extracellular matrix to the nucleus. Werb et al.,(1989) have shown that

Figure 1   Three step invasion hypothesis

Step 1: The tumor cell attaches to the basement membrane via cell surface receptors called integrins.

Step 2: The tumor cell releases proteolytic enzymes which degrade the components of the basement membrane.

Step 3: The tumor cell actively migrates through the weakened area of the basement membrane.

Figure from Liotta et al., 1986

LAMININ RECEPTOR

LAMININ

TYPE IV COLLAGEN

A

TUMOR CELL

BASEMENT MEMBRANE

STEP 1: ATTACHMENT

TYPE IV COLLAGENASE

B

TYPE IV COLLAGEN CLEAVAGE
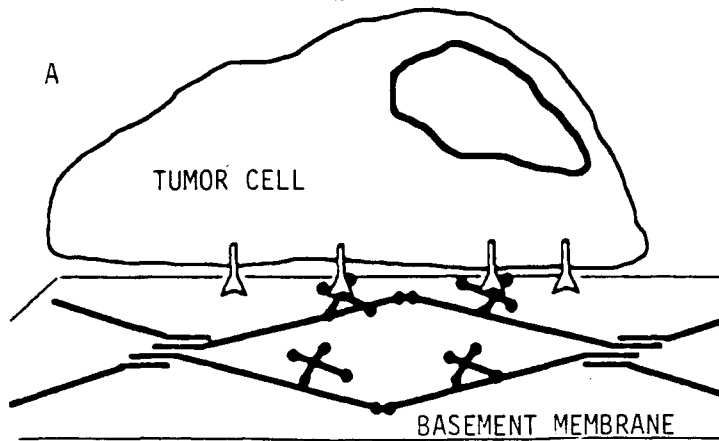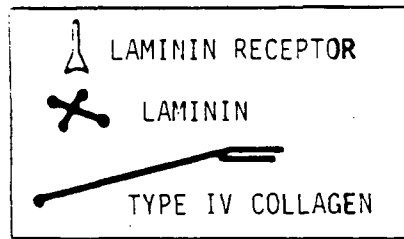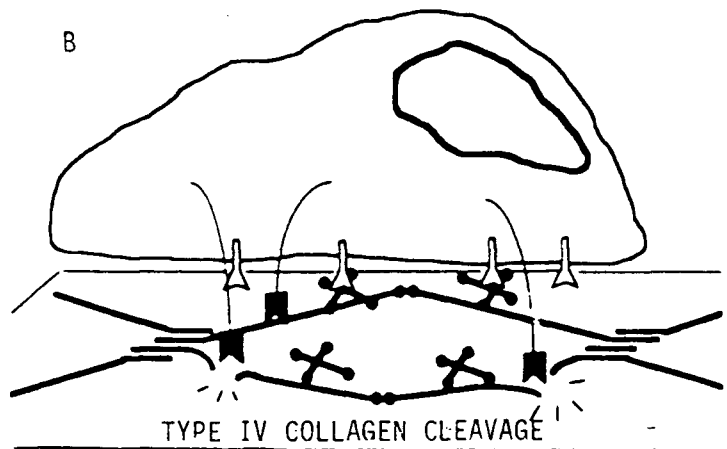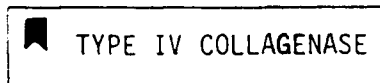
STEP 2: DISSOLUTION

C
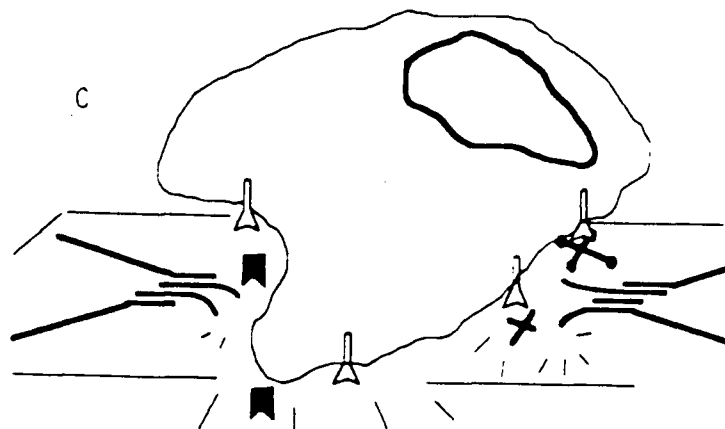
STEP 3: LOCOMOTION

antibodies to the integrin α_5 subunit and RGD containing peptides from fibronectin stimulate the release of proteolytic enzymes from rabbit synovial fibroblasts when coated to plastic. Turpeenniemi-Hujanen et al.(1986) have shown that the attachment of human and murine melanoma cells to laminin promotes the release of type IV collagenase. Kanemoto et al., (1990) have shown that a segment in the E8 segment of the A chain, called PA22-2, may be responsible for the release of type IV collagenase. As well, the integrins $\alpha_4\beta_1$ and $\alpha_5\beta_1$ have been found to be important in facilitating CD3 mediated T cell proliferation (Shimizu et al., 1989, Davis et al., 1990). Lastly, during the migration of tumor cells through the degraded basement membrane, there is successive attachment and release of integrins to extracellular matrix proteins which enables the tumor cells to migrate through the basement membrane and metastasize to other organs.

METASTATIC PHENOTYPE

Proteases

Although the mechanisms which cause cells to become metastatic are not known, malignant cells have several characteristic features which are crucial to the cell's ability to metastasize through the host tissues and establish new lesions. One important characteristic of invasive cells is their ability to release proteolytic enzymes which degrade the extracellular matrix (Mignatti et al., 1986, Liotta et al., 1979). The three major classes of degradative enzymes found in tumor cells are the serine proteases (tissue-type and urokinase-type plasminogen activators), the matrix metalloproteinases (interstitial collagenases, type IV collagenase, gelatinase and stromelysin), and the cysteine proteases (cathepsin B,L and H)(Nicolson, 1989). Although metalloproteinases are important in the invasion of tumor cells and the degradation of basement

membrane components, not all tumor cells with an invasive phenotype express elevated levels of metalloproteinases. Some cells use the serine protease, plasminogen activator, almost exclusively to degrade the extracellular matrix (Mackay et al., 1990).

The tissue inhibitor of metalloproteinases (TIMP) is also an important factor in tumor cell invasion. The regulation of protease activity is partially regulated by the expression of protease inhibitors (Khokha and Denhardt, 1989). In some cases, the cell's increased ability to degrade its surrounding matrix may not be caused by increased protease secretion but by decreased levels of protease inhibitors. Khokha et al. (1989) have shown an inverse correlation between the expression of TIMP and the invasive potential of tumor cells. By transfecting the plasmid pNMH-aT, designed to produce antisense TIMP RNA into Swiss 3T3 cells, they were able to show a decrease in TIMP expression and an increase in metastatic potential. They also observed that cells with TIMP antisense mRNA in the cytoplasm were able to produce as much TIMP as the controls and therefore concluded that the antisense mRNA exerted its inhibitory effect in the nucleus and not in the cytoplasm. TIMP may also affect tumor cell invasion indirectly by influencing the microenvironment. Reduction of proteases permits the tumor cells to lay down and adhere to extracellular matrix (Khokha and Denhardt, 1989). Edwards et al. (1987) have shown that the expression of TIMP can be regulated by TGF-ß and that TGF-ß alone did not have an effect on the expression of TIMP. However, in the presence of other growth factors such as FGF or EGF, TGF-ß was shown to upregulate the expression of TIMP in tumor cells.

MHC antigens

Another important characteristic of metastatic cells is their ability to evade the host immune system. A key component in immunogenic recognition of tumor cells are the class I major histocompatibility (MHC) proteins. In the murine system the surface expression of the H-2K/H-2D gene products have been correlated to metastatic capacity in vivo (Eisenbach et al., 1986). It was found that the ratio of H-2K/H-2D expressed on the cell surface was more important than the overall expression of the two proteins. Eisenbach et al., (1986) concluded from their experiments that cells with a high H-2K/H-2D ratio did not form many metastatic lesions when injected into mice while cells with a low H-2K/H-2D ratio were able to form numerous metastatic lesions. Increasing the expression of H-2K with interferon α or ß or retinoic acid or by gene transfection of the H-2K gene in the highly metastatic murine 3LL tumor cell line reduced the cell's ability to form metastases (Gelber et al., 1989, Wallich et al., 1985). Thus the increase or decrease in metastatic ability of the tumor cells in vivo was attributed to the increased or decreased immunogenicity of the cells.

Oncogenes

Oncogenes are a group of genes, whose products, when altered or ectopically expressed, are capable of causing cell immortalization and transformation (Weiss, 1986). They normally control a wide variety of cellular functions such as proliferation, differentiation, morphology, intercellular communication and motility (Greenberg et al., 1989). Although the expression of cellular or viral oncogenes is associated with the transformed phenotype, there is no conclusive evidence that any particular oncogene(s) is/are consistently associated with the malignant phenotype; however, certain oncogenes such as the ras and myc oncogenes have been associated with the metastatic phenotype in some tumor cells.

There are three varieties of the ras oncogene, the Harvey-ras, Kirsten-ras, and N-ras genes. All three encode 21 kDa proteins that display homology to G proteins which act as second messengers in the transduction of signals in the cell. The 21 kd protein encoded by the ras oncogene is a guanine nucleotide binding protein associated with the inner surface of the plasma membrane and binds to GTP or GDP with high affinity (Greenberg et al., 1989). This protein was also found to have an intrinsic GTPase activity that catalyzed the hydrolysis of GTP to GDP, and the coincident inactivation of ras.

NIH/3T3 cells transfected with DNA containing the activated H-ras or K-ras oncogene were able to form metastases in nude mice while the parent or spontaneously transformed NIH/3T3 cells could not form metastases (Thorgeirsson et al., 1985). Other cell lines such as rat embryo cells and mouse lymphoma cells transfected with the ras oncogene were also able to form metastases in nude mice (Muschel and Liotta, 1988, McKenna et al., 1990). Although the ras oncogene is able to induce the metastatic phenotype in some cells, not all metastatic cells express the ras oncogene. This suggests that other oncogenes may be required to induce the metastatic phenotype or that the ras oncogene is only able to induce a phenotypic change in some cells (Greenberg et al., 1989).

The myc family of oncogenes are nuclear oncogenes, originally isolated from the avian myelocytomatosis virus. Although little is known about the biochemistry of its 65 kDa protein, the myc oncogene has been associated with the metastatic phenotype in some tumors (Nicolson, 1986). Human signet ring gastric carcinoma cells capable of forming metastasis in nude mice were found to have an amplified myc oncogene (Yanagihara, et al., 1991). Ras and myc are the oncogenes commonly associated with a metastatic phenotype. However, occasionally other oncogenes such as raf, src and fms have been correlated with metastatic progression (Greenberg et al., 1989)

Extracellular matrix

The extracellular matrix plays a substantial role in cell differentiation, embryogenesis, wound repair and tumor cell invasion (McDonald, 1989, Ekblom et al., 1986). The major components of the extracellular matrix include collagens, elastins, proteoglycans, and glycoproteins (Labat-Robert et al., 1990). The more common extracellular matrix proteins include the glycoproteins fibronectin, laminin, vitronectin, and collagens type I-VII. Collagens I-III are found primarily in bone, cartilage and the extracellular space. Type IV collagen and laminin are found exclusively in basement membranes (Laurie et al., 1982). Types V and VII collagen are associated with the attachment of the basement membrane to the underlying connective tissue (Bachinger et al., 1990). Fibronectin is a very abundant extracellular matrix protein which is found in the extracellular space of numerous cell types while vitronectin is predominantly found in the serum. There are three important molecular interactions associated with extracellular matrix proteins: (1) self-aggregation to form ordered structures, (2) interactions with other matrix proteins forming large complexes and (3) interactions with the cell surface to promote cell adhesion (Ruoslahti et al., 1985). The intrinsic interconnecting network of extracellular matrix proteins in basement membranes includes all three types of intermolecular interactions (Yurchenco and Schittny, 1990, Yurchenco et al., 1986).

Several of the extracellular matrix proteins, such as laminin and fibronectin have more than one cell binding domain. The most studied is the cell binding domain mediated by the tripeptide RGD, found in fibronectin. The RGD sequence was determined by progressively trying smaller and smaller synthetic peptides from the fibronectin molecule until binding affinity was lost. It was found that only the three amino acids, Arginine-Glycine-Aspartic acid (RGD) were required for cell attachment (Pierschbacher and Ruoslahti, 1984, Yamada and Kennedy, 1985).

Since its role in cell adhesion was described in fibronectin, the RGD sequence was found to have similar properties in several other extracellular matrix molecules including, vitronectin (Suzuki et al., 1985), von Willebrand factor (Cheresh and Spiro, 1987, Dejana et al., 1989), tenascin (Bourdon and Ruoslahti, 1989), laminin (Sasaki et al., 1988), thrombospondin (Lawler et al., 1988) and fibrinogen (Ruoslahti and Pierschbacher, 1986). The RGD peptide in many of these extracellular matrix proteins is recognized by integrin receptors (D'Souza et al., 1988, Smith and Cheresh, 1988). RGD peptides in solution can inhibit cell attachment and tumor cell invasion by interfering with the interaction of the cell surface receptors with the extracellular matrix proteins (Gehlsen et al., 1988a, Bretti et al., 1989, Saiki et al., 1989). Hence the extracellular matrix-receptor interactions are important in cell adhesion and tumor cell invasion.

BASEMENT MEMBRANES

Structure and Function

Basement membranes are specialized extracellular matrix structures which are composed of three layers. Directly underlying the cells is the electron-lucid lamina rara, then a more electron-dense layer called the lamina densa followed by a second electron-lucid layer which interfaces with the underlying extracellular matrix (Abrahamson,1986). Basement membranes are found underlying epithelial cells and endothelial cells and surrounding muscle cells, adipocytes, and Schwann cells. These membranes are almost exclusively produced by the cells apposing them. The basement membrane is anchored to its underlying matrix by fibrils composed of type V and VII collagens (Keene et al., 1987). Basement membrane components are the first extracellular matrix products produced during development and are required for the adhesion, migration, growth and differentiation of cells (Timpl and Dziadek, 1986). Several functions have been described for basement membranes including maintenance of tissue architecture by providing a sheet-like support to which cells attach, serving as a physical boundary for cells, and functioning as a molecular filter preventing passage of proteins (Martin et al., 1988). There is some variability in the composition and thickness (30-300nm) of basement membranes depending on their location (Timpl and Dziadek, 1986). However a typical basement membrane is composed of several ubiquitous components; type IV collagen, laminin, Nidogen/Entactin and various sulfated proteoglycans, the predominant one being Heparan sulfate proteoglycan (Martin et al., 1988). Fibronectin is an important and abundant extracellular matrix molecule but is only occasionally found in basement membranes (Laurie et al., 1982). It is probable that basement membranes contain a number of less abundant components which are yet to be isolated and characterized. Components such as BM 40/osteonectin/SPARC, amyloid P component, Bullous pemphigoid antigen, AE26 antigen and EBA antigen are present in low amounts and are not

found in all basement membranes (Kolega and Manabe, 1990).

Changes in basement membranes are hallmarks of several diseases such as a thickened renal glomerular basement membrane in diabetes, autoimmune diseases such as Goodpasture's syndrome (Spargo and Taylor, 1988) and neoplastic lesions. The basement membrane surrounding a neoplastic lesion may appear structurally normal. However, in many cases the basement membrane components are either reduced in number or simply not properly assembled, weakening the rigid structure of the basement membrane and facilitating the passage of tumor cells (Ingber et al., 1981). Occasionally, the most important histological distinguishing feature between malignant and benign tumors is the presence of a basement membrane surrounding the lesion, benign tumors being encapsulated by a basement membrane and malignant tumors having degraded and invaded through some areas of the basement membrane (Liotta et al., 1986).

A murine tumor which produces large amounts of basement membrane is the EHS (Engelbreth Holm-Swarm) tumor. It has provided valuable information in the isolation and characterization of basement membrane proteins (Inoue and Leblond, 1985). The matrix produced by the EHS tumor can be reconstituted and used in vitro as a basement membrane substitute. Reconstituted basement membrane, sold commercially as Matrigel, has been shown to be structurally and functionally similar to the basement membranes found in vivo and is now commonly used in in vitro invasion assays.(Kleinman et al., 1982, Kleinman et al., 1983).

Type IV collagen

Type IV collagen, found exclusively in basement membranes, is a heterotrimer composed of two $\alpha1(IV)$ chains and an $\alpha2(IV)$ chain. Each heterotrimer (monomer) is approximately 400 nm in length and contains a protease resistant NC1 (noncollagenous) domain at the carboxyl end followed by a large helical region interrupted by several non-helical regions. Type IV collagen self-assembles in a time and temperature dependant manner. The monomers bind end-to-end via the NC1 domain and laterally via the 7S domain located at the amino terminal end to form large chicken-wire-like networks (Fig 2a,p.20), providing a structural framework to the basement membrane (Babel and Glanville, 1984). Type IV collagen can also bind to other extracellular matrix proteins such as heparin sulfate proteoglycans, heparin, laminin and fibronectin (Laurie et al., 1986, Koliakos et al., 1989). Herbst et al., (1988) have shown that type IV collagen and a pepsin-generated triple-helical fragment of type IV collagen were much more effective in mediating cell attachment and migration of aortic endothelial cells than were laminin and the NC1 domain of type IV collagen. Type IV collagen not only provides a framework for the basement membrane but is also important in cell adhesion.

Laminin

Laminin is a large glycoprotein (Mr 850,000) ubiquitous to basement membranes. It was originally isolated from neutral extracts of the mouse Engelbreth-Holm-Swarm (EHS) tumor cell line (Timpl et al., 1979). Laminin is a cross-shaped molecule composed of three different polypeptide chains, an A chain (400,000), a B1 chain (210,000) and a B2 chain (200,000), linked together by inter and intrachain disulfide bonds (Pikkarainen et al., 1988, Sasaki et al., 1987, Vuolteenaho et al., 1990)(Fig. 2b). The B1 and B2 chains of laminin show considerable

homology suggesting that they were derived from the same ancestral gene (Sasaki and Yamada, 1987).

Laminin is a multifunctional molecule which promotes cellular adhesion, growth, migration (Wewer et al., 1987), tumor cell invasion and differentiation of cells (Ekblom et al., 1980, Kleinman et al., 1985, Vukicevic et al., 1990, Ocalan et al., 1988). At the 2 cell stage of embryogenesis only the B1 chain of laminin is synthesized. Laminin does not appear in intact form until the morula stage (Ekblom et al. 1986). The expression of the laminin A chain was found to correspond with the development of cell polarity during embryonic development of murine kidney tubules (Klein et al., 1988).

Laminin can also bind to a number of matrix proteins found in the basement membrane such as entactin, type IV collagen via the globular domains at the end of the long and short arms, and to heparan sulfate proteoglycans. Laminin can also bind to other laminin molecules, via the E4 and E1 domains, forming large aggregates (Martin and Timpl, 1987).

Laminin is a multidomain molecule possessing several functional binding sites. The 20 amino acid F9 site, located on the internal globular domain of the B1 chain, and the YIGSR sequence, located in a cysteine rich region of the B1 chain are both involved in cell attachment (Kleinman and Weeks, 1989) (Fig. 2c). The YIGSR peptide also promotes cell migration and inhibits the formation of lung metastases when injected with B16F10 melanoma cells in vivo (Iwamoto et al., 1987, Kanemoto et al., 1990). The RGD sequence on the A chain is also involved in cell attachment (Kleinman et al., 1990). Also found on the A chain is the 19 amino acid PA22-2 segment which promotes cell adhesion, neurite outgrowth and induces collagenase IV activity (Kanemoto et al., 1990). Laminin also has three known binding sites for heparin, the F9, E8 and AC15 domains (Kouzi-Koliakos et al., 1989) (Fig. 2b). The laminin-heparin interaction is a calcium dependent interaction which modulates the polymerization of laminin

molecules (Yurchenco et al., 1990).

Recently, other molecules which are homologous to laminin have been found in basement membranes. Merosin, which is homologous to the carboxy terminal end of the laminin A (Fig. 2d) chain appears in the basal lamina of Schwann cells, striated muscle and trophoblasts (Leivo and Engvall, 1988, Ehrig et al., 1990). In mice, merosin only appears later in development suggesting that it may have a role in the differentiation or maturation of tissues (Hunter et al., 1989a). Another laminin-like molecule, called S-laminin (Fig. 2e), shares a 40% sequence homology with the B1 chain of laminin and is concentrated at synaptic sites in muscles and is also present at other locations such as peripheral nerve and glomerular basement membranes (Hunter et al., 1989b). Neurons from embryonic chick ciliary ganglia were able to adhere to plates coated with S-laminin. The use of successively smaller peptides revealed the cell binding domain of S-laminin as a tripeptide sequence called LRE (Hunter et al., 1989b).

Fibronectin

Fibronectin is an important multifunctional extracellular matrix glycoprotein (Mr 400,000) which has many biological functions similar to laminin such as cell migration (Lacovara, et al., 1984), adhesion, cell invasion (Ruoslahti, 1984), morphogenesis and development (Ruoslahti,1988). Fibronectin can be present in both soluble forms in plasma and other body fluids or in insoluble forms in the extracellular matrix (Rocco et al., 1987). It is present in some basement membranes but has not been isolated in the EHS tumor extracellular matrix or the glomerular basement membrane (Laurie et al., 1982, Kleinman et al., 1986). Fibronectin is composed of two similar chains made of three different type repeats which are linked near the carboxyl end by disulfide bonds (Ruoslahti, 1988)(Fig. 2f). The molecule possesses several binding domains including two fibrin and heparin binding domains, a collagen and gelatin binding domain, and two cell binding

domains, one found in the central region of all the fibronectin molecules and containing the RGD sequence (Ruoslahti, 1988). The other cell binding site is found in an alternatively spliced region of the fibronectin molecule and is called the IIICS domain (Guan and Hynes, 1990, Schwarzbauer et al., 1989).

Entactin/Nidogen and heparan sulfate proteoglycans

Entactin (nidogen) is a 150 kDa sulfated glycoprotein found only in basement membranes. It is dumbbell shaped having a globular domain at each end (Carlin et al., 1981, Timpl et al., 1983)(Fig. 2g). Entactin also contains an RGD sequence which is partially responsible for cell adhesion (Chakravarti et al., 1990). Entactin binds most strongly to the B2 chain of laminin near the centre of the cross in a 1:1 ratio but can also bind to the triple helix of the type IV collagen (Mann et al., 1989). The complexes that laminin forms with entactin in equimolar proportions are very stable and divalent cation dependent (Paulsson, 1988). Heparan sulfate proteoglycans are the most predominant proteoglycan found in basement membranes and in the extracellular matrix. Other proteoglycans found in basement membranes are chondroitin and dermatin sulfate proteoglycans (Fujiwara et al., 1984). Proteoglycans range in size from 75-350 kDa, however, the majority of those found in basement membranes are 130 kDa (Martin et al., 1988). Heparan sulfate proteoglycans function as selective filtration barriers and may form large aggregates which bind to other basement membrane components helping to maintain the structural integrity of the matrix (Yurchenco et al., 1987).

Vitronectin

Vitronectin/serum spreading factor/S protein is a 75 kDa glycoprotein found predominantly in the serum and occasionally in the extracellular space (Hayman et al., 1983). Vitronectin functions as a complement regulatory protein in plasma (Preissner, 1989), plays a role in the aggregation of platelets (Asch and Podack, 1990) and promotes cell adhesion and spreading. Vitronectin also contains an active RGD sequence which is recognized by integrin receptors (Thiagarajan and Kelly, 1988, Pytela et al., 1985b).

Figure 2   Schematic diagrams of extracellular matrix proteins

(a) Type IV collagen

(b,c) laminin. Figure from Anderson, 1990

(d) S-laminin. Figure from Anderson, 1990

(e) Merosin Figure from Anderson, 1990

(f) fibronectin. Figure from Ruoslahti, 1988

(g) Entactin. Figure from Mann et al., 1989

Type IV Collagen

a

monomer

NC1

7S

dimer

polymer

Laminin

b

A

NH₂

VI

V

IV

III

VI V IV III

B1

NH₂

III IV V VI

B2

α

II II

I I

G1

G2

G3 G4 G5

A

Laminin

c

NH₂

E1

E4

collagen

YIGSR

collagen

B1

NH₂

RGD

NH₂

B2

AC15

F9

S—S—S
S—S—S

cell binding

α —

PA22-2

E8

neurite
outgrowth
activity

E3

heparin
binding

S-Laminin

d

NH₂

S
S

α
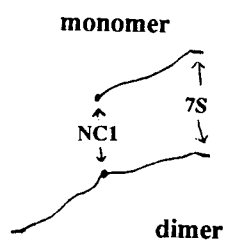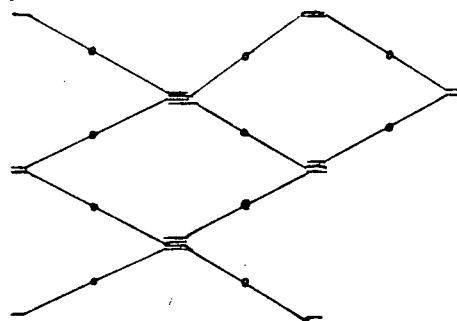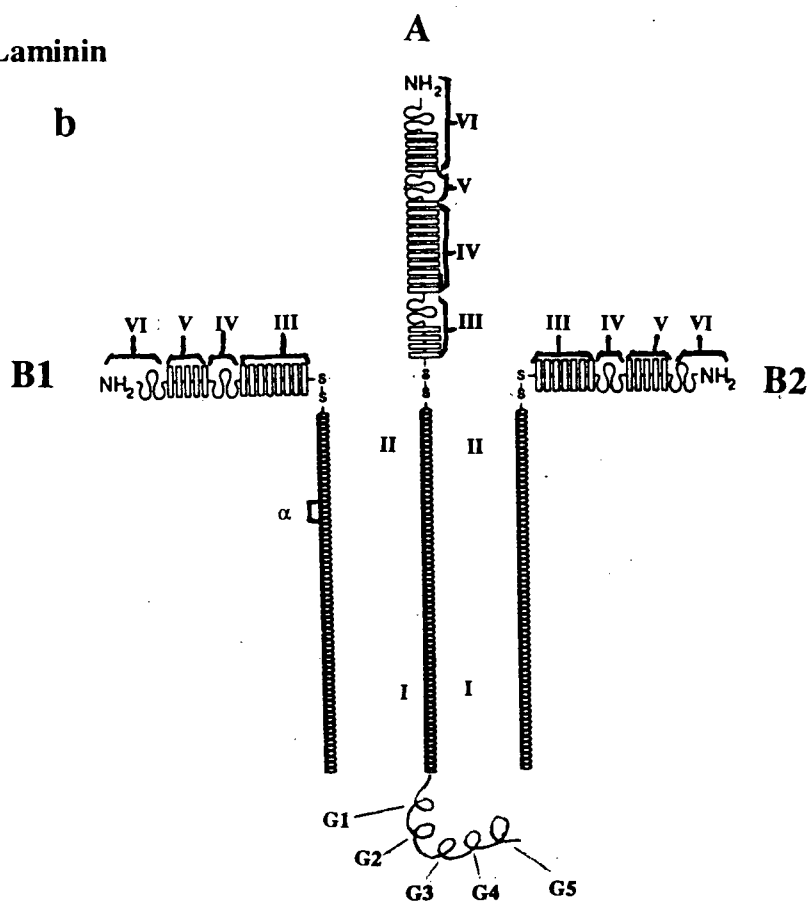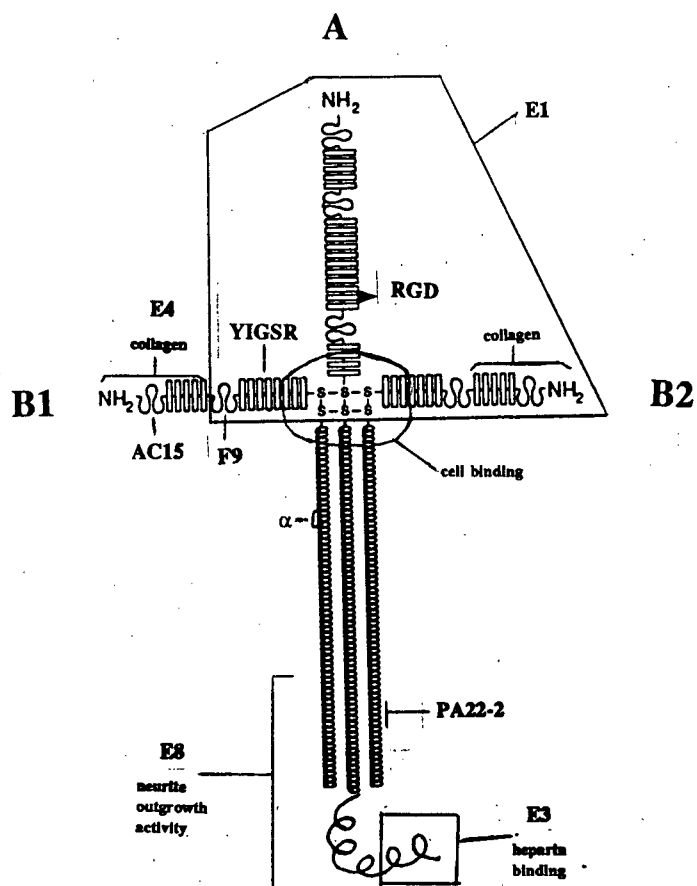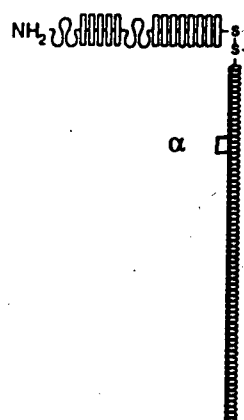
Merosin

e

Fibronectin

f
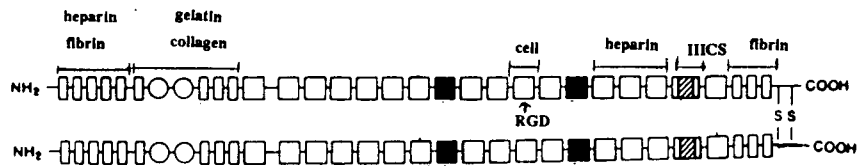
Entactin

g
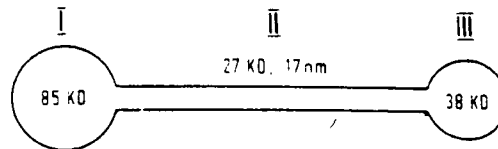
EXTRACELLULAR MATRIX RECEPTORS

The interactions between cells and the extracellular matrix is mediated by cell surface receptors many of which belong to a superfamily of adhesion receptors called integrins.

Integrins are a family of transmembrane glycoprotein heterodimers composed of an α and β subunit noncovalently bound together which mediate cell-cell and cell-matrix interactions (Hynes, 1987, Buck, 1987, Albelda and Buck, 1990) and play a key role in embryogenesis, wound healing, cell differentiation, cell migration, bacterial cell invasion (Isberg and Leong, 1990) and tumor cell invasion (Dedhar, 1990, Ruoslahti and Pierschbacher, 1987). Both the α and β subunits possess large extracellular domains, a transmembrane domain and short cytoplasmic domains, with the exception of the β_4 subunit which has a 118 kDa cytoplasmic domain (Hogervorst et al., 1990, Suzuki and Naitoh, 1990).

The superfamily of integrins was initially classified according to their β subunits and formed three major groups. The integrins sharing the β_1 subunit are the largest group having six different α subunits and bind fibronectin, collagens and laminin (table 1). The leucocyte adhesion molecules, LFA-1, MAC-1, and P150,95 are only found on lymphoid and myeloid cells and share a common β_2 subunit (Larson and Springer, 1990). The third group of integrins, the cytoadhesion molecules, consists of the vitronectin receptor ($\alpha_v\beta_3$) and the platelet gpIIb/IIIa receptor which share a common β_3 subunit.

The isolation and characterization of several new β subunits, β_p (Holzmann and Weissman, 1989), β_s (Freed et al., 1989), β_5 (Ramaswamy and Hemler, 1990) and β_6 (Sheppard et al., 1990) and the finding that α subunits could associate with multiple β subunits has forced a new classification for integrins (Fig. 3).

The members of the β_1 family possess mainly extracellular matrix molecules as ligands, many of which are components of basement membranes. Integrin $\alpha_1\beta_1$ was initially isolated from

the surface of T lymphocytes two weeks after in vitro activation (Hemler et al., 1984) but it is also expressed on a number of other cell types (Dejana and Lauri, 1990). It is composed of an α subunit of Mr 200,000 and a Mr 110,000 β_1 subunit and functions as a collagen and laminin receptor (Tawil et al., 1990). The rat analogue of the α_1 subunit contains an I domain, which is an additional 200 amino acids inserted in the α subunits of the leukocyte adhesion molecules and the α_2 subunit of the integrin $\alpha_2\beta_1$. The α_1 subunit also contains three divalent cation binding sites within its extracellular domain (Ignatius et al., 1990). The integrin α_2 subunit, Mr 150,000, was initially isolated on activated T cells but is also expressed on platelets, fibroblasts, and endothelial and epithelial cells from many different tissues (Zutter and Santoro, 1990, Hemler et al., 1984).The α_2 subunit also binds to collagen and laminin (Elices and Hemler, 1989). However its ligand specificity varies with different cell lines, binding to collagen on some cell lines and to laminin on others (Languino et al., 1989, Kirchhfer et al., 1990). The $\alpha_2\beta_1$ complex also possesses an I-domain and three divalent cation binding sites (Takada and Hemler, 1989). The $\alpha_3\beta_1$ complex is a promiscuous receptor capable of binding to laminin, fibronectin and collagen (Sanchez-Madrid et al., 1986). It is located on a large number of cell types and functions in cell-matrix adhesion (Carter et al., 1990b). It is also located at intercellular contact sites and therefore may also function in cell-cell interactions (Kaufmann et al., 1989). Gehlsen et al.(1989) have shown that the $\alpha_2\beta_1$ complex binds to the B1 chain of laminin in an RGD independent manner. The integrin $\alpha_4\beta_1$ differs from the other integrins of the β_1 family in that it is only weakly associated to its β subunit and easily undergoes partial cleavage into 80 and 70 kDa fragments (Hemler et al., 1987). It is expressed most abundantly on thymocytes, peripheral blood lymphocytes, monocytes, T and B cell lines and

Table 1   Integrin superfamily of cell adhesion receptors

integrin subunits	molecular mass	ligands
$\alpha_1\beta_1$	200/110	laminin (E1), collagen
$\alpha_2\beta_1$	160/110	laminin, collagen
$\alpha_3\beta_1$	150/110	laminin, collagen (I,IV,VI), fibronectin
$\alpha_4\beta_1$	140/110	cell-cell, fibronectin (CS-1)
$\alpha_4\beta_p$	140/100	
$\alpha_5\beta_1$	155/110	fibronectin
$\alpha_6\beta_1$	140/110	laminin (E8)
$\alpha_6\beta_4$	140/210	laminin ?
$\alpha_L\beta_2$	170/90	ICAM-1, ICAM-2
$\alpha_M\beta_2$	165/90	C3bi, fibrinogen
$\alpha_{150}\beta_2$	145/90	
$\alpha_v\beta_1$	160/110	fibronectin, vitronectin
$\alpha_v\beta_n$	160/110	fibronectin, collagen
$\alpha_v\beta_5$	160/100	fibronectin, vitronectin
$\alpha_v\beta_s$	160/105	
$\alpha_v\beta_3$	160/105	laminin, Von Willebrand factor vitronectin, fibrinogen, osteopontin,thrombospondin
$\alpha_{IIb}\beta_3$	140/105	fibrinogen, fibronectin,vitronectin, Von Willebrand factor, thrombospondin

Figure 3   Association of α and ß subunits within the integrin family of cell surface receptors
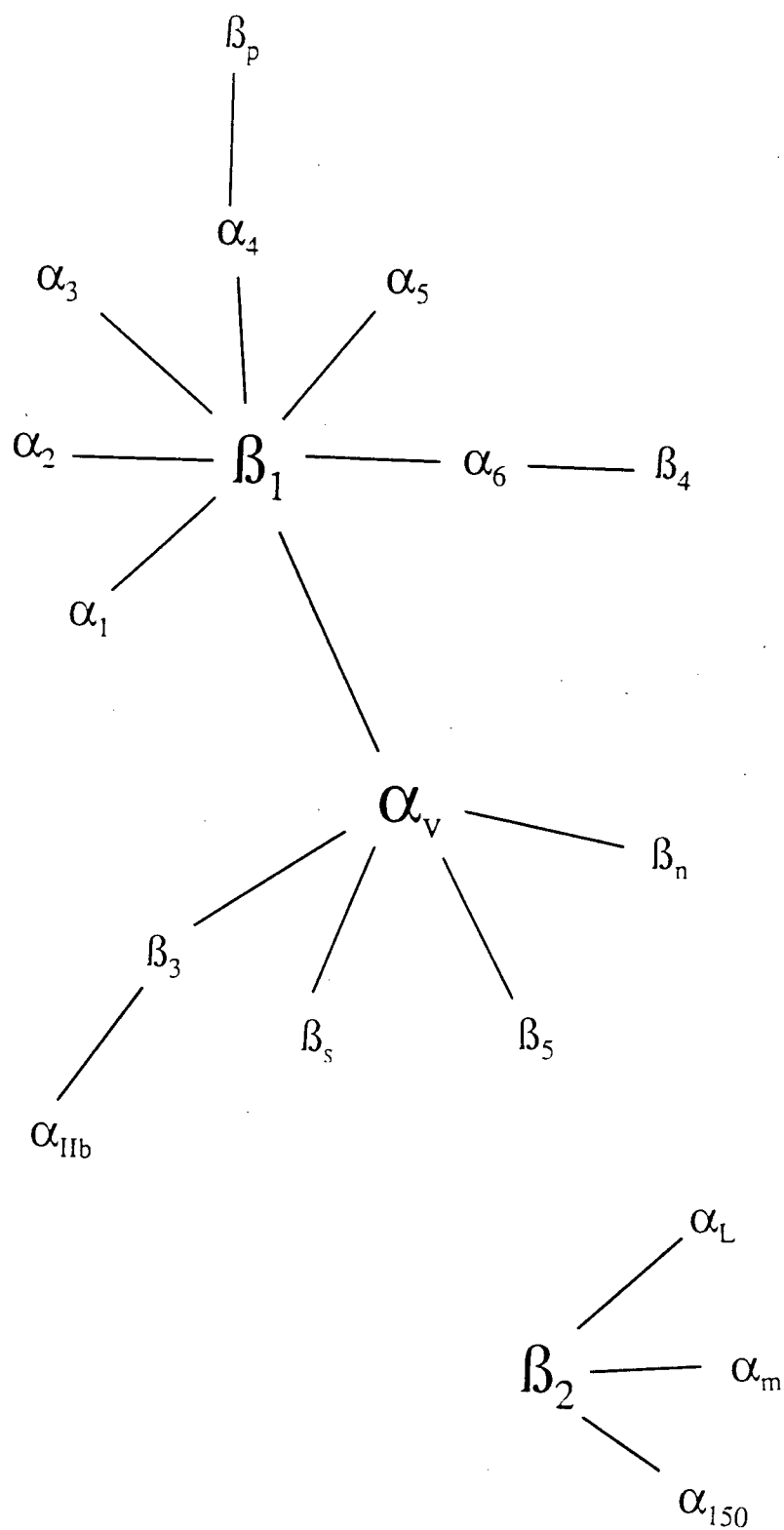
myelomonocytic cell lines (Takada et al., 1989) but is expressed on most adhesive cells in low amounts. The integrin $\alpha_4\beta_1$ functions in both cell-cell and cell-matrix adhesion (Hemler et al., 1990). Its ligands are the VCAM-1 (vascular cell adhesion molecule) and the IIICS region of the fibronectin molecule which is an alternatively spliced region (Elices et al., 1990, Mould et al., 1990). The α_4 subunit is also able to associate with the β_p subunit and functions as a lymphocyte homing receptor (Holzmann and Weissman, 1989). The classical fibronectin receptor, $\alpha_5\beta_1$ has a 155 kDa α-subunit and binds to the RGD sequence of fibronectin (Pytela et al., 1985a). During keratinocyte differentiation the loss of adhesiveness precedes the loss of $\alpha_5\beta_1$ from the surface of the cells (Adams and Watt, 1990). Giancotti and Ruoslahti (1990) transfected the α_5 and β_1 cDNA into transformed Chinese hamster ovary cells and found that the cells which overexpressed $\alpha_5\beta_1$ also secreted more fibronectin and were nontumorigenic as compared to the control cells, associating the increase in the expression of $\alpha_5\beta_1$ with a higher degree of cell differentiation (Dedhar et al., 1987). The integrin $\alpha_6\beta_1$ has been found on platelets and a large number of other cell types (Sonnenberg et al., 1988, Gehlsen et al., 1988a). This is the classical laminin receptor and binds to the E8 fragment of laminin (Sonnenberg et al., 1990) as opposed to $\alpha_1\beta_1$ which binds to the E1 domain (Hall et al., 1990). The α_6 subunit is also able to bind the β_4 subunit (Hemler et al., 1989, Kennel et al., 1989). The β_4 subunit, primarily expressed in epithelial cells, can be expressed in three forms: 200 kDa, 180 kDa and 125 kDa. The difference in size is a result of alternative splicing of the cytoplasmic domain (Suzuki and Naitoh, 1990). The $\alpha_6\beta_4$ complex also called TSP-180 is found in epithelial carcinoma cell lines but its ligand still remains uncertain (Hogervorst et al., 1990).

The α_v subunit is associated with multiple β subunits. It was first described as associating with the β_3 subunit which behaves as a vitronectin receptor. This heterodimer also binds to fibrinogen, von Willebrand factor, osteopontin and thrombospondin in an RGD-dependent manner

(Smith and Cheresh, 1990). The α_v subunit has since been shown to associate with the β_s (Freed et al., 1989), β_5 (Ramaswamy and Hemler, 1990), β_n and β_1 (Dedhar and Gray, 1990) subunits.

INVASION ASSAYS

In vitro invasion assays are now commonly used to determine the invasive potential of tumor cell lines instead of in vivo invasion assays (Albini et al., 1987, Kramer et al., 1986, Terranova et al., 1986). They have advantages over the in vivo assays in that the experimental conditions can be more closely regulated and the assay can be performed in several hours as opposed to the 15-30 days required for in vivo experiments. Although simpler and quicker, in vitro invasion assays are not an adequate substitute for in vivo experiments. In vitro invasion assays are designed to look only at the invasive potential of cells, i.e. only one step in the complex metastatic process (Yagel et al., 1989). Several different in vitro invasion assays are in use today including an amnion invasion assay, a transwell (Repesh, 1989) and Boyden chamber or a modified Boyden chamber assay (Albini et al., 1987, Terranova et al., 1986). The introduction of matrigel in in vitro invasion assays is now widely used by researchers looking at tumor cell invasion and metastatic potential of tumor cells (Kramer et al., 1986, Repesh, 1989). A comparison between the amnion and the reconstituted basement membrane revealed that fewer cells were able to invade amnion membranes and that the results were more consistent when using matrigel. Cells invading through the reconstituted basement membrane can be isolated and recultured to select a more invasive cell line. This method of isolation is more feasible compared to using the human amnion assay because the cells invading through the amnion are difficult to isolate (Hendrix et al., 1989).

To understand the role of integrins in tumor cell invasion better, an in vitro invasion assay was developed and used to determine the invasive potential of two osteosarcoma cell lines and the effect of anti-integrin antibodies on their invasion. An invasive cell population was isolated using the in vitro invasion assay and its adhesive properties to extracellular matrices and expression of integrins investigated using cell attachment assays and immunofluorescence.

Materials and Methods

Cells

Prostate carcinoma cell lines, PC-3 and DU145, human lung fibroblasts, IMR90, and human osteosarcoma cell lines, HOS and MNNG-HOS were obtained from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (with 1% glutamine, penicillin 100 μg/ml and streptomycin 100 μg/ml) supplemented with 10% heat inactivated fetal calf serum. The cells were passaged every five days. To detach the cells, the medium was aspirated and replaced with 5 mM EDTA in PBS for 2-5 min. The EDTA was then aspirated and the cells removed from the flask by pipetting media directly onto the cells.

Antibodies

Anti-fibronectin receptor and anti-vitronectin receptor polyclonal antibodies as well as anti-α_2 (P1E6) and anti-α_3 (P1B5) monoclonal antibodies were purchased from Telios. Anti-α_5 (BIIG2), anti-α_6 (GoH3) and anti-β_1 (A2BII) monoclonal antibodies were a gift from C. Damsky (U. of California, San Francisco). Normal preimmune serum was obtained from our own New Zealand white rabbits. Rhodamine conjugated antibodies (Goat anti-rat, Goat anti-rabbit and Rabbit anti-mouse) were purchased from Jackson ImmunoResearch.

Matrix proteins

Collagen types I and IV were obtained from Sigma Chemical Company. Human fibronectin and vitronectin were purchased from Telios. Mouse Laminin was purchased from Gibco. BSA was obtained from Sigma.

Matrigel and ITS+ were purchased from Collaborative Research (Boston Mass.)

Surface labelling and immunoprecipitation

Cells were detached from tissue culture flasks with 5 mM EDTA in PBS, washed twice in PBS, and surface labelled with 0.05 mCi ^{125}I in the presence of Iodogen (Pierce) for 30 min at room temperature. The cells were then washed three times in PBS to remove free label and lysed in 500 µl Radioimmunoprecipitation assay (RIPA) lysis buffer containing 2.0 mM phenylmethylsulfonyl fluoride (PMSF) for at least 30 min on ice with agitation. The lysate was centrifuged in a microcentrifuge (12,000 rpm) and the pellet discarded. The number of cpm in the supernatant was determined by counting a 5 µl aliquot in a Beckman Gamma 7000 gamma counter. The supernatant (lysate) was then aliquoted such that $6x10^6$ cpm were used in each reaction. Anti-integrin antibodies were added to the lysate aliquots and incubated overnight at 4°C on a tube rotator. The primary antibodies of mouse origin required the addition of a rabbit antimouse secondary antibody which was added several hours after the primary antibody. The following day, 50 µl protein A Sepharose in PBS was added to the lysate and incubated at 4°C on a tube rotator for at least 2 h. Samples were then washed twice with RIPA containing 0.5 M NaCl and twice with RIPA (no NaCl) both containing 1.0 mM PMSF. The protein A Sepharose beads containing the antibody-integrin complexes were resuspended in 60 µl sample buffer

(62.5mM Tris, 10% glycerol, 2.3% SDS and bromphenol blue) and boiled for 3 min. The samples were then loaded on a 7.5% SDS-polyacrylamide gel and electrophoresed under nonreducing conditions. Once the dye front had migrated off the gel, the gel was fixed in gel fixative (10% glacial acetic acid, 37.5% methanol in dH_2O, and bromophenol blue) for at least 3 h with agitation. The gel was then rinsed in gel fixative without bromophenol blue for several minutes, then rinsed with dH_2O and placed on Whatman filter backing paper. The gel was then dried in a Bio-Rad model 583 Gel Dryer for 1.5 h and subjected to autoradiography using Kodak Diagnostic X-OMAT AR Film.

In vitro Invasion assay

Transwell membranes (Costar) were coated with type I collagen (100 µl/ml) for 24 h prior to the assay and kept at 4°C . The matrigel was thawed several hours prior to the assay at 4°C. Once thawed the matrigel was diluted 1:5 in cold PBS and mixed thoroughly by pipetting and kept on ice until ready to use. Excess type I collagen solution was pipetted off the membrane and 6.0 mm diameter, alcohol sterilized polycarbonate membranes with 12 µm pores were placed on the collagen coated membrane. The diluted matrigel (40 µl) was carefully pipetted into each well and then placed in a 37°C, 5.0% CO_2 incubator for 30 min while cells were harvested.

Cells, prelabelled with 0.05 mCi ^{3}H-thymidine (20 Ci/mmol) for 24 h, were harvested with 5 mM EDTA, then washed twice in DMEM containing 0.5ml ITS+\100 ml DMEM and diluted to 5×10^5 cells/ml in the same medium. Medium containing cells (200 µl) was placed in the top chamber and 1 ml DMEM containing 0.5 ml ITS+\100 ml medium in the bottom chamber. Two aliquots of cells (200 µl) were placed in 10 ml scintillation fluid and counted in a Beckman LS 6800 scintillation counter for initial counts. The transwells were incubated at 37°C, 5.0% CO_2

for 24 h. Once the assay was completed media from the top and bottom chambers were pipetted off and the cells fixed in methanol for 5-10 min. The methanol was pipetted off and the top membrane carefully removed with forceps. Once the methanol had dried, the transwell membrane was cut with a sharp scalpel. The membranes were then placed in 10 ml scintillation fluid and the radioactivity measured in a scintillation counter and the percent invasion calculated using the following equation.

$$\frac{\text{average dpm of cells having invaded}}{\text{average dpm of cells placed in top chamber}} \times 100 = \text{percent cells}$$

Cell adhesion assay

Cell attachment assays to extracellular matrix molecules were performed in 96-well, non-tissue culture, flat bottom plates (Libro). The plates were coated overnight at 4°C with 0.156-20 µg/ml laminin, fibronectin, vitronectin, type I collagen, type IV collagen and Bovine Serum Albumin (BSA) as a control. Two hours prior to the assay all coated wells were blocked with DMEM containing 2.5 mg/ml BSA. Cells prelabelled for 24 h with ^{3}H-thymidine were harvested with 5 mM EDTA and washed in DMEM containing 2.5 mg/ml BSA. PC-3 cells were plated at a concentration of 4×10^4 cells/ well and IPC-3 at a concentration of 8×10^4 cells/ well in a total of 100 µl DMEM containing 2.5 mg/ml BSA. After 1 h at 37°C, 5.0% CO_2 all wells were gently rinsed with PBS to remove unattached cells. Attached cells were removed with 50 µl 10mM

EDTA, 0.1% Triton-X100 and the radioactivity counted in a scintillation counter.

Morphology

The morphology of PC-3 and IPC-3 cells on extracellular matrix proteins were studied in 96-well, flat bottom, non-tissue culture titer plates (Linbro). The plates were coated with 10 µg/ml BSA, fibronectin, vitronectin, laminin, and collagen types I and IV overnight at 4°C. 2 h prior to the assay, the wells were blocked with 100 µl DMEM containing 2.5 mg/ml BSA. PC-3 cells were plated at a concentration of 30,000 cells/well and IPC-3 cells at a concentration of 50,000 cells/well and then incubated at 37°C. The cells were photographed on a Wild M40 inverted biological microscope after 24 h.

Immunofluorescence

PC-3 and IPC-3 cells grown in DMEM containing 10% FCS were harvested with 5 mM EDTA, washed in DMEM and then diluted to 2×10^5 and 4×10^5 cells/ml respectively in DMEM containing 10% FCS. Alcohol rinsed and heat sterilized 12 mm circular coverslips (Fisher) were placed in the bottom of 24 well plates. Medium containing cells (0.5 ml) was placed in each well and incubated at 37°C for 48 h. The cells were gently washed twice in PBS and fixed in 2.0% paraformaldehyde in PBS (pH 7.2) for 1 h at 4°C. The cells were then washed twice with PBS and incubated for 5-10 min in 0.1% Triton-X100 in PBS to permeabilize the cells. The coverslips and cells were blocked with 1% BSA in PBS for 30 min at room temperature and then washed once in PBS. The cells were covered with a 1:200 dilution of anti-integrin antibody in PBS containing 1% BSA for 1 h at room temperature. The cells were washed extensively (1 h)

with PBS, then incubated with a 1:100 dilution of rhodamine conjugated antibody for 1 h at room temperature, in darkness. The cells were then washed 3 times and mounted on slides (Micromaster) and sealed with clear nail polish and kept at 4°C in the dark. Control slides were incubated for the same time periods as the treated slides with rabbit or mouse preimmune serum. Slides were photographed on a Zeiss Axiophot epifluorescence microscope. Photographs of the controls were printed such that the cells were barely visible and those of the treated cells were printed under the same light and aperture conditions as the controls.

Determination of Proliferation rate

PC-3 and IPC-3 cells grown in DMEM containing 10 % FCS were harvested with 5 mM EDTA and washed in DMEM containing 10% FCS. The cells were diluted to 1×10^5 cells/ml in DMEM containing 10% FCS. Medium containing cells (1 ml) was added to each T25 and incubated at 37°C. Every 24 h, 3 T25 flasks of each cell line were harvested using 5 mM EDTA, resuspended in PBS and counted in a Coulter counter.

Adhesion Kinetics

Adhesion kinetic assays were performed in 96-well, non-tissue culture, flat bottom plates (Libro). The plates were coated with 10 µl/ml BSA, fibronectin, laminin, type IV collagen and 5 µl/ml vitronectin for 2 h at 37°C. Two hours prior to the assay all wells were blocked with 100 µl DMEM containing 2.5 mg/ml BSA to prevent nonspecific binding. PC-3 cells were plated at a concentration of 30,000 cells/well and IPC-3 cells at a concentration of 50,000 cells/well. Plates were then centrifuged at 1200 rpm for 1 min. At designated time points duplicate wells were

gently rinsed with PBS to remove unattached cells and then fixed with 3.7% paraformaldehyde

in PBS. Plates were kept at 37°C between time points. After completion of the assay the fixative

was replaced with 3.7% paraformaldehyde in

PBS containing 0.25% toluidine blue and allowed to stain overnight. The plates were then

thoroughly rinsed with distilled water and the absorbance measured in an ELISA plate reader at

492 OD.

RESULTS

In vitro invasion assay

In order to investigate the invasive potential of tumor cells and the role of integrins in tumor cell invasion, an in vitro invasion assay using transwells (Costar) (Fig. 4) and reconstituted basement membrane, matrigel (Collaborative Research), was developed. The first problem encountered during the development of the assay was the removal of the matrigel from the transwell membrane. The matrigel remaining on the membrane with the invasive cells after the invasion assay retained the Toluidine blue, causing high background staining and making it difficult to count the cells. Another possible method to quantitate the number of cells having invaded was to prelabel them with ^{3}H-thymidine. In this case, some cells which had not invaded or partially invaded and remained in the matrigel which could not be removed from the membrane and were counted as part of the invasive cells. A third method investigated was digesting the matrigel with Dispase (Collaborative Research). The enzyme would also digest the extracellular matrix to which the invasive cells were attached, causing them to detach during washings. In order to rectify these problems, another membrane with 12 μm pores was cut to the size of the transwell and placed over the transwell membrane (8.0 μm pores) which was previously covered with 100 μl/ml type I collagen to permit the invasive cells to attach.

The in vitro invasion assay that we finally settled on is illustrated in figure 5. In the final procedure, the matrigel was diluted in cold PBS, placed in the transwell and allowed to gel at 37°C. Cells prelabeled for 24 h with ^{3}H-thymidine were suspended in DMEM containing 1% ITS+ (Collaborative Research) and placed in the top chamber. The cells were allowed to invade for 24 h at 37°C, 5.0% CO_2.

Figure 4   **Photograph of transwells used in the <u>in</u> <u>vitro</u> invasion**

assay

Figure 5   Schematic diagram of the <u>in</u> <u>vitro</u> invasion assay using transwells and Matrigel
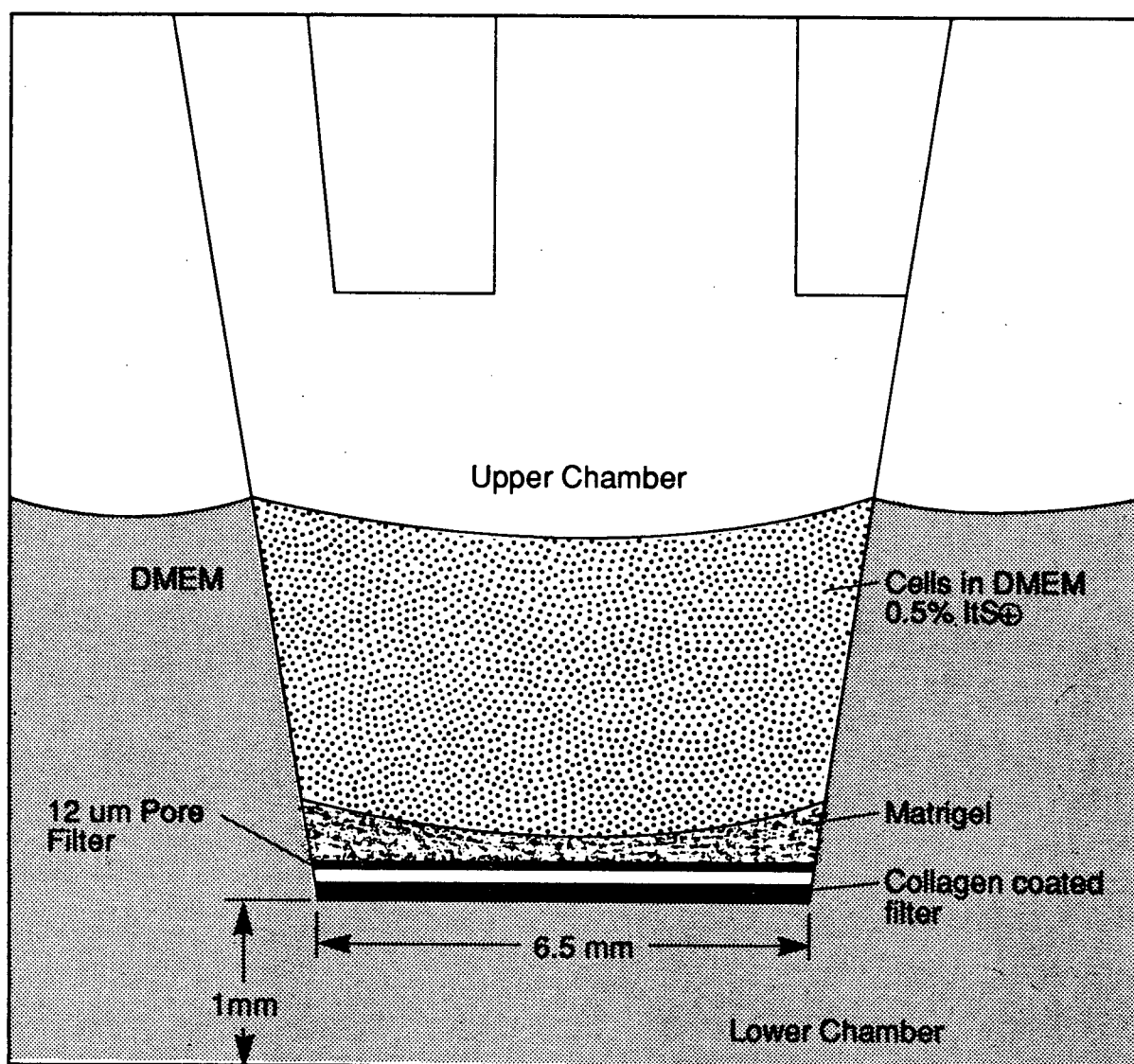
During this time, the invasive cells penetrated through the matrigel and the 12 μm pores of the upper membrane to attach to the bottom collagen coated membrane. After fixation with methanol, the top membrane and matrigel could be easily removed with curved forceps leaving behind the bottom membrane with the invasive cells. The bottom membrane was then removed and the radioactivity counted in a scintillation counter. Aliquots of the cells placed in the top chamber were taken prior to the assay and the percent invasion calculated.

The first objective after having developed the assay was to optimize the conditions for invasion. The parameters examined were (i) the number of cells, (ii) the concentration of matrigel, and (iii) the time required for the cells to invade. For these experiments, a prostate carcinoma cell line PC-3 was used. PC-3 cells are an undifferentiated cells which are known to be invasive in vitro (Albini et al., 1987).An adequate number of cells was required in the assay such that a quatitiative number of cells would invade through the matrigel and the top membrane. By varying the number of cells, an optimal cell number of 1×10^5 cells/well allowed a sufficient number of cells to invade and did not result in overcrowding during invasion. Several dilutions of matrigel in PBS were also tried to determine which concentration would permit only the invasive cells to invade in a reasonable period of time. An optimal concentration of matrigel would discriminate between the invasive and non-invasive cells. The relationship between the number of cells having invaded through the matrigel and the dilution of matrigel is shown in figure 6. Stock matrigel (8.8 mg/ml) and a 1:2 dilution did not permit any cells to invade, even after 48 h, while concentrations of less than 1mg/ml did not gel properly and permitted a large number of cells to invade. A dilution of 1:5 (1.75 mg/ml) was optimal, allowing a reasonable number of cells to invade during a 24 h incubation period.

Figure 6   Relationship between matrigel concentration and invasion of PC-3
cells

1×10^5 cells were placed in each well with varying concentrations
of matrigel and allowed to invade for 24 h at 37°C, 5.0% CO_2. The
cells were then fixed and stained with methanol containing 0.5%
toluidine blue. The cells were counted visually using a Zeiss
inverted microscope.

Matrigel Concentration vs invasion
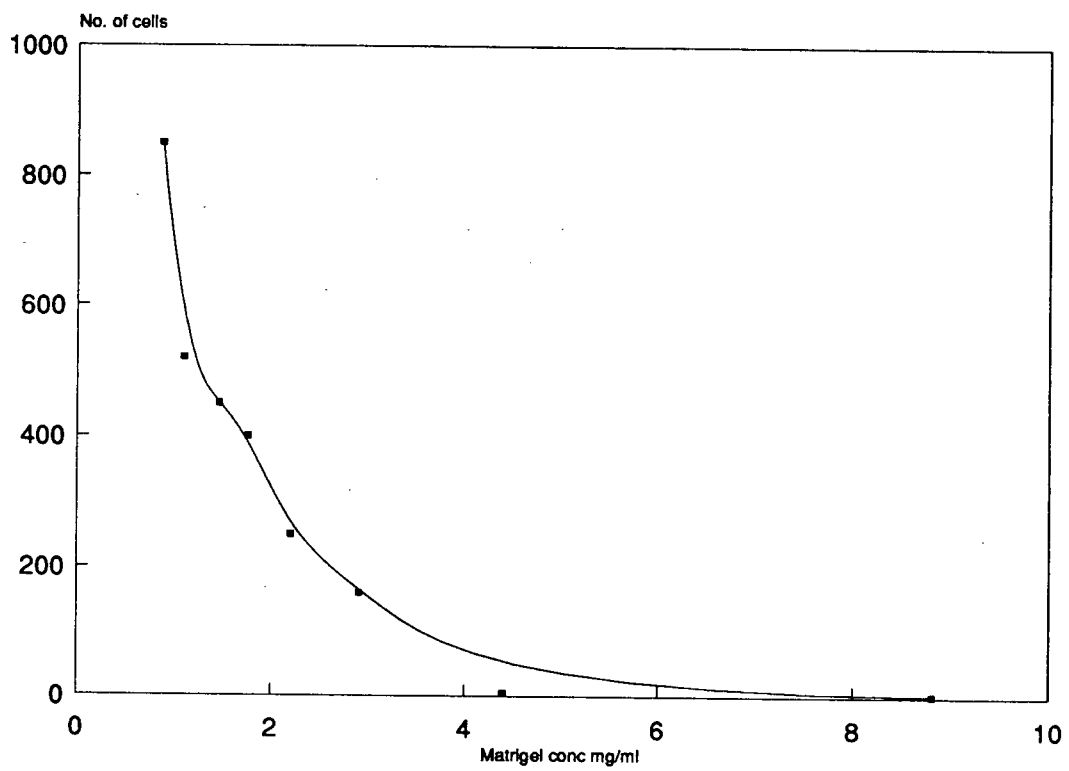
Invasion of PC-3 and IMR90 Cells

Once the conditions of the invasion assay were optimized it was necessary to compare different cell lines and verify the ability of the invasion assay to discriminate between invasive and non-invasive cells. An undifferentiated malignant prostate carcinoma cell line, PC-3 and a normal human lung fibroblast cell line, IMR90, were assayed simultaneously as described in the Materials and Methods. Although the actual percent of invasive cells varied between experiments, a higher percentage of PC-3 cells invaded through the matrigel in every case (Table 2). Since normal fibroblasts are able to migrate, the small degree of invasion observed with IMR90 was expected.

Invasion of HOS and MNNG-HOS cells

After having developed and optimized the conditions of the invasion assay, two osteosarcoma cell lines with known in vivo potential were assayed. The human osteosarcoma cell line (HOS) is a non-tumorigenic tumor cell line which is unable to form tumors in nude mice (Rhim et al.,1975). Its chemically transformed counterpart, MNNG-HOS, is very tumorigenic and is able to form tumors in nude mice. The two cell lines were assayed simultaneously under the same conditions described for PC-3 and IMR90 cells. Although there was some interassay variability MNNG-HOS cells were consistantly and considerably more invasive than HOS cells (Table 3).

Table 2

Invasion of PC-3 and IMR90 cell lines in vitro

	percent invasion	
cells	experiment 1	experiment 2
PC-3	3.70 ± 0.80	1.54 ± 0.21
IMR90	0.38 ± 0.14	0.58 ± 0.12

Values are stated as percent invasion ± standard error.
Difference in invasiveness between PC-3 and IMR90 cells are statistically significant (p<0.05) as determined by the students T-test
In all experiments n=4

Table 3

Invasion of osteosarcoma cell lines, HOS and MNNGHOS

|	Percent cells invaded		
Experiment	HOS	MNNG-HOS	IMR90
1	1.71 ± 0.84	4.65 ± 1.2	0.70 ± 0.16
2	4.97 ± 1.71	8.90 ± 1.62	0.82 ± 0.20
3	1.81 ± 1.31	6.79 ± 1.05	0.59 ± 0.13
4	1.87 ± 0.43	2.94 ± 0.85	--

Values are given in percent invasion ± standard error
Difference in invasion between HOS and MNNG-HOS cells are statistically significant ($p<0.05$) as determined by the Mann-Whitney U test.
In all experiments n=4

Expression of integrins on HOS and MNNG-HOS Cells

Having observed a difference in the invasive potential between HOS and MNNG-HOS, the expression of integrins was investigated on the two cell lines. The integrins were immunoprecipitated using anti-integrin antibodies. Immunoprecipitations with the anti-β_1 monoclonal antibody generated two bands, a 110 kDa band (β_1 subunit) and a 150 kDa band containing the α_2, α_3, α_5, and α_6 subunits. Both bands were present in both cell lines but were upregulated in MNNG-HOS cells (Fig. 7a, lanes 1 and 2). Anti-α_3 monoclonal antibody generated similar amounts of the 110 kDa β_1 and 150 kDa α_3 subunits in both cell lines (lanes 3 and 4). Neither HOS nor MNNG-HOS cells expressed high levels of the α_5 subunit in immunoprecipitations using anti-α_5 monoclonal antibodies (lanes 5 and 6). Immunoprecipitations using anti-α_6 monoclonal antibodies demonstrated that MNNG-HOS cells upregulated the 140 kDa α_6 subunit compared to the HOS cells (lanes 7 and 8). The 180 kDa α_1 subunit was also upregulated in MNNG-HOS cells when immunoprecipitated with anti-α_1 monoclonal antibodies (lanes 9 and 10). MNNG-HOS cells also upregulated the 150 kDa α_2 subunit in immunoprecipitations using anti-α_2 monoclonal antibodies (lanes 11 and 12). Immunoprecipitations with anti-vitronectin receptor polyclonal antibody generated 3 bands. A 160 kDa α_v subunit, its 97 kDa associated β_3 subunit and the β_1 subunit which cross reacts with the antibody. MNNG-HOS cells strongly downregulated the vitronectin receptor, especially the β_3 subunit (Fig. 7b, lane 2).

To investigate the role of the integrins, in particular the integrins of the β_1 family in the invasion process through matrigel, antibodies directed against the integrin subunits were incubated with the cells prior to and during the invasion

Figure 7a,b   Autoradiograph of a 7.5% sodium dodecyl sulfate polyacrylamide
gel. Integrins from ^{125}I surfaced labeled HOS (odd numbered lanes)
and   MNNG-HOS   (even   numbered   lanes)   cells   were
immunoprecipitated under non-reducing conditions with anti-ß$_1$,
lanes 1 and 2; anti-α_3, lanes 3 and 4; anti-α_5, lanes 5 and 6; anti-α_6,
lanes 7 and 8; anti-α_1, lanes 9 and 10; α_2, lanes 11 and 12 and anti-
VNR (Fig. 7b, lanes 1 and 2). After electrophoresis the gel was
fixed and stained in gel fixative containing Coomassie blue then
dried and exposed to Kodak diagnostic film.
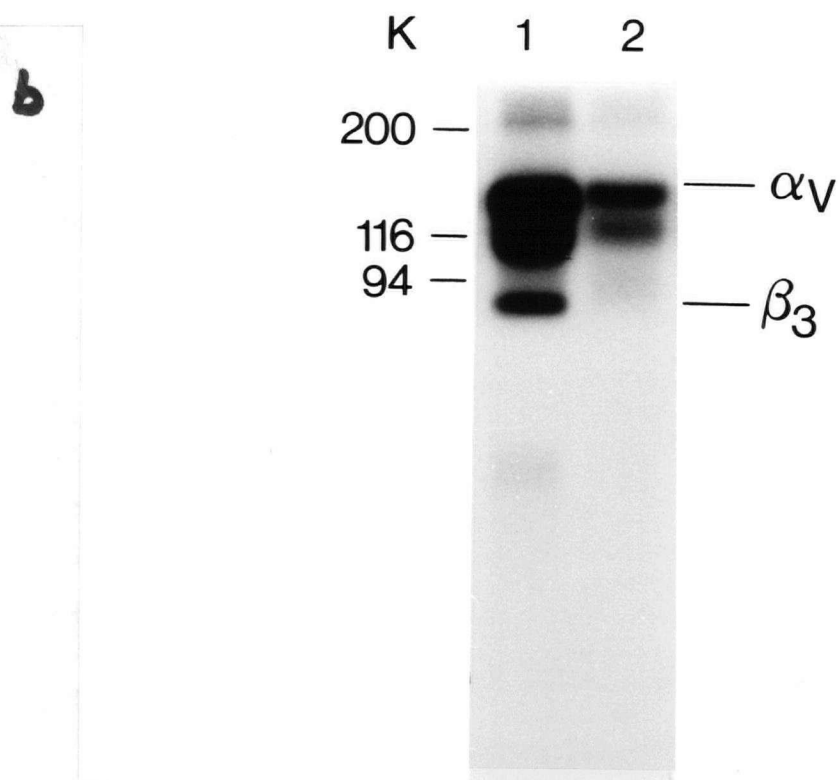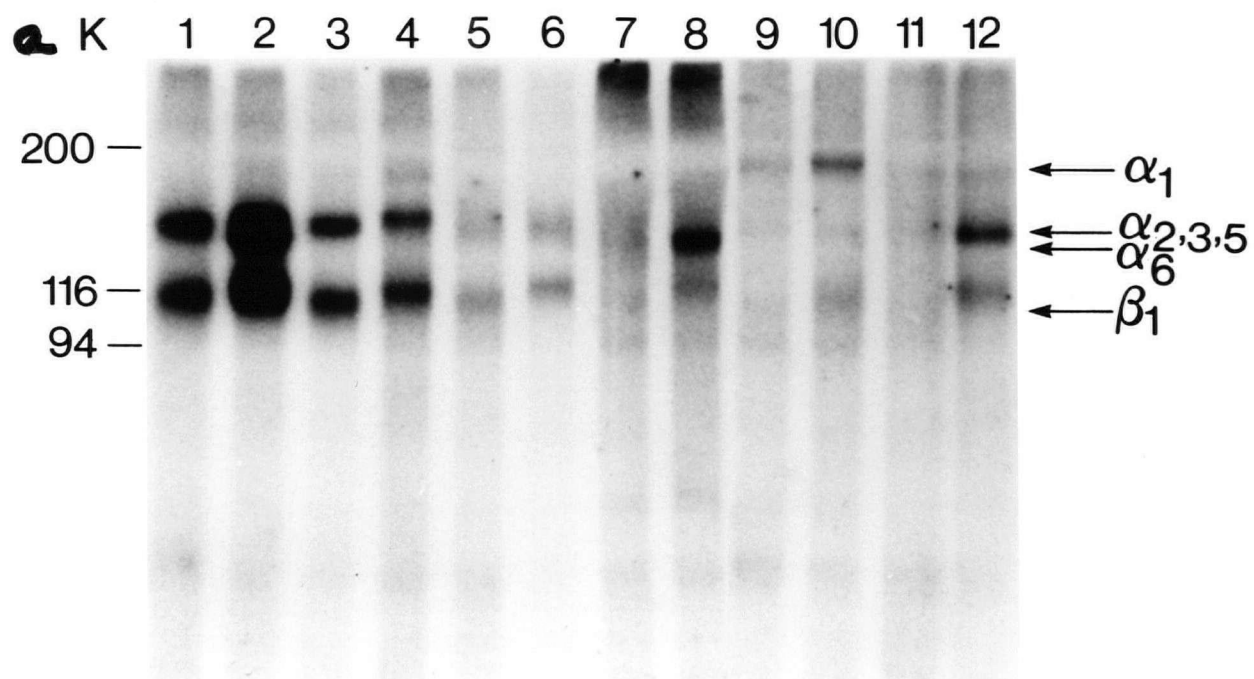
assay. A polyclonal antibody to the fibronectin receptor (directed to the β_1 subunit) and a monoclonal antibody directed to the α_6 subunit (GoH3) of $\alpha_6\beta_1$ were preincubated for 20 min with the cells prior to the assay and were present during the assay. Table 4 shows the invasion of HOS and MNNG-HOS cells in the presence of anti-integrin antibodies. The invasion of HOS cells was inhibited approximately 46% and MNNG-HOS cells 36% in the presence of a polyclonal anti-fibronectin receptor (1:50 dilution) as compared to controls using rabbit preimmune serum.

The integrin laminin receptor $\alpha_6\beta_1$ was of particular interest because it was strongly upregulated on the more invasive MNNG-HOS cells. Preincubation of HOS and MNNG-HOS cells with the monoclonal antibody GoH3 (1:2 dilution) inhibited invasion of HOS cells by approximately 70% but had no inhibitory effect on the invasion of MNNG-HOS cells. Suspecting that there was not sufficient antibody present to affect receptor-ligand interactions, we concentrated the antibody five fold and repeated the experiment. After concentration of the GoH3 antibody the invasion of MNNG-HOS was reduced by 41% (Table 4).

Isolation of an invasive cell line (IPC-3)

In order to study the properties of invasive cells, it is important to obtain a homogenous population of invasive cells. The in vitro invasion assay serves a dual purpose in that the cells having invaded through the matrigel can be easily isolated from the non- invasive cells and recultured. A more invasive cell line from the prostate carcinoma cell line, PC-3, was isolated using the in vitro invasion assay. After a 36 h incubation period, the membrane containing the matrigel and non-invasive cells was removed leaving the membrane containing the invasive cells.

Table 4
Inhibition of invasion of HOS and MNNGHOS using αFNR and anti-α$_6$ antibodies

Cells	% cells invaded	% inhibition
HOS		
Contol	5.0 ± 1.2	--
Anti-FNR antibody (1:50)	2.7 ± 0.8	46
Anti-α$_6$ antibody (1:2)	1.5 ± 0.4	70
MNNG-HOS		
Control	8.1 ± 1.5	--
Anti-FNR antibody (1:50)	5.2 ± 1.1	36
Anti-α$_6$ antibody (1:2)	8.3 ± 1.7	--
Anti-α$_6$ antibody (5 fold more concentrated)	4.8 ± 1.2	41

Values are given as percent invasion ± standard error
The difference in invasive potential between control and treatment groups
were statistically significant ($p < 0.05$) as determined by the Mann-Whitney
U test.
In all experiments n=4

The membranes were cut from the transwell and placed in DMEM containing 10% FCS and allowed to grow at 37°C, 5% CO_2. Once a sufficient number of cells was obtained they were assayed again and those having invaded recultured as before. After three successive passages through the matrigel, a more invasive cell line called IPC-3 was obtained. The difference in the morphology and size of the parent PC-3 and more invasive IPC-3 cell lines in tissue culture was studied (Fig. 8). The more invasive cells were smaller in size and remain spherical in shape (Fig. 8b) as compared to the PC-3 cells which were flatter and spindle or triangular shaped (Fig. 8a). When both cell lines were assayed simultaneously, the invasive IPC-3 cells were several times more invasive than the parent PC-3 cells. However, after several weeks in tissue culture the IPC-3 cells gradually lost their invasive potential and became similar to the PC-3 cells (Table 5). Because the IPC-3 cells initially behaved different from the PC-3 cells therefore the possibility of cell contamination was investigated. A fresh freezer stock of PC-3 cells (passage 8) was cultured and assayed under the same conditions. A cell line similar in morphology, growth rate, and integrin expression was isolated a second time (Fig. 8c).

Growth rate of PC-3 and IPC-3 cells

Typical growth curves for PC-3 and IPC-3 cells are shown in Figure 9. On subculturing, both PC-3 and IPC-3 cells started in a lag phase followed by an exponential phase. IPC-3 cells began the exponential phase sooner than PC-3 and remained in log phase for the 7 days recorded. PC-3 cells had a smaller log phase and started to level off after as they became confluent. The doubling time for PC-3 cells during log phase was 20 h and 28 h in prelog phase. IPC-3 cells had a slightly shorter doubling time of 22 h during log phase and 24 h during prelog phase.

Figure 8   Photograph of PC-3(a), the first IPC-3 isolate(b) and the second isolate (c). The cells were cultured in DMEM containing 10% FCS.

magnification 40x
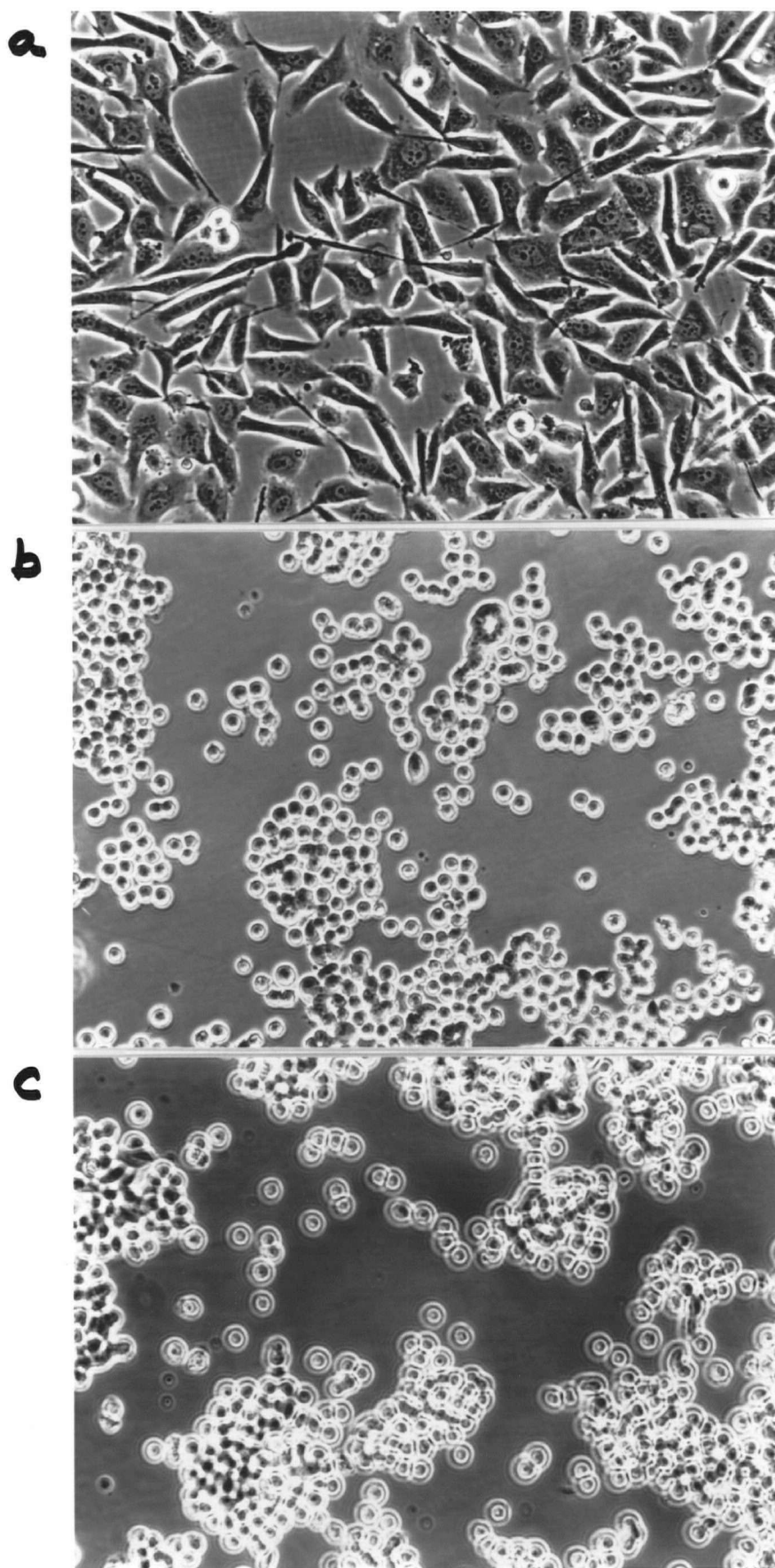
Table V   <u>Difference in invasive potential between PC-3 and IPC-3</u>

Weeks after isolation	% invasion PC-3	% invasion IPC-3	ratio
2	0.64 ± 0.12	5.4 ± 1.29	8.4
4	0.32 ± 0.06	1.1 ± 0.12	3.4
10	1.98 ± 0.73	3.55 ± 0.91	1.8
14	1.51 ± 0.21	2.35 ± 0.60	1.6

Values are given in percent invasion ± standard error
In all experiments n=4

Figure 9   Growth curves for PC-3 and IPC-3 cells

PC-3 and IPC-3 cells were plated at a concentration of 1×10^5 cells/T25 in DMEM containing 10% FCS and incubated at 37°C, 5.0% CO_2. Three flasks of each cell line were harvested every 24 h and counted in a Coulter counter.

PROLIFERATION RATE OF PC3 & IPC3
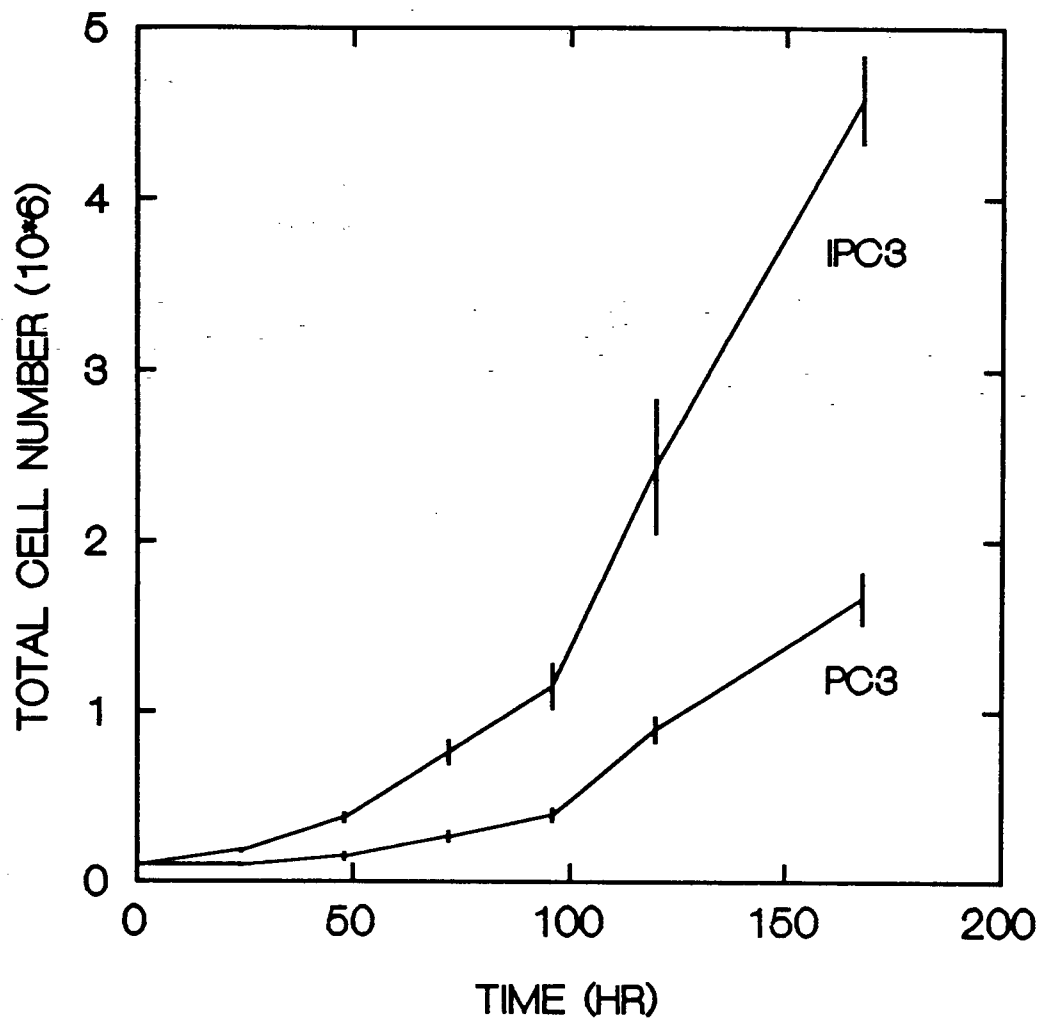
Adhesion of PC-3 and IPC-3 cells to extracellular matrix proteins

The adhesion of tumor cells to the extracellular matrix is the first step in the invasion process. Therefore the adhesion of PC-3 and IPC-3 cells to fibronectin, vitronectin, laminin, and collagens type I and IV was investigated (Figs. 10-14). PC-3 and IPC-3 did not adhere well to concentrations of fibronectin below 1.25 µg/ml However, at concentrations greater than 2.5 µg/ml the adhesion of PC-3 cells to fibronectin was greater than that seen with IPC-3 cells (Fig. 10). The adhesion of IPC-3 cells to vitronectin was greater than PC-3 at concentrations below 2.5 µg/ml but both cell lines had similar adhesion profiles at protein concentrations between 5 µg/ml and 20 µg/ml (Fig. 11). There was very little difference in the adhesion of PC-3 and IPC-3 cells to laminin at all concentrations assayed (0.156-20 µg/ml) with the exception of 1.25 µg/ml where a greater percentage of PC-3 cells attached to the substrate (Fig. 12). Both PC-3 and IPC-3 cells attached well to collagens type I and IV. For both sustrates a greater percentage of PC-3 cells adhered than IPC-3 cells at all protein concentrations studied (Figs. 13 and 14). Both PC-3 and IPC-3 reached maximal binding between 5-10 µg/ml for fibronectin, laminin and collagen types I and IV while on vitronectin they bound maximally at approximately 2.5-5.0 µg/ml.

Comparing the adhesion of PC-3 to the five extracellular matrix components assayed (Fig. 15), a greater percentage of PC-3 cells attached to type IV collagen at very low concentrations. At concentrations above 2.5 ug/ml, PC-3 cells had similar adhesion curves for all five substrates. A greater percentage of IPC-3 cells attached to vitronectin at low concentrations and to laminin at substrate concentrations above 5.0 µg/ml (Fig. 16). Fewer IPC-3 cells attached to fibronectin at both high and low protein concentrations.

Figure 10   Adhesion of PC-3 and IPC-3 to fibronectin

> 96 well, flat bottom, microtiter plates were coated with two fold
> serial dilutions of fibronectin (20-0.156 µg/ml). Cells, prelabeled
> with ^{3}H-thymidime were plated at a concentration of 4×10^4 (PC-3)
> and 8×10^4 (IPC-3) cells/well, then incubated at 37°C, 5.0% CO_2 for
> 1 h. Attached cells were removed with 50 µl 10 mM EDTA, 0.1%
> Triton-X100 and the radioactivity counted in a ß-scintillation
> counter.

Figure 11   Adhesion of PC-3 and IPC-3 to vitronectin

> 96 well, flat bottom, microtiter plates were coated with two fold
> serial dilutions of vitronectin (20-0.156 µg/ml). Cells, prelabeled
> with ^{3}H-thymidime were plated at a concentration of 4×10^4 (PC-3)
> and 8×10^4 (IPC-3) cells/well, then incubated at 37°C, 5.0% CO_2 for
> 1 h. Attached cells were removed with 50 µl 10 mM EDTA, 0.1%
> Triton-X100 and the radioactivity counted in a ß- scintillation
> counter.

IPC-3/PC-3 ADHESION ON FIBRONECTIN
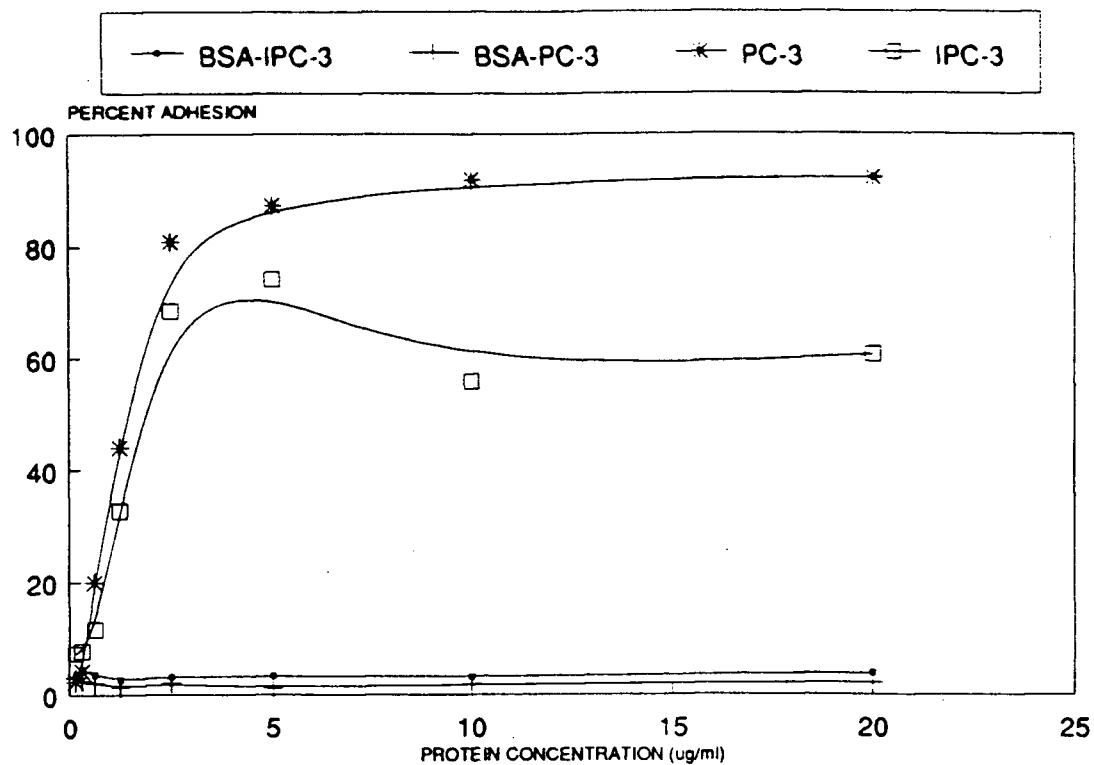
PC-3/IPC-3 ADHESION TO VITRONECTIN

Figure 12   Adhesion of PC-3 and IPC-3 to laminin

96 well, flat bottom, microtiter plates were coated with two fold serial dilutions of laminin (20-0.156 μg/ml). Cells, prelabeled with ^{3}H-thymidime were plated at a concentration of 4x10^4 (PC-3) and 8x10^4 (IPC-3) cells/well, then incubated at 37°C, 5.0% CO_2 for 1 h. Attached cells were removed with 50 μl 10 mM EDTA, 0.1% Triton-X100 and the radioactivity counted in a ß- scintillation counter.

Figure 13   Adhesion of PC-3 and IPC-3 to type I collagen

96 well, flat bottom, microtiter plates were coated with two fold serial dilutions of type I collagen (20-0.156 μg/ml). Cells, prelabeled with ^{3}H-thymidime were plated at a concentration of 4x10^4 (PC-3) and 8x10^4 (IPC-3) cells/well, then incubated at 37°C, 5.0% CO_2 for 1 h. Attached cells were removed with 50 μl 10 mM EDTA, 0.1% Triton-X100 and the radioactivity counted in a ß- scintillation counter.
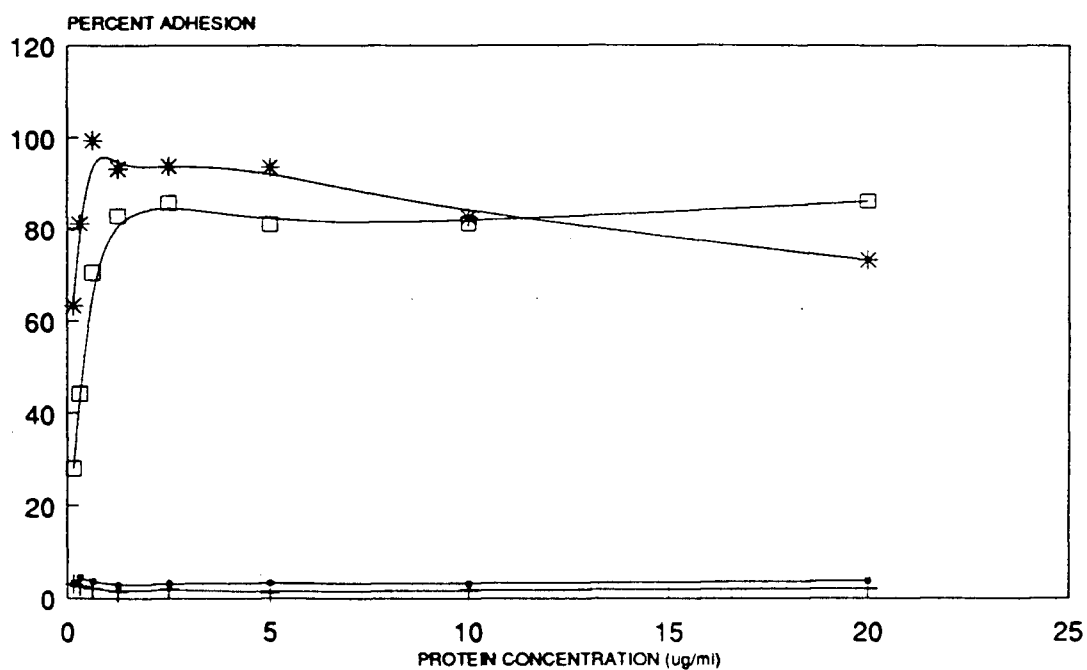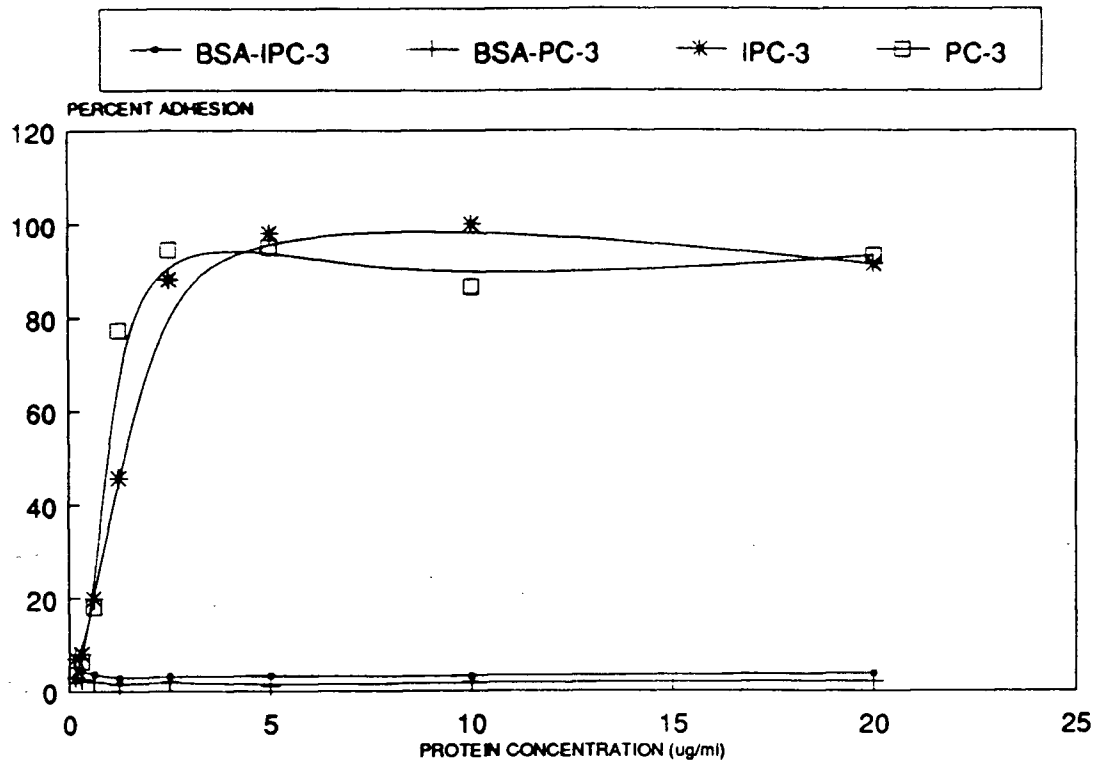
PC-3/IPC-3 ADHESION TO LAMININ

—•— BSA-IPC-3	—+— BSA-PC-3	—*— IPC-3	—□— PC-3

PC-3/IPC-3 ADHESION TO TYPE I COLLAGEN

Figure 14   Adhesion of PC-3 and IPC-3 to type IV collagen

96 well, flat bottom, microtiter plates were coated with two fold serial dilutions of type IV collagen (20-0.156 µg/ml). Cells, prelabeled with ^{3}H-thymidime were plated at a concentration of 4×10^4 (PC-3) and 8×10^4 (IPC-3) cells/well, then incubated at 37˚C, 5.0% CO_2 for 1 h. Attached cells were removed with 50 µl 10 mM EDTA, 0.1% Triton-X100 and the radioactivity counted in a ß-scintillation counter.

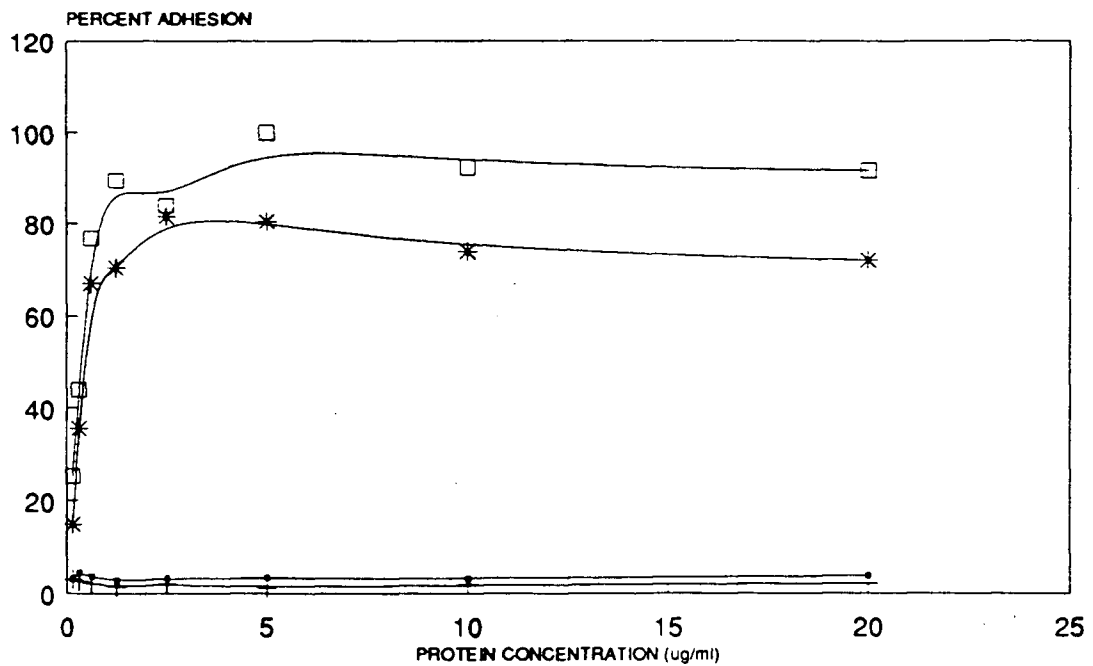PC-3/IPC-3 ADHESION TO TYPE IV COLLAGEN
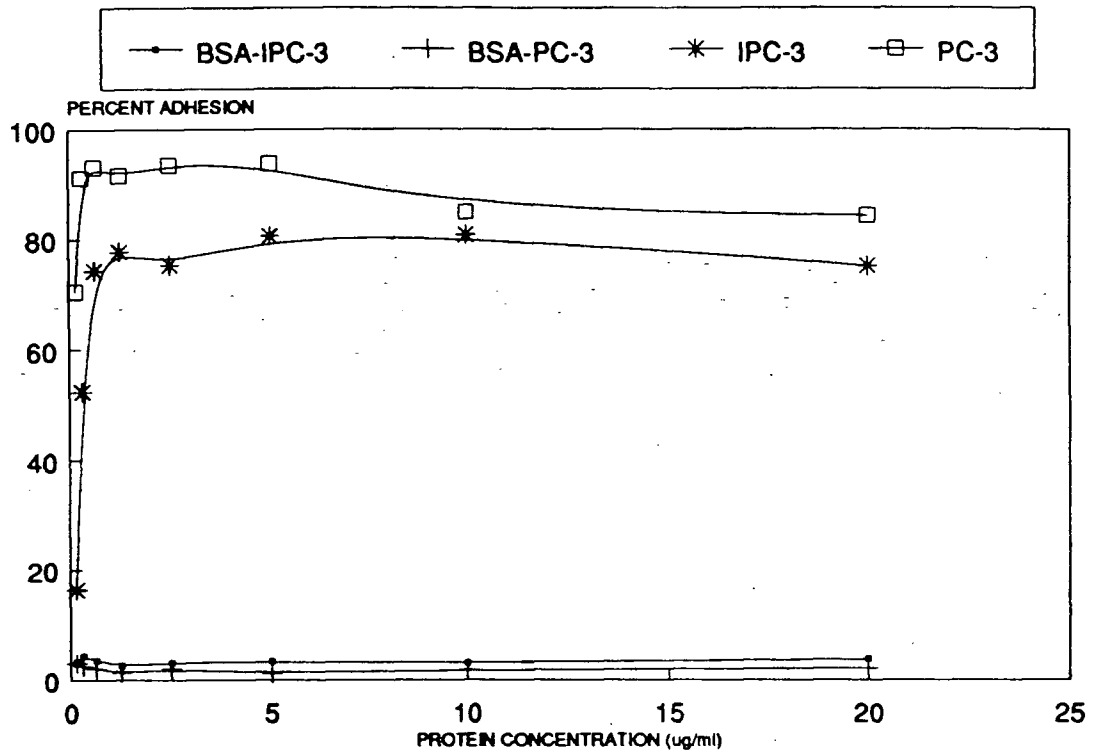
Figure 15   Adhesion of PC-3 to fibronectin, vitronectin, laminin, and collagen types I and IV.

96 well, flat bottom, microtiter plates were coated with two fold serial dilutions of fibronectin, vitronectin, laminin, and collagen types I and IV (20-0.156 µg/ml). PC-3 cells, prelabeled with ^{3}H-thymidime were plated at a concentration of 4×10^4 cells/well, then incubated at 37°C, 5.0% CO_2 for 1 h. Attached cells were removed with 50 µl 10 mM EDTA, 0.1% Triton-X100 and the radioactivity counted in a ß-scintillation counter.

Figure 16   Adhesion of IPC-3 to fibronectin, vitronectin, laminin, and collagen types I and IV.

96 well, flat bottom, microtiter plates were coated with two fold serial dilutions of fibronectin, vitronectin, laminin, and collagen types I and IV (20-0.156 µg/ml). IPC-3 cells, prelabeled with ^{3}H-thymidime were plated at a concentration of 8×10^4 cells/well, then incubated at 37°C, 5.0% CO_2 for 1 h. Attached cells were removed with 50 µl 10 mM EDTA, 0.1% Triton-X100 and the radioactivity counted in a ß-scintillation counter.

PC-3 CELL ADHESION
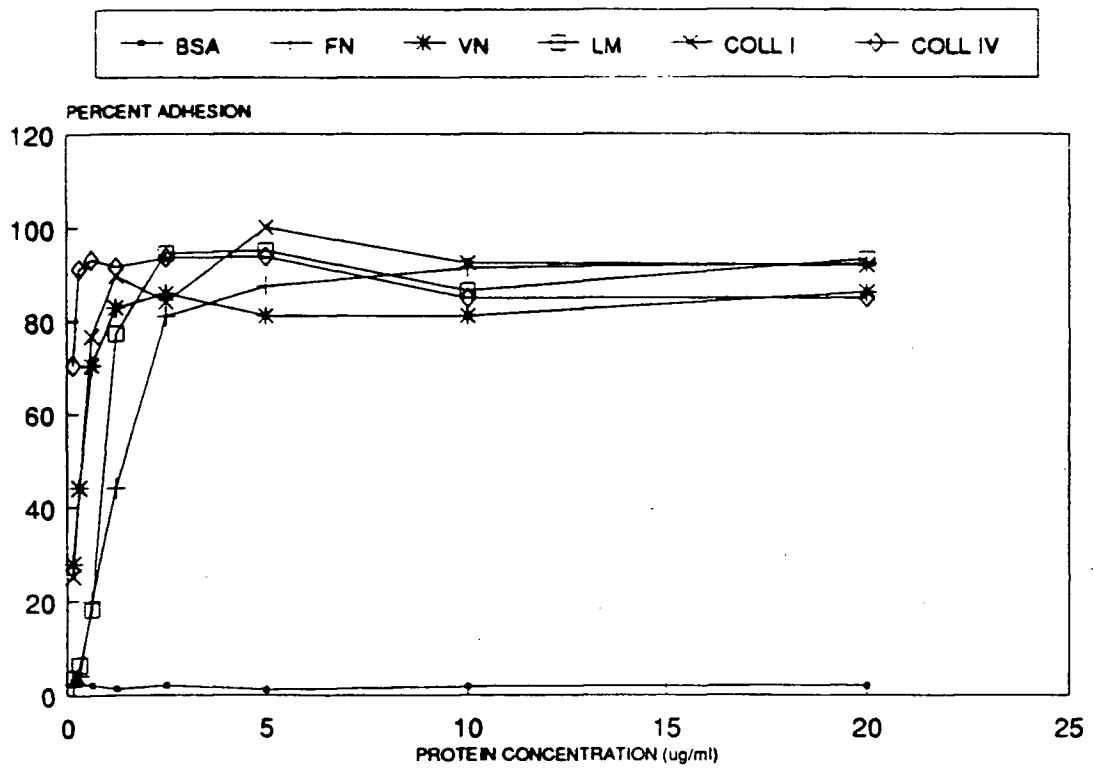
IPC-3 CELL ADHESION
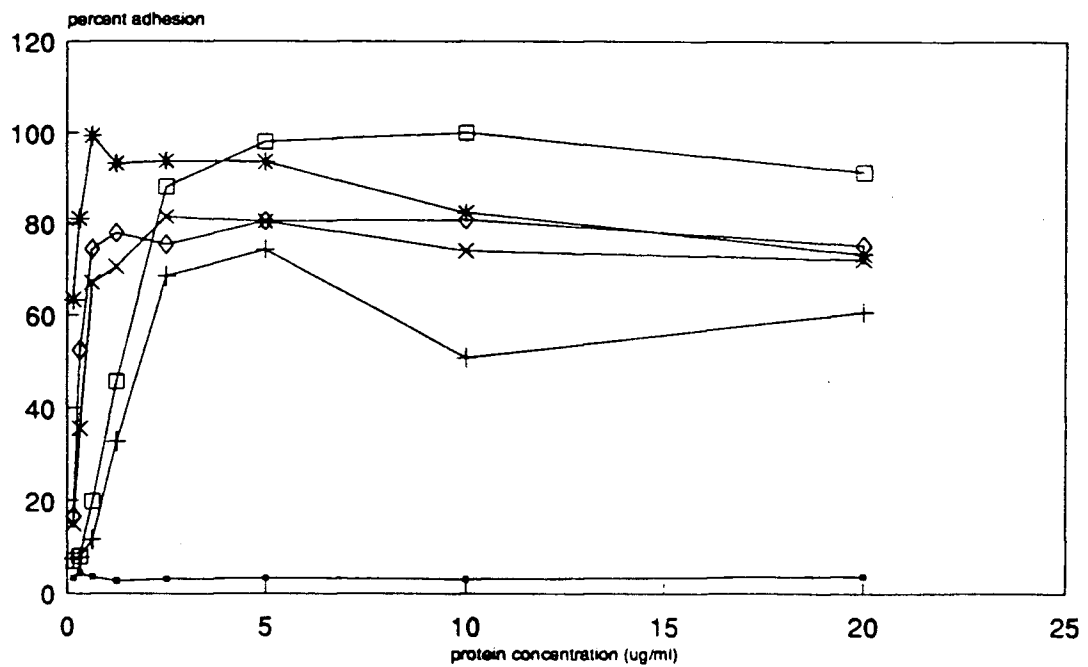
Adhesion kinetics of PC-3 and IPC-3 cells

Having observed a difference in the attachment of PC-3 and IPC-3 cells to the different extracellular matrix proteins, the rate of attachment of PC-3 and IPC-3 cells to the matrix proteins, fibronectin, laminin, vitronectin and type IV collagen was investigated (Figs 17 and 18). The rate of attachment of PC-3 cells to vitronectin was slower than to laminin, type IV collagen and fibronectin (which all have similar rates of adhesion) (Fig. 17). The rate of attachment of IPC-3 cells was slower to fibronectin but leveled off sooner on type IV collagen (Fig. 18). The rate of attachment of PC-3 and IPC-3 cells did not increase after 30 minutes on laminin, vitronectin and type IV collagen. However, the rate of attachment on fibronectin did not level off until after 60 minutes on both cell lines. Both PC-3 and IPC-3 cells showed a very rapid attachment to laminin with little change in attachment after 10 minutes.

Morphology of PC-3 and IPC-3 cells on extracellular matrix proteins

There are several factors which can affect the shape of cells in culture. The cytoskeleton, intermediate filaments, extracellular matrix, and growth factors all contribute in determining the shape of the cell (Ingber and Folkman, 1989).

The cell morphology of PC-3 and the invasive IPC-3 cells on 10 μg/ml fibronectin, laminin, collagen type I and IV, 5 μg/ml vitronectin and stock, 1:2 and 1:10 dilutions of matrigel were studied to investigate the effect of these extracellular matrix proteins on cell morphology and spreading (figs. 19 and 20). After 1 h incubation at 37°C, PC-3 cells had a similar morphology on fibronectin (Fig. 19a), vitronectin (Fig. 19c) and collagen type IV (Fig. 19g), showing a triangular or spindle shaped morphology. PC-3 cells attached very quickly to laminin and had a slightly different morphology in

Figure 17   Adhesion kinetics of PC-3 cells to fibronectin, vitronectin, laminin
             and type IV collagen.

             96 well. flat bottom, microtiter plates were coated with 10 µg/ml
             fibronectin, laminin and type IV collagen and 5 µg/ml vitronectin.
             PC-3 cells were plated at a concentration of 40,000 cells/well and
             centrifuged at 1200 rpm for 1 min. Duplicate wells were rinsed and
             the attached cells fixed at 5,10,20,40,60,90, and 120 min time
             points. Fixed cells were then stained with Coomassie blue and the
             absorbance measured in an ELISA plate reader at OD 492.

Figure 18   Adhesion kinetics of IPC-3 cells to fibronectin, vitronectin, laminin
             and type IV collagen.

             96 well. flat bottom, microtiter plates were coated with 10 µg/ml
             fibronectin, laminin and type IV collagen and 5 µg/ml vitronectin.
             IPC-3 cells were plated at a concentration of 80,000 cells/well and
             centrifuged at 1200 rpm for 1 min. Duplicate wells were rinsed and
             the attached cells fixed at 5,10,20,40,60,90, and 120 min time
             points. Fixed cells were then stained with Coomassie blue and the
             absorbance measured in an ELISA plate reader at OD 492.

PC-3 Adhesion kinetics
Adhesion vs Time
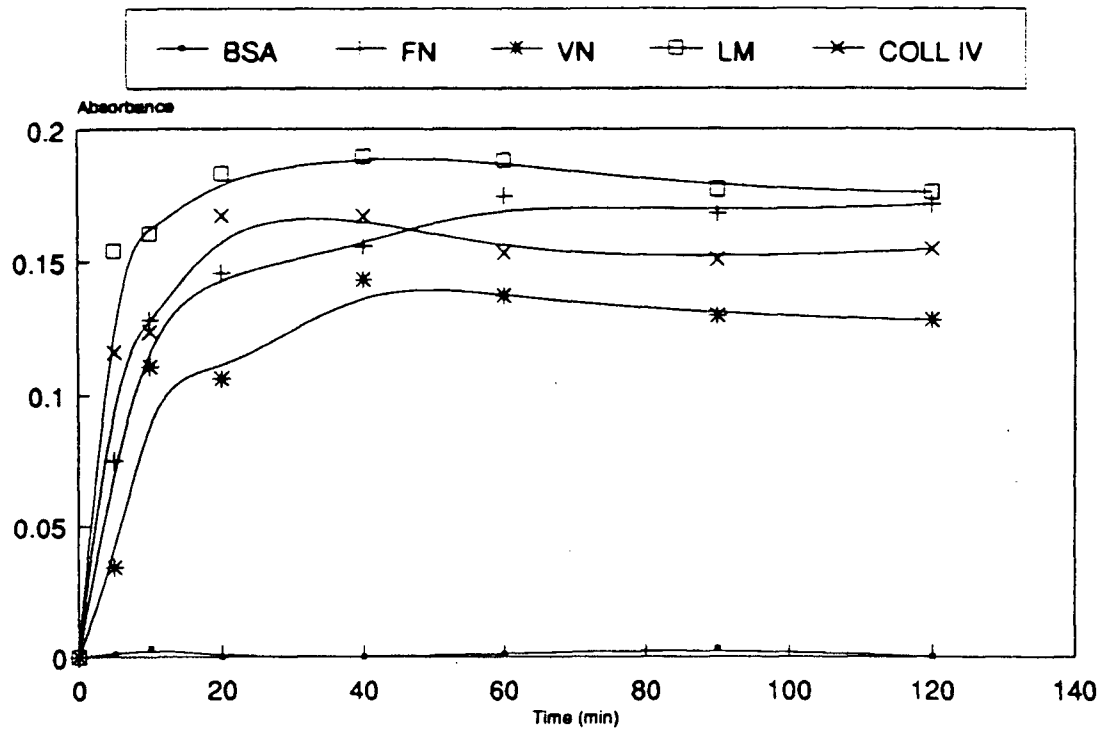
IPC-3 Adhesion Kinetics
Adhesion vs Time
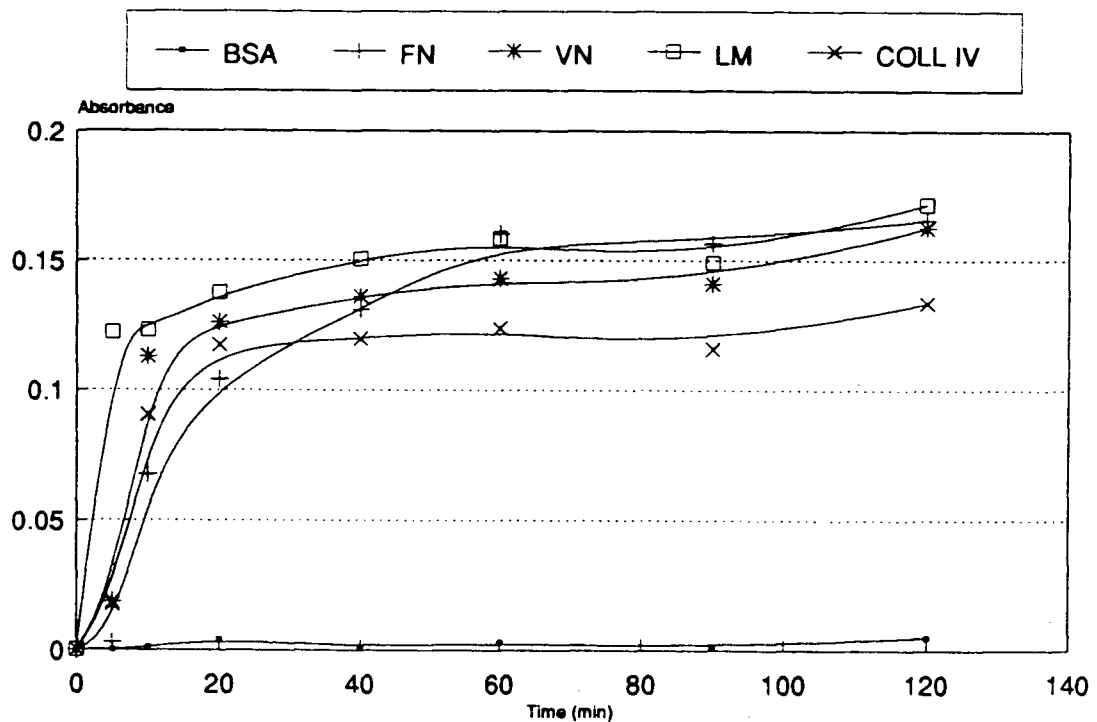
Figure 19   Morphology of PC-3 and IPC-3 cells on fibronectin, vitronectin, laminin, and collagen type IV.

96 well, flat bottom, microtiter plates were coated with 10 µg/ml fibronectin, vitronectin, laminin, and collagen types I and IV. Cells were plated at a concentration of $3x10^4$ (PC-3) and $5x10^4$ (IPC-3), then incubated at 37°C, for 24 h and photographed

(a) PC-3 on fibronectin
(b) IPC-3 on fibronectin
(c) PC-3 on vitronectin
(d) IPC-3 on vitronectin
(e) PC-3 on laminin
(f) IPC-3 on laminin
(g) PC-3 on type IV collagen
(h) IPC-3 on type IV collagen

magnification 40X
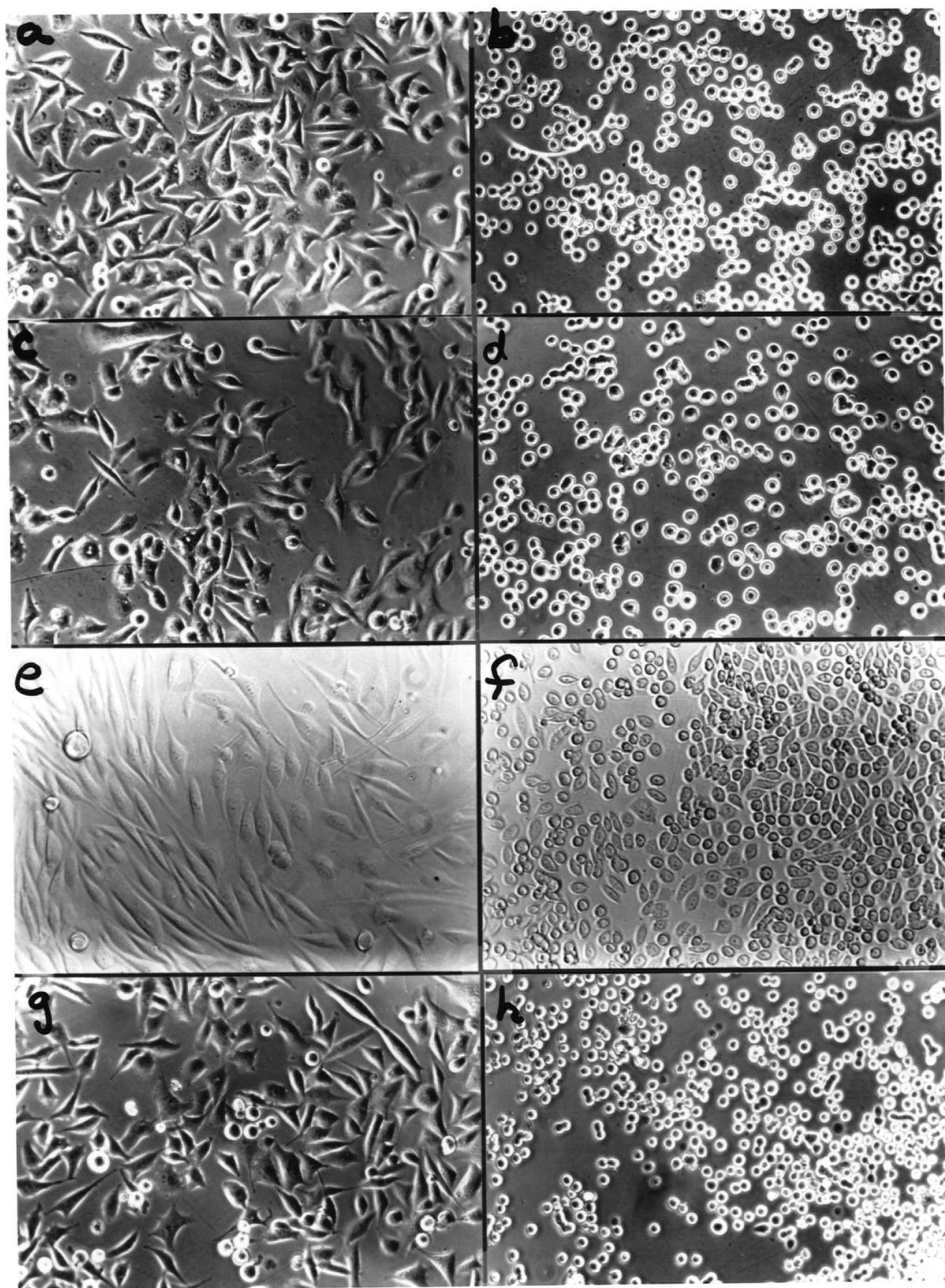
Figure 20   Morphology of PC-3 and IPC-3 cells on matrigel

96 well, flat bottom, microtiter plates were coated with stock, 1:2, and 1:10 dilution of matrigel at a concentration of 3×10^4 (PC-3) and 5×10^4 (IPC-3), then incubated at 37°C for 24 h and photographed

(a) PC-3 on stock matrigel
(b) IPC-3 on stock matrigel
(c) PC-3 on a 1:2 dilution of matrigel
(d) IPC-3 on a 1:2 dilution of matrigel
(e) PC-3 on a 1:10 dilution of matrigel
(f) IPC-3 on a 1:10 dilution of matrigel

Magnification 40X
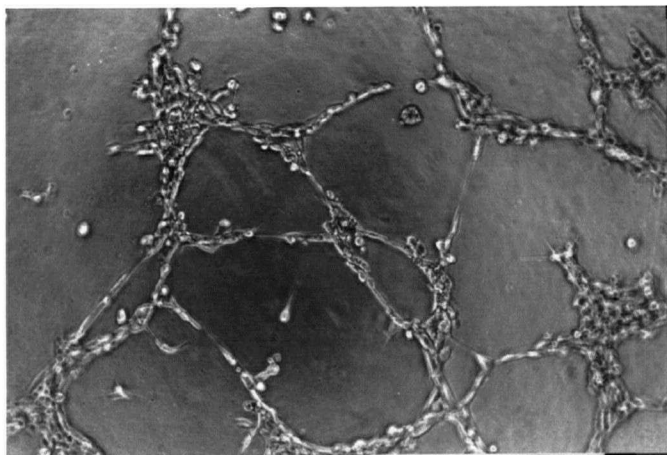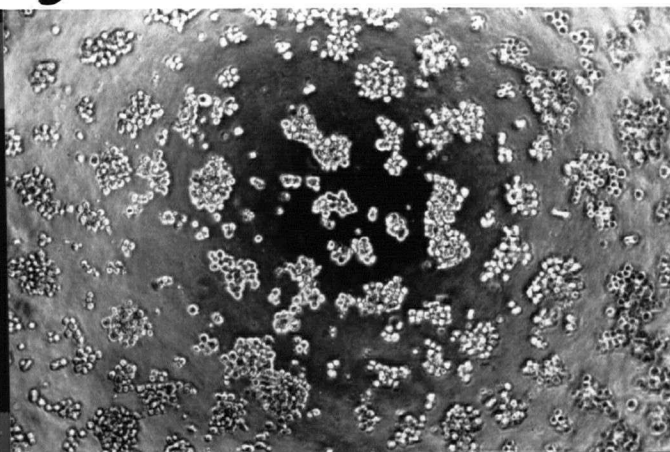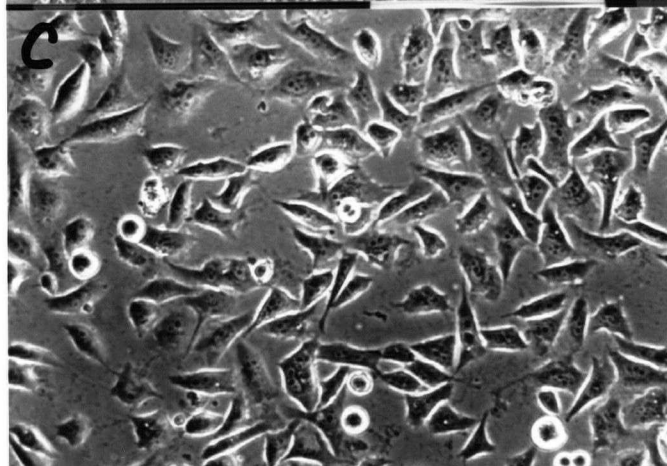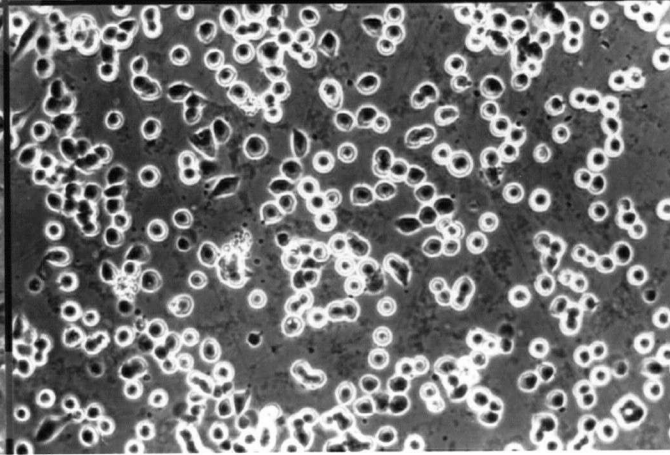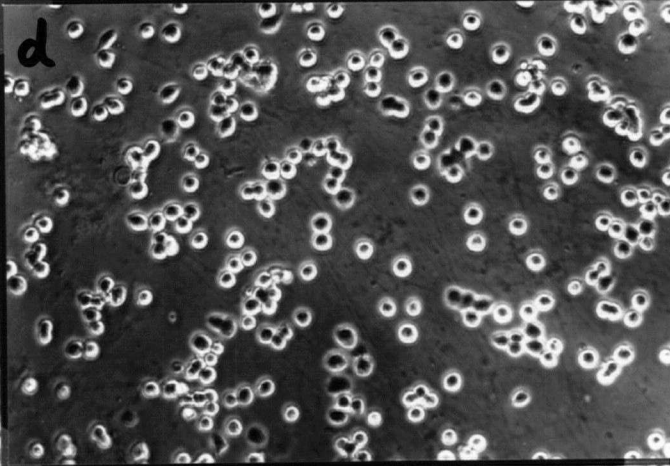
that the cells were more flattened making the cell borders diffficult to distinguish (Fig. 19e). On stock matrigel (8.8 mg/ml), PC-3 cells spread only slightly and lined up end to end. After 2-3 h incubation they formed tube-like structures (Fig. 20a). The organized structure of PC-3 cells was not present on 1:2, 1:5 and 1:10 dilutions of matrigel. However, the cells did spread on 1:5 and 1:10 dilutions (Fig. 20c and e). The invasive IPC-3 cells firmly attached to fibronectin (Fig. 19b), vitronectin (Fig. 19d), and collagen type IV (Fig. 19g) but did not show any signs of cell spreading. A difference in the morphology of IPC-3 on laminin was noted. After a couple of hours of incubation at 37°C, a few IPC-3 cells showed some signs of spreading, giving the cell a spindle or cuboidal shaped appearance. After 24 h all but a few IPC-3 cells had spread on laminin (Fig. 19f). IPC-3 cells remained smaller in size than PC-3 even after spreading. On stock matrigel IPC-3 cells did not spread and did not show any tube-like structures as did PC-3 cells (Fig. 20b). After 24 h on matrigel, IPC-3 cells were still round in shape and had aggregated in clusters. On a 1:10 dilution of matrigel, a few IPC-3 cells did show some signs of spreading (Fig. 20f).

Expression of integrins on PC-3 and IPC-3 cells

Having observed a difference in morphology and invasive potential between PC-3 and IPC-3 cells, the expression of integrins from both cell lines were examined. The integrins were immunoprecipitated from ^{125}I-labelled PC-3 and IPC-3 cell lysates using anti-integrin antibodies and analysed by SDS-PAGE under nonreducing conditions followed by autoradiography. Immunoprecipitations with a polyclonal antibody against the fibronectin receptor generated 3 bands, a 110 (β_1), 150 (α_2,α_3,α_5,and α_6) and 210 (α_1) kDa subunits (fig 21, lane 1). The expression of all three

Figure 21   Autoradiograph of a 7.5% sodium dodecyl sulfate polyacrylamide
gel. Integrins from ^{125}I-labeled PC-3 and IPC-3 cell were
immunoprecipitated under nonreducing conditions with anti-
fibronectin receptor, lanes 1 and 2 respectively; anti-vitronectin
receptor , lanes 3 and 4 respectively; anti-α_2, lanes 5 and 6
respectively; anti-α_3, lanes 7 and 8 respectively; anti-α_5, lanes 9 and
10 respectively and; anti-α_6, lanes 11 and 12 respectively. After
electrophoresis the gel was fixed and stained in gel fixative
containing Coomassie blue, then dried and exposed to Kodak
diagnostic film.

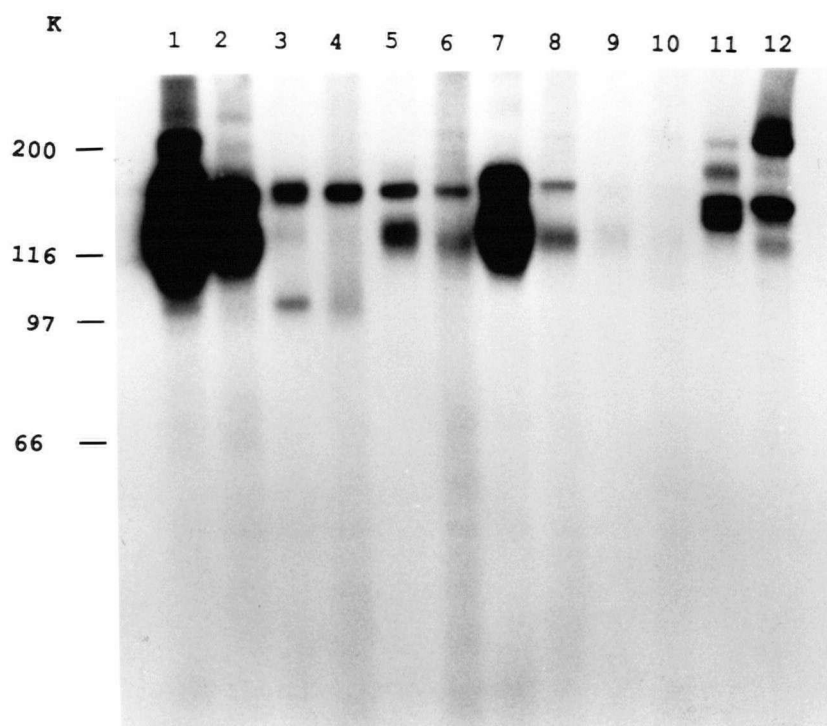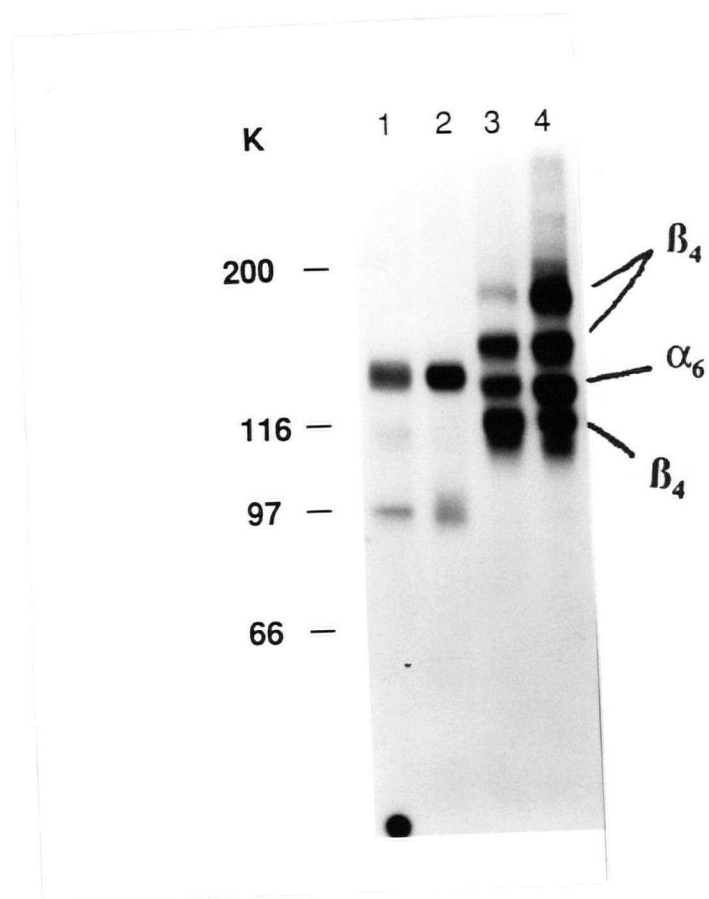# Immunoprecipitation of integrins from PC-3 and IPC-3 cells

Figure 22   Autoradiograph of a 6.0% sodium dodecyl sulfate polyacrylamide gel. Integrins from ^{125}I-labeled PC-3 and IPC-3 cell were immunoprecipitated under nonreducing conditions with anti-vitronectin receptor, lanes 1 and 2 respectively; anti-α_6 lanes 3 and 4 respectively.

bands were downregulated in IPC-3 cells (Fig. 21, lane 2). Immunoprecipitations using a polyclonal anti-vitronectin receptor antibody generated the 150 and 95 kda bands forming the $\alpha_v\beta_3$ complex respectively (lanes 3 and 4). Also present in anti-vitronectin receptor immunoprecipitations with PC-3 but not IPC-3 cells were low amounts of β_1 (Fig 22, lane 1). The expression of the $\alpha_1\beta_1$ seen in anti-fibronectin receptor immunoprecipitations was reduced in IPC-3 cells as well the expression of $\alpha_2\beta_1$, immunoprecipitated with the monoclonal antibody P1E6 (Fig. 21, lanes 5 and 6). There was a large downregulation of the integrin $\alpha_3\beta_1$ in IPC-3 cells which is normally expressed in high amounts in PC-3 cells (Fig. 21, lanes 7 and 8). The classical fibronectin receptor, $\alpha_5\beta_1$ which generates bands of 150 and 110 kDa demonstrated little change between PC-3 and IPC-3 (Fig. 21, lanes 9 and 10). Immunoprecipitations with the monoclonal anti-α_6 antibody (GoH3) generated 4 bands, the 150 kDa α_6 subunit and three β_4 subunits of 180, 130 and 115 kDa. IPC-3 cells strongly upregulated the 200 kDa subunit and slightly upregulated the 180 and 120 kDa β_4 subunits and the α_6 (Fig. 21,lane 12 and Fig. 22 lane 4).

Immunofluorescence of PC-3 and IPC-3 cells using anti-integrin antibodies

It was hoped that analysis of the integrins by immunofluorescence might give some indication of the distribution of integrins on the cell surface as well as the quantity of integrins. PC-3 and IPC-3 cells were grown on circular coverslips for 48 h, fixed in 2% paraformaldehyde and then incubated in a 1% solution of BSA to block nonspecific binding. This was followed by an incubation in a 1:200 dilution of the monoclonal antiboby P1B5 (anti-α_3) and an incubation with a rhodamine conjugated secondary antibody (1:100 of Rabbit anti-mouse).

Immunofluorescence staining of PC-3 and IPC-3 cells with anti-α_3 antibodies confirmed the almost complete downregulation of the $\alpha_3\beta_1$ integrin in IPC-3 cells (Fig. 23). The staining on PC-3 cells was diffusely distributed on the surface of the cell with a stronger staining along the

edge (Fig. 23c). The staining on IPC-3 cells was considerably reduced showing a circular pattern

on several cells. A large number of cells were completely devoid of the $\alpha_3\beta_1$ integrin (Fig. 23d).

Similar treatment of PC-3 and IPC-3 cells with a polyclonal anti-vitronectin receptor

antibody demonstrated a different staining pattern. The vitronectin receptor on PC-3 cells

localized at numerous adhesion plaques located primarily at the periphery of the cells (Fig. 24d)

while the vitronectin receptor on IPC-3 cells demonstrated the same circular staining observed

with anti-α_3 antibodies except the staining was much more prevalent and all the cells stained

positively (Fig. 24f). PC-3 cells stained with J1B5 antibodies (anti-α_6) showed in staining

primarily along the edge of some cells and at the tip of some cytoplasmic processes (Fig 25c).

Similar treatment of IPC-3 cells with anti-α_6 antibodies resulted in circular shaped staining seen

with anti-α_3 and anti-vitronectin receptor antibodies. A larger proportion of the cells of IPC-3

cells demonstrated positive staining for the integrin α_6 subunit than did the PC-3 cells (Fig. 25d).

Figure 23   Immunofluorescence and phase photographs of PC-3 and IPC-3
cells stained with anti-α_3 antibodies. Cells were cultured on round
glass coverslips in DMEM containing 10% FCS at 37°C, for 48 h.
Cells were washed in PBS, blocked with 1.0% BSA in PBS for 30
min, then incubated with a 1:200 dilution of anti-α_3 (P1B5) for 1
h at room temperature. Cells were then washed extensively and
incubated with a 1:100 dilution of rhodamine conjugated rabbit
anti-mouse antibody for 1 h at room temperature. Coverslips were
washed and mounted onto slides and sealed with clear nailpolish.

(a) PC-3 control
(b) IPC-3 control
(c) PC-3 stained with anti-α_3
(d) IPC-3 stained with anti-α_3
(e) PC-3 phase contrast
(f) IPC-3 phase contrast

Magnification 120X
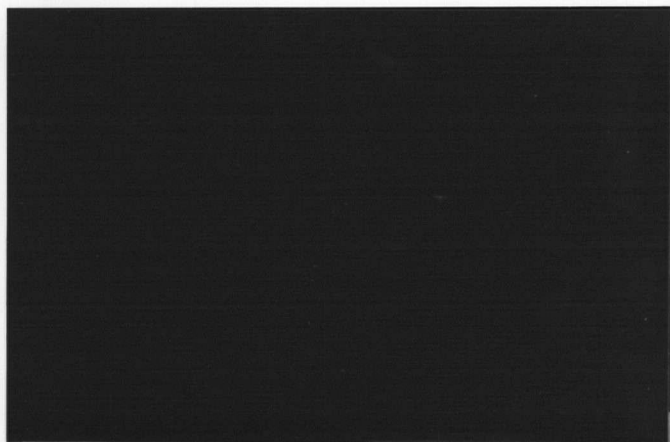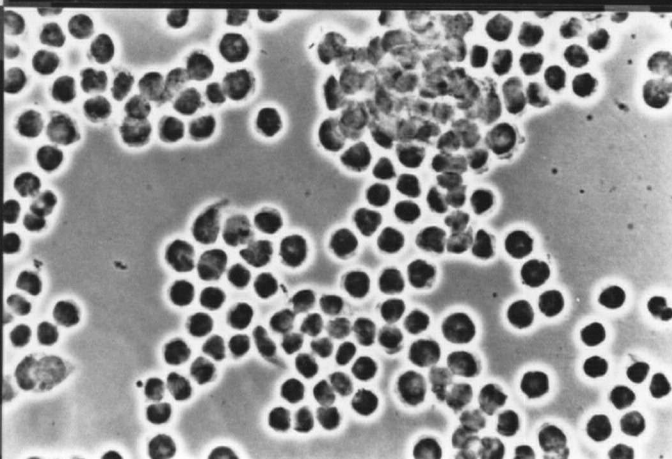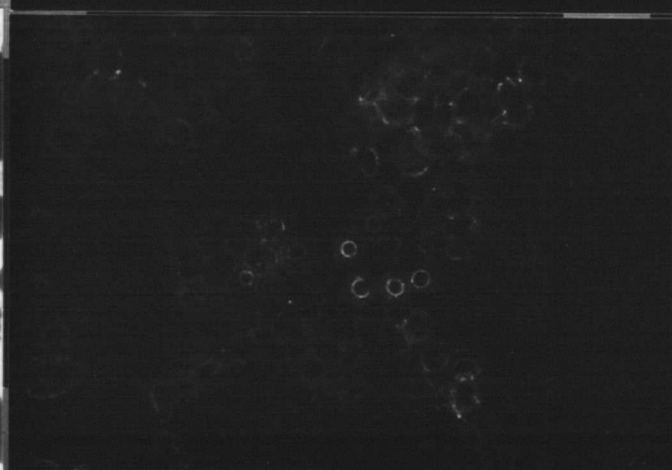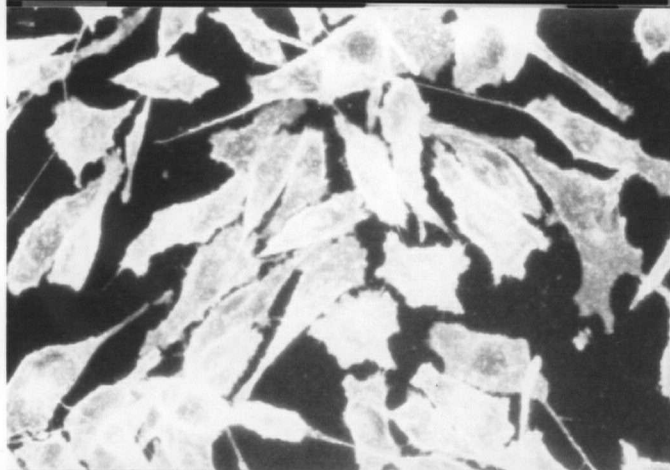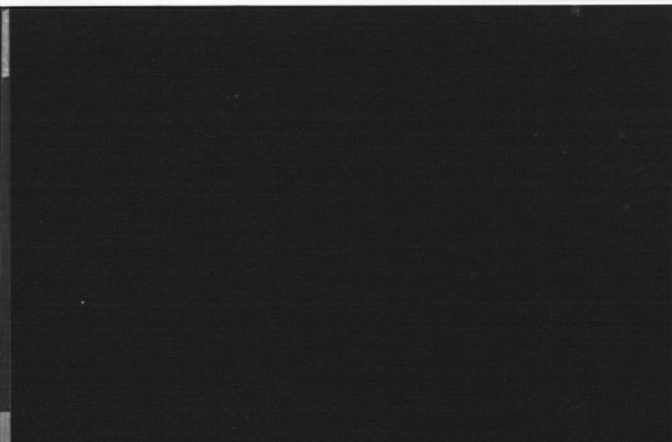
Figure 24   Immunofluorescence and phase photographs of PC-3 and IPC-3
cells stained with anti-vitronectin receptor antibodies. Cells were
cultured on round glass coverslips in DMEM containing 10% FCS
at 37°C, for 48 h. Cells were washed in PBS, blocked with 1.0%
BSA in PBS for 30 min, then incubated with a 1:200 dilution of
anti-vitronectin receptor for 1 h at room temperature. Cells were
then washed extensively and incubated with a 1:100 dilution of
rhodamine conjugated Goat anti-Rabbit antibody for 1 h at room
temperature. Coverslips were washed and mounted onto slides and
sealed with clear nailpolish.

(a) PC-3 control
(b) IPC-3 control
(c) PC-3 phase contrast
(d) PC-3 stained with anti-vitronectin receptor
(e) IPC-3 phase contrast
(f) IPC-3 stained with anti-vitronectin receptor

Magnification 1200X
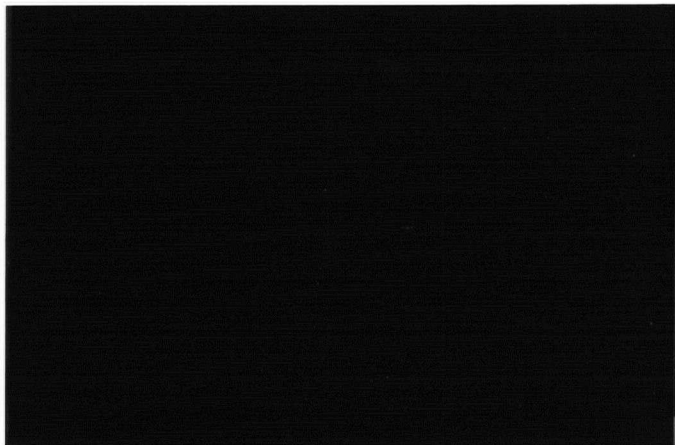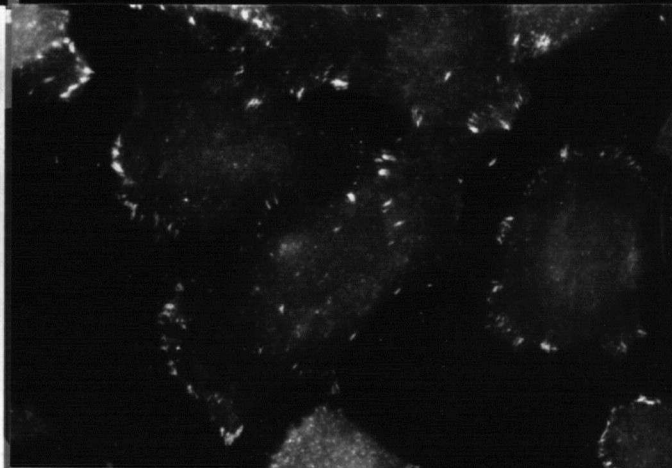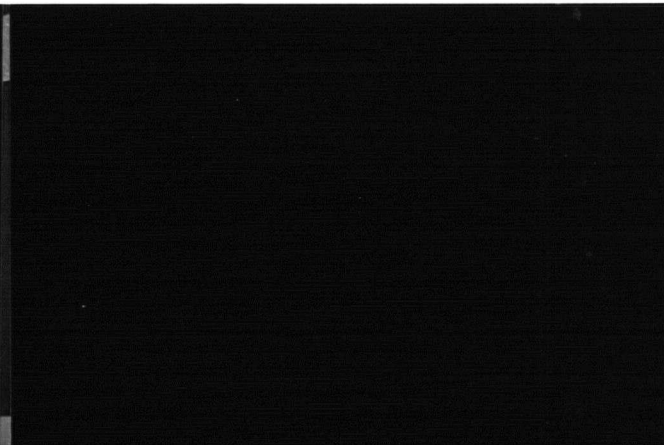
Figure 25   Immunofluorescence and phase photographs of PC-3 and IPC-3 cells stained with anti-α_6 antibodies. Cells were cultured on round glass coverslips in DMEM containing 10% FCS at 37°C, for 48 h. Cells were washed in PBS, blocked with 1.0% BSA in PBS for 30 min, then incubated with a 1:200 dilution of anti-α_6 receptor for 1 h at room temperature. Cells were then washed extensively and incubated with a 1:100 dilution of rhodamine conjugated Goat anti-Rat antibody for 1 h at room temperature. Coverslips were washed and mounted onto slides and sealed with clear nailpolish.

(a) PC-3 control
(b) IPC-3 control
(c) PC-3 stained with anti-α_6
(d) IPC-3 stained with anti-α_6
(e) PC-3 phase contrast
(f) IPC-3 phase contrast

Magnification 120X
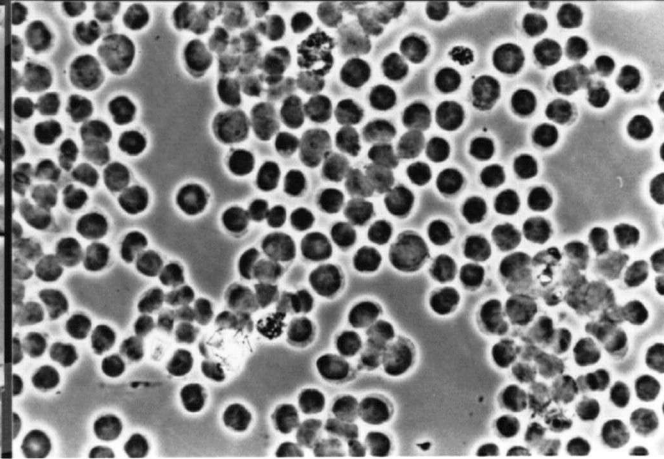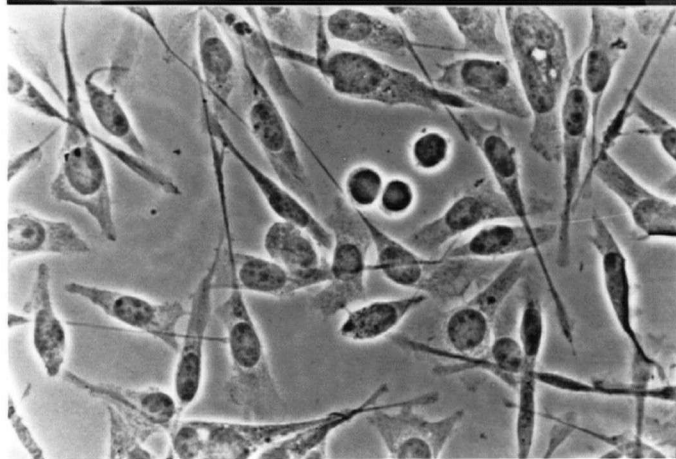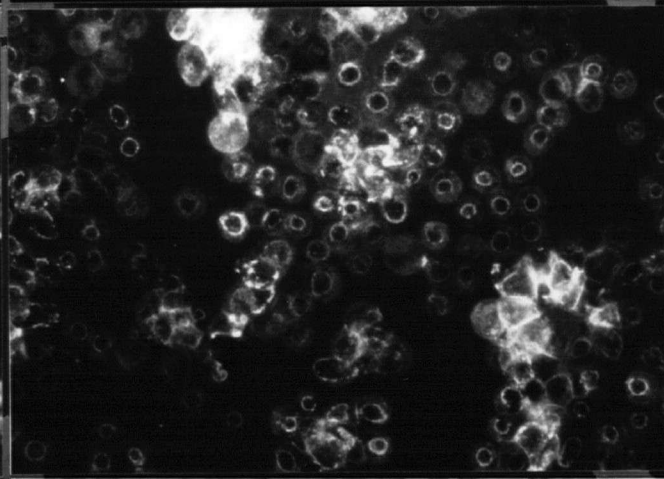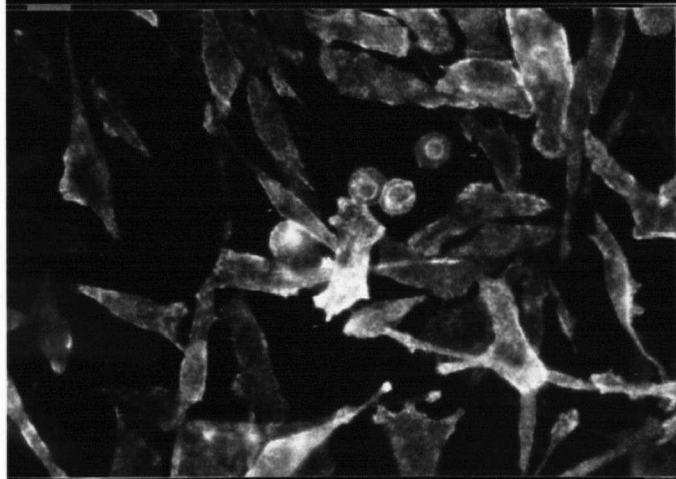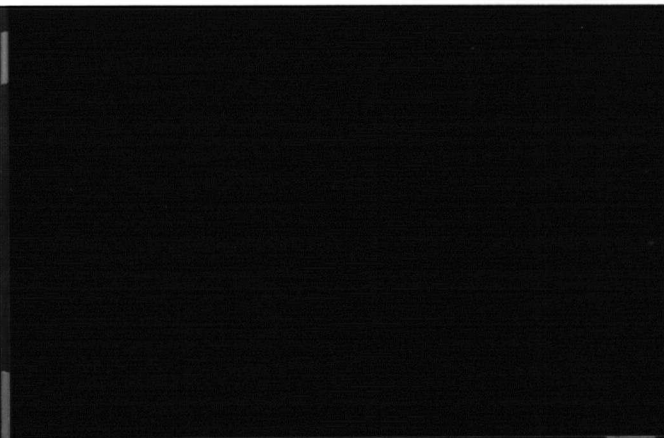
DISCUSSION

The objectives of this study were to develop an in vitro invasion assay in order to investigate the invasive potential of tumor cells and the expression of integrins on tumor cells with different invasive potentials.

The use of an in vitro invasion assay to determine the invasive potential of tumor cells has gained recognition in the past few years. Several different types of in vitro invasion assay are in use today. The most commonly utilized are the amniotic membranes, Boyden chambers and transwells (Albini et al., 1987, Terranova et al., 1986). Although it is important to perform in vivo experiments to determine the metastatic potential of cells, the advantages of an in vitro invasion assay are numerous: (i) It allows one to focus on a very important step in the multi-step process of metastasis (invasion across the basement membrane), (ii) the conditions of the assay can be more closely regulated, (iii) the cells which have invaded can be easily isolated for reculturing, (iv) the results obtained from in vitro invasion assays are often more consistent than those obtained in vivo, and (v) they are much less time consuming. Another important advantage of an in vitro invasion assay is that the role of specific biochemical processes such as protein phosphorylation can be studied using compounds which inhibit or stimulate protein kinases. Recently, staurosporine, a protein kinase C inhibitor, has been shown to inhibit tumor cell invasion in vitro without affecting cell attachment to the extracellular matrix (Schwatz et al., 1990). Compounds such as staurosporine are not as useful in vivo Because of the complex multi-step process that tumor cells undergo during metastasis, the results from such in vivo experiments are difficult to interpret. One disadvantage of an in vitro invasion assay is that results obtained cannot be expanded to an in vivo situation. There are numerous components such as growth factors and other serum proteins present in the circulatory system and tissues of animals used in

in vivo invasion experiments which are not present in vitro. Some of these components may contribute to tumor cell invasion.

The use of transwells and matrigel for in vitro invasion assays is not novel (Repesh, 1989). The invasion assay described in this thesis is the first in vitro invasion assay using two membranes. The advantage of having two membranes is that it greatly facilitates the removal and reculturing of cells that have invaded through the matrigel and top membrane. It is also much easier to remove the matrigel and non-invasive cells with this two membrane system. Several laboratories use reconstituted basement membranes in in vitro invasion assays. However, the conditions they use are different from ours. There are variations in the concentration and thickness of matrigel used as well the time period allowed for invasion, varying from 6-72 h. All the different invasion assays used are able to discriminate between invasive and non-invasive cells, showing either flexibility or a disadvantage in the use of matrigel in invasion assays.

Using the in vitro invasion assay described in the Material and Methods, the invasive potential of two osteosarcoma cell lines were compared. MNNG-HOS cells which are tumorigenic and metastatic in vivo (Rhim et al., 1975) demonstrated greater invasive potential than HOS cells, which are not tumorigenic or metastatic in nude mice. This data suggest that there is a correlation between the invasive potential in vitro and the metastatic potential in vivo. However, Noel et al.(1991) have accumulated evidence against such a correlation. They showed that both normal and transformed fibroblasts were able to invade the reconstituted matrix while only the transformed cells were metastatic in vivo and concluded that there was no correlation between the in vitro and in vivo invasive potential. Our results show that HOS cells, which do not metastasize in vivo were able to invade the matrigel in vitro. There are several possible explanations for the invasion of non-metastatic cells in vitro. It is possible that the matrigel does not provide as stringent a barrier as the basement membrane in vivo. Another reason may be that

the transformed but nonmalignant HOS cells are invasive in vivo but lack the ability to form

distant metastases. Another contributing factor may be the microenvironment of the cells.

Nakajima et al. (1990) have shown that the human colon carcinoma cells, KM12, do not form

metastases when injected subcutaneously in nude mice but do metastasize to the liver when

injected in the caecal wall. They also noted that the intracaecal tumors secreted three times the

amount of the 92-kd type IV collagenase. They concluded from their experiments that organ

specific factors, such as growth factors, contributed to the increased secretion of proteases and

the metastatic potential of the cells.

The ability of both HOS and MNNG-HOS cells to invade through the reconstituted matrix

was significantly inhibited when assayed in the presence of a polyclonal anti-fibronectin receptor

antibody or a monoclonal antibody to the α_6 subunit of the $\alpha_6\beta_1$ complex. Others have shown that

the invasion of tumor cells can also be inhibited using fragments of laminin containing the

YIGSR peptide or fragments of fibronectin containing the RGD peptide. (McCarthy et al., 1988,

Humphries et al., 1988, Iwamoto, et al., 1987). In this study, integrin profiles of both cell lines

demonstrated that the expression of $\alpha_1\beta_1$, $\alpha_2\beta_1$ and especially the $\alpha_6\beta_1$ integrin (laminin receptor)

were upregulated on the more invasive MNNG-HOS cells. Inhibition of invasion with anti-

integrin antibodies further support the evidence that the β_1 integrins, particularly the laminin

receptors play an important role in the invasion of tumor cells across basement membranes. A

69 kDa laminin receptor is also present on a large number of cell types and is often upregulated

on tumor cells (Hand et al., 1985). This 69 kDa nonintegrin peripheral protein binds to the B1

chain of laminin with high affinity and may bind to basement membrane laminin during the

initial stages of invasion However, it is not an integral protein and most likely does not transmit

any signals intracellularly. Yannariello-Brown et al., (1988) argue against this hypothesis. Their

data show that the 69 kDa protein colocalizes with actin microfilaments and that it may be a

candidate involved in signal transduction.

The expression of integrins varies considerably between cell lines. There is no pattern for the expression of integrins on tumor cells. However, on normal cell lines of epithelial and endodermal origin, certain integrins are prevalent. They express moderate amounts of $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$ and the α_v subunit and negligible amounts $\alpha_4\beta_1$, $\alpha_5\beta_1$ and β_3. Albelda and Buck (1990) noted that the expression of $\alpha_1\beta_1$ was lost in tissue culture. Growth factors such as TGF-ß have a regulatory role in the expression of integrins and extracellular matrix proteins. The presence of TGF-ß often results in increased expression of integrins and the secretion of ECM proteins (Roberts et al., 1990, Heino and Massague, 1989, Heino et al., 1989). It is possible that the downregulation of integrins is favourable for migration and invasion by making the cells less adhesive. It is also possible that the functional ability of integrins is altered during the various stages of the metastatic cascade to accommodate the multiple steps the cells must pass through.

Comparing the expression of integrins on PC-3 and IPC-3 cells shows a reduction in the expression of $\alpha_1\beta_1$, $\alpha_2\beta_1$ and a dramatic decrease in the expression of $\alpha_3\beta_1$ on IPC-3 cells. The vitronectin receptor, $\alpha_v\beta_3$ and the fibronectin receptor remains unchanged while only the $\alpha_6\beta_4$ integrin receptor was upregulated on the invasive cells. The importance of the vitronectin receptor on invasive cells is still unclear. Albelda et al. (1990) have shown that the expression of β_3 was upregulated in metastatic melanomas and primary melanomas in a vertical growth phase as compared to melanocytes and benign melanocytic nevi. No experiments have been conducted to determine whether the vitronectin receptor plays a role in the invasion of tumor cells across the basement membrane. However, the vitronectin receptor does not interact strongly with any of the proteins present in the basement membrane. Vitronectin, being most abundant in the serum and functioning in helping platelets aggregate (Asch and Podack, 1990) may serve a purpose in

helping tumor cells aggregate while they are in the circulatory system. It is interesting that the invasive IPC-3 cells retain the expression of the vitronectin receptor while it is downregulated in the invasive MNNG-HOS, especially the expression of the β_3 subunit. It is possible that the difference in the expression of the vitronectin receptor is a result of the different histologic origin of the cells, IPC-3 being of epithelial origin and MNNG-HOS of stromal origin. MG63 cells, which are osteosarcoma cells, also express much higher levels of α_v than β_3 (Freed et al., 1989). Cells of epithelial origin commonly express large amounts of α_v but the expression of β_3 varies in each cell line (Albelda and Buck, 1990). MNNG-HOS cells upregulate their laminin/collagen receptors $\alpha_1\beta_1$, $\alpha_2\beta_1$, and retain $\alpha_3\beta_1$, while IPC-3 cells downregulate all three integrin receptors. One reason for having a difference on two invasive cell lines may be the difference in histologic origin. Stromal cells such as MNNG-HOS are not ordinarily on basement membranes and may therefore require upregulation of integrins to bind to basement membrane proteins. Epithelial cells, however, are ordinarily found on basement membranes and already possess integrins which bind to the basement membrane components. Another possible reason is the method of transformation. No studies have been performed to determine whether changes in the expression of integrins are similar on a cell line which has been transformed by different methods. MNNG-HOS cells were chemically transformed, while IPC-3 cells were selected from a heterogenous population of cells. The effect of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) on the expression of integrins on PC-3 cells is unknown.

One interesting phenomenon that occurred with the invasive cell line isolated in the in vitro invasion assay was that after 12-15 weeks in tissue culture, the invasive cells lost their invasive potential but retained their spherical morphology and their expression of integrins. This suggests that components of the basement membrane may have a role in inducing a temporary invasive phenotype in some cells. Osssowski and Reich (1980 and 1983) and Kleinman (personal

comm.,1990) have also observed a loss in invasiveness of tumor cells which were isolated in vitro. The loss of invasive potential without change in morphology, growth rate, or integrin expression suggests that other properties required for invasion are more unstable. Expression of proteases, oncogenes and growth factors may be the contributing factors in the loss of invasive potential of IPC-3 cells. More experiments are required to determine the cause of this loss of invasivness.

It is interesting that laminin was the only extracellular matrix protein able to induce spreading of IPC-3 cells. The fact that the cells attached to the substrate in only a few minutes but did not spread for several hours suggests that spreading may require the synthesis of proteins. Shaw et al., (1990) have shown that in PMA activated macrophages, cell spreading on laminin was not caused by an increase in the number of laminin receptors but by an increase in the number of receptors associating with the cytoskeletal elements. This in turn was caused by the phosphorylation of the α_6 subunit. IPC-3 cells do not express any detectable levels of $\alpha_6\beta_1$. Only the $\alpha_6\beta_4$ integrin is expressed and it is therefore possible that phosphorylation of the $\alpha_6\beta_4$ integrins also occurs in IPC-3 cells. Several integrin receptors are able to bind to laminin. $\alpha_1\beta_1$ binds to the E1 fragment, $\alpha_6\beta_1$ binds to the E8 fragment of laminin (Sonnenberg et al., 1990, Hall et al., 1990). $\alpha_2\beta_1$ and $\alpha_3\beta_1$ are also low affinity receptors for laminin. The ligand for $\alpha_6\beta_4$ was only recently tentatively determined as laminin (Tamura et al., 1990). However, Carter et al.,(1990a) concluded from their experiments that in human keratinocytes, $\alpha_6\beta_4$ localizes in new stable anchoring contacts, possibly associated with the bullous pemphigoid antigen, which cooperates with $\alpha_3\beta_1$ to mediate adhesion to the extracellular matrix.

The rapid adhesion of IPC-3 cells to fibronectin, collagen and vitronectin with the absence of spreading indicates that the receptors are functional but fail to interact properly with the cytoskeleton or may organize their cytoskeletal elements in a way such that the cell remains

spherical. Because IPC-3 cells can spread on laminin but not on other substrates, it suggests that not all the integrins interact in an identical manner with the cytoskeleton showing evidence that different integrins mediate different signals. Another possible explanation is that the β_4 subunit, which possesses a large intracellular domain (Hogervorst et al., 1990), may be the only integrin able to interact with the cytoskeletal components. Figures 21 and 22 show that the 180 kDa subunit of β_4 which contains the largest intracellular segment, is most strongly upregulated.

The morphology of PC-3 cells cultured on stock matrigel was also observed by Albini et al. (1987) and by Kramer et al. (1986) with HT1080 fibrosarcoma cells. Grant et al.,(1989) noted that human umbilical vein endothelial cells would stop proliferating when they formed tube-like structures on matrigel. They concluded that matrigel induces differentiation of endothelial cells. PC-3 cells did not stop proliferating when organized in tube-like structures on matrigel, since after several days in culture the entire surface of the matrix was confluent with cells. The IPC-3 cells cultured on matrigel aggregate in clusters and also continue to proliferate on matrigel. The clustering of IPC-3 cells resembles homotypic aggregation of tumor cells which occurs in vivo.

The invasion of tumor cells is a complex process requiring a multitude of events all of which are required for the tumor cell to metastasize and form colonies in distant tissues. If any one of several steps are inhibited the tumor cells are unable to invade. In this thesis, the invasion of two osteosarcoma cell lines were inhibited using antibodies directed to the integrins of the β_1 family. Inhibition of other processes also affect the invasion of tumor cells. For example, the inhibition of proteases also have a profound effect in reducing the invasive potential of tumor cells. The presence of a chemically designed collagenase IV inhibitor, SC-444463, inhibited the invasion of both murine and human fibrosarcoma cells (Terranova et al., 1989, Reich et al., 1988). The presence of estramustine, an antitumorigenic, antiprostatic cancer compound also inhibited the invasion of DU145 cells in vitro by disrupting microtubule-associated proteins and

the secretion of type IV collagenase (Wang and Stearns, 1988, Stearns, et al., 1991). Treatment with cytochalasin, which inhibits attachment by disrupting the actin cytoskeleton but does not block collagenase IV secretion, did inhibit invasion indicating the importance of the actin cytoskeleton in invasion (Wang and Stearns, 1988). The invasion process is not the result of one biochemical change but the cooperative effect of many cellular processes, all of which are required for invasion.

In conclusion, this thesis has focused on two points (i) The development of an in vitro invasion assay and the isolation of an invasive cell line with unique properties from its parent cell line and, (ii) the inhibition of invasion of two osteosarcoma cell lines with anti-integrin antibodies. From these experiments we have shown the importance of integrins in tumor cell invasion across the basement membrane.

Future directions

There remain many unanswered questions about the invasive phenotype of tumor cells. These include (1) The role of the dramatic downregulation of α_3 expression on IPC-3 cells. This can be investigated by transiently increasing the expression of α_3 (by transfecting a full length α_3-cDNA into IPC-3 cells) to determine the role of $\alpha_3\beta_1$ on the morphology, spreading, and invasive potential of IPC-3 cells, (2) Whether the $\alpha_6\beta_4$ complex is involved in the invasion process. This can be looked at using IPC-3 cells, which, like MNNG-HOS, have elevated levels of α_6. Invasion of IPC-3 cells in the presence of anti-α_6 antibodies could therefore be conducted. (3) Whether the spherical morphology and lack of spreading of IPC-3 cells on fibronectin, vitronectin and collagen is a result of improper cytoskeletal-integrin interactions. Double immunofluorescent labelling of cytoskeletal proteins and integrins would reveal which cytoskeletal proteins (if any) are coupled with the integrins at the plasma membrane. (4) What are the specific ligands of each integrin expressed on IPC-3 cells is not known. Affinity

chromatography using laminin, fibronectin, and collagen columns could indicate which integrins bind to each ligand and whether the interactions are RGD dependent, (5) what is the in vivo invasive potential of IPC-3 cells. These experiments are being carried out in collaboration with Dr. Nagle of the University of Arizona Health Sciences Centre. (6) Finally, experiments should be conducted to determine the expression of proteases and oncogenes in IPC-3 cells compared with PC-3 cells.

REFERENCES

Abrahamson,D. Recent studies on the structure and pathology of basement membranes. J. of Pathology. 149:257-278, 1986.

Adams,J., and Watt,F. Changes in keratinocyte adhesion during terminal differentiation: reduction in fibronectin binding precedes $\alpha_5\beta_1$ integrin loss from the cell surface. Cell 63:425-435, 1990.

Albelda,S., and Buck,C. Integrins and other cell adhesion molecules. FASEB J. 4:2868-2880, 1990.

Albelda,S., Mette,S., Elder,D., and Stewart,R.M., Damjanovich,L., Herlyn,M., and Buck,C. Integrin distribution in malignant melanoma: association of the β_3 subunit with tumor progression. Cancer Res. 50:6757-6764, 1990.

Albini,A., Iwamoto,Y., Kleinman,H., Martin,G., Aaronson,S., Kozlowski,J., and McEwan,R. A rapid in vitro assay for quantitating the invasive potential of tumor cells. Cancer Res. 47:3239-3245, 1987.

Anderson,H. Adhesion molecules and animal development. Experientia. 46:2-13, 1990.

Asch,E., and Podack,E. Vitronectin binds to activated human platelets and plays a role in platelet aggregation. J. Clin. Invest. 85:1372-1378, 1990.

Babel,W., and Glanville,R. Structure of human-basement membrane(type IV) collagen. Complete amino-acid sequence of a 914-residue-long pepsin fragment from the $\alpha 1$(IV) chain. Eur. J. Biochem. 143: 545-556, 1984.

Bachinger,H.P., Morris,N., Lunstrum,G., Keene,D., Rosenbaum,L., Compton,L., and Burgeson,R. The relationship of the biophysical and biochemical characteristics of type VII collagen to the function of anchoring fibrils. J. Biol. Chem. 265:10095-10101, 1990.

Bourdon,M., and Ruoslahti,E. Tenascin mediates cell attachment through an RGD-dependent receptor. J. Cell Biol. 108:1149-1155, 1989.

Bretti,S., Neri,P., Lozzi,L., Rustici,M., Comoglio,P., Giancotti,F., and Tarone,G. Inhibition of experimental metastasis of murine fibrosarcoma cells by oligopeptide analogues to the fibronectin cell-binding domain. Int. J. Cancer. 43:103-106, 1989.

Buck,C. Cell Surface receptors for extracellular matrix molecules. Ann. Rev. Cell Biol. 3:179-205, 1987.

Carlin,B., Jeffe,R., Bender,B., and Chung,A. Entactin, a novel basal lamina-associated sulfated glycoprotein. J. Biol. Chem. 256:5209-5214, 1981.

(a)   Carter,W., Kaur,P., Gil,S., Gahr,P., and Wayner,E. Distinct functions for integrins $\alpha_3\beta_1$ in focal adhesions and $\alpha_6\beta_4$/bullous pemphigoid antigen in a new stable anchoring contact (SAC) of keratinocytes: relation to hemidesmosomes. J. Cell. Biol. 111:3141-3154, 1990.

(b)   Carter,W., Wayner,E., Bouchard,T., and Kaur,P. The role of integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$ in cell-cell and cell-substrate adhesion of human epithelial cells. J. Cell Biol. 110:1387-1404, 1990.

Chakravarti,S., Tam,M., and Chung,A. The basement membrane glycoprotein entactin promotes cell attachment and binds calcium ions. J. Biol. Chem. 265: 10597-10603, 1990.

Cheresh,D. and Spiro,R. Biosynthetic and functional properties of an Arg-Gly-Asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibrinogen, and von Willebrand factor. J. Biol. Chem. 262:17703-17711, 1987.

Davis,L., Oppenheimer-Marks,N., Bednarczyk,J., McIntyre,B., and Lipsky,P. Fibronectin promotes proliferation of naive and memory T cells by signaling through both the VLA-4 and VLA-5 integrin molecules. J. of Immunology. 145:785-793, 1990.

Dedhar,S. Integrins and tumor invasion. Bioassays 12:1-8, 1990.

Dedhar,S., and Saulnier,R. Alterations in integrin receptor expression on chemically transformed human cells: specific enhancement of laminin and collagen receptor complexes. J. Cell Biol. 110:481-489, 1990.

Dedhar,S., Argraves,S., Suzuki,W., Ruoslahti,E., and Pierschbacher,M. Human osteosarcoma cells resistant to detachment by an Arg-Gly-Asp containing peptide overproduce the fibronectin receptor. J. Cell Biol. 105:1175-1187, 1987.

Dedhar,S., and Gray,V. Isolation of a novel integrin receptor mediating Arg-Gly-Asp-directed cell adhesion to fibronectin and type IV collagen from human neuroblastama cells. Association of a novel β_1 related subunit with α_v. J. Cell Biol. 100:2185-2193, 1990.

Dejana,E., Lampugnani,M.G., Giorgi,M., Gaboli,M., Federici,A., Ruggeri,Z., and Marchisio,P.C. Von Willebrand Factor promotes endothelial cell adhesion via an Arg-Gly-Asp-dependent mechanism. J. Cell Biol. 109:367-375, 1989.

Dejana,E., and Lauri,D. Biochemical and functional characteristics of integrins: A new family of adhesive receptors present in haematopoietic cells. Haematologica. 75:1-6, 1990.

De Luca,M., Tamura,R., Kajiji,S., Bondanza,S., Rossino,P., Cancedda,R., Marchisio,P., and Quaranta,V. Polarized integrins mediate human keratinocyte adhesion to basal lamina. Proc. Natl. Acad. Sci. USA. 87:6888-6892, 1990.

D'Souza,S., Ginsberg,M., Burke,T., Lam,S., and Plow,E. Localization of an Arg-Gly-Asp recognition site within an integrin adhesion receptor. Science 242:91-93, 1988.

Edwards,D., Murphy,G., Reynolds,J., Whitham,S., Docherty,A., Angel,P., and Heath,J. Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. EMBO J. 6:1899-1904, 1987.

Ehrig,K., Leivo,I., Argraves,S., Ruoslahti,E., and Engvall,E. Merosin, a tissue-specific basement membrane protein, is a laminin-like protein. Proc. Natl. Acad. Sci. USA 87: 3264-3268, 1990.

Eisenbach,L., Kushtai,G., Plaksin,D., and Feldman,M. MHC·genes and oncogenes controlling the metastatic phenotype of tumor cells. Cancer Rev. 5:1-18, 1986.

Ekblom,P., Alitalo,K., Veheri,A., Timpl,R., and Saxen,L. Induction of a basement membrane glycoprotein in embryonic kidney: possible role of laminin in morphogenesis. Proc. Natl. Acad. Sci. USA. 77:485-489, 1980.

Ekblom,P., Vestweber,D., and Kemler,R. Cell-matrix interactions and cell adhesion during development. Ann. Rev. Cell Biol. 2:27-47, 1986.

Elices,M., Osborn,L., Takada,Y., Crouse,C., Luhowskyj,S., Hemler,M., and Lobb,R. VCAM-1 on activated endothelium interacts with the leucocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. Cell. 60: 577-584, 1990.

Elices,M. and Hemler,M. The human integrin VLA-2 is a collagen receptor on some cells and a collagen/laminin receptor on others. Proc. Natl. Acad. Sci. U.S.A. 86: 9906-9910,1989.

Fidler,I., Gersten,D., and Hart,I. The biology of cancer invasion and metastasis. Adv. Cancer Res. 28: 149-250, 1978.

Fidler,I. and Hart,I. Biological diversity in metastatic neoplasms: origins and implications. Science 217: 998-1003, 1982.

Freed,E., Gailit,J., Van der Geer,P., Ruoslahti,E., and Hunter,T. A novel integrin ß subunit is associated with the vitronectin receptor α subunit (α$_v$) in a human osteosarcoma cell line and is a substrate for protein kinase C. EMBO J. 8: 2955-2965, 1989.

Fujiwara,S., Wiedemann,H., Timpl,R., Lustig,A., and Engel,J. Structure and interactions of heparan sulfate proteoglycans from a mouse tumor basement membrane. Eur. J. Biochem. 143:145-157, 1984.

(a)   Gehlsen,K., Argraves,S., Pierschbacher,M. and Ruoslhti,E. Inhibition of in vitro tumor cell invasion by Arg-Gly-Asp-containing synthetic peptides. J. Cell Biol. 106:925-930, 1988.

(b)   Gehlsen,K., Dillner,L., Engvall,E., and Ruoslahti,E. The human laminin receptor is a member of the integrin family of cell adhesion receptors. Science. 241:1228-1229, 1988.

Gehlsen,K., Dickerson,K., Argraves,S., Engvall,E., and Ruoslahti,E. Subunit structure of a laminin-binding integrin and localization of its binding site on laminin. J. Biol. Chem. 264:19034-19038, 1989.

Gelber,C., Plaksin,D., Vadai,E., Feldman,M., and Eisenbach,L. Abolishment of metastasis formation by murine tumor cells transfected with "foreign" H-2K genes. Cancer Research. 49:2366-2373, 1989.

Giancotti,F., and Ruoslahti,E. Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of chinese hamster ovary cells. Cell 60:849-859, 1990.

Grant,D., Tashiro,K.I., Segui-Real,B., Yamada,Y., Martin,G., and Kleinman,H. Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures in vitro. Cell. 58:933-943, 1989.

Greenberg,A., Egan,S., and Wright,J. Oncogenes and Metastatic progression. Invasion Metastasis. 9:360-378, 1989.

Guan,J.L. and Hynes,R. Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor. Cell. 60:53-61, 1990.

Hall,D., Reichardt,L., Crowley,E., Holley,B., Moezzi,H., Sonnenberg,A., and Damsky,C. The $\alpha_1\beta_1$ and $\alpha_6\beta_1$ integrin heterodimers mediate cell attachment to distinct sites on laminin. J. Cell Biol. 110:2175-2184, 1990.

Hand,P., Thor,A., Rao,C., and Liotta,L. Expression of laminin receptor in normal and carcinomatous human tissues as defined by a monoclonal antibody. Cancer Research 45:2713-2719, 1985.

Hayman,E., Pierschbacher,M., Ohgren,Y., and Ruoslahti,E. Serum spreading factor (vitronectin) is present at the cell surface and in tissues. Proc. Natl. Acad. Sci. USA. 80:4003-4007, 1983.

Heino,J., Ignotz,R., Hemler,M., Crouse,C., and Massague,J. Regulation of cell adhesion receptors by transforming growth factor-ß. Concomitant regulation of integrins that share a common ß₁ subunit. J. Biol. Chem. 264:380-388, 1989.

Heino,J., and Massague,J. Transforming growth factor-ß switches the pattern of integrins expressed in MG-63 human osteosarcoma cells and causes a selective loss of cell adhesion to laminin. J. Biol. Chem. 264:21806-21811, 1989.

Hendrix,M., Seftor,E., Seftor,R., Misiorowski,R., Soba,P., Sundareshan,P., and Welch,D., Comparison of tumor cell invasion assays: Human amnion versus reconstituted basement membrane barriers. Invasion Metastasis 9:278-297, 1989.

Hemler,M., Sanchez-Madrid,F., Flotte,J., Krensky,A., Burakoff,S., Bhan,A., Springer,T., and Strominger,J. Glycoproteins of 210,000 and 130,000 M.W. on activated T cells: Cell distribution and antigenic relation to components on resting cells and T cell lines. J. of Immunology. 132: 3011-3018, 1984.

Hemler,M., Elices,M., Parker,C., and Takada,Y. Structure of the integrin VLA-4 and its cell-cell and cell-matrix adhesion functions. Immunological Rev. 114:45-65, 1990.

Hemler,M., Huang,C., Takada,Y., Schwarz,L., Strominger,J., and Clabby,M. Characterization of the cell surface heterodimer VLA-4 and related peptides. J. of Biol. Chem. 262: 11478-11485, 1987.

Hemler,M., Crouse,C., and Sonnenberg,A. Association of the VLA α_6 subunit with a novel protein. a possible alternative to the common VLA ß₁ subunit on certain cell lines. J. Biol. Chem. 264: 6529-6535, 1989.

Herbst,T., McCarthy,J., Tsilibary,E., and Furcht,L. Differential effects of laminin, intact type IV collagen and specific domains of type IV collagen on endothelial cell adhesion and migration. J. Cell Biol. 106: 1365-1373, 1988.

Hogervorst,F., Kuikman,I., Kr von dem Borne,A., and Sonnenberg,A. Cloning and sequence analysis of beta-4 cDNA: an integrin subunit that contains a unique 118kd cytoplasmic domain. EMBO J. 9: 765-770, 1990.

Holzmann,B., and Weissman,I. Payer's patch-specific lymphocyte homing receptors consists of a VLA-4 like α chain with either of two integrin ß chains, one of which is novel. EMBO J. 8:1735-1741, 1989.

Humphries,M., Yamada,K., and Olden,K. Investigation of the biological effects of anti-cell adhesive synthetic peptides that inhibit experimental metastasis of B16-F10 murine melanoma cells. J. Clin. Invest. 81:782-790, 1988.

(a)   Hunter,D., Shah,V., Merlie,J., and Sanes,J. A laminin-like adhesive protein concentrated in the synaptic cleft of the neuromuscular junction. Nature. 338: 229-234, 1989.

(b)   Hunter,D., Porter,B., Bulock,J., Adams,S., Merlie,J., and Sanes,J. Primary sequence of a motor neuron-selective adhesive site in the synaptic basal lamina protein s-laminin. Cell. 59: 905-913, 1989.

Hynes,R. Integrins: A family of cell surface receptors. Cell 48: 549-554, 1987.

Ignatius,M., Large,T., Houde,M., Tawil,J., Barton,A., Esch,F., Carbonetto,S., and Reichardt,L. Molecular cloning of the rat integrin α_1-subunit: a receptor for laminin and collagen. J. Cell Biol. 111:709-720, 1990.

Ingber,D., Madri,J., and Jamieson,J. Role of Basal Lamina in Neoplastic Disorganization of Tissue Architecture. Proc. Natl. Acad. Sci. USA. 78:3901-3905, 1981.

Ingber,D. and Folkman,J. Tension and compression as basic determinants of cell form and function: utilization of a cellular tensegrity mechanism. Cell Shape. Ed. Stein,W. and Bronner,F. Academic Press Inc., San Diego, USA. p.3-31, 1989.

Inoue,S., and Leblond,C. The basement-membrane-like matrix of the mouse EHS tumor:I ultrastructure. Amer. J. Anat. 171:373-386, 1985.

Isberg,R., and Leong,J. Multiple β_1 chain integrins are receptors for invasion, a protein that promotes bacterial penetration into mammalian cells. Cell 60: 861-871, 1990.

Iwamoto,Y., Robey,F., Graf,J., Sasaki,M., Kleinman,H., Yamada,Y, and Martin,G. YIGSR, a synthetic laminin pentapeptide, inhibits experimental metastasis formation. Science. 238:1132-1134, 1987.

Kanemoto,T., Reich,R., Royce,L., Greatorex,D., Adler,S., Shiraishi,N., Martin,G., Yamada,Y., and Kleinman,H. Identification of an amino acid sequence from the laminin A chain that stimulates metastasis and collagenase IV production. Proc. Natl. Acad. Sci. USA. 87: 2279-2283, 1990.

Kaufmann,R., Frosch,D., Westphal,C., Weber,L., and Klein,E. Integrin VLA-3: ultrastructural localization at cell-cell contact sites of human cell cultures. J. of Cell Biol. 109:1807-1815,1989.

Keene,D., Sakai,L., Lunstrum,G., Morris,N., and Burgeson,R. Type VII collagen forms an extended network of anchoring fibrils. J. Cell Biol. 104: 611-621, 1987.

Kennel,S., Foote,L., Falcioni,R., Sonnenberg,A., Stringer,C., Crouse,C., and Hemler,M. Analysis of the tumor-associated antigen TSP-180. Identity with $\alpha_6\beta_4$ in the integrin superfamily. J. Biol. Chem. 264: 15515-15521, 1989.

Khokha,R., and Denhardt,D. Matrix metalloproteinases: A review of their role in tumorigenesis and tissue invasion. Invasion Metastasis 9:391-405, 1989.

Khokha,R., Waterhouse,P., Yagel,S., Lala,P., Overall,C., Norton,G., and Denhardt,D. Antisense RNA-induced reduction in murine TIMP levels confers oncogenicity on Swiss 3T3 cells. Science. 243:947-950, 1989.

Kirchhfer,D., Languino,L., Ruoslahti,E., Pierschbacher,M. $\alpha_2\beta_1$ integrins from different cell types show different binding specificities. J. of Biol. Chem. 265: 615-618, 1990.

Klein,G., Lengegger,M., Timple,R., and Ekblom,P., Role of laminin A chain in the development of epithelial cell polarity Cell 55: 331-341, 1988

Kleinman,H., Cannon,F., Laurie,G., Hassell,J., Aumailley,M., Terranova,V., Martin,G., and DuBois-Dalcq,M. Biological activities of laminin. J. of Cell. Biochem. 27:317-325, 1985.

Kleinman,H., McGarvey,M., Hassell,J., and Martin,G. Formation of a supramolecular complex is involved in the reconstitution of basement membrane components. Biochemistry. 22:4969-4974, 1983.

Kleinman,H., McGarvey,M., Hassell,J., Star,V., Connon,F., Laurie,G., and Martin,G. Basement membrane complexes with biological activities. Biochemistry. 25:312-318, 1986.

Kleinman,H., McGarvey,M., Liotta,L., Robey,P.G., Tryggvason,K., and Martin,G. Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry. 21:6188-6193, 1982.

Kleinman,H., Sephel,G., Tashiro,K., Weeks,B., Burrous,B., Adler,S., Yamada,Y., and Martin,G. Laminin in Neuronal development. In Structure, Molecular Biology, and Pathology of Collagen. Ed. Fleischmajer,R., Olsen,B., and Kuhn,K. Ann. New York Acad. Sci. New York, New York. 580:302-310, 1990

Kleinman,H. and Weeks,B. Laminin: structure, function and receptors. Current Opinion in Cell Biol. 1:964-967, 1989.

Koliakos,G., Kouzi-Koliaakos,K., Furcht,L., Reger,L., and Tsilibary,E. The binding of heparin to type IV collagen: domain specificity with identification of peptide sequences from the $\alpha1(IV)$ and $\alpha2(IV)$ which preferentially bind heparin. J. Biol. Chem. 264:2313-2323, 1989.

Kolega,J., and Manabe,M. Tissue-specific distribution of a novel component of epithelial basement membranes. Exp. Cell Res. 189:213-221, 1990.

Kouzi-Koliakos,K., Koliakos,G., Tsilibary,E., Furcht,L., and Charonis,A. Mapping of three heparin-binding sites on laminin and identification of a novel heparin-binding site on the B1 chain. J. Biol. Chem. 264:17971-17978, 1989.

Kramer,R., McDonald,K., Crowley,E., Ramos,D., and Damsky,C. Melanoma cell adhesion to basement membrane mediated by integrin-related complexes. Cancer Res. 49:393-402, 1989.

Kramer,R., Bensch,K., and Wong,J. Invasion of reconstituted basement membrane matrix by metastatic human tumor cells. Cancer Res, 46:1980-1987, 1986

Labat-Robert,J., Bibari-Varga,M., and Robert,L. Extracellular matrix. FEBS Letters, 268:386-393, 1990.

Lacovara,J., Cramer,E., and Quigley,J. Fibronectin enhancement of directed migration of B16 melanoma cells. Cancer Research. 44:1657-1663, 1984.

Languino,L., Gehlesen,K., Wayner,E., Carter,W., Engvall,E., and Ruoslahti.E. Endothelial cells use $\alpha_2\beta_1$ integrin as a laminin receptor. J. of Cell Biol. 109: 2455-2462,1989.

Larson,R., and Springer,T. Structure and function of leukocyte integrin. Immunological Rev. 114:181-217, 1990.

Laurie,G., Leblond,C., and Martin,G. Localization of type IV collagen, laminin, heparan sulfate proteoglycan, and fibronectin to the basal lamina of basement membranes. J Cell Biol. 95:340-344, 1982.

Laurie,G., Bing,J., Kleinman,H., Hassell,J., Aumailley,M., Martin,G., and Feldmann,R. Localization of binding sites for laminin, heparan sulfate proteoglycan and fibronectin on basement membrane (type IV) collagen. J. Mol. Biol. 189: 205-216, 1986.

Lawler,J., Weinstein,R., and Hynes,R. Cell attachment to thrombospondin: the role of ARG-GLY-ASP, calcium, and integrin receptors. J. Cell Biol. 107:2351-2361, 1988.

Leivo,I., and Engvall,E. Merosin, a protein specific for basement membranes of schwann cells, striated muscle, and trophoblasts, is expressed late in nerve and muscle development. Proc. Natl. Acad. Sci. USA 85: 1544-1548, 1988.

Liotta,L., Abe,S., Robey,P., and Martin,G. Preferential digestion of basement membrane collagen by an enzyme derived from a metastatic murine tumor. Proc. Natl. Acad. Sci. USA. 76:2268-2272, 1979.

Liotta,L., Rao,N., and Wewer,U. Biochemical interactions of tumor cells with the basement membrane. Ann. Rev. Biochem. 55: 1037-1057, 1986.

Mackay,A., Corbitt,R., Hartzler,J., and Thorgeirsson,U. Basement membrane type IV collagen degradation: Evidence for the involvement of a proteolytic cascade independent of metalloprotinases. Cancer Res. 50:5997-6001, 1990.

Mann,K., Deutzmann,R., Aumailley,M., Timpl,R., Raimondi,L., Yamada,Y., Pan,T., Conway,D., and Chu,M.L. Amino acid sequence of mouse nidogen, a multidomain basement membrane protein with binding activity for laminin, collagen IV and cells. EMBO J. 8:65-72, 1989.

Martin,G., and Timpl,R. Laminin and other basement membrane components. Ann Rev. Cell Biol. 3:57-85, 1987.

Martin,G., Timpl,R., and Kuhn,K. Basement membrane proteins: molecular structure and function. Advances in Protein Chemistry. 39: 1-50, 1988.

McCarthy,J., Skubitz,A., Plam,S., and Furcht,L. Metastasis inhibition of different tumor types by purified laminin fragments and a heparin-binding fragment of fibronectin. J. Natl. Cancer Institute. 80:108-116, 1988.

McDonald,J. Matrix regulation of cell shape and gene expression. Current Opinion in Cell Biol. 1:995-999, 1989.

McKenna,G., Weiss,M., Bakanauskas,V., Sandler,H., Kelsten,M., Biaglow,J., Tuttle,S., Endlich,B., Ling,C., and Muschel.R. The role of the H-ras oncogene in radiation resistance and metastasis. Int. J. Radiation Oncology Biol. Phys. 18:849-859, 1990.

Mignatti,P., Robbins,E., and Rifkin,D. Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. Cell. 47:487-498, 1986.

Mould,P., Wheldon,L., Komoriya,A., Wayner,E., Yamada,K., and Humphries,M. Affinity chromatographic isolation of the melanoma adhesion receptor for the IIICS region of the fibronectin and its identification as the integrin $\alpha_4\beta_1$. J. of Biol. Chem. 265: 4020-4024, 1990.

Muschel,R. and Liotta,L. Role of oncogenes in metastases. Carcinogenesis. 9:705-710, 1988.

Nakajima,M., Morikawa,K., Fabra,A., Bucana,C., and Fidler. Influence of organ environment on extracellular matrix degradative activity and metastasis of human colon carcinoma cells. J. Natl. Cancer Institute. 82:1890-1898, 1990.

Nicolson,G., Tumor oncogene expression and the metastatic phenotype. Cancer Rev. 3:25-57, 1986.

Nicolson,G., Metastatic tumor cell interactions with endothelium, basement membrane and tissue. Current Opinion in Cell Biology. 1:1009-1019, 1989.

Noel,A., Calle,A., Emonard,H., Nugens,B., Simar,L., Foidart,J., Lapiere,C., and Foidart,J.M. Invasion of reconstituted basement membrane matrix is not correlated to the malignant metastatic cell phenotype. Cancer Research. 51:495-414, 1991.

Ocalan,M., Goodman,S., Kuhl,U., Hauschka,S., and von der Mark,K. Laminin alters cell shape and stimulates motility and proliferation of murine skeletal myoblasts. Developmental Biol. 125:158-167, 1988.

Ossowski,L., and Reich,E. Changes in malignant phenotype of human carcinoma conditioned by growth environment. Cell. 33:323-333, 1983.

Ossowski,L., and Reich,E. Loss of malignancy during serial passage of human carcinoma in culture and discordance between malignancy and transformation parameters. Cancer Research. 40:2310-2315, 1980.

Paulsson,M. The role of Ca^{2+} binding in the self-aggregation of laminin-nidogen complexes. J. Biol. Chem. 263:5425-5430, 1988.

Pierschbacher,M., and Ruoslahti,E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature. 309:30-33, 1984.

Pikkarainen,T., Kallunki,T., and Tryggvason,K. Human laminin B2 chain. comparison of the complete amino acid sequence with the B1 chain reveals variability in sequence homology between different structural domains. J. Biol. Chem. 263:6751-6758, 1988.

Poste,G. and Fidler,I. The pathogenesis of cancer metastasis. Nature 283:139-145, 1980.

Preissner,K. The Role of vitronectin as multifunctional regulator in the haemostatic and immune systems. Blut. 59:419-431, 1989.

(a)   Pytela,R., Pierschbacher,M., and Ruoslahti,E. Identification and isolation of a 140 kd cell surface receptor glycoprotein with properties expected of a fibronectin receptor. Cell 40:191-198, 1985.

(b)   Pytela,R., Pierschbacher,M., Ruoslahti,E. A 125/115-kDa cell surface receptor specific for vitronectin interacts with the arginine-glycine-aspartic acid adhesion sequence derived from fibronectin. Proc. Natl. Acad. Sci. USA 82:5766-5770, 1985.

Ramaswamy,H., and Hemler,M., Cloning, primary structure and properties of a novel human laminin integrin ß subunit. EMBO J. 9:1591-1568, 1990.

Reich,R., Thompson,E., Iwamoto,Y., Martin,G., Deason,J., Fuller,G., and Miskin,R. Effect of inhibitors of plasminogen activator, serine protinases, and collagenase IV on the invasion of basement membranes by metastatic cells. Cancer Res. 48:3307-3312, 1988.

Repesh,L. A new in vitro assay for quantitating tumor cell invasion. Invasion Metastasis 9:192-208,1989.

Rhim,J., Park,D., Arnstein,P., Huebner,R., Weisburger,E., and Nelson-Rees,W. Transformation of human cells in culture by N-methyl-N'-nitroguanidine. Nature. 256:751-753, 1975.

Rocco,M., Infusini,E., Giovanna Daga,M., Gogioso,L., and Cuniberti,C. Models of fibronectin. EMBO J. 6: 2343-2349, 1987.

Roberts,A., Heine,U., Flanders,K., and Sporn,M. transforming growth factor-ß Major role in regulation of extracellular matrix. Annals N.Y. Acad. Sci. 580:225-232, 1990.

Ruoslahti,E., Fibronectin and its receptors. Ann. Rev. Biochem. 57:375-413, 1988.

Ruoslahti,E., Fibronectin in cell adhesion and invasion. Cancer Metastasis Rev. 43-51, 1984.

Ruoslahti,E., and Pierschbacher,M. Arg-Gly-Asp: A versatile cell recognition signal. Cell 44:517-518, 1986.

Ruoslahti,E., and Pierschbacher,M. New perspective in cell adhesion: RGD and integrins. Science 238: 491-497, 1987.

Ruoslahti,E., Hayman,E., and Pierschbacher,M. Extracellular matrices and cell adhesion. Arteriosclerosis, 5:581-594, 1985.

Saiki,I., Iida,J., Murata,J., Ogawa,R., Nishi,N., Sugimura,K., Tokura,S., and Azuma,I. Inhibition of the metastasis of murine malignant melanoma by synthetic polymeric peptides containing core sequences of cell-adhesive molecules. Cancer Research. 49:3815-1822, 1989.

Sanchez-Madrid,F., Landazuri,M., Morago,G., Cebrian,M., Acevedo,A., and Bernadeau,C. VLA-3: A novel polypeptide associated within the VLA molecular complex: cell distribution and biochemical characterization. Eur. J. Immunol. 16:1343-1349, 1986.

Sasaki,M., Kato,S., Kohno,K., Martin,G., and Yamada,Y. Sequence of the cDNA encoding the laminin B1 chain reveals a multidomain protein containing cysteine-rich repeats. Proc. Natl. Acad. Sci. USA. 84:935-939, 1987.

Sasaki,M., Kleinman,H., Huber,H., Deutzmann,R., and Yamada,Y. Laminin, a Multidomain Protein. The A chain has a unique globular domain and homology with the basement membrane proteoglycan and the laminin B chain. J. Biol. Chem. 263:16536-16544, 1988.

Sasaki,M., and Yamada,Y., The laminin B2 chain has a mutidomain structure homologous to the B1 chain. J. Biol. Chem. 262: 17111-17117, 1987.

Schwarzbauer,J., Spencer,C., and Wilson,C. Selective secretion of alternatively spliced fibronectin varients J. Cell Biol. 109:2445-3453, 1989.

Shaw,L., Messier,J., and Mercurio,A. The activation dependent adhesion of macrophages to laminin involves cytoskeletal anchoring and phosphorylation of the $\alpha_6\beta_1$ integrin. J. Cell Biol. 110:2167-2174, 1990.

Sheppard,D., Rozzo,C., Starr,L., Quaranta,V., Erle,D., and Pytela,R., Complete amino acid and sequence of a novel integrin ß subunit (β_6). identified in epithelial cells using the polymerase chain reaction. J. Biol. Chem. 265:11502-11507, 1990.

Shimizu,Y., van Seventer,G., Horgan,K., and Shaw,S. Costimulation of proliferative responses of resting $CD4^+$ T cells by the interaction of VLA-4 and VLA-5 with fibronectin or VLA-6 with laminin. J. Immunology. 145:59-67, 1990.

Smith,J. and Cheresh,D. The Arg-Gly-Asp binding domain of he vitronectin receptor. J. Biol. Chem. 263:18726-18731, 1988.

Smith,J. and Cheresh,D. Integrin ($\alpha_v\beta_3$)-ligand interaction. Identification of a heterodimeric RGD binding site on the vitronectin receptor. J. Biol. Chem. 265:2168-2172, 1990.

Sonnenberg,A., Linders,C., Modderman,P., Damsky,C., Aumailley,M., and Timpl,R. Integrin recognition of different cell-binding fragments of laminin (P1,E3,E8) and evidence that $\alpha_6\beta_1$ but not $\alpha_6\beta_4$ functions as a major receptor for fragment E8. J. Cell Biol. 110:2145-2155, 1990.

Sonnenberg,A., Modderman,P., and Hogervorst,F. Laminin receptor on platelets is the integrin VLA-6. Nature. 336:487-489, 1988.

Spargo,B., and Taylor,J. The kidney. In Pathology Ed. Rubin,E. and Farber,J. J.B. Lippincott Company, Philadelphia, USA. p.857-858, 1988.

Stearns,M., Wang,M., and Sousa,O. Evidence that estramustine binds MAP-1A to inhibit type IV collagenase secretion. J. Cell Science. 98: 55-63, 1991.

Suzuki,S. and Naitoh,Y. Amino acid sequence of a novel integrin β_4 subunit and primary expression of the mRNA in epithelial cells. EMBO J. 9:757-763, 1990.

Suzuki,S., Oldberg,A., Hayman,E., Pierschbacher,M., and Ruoslahti,E. Complete amino acid sequence of human vitronectin deduced from cDNA. Similarity of cell attachment sites in vitronectin and fibronectin. EMBO J. 4:2519-2524. 1985.

Schwartz,G., Redwood,M., Ohnuma,T., Holland,J., Droller,M., and Liu,B. Inhibition of invasion of invasive human bladder carcinoma cells by protein kinase C inhibitor Staurosporine. J. Natl. Cancer Institute. 82:1753-1756, 1990.

Takada,Y., Elices,M., Crouse,C., and Hemler,M. The primary structure of the α_4 subunit of VLA-4: homology to the other integrins and a possible cell-cell adhesion function. EMBO J. 8: 1361-1368, 1989.

Takada,Y., and Hemler,M. The primary structure of the VLA-2/collagen receptor $\alpha2$ subunit (platelet GPIa): homology to other integrins and the presence of a collagen binding domain. J. Cell Biol. 109:397-407, 1989.

Tamura,R., Rozzo,C., Starr,L., Chambers,J., Reichardt,L., Cooper,H., and Quaranta,V. Epithelial integrin $\alpha_6\beta_4$: complete primary structure of α_6 and forms of β_4. J. Cell Biol. 111:1593-1604, 1990

Tawil,N., Houde,M., Blacher,R., Esch,F., Reichardt,L., Turner,D., and Carbonetto,S. $\alpha_1\beta_1$ integrin heterodimer functions as a dual laminin/collagen receptor in neural cells. Biochemistry. 29:6540-6544, 1990.

Terranova,V., Maslow,D., and Markus,G. Directed migration of murine and human cells to collagenase and other proteses. Cancer Res. 49:4835-4841, 1989.

Terranova,V., Hujanen,E., Loeb,D., Martin,G., Thornberg,L., and Glushko,V. Use of a reconstituted basement membrane to measure cell invasivness and select for highly invasive tumor cells. Proc. Natl. Acad. Sci. USA. 83:465-469, 1986.

Thiagarajan,P., and Kelly,K. Exposure of binding sites for vitronectin on platelets following stimulation. J. Biol. Chem. 263:3035-3038, 1988.

Thorgeirsson,U., Turpeenniemi-Hujanen,T., Williams,J., Westin,E., Heilman,C., Talmadge,J., and Liotta,L. NIH/3T3 cells transfected with human tumor DNA containing activated ras oncogenes express the metastatic phenotype in mude mice. Mol. Cell. Biol. 5:259-262, 1985.

Timpl,R., and Dziadek,M. Structure, development, and molecular pathology of basement membranes. International Rev. Exp. Path. 29: 1-112, 1986.

Timpl,R., Dziadek,M., Fujiwara,S., Nowack,H., and Wick,G. Nidogen: a new, self-aggregating basement membrane protein. Eur. J. Biochem. 137:455-465, 1983.

Timpl,R., Rohde,H., Robey,G.P., Rennard,S., Foidart,J.M., and Martin,G. Laminin-A glycoprotein from basement membranes. J. Biol. Chem. 254:9933-9937, 1979.

Turpeenniemi-Hujanen,T., Thorgeirsson,U., Rao,C., and Liotta,L. Laminin increases the release of type IV collagen from malignant cell. J. Biol. Chem. 261:1883-1889, 1986.

Vukicevic,S., Luyten,F., Kleinman,H., and Reddi,A. Differentiation of canalicular cell processes in bone cells by basement membrane matrix components: regulation by discrete domains of laminin. Cell 63:437-445, 1990.

Vuolteenaho,R., Chow,L., and Tryggvason,K. Structure of the human laminin B1 chain gene. J. Biol. Chem. 265:15611-15616, 1990.

Wallich,R., Bulbuc,N., Hammerling,G., Katzav,S., Segal,S., and Feldman,M. Aboration of metastatic properties of tumour cells by de novo expression of H-2K antigens following H-2 gene transfection. Nature. 315:301-305, 1985.

Wang,M., and Stearns,M., Blocking of collagenase Secretion by estramustine during in vitro tumor cell invasion. Cancer Res. 48:6262-6271, 1988.

Weiss,R. The oncogene concept. Cancer Rev. 2:1-17, 1986.

Weiss,L. Random and nonrandom processes in metastasis, and metastatic inefficiency. Invasion Metastasis 3:193-207, 1983.

Werb,Z., Tremble,P., Behrendtsen,O., Crowley,E., and Damsky,C. Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J. Cell Biol. 109: 877-889, 1989.

Wewer,U., Taraboletti,G., Sobel,M., Albechtsen,R., and Liotta,L. Role of laminin receptor in tumor cell migration. Cancer Research 47: 5691-5698, 1987.

Yagel,S., Khokha,R., Denhardt,D., Kerbel,R., Parhar,R., and Lala,P. Mechanism of cellular invasivness: A comparison of amnion invasion in vitro and metastatic behavior in vivo. J. Natl. Cancer Inst. 81:768-775, 1989.

Yamada,K., and Kennedy,D. Amino acid sequence specificities of an adhesive recognition signal. J. Cell Biochem. 28:99-104, 1985.

Yanagihara,K., Seyama,T., Tsumuraya,M., Kamada,N., and Yokoro,K. Establishment and characterization of human signet ring cell gastric carcinoma cell lines with amplification of the c-myc oncogene. Cancer Research. 51:381-386, 1991.

Yannariello-Brown,J., Wewer,U., Liotta,L., and Madri,J. Distribution of a 69-kD laminin-binding protein in aortic and microvascular endothelial cells: modulation during cell attachment, spreading, and migration. J. Cell Biol. 106:1773-1786, 1988.

Yurchenco,P., Cheng,Y.S., and Ruben,G. Self-assembly of a high molecular weight basement membrane heparan sulfate proteoglycan into dimers and oligomers. J. Biol. Chem. 262:17668-17676, 1987.

Yurchenco,P., Cheng,Y.S., and Schittny,J. Heparin modulation of laminin polymerization. J. Biol. Chem. 265:3981-3991, 1990.

Yurchenco,P., and Schittny,J. Molecular architecture of basement membranes. FASEB J. 4:1577-1590, 1990.

Yurchenco,P., Tsilibary,E., Charonis,A., and Furthmayr,H. Models for the self-assembly of basement membrane. J. Histochem. Cytochem. 34:93-102, 1986.

Zutter,M., and Santoro,S. Widespread histologic distribution of the $\alpha_2\beta_1$ integrin cell-surface collagen receptor. American J. Path. 137: 113-120, 1990.