ALTERATIONS IN INTEGRIN EXPRESSION, DISTRIBUTION AND REGULATION DURING CARCINOGENESIS

by

Colette Mae Witkowski

A Dissertation Submitted to the Faculty of the

COMMITTEE ON GENETICS (GRADUATE)

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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Alterations in integrin expression, distribution and regulation during carcinogenesis

Witkowski, Colette Mae, Ph.D.

The University of Arizona, 1994

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Colette Mae Witkowski

entitled ALTERATIONS IN INTEGRIN EXPRESSION, DISTRIBUTION AND REGULATION DURING CARCINOGENESIS

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Dr. Anne E. Cress	Date 11/17/94
Dr. Harris Bernstein	Date 11/17/94
Dr. John R. Davis	Date 11-17-94
Dr. Roger Miesfeld	Date 11/17/94
Dr. Eugene W. Gerner	Date 11/17/94

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director Dr. Anne E. Cress	Date 11/17/94

STATEMENT BY AUTHOR

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SIGNED: Colette M. Witkowski

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I want to also thank my peers and collegues Margaret Tome, Pat Bauman, Vickie Peck, Isaac Rabinovitz and Dorothea von Bredow for discussions and support.

DEDICATION

This written dissertation is dedicated with respect and admiration to my grandfather, Gordon Bayless Robison, Bachelor Degree, Science and Math, Wabash College, Wabash, IN; Master of Science, Biochemistry, Northwestern University, Chicago IL. He has supported my interest in science since I can remember. From cutting a piece of log for a third grade science fair project to stressing to me in college the importance of "necessary evils" such as English and History (these too needed to be passed along with the science) he has supported my endeavors.

As Science Coordinator for the Michigan City, Indiana school system he organized the science fairs in which I participated. On a couple of occasions my grandfather was able to present to me the awards I had received in the competition.

He has been an ardent supporter throughout the course of my graduate education. I hope I am as successful as he in stimulating scientific interest in others. Thanks Grandfather.

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ABSTRACT

Metastatic cancer is the most difficult aspect in the treatment of cancer. Cancer cells that have metastasized show altered adhesion compared to normal cells of the same origin. Altered cell adhesion of cancer cells is central to the process of invasion and metastasis. A family of cell adhesion molecules termed integrins have been shown to be the cell adhesion molecules that are altered on cancer cells. These alterations and the mechanisms responsible for altering integrin expression, function and distribution during tumor progression currently are not fully understood.

Altered integrin expression was found to affect the tumorigenicity of human prostate carcinoma cells. The integrin profiles on four human prostate cell lines were analyzed using fluorescence activated cell sorting and immunoprecipitation of surface labeled proteins. The tumorigenicity was tested by injection into SCID mice. Modification of integrin profiles affected tumor formation differently. Increased expression of $\alpha 5\beta 1$ and $\alpha 6\beta 4$ decreased invasion. Altered non-functioning receptors also contributed to the lack of tumorigenicity. These data show that alterations in integrin expression affect the tumorigenic potential of human prostate cells.

A mouse model of skin carcinogenesis comprising normal, benign and malignant stages from a common lineage, was

analyzed to determine at which stage integrin expression was altered. The malignant carcinoma stage demonstrated a decrease in the surface expression of integrin $\alpha 6\beta 4$ and loss of the normal basal lateral distribution of this integrin. The changes in the $\alpha 6\beta 4$ on the malignant carcinoma cells parallels integrin expression results *in situ*.

This *in vitro* cell model system provided an opportunity to study the mechanisms of aberrant integrin expression. The results show the $\beta 4$ subunit expression in this model is altered by downregulation of the mRNA and posttranslational cleavage resulting ultimately in decreased surface expression. The $\beta 4$ in the malignant cells appears to be susceptible to degradation due to loss of protein-protein interactions involving the 100 kD $\beta 4$ cytoplasmic domain. These protein interactions localize the $\alpha 6\beta 4$ to the basal surface. Loss of these interactions results in non-polar distribution and potential susceptibility of the $\alpha 6\beta 4$ to proteolytic degradation. These mechanisms contribute to altered surface expression on cancer cells.

I. INTRODUCTION

A major obstacle in the treatment of cancer in general is the ability of the tumor cells to invade surrounding tissue and metastasize to distant sites. Treatment with various therapies results in eradication of the primary tumor, but clinical data show a fairly large percentage of patients ultimately develop clinical metastasis after treatment (Liotta and Stetler-Stevenson, 1993).

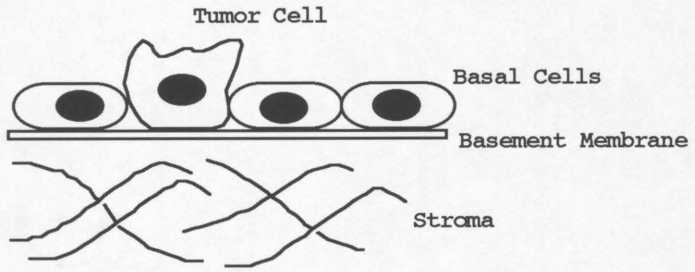
The intricate complexity of metastasis as a whole has impeded progress in understanding this phenomenon of cancer. The metastatic process is divided into multiple successive steps of tumor cell-host interactions to facilitate investigation. These steps are summarized from Liotta and Stettler-Stevenson (1993).

The first step defined in this process is tumor initiation. This step requires a carcinogenic insult, oncogene activation, or a chromosome rearrangement that produces an irreversible somatic mutation. The second and third step, promotion, progression and uncontrolled growth of the initiated tumor cell result in the expansion of the tumor cell population. Some of the factors and alterations that contribute to this expansion include: gene amplification, genetic and epigenetic instability or alteration, and growth factors and receptors acting to stimulate proliferation.

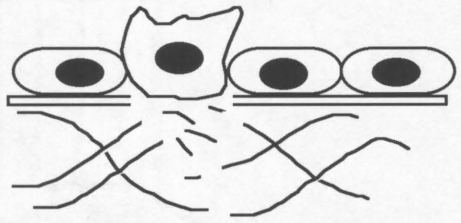
The fourth step is angiogenesis, the process of revascularization. Tumor cells secrete angiogenic factors that stimulate the growth of new capillaries. Angiogenesis attracts a blood supply to the tumor to maintain its growth. This increased vascularization sets the stage for the rest of the events of the metastatic cascade. In step five, cells from the tumor can invade normal tissue boundaries, basal lamina, blood and lymph vessels. Single cells or aggregates of cells can enter the circulation, interact with fibrin, platelets, and clotting factors in the blood, allowing transport of the tumor cells to a distant site in the body.

Tumor cells use many cellular processes in accomplishing all the events in the next steps of the metastatic cascade. These processes include: secretion of proteases, such as, type IV collagenase and heparinase that degrade the basement membranes of tissue, motility factors usually expressed in an autocrine fashion and the use of cell surface receptors involved in adhesion to the basement membrane components (reviewed in Long et al., 1989; Zetter and Brightman, 1990; Ruoslahti, 1992; Liotta and Stetler-Stevenson, 1993). Expression of these processes by the tumor cells leads to the final steps in the metastatic cascade: arrest at a distant site, colonization of the site and ultimately growth that has escaped host surveillance systems which is usually resistant to cancer therapies.

1. Attachment



2. Proteolysis of ECM Proteins



3. Migration through the ECM

Figure 1. Three step hypothesis of tumor cell invasion (Modified from Liotta and Kohn, 1990).

Liotta (Liotta et al., 1987; Liotta and Kohn, 1990) has proposed a model of tumor cell invasion to relate the sequence of biochemical events that takes place during this process. Figure 1 schematically represents this three step process of tumor cell invasion (Liotta and Kohn, 1990). Initially the tumor cell has to come in contact with and attach to the basement membrane matrix. Tumor cell associated proteases then degrade the basement membrane in the area adjacent to the tumor cell. The last proposed step is the migration of the tumor cell into and through the area of degraded basement membrane proteins such as laminin, fibronectin and collagen. It was proposed and has recently been shown that proteolytic fragments of the matrix can stimulate cellular migration (Werb et al., 1989).

Cell-extracellular matrix interactions, attachment and migration, of tumor cells to and through basement membranes is central to the process of invasion and metastasis (Juliano, 1987; Dedhar and Saulnier, 1990; Chammas and Brentani, 1991; Albelda, 1993; Juliano and Varner, 1993). The basement membrane is a major barrier to tumor cells at several stages during the process of metastasis. The proteolytic basement membrane and stromal fragments were originally thought to be simply chemotactic stimulants to the cells. It is now realized that attachment and migration of cells on basement membrane proteins and fragments is an active cellular process. Cell matrix interactions are known

to effect cellular phenotype (Hay, 1984). The transduction of signals as a result of extracellular matrix interactions conveys information that governs the shape, morphology and migratory capacity of cells. This active process of attachment and migration is mediated by a family of cell surface receptors for the extracellular matrix proteins and fragments called integrins (Tamkun et al.,1986; Hynes, 1987; Hynes, 1992).

The integrins are a recently described family of cell surface receptors involved in cell-cell and cell-substratum adhesion (Hynes, 1987; Hynes, 1992). Ligands for the integrins involved in cell-substrate adhesion include components of basement membranes, such as, laminin, collagen and fibronectin. Integrins are ubiquitous surface receptors with all cells expressing a subset of the 22 possible integrin combinations. Some integrins are expressed in a cell type specific fashion. Examples of this specific cell expression include α11bβ3 expression exclusively on platelets (Ginsberg et al., 1993), the β2 subfamily expressed only on leukocytes (Kishimoto et al., 1987), and the expression of α6β4 exclusively on epithelial cells (Hemler et al., 1989; Kajiji et al., 1989).

Each integrin is a heterodimer that consists of an α and β subunit noncovalently associated. Each subunit has a large extracellular domain, transmembrane domain and cytoplasmic domain (reviewed in Hynes, 1992). Figure 2 illustrates the

important features of the α and β subunits of the integrin molecules inferred from the complimentary DNA sequences (α and β references Argraves et al., 1987; Takada et al., 1989; Takada and Hemler, 1989; Hogervorst et al., 1990; Sheppard et al., 1990; Suzuki and Naitoh, 1990; Hogervorst et al., 1991).

The 8 β subunits (designated β1-8) share the following common features as listed. Within the extracellular domain primary amino acid sequence is a region rich in cysteines. These are conserved in each β subunit and form a series of disulfide bonds to form the accordion-like tertiary structure. This region is thought to be an area involved in the activation and deactivation (possibly by integrin-associated proteins) of the integrin molecules. The N-terminus of the extracellular domain folds back and forms a disulfide bond to maintain a loop structure. The internal side of the loop contains the binding site for the ligands. For fibronectin and vitronectin this involves the amino acid sequence, arginine, glycine and aspartic acid (RGD) (Ruoslahti and Pierschbacher, 1987; Ruoslahti et al., 1990). The C-terminus contains the cytoplasmic domain which is unique to each β subunit. These cytoplasmic domains are approximately 50 amino acids in length except for the β4 subunit which has a cytoplasmic domain of 1100 amino acids (Suzuki and Naitoh, 1990).

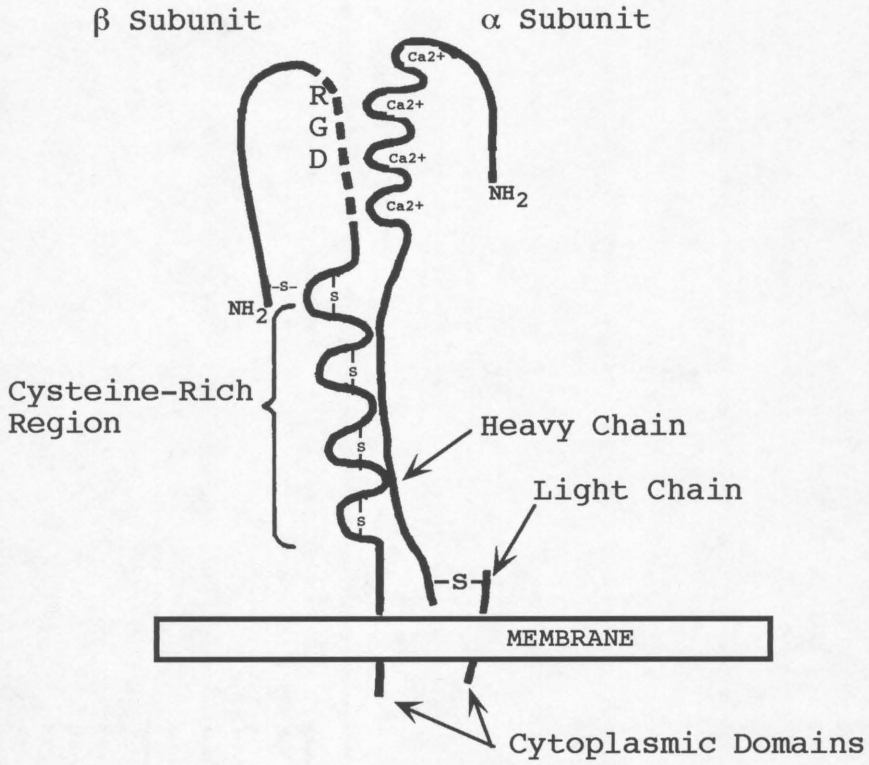


Figure 2. Schematic diagram of an integrin molecule (modified from D'Souza et al., 1991).

The cytoplasmic domain of the β subunits has recently been shown to be involved in both "outside-in" signaling, signaling upon binding to the ligand, and "inside-out" signaling, signaling involved in activation of the integrin (Ginsberg et al., 1992; Akiyama et al., 1994; Crowe et al., 1994; Lukashev et al., 1994).

The 14 α subunits have the following common features as listed. The N-terminus of the extracellular sequence is a series of 3 to 4 calmodulin-type divalent cation binding sites. This tertiary conformation is required for integrin binding (Hynes, 1992). Some of the α (3,4,5,6,v and 8) subunits are cleaved just proximal to the transmembrane domain into a heavy and light chain. The purpose of this processing is not yet known. Several of the α subunits, especially those found on white blood cells, have an extra inserted sequence within the extracellular domain termed the I domain that has been demonstrated to be involved in ligand binding (Randi and Hogg, 1994). The cytoplasmic tails of the α subunits are unique and relatively short. Two subunits, α3 and α6, undergo cell type specific alternative splicing generating different cytoplasmic domains (Tamura et al., 1990; Hogervorst et al., 1991; Tamura et al., 1991). At this time no specific function has been ascribed to the α subunit domains. Recent evidence suggests that the α subunit

cytoplasmic domain is involved in post-ligand binding events (Hemler et al., 1992).

The evidence for involvement of integrins during the process of tumor progression has been increasing (Dedhar et al., 1987; Dedhar and Saulnier, 1990; Albelda 1993; Juliano and Varner, 1993). Initial observations were made on normal versus transformed cell lines. The earliest change detected on the transformed cells was that the deposition of the fibronectin matrix was disrupted (Hynes and Yamada, 1982). The fibronectin receptor, integrin $\alpha 5\beta 1$, is involved in the process of fibronectin matrix assembly. The altered phenotype of the transformed cells compared to the normal cells suggested an alteration in this integrin. Indeed overexpression of the $\alpha 5\beta 1$ in transformed cells was shown to suppress the transformed phenotype (Giancotti and Ruoslahti, 1990). Other integrins have been shown to have altered expression on transformed cells compared to their normal counter parts (reviewed in Juliano and Varner, 1993).

Several recent studies of <u>in vivo</u> integrin expression on carcinomas of the breast, skin, cervix, and renal tissue have shown that there is heterogeneity concerning the changes in the levels of expression of the integrin molecules. There is however one uniform finding on polarized epithelial cells, the integrins lose their spatial orientation on the cell surface (Tennenbaum et al., 1992; Carico et al., 1993;

Juliano and Varner, 1993). All types of squamous cell carcinoma analyzed *in vivo* show a pattern of non-polar distribution of integrins compared to the normal polarized expression (Carico et al., 1993; Juliano and Varner, 1993). It is still not known what mechanisms are responsible for altering integrin expression, function and distribution during the process of tumor progression.

The research described in this dissertation centers around gaining an understanding of the changes that occur in the integrin profiles on epithelial cells which contribute to aberrant cell behavior, i.e. the formation of cancer. Two epithelial cell models were utilized to answer questions concerning these changes in the integrins as well as gain insight into the potential mechanisms involved in causing these changes on cells during tumor progression.

Chapter 3 discusses the alterations in the integrin profiles on 4 human prostatic tumor cell lines and how the individual profiles contribute to the metastatic capacity and behavior of the cell lines.

Chapter 4 addresses the following questions: At what stage does an alteration in integrin expression occur and what potential biochemical mechanisms are responsible for the alterations? An *in vitro* model of mouse skin carcinogenesis having the normal, benign and malignant stages represented was used to address these questions.

II. MATERIALS AND METHODS

Cell Lines and Culture Conditions

Human prostate cells

DU145 (ATCC HTB 81) originally was isolated from a lesion of the brain in a patient with metastatic carcinoma of the prostate (Stone et al., 1978). The PC3 (ATCC CRL 1435) cell line was initiated from a grade IV prostatic adenocarcinoma (Kaighn et al., 1979). The LNCaP (ATCC CRL 1740) cell line was isolated from a needle aspiration biopsy of a supraclavicular lymph mode metastatic prostate adenocarcinoma (Horoszewicz et al., 1983). All cell lines were obtained from the American Type Tissue Culture Collection, Rockville, MD. The 431P cell line is a variant of the PC3 cell line that was obtained by serially passaging PC3 in nude mice (a gift from Dr. D.H. Shevrin, Shevrin et al., 1986; Shevrin et al., 1988). Cells were maintained in Iscove's modified Dulbecco's medium, 10% fetal bovine serum, 1% L-glutamine (2300 mM Gibco, Grand Island, NY) and 1% penicillin/streptomycin (10,000 U/ml each, Gibco)

Keratinocyte cells

The keratinocyte cell line (291) was originally derived in low calcium Eagle medium containing 0.02 to 0.04 mM calcium, 5% chelated fetal bovine serum (FBS), 5% newborn

dermal fibroblast conditioned media and 10 ng/ml epidermal growth factor (EGF). This keratinocyte cell line exhibits properties of primary cultures of mouse keratinocytes (Kulesz-Martin et al., 1985). These properties include characteristic epidermal morphology along with keratin immunofluorescence patterns, formation of cornified envelopes in response to increased calcium and lack of tumorgenicity when tested in in vivo assays.

The growth characteristics of the keratinocyte (291) cell line were used to establish a mouse epidermal cell culture model of carcinogenesis. Primary epidermal cultures respond to changes in the calcium concentration in the media. These cells proliferate as a monolayer in low calcium media (0.02-0.04 mM). When calcium level is increased to that of normal media (1.4 mM) the primary epidermal cells respond by terminally differentiating and sloughing from the dish (Hennings et al., 1980). This phenotypic response was utilized to establish "initiated" cell lines. After treatment of the keratinocyte cells with the carcinogen, 7,12-Dimethylbenz[a]anthracene (DMBA), clones were isolated that no longer responded to the increase in calcium concentration, exhibiting an altered terminal differentiation response (Kulesz-Martin et al., 1980). Cells that exhibited altered terminal differentiation, proliferation in 1.4 mM calcium medium, were tested for tumorgenicity and

Table 1. Mouse keratinocyte cell line descriptions.

Cell Lines	Origin	Morphology / Histology	Tumorigenicity Assay
Keratinocyte (cell line 291)	BALB/c Ros	No Tumors	s.c., s.g.,i.p.
Benign Papilloma (line 291.09)	Derived from DMBA treatment of 291 line	Papilloma	s.g.
Squamous Carcinoma (line 291.03)	Derived from DMBA treatment of 291 line	Anaplastic Carcinoma	s.c., s.g.

s.c., subcutaneous injection, i.p., intraperitoneal injection, s.g., skin graft (Kulesz-Martin et al., 1988)

approximately 90% of the cell lines produced squamous cell carcinomas.

The benign and malignant derivative cell lines of 291 were produced using this technique established with primary keratinocyte cultures. The keratinocyte (291) cell line at passage eleven was treated with DMBA for 24 hours and putative initiated colonies were isolated in high calcium media (1.4 mM) (Kulesz-Martin et al., 1988). This produced three initiated cell lines that, when injected subcutaneously (5×10^6 cells) into athymic nude mice, resulted in tumors. Cell line 291.09 produced benign papillomas and cell line 291.03 produced anaplastic squamous cell carcinomas. Table 1 outlines the cell lines, their origin and histology. The observation of the characteristics of squamous cell tumors suggests that the process of transformation observed in the keratinocyte cell line is analogous to epidermal transformation *in vivo*. This *in vivo*/*in vitro* mouse skin carcinogenesis is advantageous for the study of the mechanisms of carcinogenesis.

Human specific integrin antibodies

The antibodies to human integrin subunits listed in Table 1 included monoclonal antibodies to α2 (P1E6, Telios, San Diego, CA) (Wayner et al., 1988), α3 clone P1B5 (Telios, Wayner et al., 1988), α4 clone P4G9 (Telios, Wayner et al., 1988), α5 clone P1D6 (Telios, Wayner et al., 1988), α6 clone

Table 2. Antibodies specific to human integrin subunits

Antibody	Source
α2 (P1E6)	Telios
α3 (P1B5	Telios
α4 (P4G9)	Telios
α5 (P1D6)	Telios
α6 (GoH3)	Dr.Sonnenberg, Accurate Chemical and Scientific Corp.
αv (VNR147)	Telios
β1 (A-1A5)	Dr. M.E. Hemler, T-Cell Diagnostics
β2 (P4H9)	Telios
β3 (4B12)	Genentech, Inc.
β4 (3E1)	Telios
αvβ3	Calbiochem

GoH3, a gift from Dr. A. Sonnenberg (Sonnenberg et al., 1987), αv clone VNR 147 (Telios, Freed et al., 1989; Vogel et al., 1990), β1 clone A-1A5, a gift from Dr. M.E. Hemler (Hemler et al., 1983) and T-Cell Diagnostics, β2 clone 3E1 (Telios, Hessle et al., 1984), β3 clone 4B12 (Genentech, Inc.), β4 clone 3E1 (Telios, Hessle et al., 1984) and a polyclonal antibody to αvβ3 (Calbiochem, La Jolla, CA). These integrin antibodies recognize external epitopes and are specific for the human integrin proteins. The antibody 10.11 is specific for human keratins 8 and 18 (Chan et al., 1986) (a gift from Dr. Robert Cardiff).

Secondary antibodies used were fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-rat IgG and FITC-conjugated goat anti rabbit IgG (Cappel, Durham, NC). Primary antibodies were used at a dilution of 1:500 for immunofluorescence and at a 1:1000 dilution for FACS analysis.

Integrin antibodies which recognize mouse proteins

Table 2 lists the integrin antibodies that were used in the mouse keratinocyte study. This table lists the techniques that the antibody can be used for and the source of each antibody. The techniques are abbreviated as follows: BF, blocks functional attachment to the ligand; IB, antibody can be used for immunoblotting; IF, antibody can be used for

immunohistochemical staining; IP, this antibody can be used to immunoprecipitate the protein that it recognizes.

The α2 polyclonal antibody, AB1936, recognizes an epitope on the intracellular carboxy-terminal sequence of α2 and shows no cross-reactivity to other α subunits. This antibody recognizes the mouse protein and was used in Western blotting and immunofluorescence assays.

The α3 polyclonal antibody, AB1920, recognizes an epitope on the intracellular carboxy-terminal domain of the α3 subunit. There is no cross-reactivity to other α subunits. This antibody was used in Western blot and immunofluorescence assays.

The α5β1 polyclonal antibody (FNR) (Argraves et al., 1987; Cardarelli and Pierschbacher, 1987; Dedhar et al., 1987) shows specific reactivity with an external epitope of α5β1, the fibronectin receptor with no cross-reactivity to αvβ3 or αvβ5 (vitronectin receptors). This antibody was used in immunoprecipitation assays, Western blotting and attachment inhibition assays.

Monoclonal α6 antibody, GoH3, (a gift from Dr. A. Sonnenberg) and purchased from Accurate Chemical and Scientific Corp. recognizes an external epitope on the α6 subunit (Sonnenberg et al., 1987). This α subunit complexes with the β1 or β4 subunit. This antibody was used in attachment inhibition assays, immunoprecipitation assays and immunofluorescence.

Table 3. Polyclonal antibodies to integrin subunits used to analyze the keratinocyte cell lines

ANTIBODY	TECHNIQUE*	SOURCE
α2 (AB1936)	IB, IF	Chemicon
α3 (AB1920)	IB, IF	Chemicon
α5β1 (FNR)	BF,IB,IF	Gibco-BRL
α6(GoH3)	BF,IF,IP	Sonnenberg
β1 (AB1938)	IB,IF	Chemicon
β4 (346-11A)	BF,IP,IF,IB	S.J. Kennel
β4 (AB1922)	IF, IP	Chemicon
αvβ3 (VNR)	IB,IF	Calbiochem

*BF=Blocks Function; IB= Immunoblotting; IF=Immunofluorescence; IP=Immunoprecipitation

The β1 polyclonal antibody, AB1938, (Chemicon International, Inc., Temecula, CA) recognizes an epitope on the intracellular carboxy-terminal domain of the β1 subunit. This antibody was used in Western blotting, immunofluorescence and immunoprecipitation assays.

The monoclonal β_4 antibody to the mouse subunit, 346-11A (a gift from Dr. Steven J. Kennel), recognizes an epitope on the external domain of the mouse β4 subunit (Kennel et al., 1989). This antibody shows no cross-reactivity to the human β4 subunit and was used in immunoprecipitation and indirect immunofluorescence microscopy.

The polyclonal antibody to the β4 subunit, AB1922 (Chemicon International, Inc.), recognizes the cytoplasmic sequence NH_2-(K)GTLSTHMDQQFFQT-amide of the β4 subunit.

Polyclonal antibody, to αvβ3, (clone VNR), recognizes external epitopes of the vitronectin receptor. This antibody was used for immunofluorescence assays.

The monoclonal antibody recognizing a common domain of μ and m calpain was provided by Dr. Darrel Goll's laboratory, University of Arizona, Tucson, AZ.

The polyclonal antibody that recognizes calpastatin was also provided by Dr. Darrel Goll's laboratory. These analysises were done on a Ziess Confocal microscope in the University of Arizona Department of Pathology bioimaging laboratory.

Indirect immunofluorescence microscopy

Human prostate cells

Cells were plated onto coverslips in 35mm tissue culture dishes and allowed to replicate through 1 to 2 cell doublings. Coverslips were washed three times at room temperature with Dulbecco's Phosphate Buffered Saline (DPBS), cells were permeabilized with 0.1% Saponin at room temperature for 5 minutes and washed with DPBS/1% FBS/10 mM Sodium azide. Coverslips were then incubated at room temperature for 30 min with anti-integrin antibodies, washed with DPBS/1% FBS/10 mM sodium azide and incubated with appropriate secondary antibodies, either a FITC-conjugated goat anti-rat (Tago) or a FITC-conjugated goat anti-rabbit (Cappel, Durham, NC). Coverslips were then washed and fixed with 3% formaldehyde and mounted with 90% glycerol containing the quenching agent, ρ-phenylenediamine (PPD) (Johnson and Nogueira Araujo, 1981; Johnson et al., 1982). Slides were viewed with a Ziess Photomicroscope II equipped for fluorescence microscopy and photographed using Ilford HP5 film.

Experimental tumors were excised from the SCID mice and snap-frozen in liquid nitrogen cooled isopentane. Serial 3-5 μm sections were put on slides and acetone fixed for 5 min. then allowed to dry in air. Sections were rehydrated in

DPBS for 5 min. and excess PBS was drained from the slide. The slides were incubated with 50 µl of a 1:100 dilution of integrin monoclonal antibodies or monoclonal antibody 10.11 for 30 min. at room temperature in a humidified chamber. The slide was flooded with PBS and allowed to rinse for an additional 5 min., then the excess was drained from the slide. The slides were flooded with the appropriate FITC-conjugated or rhodamine-conjugated secondary antibody for 30 min. at room temperature in a humidified chamber. Slides were again flooded with PBS and allowed to rinse for 5 min. in a Coplin jar. Coverslips were mounted on slides using 10% Gelvatol (Monsanto, St. Louis, MO) and stored at 4°C.

Keratinocyte cells

Cells were plated onto coverslips in 35 mm tissue culture dishes and allowed to replicate through 1 to 2 cell doublings. To switch cells to high calcium conditions, cells were plated in low calcium media and after 1-2 cell doublings the media was changed to high calcium media. The cells were then maintained for up to 5 days. Coverslips were washed three times at room temperature with DPBS, cells were permeabilized with 0.1% Saponin at room temperature for 5 minutes and washed with DPBS/1% FBS/10 mM Sodium azide. Coverslips were then incubated at room temperature for 30 min. with anti-integrin antibodies, washed with DPBS/1%

FBS/10 mM sodium azide and incubated with appropriate secondary antibodies, either a FITC-conjugated goat anti-rat (Tago) or a FITC-conjugated goat anti-rabbit (Cappel). Coverslips were then washed and fixed with 3% formaldehyde and mounted with 90% glycerol containing the quenching agent, ρ-phenylenediamine (PPD) (Johnson and Nogueira Araujo, 1981; Johnson et al., 1982). Slides were viewed with a Ziess Photomicroscope II equipped for fluorescence microscopy and photographed using Ilford HP5 film.

Flow Cytometric Analysis

Cells were trypsinized and washed in Hank's Balanced Salt Solution (HBSS) containing 0.5 mg/ml soybean trypsin inhibitor (Sigma). Cell number was adjusted to 1.5 x 10^6 cells/ml and aliquoted into small culture tubes. 1.5 x 10^6 cells were incubated on ice in 300 µl of integrin antibody for 30 minutes and then washed in HBSS/1% FBS/10 mM sodium azide. Cells were incubated on ice in 500 µl of appropriate secondary antibody at a dilution of (1:500) for 30 minutes. The human prostate cells were washed and fixed in 1% formaldehyde and could be held at 4°C for a maximum of 5 days before analysis.

The keratinocyte cells were washed and resuspended in HBSS, held on ice and analyzed within two hours. These cells could not be put into any fixative, formaldehyde or paraformaldehyde, without lysis of the cells. Cells were

analyzed through a Becton/Dickson FACStar Plus. Approximately 10,000 events were analyzed for each cell line/antibody combination. Histograms were collected in log scale. Cross-calibration between log and linear scales indicates 75 channels is equal to a doubling in fluorescence intensity.

Western Blot Analysis

Western blot analysis was carried out on immunoprecipitated $\alpha 5\beta 1$ and $\alpha 6$ integrin proteins from the keratinocyte cells. The blots were blocked for 1 to 2 hours with 5% powdered milk in DPBS/0.06% Tween 20, washed three times with DPBS/Tween, incubated 1 hour in 3% powdered milk containing integrin antibody $\beta 1$ (1:1000 dilution). The blot was washed and incubated in 3% powdered milk containing the secondary goat anti-rabbit horseradish peroxidase-conjugated antibody (Pierce, Rockford, IL). The blots were detected with ECL Western blotting detection reagents (Amersham, Arlington Heights, IL).

RNA Preparation and Northern Analysis

Total cellular RNA was isolated from the keratinocyte cells using the Acid Guanidine Thiocyanate-Phenol Chloroform (AGPC) extraction (Chomczynski and Sacchi, 1987). Approximately 20 μg of total cellular RNA was electrophoresed in a 1% formaldehyde agarose gel and transferred via

capillary method to Gene Screen (New England Nuclear, NEN Dupont, Burbank, CA). Blots were prehybridized overnight in 50% formamide, 10 x Denhardt's (.4% BSA, .4% PVP, .4% Ficoll), 50 mM Tris-Cl, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 1 M NaCl, and 200 ug/ml salmon sperm DNA. Subsequent hybridizations were carried out in the same buffer. Specific cDNA inserts, 20-30 ng, were ^{32}P-labeled using the random prime method (Feinberg and Vogelstein, 1983) with a Gibco-BRL kit. The blots were hybridized at 42^{o}C for 16 hours. The filters were then washed one time in 2 x SSC at room temperature for 30 min., one time in 2 x SSC/1% SDS heated to 50^{o}C at room temperature for 30 min. and one final wash in 2 x SSC at room temperature. These reduced stringency conditions were used because of the 20 percent mismatch determined between the mouse sequences and the sequences of the human probes. This mismatch was determined using the Genbank database. Blots were exposed to Kodak XAR-5 X-ray film at -70^{o}C with Cronex-Hi Plus (Dupont) intensifying screens. The blots were also exposed to a phosphorimaging screen (Molecular Dynamics) overnight and scanned on a phosphorimager. Band intensity was determined by integrated volume using ImageQuant software (Molecular Dynamics).

Probes

The α6 cDNA probe (clone 1363) 680 bp EcoRI insert in PUC18 and was a gift from Dr. A Sonnenberg (Hogervorst et

al., 1991). The β4 (Clone K163) 600 bp PstI insert in PUC18 was also a gift from Dr. A Sonnenberg (Hogervorst et al., 1990). The β1 probe is a 3.2 Kb EcoRI insert was a gift from Joanna Solowska (Solowska et al., 1991). The GAPD (glyceraldehyde 3-phosphate) probe was used to assess RNA quantities on the Northern blots.

Polymerase Chain Reaction

Reverse transcriptase coupled with PCR amplification (RT-PCR) was used to determine the α6 message splice variants expressed by the keratinocyte and carcinoma cells. The primers and basic protocol were followed directly from a previously published method (Hierck et al., 1993). Total RNA was isolated as described above. An aliquot of 2 μg of total RNA was used in a reverse transcription (RT) reaction (kit from Boehringer Mannheim, Indianapolis, IN) to synthesize cDNA. The reaction included reverse transcriptase buffer (1 x), dNTP mix (stock 8 mM each, Pharmacia, Piscataway, NJ), random hexamers (100 pmoles/μl, Pharmacia), RNasin (recombinant, Promega, Madison, WI), and reverse transcriptase. The Perkin Elmer Cetus (Irvine, CA) thermal cycler was used to cycle through the RT reaction steps. Samples were denatured at 90°C for 5 min., 23°C for 10 min., 42°C for 60 min., 95°C for 10 min., and a final soak at 4°C. An aliquot of 2 μl of the RT reaction was used immediately

for PCR amplification. The rest of the cDNA was precipitated by adding 0.1 volume of sodium acetate 3 M, and an equal volume of isopropanol and 1 hour at -80°C.

The mouse α6 sequence specific primers (Hierck et al., 1993) are as follows: the sense primer designated Sen2975, 5'GTCAGGTGTGTGAACATCAGG3", the antisense primer, Anti3597, 5'CTGGAAAAAATAAGGGGGGGC3' (both from Biosynthesis, Inc., Lewisville, TX). The RT reaction aliquot was added to 1 x PCR buffer (kit from Boehringer Mannhiem), Sen2975 (50 pmoles, stock 100 pmoles/μl), Anti3597 (50 pmoles, stock 100 pmoles/μl), Taq polymerase, dNTP mix and sterile distilled, deionized water to bring the final volume to 100 μl for each reaction. Mineral oil was layered on top of the reactions and cycled on a Perkin Elmer Cetus thermocycler. The amplification protocol was as follows: samples were denatured at 94°C for 5 min., then 35 cycles of amplification of 94°C for 1 min., 55°C for 2 min., 72°C for 2 min., a final 72°C 15 min. extension and a final soak at 4°C. Samples were separated from the oil using parafilm. DNA loading buffer was added to each sample and then denatured at 65°C for 5 min. Each sample was then electrophoresed along with ϕX-174-RF DNA HincII digest (Pharmacia) on a 1.25% NuSieve GTG agarose (FMC Bioproducts, Rockland, ME) in 1% TAE (Tris-Acetate) buffer (Sambrook et al., 1989) at 60 volts. Gels were stained with ethidium bromide and photographed over a UV light source. The sizes of the PCR products were determined

by linear regression analysis of the migration distance of each band. The reported size of the two α6 amplified fragments are α6A, 673bp and α6B, 543bp (Hierck et al., 1993). The calculated size of the α6A generated from the keratinocyte and carcinoma cells was 681bp. The 8bp difference from the reported size can be expected when the salt/buffer concentrations are different in the samples and size standards.

Labeling and Immunoprecipitation of Cell Surface Proteins

Surface labeling of cells.

The cells were trypsinized and washed 2 times in DPBS/0.5 mg/ml soybean trypsin inhibitor and cell concentration was adjusted to 5 x 10^6 cells/ml. The surface exposed proteins were labeled with NHS-LC biotin (Pierce) according to the protocol of Isberg and Leong (1990). Stock biotin in DMSO (10 mg/ml) was used at a 1:100 dilution (final concentration of 100 μg/ml). Cell suspensions were incubated at room temperature with occasional mixing. Cells were washed and dispensed into 1 ml aliquots containing 5 x 10^6 cells. Cells were immediately lysed in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 1% NP-40, 1 mM phenylmethane-sulfonyl fluoride (PMSF), 17 μg/ml Calpain I inhibitor, 0.3 μM aprotinin, and 1 μM leupeptin.

Metabolic labeling of cells.

Cells were grown to approximately 70% confluency, washed in media without serum and without methionine and then starved for 30 minutes. The methionine deficient media was exchanged with fresh media containing 100 μCi/ml ^{35}S methionine (NEN Express Labeling Mix) for three hours. Cells were washed with complete media and immediately lysed. Whole cell lysates were subjected to immunoprecipitation with antibodies to the integrin subunits.

Immunoprecipitation

Lysates were mixed and incubated on ice for 15 min. and centrifuged at 12,000 rpm for 15 min. to remove insoluble proteins. The supernatants were incubated with integrin antibodies for 1 to 2 hours at 4°C with constant mixing. Antigen-antibody complexes were precipitated with protein G-4FF or A CL-4B Sepharose (Pharmacia) with constant mixing at 4°C for 1 hour. Washed Sepharose beads were resuspended in SDS sample buffer with or without β-mercaptoethanol and boiled for 7 min. Samples were analyzed by SDS-PAGE. Gels containing biotinylated proteins were transblotted to nitrocellulose and detected using streptavidin-conjugated horse radish peroxidase (Pierce) and the ECL chemiluminescence Western blotting detection kit (Amersham). Gels containing ^{35}S-labeled proteins were dried and exposed to Kodak XAR-5 film or exposed to a phosphorimaging screen

(Molecular Dynamics) overnight and scanned on the phosphorimager. Band intensity (integrated volume) was determined using ImageQuant software (Molecular Dynamics).

Substrate Attachment and Inhibition of Attachment

Experimental substrate attachment was followed as previously described (Witkowski et al., 1993). Briefly, 96-well tissue culture plates (Falcon, Franklin Lakes, NJ) were coated with 100 µl of mouse EHS laminin (Collaborative Research, Badford, MA) at 20 µg/ml overnight at 4°C to test keratinocyte cell attachment. Non-specific cell attachment was inhibited by coating wells with 200 µl of 1% BSA in DPBS. All conditions were done in triplicate.

Cells were metabolically labeled with 5 µCi/ml ^{35}S-methionine (NEN express labelling mix) overnight for the keratinocyte cells and 30 µCi/ml for the human prostate cells, harvested and approximately 60,000 cells were plated into each well. For keratinocytes the assay time was 12 hours (De Luca et al., 1990) in a humidified incubator with 5% CO_2 at 37°C. The human prostate cells were allowed to attach for 1 hour.

To assess inhibition of attachment to laminin using the α6 antibody, cell suspensions were incubated with the antibody to α6 for 15 min. at 4°C prior to plating onto coated wells. Non adherent cells were removed and the remaining cells were solubilized with 200 µl of 2% SDS

(sodium dodecyl sulfate) in DPBS. Cellular radioactivity was determined for each well and results are presented as percent attachment (cellular radioactivity of adherent cells/total plated onto each well).

Experimental tumorigenicity assay

Human prostate cells from each cell line were injected intraperitoneally into SCID mice. Approximately 1 x 10^7 viable cells (viability determined by trypan blue dye exclusion test). The mice were submitted to autopsy approximately 25 days after injection. The animals were screened for immunodeficiency by determining the concentration of IgG present in the serum using an enzyme-linked immunoadsorbent assay (ELISA). Male and female mice with IgG less than 1.0 ng/dl were used (Ansell, 1989; Bosma and Carroll, 1991).

Biological half-life determination/Protein turnover

Keratinocyte cells, 291, and the squamous carcinoma cells, 291.03 were grown to approximately 60-70% confluency in 100 mm tissue culture dishes (Costar,Pleasanton, CA). Monolayers were washed 1 time with HBSS and then methionine starved in methionine-deficient media for 10-15 minutes. This media was replaced with methionine deficient media containing 100 μCi/ml ^{35}S-methionine (NEN-Dupont) for three hours.

After the three hour pulse, the plates were washed twice with complete media containing 10 mM methionine. The monolayers were incubated in complete media containing 10 mM methionine for various times specifically 0, 8, 12, 18, 21 and 24 hours. The cells were lysed in 400 µl lysis buffer, 50 mM Tris-HCl, pH 7.5; 0.15 M NaCl; 1 mM $MgCl_2$; 10 mM EDTA, pH 8.0; 1% NP-40, on the plate at the completion of the chase time. The following protease inhibitors were added to the lysis buffer, aprotinin (0.1 µM final concentration, 100 x stock of 66 µg/ml), leupeptin (1 µM final concentration, 100 x stock of 50 µg/ml), PMSF (0.2 to 2 mM final concentration, stock 200 mM is 0.348 g/10ml in isopropanol) and pepstatin (1 µM final concentration, 100 x stock of 70 µg/ml in methanol). Cells were scraped from the dish and the 400 µl suspension was transferred to 15 ml conical centrifuge tubes. The cell lysates were sonicated and held on ice for 10 min. Insoluble proteins were then pelleted with a 7 min. centrifugation at 14000 rpm (13,500 x g).

The total amount of protein in the cell lysates was determined using the BCA protein determination reagent following the standard 37°C protocol (Pierce). The incorporation of ^{35}S-methionine into total protein was assayed by trichloroacetic acid (TCA) precipitation of cell lysate (Harlow and Lane, 1988). The µg protein/µl cell lysate was determined using linear regression analysis and 200 µg of

whole cell lysate was reacted with 16 µl (3.2 µg) of the α6 antibody (GoH3).

The above saturating antibody conditions were determined using labeled 291 keratinocyte protein. The total protein was kept constant and the dilution of the antibody varied from 2 µl (0.4 µg) to 20 µl (4 µg). The determined ratio of antibody to whole cell lysate which was more than sufficient to precipitate all of the α6 protein from solution was 16 µl (3.2 µg) to 200 µg whole cell lysate.

The antibody was allowed to react with the whole cell lysate for 1 hour at 4°C with constant rotating. Approximately 100 µl of a 1:1 suspension of Protein G 4FF sepharose (Pharmacia) in 50 mM Tris-HCl, pH7.5, 0.5 M NaCl, 1 mM $MgCl_2$, 0.1%Tween-20 (IP wash buffer) with 0.5% bovine serum albumin (BSA), and 0.02% sodium azide was added for 1 hour to precipitate the antigen/antibody complex from the solution. A secondary antibody, rabbit anti-rat IgG (Sigma), was tried as an intermediate step but did not significantly increase the yield of precipitated α6 and associated proteins.

The sepharose beads and antigen/antibody complex were washed six times in 200 µl of IP wash buffer (as above) without BSA or sodium azide. Suspensions were vortexed briefly after each addition of fresh wash buffer. The last wash was aspirated using a 1 ml syringe with a 25G needle to remove as much wash buffer as possible.

To each sample 50 μl of 4 x cracking buffer (8% sodium dodecyl sulfate (SDS), 0.2 M Tris-HCl, pH7.0, 12% sucrose) without β-mercaptoethanol (non-reducing conditions) was added, samples were vortexed, centrifuged briefly and boiled for 10 min. then stored at -20°C.

The samples were reboiled after thaw before electrophoresis on a 7% SDS polyacrylamide gel under non-reducing conditions. The 0.5 mm spacers were used and the gel solutions were degassed under vacuum to give the best resolution of the protein bands. The non-reducing conditions gave the simplest protein band pattern with the α6 and β4 bands migrating at 140 and approximately 187-200 kD respectively. The total 50 μl of sample was electrophoresed.

Gels were run at 15-20 mAmps until the dye front was approximately 2-3 mm from the bottom of the gel. Gels were marked for orientation and fixed for approximately 10 min. until the color of the bromophenol blue dye changed to yellow in 25% methanol/7% acetic acid. This gel fix solution did not alter the size of the gel. After fixation, the gels were floated onto gel drying paper (BioRad, Hercules, CA) and dried overnight with 2 hours of heat under vacuum.

The dried gels were exposed directly to Kodak XAR-5 film for 5 days at room temperature. The gels were also exposed to a phosphorimaging screen (Molecular Dynamics) for 2 days and scanned on the phosphorimager. Protein band intensity

(integrated volume) was determined using ImageQuant software (Molecular Dynamics).

The biological half life was determined for the α6 and β4 subunits from both the keratinocytes (291) and carcinoma cells (291.03). The 0 chase time was used as control (100%) and the integrated volume of the bands was graphed as percent control for each chase time point (0, 8, 12, 18, 21, 24 hours). The biological half life, the time at which the ^{35}S-methionine activity in the protein bands is decreased by 50%, was determined from these data using a Best Fit analysis.

III. CHARACTERIZATION OF INTEGRIN EXPRESSION AND CORRELATION WITH TUMORGENICITY ON FOUR HUMAN PROSTATE CELL LINES.

Introduction

The process of tumor formation, invasion and metastasis is an intricate one that involves cellular attachment to the extracellular matrix, local proteolysis of the basement membrane and migration through the stroma to gain access to the circulation for the establishment of a metastatic colony (Liotta and Kohn, 1990). Within this cascade, aberrant adhesion is known to be involved in the metastatic process in human cancers (Liotta and Kohn, 1990); migratory cells display in part a loss of cellular adhesiveness (Ruoslahti and Giancotti, 1989). The process of cellular adhesion to extracellular matrix proteins (collagen, fibronectin, laminin, and vitronectin) as well as cell-cell adhesion is mediated in part by transmembrane proteins that have been termed integrins (Tamkun et al., 1986). A functional integrin is a heterodimer composed of an α and β subunit non-covalently linked (Hynes, 1992). Both subunits are integral membrane proteins with a large extracellular domain, a transmembrane domain and a cytoplasmic domain (Albelda and Buck, 1990). The $\beta 1$ subunit communicate with the actin cytoskeleton via talin, vinculin, fibulin and α-actinin

(Burridge et al., 1987; Burridge and Fath, 1989; Albelda and Buck, 1990).

An alteration of integrin composition has been observed to correlate with metastatic potential. For example, an investigation of integrin expression in histological sections of human malignant melanoma showed that the expression of β3 was most prevalent on melanoma cells in the vertical growth phase and that β3 expression correlated with increased metastatic potential (Albelda et al., 1990). In addition, alternative integrin subunits have been found to be expressed on melanoma cells in contrast to the normal melanocyte integrin profile (Kramer et al., 1991). These changes in integrin expression on cancer cells suggested an altered ability to adhere and invade the basement membrane in conjunction with its degradation ultimately metastasizing to distant sites (Costantini et al., 1990; Feldman et al., 1991). More recent studies have shown that overexpression of α2β1 in rhabdomyosarcoma cells in vitro causes an increase in formation of metastatic tumors after injection of the cells into nude mice (Bosco et al., 1991). An increase in expression of α6β1, a laminin receptor, has been correlated with an increase in basement membrane invasion by chemically transformed human HOS cells (Dedhar and Saulnier, 1990). However in other studies, an increase in expression of α5β1, the fibronectin receptor, correlates with an increased adhesiveness to fibronectin and less aggressive behavior

related to metastasis in transformed rodent cells (Plantefaber and Hynes, 1989).

We characterized the cell surface integrins expressed and the adhesive properties to specific substrates of human prostate cell lines isolated from human metastatic tumors. The tumorigenicity of these cells was determined by intraperitoneal injection into mice containing the severe combined immunodeficiency (SCID) mutation.

Results

Indirect immunofluorescence microscopy

Indirect immunofluorescence microscopy was used to detect the presence and distribution of the integrin subunits expressed by the human prostate cell lines. Figure 3 shows a representative example of indirect immunofluorescence microscopy on all four cell lines using monoclonal antibodies to the α6 and β1 subunits. All the cell lines show expression of surface α6 but the distribution of the integrins on each cell line is different. The DU145 cells exhibit strong staining of α6 and β1 at the periphery of the cell surface. The PC3 cell line displays a generally diffuse cytoplasmic staining with some peripheral staining apparent especially in the β1 stained panel. The 431P cells show a generally diffuse staining of the α6 with peripheral staining evident. The β1 subunit showed a diffuse cytoplasmic

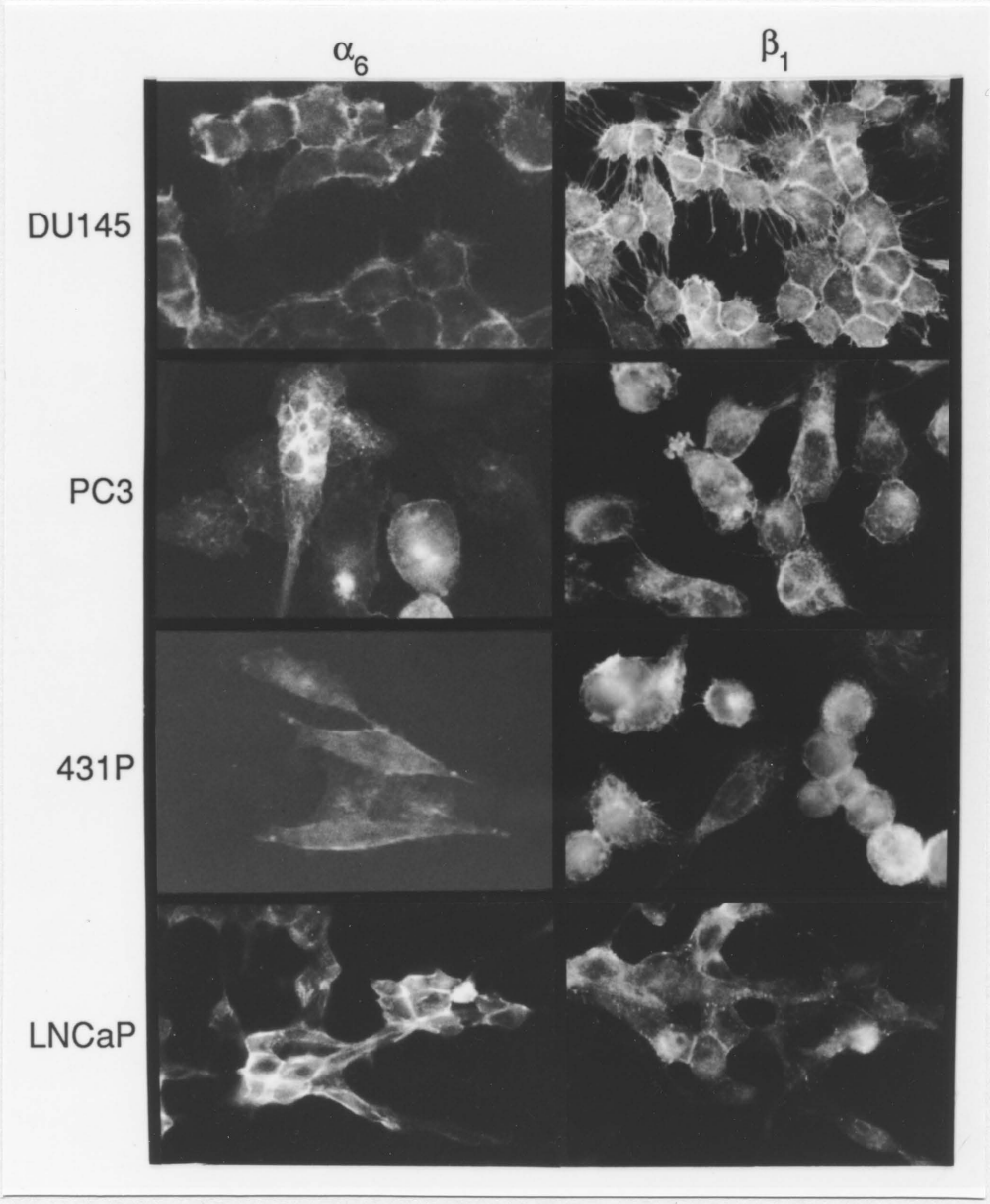


Figure 3. Indirect immunofluorescence detection of the integrin subunits α6 and β1 on human prostate cell lines.

The presence of the antigen-antibody complex was detected by fluorescein-isothiocyanate conjugated goat anti-(mouse IgG) or anti-(rat IgG). Magnification x400.

staining. The LNCaP cell line showed a similar distribution of the α6 and β1 subunits as on the DU145 cells. Within each cell line population variability was noted in the visible amount and distribution of integrins present on individual cell surfaces.

Fluorescence-activated cell sorter (FACS) analysis

Figure 4 shows FACS histograms for each α subunit antibody on the four human prostate cell lines. Composite histograms were produced to compare the negative-control histogram, cells incubated with only the appropriate FITC-conjugated second antibody (solid line), to the same cells incubated with monoclonal antibodies for specific α integrin subunits (dotted line).

The DU145 cell line showed a strong positive shift in fluorescence intensity with the monoclonal antibodies (mAb) to α3, α5, α6, and αv and a weak positive shift with the α2 mAb. The PC3 cell line showed a strong positive shift in fluorescence intensity with α3 and α6 mAbs and a moderate shift with α2, α5, αv. The 431P cell line which has been characterized as a highly metastatic derivative of the PC3 cell line, produced a moderate reaction with α3, α5, α6 and αv. All three of these cell lines showed no positive reaction with the α4 mAb. The LNCaP cell line was different in that it reacted weakly with α2 and α3 mAb.

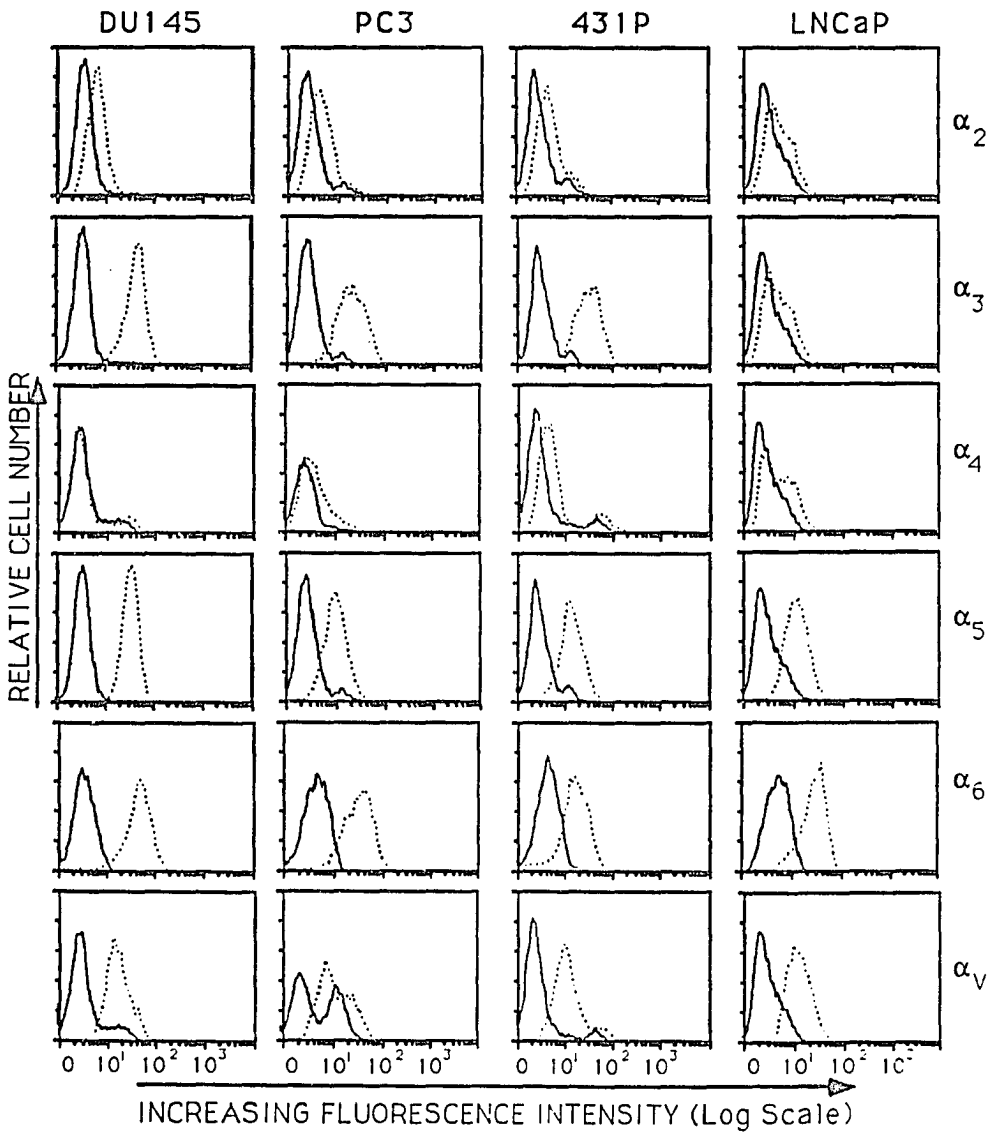


Figure 4. Fluorescence activated cell sorter (FACS) analysis of the integrin subunits α2, α3, α4, α5, α6 and αv on all four human prostate carcinoma cell lines.

The cells were reacted with each monoclonal antibody. The composite histograms shown for each α subunit contains the secondary only control histogram (____) and the histogram obtained after incubation with a monoclonal antibody to an α subunit (·····). Cell lines are indicated at the top and specific α subunit antibodies to the right of the histograms.

Table 4. Median channel of peak fluorescence of the α subunits

	α2	α3	α4	α5	α6	αv
DU145	7.03	51.26	2.47*	38.40	46.00	15.01
PC3	5.08	22.34	2.75*	9.73	46.00	8.42
431P	4.90	26.7	4.09	12.53	12.99	11.25
LNCaP	3.54	3.06	3.29	12.53	49.44	10.85

*Critical D value is 0.0326 for the 99% confidence level, D values not significant.

Table 4 shows the median channel of peak fluorescence from the antibody-treated histograms (dotted histograms from Figure 4) for all four human cell lines using the monoclonal antibodies to the α subunits. The relative quantities of each α subunit on each cell line can be compared.

Figure 5 shows FACS histograms of the human prostate cell lines using monoclonal antibodies to the β subunits. The DU145, PC3 and 431P cell lines show a positive shift in fluorescence intensity with mAbs to β1, β3, and β4. LNCaP cells show very minor peak shifts with β1, β2, β3 and β4. Table 5 shows the median channel of peak fluorescence from the β antibody-treated histograms (dotted histograms from Figure 5) for all four human cell lines. The relative quantities of each β subunit can be compared.

The Kolomogorov-Smirnorv (K-S) test (Young, 1977) was used to test whether the negative control histogram and the histogram produced by incubation of cells with the monoclonal antibodies to the integrin subunits were significantly different. The composite histograms for α and β subunits (Figures 4 and 5) were subjected to the K-S two sample test. The histograms produced by the monoclonal antibodies that were not significant are indicated in Tables 4 and 5 by asterisks.

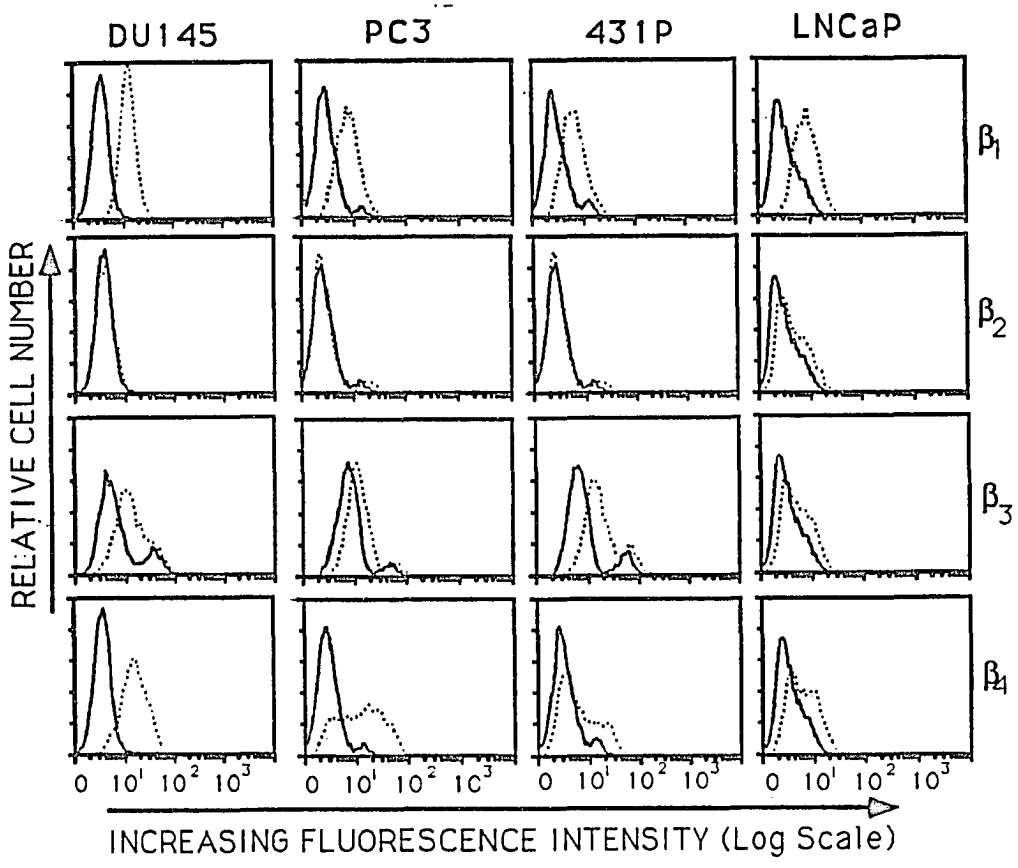


Figure 5. Fluorescence activated cell sorter (FACS) analysis of the integrin subunits β1, β2, β3, and β4 on all four human prostate carcinoma cell lines.

The cells were reacted with each monoclonal antibody. The composite histograms shown for each β subunit contain the secondary only control histogram (____) and the histogram obtained after incubation with a monoclonal antibody to a β subunit (·····). Cell lines are indicated at the top and specific β subunit antibodies to the right of the histograms.

Table 5. Median channel of peak fluorescence of the β subunits

	β1	β2	β3	β4
DU145	13.42	3.81*	9.03	17.98
PC3	7.29	2.30*	12.53	15.57
431P	5.66	2.65*	13.47	2.85
LNCaP	8.42	3.81	2.75	3.67

*Critical D value is 0.0326 for the 99% confidence level, D values not significant.

Immunoprecipitation of cell surface integrins

Figure 6 shows the immunoprecipitation of the integrin subunits α3, α5, α6 and αvβ3 along with co-precipitating proteins from DU145 and LNCaP cells. Both cell lines showed surface expression of the α5β1, α6β1 and αvβ3 integrins. These data are consistent with the data obtained by the FACS analysis shown in Figures 4 and 5. The DU145 cells showed the presence of the co-precipitating β4 subunit as well as the β1 subunit using the anti-α6 antibody. Present also were co-immunoprecipitating proteins of 70 kDa and 50 kDa. The identity and function of these proteins is currently unknown.

Using a polyclonal antibody specific for αvβ3, an integrin subunit of approximately 100 kDa was detected on both cell lines. The molecular weight is consistent with the surface expression of the β5 subunit of the αvβ5 heterodimer (Smith et al., 1990; Wayner et al., 1991). The LNCaP cell line did not express α3β1 on the cell surface compared to the DU145 cell line. These data confirmed the FACS analysis (Figures 4 and 5). The α integrin subunits from the LNCaP line migrated slightly faster than those from the DU145 cell line. These data suggest that the LNCaP integrins may have altered posttranslational processing of the integrins.

Substrate attachment and inhibition of attachment

Experimental substrate attachment was used to test the function of α5β1 (fibronectin receptor), α6β1 (laminin

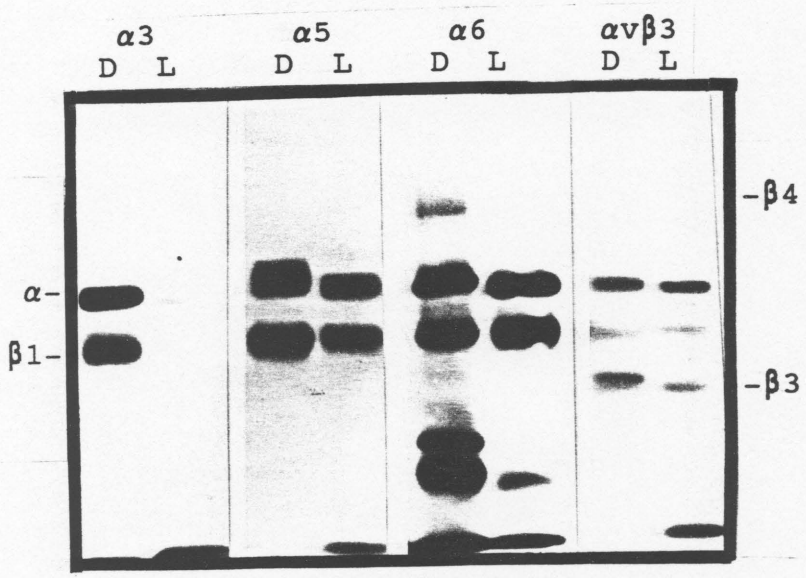


Figure 6. Immunoprecipitation of integrin subunits.

The surface expressed integrins on DU145 (D) and LNCaP (L) cells were labeled with biotin and immunoprecipitated with the monoclonal antibodies to α3, α5, α6 and the polyclonal antibody to αvβ3. Immunoprecipitated proteins were separated on a 7.5% SDS-Polyacrylamide gel, transferred to nitrocellulose and detected by chemiluminescence. Analysis was completed by collaborator Dr. Isaac Rabinovitz.

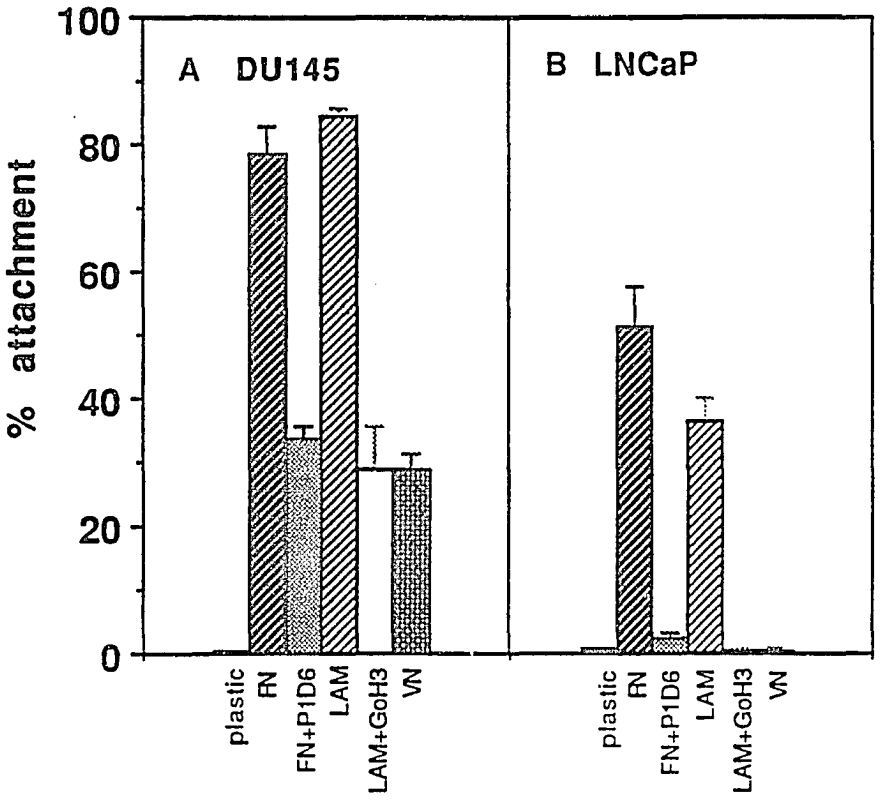


Figure 7. Experimental substrate attachment assay.

The percentage of attachment is defined as the cells attached/cells loaded per well x 100. The standard error of the mean is shown for each substrate. DU145 cells (A) or LNCaP (B) were tested for attachment to uncoated plastic (plastic), fibronectin (FN), laminin, or vitronectin (VN). The inhibition of attachment was tested by incubating the cells with anti-$\alpha 5\beta 1$ or anti-$\alpha 6$ antibody prior to exposing the cells to the matrix. Analysis was completed by collaborator Dr. Isaac Rabinovitz.

receptor) and αvβ3 or αvβ5 (vitronectin receptor). These results are shown in Figure 7. Both the DU145 and LNCaP cell lines attached to human fibronectin and human laminin. Approximately 50-60% of the attachment of DU145 cells to fibronectin and laminin is inhibited by the anti-α5β1 antibody and anti-α6 antibody, respectively. The remaining attachment to fibronectin and laminin may occur through α3β1 since this integrin is observed by FACS analysis (Figure 4 and Table 4) or attachment may be integrin independent. Approximately 97% of the attachment of LNCaP cells to fibronectin and laminin is inhibited by the anti-α5β1 antibody and the anti-α6 antibody, respectively. The LNCaP cells showed no attachment to vitronectin within the 1 hour incubation period. This is an unexpected result since the cells contain surface expressed αvβ3 as detected by FACS analysis (Figure 4) and immunoprecipitation (Figure 6).

Experimental tumorgenicity assay

The DU145 cells formed multiple microscopic tumors in the SCID mice with occasional bloody ascites (Table 6). The tumors formed from this cell line were small and found to be non invasive. PC3 and 431P cells formed grossly evident peritoneal tumors. Both PC3 and 431P tumors grew aggressively and upon histological analysis were shown to be invading the muscle tissue of the diaphragm. No metastases

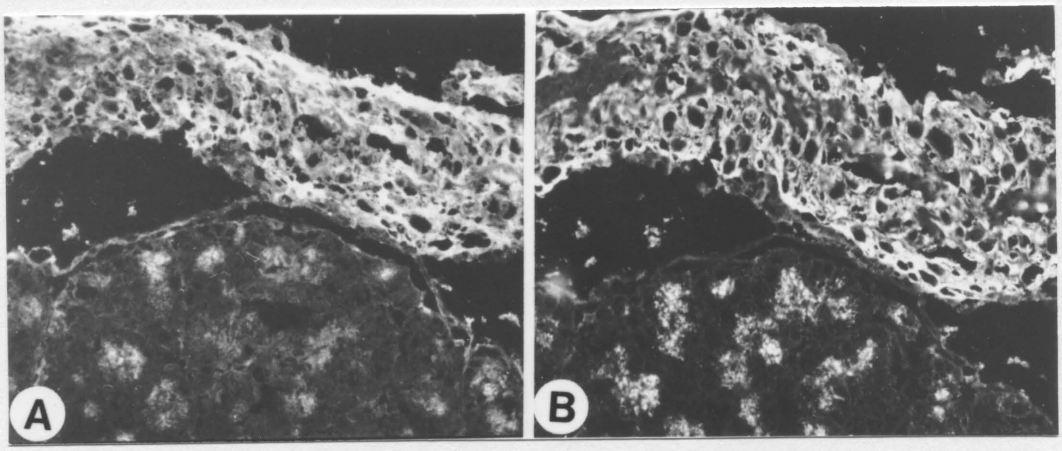


Figure 8. Double immunofluorescence of a tumor section of DU145 cells from a severe combined immunodeficient (SCID) mouse.

A. Distribution of β1 integrin subunit on the human prostate cell. B. Distribution of human cytokeratin using a monoclonal antibody designated 10,11. The keratin-specific fluorescence verified human prostate cells in the tumor within the SCID mouse.

Table 6. Human prostate cell line tumorigenicity and integrin content in murine tumors[a]

Cell Lines	SCID mouse tumors	Integrin subunits
DU145	Multiple, microscopic	α3, β1, β4
PC3	Gross, peritoneal, invasive	α6, β1, β4
431P	Gross, peritoneal, invasive	α3, α6, β1
LNCaP	None	

[a]Four mice were injected for each cell line. The presence of integrin subunits was tested by indirect immunofluorescence microscopy.

were found outside the peritoneal cavity. Both of these cell lines were reduced in the α5 subunit compared to DU145 cells (Figure 4 and Table 4). The LNCaP cell line up to 33 days after injection did not produce tumors.

Immunofluorescence microscopy was used to determine the integrin expression on the human tumor tissue from the SCID mice. Figure 8 shows that human tumor cells were growing within the SCID mice. Serial sections from all resulting tumors were sectioned and were incubated with a human specific keratin monoclonal antibody and an integrin subunit monoclonal antibody. This technique was used to verify that the tumor contained human cells and to analyze the integrin expression in the resulting tumors. The integrin monoclonal antibodies used on the tumor sections revealed positive staining with α3 on DU145 and 431P tumors and β1 on all tumors (Table 6). The α6 integrin was found primarily on the tumors formed by injection of the metastatic cell line variants PC3 and 431P. Similar to the tissue culture lines (Table 4), β1 was positive on all human tumors.

Discussion

Integrins are thought to be involved in the process of invasion and metastasis either by inappropriate or altered integrin receptor expression (Chelberg et al., 1989; Long et al., 1989; Ruoslahti and Giancotti, 1989). Little is known about integrin expression in human prostate cells and how

this effects tumorigenicity. We have initially chosen a model system that includes four human prostatic cell lines isolated from different metastatic sites.

All four cell lines were found to express relatively similar amounts of α2, α4, α6, αv and β1 consistently. The DU145 cells expressed the greatest amount of α5 compared to the other cell lines. The DU145 cells formed microscopically sized tumors which were not invasive. The PC3 and 431P cells however, expressed less α5 and were found to be invading surrounding tissues. Ruoslahti and Giancotti (1989; Giancotti and Ruoslahti, 1990) have suggested that expression of high levels of the α5β1 integrin reduces cell migration. Our data are consistent with this observation.

Dedhar and Saulnier (1990) have demonstrated the role of α6β1 in basement membrane invasion by using a monoclonal antibody against α6 to inhibit invasion of chemically transformed cells through a reconstituted membrane. All three tumor-forming cell lines expressed similar levels of α6 on the cell surfaces before injection into the SCID mice (Figure 4). After injection, the invasive tumors formed by the PC3 and 431P cells were found to express α6 and β1. The preservation of expression within the tumors of this integrin combination may indicate a role for this heterodimer in tumor progression.

We also find it interesting that the DU145 cells, which were not invasive, formed microscopic tumors and expressed

the greatest amount of β4 compared to the other cell lines (Figure 5). The α6 is known to complex with β1 and β4. Both heterodimers are thought to be receptors for laminin (Sonnenberg et al., 1988; Lee et al., 1992). Our data indicate the α6 on these cells is present as both α6β1 and α6β4 complexes (Figure 6). In comparison, the invasive PC3 and 431P cell lines do not contain surface expressed β4 (Figures 5 and 6) suggesting primarily α6β1 complexes. The role of α6 in tumor progression may depend upon its association with β1.

The LNCaP cells show surface expression of αvβ3 as detected by immunofluorescence and immunoprecipitation, but these cells do not functionally attach to vitronectin (Figures 4, 5, 6, and 7). This is an interesting result since vitronectin is known to be the ligand for αvβ3 (Cheresh et al., 1989; Wayner et al., 1991). These data suggest the presence of inactive integrin subunits on the LNCaP cell surface which may be related to the lower apparent molecular weight of the integrin subunits observed by immunoprecipitation (Figure 6). The activation of integrin subunits has been previously described as a mechanism to regulate integrin function (Du et al., 1991).

The activation of integrin receptors may require cooperativity between receptors mediated by an extracellular ligand (Du et al., 1991; Bauer et al., 1992; La Flamme et

al., 1992). The activation of the laminin receptors by the ligand may explain the observations that incubation of the LNCaP cells with extracellular matrix components prior to injection allows tumor formation (Pretlow et al., 1991). The extracellular matrix may play a role in prostate cancer growth and metastasis (Chung, 1991; Chung et al., 1991; Freeman et al., 1991; Geave et al., 1991; Geave et al., 1992; Passaniti et al., 1992) via altered expression and activation of integrin heterodimers.

IV. DECREASED SURFACE EXPRESSION, NON-POLAR DISTRIBUTION AND ALTERATION IN THE REGULATION OF THE α6β4 INTEGRIN IN A MOUSE MODEL OF SKIN CARCINOGENESIS

Introduction

Integrins are ubiquitous cell surface receptors with all cells expressing a subset of the possible integrin combinations. Several integrins have restricted cell and tissue expression. One integrin that has been shown to have a tissue specific pattern of expression is the α6β4 integrin. This integrin is primarily expressed on tissues of epithelial origin in both human and mouse which include epidermis, gut-lining epithelia, mammary glands and the amniotic sac (Hemler et al., 1989; Kajiji et al., 1989; Sonnenberg et al., 1990a; Suzuki and Naitoh, 1990; Ryynanen et al., 1991; Kennel et al., 1992).

The α6β4 integrin is a unique heterodimer. Both subunits have unique features. The cytoplasmic domain of the β4 is the largest of the β subunits, 1100 amino acids in length and approximately 100 kD (Suzuki and Naitoh, 1990). The external domain of the β4 is homologous to the other β subunits including the cysteine repeats and the transmembrane domain. The sequence of the β4 subunit was determined independently by three groups (Hogervorst et al., 1990; Suzuki and Naitoh, 1990; Tamura et al., 1990). The resulting

sequences of β4 when compared to each other, only differed in the sequence of the cytoplasmic tail producing what is interpreted as three variants of this subunit. There are two possible regions where inserts of 70 and 53 residues were found as well as the sequence with neither insert in the cytoplasmic tail. Also within the cytoplasmic domain were sequences found to be homologous to type III fibronectin repeats (Suzuki and Naitoh, 1990; Tamura et al., 1990). These type III fibronectin repeats recently have been shown to be involved in targeting the β4 to the basolateral cell surface (Spinardi et al., 1993). Experiments were conducted using truncated β4 cDNAs expressed in 804G cells which form hemidesmosomes in culture (Riddelle et al., 1992).

The α6 subunit is one of two α subunits (the other is the α3 subunit) that have been shown to undergo alternative mRNA splicing as well as proteolytic cleavage into two polypeptides linked by a disulfide bond (Sonnenberg et al., 1988a; Tamura et al., 1990; Hogervorst et al., 1991; Tamura et al., 1991). The alternative splicing of these two subunits yields unique cytoplasmic domains which show cell-type specific expression (Tamura et al., 1991). The variants of α6 are termed α6A and α6B. Most normal tissues were found to be expressing both variants but several tissues only express one variant or the other (Tamura et al., 1991; Hogervorst et al., 1993a). For example, keratinocytes were

found to be expressing only the α6A subunit and undifferentiated embryonic cells expressed the α6B and switched to the α6A when allowed to differentiate. The α6A was shown to be a target of phorbol 12-myristate 13-acetate (PMA)-induced phosphorylation but this condition did not apparently affect function (Hogervorst et al., 1993b). It is not yet known what function the alternative cytoplasmic domains or the phosphorylation of the α6A play in the expression and function of these subunits. The current hypothesis is that this is another level of integrin complexity possibly related to the regulation of function during development and differentiation (Tamura et al., 1991; Hogervorst et al., 1993b).

The α6 subunit can complex with β1 or β4 (Kennel et al., 1986; Hemler et al., 1989; Kajiji et al., 1989; Kennel et al., 1989; Sonnenberg et al., 1990b). In tissues that express both the β1 and β4 subunits, such as skin, the α6 preferentially forms a heterodimer with the β4 subunit (Sonnenberg et al., 1988a and b; Kennel et al., 1989; Sonnenberg et al., 1990a and b; Giancotti et al., 1992). The α6β1 integrin is a known laminin receptor specifically recognizing the E8 fragment of this molecule (Aumailley et al., 1990; Sonnenberg et al., 1990b). The α6β4 heterodimer has been shown to be a receptor for both laminin and a unique laminin isoform, kalinin, a specific component of basement

membranes of stratified epithelia (Niessen et al., 1994; Rousselle and Aumailley, 1994).

The unique tissue distribution of the α6β4 led to the observation that this integrin is part of the specialized structure of epithelial cells, the hemidesmosome or stable anchoring contact (SAC) (Carter et al., 1990; Stepp et al., 1990; Sonnenberg et al., 1991). The hemidesmosomes are located on the basolateral surface of basal cells on simple epithelium and function in adhesion to the basement membrane. One of the functions of the hemidesmosome is in the maintenance of the structural integrity of the epithelial tissues.

The integrin α6β4 has been shown to be altered in expression on squamous carcinomas. As early as 1979, expression of tumor associated antigens (TAA), A9 (human) and TSP-180 (mouse), were known to correlate with metastatic potential in both murine and human tumor models (Falcioni et al., 1986; Falcioni et al., 1988; Wolf et al., 1990). The protein complexes recognized by antibodies to these tumor specific antigens were shown to be identical to the α6β4 expressed by epidermal cells and other cells of epithelial origin (Kennel et al., 1989; Van Waes et al., 1991).

The evidence for involvement of integrins during the process of tumor progression has been increasing (Albelda, 1993, Juliano and Varner, 1993). Several recent studies of

in vivo integrin expression on carcinomas of the breast, skin, cervix, prostate and renal tissue have shown that the expression of the α6β4 integrin, independent of expression level, is altered in its distribution (reviewed in Carico et al., 1993; Juliano and Varner, 1993). All types of squamous cell carcinoma analyzed in vivo show a pattern of non-polar distribution of α6β4 compared to the normal polarized basolateral expression. It is still not known what mechanisms are responsible for altering α6β4 integrin expression, function and distribution during the process of tumor progression.

I have analyzed the expression of α6β4 on an established in vitro model of skin carcinogenesis. This model represents the three recognized stages of skin carcinogenesis as depicted in Figure 9. This model has been previously described in detail (Kulesz-Martin et al., 1985; Kulesz-Martin et al., 1988; Schneider et al., 1993). Briefly, the model consists of a keratinocyte cell line (291) that phenotypically and biochemically resembles a primary keratinocyte culture which was treated in vitro with the carcinogen 7, 12- dimethylbenz[a]anthracene (DMBA). This treatment independently produced two cell lines. One cell line is phenotypically a benign papilloma (291.09) and forms papillomas when tested in tumorgenicity assays. The other cell line is phenotypically an anaplastic squamous cell

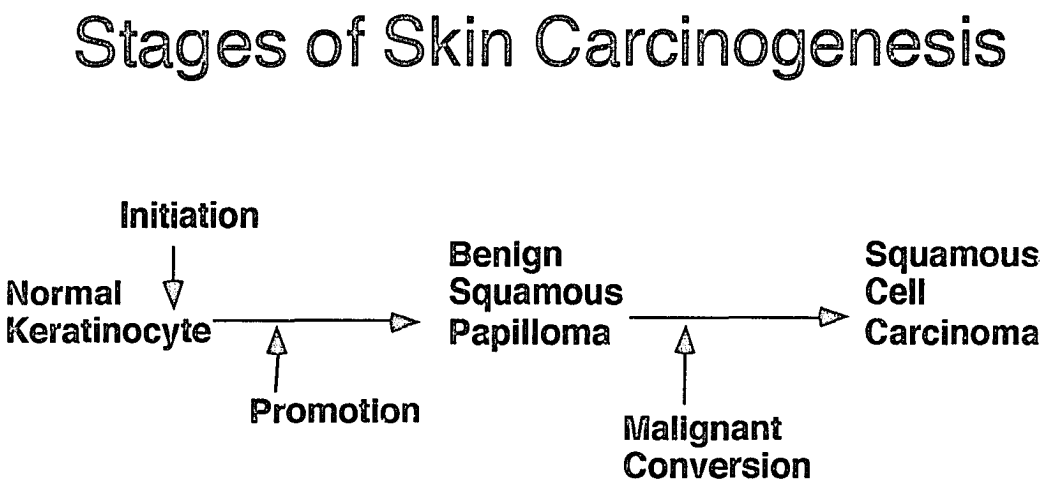


Figure 9. Stages of skin carcinogenesis.

carcinoma (291.03). These cells produce squamous carcinomas that migrate through the basement membrane and are locally invasive when tested in tumorgenicity assays (Kulesz-Martin et al., 1985; Kulesz-Martin et al., 1988; Schneider et al., 1993).

This mouse model of skin carcinogenesis consisting of the normal, benign and malignant stages was utilized to answer the following questions which are aimed at gaining an insight into the potential mechanisms of alteration in integrin expression on carcinomas: (1) Is there a differential surface expression and or distribution of integrins during tumor progression? (2) Is there an alteration in the integrin subunit composition on carcinomas that is associated with a more migratory phenotype? (3) Is there a functional difference of $\alpha 6\beta 4$ on the tumor cells compared to the normal cells? and finally (4) Is $\alpha 6\beta 4$ regulated differently on carcinoma cells compared to the normal cells?

Results

Integrin expression on mouse keratinocytes

The surface expression of $\alpha 6\beta 4$ on the mouse keratinocyte cell lines was initially analyzed by flow cytometry. Each cell line was analyzed with monoclonal antibodies to $\alpha 6$ (GoH3), mouse $\beta 4$ (346-11A) and the polyclonal antibody to

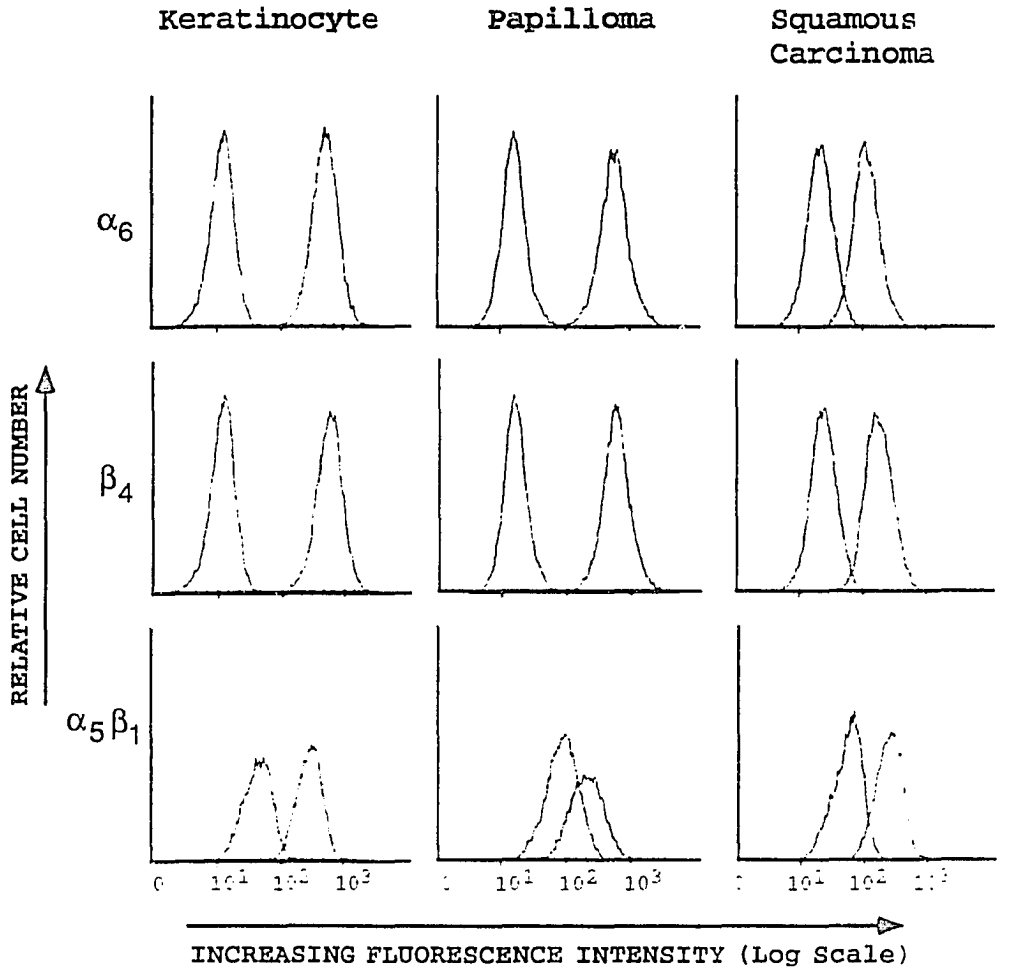


Figure 10. Flow cytometric analysis (FACS) on the mouse cell lines.

Keratinocyte, papilloma, and carcinoma cells were incubated with antibodies to $\alpha 6$, $\beta 4$, or $\alpha 5\beta 1$ (polyclonal). The composite histograms for each antibody/cell line combination contains two histograms representing the secondary-only control (left most histogram) and the antibody-antigen complex generated fluorescence (right most histogram). The Y axis represents relative cell number and the X axis represents the fluorescence intensity in log scale from 0 to 10,000. The cell lines are in the indicated columns and antibodies indicated to the left in the rows. The fluorescence data was collected and plotted in log scale.

$\alpha 5\beta 1$. The results are shown in Figure 10. The two histograms within each graph represent the cells incubated with the secondary antibody only, far left histogram (either goat anti-rat conjugated FITC or goat anti-rabbit FITC) and the right histogram represents cells incubated with both the integrin antibody and secondary antibody. All histograms are presented in log scale.

These results show the normal keratinocyte and papilloma cell lines were expressing similar amounts of $\alpha 6$ and $\beta 4$ as detected by this technique. In fact the $\alpha 6$ and $\beta 4$ histograms of each cell line were superimposable which was an early indication that the $\alpha 6\beta 4$ was the only $\alpha 6$ heterodimer expressed by these cells. The carcinoma cell line showed a decreased surface expression of both $\alpha 6$ and $\beta 4$ compared to the other two cell lines. In contrast, the integrin $\alpha 5\beta 1$ was expressed at similar levels in all cell lines.

The shift in peak fluorescence was calculated (secondary-only histogram mean subtracted from the antibody produced histogram and averaged). These results are presented in Table 7. The mean peak of fluorescence generated by the antibody-antigen complex was of lesser intensity on the carcinoma cells. A difference of 75 channels is equal to a doubling in fluorescence intensity. Using this parameter, the surface expression on the carcinoma cells was

Table 7. The average magnitude of the fluorescence peak shift

	α6	β4	α5β1
Keratinocyte (291)	318	320	168
Papilloma (291.09)	330	333	122
Carcinoma (291.03)	154	163	153

The average magnitude of the fluorescence peak shift from the secondary only antibody-antigen detected fluorescence peak using the median channel of peak fluorescence.

approximately four fold less compared to the keratinocyte and papilloma cell lines.

To further evaluate the decreased surface expression on the carcinoma cells, surface proteins were biotinylated and immunoprecipitated with antibodies to the α6 and β4 subunits. Initial cell concentrations were standardized and equal cell numbers were immunoprecipitated. Equal volumes were electrophoresed on a 7% SDS polyacrylamide gel. The results are shown in Figure 11. The α6 specific band is indicated at 143 kD. The two bands above the α6 band are the β4 specific bands at 180 and 200 kD (Giancotti et al., 1992). These results confirm a decreased surface expression of the α6β4 integrin heterodimer on the carcinoma cells.

A dilution series experiment was conducted using surface biotinylated proteins. Two different sample volumes of keratinocyte α6 immunoprecipitation reactions (15 μl and 20 μl) were compared to several different volumes of the carcinoma α6 immunoprecipitation reaction (15 μl, 20 μl, 30 μl, 40 μl, 50 μl). Figure 12 shows the results of these experiments. Comparing dilution 2 (20 μl) of the keratinocyte to dilution 5 (50 μl) of the carcinoma, approximately three times more sample volume from the carcinoma was required to equal the band intensity of the α6 and β4 proteins of the keratinocyte. Confirming the decrease in surface expression of α6β4 on the carcinoma cells.

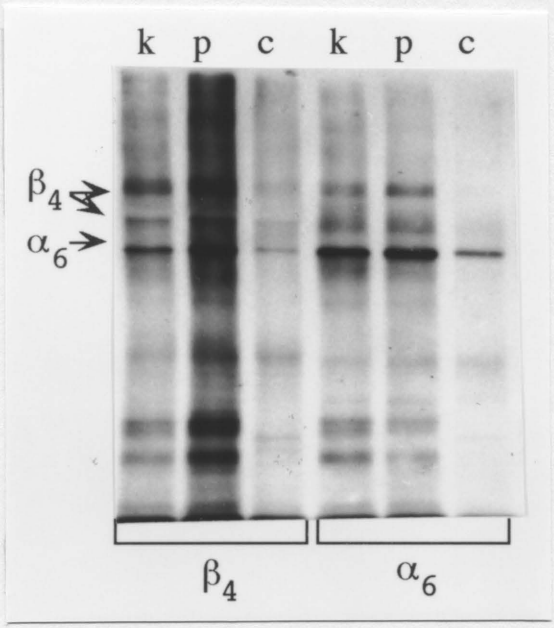


Figure 11. Immunoprecipitation of surface biotinylated integrin α6 and β4 subunits.

Keratinocyte, papilloma and carcinoma cells were surface biotinylated and immunoprecipitated with antibodies to α6 and β4. Precipitated proteins were subjected to a non-denaturing 7% SDS-PAGE and transferred to nitrocellulose. Biotin labeled proteins were incubated with streptavidin-conjugated horseradish peroxidase and detected using chemiluminescence. The first three lanes were immunoprecipitated with β4 from the keratinocyte cells, papilloma cells, and carcinoma cells respectively. The right three lanes were immunoprecipitated with α6 with the same order of cell lines. The α6 specific band is indicated at 143kD and two β4 bands are above at 180 and 200kD.

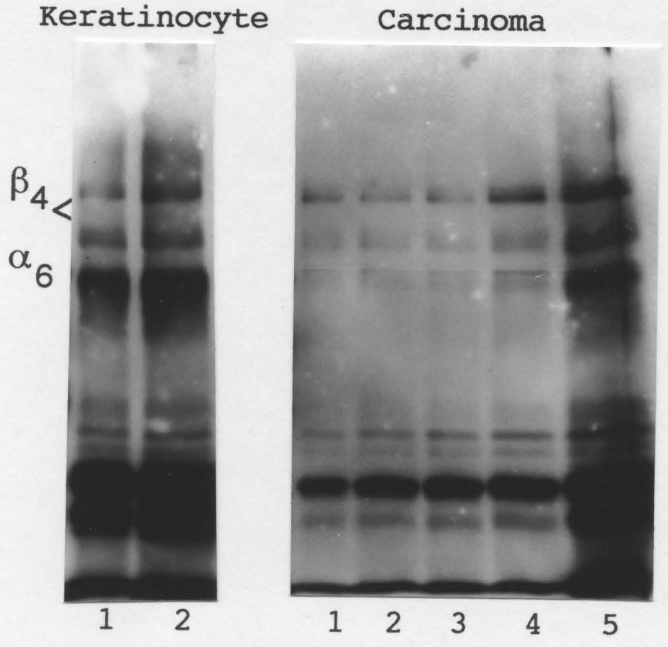


Figure 12. Dilution series comparing the amount of α6β4 on the surface of the keratinocytes and carcinoma cells.

Keratinocyte and carcinoma cells were surface biotinylated and immunoprecipitated with antibodies to α6. Precipitated proteins were subjected to a non-denaturing 7% SDS-PAGE and transferred to nitrocellulose. Biotin labeled proteins were incubated with streptavidin-conjugated horseradish peroxidase and detected using chemiluminescence. The two cell lines were initially standardized with an equal number of cells biotinylated. The first two lanes are from the keratinocyte cells with 15 μl(1) and 20 μl(2) of precipitated proteins loaded per lane. The series of lanes to the right is precipitated proteins from the carcinoma cells with 15 μl(1), 20 μl(2), 30 μl(3), 40 μl(4) and 50 μl(5) of precipitated proteins per lane. The α6 specific band is indicated at 143 kD and the two β4 bands are above at 180 and 200 kD.

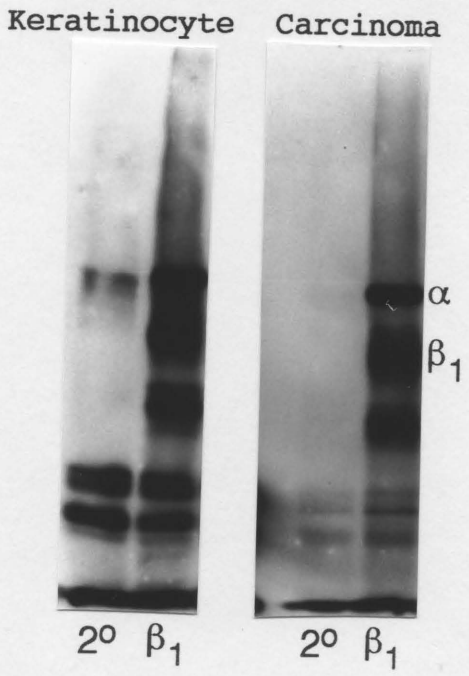


Figure 13. Immunoprecipitation of surface biotinylated β1 and associated α subunits.

Keratinocyte and carcinoma cells were surface biotinylated and immunoprecipitated with the antibody to the β1 subunit (AB1938). Precipitated proteins were subjected to a non-denaturing 7% SDS-PAGE and transferred to nitrocellulose. Biotin labeled proteins were incubated with streptavidin-conjugated horseradish peroxidase and detected using chemiluminescence. For each cell line the secondary only (2°) is shown along with the β1 precipitated proteins. The α subunit band (α) is indicated to the right the proteins range in molecular weight from 130-160 kD. The β1 band (β1) is also indicated to the right. The mature β1 has an approximate molecular weight of 110 kD.

To confirm that this decrease in the integrin receptor was not a general phenomenon on the carcinoma cells, surface biotinylated proteins were immunoprecipitated with the antibody to the β1 subunit (AB1938) under the same standardized conditions described above. The β1 coprecipitates all associated α subunits. Figure 13 shows the results of the immunoprecipitation reactions from the keratinocyte and carcinoma cells. The β1 band is indicated to the right of the lanes as is the α subunits. The mature β1 has a molecular weight of approximately 110kD and the associated α subunits range in molecular weight from 130 to 160kD. From this analysis the carcinoma cells are expressing similar amounts of the β1 integrins on the cell surface. The surface decrease in the α6β4 integrin is specific for this receptor and not a general phenomena for the integrin receptors on the carcinoma cells.

Integrin α6 composition

Since integrin expression has been shown to be altered on cancer cells, there was a possibility that there was a switch in the α6 integrin heterodimer composition on the surface of the carcinoma cells compared to the keratinocyte cells. Normal keratinocytes are known to express only the α6A mRNA splice variant and the α6β4 heterodimer. Because the expression of α6β1 is known to be involved in cell migration and invasion and a switch or dual expression of

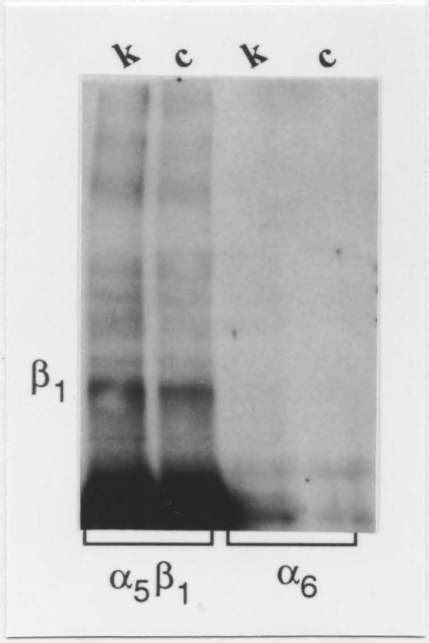


Figure 14. Western blot analysis with an antibody to β1.

The α5β1 and α6 proteins were immunoprecipitated from the keratinocyte and carcinoma cells, the precipitated proteins were resolved on reducing 7.5% SDS-PAGE and transferred to nitrocellulose. Blots were incubated with the β1 antibody (AB1938) which recognizes the cytoplasmic domain of this subunit. The keratinocyte cell line and carcinoma cell line are indicted at the top of the lanes. The left two lanes were immunoprecipitated with α5β1 and show coprecipitating β1 protein, whereas the α6 immunoprecipitated proteins, right two lanes, do not show presence of β1 coprecipitating with the α6 subunit. The location of the β1 protein at 110 kD is indicated to the left of the lanes.

both α6 splice variants have been shown in other carcinoma cells lines, we analyzed the possibility that the carcinoma cells were expressing the α6β1 heterodimer or were now expressing an alternative α6 message.

Figure 14 shows a Western blot analysis using the polyclonal antibody to the cytoplasmic domain of β1 on immunoprecipitated α6 and α5β1 proteins from the keratinocyte and carcinoma cells. The results show that no detectable β1 co-precipitated with the α6 subunit from either the keratinocytes or carcinoma cells. Therefore there is no switch in the α6 heterodimer composition on the carcinoma cells that could result in an altered adhesion phenotype.

The polymerase chain reaction (PCR) was used to amplify the potential α6 mRNAs expressed by the keratinocyte and carcinoma cells. Mouse sequence specific primers were used in this reaction (Hiercket al., 1993). The primers are positioned so that the region containing the 130bp deletion is amplified and the different splice variant products can be distinguished. Total RNA was reverse-transcribed according to general protocol (PCR A Practical Approach, 1991) and the cDNA was combined with the primer pair specific for the mouse α6A and α6B. The reported size of the α6A is 673bp and the size of the α6B is 543 bp. Figure 15 shows the results of the PCR amplification of the α6A and B mRNA. Presence of only the α6A mRNA could be detected in both the keratinocyte

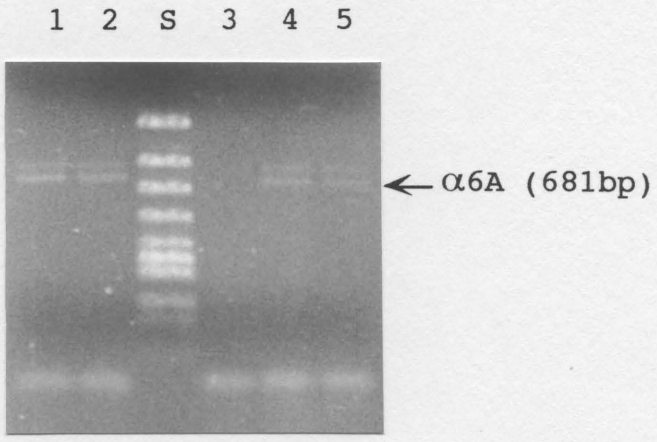


Figure 15. Polymerase chain reaction amplification of the α6 A and B variants. Lanes 1 and 2 are PCR reactions from the keratinocytes cells on two different aliquots of cDNA, the standard lane (S), Lane 3 is a mock PCR reaction with no cDNA, and Lanes 4 and 5 are PCR reactions from the carcinoma cells on two different aliquots of cDNA. The α6A variant that was amplified is indicated to the right with the calculated molecular weight (681bp) from this gel. The band above the α6A band is a PCR amplified artifact (Hierck,1993)

Total RNA was isolated from keratinocyte and carcinoma cells and 2.0μg was reverse transcribed. Primers specific for the mouse a6 sequences were used to detect and amplify corresponding cDNAs. The reaction products were electrophoresed on a 1.25% NueSieve GTG agarose in 1% TAE buffer at 60 volts. The standard bands are in the middle of the gel (S). The first 5 bands range in size from 1057bp (top), 770bp, 612bp, 495bp, and 392bp. The reported sizes of the α6A is 673bp and α6B is 543bp.

and carcinoma cells by this technique. The α6A fragment at 681 bp is indicated to the left of the Figure. The band above is a PCR artifact described by Hierckand coworkers (1993). The discrepancy of 8bp between the reported fragment size and the fragment obtained from the keratinocyte and carcinoma cells could possibly be due to the different buffer/salt concentrations between the samples and the size standards. These data show that the carcinoma cells have not switched the α6 variant expressed.

Functioning of the α6β4 Integrin

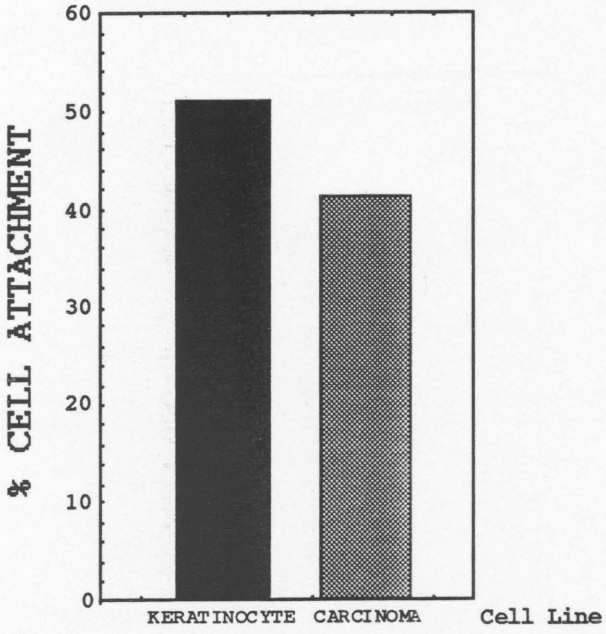
Laminin is the proposed ligand for α6β4 (Lotz et al., 1990; Lee et al., 1992). More recently the α6β4 integrin had been shown to bind to kalinin, a laminin isoform expressed by keratinocytes (Niessen et al., 1994; Rousselle and Aumailley, 1994). Experimental substrate attachment assays were used to assess the ability of the α6β4 on the keratinocyte and carcinoma cell lines to adhere to mouse EHS laminin. Figure 16A shows the carcinoma and keratinocyte cell attachment to laminin, 40% and 50%, respectively. Figure 16B shows inhibition of attachment to laminin by preincubating the cells with antibodies to the α6 subunit. The antibody to α6 inhibits the functional attachment of the keratinocytes greater than 40%. The carcinoma cells show a 25% inhibition of attachment by blocking the α6 subunit. The

Figure 16. Substrate attachment to mouse EHS laminin.

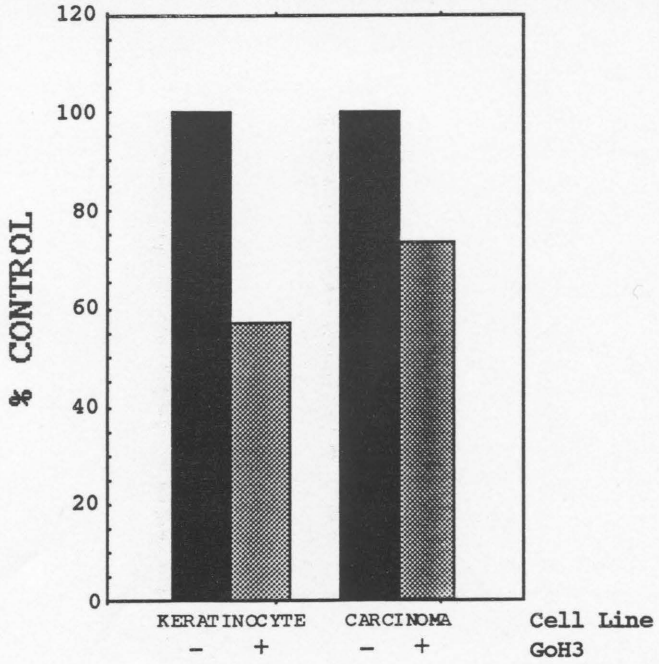
A. Attachment to laminin. Cell attachment is presented as percent cells attached (remaining radioactivity of attached cells/radioactivity of cells plated x 100). The standard error of the mean SEM for triplicates was calculated to be approximately 0 so the error bars are contained within the columns.

B. Inhibition of attachment to laminin. Attachment conditions were as above. Inhibition of attachment is presented as percent control (cells inhibited/cells attached x 100). Both attachment and inhibition are presented for comparison, (-) without antibody treatment, (+) prior incubation with GoH3. The standard error of the mean for triplicates was calculated to be approximately 0 so the error bars are contained within the columns.

A. ATTACHMENT TO LAMININ



B. INHIBITION OF ATTACHMENT TO LAMININ



attachment to laminin was not completely inhibited by the GoH3 antibody indicating non-integrin mediated interaction with laminin as well as possible other integrin involvement. These cells do express α3β1, an alternative laminin receptor (data not shown). These data indicate that the α6β4 on the carcinoma cells is functioning as a laminin receptor.

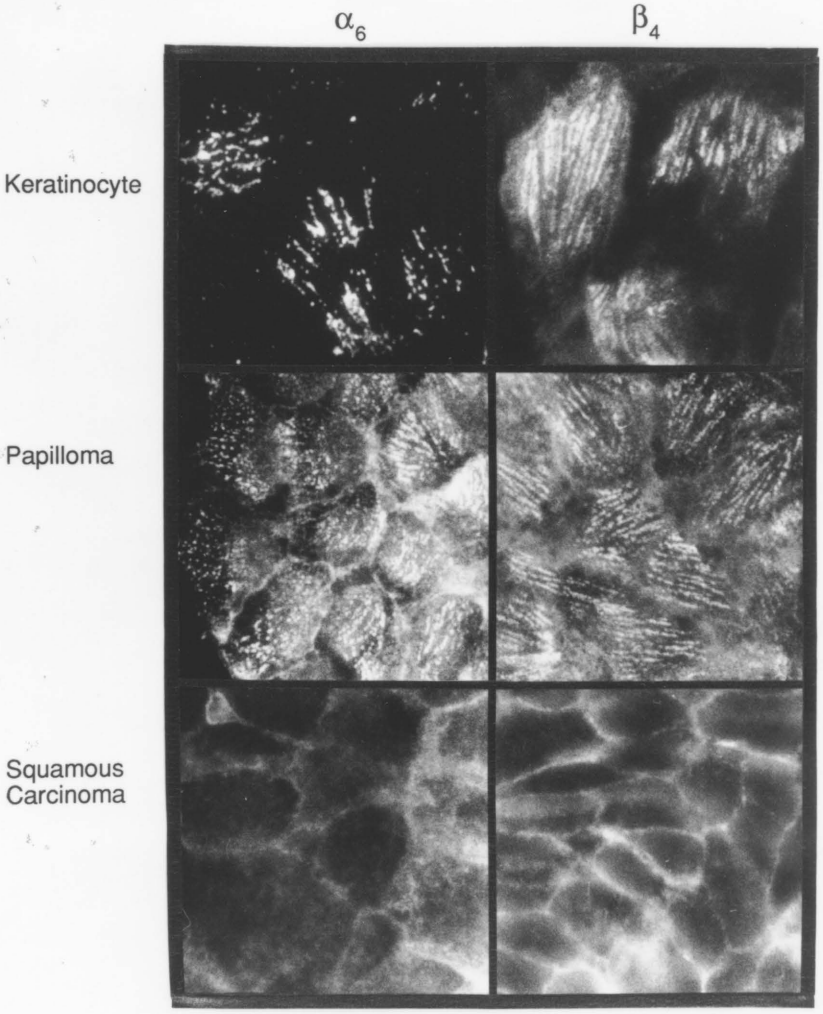
Ability of α6β4 to Polarize on the Mouse Cell Lines

Keratinocytes proliferate in culture when maintained in low calcium medium. When the calcium level is increased the keratinocytes are stimulated to initiate differentiation which is characterized by expression of several keratinocyte marker proteins (Boyce and Ham, 1983). The papilloma and carcinoma cells were isolated on the basis of their altered response to high calcium levels (i.e., proliferate in high calcium without initiating expression of marker proteins).

Indirect immunofluorescence staining of the three cell lines with antibodies to α6 and β4 under conditions of low calcium and high calcium revealed an interesting redistribution of the α6 and β4 subunits. Figure 17 shows staining with antibodies to α6 and β4 on all three cell lines under conditions of high calcium. The keratinocyte and the papilloma cell lines demonstrated a streaking pattern that indicated polarization and clustering of this integrin. This pattern has been shown by others on human keratinocytes and colon carcinoma cells under similar conditions

Figure 17. Indirect immunofluorescence of α6β4.

All three cell lines were grown on coverslips and stained with the antibodies to α6 (GoH3) and β4 (346.11A) and goat anti-rat FITC secondary antibody. The left column was stained with α6, the right column with β4. Cells lines are indicated on the left. Cells were photographed at 63 x magnification.


α_6
β_4
Keratinocyte
Papilloma
Squamous Carcinoma

(Ryynanen et al.,1991; Simon-Assmann et al., 1994). The carcinoma cells, under the same high calcium conditions, could not be induced to polarize the α6β4 integrin in the same fashion as the keratinocyte and papilloma cells. The carcinoma cells displayed diffuse and peripheral staining of the α6 and β4 subunits. Under conditions of low calcium, the pattern of α6 and β4 staining on all three cell lines was diffuse and generally non-polarized (data not shown) similar to the staining on the carcinoma cells in Figure 17.

Regulation of α6β4 Expression

The data show that the amount of α6β4 on the surface of the carcinoma cells is decreased and does not redistribute or cluster. These data suggest that there is a mechanism that results in this decreased expression.

The mRNA levels of the α6, β4 and β1 integrin subunits were analyzed by Northern blot analysis. Total RNA was isolated from keratinocyte (291) and carcinoma cells (291.03). A total of 25 μg of RNA from each cell line was hybridized with probes recognizing the α6, β4 and β1 subunits. The message size of both the α6 and β4 mRNA is approximately 6 Kb. The β1 message in mouse is 3.8 Kb with a 3.1 Kb band also detected. The 3.1 Kb message is interpreted as the same message with different polyadenylation signals. The blots were stripped and

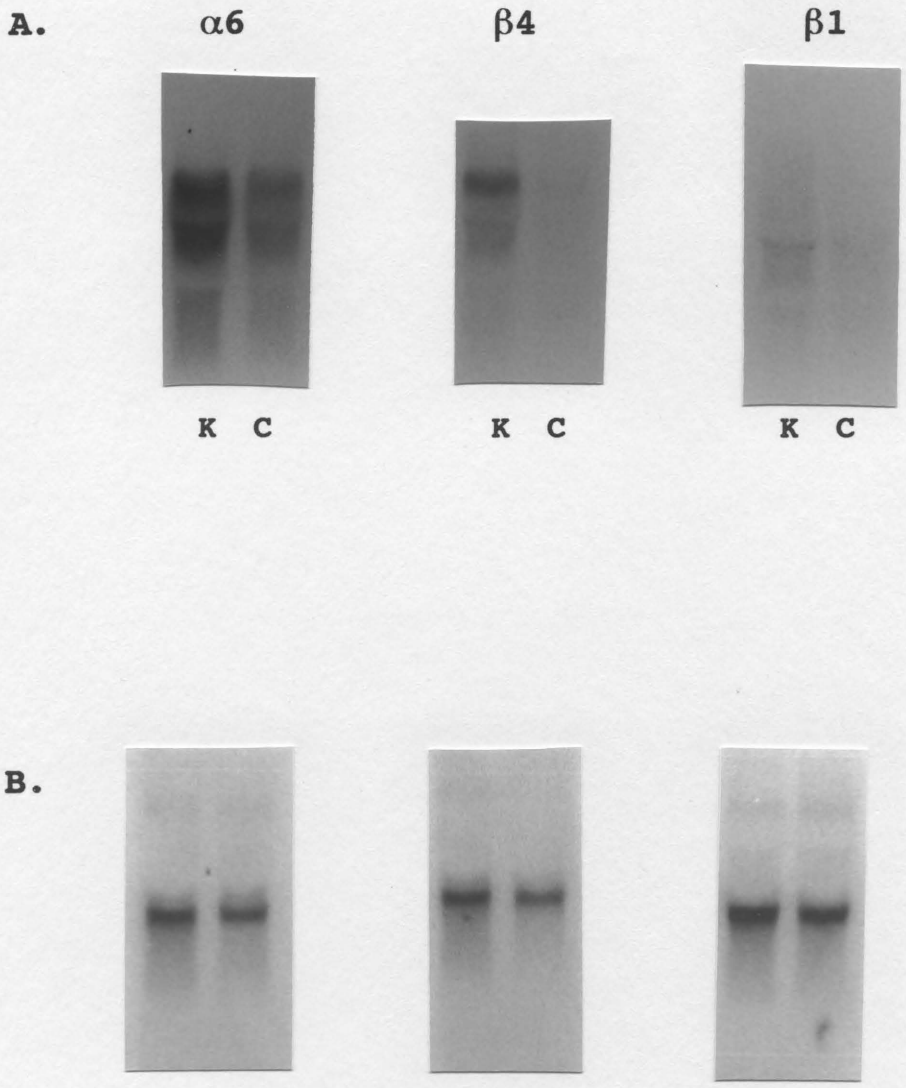


Figure 18. Northern analysis of mRNA expression of α6, β4, and β1 messages.
A. Total RNA was isolated from keratinocytes (K) and carcinoma cells (C), 25 μg were hybridized with probes recognizing the α6 (6Kb), β4 (6Kb), and β1 (3.8 and 3.1Kb) messages
B. Corresponding Northern blots were stripped and reprobed with the probe recognizing GAPD to assess RNA sample loading.

reprobed with the GAPD probe to assess RNA loading. All blots were exposed to a phosphorimaging screen and densitometry was used to analyse and compare message quantities. Figure 18 shows the results of this analysis. Both the keratinocyte and carcinoma cells express a significant amount of the α6 message (Figure 18A). Densitometric analysis on the relative amount of GAPD present in each lane indicates that the carcinoma lane contains approximately 29% less total RNA (Figure 18B). Correcting for this discrepancy shows the carcinoma cells to be expressing 87% of the α6 mRNA compared to the keratinocyte cells.

Analysis of the β4 message level in both cell lines reveals that the carcinoma is transcribing significantly less mRNA from this gene than the keratinocytes (Figure 18A). Densitometric analysis on these two aliquots of RNA shows the carcinoma lane to contain 29% less RNA (Figure 18B). Correcting for the loading difference shows the carcinoma cells to be expressing only 25% of the keratinocyte message.

The analysis of the β1 message in these two cell lines (Figure 18A) shows a weak signal for both the keratinocyte and carcinoma cell line. The probe used to detect β1 messages in the mouse was derived from the chicken β1 sequence. It is possible that the wash out conditions for this probe were not quite correct although significantly reduced stringency conditions were used. Analysis of band

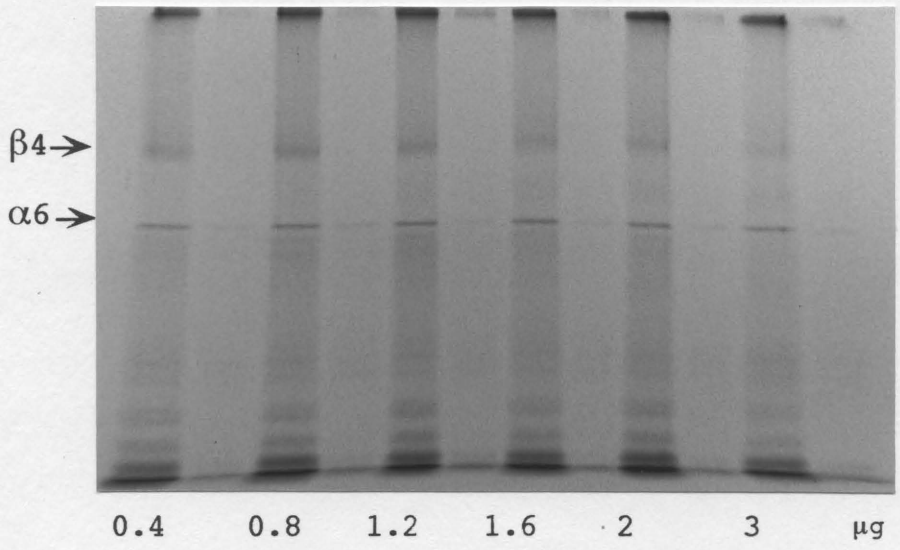


Figure 19. Determination of saturating α6 monoclonal antibody conditions on the keratinocyte cells.

Keratinocyte cells were labeled with ^{35}S-methionine and immunoprecipitated with the α6 monoclonal antibody. All reactions contained 100 μg of protein from whole cell lysate with increasing concentrations of α6 antibody to precipitate the α6 and β4 proteins. Proteins were resolved on a 7% polyacrylamide gel under non-reducing conditions.

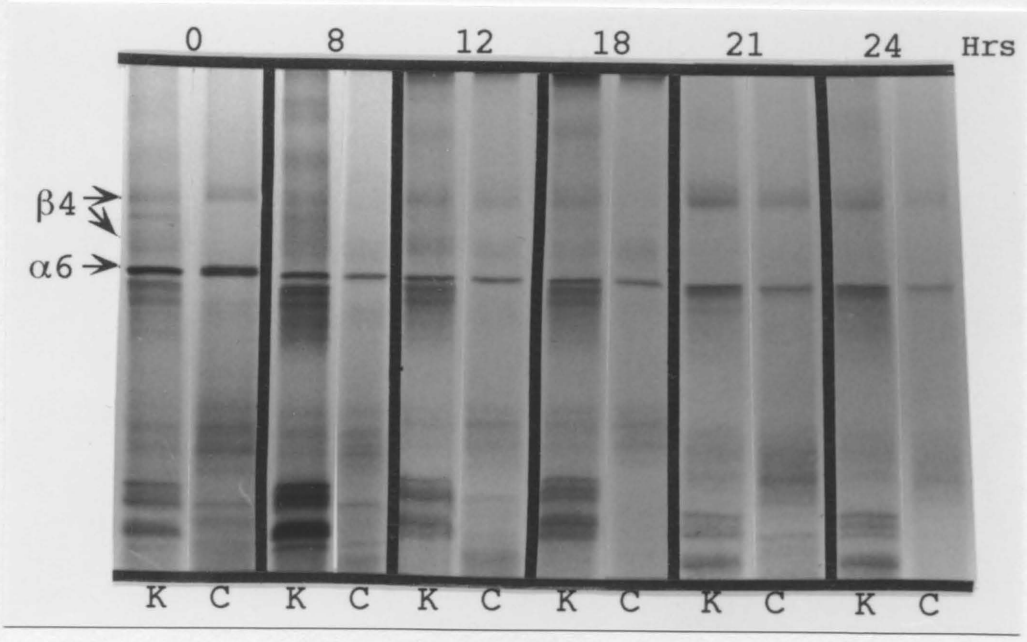
intensity on the GAPD signal in these two lanes detected 17% less RNA from the carcinoma cells present in the lane. Correcting for this difference, the carcinoma cells are expressing 71% of the β1 message level of the keratinocyte.

The integrity and amount of the the α6 and β4 integrin subunit proteins were analyzed in the keratinocyte and carcinoma cells. Pulse chase experiments were used to assess the relative protein turnover rates of the α6 and β4 in the two cell lines. Both cell lines were pulsed with 50 μCi/ml ^{35}S-methionine in methionine-deficient media for 3 hours (the determined optimal pulse time for the α6 and β4 molecules on these cells). After the pulse of ^{35}S-methionine, the media was replaced with fresh media containing 10 mM cold methionine for 0, 8, 12, 18, 21, and 24 hours.

At the completion of the chase time, cells were lysed, protein determinations were completed and equal amounts of protein from each cell line was added to immunoprecipitation reactions with the monoclonal antibody to α6. The ratio of whole cell lysate to antibody for saturating antibody conditions was determined to be 200 μg/3.2 μg α6 monoclonal antibody. Figure 19 shows the determination of the saturating antibody conditions. The keratinocyte cells were used to establish these conditions because previous data shows these cells to be expressing more α6 and β4 than the carcinoma cells. Whole cell lysate protein was kept constant at 100 μg and the amount of antibody recognizing α6 was

Figure 20. Biosynthesis of the α6 and β4 integrin subunits

Cells were labeled for 3 hours with ^{35}S-methionine, fresh media containing 10 mM methionine was replaced for the times indicated and immunoprecipitated. Proteins were resolved on a 7% polyacrylamide gel under non-reducing conditions. The hours of chase are indicated at the top of each pair of lanes representing resolved proteins from the keratinocyte (K) and carcinoma (C) cells. The α6 band as indicated has a molecular weight of approximately 140kD. The β4 band migrates at 187 to 200kD.


0
8
12
18
21
24
Hrs
β4
α6
K C K C K C K C K C K C

increased. The α6 and β4 bands are indicated to the left of the figure and the μg of antibody used is indicated below the appropriate lane. Intensity (integrated volume) of the α6 and β4 bands was estimated on a Phosphorimager (Phosphorimager, ImageQuant software, Molecular dynamics) (data not shown). This analysis indicated that the intensity of the α6 and β4 bands increased at 1.2 μg of antibody/100 μg and reached a plateau with increase in amount of antibody. For subsequent experiments 3.2 μg of the α6 antibody was used to precipitate protein from 200 μg of whole cell lysate.

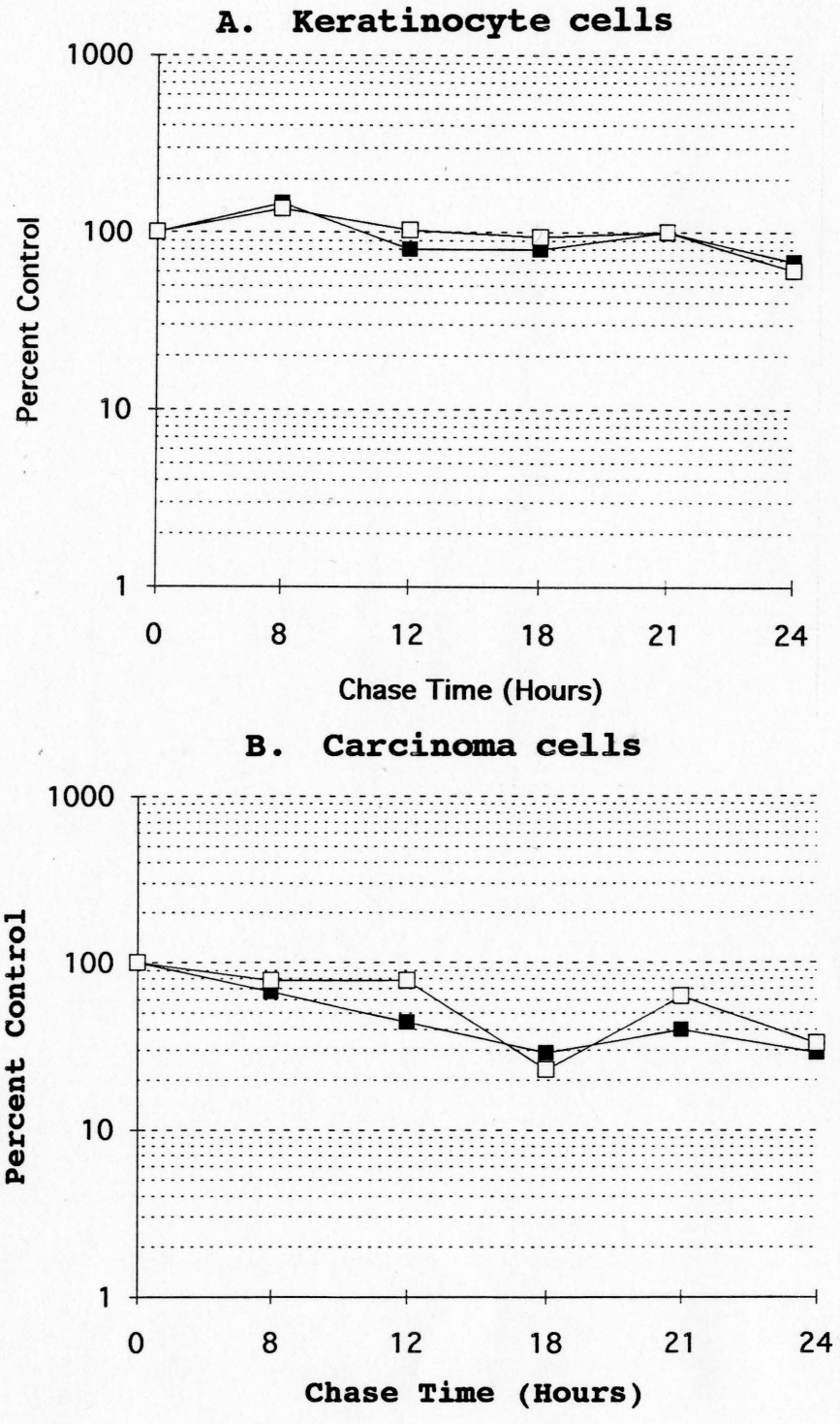
These saturating antibody conditions were used to immunoprecipitate the α6 and β4 from keratinocyte and carcinoma cells which were pulse-chased to assess the rate of turnover of these two proteins. For each chase time duplicate samples were analyzed for each cell line. Figure 20 shows the results of this analysis. The data shown represents one immunoprecipitation reaction from duplicate samples electrophoresed on 7% polyacrylamide gels under non-reducing conditions. Under these conditions the α6 band migrates as a tight single band at approximately 140 kD. The β4 band migrates between 187 to 200 kD and is a broad band under non-reducing conditions.

The chase times are indicated above each pair of lanes. With a chase time of 0 hours after a 3 hour pulse similar

Figure 21. Turnover of the α6 and β4 subunits.

A. Profile of the α6 and β4 subunit turnover rate in the keratinocyte cells during the 24 hour time course . The intensity of the immunoprecipitated bands was measured by densitometry (Phosphorimager and ImageQuant software) and graphed as percent control (0 chase time point) for comparison over all time points. The α6 (——▫——) and β4 (——□——) are graphed together for comparison.

B. Profile of the α6 and β4 subunit in the carcinoma cells over the 24 hour time course . The intensity of the immunoprecipitated bands was measured by densitometry (Phosphorimager and ImageQuant software) and graphed as percent control (0 chase time point) for comparison over all time points. The α6 (——▫——) and β4 (——□—)are graphed together for comparison.


A. Keratinocyte cells
1000
100
10
1
Percent Control
0
8
12
18
21
24
Chase Time (Hours)
B. Carcinoma cells
1000
100
10
1
Percent Control
0
8
12
18
21
24
Chase Time (Hours)

amounts of the α6 and β4 proteins are immunoprecipited from both the keratinocyte and carcinoma cells. This analysis indicates the carcinoma cells are initially producing the same amount of α6 and β4 protein as the keratinocytes but ultimately, the majority of α6 and β4 is not detected on the surface. This is an interesting finding given the Northern analysis (Figure 18) reveals significantly reduced levels of β4 mRNA.

After an 8 hour chase, the carcinoma cells already show a significant decrease in both the α6 and β4 subunits compared to the keratinocyte cells (Figure 20). Also note the presence of other bands in the keratinocyte lanes. The α6β4 integrin is a known component of the hemidesmosome which comprises to date many other potential precipitating proteins some of which could be co-precipitating with the α6 and β4 proteins. It is also possible that the prominent α6 band and those bands present migrating just ahead represent the maturation from precursor to processed mature α6 subunit (Kennel et al., 1989).

After 12 and 18 hours chase the α6 and β4 from the carcinoma cells continue to decrease at an increased rate compared to the α6 and β4 from the keratinocytes. The largest α6 band immunoprecipitated from the keratinocyte cells appears to decrease (compare keratinocyte α6 band across all time points) and the lower molecular weight bands

are increasing in intensity. The carcinoma cells do not show the presence of these co-precipitating bands or potential processing of the α6 subunit at any of the time points compared to the keratinocytes.

At 21 hours of chase the α6 and β4 in the keratinocytes still show significant intensity. Again note the apparent processing of the potential precursor α6 to mature forms. These bands in the keratinocyte cells appear to have decreased distance between them and are now migrating more as a single band. As result this whole area was included in the area analyzed on the phosphorimager. The carcinoma α6 and β4 subunits at 21 hours are still decreasing and show no potential α6 maturation products. At 24 hours both keratinocyte and carcinoma are decreased. The keratinocyte is still retaining significant amount of the α6 and β4 subunits compared to the carcinoma cells. The carcinoma cells show an altered turnover rate of the α6 and β4 subunits compared to the keratinocytes.

The α6 and β4 band intensity from each chase time was analyzed on Phosphorimager-scanned images. The intensity (integrated volume in arbitrary units of density) of each band was estimated. These data were graphed as percent control with the 0 chase time as the control (100%). The analysis of the α6 band by densitometry included the lower bands present below the 143 Kd α6 band. This whole area was

included because by 18 hours chase these bands were migrating very close together and it was difficult to isolate only the 143Kd band for analysis. The same area was analyzed in the carcinoma lanes for comparison. The densitometric values were averaged from both duplicates and graphed as percent control. Both the keratinocyte and carcinoma cells showed the same level of ^{35}S-methionine incorporation into total protein as determined by TCA precipitation (data not shown).

The profile of the α6 and β4 turnover rate in the keratinocyte cells is shown in Figure 21A. The turnover rates of the α6 and β4 in the keratinocyte cells are very similar. The integrated volume averaged values for the 0 chase time for the α6 and β4 from the keratinocyte are 9.7 x 10^4 and 2.8 x 10^4, respectively and designated as 100% for each respective subunit.

Both subunits show an increase at 8 hours. This is possibly due to processing and epitope recognition. The α6 and β4 are at 147% and 137% of control respectively. The levels of these two subunits remains steady until 24 hours at which the decrease is 68% and 61% of control for the α6 and β4 subunits respectively. During this 24 hour time course the radioactivity incorporated into the α6 and β4 proteins was not reduced to 50%. Therefore the estimated biological half-life is greater than 24 hours for these two proteins in the keratinocyte cells.

The phosphorimager data were also used to estimate the biological half life of the α6 and β4 proteins. Using linear regression, a line was estimated for the α6 and β4 turnover profiles. The measure of association for the α6 was 0.5149 and β4 was 0.5931. The estimated half life of the α6 subunit from these data was 42 hours. The estimated half life of the β4 molecule from these data was 45 hours.

The turnover rate of the α6 and β4 of the carcinoma cells were also analyzed in the same manner. These results are presented in Figure 21B. The rates of turnover of the α6 and β4 in these cell is also similar to one another. The averaged densitometric value at chase time 0 hour was designated 100%. The density values for α6 and β4 are 9.8×10^4 and 2.7×10^4, respectively. Both the α6 and β4 immunoprecipitated from the carcinoma cells shows an immediate decrease in relative quantity.

At 8 hours chase, the α6 and β4 are at 67% and 78% of control, respectively. The level of these two proteins show a steady decrease in amount over the 24 hour time course. At 24 hours the α6 and β4 have decreased to 30% and 33% of control respectively.

The biological half life of the α6 and β4 proteins on the carcinoma cells was estimated from the densitometric data. Using linear regression, a line was estimated independently for the α6 and β4 turnover profiles. The

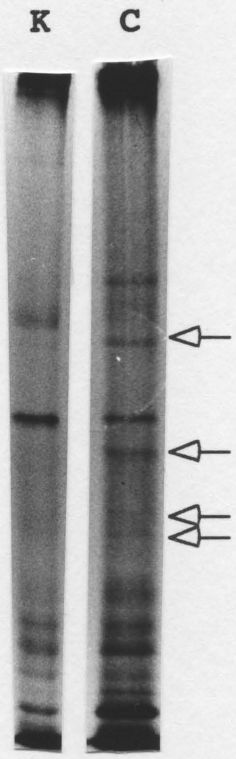


Figure 22. Demonstration of proteolytic fragments of the β4 subunit in the carcinoma cells.

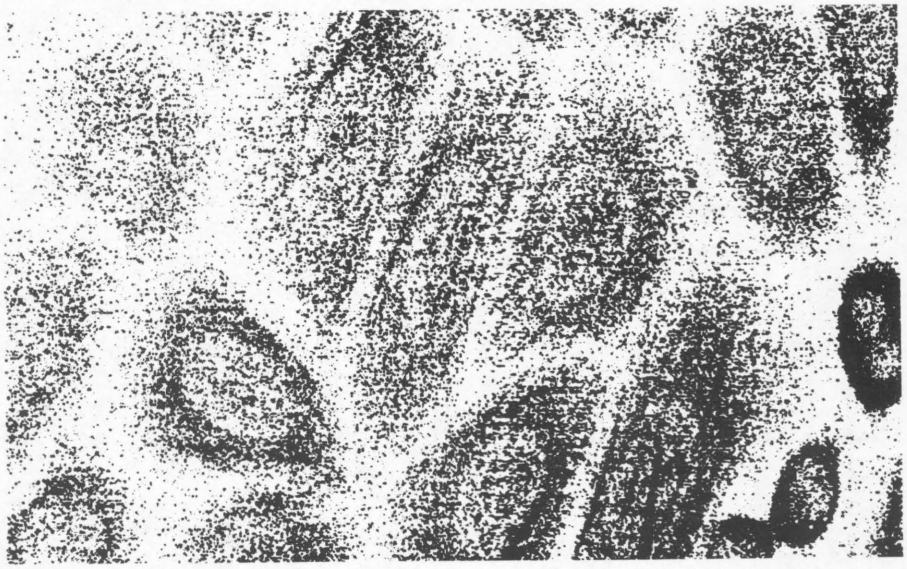
The keratinocyte and carcinoma cells were labeled for 3 hours in 35S-methionine containing media. The α6 subunit was immunoprecipitated from the whole cell lysates in an 18 hour reaction. The proteins were resolved on a 7% polyacrylamide non-reducing gel. The arrowheads to the right of the figure identify distinct potential fragments of the β4 subunit produced by proteolytic digestion. The molecular weights denoted by the arrows are from top to bottom: 187 kD, 100 kD, 75 kD and 70 kD.

Figure 23. Indirect immunofluorescence of calpain and calpastatin.

A. Keratinocyte cells were grown on coverslips and stained with the monoclonal antibody for calpain (top panel) and the polyclonal recognizing calpastatin (bottom panel). The antibodies were tagged with the appropriate secondary and analyzed by confocal microscopy.
B. Carcinoma cells stained with the antibodies to calpain (top panel) and calpastatin (bottom panel) as above.

A. Keratinocyte Cells

Calpain

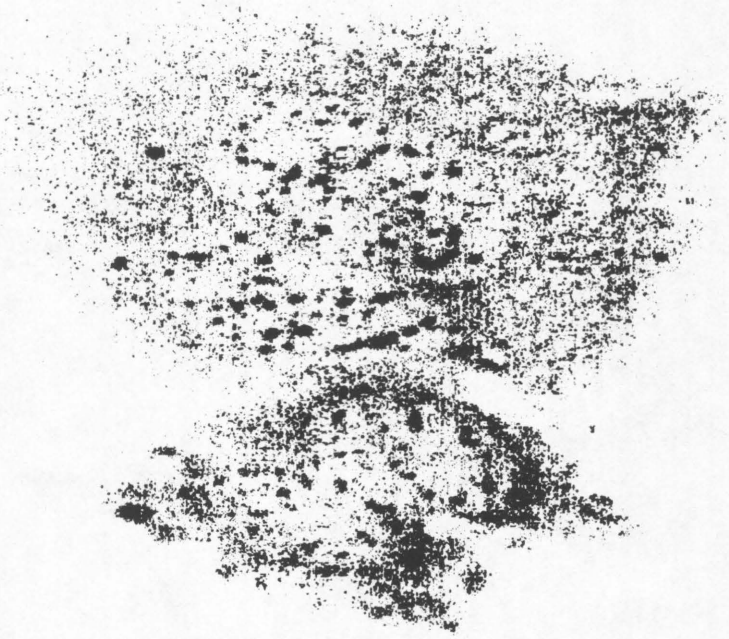


Calpastatin

B. Carcinoma Cells

Calpain

Calpastatin



measure of association for the α6 was 0.9346 and β4 was 0.835. The estimated biological half lives of the α6 and β4 subunits on the carcinoma were 14 and 18 hours, respectively.

The β4 integrin subunit of the carcinoma cells is more susceptible to degradation in vitro. The keratinocyte and carcinoma cells were immunoprecipitated for 18 hours using the monoclonal antibody to α6. Figure 22 shows presence of potential degradation fragments of the β4 subunit. The arrows to the right of the figure identify bands that correspond to previously published fragment sizes of the β4 subunit generated by proteolytic digestion by calpain (see discussion). The 200 kD β4 has been show to be digested into 187, 100, 75, and 70 kD fragments. The keratinocyte cells show fragments of these sizes in the immunoprecipitation but not to the extent of degradation present in the carcinoma lysates.

Since cleavage by calpain has been shown to generate the degradation fragments of the β4 subunit. The distribution of calpain and the calpain inhibitor, calpastatin, was analyzed in the keratinocyte and carcinoma cells. Indirect immunofluorescence using a monoclonal antibody to calpain and a polyclonal antibody to calpastatin was used to localize these two proteins in the cells. The cells were analyzed by confocal microscopy which revealed interesting results. Figure 23 shows the distribution of the calpain and

calpastatin are distinctly different in the carcinoma cells compared to the keratinocytes. Calpain and calpastatin on the keratinocytes shown a similar pattern of cellular distribution (Figure 23A). This distribution is cytoplasmic as well as nuclear. The carcinoma cells on the other hand show opposite distribution. Figure 23B shows the calpain staining almost exclusively in the cytoplasm where as the inhibitor, calpastatin, showed staining only in the nucleus.

Discussion

The normal distribution of α6β4 on basal epithelial cells is polarized to the basolateral plasma membrane surface juxtaposed to the basement membrane (Kajiji et al., 1989; Carter et al., 1990; De Luca et al., 1990; Jones et al., 1991; Hormia et al., 1992; Kennel et al., 1992; Hogervorst et al., 1993a). On carcinomas in vivo this polarized expression is lost and cells show diffuse distribution of this integrin (Tennenbaum et al., 1992).

Analysis of the surface expression of α6β4 on all three mouse cell lines in this model of carcinogenesis showed the α6β4 expression to be decreased on the cell surface of the carcinoma cells. FACS analysis of fluorescence labeled α6 and β4 proteins revealed that the α6β4 integrin on the surface of the carcinoma cells was decreased by approximately four fold (Figure 10 and Table 7). This decrease in surface expression of the α6β4 on the carcinoma cells was verified by

immunoprecipitation and detection of surface biotinylated α6 and β4 from all three cell lines (Figures 11 and 12). Analysis of other integrins, α2β1, α3β1, and α5β1 expressed by epidermal cells, by FACS, immunoprecipitation and indirect immunofluorescence microscopy show the expression of these molecules to be similar on keratinocyte, papilloma and carcinoma cells (Figures 10, 13, Table 7 and data not shown). Therefore, the decrease in the α6β4 integrin observed on the carcinoma cells is not a general decrease in integrin surface expression overall but specific for this integrin.

The decrease in the surface α6β4 on the carcinoma cells was not due to an altered α6 integrin subunit composition. Since the integrin α6β1 has been implicated in migration and invasion (Mercurio and Shaw, 1988; Dedhar and Saulnier, 1990), there was the possibility that the carcinoma cells were expressing α6β1 as well as α6β4 on the cell surface. Western blot analysis of α6 immunoprecipitated proteins with an antibody to the β1 subunit showed no co-precipitation of the β1 subunit with α6 (Figure 14). This analysis ruled out an α6 subunit compositional alteration on the carcinoma cells that had the potential to contribute to the invasive phenotype. The carcinoma cells, as well as, the other two cell lines do express α5β1, α2β1 and α3β1. The β1 subunit is available to complex with the other α subunits on the carcinoma cells. These data show that the carcinoma cells

retain the preferential association of α6 with β4 but show decreased surface expression of this specific integrin heterodimer.

There are two reported splice variants of the α6 messenger RNA termed α6A and B (Hogervorst et al., 1991; Tamura et al., 1991). Normal keratinocytes have been shown to express only the α6A cytoplasmic domain splice variant (Hogervorst et al., 1991). Various carcinomas were shown to express both the α6A and B cytoplasmic splice variants (Hogervorst et al., 1991). The α6B was demonstrated to be a target of PMA-induced phosphorylation (Hogervorst et al., 1993b). A switch in the variant expressed or co-expression of the α6B in the carcinoma cells could lead to an altered adhesion phenotype.

The α6 message(s) expressed by both the keratinocyte and carcinoma cells was analyzed by RT-PCR using mouse sequence specific primers for the α6 messages (Hierck et al., 1993). These results show that only the α6A mRNA could be detected in both the keratinocyte and carcinoma cells (Figure 15). The α6 variant expressed by the carcinoma cells has not been altered. This demonstrates that the carcinoma cells do not exhibit an altered α6 integrin composition compared to the keratinocytes that could contribute to the invasive potential of these cells.

Northern analysis of α6, β4 and β1 message levels show the carcinoma cells to be transcribing significantly less of the β4 message. Densitometry indicates the level of expression in the carcinoma cells is 25% of the keratinocyte expression. The β4 subunit expression is specifically regulated and the expression is limited to cells of epithelial origin and nerve sheath cells. This decrease in transcription is possibly related to loss of differentiation capacity of the carcinoma cells.

The carcinoma cells synthesized α6 protein to the same level as the keratinocyte cells detected by immunoprecipitation of metabolically labeled proteins and the a6 complexed initially with similar amounts of β4 subunit (Figure 20, 0 chase time). These data suggest that atlest two different types of mechanisms are responsible for the reduced levels of the α6β4 integrin heterodimer on the surface of the carcinoma cells. Altered posttranscriptional mechanisms could result in significant levels of protein with reduced message levels. Potential mechanisms include increased mRNA stability and/or message recoding producing multiple protein molecules from a single message. Another possibility is that there is a cytoplasmic pool of β4 maintained by cells similar to the β1 cytoplasmic pool observed in other cell types. This could possibly account

for similar levels of protein under conditions of reduced mRNA levels.

Pulse/chase experiments were used to estimate the rate of turnover of the α6 and β4 proteins of the keratinocyte and carcinoma cells. These results reveal over a time course of 24 hours that the rate of protein turnover and overall levels in the carcinoma cells is significantly altered compared to the keratinocyte cells (Figures 20 and 21). The biological half-life of the keratinocyte proteins from these data is greater that the 24 hour and could not be estimated within the 24 hour time course. The biological half-life of both the α6 and β4 proteins in the carcinoma cells was estimated within this time course. The α6 had a half-life of 16 hours and the β4 18 hours, respectively. The biological half-life of the α6 and β4 proteins is reduced in the carcinoma cells (Figure 21). The altered turnover rates suggest that posttranslational mechanisms are affecting the α6β4 integrin expressed on the surface of the carcinoma cells. These data show that initially similar levels of α6 and β4 subunit proteins are present in the carcinoma but the majority of the α6 and β4 subunits are not being fully processed to expression on the cell surface as heterodimers.

This information suggests that a posttranslational mechanism is also responsible for the reduced surface expression of α6β4 on the surface of the carcinoma cells. It

is possible that the α6 and β4 proteins in the carcinoma cells are degraded before heterodimer formation or that the process of heterodimer formation is inefficient in these cells and degradation occurs as a consequence.

One potential hypothesis is that the large β4 subunit is degraded which ultimately affects heterodimer formation or it is degraded after complexing with the α6 subunit. In support of this hypothesis, the large cytoplasmic domain of β4 has been shown to contain sites that are calpain sensitive (Giancotti et al., 1992). Calpain, a calcium dependent protease, is thought to be involved in protein processing (Goll et al., 1992; Saido et al., 1994). Calpain has been shown to cleave specific sites in proteins resulting in large fragments. It is possible that the β4 of the carcinoma cells is susceptible to degradation by this enzyme or other enzymes in processing either prior to or after being incorporated into a heterodimer.

The susceptibility of the α6 and β4 subunits to degradation from the keratinocyte (291) and carcinoma (291.03) cells was analyzed in vitro. Immunoprecipitation of α6 and β4 proteins were subjected to an 18 hour incubation in each cell line's respective whole cell lysate. Under identical conditions (i.e., lysis buffer) the immunoprecipitated proteins from the carcinoma cells are significantly more susceptible to degradation than the proteins from the keratinocyte cells (Figure 22).

The resultant fragments from the carcinoma cells were identical in size to fragments of the β4 subunit digested by the enzyme calpain described by Giancotti et al. (1992). These data do not definitively demonstrate that calpain digestion has resulted in the fragments observed from the carcinoma immunoprecipitated α6 and β4. Clearly these results need to be extended, analyzing other potential degradation pathways. Nonetheless, there is supporting evidence to suggest that this enzyme is potentially involved in the digestion of the β4 in the carcinoma cells. This enzyme as stated above is primarily involved in protein processing and its activity is very tightly regulated by inhibitor calpastatin (Goll et al., 1992; Saido et al., 1994). The substrates for calpain include cytoskeletal proteins, membrane proteins, enzymes (especially those involved in signal transduction), cytokines and transcription factors. The largest class of substrates are the membrane associated proteins. This class includes the integrin family of proteins.

In the normal tissues examined, the relative amount of calpastatin activity is comparable to the total calpain activity (Saido et al., 1994). This balance of inhibitor to enzyme has been shown to be altered in several pathological conditions. Some of these conditions are ischemia, aging, inflammation and arthritis (reviewed in Saido et al., 1994).

An alteration in the calpastatin/calpain balance has precedence in the condition of cancer. Using antibodies to μ and m calpain and calpastatin, the distribution was compared in the keratinocytes (291) cells and carcinoma (291.03) cells (Figure 23). These results show the keratinocyte cells have a similar cellular distribution of both the inhibitor and enzyme. The carcinoma cells, on the other hand, demonstrated reduced staining, but the distribution of the inhibitor and enzyme was distinctly altered. The calpain staining was primarily located in the cytoplasm and the inhibitor staining was found in the nucleus. This imbalance of inhibitor/enzyme suggests a state in which the calpain is not as tightly regulated in these cells and could potentially participate in the degradation of the β4 subunit.

Degradation by calpain is thought to involve the amino acid sequence proline, glutamic acid, aspartic acid, serine and threonine (PEST) (Wang et al., 1989). These PEST sequences are located some distance from the actual cleavage site and are hypothesized to bind calcium which is possibly involved in the interaction of the enzyme and substrate. The amino acid sequence of the β4 subunit was analyzed for the presence of PEST sequences (Giancotti et al., 1992). Two were found within the cytoplasmic domain evidence that calpain could be involved.

The hypothesis formulated from these data is that the β4 in the carcinoma cells is not necessarily targeted for

degradation but is degraded as a consequence of initial cleavage by calpain. This cleavage is possibly a result of alteration or loss of protein/protein interactions or alterations in the processing involving the carcinoma β4 cytoplasmic domain which could expose the calpain cleavage sequences.

The β4 subunit cytoplasmic domain has been shown to be involved in protein protein interaction (Spinardi et al., 1993). Within the β4 cytoplasmic domain is a 303 amino acid region that contains the first in a series of fibronectin type III repeats. These sequences are known to be involved in protein protein interactions. Spinardi et al. (1993) demonstrated that this 303 amino acid segment was enough information to target localization of this segment at the basal lateral surface of the transfected cells into hemidesmosome-like structure in vitro.

Conditions that result in polarized integrin α6β4 on the surface of the keratinocyte and benign cells fails to polarize the α6β4 on the carcinoma cells. Data presented in this dissertation show that the carcinoma cells have lost the ability to polarize the α6β4 integrin on the cell surface (Figure 17). The carcinoma cells have been shown not to express at least one of the components of the hemidesmosome, the keratin pair 5/14, involved in the hemidesmosome structure (Schneider et al., 1993 and data not shown).

This loss of protein-protein interaction involving the carcinoma β4 subunit and hemidesmosome components could leave it vulnerable to the initial cleavage of calpain by exposing the PEST sequences. This would allow access of the enzyme and ultimately degradation. The data presented suggest that the carcinoma cells have lost expression of other cytoplasmic components involved in the targeting of the α6β4 to the hemidesmosomes. The keratinocyte cells not having lost the ability to polarize the α6β4 retain the important protein protein interactions which protect the calpain cleavage sites.

The 303 amino acid region that contains the localizing information also contains the location of the alternative splice variants of the β4 (discussed in the introduction). The 53 or 70 amino acid insert is located in the region between the second and third fibronectin type III repeats. It is possible that the alternate cytoplasmic domains have an affect on the interaction with the cytoplasmic proteins. Spinardi et al. (1993) suggest this as a mechanism for modulation of α6β4 activities in different cell types. It is possible that the carcinoma β4 subunit sequence is altered compared to the keratinocyte cells. This suggests future studies to analyze and compare the cytoplasmic domains between the keratinocyte and carcinoma cells.

The alterations in the associations with the β4 cytoplasmic domain in the carcinoma cells did not appear to

affect the ability of α6β4 heterodimers that were eventually expressed on the surface to adhere to the proposed ligand laminin (Lotz et al., 1990). Our results show that the α6β4 on the carcinoma cells can function as a laminin receptor in adherence. The ability of the α6β4 to adhere to the proposed ligand, laminin, was tested in experimental substrate attachment assays. These results show that the α6β4 expressed on the carcinoma cells was able to attach to laminin and this attachment could be significantly inhibited by pre-incubation with the α6 antibody, GoH3, which functionally blocks the integrin receptor (Figure 16). The decrease in the ability of the carcinoma to attach to laminin was not proportional to the detected receptor decrease on the surface. This phenomena has been shown by others (Sugiyama et al., 1993; Simon-Assmann et al., 1994). In the study by Sugiyama et al. (1993) several squamous carcinoma cell lines, showed reduced surface integrin expression, but did not generally show reduced adhesion to the ligands of those receptors. The carcinoma cells also express α3β1 (data not shown) which has been shown to be a laminin receptor (Gehlsen et al., 1988).

Summary

In this study, I have documented the α6β4 integrin expression on a mouse model of skin carcinogenesis. The

altered α6β4 integrin expression on the carcinoma cells in this mouse model system represents a pattern of expression observed on human carcinomas in situ. The α6β4 integrin is reduced on the cell surface and has lost the normal polarized distribution on the basolateral surface. These alterations in this model system take place presumably after or concurrent with the malignant conversion which results in the conversion of a benign tumor to a malignant carcinoma.

The alteration of the α6β4 expression on the mouse carcinoma cells does not involve an integrin subunit composition that is associated with a migratory phenotype.

There is a functional difference in the ability of the α6β4 on the carcinoma cells to polarize on the basolateral surface compared to the keratinocyte cells. The carcinoma cells have lost this function associated with hemidesmosome formation.

Analysis of the different levels of potential regulation of the α6β4 reveal that the carcinoma cells have altered transcription and processing of the β4 subunit and the α6β4 heterodimer.

Prior to this study the processing of the α6β4 integrin had not been studied in normal cells nor during tumor progression. This is the first study focusing on identifying potential mechanisms involved in the alteration of integrin expression on the cell surface of tumor cells. The data

presented in this dissertation have begun to analyze the mechanisms involved in regulation of integrin expression on the surface of cells. The complexity of the $\alpha 6\beta 4$ heterodimer makes the analysis of this integrin a difficult task. This cell culture model, representative of the three stages of mouse skin carcinogenesis, provide a relevant experimental model system to study the biochemical changes taking place that result in altered integrin expression during tumor progression.

The mechanisms and consequences of cellular adhesion are very complicated. These studies contribute information on alterations in integrin expression and function during the process of skin carcinogenesis. The alterations in integrin expression on cancer cells can affect all levels of integrin expression, function and signaling.

V. CONCLUSIONS

The research presented in this dissertation documents the altered expression of integrins on cancer cells, how these changes contribute to altered adhesion and addresses potential mechanisms involved in altering specific integrin expression during the process of tumor progression.

Altered integrin expression on tumor cells has been shown to alter the adhesive capacity of these cells. The alterations are more complicated than simply affecting cell adhesion since integrins convey information about the extracellular matrix via signal transduction. These signals affect gene expression and control cell growth. Alteration in these signals alters the functions governed by these signals.

The analysis of integrin expression on human prostate tumor cells documented changes in integrin profiles on this cell type that were important participants in the tumorigenic potential of each cell line. Integrin expression can be reduced or increased on the surface of the cancer cells compared to normal cells. Integrins can also be present on cancer cells in normal levels but they may no longer function in the same capacity, i.e., altered affinity for specific ligand, altered heterodimer formation and altered ligand expression. The human prostate cell line, LNCaP, expressed

non-functional integrins on the cell surface. These cells exhibited altered adhesion in experimental assays and were non-tumorigenic when injected into SCID mice.

The other three human prostate cell lines analyzed were tumorigenic. The DU145 cells were the least aggressive of the three. Analysis of the integrin repertoire on this cell line revealed expression of integrin heterodimers that are involved in focal contacts ($\alpha5\beta1$) and hemidesmosomes ($\alpha6\beta4$). The focal contacts and hemidesmosomes are stabilizing structures. The hemidesmosomes are involved in the maintenance of the integrity of the epidermis and other epithelial linings. The presence of these integrins on the surface of the DU145 cells possibly decreased the invasiveness of this cell line. This reduced its capacity for migration compared to the other tumorigenic human prostate cell lines which were expressing $\alpha6\beta1$ an integrin known to participate in migration on laminin.

A decrease in $\alpha6\beta4$ is an alteration that takes place after or concurrent with malignant conversion of benign tumor cells. Analysis of the expression of the $\alpha6\beta4$ integrin on the three cell lines of a mouse model of tumor progression identifies altered expression on the carcinoma cells. The decrease and altered regulation, i.e., loss of polarization of the $\alpha6\beta4$ potentially results in decreased or no hemidesmosome formation on the carcinoma cells. It is this potential loss of hemidesmosomes, the stable anchoring

contacts, which contributes to the invasive potential of these cells.

The cancer cells, no longer having the cellular components to assemble the hemidesmosome (or having reduced hemidesmosome formation), are not restricted or governed by the epidermal extracellular matrix and the correct signals important to the normal maintenance of the basal cell layer. As a result, the carcinoma cells can invade the basement membrane and ultimately metastasize to distant sites.

Understanding cell adhesion and the ultimate consequences as the result of signals transduced from the extracellular matrix will further the understanding of the alterations on cancer cells that contribute to the invasive phenotype of malignant disease.

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