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Integrin $\alpha 6$ expression in human prostate carcinoma cells is associated with a migratory and invasive phenotype *in vitro* and *in vivo*

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Abstract

Cell adhesion and migration are important features in tumor invasion, being mediated in part by integrins (extracellular matrix receptors). Integrins are significantly decreased in human prostate cancer. An exception is $\alpha 6$ integrin (laminin receptor) which persists during prostate tumor progression. We have selected high (DU-H) and low (DU-L) expressors of $\alpha 6$ integrin from a human prostate tumor cell line, DU145, to assess experimentally the importance of $\alpha 6$ integrin in tumor invasion. DU-H cells exhibited a four-fold increased expression of $\alpha 6$ integrin on the surface compared to DU-L cells. Both cell types contained similar amounts of $\alpha 3$ and $\alpha 5$ integrin. The DU-H cells contained $\alpha 6$ subunits complexed with both the $\beta 1$ and $\beta 4$ subunits whereas DU-L cells contained $\alpha 6$ complexed only with $\beta 4$. DU-H cells were three times more mobile on laminin as compared to DU-L, but adhered similarly on laminin. Adhesion and migration were inhibited with anti- $\alpha 6$ antibody. Each subline was injected intraperitoneally into SCID mice to test its invasive potential. Results showed greater invasion of DU-H compared to DU-L cells, with increased expression of $\alpha 6$ integrin on the tumor at the areas of invasion. These data suggest that $\alpha 6$ integrin expression is advantageous for prostate tumor cell invasion.

Keywords

basement membrane; cell adhesion; extracellular matrix; laminin; prostate cancer

Introduction

Cell adhesion and migration are important features that influence the ability of malignant cells to invade and metastasize to distant tissues [1]. Cell adhesion to the extracellular matrix (ECM) is mediated largely through the integrins, a group of heterodimeric transmembrane proteins [2]. These heterodimers are formed by a combination of an α and a β subunit. There are currently 14 α and 8 β subunits [2,3]. Ligand specificity is mainly defined by this subunit combination though it may depend also on the cell type and environmental factors [2].

Cell binding to laminin (LM), a major component of the basal lamina, has been implicated in the invasive and metastatic processes [1,4]. Laminin binding is mediated through several

integrins, primarily $\alpha 6\beta 1$ and $\alpha 6\beta 4$ [5–8], but also $\alpha 7\beta 1$, $\alpha 3\beta 1$, $\alpha 2\beta 1$, and $\alpha 1\beta 1$ have been implicated in certain cell types [9–12].

The $\alpha 6$ integrin subunit comprises 1050 amino acids synthesized as a single chain, which is cleaved into a heavy and a light chain [13,14]. As a result of alternative splicing, $\alpha 6$ -A and $\alpha 6$ -B variants are formed, differing from each other only in their short cytoplasmic tail [13, 15].

Integrin $\alpha 6\beta 1$ is expressed in a wide variety of cells, including epithelial and endothelial cells, where it usually shows a polarized distribution to the basolateral surface [16–18]. This integrin is linked indirectly to the actin cytoskeleton. In contrast, integrin $\alpha 6\beta 4$ is localized to hemidesmosomes in several epithelia [19,20], where it is linked to the cytokeratin cytoskeletal network [21,22].

The role of $\alpha 6$ integrins in tumor invasion and metastasis has been assessed from different standpoints [23–25]. For example, human melanoma cells injected into nude mice were diminished in their metastatic potential by anti- $\alpha 6$ antibodies, probably by decreasing the rate of cellular retention in the lungs [26]. Neoplastic transformation of fibroblasts [27] or chemically transformed cells [28] resulted in an increased expression of $\alpha 6$ integrin. Histopathologically, the picture is varied. Tumors such as human breast and renal carcinomas are reported to show a decrease of several types of integrins including $\alpha 6$ and $\beta 4$ and to show an unpolarized pattern [29–32]. On the other hand, breast carcinomas that preserve $\alpha 6$ integrin expression are associated with reduced survival [33], suggesting an important role in tumor progression. Other human tumors show an increase in $\alpha 6$ but usually in a non-polar distribution: pancreatic carcinoma, head and neck squamous cell carcinomas, seminomas, and melanomas [34–38]. In prostate cancer, we have found previously that several of the integrins are down-regulated [39]. However, the $\alpha 6$ integrin persists during tumor progression and in some cases increases its expression in an altered unpolarized pattern on the tumor cell surfaces [39,40]. The $\beta 4$ integrin subunit is undetectable in primary prostate carcinoma, suggesting that the $\alpha 6\beta 1$ heterodimer is predominant in progressing tumors. Integrin $\alpha 6$ may be increased in lymph-node metastatic lesions, suggesting that it could play a role in this process [40].

In this study we have analysed the role of $\alpha 6$ in the invasive phenotype of human prostate carcinoma cells (DU145) by selecting cell subpopulations with high and low surface expression of the $\alpha 6$ integrin. We have used these subpopulations to compare the effect of the $\alpha 6$ integrin on several invasive features: cell adhesion and migration *in vitro*, breaching of a basal lamina and migrating through the stroma *in vivo*.

Materials and methods

Cell culture

DU145 human prostate carcinoma [41] and JAR choriocarcinoma [42] cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). DU145 cells were originated from a prostate carcinoma brain metastasis. Cells were maintained in IDMEM medium with 10% fetal bovine serum (all from Gibco) at 37°C in a humidified atmosphere of 95% air, 5% CO₂.

Antibodies

The GoH3 anti- $\alpha 6$ integrin antibody was obtained from Accurate Chemical & Scientific (Westbury, NY, USA). Anti- $\beta 1$ (P4C10), anti- $\alpha 5$ (PID6), anti- $\beta 4$ (3E1) and anti- $\alpha 3$ (PIB5) integrin antibodies were obtained from Gibco BRL (Gaithersburg, MD, USA).

Fluorescence-activated cell sorting (FACS)

DU145 cells in suspension were incubated with GoH3 anti- $\alpha 6$ antibody (1:100) for 30 min at 4°C, washed several times with medium, then incubated with FITC-conjugated anti-rat antibody for 30 min at 4°C, and washed several times. Cells were selected with a FACS star^{plus} cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA) for their higher or lower $\alpha 6$ expression by collecting the cells from the 2% 'tails' as determined by FACS. The sorted cells were expanded *in vitro* and resorted seven times following the same procedure.

Biotinylation and immunoprecipitation of cell surface integrins

Using modifications from previous protocols [43,44], cells were grown to early confluency in 100 mm tissue culture dishes and washed with HEPES buffer (20 mM HEPES, 130 mM NaCl, 5 mM KCl, 0.8 mM MgCl₂, 1.0 mM CaCl₂, pH 7.45). The cells were then incubated with 2 ml of HEPES buffer supplemented with Sulfo-succinimidyl hexanoate conjugated biotin (500 μ g/ml) (NHS-LC-Biotin, Pierce, Rockford, IL, USA) to label cell surface proteins for 30 min at 4°C. Cells were washed three times and lysed in lysis buffer (PBS containing 1% NP-40, 10 mM EDTA and antiproteases: PMSF, 2 mM; leupeptin and aprotinin, 1 μ g/ml). The extract was briefly sonicated and centrifuged to remove insoluble material, then precleared with protein G-Sepharose (Pharmacia, Uppsala, Sweden). Anti-integrin antibodies (5 μ l/ml) were added and incubated overnight at 4°C. The integrin/antibody complexes were immunoprecipitated with protein G-Sepharose, washed with lysis buffer three times and eluted by boiling in sample buffer for 5 min. Proteins were separated in 7.5% SDS-PAGE, electrotransferred to nitrocellulose, incubated with peroxidase-conjugated streptavidin and visualized by chemiluminescence (ECL Western Blotting Detection System, Amersham, Arlington Heights, IL, USA).

Microscopy

For indirect immunofluorescence, cells were grown on glass coverslips, washed with PBS, incubated with anti- $\alpha 6$ antibody for 30 min at 4°C, washed and then incubated with FITC-conjugated anti-rat antibody for 30 min at 4°C. After the incubation, the cells were fixed with 4% formaldehyde and the coverslips mounted onto the slides with Elvanol. The preparations were analysed by confocal fluorescence microscopy using a Zeiss LSM10 instrument using a scan time of 16 s.

Fresh frozen samples of mouse diaphragm were obtained at necropsy and snap frozen at -140°C in isopentane, supercooled in liquid nitrogen and sectioned on a cryostat and immunoreacted with antibody specific for $\alpha 6$ integrin. Additional specimens fixed in 10% formalin and paraffin-embedded were sectioned, rehydrated and stained with hematoxylin and eosin.

Adhesion assays

ELISA Multiwell plates (96) were coated with 10 μ g or 5 μ g of EHS laminin or fibronectin (Collaborative Research, Bedford, MA, USA), respectively. Cells (3×10^4) were overlaid on coated wells and incubated with or without antibody for 1 h at 37°C. Unattached cells were removed by washing three times with HEPES buffer. Cells were fixed with 4% formaldehyde and dried. Attached cells were stained with 0.1% Crystal Violet solution, washed, and the retained dye eluted with Sorensen solution (9 mg of trisodium citrate in 305 ml of H₂O, 195 ml of 0.1 N HCl and 500 ml of ethanol). Absorbance at 540 nm was determined with an ELISA reader.

Cell migration assays

Non-tissue culture Petri dishes were coated with laminin (Collaborative Research) at different concentrations (0–20 μ g/cm²) for 2h and then blocked with 1% denatured albumin for 2 h. The

cells were added and allowed to attach overnight. The Petri dish was sealed and transferred to a heated stage microscope (Nikon) adapted for video microscopy using an Hitachi solid state camera interfaced with a Macintosh Quadra 800 computer. Random cell migration was determined by measuring displacement of cell centroids as a function of time. Seventy to 100 cells were analysed for each condition.

Tumor growth in SCID mice

Male SCID mice, 5–6 weeks old, were inoculated intraperitoneally with $5\text{--}20 \times 10^6$ cells resuspended in PBS. Necropsy was done 31 days later. The diaphragm was dissected out and weighed. The specimen was divided with portions fixed in 10% neutral buffered formalin and processed according to standard procedures for hematoxylin and eosin staining. An additional portion was snap frozen, sectioned in a cryostat and processed for indirect immunofluorescence. Several transverse sections were examined for breaching of mesothelial basement membrane and the depth of diaphragm invasion.

Results

Selection of stable subpopulations of DU145 cells expressing high and low amounts of $\alpha 6$ integrin

Cell subpopulations of DU145 human prostate carcinoma cells containing high and low amounts of $\alpha 6$ integrin were selected by repeated sorting in a FACS system (Figure 1). After seven sortings, the high $\alpha 6$ integrin expressors (DU-H) showed an approximate four-fold increase in surface expression of $\alpha 6$ as compared to the low $\alpha 6$ integrin expressors (DU-L), as estimated by the difference in the fluorescence mean channel of the two populations (representing a log scale, there is a doubling in the fluorescence intensity for each 75 channel difference approximately) (Figure 1C). Two months later, the cell population maintained the selected phenotype indicating that a stable difference in integrin expression was obtained (Figure 1D). The distribution of $\alpha 6$ integrin in DU-H cells was focal, intracellular and intercellular while that in DU-L cells was mostly negative (Figure 2).

The DU-H cells express both $\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrins and the DU-L cells only express $\alpha 6\beta 4$

The integrin composition of the high and low $\alpha 6$ integrin expressors was determined by immunoprecipitation of surface biotinylated integrins and analysis by gel electrophoresis. The JAR choriocarcinoma cell line was used as a positive control (Figure 3A, lane 1; Figure 3B, lane 1). The DU-H cells showed higher amounts of $\alpha 6$ than the DU-L cells, consistent with FACS analysis (Figure 3A, lanes 2 and 3). The composition of the heterodimer was different between the cell lines. The high expressors contained both $\beta 1$ and $\beta 4$ integrin chains coupled to $\alpha 6$; in contrast, the DU-L cells contained only the $\beta 4$ chain coupled to the $\alpha 6$ chain. A five-fold increase in the amount of immunoprecipitated $\alpha 6$ integrin from the DU-L cells still contained no $\beta 1$ (data not shown). Lower molecular weight bands (approximately 80 kDa) were co-immunoprecipitated with the anti- $\alpha 6$ antibody in the three cell lines. These bands have not yet been identified but may probably represent proteolytic products of $\alpha 6$ integrin [45, 46]. Similar cleavage products of integrins have also been found in $\alpha 4$ integrins [47]. Immunoprecipitation of the $\beta 4$ integrin was consistent with the $\alpha 6$ immunoprecipitations.

Both cell sublines express similar amounts of $\alpha 3$, $\alpha 5$ and $\beta 1$

Other integrins were immunoprecipitated to determine if the selection was specific for the $\alpha 6$ integrin. Figure 3C indicates that the $\alpha 3$ (lanes 1 and 2), $\alpha 5$ (lanes 3 and 4) and $\beta 1$ (lanes 5 and 6) integrin content was similar in both cell sublines, confirming that our selection for differential expression was restricted to the $\alpha 6$ integrin.

Both cell sublines attach to laminin and fibronectin to a similar extent but by different mechanisms

The consequences of $\alpha 6$ expression in the selected sublines was assessed by three different functional assays. These included determining changes in cell adhesion to laminin or other ECM molecules, changes in cell migration and invasion of the cells through a SCID mouse diaphragm *in vivo*.

The extent of cellular adhesion to laminin was similar for both cell populations (Figure 4). However, the adhesion of the DU-H cells depended completely on the $\alpha 6$ integrin since the anti- $\alpha 6$ antibody inhibited adhesion to laminin. On the other hand, the DU-L cells were only partially inhibited with the anti- $\alpha 6$ antibody. We investigated which other integrins participated in the DU-L cell adhesion to laminin and found that a $\beta 1$ integrin (probably $\alpha 3\beta 1$) was participating in laminin adhesion. A partial inhibition of DU-L cells to laminin occurred by incubation of the cells with either $\beta 1$ or $\alpha 3$ antibodies. The anti- $\alpha 6$ antibody combined with either anti- $\beta 1$ or anti- $\alpha 3$ inhibited adhesion to laminin completely, confirming the sufficiency of the interaction. The DU-H cells were inhibited by an anti- $\beta 1$ antibody but not by an anti- $\alpha 3$ antibody. The adhesion to fibronectin was similar in both cell sublines.

The high $\alpha 6$ integrin expressors migrate faster than $\alpha 6$ low expressors on laminin

The importance of $\alpha 6$ integrin in cell movement on laminin was assessed by comparing the random migration speeds of the high and low $\alpha 6$ integrin sublines. Videomicroscopic analysis of cells moving on different amounts of coated laminin (Figure 5A) showed that the low $\alpha 6$ expressors moved 2.5-fold slower than the high expressors. The involvement of $\alpha 6$ was confirmed by inhibiting cell movement with anti- $\alpha 6$ antibody (Figure 5B). The DU-H cells were largely inhibited, most of the cells rounding up soon after the antibody addition. The remaining movement seen here was probably due to cell rolling more than amoeboid movement, as observed in time-lapse video recording. The $\alpha 6$ antibody alone or the $\alpha 3$ antibody alone did not have any effect on the low expressors, which preserved both their speed and spreading.

Increased invasive ability of DU-H tumors into the mouse diaphragm

The $\alpha 6$ sublines were assessed in their invasive ability in SCID mice by determining their capacity to breach the diaphragm basement membrane and invade its stroma. This model is practical as it allows evaluation of the frequency of basement membrane breaching and of the depth of invasion by the tumor (Figure 6A and B). Initial experiments indicated that the low $\alpha 6$ expressors showed an increased proliferation rate in comparison to the high $\alpha 6$ expressors after injection into the mice (data not shown). Accordingly, the number of cells injected into the mice was adjusted to achieve a similar tumor burden and size on the diaphragm. At a similar tumor burden, the high expressors resulted in an increased invasion (Table 1, Figure 6A). The increased invasion was characterized by multiple basement membrane breaching points and penetration into the underlying muscle in 80% of the cases. The cells were observed between the muscle fibers, suggesting a migration path along the surface of the muscle. The low expressors showed superficial invasion in one case (20%) and were negative in the other mice (Table 1, Figure 6B).

Microscopic analysis of the frozen sections of the mouse diaphragm by indirect immunofluorescence indicated that the tumor arising from the DU-H cells was positive for $\alpha 6$ (Figure 6C and E). Of particular interest was the increased expression of the integrin on the surface of the cells at the invasion sites as compared to the expression level within the tumor mass. Analysis of the digital images by quantitation of the pixel density indicated a three-fold elevation in expression (data not shown). The DU-L cells showed also moderate positivity for $\alpha 6$ (Figure 6D and F) but no evidence of a preferential pattern of expression along the boundaries of the diaphragm was noted.

Discussion

We have investigated the influence that $\alpha 6$ integrin has on the invasive potential of prostate tumor cells. We chose as a model a human prostatic carcinoma cell line, DU145, which was previously characterized for its adhesive properties and integrin profile [45]. We selected cell populations from the original cell line which contained high and low surface expression of the $\alpha 6$ integrin. The high $\alpha 6$ integrin expressors (DU-H) showed a four-fold increase in $\alpha 6$ as compared to the low $\alpha 6$ expressors (DU-L). The selected phenotype continues to be stable 2 months after the selection.

A biochemical analysis of the integrin composition showed that DU-H cells expressed both $\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrins in contrast to DU-L which only expressed lower levels of $\alpha 6\beta 4$. Yet both cell sublines produced similar amounts of $\alpha 3$, $\alpha 5$ and $\beta 1$ integrins, showing that the integrin difference was restricted to $\alpha 6$, mainly $\alpha 6\beta 1$.

The ability to adhere to laminin was similar in both cell sublines, suggesting that either lower amounts of $\alpha 6$ integrins can accomplish attachment, or that other laminin receptors may compensate in attachment for the low $\alpha 6$ expression. The latter seems likely as suggested by antibody inhibition assays. DU-L cells were only partially inhibited with anti- $\alpha 6$ integrin antibody, suggesting the participation of other adhesion molecules. Further investigation showed that $\alpha 3$ integrin was also involved in the adhesion of DU-L to laminin as it was possible to partially inhibit adhesion with anti- $\alpha 3$ antibody. Taking into consideration that the combination of anti- $\alpha 6$ and anti- $\alpha 3$ antibodies (or anti- $\alpha 6$ and anti- $\beta 1$) could inhibit most of the adhesion in the low expressors, one may conclude that adhesion to laminin is predominantly mediated through $\alpha 6\beta 4$ and $\alpha 3\beta 1$ integrins in DU-L cells. Integrin cooperation in laminin binding has been found in other cell lines as well [48–50]. It is not clear why $\alpha 3$ does not have a laminin binding role in the high expressors, but may reflect a competition for intracellular factors that mediate the response to laminin binding or the connection of the integrin to the cytoskeleton. For example, a recent study shows that chimeric integrins (with functional cytoplasmic tails but non-functional chimeric extracellular domains) might inhibit endogenous integrin receptor function, presumably by interacting with cytoplasmic components critical for endogenous integrin function [51]. In an analogous fashion, $\alpha 6$ integrin might compete with $\alpha 3$ integrin for similar cytoplasmic factors.

In contrast to adhesion, migration on laminin was affected by $\alpha 6$ integrin expression in these sublines. DU-H cells showed an increased random migration speed on laminin in relation to DU-L cells. Since the cell sublines differ primarily in the presence of $\alpha 6\beta 1$, these data suggest that $\alpha 6\beta 1$ increases cell migration on laminin. The inhibition of migration with $\alpha 6$ antibodies in the high expressors indicates a strong dependence on the $\alpha 6$ integrins. Although there is only half the inhibition of the apparent migration speed after antibody treatment, the high expressors assumed a round morphology. The remaining measured cellular displacement may be attributed to an integrin-independent cell rolling more than the extension of pseudo or filopodia as documented by time-lapse video-microscopy. The low expressors, though showing a slow migration speed, were not altered by anti- $\alpha 6$ antibodies either in their migration or in their spreading, suggesting that $\alpha 6\beta 4$ may not participate in DU-L cell migration and that other cell adhesion molecules could be taking part in these actions. In this case, anti- $\alpha 3$ did not change the DU-L migration speed.

Previous studies have suggested the participation of $\alpha 6\beta 1$ in cell migration which is consistent with our results. For example, invasion of reconstituted basement membranes *in vitro* by fibrosarcoma or osteosarcoma cells could be inhibited by anti- $\alpha 6$ antibody [28,52]. It is interesting that the DU145 variants of $\alpha 6$ integrin studied here show only the $\alpha 6a$ integrin subtype (data not shown), which has been shown to be associated with increased migration in

comparison to $\alpha 6\beta 1$ [53]. In human foreskin keratinocytes it has been suggested that $\alpha 6\beta 4$ might restrict migration rather than promote it [49]. On the other hand, in an *in vitro* wound-healing explant model [54], migration of corneal epithelial cells was not inhibited by anti- $\beta 4$ antibodies (only hemidesmosome formation was inhibited) but anti- $\alpha 6$ antibody was completely inhibitory for migration. Some studies suggest the opposite, $\alpha 6\beta 4$ participating in migration [55].

Several factors may account for the apparent contradiction. For example, there may be a requirement for a threshold level of $\alpha 6\beta 4$ to accomplish migration. The poor participation of $\alpha 6\beta 4$ seen in DU-L cell migration might be a reflection of a small number of these receptors and could indicate that these cells require a critical amount of $\alpha 6\beta 4$ receptors to accomplish migration rather than to adhere to laminin. Alternatively, the molecular features of $\alpha 6\beta 4$ itself or its interaction with the cytoskeleton may be a factor. Others have reported splice variants of both $\alpha 6$ and $\beta 4$ which also may be a factor. The interactions of these variant receptors with the cytoskeleton and its influence on cytoskeleton-dependent migration are currently unknown.

The fact that $\alpha 6\beta 1$ is linked to the actin cytoskeleton makes it more likely to participate in migration than $\alpha 6\beta 4$ which is linked to the intermediate filament network. The $\alpha 6\beta 4$ and its associated structural proteins localize into stable anchoring structures known as hemidesmosomes. The loss of hemidesmosomes and the $\alpha 6\beta 4$ integrin in prostate carcinomas has been observed by us previously [39]. The loss of the stable anchor could facilitate movement of the cell and according to our migration data, the persistence of the alternative receptor, $\alpha 6\beta 1$, would provide a definite advantage in facilitating movement on a laminin substrate.

Our data suggest that $\alpha 6$ may have an important influence on the invasion of cells through the basement membranes and stromal tissues. We find that DU-H expressors show a considerable difference in their invasive ability of the SCID mouse diaphragm basement membrane and stroma in comparison to DU-L cells that show a low invasive ability. This is consistent with other studies done with osteosarcoma cells that showed a correlation of the *in vitro* invasion of reconstituted basement membranes with $\alpha 6$ expression [28]. In the same line of studies, mouse tail vein injections of melanoma cells have been inhibited in their metastatic potential with anti- $\alpha 6$ antibodies [26], or show a correlation between $\alpha 6$ expression and metastatic ability [56]. Our results add to these previous results that the expression of $\alpha 6$ integrins is correlated with invasion *in vivo* of basement membranes and tissue stroma.

The mechanism by which $\alpha 6$ facilitates invasion could be adhesion-related (as migration and contact with basement membranes), and/or signal transduction related, as an increase in other elements useful to traverse basement membranes. Some integrins have been shown to increase the signaling for protease production [57], but this has not been shown for the $\alpha 6$ integrins. Our data suggest that the migratory and adhesive abilities of the DU-H cells might explain in part their greater invasive ability in comparison to DU-L, but other mechanisms are likely to co-exist. It is interesting that the DU-L cells had a marked increase in their proliferation rate *in vivo*, which could be related to some regulatory signaling by the $\alpha 6$ integrins. This has been observed with other integrins. An inverse correlation between proliferation and integrin expression has been observed for $\alpha 5\beta 1$ integrin where the low expressors showed a higher proliferation and tumorigenicity than the high expressors; transfection of $\alpha 5$ may result in decreased tumorigenicity [58,59]. On the other hand the presence of certain integrins such as $\alpha v\beta 3$ may show positive effects on cell proliferation [60]. In either case it underscores the participation of integrins in the proliferation process and our results suggest the participation of $\alpha 6$ in this process.

It is interesting that $\alpha 6$ integrin is increased chiefly at the sites of invasion. This suggests that expression of $\alpha 6$ integrins may provide an advantage mainly at the front of invasion where it might interact and be regulated by the newly available ECM. Diaphragm skeletal muscle is rich in laminin-related molecules which could facilitate migration of $\alpha 6\beta 1$ -containing cells. This would be relevant in prostatic carcinoma as prostate tissue is laminin rich. Laminin in the prostate gland is present surrounding smooth muscle cells, vascular structures and perineurial nerve sheaths which could provide laminin paths for the tumor cells. Indeed nerves have been shown to participate in the extracapsular dissemination of prostate carcinoma [61].

Our results indicating that $\alpha 6$ integrin expression correlates with prostate cell migration on laminin and invasion through stroma, together with previous histopathological studies that confirm the persistence of this integrin in prostate tumor progression [26,39], suggest an important role of $\alpha 6$ integrins in this disease.

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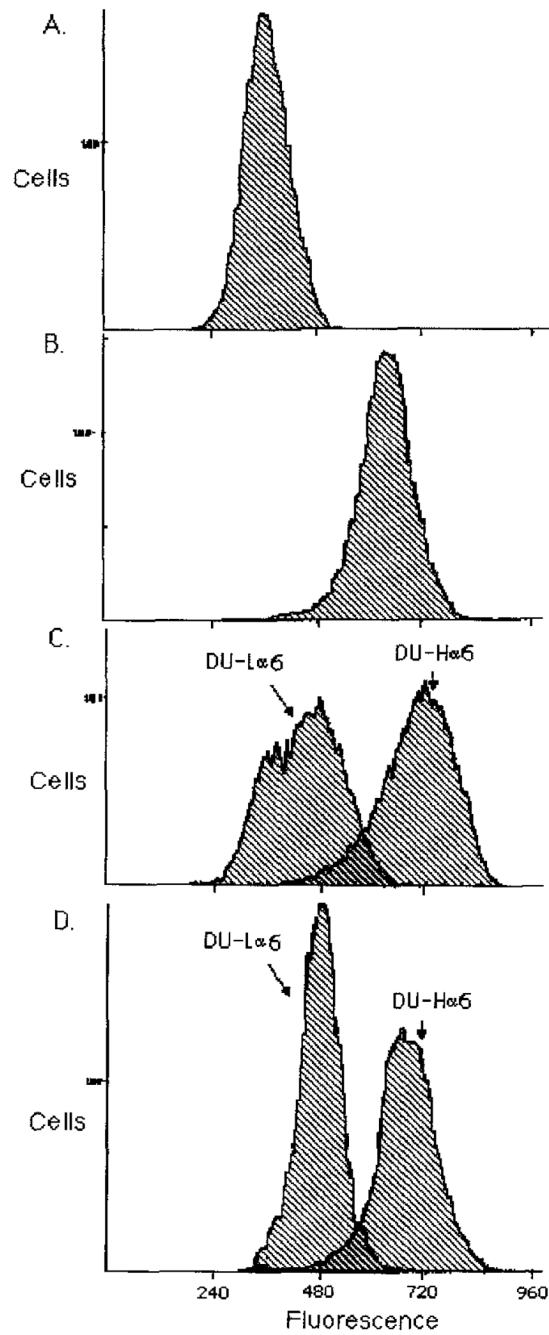


Figure 1.

Selection of high (DU-H) and low (DU-L) expressers of $\alpha 6$ integrin and FACS analysis. Cells were selected for their high or low $\alpha 6$ expression by collecting the cells from the 2% 'tails' as determined by fluorescence activated cell sorting (FACS). Sorted cells were expanded *in vitro* and resorted seven times following the same procedure. Fluorescence is represented in channels. Approximately 75 channels constitute a doubling of fluorescence intensity. (A) Fluorescent cell distribution of negative control cells (reacted with secondary antibody only). (B) Fluorescent cell distribution of pre-sorted DU145 cells. (C) Fluorescent cell distribution of high and low $\alpha 6$ integrin expressers after seven rounds of sorting. (D) Two months after the last sort.

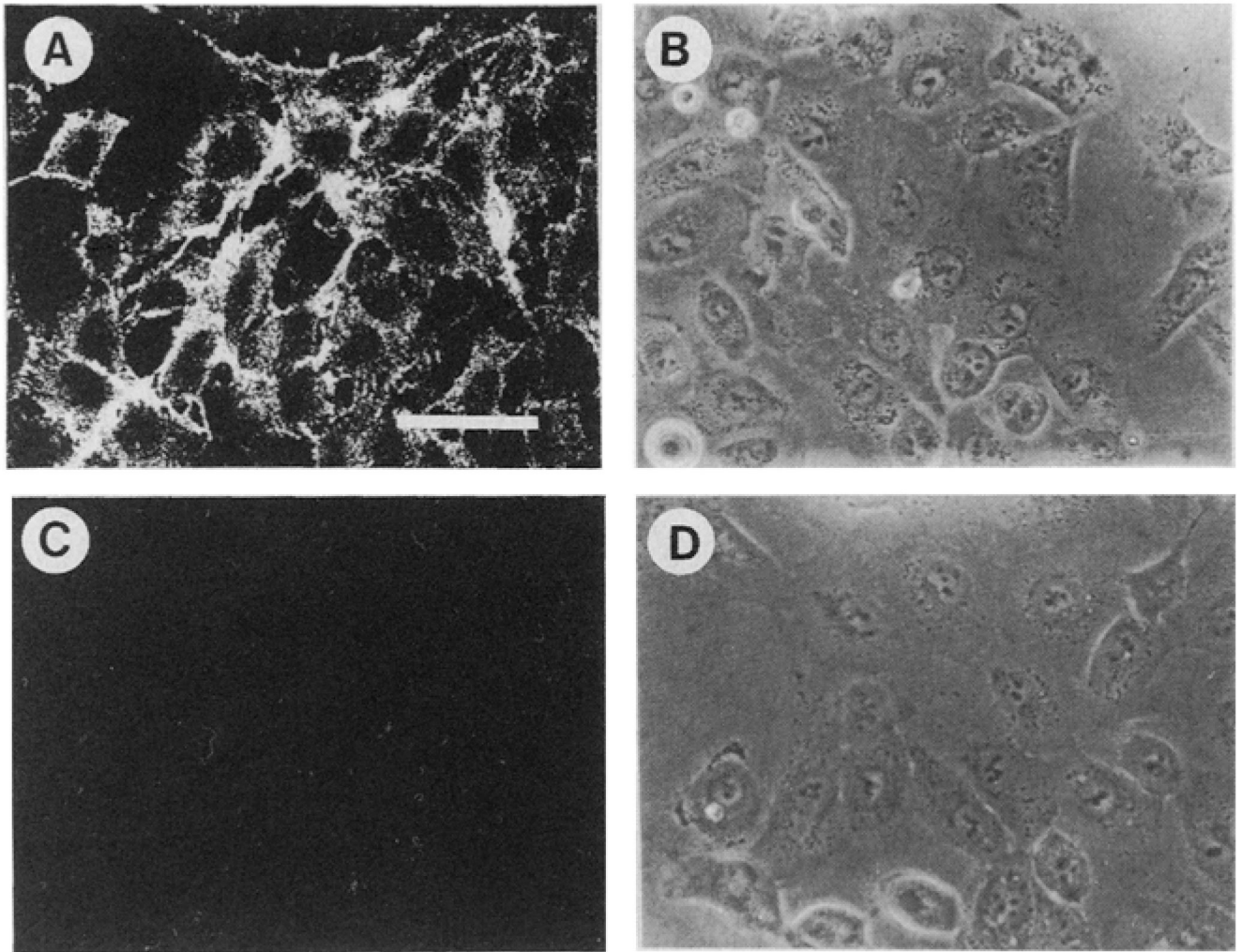


Figure 2. Distribution of $\alpha 6$ integrin by indirect immunofluorescence microscopy. Cells were grown on coverslips, washed with PBS, incubated with anti- $\alpha 6$ antibody (GoH3) for 30 min at 4°C, washed and then incubated with FITC-conjugated anti-rat antibody for 30 min at 4°C, then fixed with 4% formaldehyde and mounted with Elvanol. Preparations were analysed by confocal fluorescence microscopy (A and C) or phase contrast microscopy (B, D). (A and B) DU-H. (C and D) DU-L. Bar = 50 μ m.

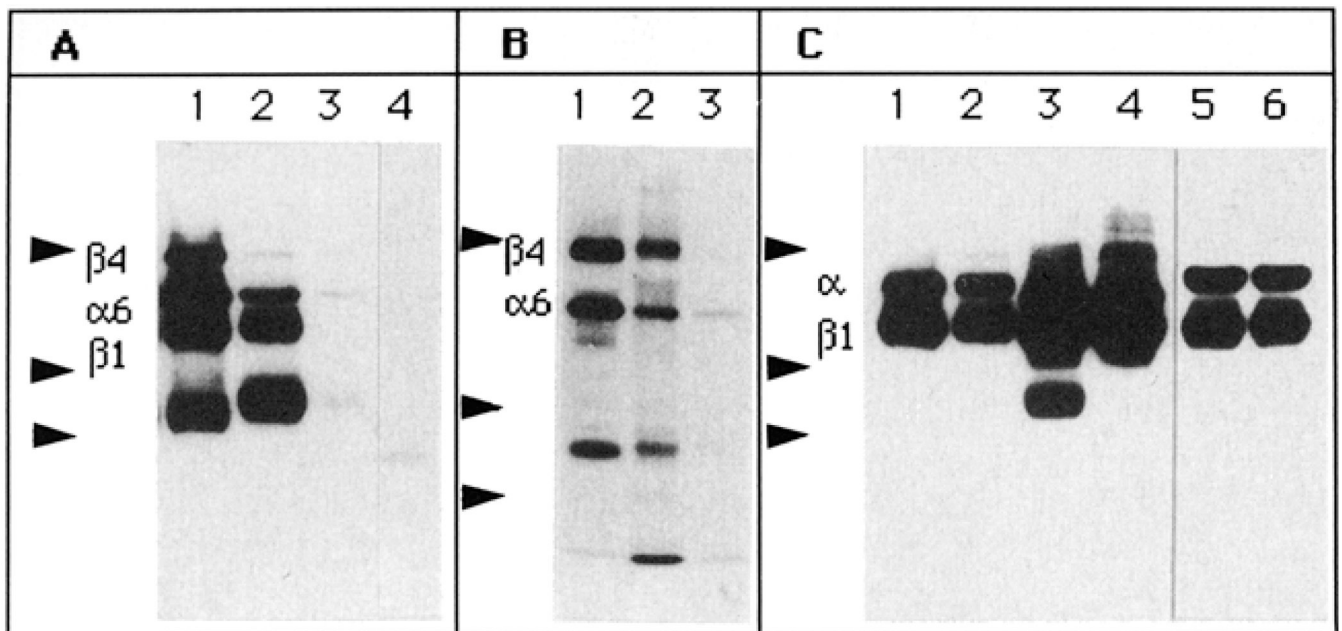


Figure 3. Biotinylation and immunoprecipitation of cell surface integrins from DU-H and DU-L cells. Integrins were immunoprecipitated from surface biotinylated cells as described in Materials and methods. The proteins were separated in 7.5% SDS-PAGE, electrotransferred to nitrocellulose, incubated with peroxidase-conjugated streptavidin and visualized by chemiluminescence. (A) $\alpha 6$ integrin was immunoprecipitated with GoH3 antibody (lanes 1–3) or control IgG antibody (lane 4). Lane 1, JAR cells (used as a positive control); lanes 2 and 4, DU-H; lane 3, DU-L. (B) $\beta 4$ integrin was immunoprecipitated with 3E1 antibody (lanes 1–3). Lane 1, JAR cells; lane 2, DU-H; lane 3, DU-L. (C) $\alpha 5$ (lanes 1 and 2), $\beta 1$ (lanes 3 and 4), and $\alpha 3$ (lanes 5 and 6) integrins were immunoprecipitated with PID6, PID6 and PIB5 antibodies, respectively. Lanes 1, 3, 5, DU-H; lanes 2, 4, 6, DU-L. Arrowheads (top to bottom): protein molecular weight markers of 200, 97 and 68 kDa.

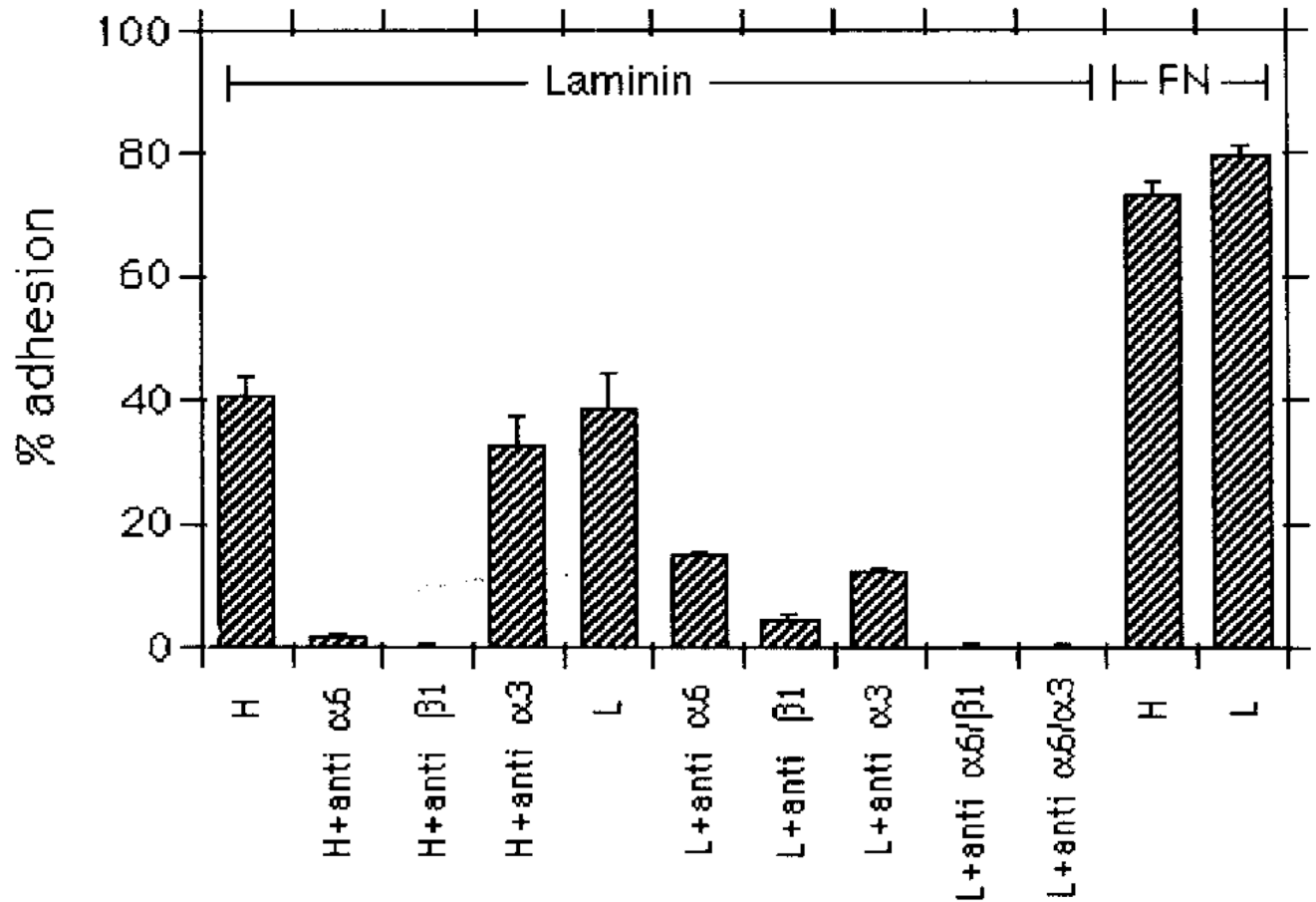


Figure 4.

Attachment of DU-H and DU-L cells to laminin and fibronectin. Cells were tested for their adhesive ability on laminin and fibronectin in the presence or absence of specific antibodies as described in Materials and methods. Results are expressed as a percentage of the total number of applied cells which were attached after 1 h of incubation. H, DU-H; L, DU-L; anti- $\alpha 6$, anti- $\alpha 6$ antibody (GoH3, 20 $\mu\text{l/ml}$); anti- $\beta 1$, anti- $\beta 1$ antibody (P4C10, 5 $\mu\text{l/ml}$); anti- $\alpha 3$, anti- $\alpha 3$ antibody (P1B5, 10 $\mu\text{l/ml}$); FN, fibronectin.

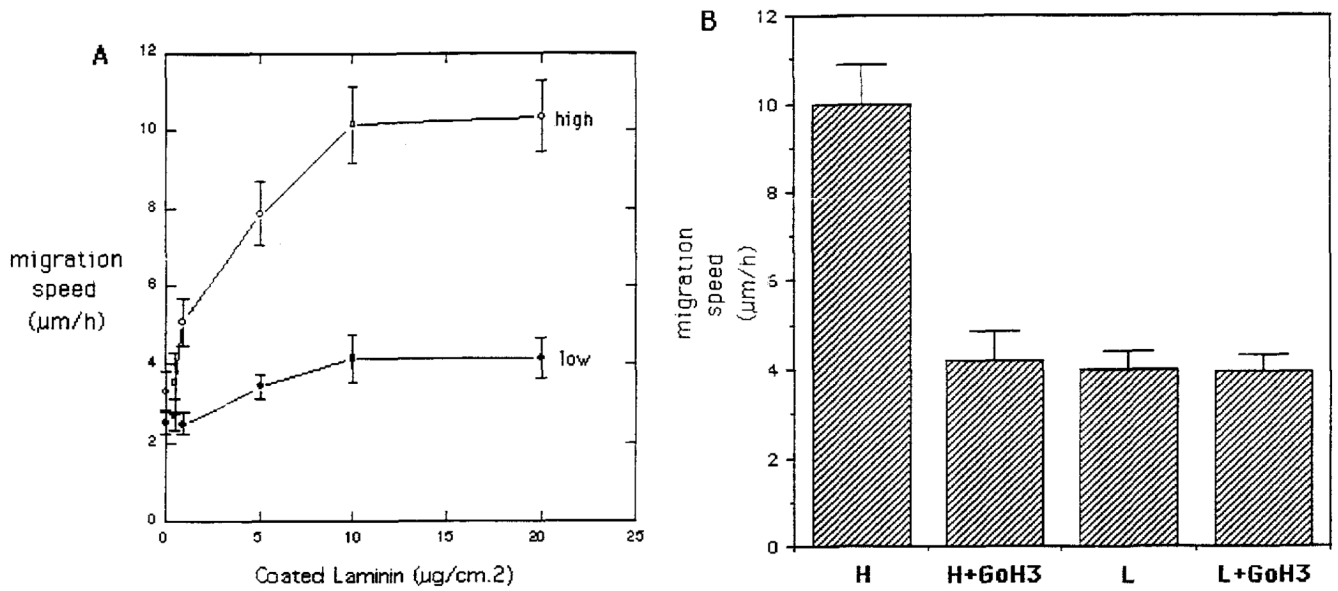


Figure 5.

Migration of DU-H cells and DU-L cells on laminin. Videomicroscopic analysis of cell migration was performed on cells grown in laminin-coated dishes ($0\text{--}20\ \mu\text{g}/\text{cm}^2$). Random cell migration was determined by measuring the displacement of centroids as a function of time. Seventy to 100 cells were analysed for each condition. (A) Random migration of DU-H and DU-L on laminin at different concentrations. (B) Inhibition of migration with anti- $\alpha 6$ antibody ($20\ \mu\text{l}/\text{ml}$) of DU-H cells and DU-L cells on laminin ($10\ \mu\text{g}/\text{cm}^2$).

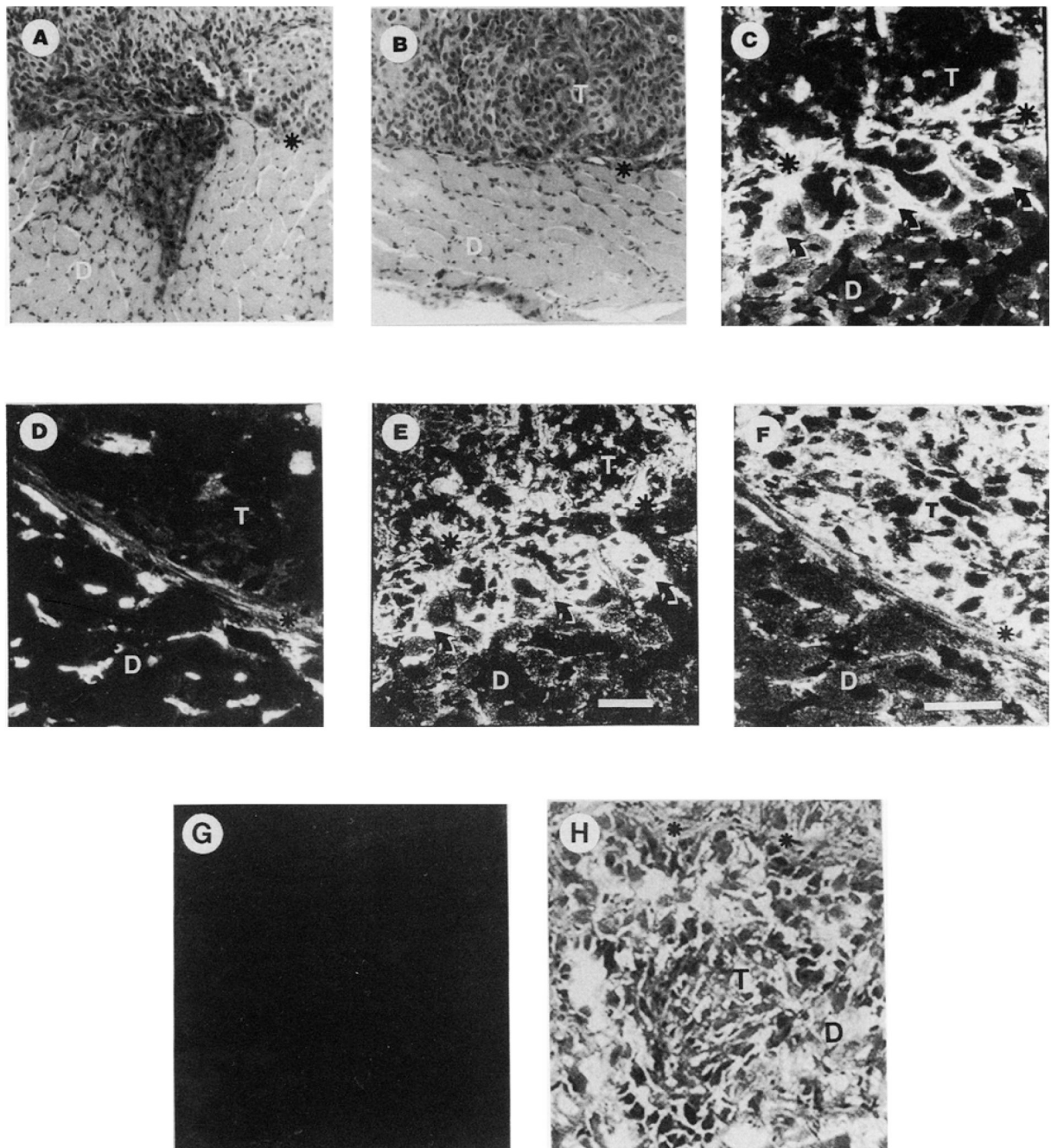


Figure 6.

Invasion of SCID mouse diaphragm by DU-H and DU-L cells. Cells were injected intraperitoneally in SCID mice and grown for 31 days. Diaphragm samples were fixed in formalin or frozen, sectioned and stained with hematoxylin and eosin. (A) Highly invasive tumor (DU-H). (B) Non-invading tumor (DU-L). (C–G) Indirect immunofluorescent analysis of DU-H and DU-L tumors. Frozen sections of tumor-invaded diaphragms from inoculated SCID mice with DU-H (C, E) and DU-L (D, F) were immunostained with anti- $\alpha 6$ integrin (C, D) or human anti-keratin (E, F) antibodies. As a control, contiguous frozen sections of DU-H tumors were stained with the secondary antibody only (G) and hematoxylin and eosin stain (H). Asterisks denote the normal diaphragm boundaries. T, tumor; D, diaphragm. Arrows

indicate the invasive edges of the tumor. Bars = 50 μ m. Note the invasion edge of the tumor is surrounding muscle fibers and expressing α 6 integrin.

Table 1

Invasive ability of DU-H and DU-L cells injected into SCID mice

	Invasion to diaphragm^a	Mean tumor weight^b
DU-H	4/5	0.32
DU-L	1/5	0.38

^aPenetration and migration through the diaphragm.

^bDiaphragm with tumor was dissected and weighed (mg). Tumor weight=total diaphragm with tumor weight–mean weight of normal diaphragm. The mean weight of a normal diaphragm was 80 mg.