

Integrin $\alpha_6\beta_4$ mediates dynamic interactions with laminin

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SUMMARY

We present here a novel form of dynamic adhesion in which both the integrin receptor and the ligand supporting dynamic adhesion have been identified. Laminar flow assays showed that laminin supported attachment of $\alpha_6\beta_4$ -positive cells in the presence of fluid shear stress ($\tau \leq 2$ dyn/cm²), indicating that these cells adhered to laminin within a fraction of a second. Further increases in flow rate (3.5 dyn/cm² $\leq \tau \leq 100$ dyn/cm²) initiated rolling of attached cells in the direction of flow, suggesting that rapidly formed adhesion is reversible and repeatable. Laminin fragment E8, which interacts with α_6 integrins, supported dynamic attachment and rolling but extracellular matrix glycoprotein fibronectin did not. In cell lines that express $\alpha_6\beta_4$ but

not $\alpha_6\beta_1$ an anti- α_6 monoclonal antibody inhibited attachment to laminin in the presence of flow and following 5 minutes of static incubation. Infusion of this antibody onto cells adherent to laminin-coated slides led to rapid detachment of cells from the substratum. An anti- β_1 monoclonal antibody diminished adhesion strength following static incubation but did not inhibit rapid attachment and flow-initiated rolling. These results indicate that in some $\alpha_6\beta_4$ -expressing epithelial and carcinoma cell lines, integrin $\alpha_6\beta_4$ mediates rapidly formed dynamic interactions with laminin.

Key words: laminin, integrin, $\alpha_6\beta_4$, dynamics, adhesion

INTRODUCTION

The extracellular matrix glycoprotein laminin mediates adhesion events that are critical to a number of fundamental biological phenomena, including cell migration, neurite outgrowth and angiogenesis (see for review Yamada and Kleinman, 1992). Although these events take hours for completion, they may be composed of a series of rapid and reversible cellular interactions with laminin. Such reversible or dynamic interactions are suggested, for example, by the rapid lamellipod extensions that occur during translocation of myoblasts on laminin (Goodman et al., 1989). The mechanisms that regulate the dynamic interactions of cells with laminin or other matrix proteins have not been examined. Based on existing data, it can be postulated that dynamic interactions involve a cycle of integrin-mediated attachment to and detachment from matrix proteins and that this cycle is regulated by the rapid modulation of integrin function (Regen and Horwitz, 1992).

The principal epithelial and carcinoma cell surface adhesion receptors that interact with laminin are integrins $\alpha_2\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$ (Sonnenberg et al., 1990; Lotz et al., 1990; Lee et al., 1992). Although some carcinoma cells also express $\alpha_3\beta_1$, an established laminin and collagen receptor (Carter et al., 1990; Kramer et al., 1990; Hynes, 1992), they do not appear to use it in adhesion to laminin (Lotz et al., 1990). The $\alpha_2\beta_1$ integrin

laminin receptor functions as a collagen receptor in a number of epithelial and carcinoma cells (Carter et al., 1990; Elices and Hemler, 1989; Lotz et al., 1990). Integrins $\alpha_6\beta_4$ and $\alpha_6\beta_1$ bind to laminin and to laminin subfragment E8, the distal stem and proximal half of the large globule of the long arm (Kramer et al., 1990; Elices et al., 1991; Lee et al., 1992). The β_4 integrin subunit, which is probably the most structurally complex of all integrins associates with variants of the α_6 integrin (Quaranta and Jones, 1991). The α_6 integrin subunit has a higher binding affinity for β_4 than for β_1 integrin (Giancotti et al., 1992). As a result, in cells expressing both β_1 and β_4 integrins, α_6 integrin preferentially associates with β_4 integrin (Hemler et al., 1989; Kajiji et al., 1989; Simon-Assmann et al., 1994).

In a variety of cell types that express large amounts of the $\alpha_6\beta_4$ integrin, it has not been possible to assign a distinct adhesive function to $\alpha_6\beta_4$ using conventional adhesion tests (Sonnenberg et al., 1990). Only recently, with the use of clone A colon carcinoma cells that express $\alpha_6\beta_4$ but not $\alpha_6\beta_1$ and are highly motile on laminin, was integrin $\alpha_6\beta_4$ shown to be a laminin receptor (Lee et al., 1992). These authors also illustrated that carcinoma cell lines that express both $\alpha_6\beta_4$ and $\alpha_6\beta_1$ or exclusively $\alpha_6\beta_1$ (RKO human rectal carcinoma cells) adhere to laminin with slower kinetics than the clone A cells. The capacity for rapid attachment and motility on laminin suggests a dynamic mode of interaction that is distinct and perhaps more significant biologically than from more static

modes of interaction. In this context it is important to establish whether epithelial and carcinoma cells can undergo dynamic interactions with laminin and whether such interactions are mediated by a specific integrin.

The successful use of laminar flow assays in deducing information on the molecular basis of leukocyte rolling in post-capillary blood venules (Abbass et al., 1991; Lawrence and Springer, 1991) indicated to us that this experimental procedure could also be used to investigate the mechanisms involved in integrin-mediated adhesion to laminin. In the present study, we used laminar flow assays to study the time course and the forces involved in the interaction of cultured epithelial cells and carcinoma cells with laminin and laminin fragments. Cells were incubated on a laminin-coated glass coverslip in a flow chamber for 1 to 30 minutes before imposition of flow at specified levels of wall shear stress. In other experiments, cells were infused into the chamber onto the laminin-coated glass coverslip at various flow rates. These experimental procedures were necessary to establish the temporal characteristics of adhesion to laminin.

Cell lines used included clone A, HT-29 and RKO carcinoma cells that exclusively express one of the α_6 integrins (Lee et al., 1992; Simon-Assmann et al., 1994), and MCF-10A human breast epithelial cells in which the large majority of α_6 integrin subunits are complexed with β_4 not β_1 integrin (present study). Additionally, a number of other carcinoma cell lines were used in adhesion experiments to explore whether dynamic interactions with laminin correlate with $\alpha_6\beta_4$ expression. In order to separate the adhesive function of integrin $\alpha_6\beta_4$ from those of β_1 integrins, cells that were treated with function blocking monoclonal antibodies were used in laminar flow assays. In experiments designed to investigate the reversibility of adhesion, these antibodies were infused onto cells already adherent to laminin. The results revealed a novel form of transient cell-matrix interaction mediated by the $\alpha_6\beta_4$ integrin. The laminin-supported dynamic cell adhesion is characterized by rapidly formed adhesive contacts that withstand large forces for brief durations. This mode of interaction was shown to occur specifically with the E8 domain of laminin and not with other laminin domains or fibronectin.

MATERIALS AND METHODS

Cells

The cell lines used in this study were obtained from the American Type Tissue Collection (Rockville, MD), unless otherwise stated. These cell lines were: (a) MCF-10A human breast epithelial cells (Tait et al., 1990); (b) clone A (provided by Dr D. Dexter; Dexter et al., 1979) and HT-29 human colon carcinoma cells (Fogh et al., 1977); (c) RKO human rectal carcinoma cells (provided by Dr M. Brattain; Boyd et al., 1988); (d) A-431 epidermoid carcinoma cells (Tamura et al., 1991); and (e) MCF-7, HS-578t, MDA-231 and T-47D breast carcinoma cells that were characterized recently for their β_4 integrin expression (Sommers et al., 1994). Subconfluent flasks of cells were trypsinized, and resuspended at 1×10^6 cells per ml in DMEM supplemented by 10% fetal calf serum (Hyclone Lab, Inc., Logan, UT). These cells were then maintained in suspension culture for 2 hours at 37°C to regenerate cell surface proteins, and then placed on ice. The results of the laminar flow assays were highly reproducible if the cells were used within six hours from the time they were placed on ice.

Substrata used in adhesion assays

Glass coverslips were coated with extracellular matrix proteins (laminin, fibronectin), laminin fragments or heat-denatured bovine serum albumin (BSA). Fragments of Englebreth-Holm-Swarm tumor laminin (E8, E1, E3 and E4), prepared as described by Schittney and Yurchenco (1990), were a generous gift from Dr Peter D. Yurchenco (Department of Pathology, Robert Wood Johnson Medical School, Piscataway, NJ 08854). The proteins and protein fragments were diluted in distilled water (or in distilled water containing acetic acid) from a stock solution and placed on the coverslip in the form of 50 μ l drops (Timpl et al., 1979). Such drops eventually coat an approximately circular region of 0.5 cm² in area. The coverslips were then incubated in a humidified CO₂ incubator for one to six hours and remained wet at all times. Laminin coating was approximately uniform as judged from the uniform distribution of cells that remained on the laminin-coated region after the completion of laminar flow.

Antibodies

The mouse monoclonal antibodies (mAb) P1D6 (anti- α_5 ; Wayner et al., 1988), P4C10 (anti- β_1 ; Carter et al., 1990) and 3E1 (anti- β_4 ; Hesse et al., 1984) were purchased from Gibco BRL (Gaithersburg, MD). The rat mAb GoH3 (anti- α_6 ; Sonnenberg et al., 1986) was purchased from AMAC, Inc. (Westbrook, Maine). The rat mAb 13 (anti- β_1 ; Akiyama et al., 1989) was kindly provided by Dr Steven K. Akiyama (National Institutes of Health, Bethesda, MD, 20892). The mouse mAb UM-A9 (anti- β_4 ; Van Waes et al., 1991) was provided by T. Carey (University of Michigan, Ann Harbor). The anti- α_5 mAb P1D6 that inhibits attachment to fibronectin and the normal rat IgG were used as controls for experiments involving antibody inhibition of adhesion to laminin.

Immunoprecipitation

MCF-10A cells were surface labeled with biotin and detergent extracts as described earlier (Lee et al., 1992). One dish of cells was extracted in lysis buffer containing 1 mM CaCl₂ and one with lysis buffer containing 10 mM EDTA (Giancotti et al., 1992). Immunoprecipitation using anti-human α_6 integrin mouse monoclonal antibody 2B7 or its control (rat IgG) was carried out as described by Lee et al. (1992) and the precipitated material run under non-reducing conditions on a 4% to 20% gradient SDS-PAGE. Following transfer to nitrocellulose the precipitated proteins were detected with alkaline phosphatase-conjugated streptavidin.

Flow chamber

A parallel plate flow chamber of uniform width was used in the laminar flow assays. The chamber consisted of: (a) an upper plate having appropriate openings for the delivery of the fluid into and out of the channel; (b) a gasket with an opening in the form of the channel; (c) a transparent bottom plate (grade no. 1 coverslip, Fisher Scientific, Pittsburgh, PA); and (d) top and bottom stainless steel cover plates with observation slots. The bottom plate, the gasket and the base plate were fastened between the cover plates. The entry port of the chamber was connected through a valve and teflon tubing to two syringes, one filled with the cell suspension, and the other filled with suspending medium. The flow chamber design is identical to those reported previously by Chien and Sung (1987) and Lawrence and Springer (1991).

Laminar flow assays

Experiments were initiated by placing the flow chamber on the stage of an inverted microscope (Diaphot; Nikon Inc., Garden City, NJ) equipped with $\times 10$ and $\times 40$ Hoffman and brightfield objective lenses. In dynamic cell attachment experiments, MCF-10A or clone A cells (10 cells/ml) were gently inserted into the chamber from a side port at the prescribed flow rate (0-1 ml/min) and the number of attached cells in the videoscreen was recorded after 1 minute of flow at three different locations on the laminin-coated region. In static attachment

experiments, cells (~100 cells/screen) were allowed to interact with the laminin-coated bottom plate for 5 minutes under static conditions. Flow was then initiated and the flow rate was increased at prescribed time intervals.

A syringe pump (Harvard Apparatus, South Natick, MA) was used to pump medium into the chamber at specified flow rates. The shear stress on the bottom plate of the chamber along the direction of flow, τ (dyn/cm²), was evaluated by using the following equation, assuming Poiseuille flow:

$$\tau = 6 \mu Q/h^2w, \quad (1)$$

where μ (~0.01 dyn-s/cm²) is the viscosity of the cell medium, Q (cm³/s) is the flow rate, h is the gap thickness of the channel (0.12 mm), and w (~1.0 cm) is the width of the chamber (Lawrence and Springer, 1991).

In experiments designed to follow the motion of individual cells as a function of time, either a $\times 40$ Hoffman objective (Nikon, NJ) or a $\times 10$ long working distance brightfield objective lens (Nikon, NJ) with two $\times 15$ eyepieces and a $\times 10$ eyepiece on the side port (Diaphot; Nikon Inc., NJ) was used. A video-camera (Dage-MTI, Michigan City, IN) was attached to the side port of the microscope to record cell attachment, cell detachment and cell rolling events in the video field. The times were displaced on the video monitor with a 'data mixer' (Vista Electronics, La Mesa, CA). The distance a cell travelled along the direction of flow was determined using a position analyzer (Vista Electronics, La Mesa, CA) that provided a digital readout proportional to the distance between two sets of vertical and horizontal lines.

A cell was said to be rolling if the cell center moved relative to the laminin-coated glass coverslip in the direction of flow while some of the cell surface projections remained in contact with the substratum. The irregularities on the cell surface (cell surface projections) were clearly visible with a $\times 40$ microscope objective. The detachment of cell surface projections from laminin at the rear of the contact region was frequently observed and videotaped at this magnification.

In experiments in which a $\times 10$ microscope objective was used, rolling cells were identified with the following procedure: (1) using the method of Goldman et al. (1967), the velocity of a spherical cell flowing at a distance of 1 μ m from a planar substratum (U (μ m/s)) was computed as a function of wall shear stress τ . A cell was considered to be rolling if it moved at least 5 cell diameters with a velocity of less than $U/10$. Although the upper bound set for rolling cell velocity is rather arbitrary, results are not sensitive to this parameter because speeds of rolling cells are typically 100- to 1,000-fold smaller than the speed of flow.

Average cell speed was determined by dividing the distance travelled in 5 s by that time period. The percentage of non-flowing cells (cells that remained adherent to the substratum or rolling on the substratum) was determined by dividing: the number of non-flowing cells in the video-monitor (~1 mm²) after the application of flow at the prescribed shear stress (a) by the number of cells in the video-screen before the initiation of flow (b).

Laminar flow assays were repeated at least five times under identical conditions at 25°C. Control experiments with MCF-10A cells showed that increasing temperature to 37°C did not lead to mechanistic differences in laminin-supported adhesion. The mean value and the standard deviation of the percentage of non-flowing cells were determined for each different case. In experiments with function-blocking mAbs and their controls, laminar flow assays were simplified so that at most two different flow rates were used in each experiment.

RESULTS

Immunoprecipitation shows that MCF-10A cells express $\alpha_6\beta_4$

Immunocytochemistry on MCF-10A cells in culture showed

that these cells express α_2 , α_3 , α_6 , β_1 and β_4 integrin subunits on their surface (Sommers et al., 1994). The expression of integrins $\alpha_6\beta_4$ and $\alpha_6\beta_1$ on the surface of MCF-10A cells was investigated. Using an antibody specific to human α_6 integrin, a complex consisting of two proteins, one at approximately 130 kDa (the size of the integrin subunit α_6) and one at approximately 200 kDa (the size of the integrin subunit β_4), was immunoprecipitated from MCF-10A cells (Fig. 1). There was no evidence of co-precipitated β_1 integrin in MCF-10A cells. However, trace amounts of $\alpha_6\beta_1$ that could not be detected by this experiment may be present in MCF-10A cells. Immunoprecipitation with control (rat IgG) showed no bands. Giancotti et al. (1992) have demonstrated that the integrin subunit β_4 in certain cells is proteolytically degraded by a calcium-dependent protease yielding several lower molecular mass fragments. In our experiments the co-precipitated β_4 integrin was largely present in the intact 200 kDa form although faint bands at 105 kDa and 70 kDa could be discerned in immunoprecipitates collected in cells lysed in the presence or absence of calcium (Fig. 1). These results indicate that the large majority of α_6 integrin subunits in these cells are complexed with β_4 not β_1 integrin. Immunoprecipitation data presented here are consistent with immunocytochemistry, which showed that α_6 and β_4 integrin subunits are localized on the undersurface of the MCF-10A cells and on thin processes extending onto the substratum (Sommers et al., 1994).

Laminar flow initiates rolling of MCF-10A and clone A cells on laminin

MCF-10A cells that had attached to a laminin-coated glass coverslip for several seconds to 20 minutes at $\tau \leq 2.0$ dyn/cm², rolled on laminin in response to an increase in the rate of flow ($\tau \leq 100$ dyn/cm²). Fig. 2 shows a series of videomicrographs of an MCF-10A cell as it moves on a laminin-coated glass coverslip in the direction of flow at $\tau = 7$ dyn/cm². The microscope objective was kept stationary for each row of the videomicrographs shown in Fig. 2. As a result, the stationary cells as well as the numbers on the bottom right-hand corner of the videomicrographs served as reference points for studying the motion of the MCF-10A cell. The surface of the moving MCF-10A cell contained large number of surface projections. These projections moved relative to the cell center in a manner consistent with rolling but not with sliding or crawling. Furthermore, videotape of the transient interactions of the MCF-10A cell with laminin showed clearly that the detachment of the cell surface projections from the laminin-coated glass coverslip as the cell moved along the direction of

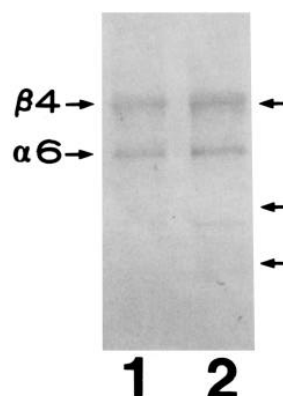


Fig. 1. α_6 Integrin immunoprecipitation. MCF-10A cells were lysed in lysis buffer containing 10 mM EDTA (lane 1) or 1 mM CaCl₂ (lane 2) and immunoprecipitated with anti- α_6 integrin antibody 2B7. α_6 and β_4 integrin bands are indicated. The arrows show the positions of the molecular mass standards at 200, 116 and 68 kDa.

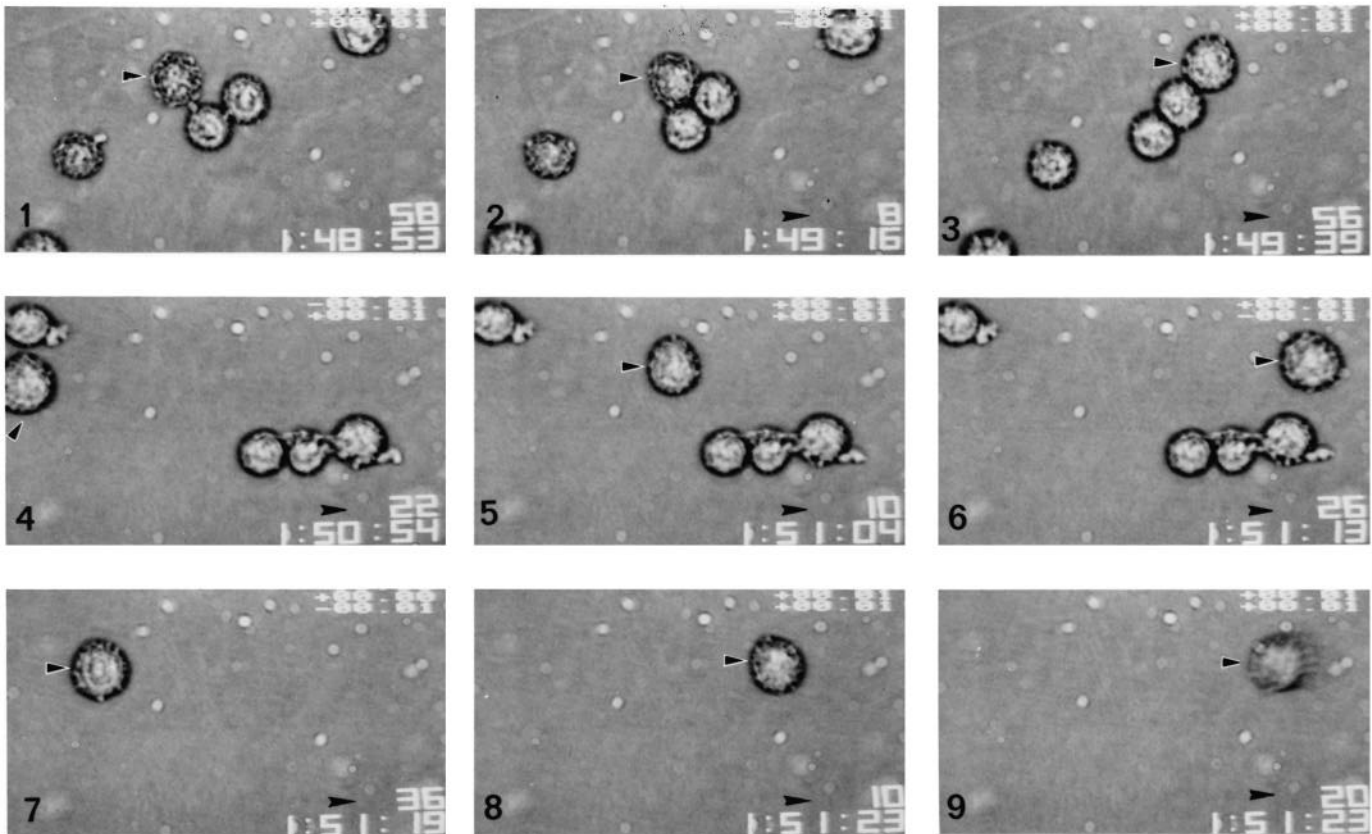


Fig. 2. Videomicrographs of an MCF-10A cell rolling on laminin-coated glass ($12.8 \mu\text{g}/\text{cm}^2$) in simple shear flow with wall shear stress (τ) equal to $7 \text{ dyn}/\text{cm}^2$. The numbers in the right-hand corner of the videomicrographs indicate the time in hours, minutes, seconds and 1/60 seconds. The arrowhead in the bottom right-hand corner in each micrograph indicates the direction of the flow imposed on cells. MCF-10A cells were incubated on laminin for 5 minutes under static conditions. The flow was initiated at 1:49:00. The cell indicated with the black arrow first rolled around the adjacent cells and then continued rolling in the direction of flow for 143 seconds. Micrograph 9 captures the cell as it ceased to interact with the laminin-coated glass and acquired the speed of flow.

flow. These observations indicated collectively that the motion of the MCF-10A cell can be characterized as rolling (Tozeren and Ley, 1992). The cell shown in Fig. 2 rolled with an average speed of $5 \text{ mm}/\text{s}$ during the first 80 seconds of rolling. The speed of the rolling cell was approximately 200-fold lower than the speed of flow. The demonstration that the cell in Fig. 2 was interacting with the planar substratum is further confirmed by the last micrograph, which shows the cell as a blurry object as it gains the speed of flow.

MCF-10A cells rolled in the direction of flow for distances ranging from $50 \mu\text{m}$ to $700 \mu\text{m}$ (Fig. 3). The duration of rolling interactions increased with increased laminin surface density (Fig. 3). The average cell speed (rolling velocity) over 5 s intervals increased with applied fluid shear stress and decreased with increasing surface density of laminin (Fig. 4), but rarely exceeded $30 \mu\text{m}/\text{s}$, a value comparable to the rolling velocity of leukocytes on P-selectins (Lawrence and Springer, 1991). When the rolling distance was monitored with a Hoffman $\times 40$ objective at smaller time intervals (fractions of seconds), rolling velocity fluctuated in cases where the cells interacted with laminin through a few cell surface projections whereas cells with smaller sized areas of surface roughness (large interphase MCF-10A cells) rolled more smoothly on laminin (not shown).

Imposition of flow ($3.5 \leq \tau \leq 100 \text{ dyn}/\text{cm}^2$) on clone A cells adherent to laminin induced instantaneous detachment of a subpopulation (20-50% of the detached cells) and initiated rolling in the direction of flow in another subpopulation. The percentage of cells that eventually detached from laminin at a certain shear stress level was lower for clone A cells than for MCF-10A cells (Fig. 5).

MCF-10A and clone A cells adhered poorly to glass or to glass coated with BSA. Cells that had attached to these substrata during 20 minutes of static incubation detached instantaneously in response to flow at $\tau = 3.5 \text{ dyn}/\text{cm}^2$. On the other hand, MCF-10A and clone A cells attached well to fibronectin, but the detachment from the fibronectin-coated glass coverslip with the imposition of flow was instantaneous (not shown).

Laminin fragment E8 supports dynamic interactions

Clone A cells were shown previously to interact with the E8 subfragment of laminin but with no other laminin subfragment (Lee et al., 1992). Adhesion of MCF-10A cells to glass coverslips coated with laminin fragments E1', E3, E4 and E8 (Beck et al., 1990; Schittney and Yurchenco, 1990) was investigated. Fragment E4 is known to be inactive in promoting cell adhesion, and thus served as a control (Yurchenco et al., 1992).

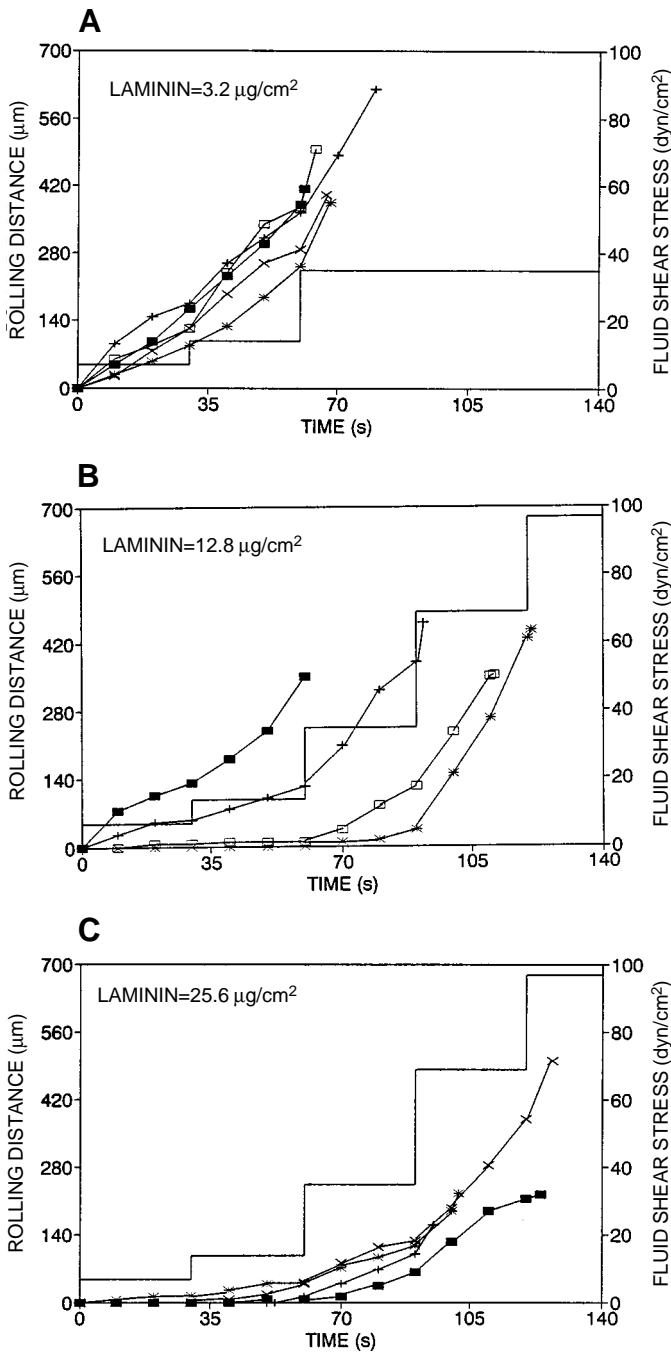


Fig. 3. The time course of fluid shear stress and distance travelled by the rolling MCF-10A cells for three different surface concentrations of laminin (A, 3.2 $\mu\text{g}/\text{cm}^2$; B, 12.8 $\mu\text{g}/\text{cm}^2$; C, 25.6 $\mu\text{g}/\text{cm}^2$). Flow into the flow chamber was increased at 30 second intervals such that the shear stress on the bottom plate of the chamber (laminin-coated glass coverslip) took the values 0, 7, 14, 35, 70 and 100 dyn/cm^2 . Each plot represents the motion of a single cell.

Among these laminin fragments, only E8 could support adhesion in the presence of flow ($\tau=1.7 \text{ dyn}/\text{cm}^2$) and flow-induced rolling following 5 minutes of static incubation (Fig. 6). Mixing E8 with the other laminin fragments did not have an effect on adhesion strength (not shown). On the other hand, attachment strength to E8 increased with increas-

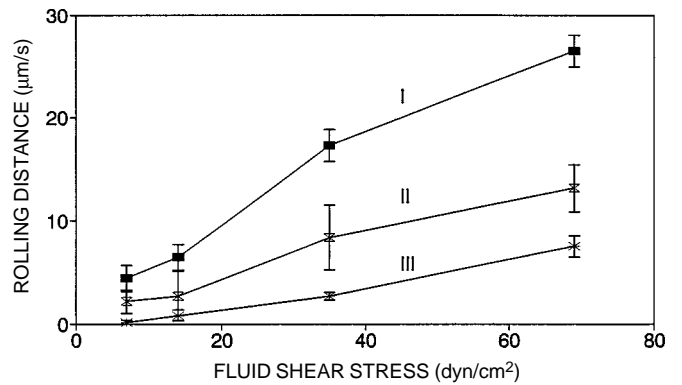


Fig. 4. Dependence of rolling velocity on fluid shear stress. Rolling velocity of MCF-10A cells, averaged over 5-second time intervals, was plotted as a function of wall shear stress for three different surface concentrations of laminin immobilized on the bottom plate of the flow chamber (curve I, 3.2 $\mu\text{g}/\text{cm}^2$; curve II, 12.8 $\mu\text{g}/\text{cm}^2$; curve III, 25.6 $\mu\text{g}/\text{cm}^2$).

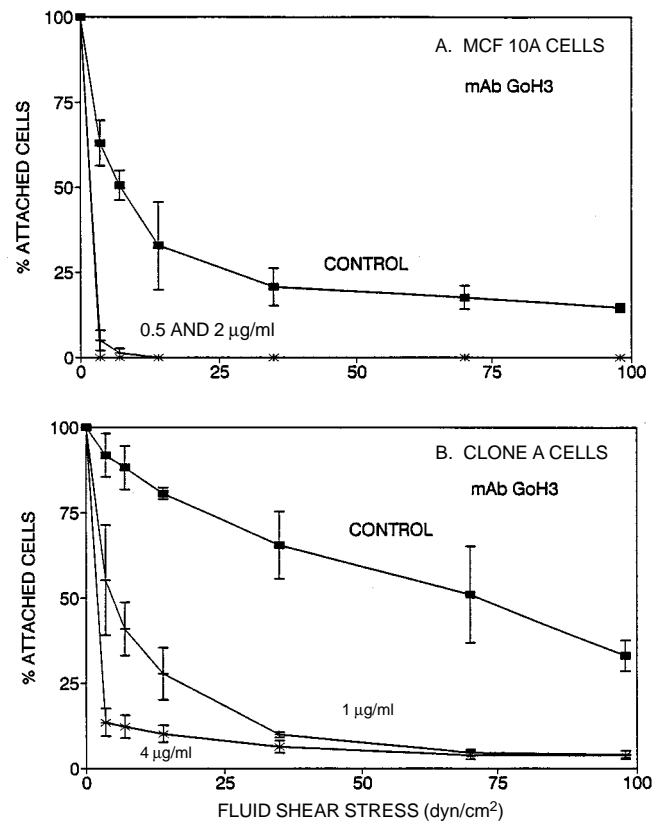


Fig. 5. The effects of the mAb GoH3 (anti- α_6 ; Sonnenberg et al., 1987) on the adhesion strength of MCF-10A (A) and clone A cells (B) to laminin (20 $\mu\text{g}/\text{cm}^2$) following 5 minutes of static incubation in the flow channel. Cells were incubated with the mAb or control (rat IgG) at the concentrations indicated in the figure for 30 minutes at 25°C. Cells were then injected into the flow channel and allowed to settle on the laminin-coated glass coverslip for 5 minutes. Subsequently, flow was introduced and increased at 30 s intervals so that the fluid shear stress on the coverslip took the values $\tau=3.5, 7, 14, 35, 70$ and 98 dyn/cm^2 . The number of non-flowing cells on the video-screen was quantified immediately before each step increase in shear stress. These numbers were then divided by the number of cells on the same screen before the application of flow to obtain the percentage of stationary or rolling cells.

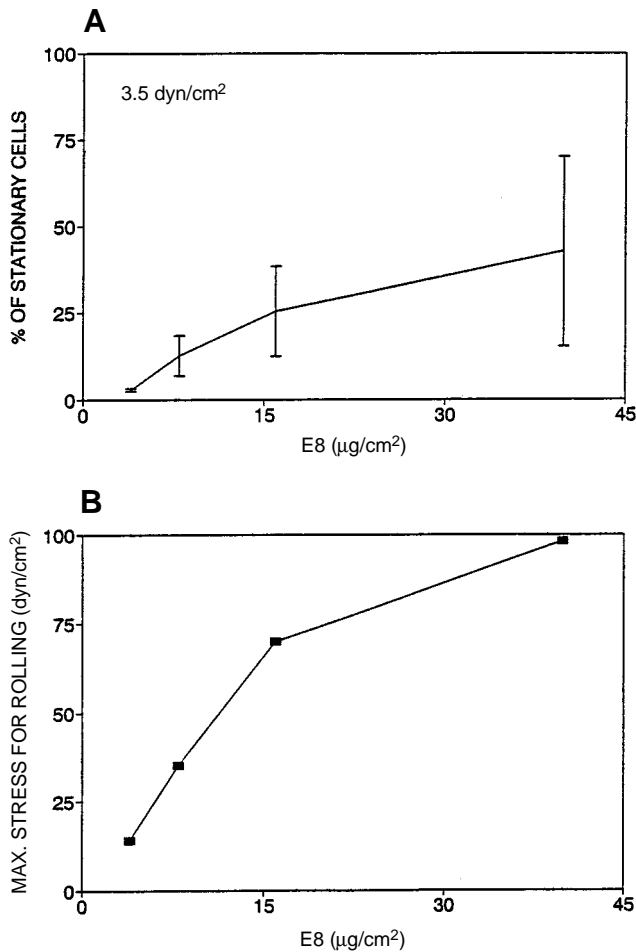


Fig. 6. MCF-10A cell binding to laminin fragment E8: effect of E8 concentration on rolling parameters. MCF-10A cells were incubated in a flow chamber on a glass coverslip coated with laminin fragment E8 for 5 minutes. Laminar flow was then imposed and flow rate was incrementally increased every 30 seconds. (A) Percentage of non-flowing MCF-10A cells following application of flow at $\tau=3.5$ dyn/cm^2 as a function of E8 surface density (note that $10 \mu\text{g}/\text{ml}$ is equal to $1 \mu\text{g}/\text{cm}^2$, since $50 \mu\text{l}$ drops deposited on glass coverslips covered an area of 0.5 cm^2). (B) The maximum fluid shear stress at which at least five cells were rolling per 1 mm^2 of the E8-decorated glass coverslip as a function of E8 surface density. MCF10 cells rolled on E8 ($40 \mu\text{g}/\text{cm}^2$) at $\tau=100 \text{ dyn}/\text{cm}^2$, the highest fluid shear stress used in this study.

ing E8 concentration (Fig. 6, top panel). The cells that detached in response to flow rolled on E8 at rolling velocities comparable to that on laminin (not shown). The maximum fluid shear stress at which rolling was observed also increased with E8 concentration (Fig. 6, bottom panel). Consistent with data obtained from static adhesion assays (Sonnenberg et al., 1990), the percentage of cells that remained stationary on E8 was significantly smaller than that on laminin at the same fluid shear stress and surface density of the ligand deposited on glass coverslips.

MCF10-A and clone A cells accumulate on laminin in the presence of laminar flow

The ability of MCF-10A and clone A cells to roll on laminin suggests high rates of bond formation (Tozere and Ley,

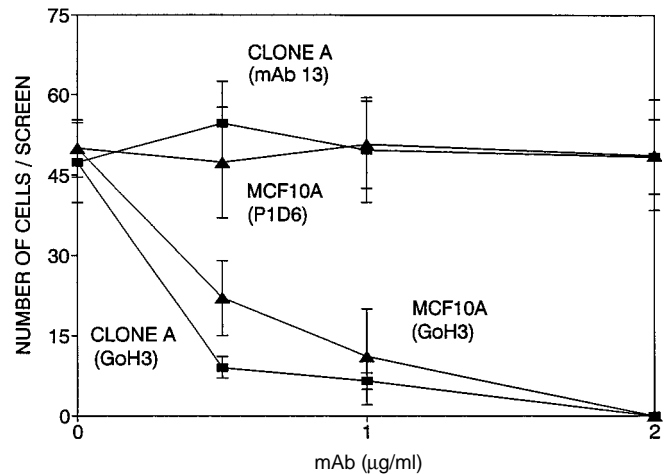


Fig. 7. The dynamic attachment of MCF-10A and clone A cells to laminin ($20 \mu\text{g}/\text{cm}^2$). Cells were treated with one of the mAbs: GoH3 (anti- α_6), mAb 13 (anti- β_1) or control (anti- α_5 mAb P1D6) for 30 minutes at 25°C , and then were infused into the flow channel at a constant flow rate ($\tau=1.85 \text{ dyn}/\text{cm}^2$) for 1 minute. The number of cells accumulated on laminin ($\sim 1 \text{ mm}^2$) during infusion was evaluated at three different locations within the laminin-coated region of the flow channel as a function of the mAb concentration. The points shown in the figure represents at least five different experiments performed under identical conditions. MCF-10A and clone A cells preincubated with mAb 13 continued to accumulate on laminin at $\tau=1.85 \text{ dyn}/\text{cm}^2$ even at high concentrations ($2\text{--}80 \mu\text{g}/\text{ml}$) (not shown).

1992). In order to test this further, the capacity of flowing cells to adhere to laminin was investigated using dynamic adhesion assays. The presence of flow restricts the time in which a cell can form bonds with a substratum at a certain location. Therefore, dynamic adhesion assays provide quantitative information about the speed with which cells formed adhesive contacts with laminin.

MCF-10A or clone A cells ($10 \text{ cells}/\text{ml}$) were infused at prescribed flow rates into the flow channel. The number of cells accumulated on laminin (1 mm^2) during one minute of infusion was determined at three locations on the laminin-coated coverslip. Both the MCF-10A and the clone A cells attached to laminin in significant numbers at $\tau \leq 2.0 \text{ dyn}/\text{cm}^2$. For both cell types, the number of cells that accumulated per 1 mm^2 of laminin ($20 \mu\text{g}/\text{cm}^2$)-coated glass during 1 minute of infusion was approximately equal to 50 at $\tau=1.85 \text{ dyn}/\text{cm}^2$ (Fig. 7). This fluid shear stress level is typically found in post-capillary blood venules and is similar to the levels of fluid shear stress ($\tau < 2.8 \text{ dyn}/\text{cm}^2$) at which leukocytes bind to P-selectin incorporated planar lipid-bilayers (Lawrence and Springer, 1991). At these creeping flow conditions, MCF-10A and clone A cells rolled on laminin for a brief period (seconds) before coming to a halt. Cells that attached to laminin remained attached despite the imposed flow. Attachment to laminin in the presence of flow is a result of a specific interaction because the cell types used in the study did not adhere to glass or to glass coated either with fibronectin ($1\text{--}30 \mu\text{g}/\text{cm}^2$) or with heat-denatured bovine serum albumin (BSA) ($50 \mu\text{g}/\text{cm}^2$) at $\tau \geq 0.25 \text{ dyn}/\text{cm}^2$.

The anti- α_6 mAb GoH3 inhibits adhesion to laminin

Preincubation of MCF-10A and clone A cells with anti- α_6

Table 1. Effects of neutralizing mAbs on strength of adhesion to laminin

Cell name	5 min on laminin			20 min on laminin		
	Rat IgG (control) (80 μ g/ml)	mAb13 (anti- β_1) (80 μ g/ml)	GoH3 (anti- α_6) (10 μ g/ml)	Rat IgG (control) (80 μ g/ml)	mAb13 (anti- β_1) (80 μ g/ml)	GoH3 (anti- α_6) (10 μ g/ml)
MCF-10A	72 \pm 6	68 \pm 7	0	82 \pm 6	61 \pm 9	10 \pm 9
Clone A	68 \pm 5	26 \pm 8	0	81 \pm 6	20 \pm 7	61 \pm 8
RKO	39 \pm 4	0	0	62 \pm 5	0	54 \pm 7

Cells, treated with a mAb or its control for 30 minutes at 25°C, were infused into the flow channel onto the laminin.

*The numbers shown indicate the mean and the standard deviation of the percentage of cells in the video screen after the completion of flow ($\tau=14$ dyn/cm² for 1 minute and $\tau=35$ dyn/cm² for the next minute).

mAb GoH3 (Sonnenberg et al., 1986) blocked their accumulation on laminin in the presence of flow ($\tau=1.85$ dyn/cm²) in a dose-dependent manner (Fig. 7). The lack of cell accumulation on laminin was not due to defects in the laminin coating of the glass coverslip because the same coverslips used in subsequent control experiments supported dynamic adhesion of cells treated with rat IgG or with mAb P1D6 (anti- α_5 ; Wayner et al., 1988) (Fig. 7). This result indicates that integrin $\alpha_6\beta_4$ is involved in initial cell interactions with laminin.

MCF-10A cells treated with GoH3 could not adhere to laminin even after 20 minutes of static incubation on laminin (Fig. 5 and Table 1). These cells detached instantaneously from laminin with the imposition of flow at low rates ($\tau=3.5$ dyn/cm²). Similarly, clone A cells treated with GoH3 did not adhere to laminin after 5 minutes of static incubation (Fig. 5). However, clone A cells incubated with this antibody could adhere to laminin after 20 minutes of static incubation (Table 1). These results indicate that integrin $\alpha_6\beta_4$ is required for the adhesion of some epithelial and carcinoma cells to laminin within a brief static incubation (minutes).

In order to investigate the reversibility of rapidly formed cell adhesion to laminin and its dependence on $\alpha_6\beta_4$ integrin, medium containing either GoH3 or the anti- β_1 mAb 13 (Akiyama et al., 1989) was infused into the flow chamber and on to MCF-10A or clone A cells that were adherent to laminin. Control experiments were conducted using medium containing rat IgG at the same concentration. MCF-10A cells were allowed to interact with laminin under static conditions in the flow channel for 2 minutes. Control laminar flow assays had shown that this duration is long enough for cells to form adhesive contacts with laminin. Medium containing mAb (10 μ g/ml) or control (10 μ g/ml) was then infused into the flow channel for 5 minutes at $\tau=3.5$ dyn/cm². The flow rate for infusion was chosen such that the cells newly arriving on to the laminin-coated region could not attach. After 3 minutes of static incubation, the medium was again infused into the flow chamber but at a higher flow rate ($\tau=7$ dyn/cm²). In the case when the medium contained GoH3 most cells detached rapidly from laminin without undergoing transient interactions (Table 2). In cases where the medium contained either mAb 13 or rat IgG the number of cells in the videoscreen hardly decreased during the course of flow (Table 2). This was because MCF-10A cells that rolled away from the screen (~25%) were replaced to a large extent by cells that rolled into the screen.

Table 2. Effect of infusion of GoH3 and mAb13 onto MCF-10A cells adherent to laminin

Time (s)	0	5	10	25	40
Rat Ig G (10 μ g/ml)	100*	98 \pm 5	94 \pm 4	86 \pm 4	84 \pm 5
GoH3 (10 μ g/ml)	100	51 \pm 7	46 \pm 10	34 \pm 9	16 \pm 5
mAb13 (40 μ g/ml)	100	94 \pm 5	90 \pm 6	84 \pm 7	81 \pm 6

GoH3, mAb13 or rat IgG was infused onto cells already in contact with laminin (2 minutes) for 5 minutes at $\tau=3.5$ dyn/cm². After 3 min of static incubation flow was initiated at $\tau=7.0$ dyn/cm². Time 0 indicates the instant before flow.

*The numbers indicate the mean and the standard deviation of the percentage of cells in the videoscreen.

The experiments with clone A cells yielded similar results (not shown). Taken together, these results showed that, at the initial stages of contact with laminin, continuous involvement of the $\alpha_6\beta_4$ integrin is required for MCF-10A and clone A cell adhesion to laminin.

Anti- β_1 mAb 13 does not inhibit dynamic attachment or rolling interactions

The effect of function-blocking anti- β_1 mAbs on the initial stages of adhesion to laminin was investigated. The treatment of cells with anti- β_1 mAb 13 had no effect on cell accumulation on laminin in the presence of laminar flow (Fig. 7). In another set of experiments, cells were incubated with mAb 13 and then were infused into the flow channel. After 5 or 20 minutes of static incubation, flow was imposed on cells that were in contact with laminin. The percentages of MCF-10A and clone A cells that remained in the videoscreen after the completion of laminar flow were lower when these cells were preincubated with mAb 13 than with rat IgG (Table 1 and Fig. 8). This weakening of adhesion was less significant in the case of MCF-10A cells. The clone A cells treated with mAb 13 gradually detached from laminin after rolling in the direction of flow (Fig. 8). The mAb P4C10 (anti- β_1 ; Carter et al., 1990) had a similar effect on clone A cell adhesion to laminin (not shown). Thus, the effects of neutralizing anti- α_6 (GoH3) and anti- β_1 mAbs (mAb 13, P4C10) on the initial stages of adhesion to laminin are quite different. GoH3 blocks adhesion whereas mAb 13 weakens adhesive strength but does not inhibit dynamic attachment and flow-initiated rolling.

To our knowledge, an antibody specific to β_4 that blocks epithelial or carcinoma cell adhesion to laminin is not available. However, β_4 -specific mAb UM-A9 (20 μ g/ml) (Van Waes et al., 1991) was shown to decrease the percentage of clone A cells bound to laminin by about 25% in a static adhesion test (Lee et al., 1992). MCF-10A and clone A cells incubated with this antibody (20 μ g/ml) attached to laminin in the presence of flow ($\tau\leq 1.5$ dyn/cm²) and rolled on laminin in response to high flow rates ($\tau\geq 7$ dyn/cm²) (not shown). Similarly, β_4 -specific mAb 3E1 (Hessle et al., 1984) did not prevent cell rolling interactions (not shown).

β_4 Expression correlates with dynamic attachment

The rectal carcinoma cell line RKO (Boyd et al., 1988), which expresses $\alpha_6\beta_1$ but not $\alpha_6\beta_4$ (Lee et al., 1992), was used to

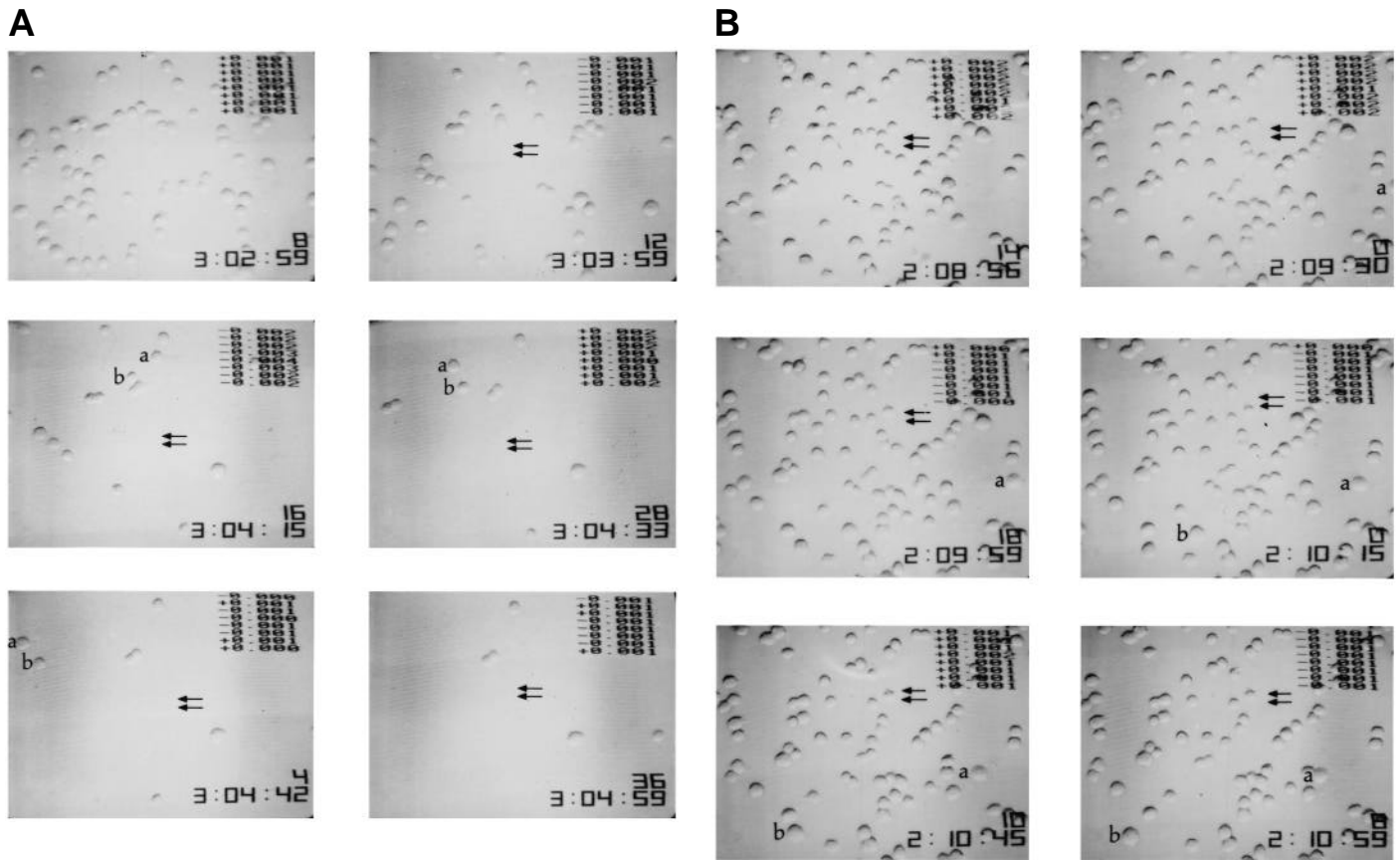


Fig. 8. The effect of anti- β_1 mAb 13 on clone A cell adhesion to laminin. Cells treated with this mAb (80 $\mu\text{g}/\text{ml}$ in 10^6 cells/ml) (A) or control (rat IgG) at the same concentration (B) for 30 minutes at 25°C were allowed to interact with laminin for 5 minutes under static conditions. Flow was initiated and incrementally increased so that the fluid shear stress τ was equal to $14 \text{ dyn}/\text{cm}^2$ during the first minute and $\tau=35 \text{ dyn}/\text{cm}^2$ during the next minute. The numbers at the lower right-hand corner show the time in hours, minutes, seconds and 1/60 seconds. The double arrows indicate the direction of flow. Flow was initiated at 3:03:00 in A and at 2:09:00 in B. Examples of rolling cells are identified in the figure by a and b.

investigate the role of $\alpha_6\beta_1$ on adhesion to laminin. RKO cells did not accumulate on laminin in the presence of flow ($\tau \geq 0.6 \text{ dyn}/\text{cm}^2$), indicating that these cells are not capable of forming rapid adhesive contacts with laminin. On the other hand, RKO cells adhered to laminin following brief static incubation. After 5 minutes of static incubation, approximately 40% of the RKO cells remained adherent to laminin when exposed to shear forces at $\tau=35 \text{ dyn}/\text{cm}^2$ for 1 minute (Table 1). Further increases in the duration of incubation increased adhesive strength (Table 1). RKO cells that detached from laminin with the imposition of flow detached instantaneously without undergoing rolling interactions (not shown).

mAb 13 and GoH3 completely inhibited adhesion of RKO cells to laminin following 5 minutes of static incubation (Table 1), indicating that integrin $\alpha_6\beta_1$ mediates initial interactions of RKO cells with laminin. RKO cells treated with GoH3 but not with mAb 13 were able to adhere to laminin after 20 minutes of static cell-substratum contact (Table 1), indicating that β_1 integrins other than $\alpha_6\beta_1$ ($\alpha_2\beta_1$; Lotz et al., 1990) are also involved in adhesion at later stages of interaction with laminin.

Laminar flow assays showed that the ability to roll on laminin is correlated with β_4 integrin expression in a number of epithelial and carcinoma cell lines (Table 3). The $\alpha_6\beta_4$ integrin-positive MCF-10A, clone A, HT-29 colon carcinoma

and A-431 epidermoid carcinoma cells underwent dynamic interactions with laminin whereas β_4 integrin-negative MCF-7, T-47D, HS-578t and MDA-231 breast carcinoma cells did not.

DISCUSSION

In this study, the use of laminar flow assays enabled us to uncover a novel mode of integrin-mediated attachment to extracellular matrix. Specifically, we observed that epithelial and carcinoma cells attached to laminin in the presence of flow ($\tau \leq 2.0 \text{ dyn}/\text{cm}^2$) and that increasing this flow ($3.5 \text{ dyn}/\text{cm}^2 \leq \tau \leq 100 \text{ dyn}/\text{cm}^2$) resulted in the rolling of these cells on laminin for relatively long distances. The ability of cells to engage in these dynamic interactions with laminin correlated with the expression of the β_4 integrin subunit. Additional experiments using function-blocking antibodies enabled us to conclude that this form of dynamic adhesion is mediated by the $\alpha_6\beta_4$ integrin and that it does not involve β_1 integrin receptors, which are also expressed by these cells. To our knowledge this is the first time an integrin has been shown to mediate rolling in the presence of flow. The results substanti-

Table 3. Correlation of β_4 integrin expression and ability to roll on laminin

Cell name	Cell type	β_4 Expression	Rolling
MCF-10A	Breast epithelial	+	+
		Present study	
Clone A	Colon carcinoma	+	+
		Lee et al. (1992)	
RKO	Colon carcinoma	-	-
		Lee et al. (1992)	
HT-29	Colon carcinoma	+	+
		Simon-Assmann et al. (1994)	
MCF-7	Breast carcinoma	-	-
		Sommers et al. (1994)	
HS 578t	Breast carcinoma	-	-
		Sommers et al. (1994)	
MDA-231	Breast carcinoma	-	-
		Sommers et al. (1994)	
T-47D	Breast carcinoma	-	-
		Sommers et al. (1994)	
A431	Epidermoid carcinoma	+	+
		Tamura et al. (1990)	

Cells were allowed to incubate in the flow chamber on a laminin-coated glass coverslip for 5 minutes under static conditions. Cell medium was then infused into the flow chamber for 1 minute at $\tau=14$ dyn/cm². The (+) signs in the 4th column indicate those cell lines for which 50% of the cells that detached from laminin rolled at least 5 cell diameters before gaining the speed of flow. The (-) signs denote those cell lines for which detachment from laminin with the imposition of flow was instantaneous.

ate the hypothesis that β_4 and β_1 integrin laminin receptors mediate distinct functions.

The fact that laminin supported cell attachment in the presence of flow indicates a rapid rate of formation of adhesive contacts because shear flow severely limits the time during which cell surface receptors form contacts with a substratum (Goldman et al., 1967). MCF-10A, clone A and other β_4 integrin-expressing cells attached to laminin in the presence of fluid shear stress comparable to that found in blood venules. This dynamic adhesion is specific, because MCF-10A and clone A cells did not attach to glass coverslips, uncoated or coated with either fibronectin or bovine serum albumin, under these conditions.

Laminin, similar to E-selectin and P-selectin, supported cell rolling in the presence of laminar flow. A most striking aspect of this rolling interaction is that the cycles of rapidly formed attachments and synchronized detachments occurred at high flow rates ($\tau=100$ dyn/cm²). These data provide useful information on the characteristics of the adhesion receptors that mediate these interactions. Specifically, they indicate that the adhesive contacts formed during rolling are capable of resisting large forces, but only for brief durations before they break. The fluid force on a MCF-10A cell rolling on laminin in simple shear flow can be calculated as described by Tozeren and Ley (1992). This force is approximately equal to 2.5×10 dyn for an MCF-10A cell (15 μ m in diameter) rolling on laminin (10 μ m/s) at $\tau=100$ dyn/cm². This is two to three orders of magnitude higher than the estimates for the maximum force resisted by molecular point-attachments (Bell, 1978; Evans et al., 1991), suggesting that a large number of adhesion bonds are formed at laminin-cell binding sites.

Cell attachment to laminin in the presence of flow and flow-initiated rolling are mediated by $\alpha_6\beta_4$ integrin, although the cell lines used in this study express both $\alpha_6\beta_4$ and $\alpha_2\beta_1$ integrin

laminin receptors. This conclusion is based primarily on the ability of α_6 -specific antibodies, but not β_1 -specific antibodies, to inhibit dynamic attachment and flow-initiated rolling. Additional evidence is provided by our finding that only those cell lines that express the β_4 subunit were capable of dynamic interactions with laminin. The possibility that laminin interaction with $\alpha_6\beta_4$ is only a signaling event and another receptor is responsible for dynamic adhesion is rather unlikely, since the infusion of an anti- α_6 mAb into the flow chamber and onto MCF-10A and clone A cells briefly incubated on laminin led to the detachment of these cells from laminin within few seconds.

The existence of a mechanical connection among extracellular matrix components, integrins and cell cytoskeleton was recently illustrated (Wang et al., 1993). Such connections may be needed for rolling interactions to occur. For example, it is possible that the cytoplasmic domain of integrin $\alpha_6\beta_4$ may play a role in the detachment of rolling cells from laminin by modulating the affinity of the β_4 integrin to laminin. Consistent with this, the β_4 subunit cytoplasmic domain was shown to mediate the interaction of $\alpha_6\beta_4$ integrin with the cytoskeleton of hemidesmosomes (Spinardi et al., 1993). The β_4 cytoplasmic domain (>1000 amino acids) contains multiple sites for phosphorylation and for interaction with other signaling molecules (Tamura et al., 1990; Giancotti et al., 1992).

Similar to variants of the β_4 integrin subunit, variants of the α_6 integrin that differ in their cytoplasmic domain have been described (Tamura et al., 1991; Cooper et al., 1991). Most adult cells express the α_6A variant and often co-express α_6B whereas embryonic stem cells express the α_6B variant, only turning on the α_6A variant upon differentiation (Cooper et al., 1991). A-431 epidermal carcinoma cells express β_4 and only the α_6A variant of the α_6 integrin subunit (Tamura et al., 1991). Since these cells attached to and rolled on laminin in the presence of flow (Table 3), these results indicate that the α_6A integrin variant can support dynamic interactions with laminin.

Epithelial and carcinoma cells are likely to utilize a variety of molecular mechanisms in flow-mediated interactions with various substrata. For example, HT-29 cells that express integrin $\alpha_6\beta_4$ but not $\alpha_6\beta_1$ (Simon-Assmann et al., 1994) roll both on laminin (Table 3) and on cytokine-stimulated human umbilical cord vein endothelial cell monolayers (IL-1-HUVEC) (Glavazzi et al., 1993). A neutralizing mAb against E-selectin inhibits dynamic interactions of HT-29 cells with IL-1-HUVEC (Glavazzi et al., 1993) whereas the same mAb has no effect on dynamic interactions with laminin (our unpublished observations). Furthermore, $\alpha_6\beta_4$ -negative T-47D breast carcinoma cells interact dynamically with IL-1-HUVEC (Glavazzi et al., 1994) but not with laminin (Table 3). These results show that some carcinoma cells express multiple cell rolling receptors and that the rolling receptor is substratum-specific.

The results obtained here permit the speculation that $\alpha_6\beta_4$ integrin has a regulatory effect on $\alpha_2\beta_1$ integrin in clone A cells. Specifically, we propose that at early stages of cell contact with laminin integrin $\alpha_6\beta_4$ facilitates the ability of $\alpha_2\beta_1$ integrin to increase the strength of adhesion. This possibility is supported by the following observations: (1) $\alpha_6\beta_4$ -mediated adhesion of clone A cells to laminin occurs more rapidly than $\alpha_2\beta_1$ -mediated adhesion. (2) The $\alpha_6\beta_1$ -negative clone A cells incubated with an α_6 -specific mAb do not adhere to laminin

after 5 minutes of incubation but clone A cells treated with a β_1 -specific mAb do adhere, although their strength of adhesion is diminished. (3) Lee et al. (1992) reported that integrin $\alpha_2\beta_1$ was active during initial interactions of clone A cells with laminin when $\alpha_6\beta_4$ was not blocked by an antibody. Integrin $\alpha_2\beta_1$ should still be active in the presence of an α_6 -specific mAb if this receptor functions independently of $\alpha_6\beta_4$. It is this paradox that raises the possibility that integrin $\alpha_6\beta_4$ exerts a regulatory effect on $\alpha_2\beta_1$. Our observation that clone A cells treated with an anti- α_6 mAb adhere to laminin after 20 minutes of static incubation indicates that given sufficient time β_1 integrins are capable of forming firm adhesive contacts with laminin, independently of β_4 integrin.

The most likely biological consequence of dynamic interactions with laminin would be the facilitation of cell migration. Integrin $\alpha_6\beta_4$ has recently been implicated to be involved in epithelial cell migration during wound healing. Kurpakus et al. (1991) showed that $\alpha_6\beta_4$ integrin is distributed throughout the cell surface in epithelial and carcinoma cells migrating from the edges of wounded sites into the bare connective tissue, rather than being polarized in fixed adhesion junctions called hemidesmosomes. Rolling interactions and cell migration both involve similar physical events, such as the formation of adhesion bonds at the leading edge of conjugation and the synchronized detachment of bonds at the trailing edge of the cell. Consistent with these observations, recent studies have suggested a correlation between maximal migration and reversibility of cell-substratum adhesion (Calof and Lander, 1991; Dubond et al., 1991; DeMilla et al., 1993).

In conclusion, laminar flow assays were used to establish that laminin supports rapidly formed and transient interactions with some $\alpha_6\beta_4$ -expressing epithelial and carcinoma cells. Adhesion of these cells to laminin assumes the form of rolling in the presence of laminar flow. Integrin $\alpha_6\beta_4$ was shown to mediate the dynamic aspects of MCF-10A and clone A cell adhesion to laminin.

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