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Role of extracellular matrix proteins in regulation of human glioma cell invasion *in vitro*

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Primary brain tumors lack the metastatic behavior that is in part believed to be promoted by the extracellular matrix (ECM) components of the basement membrane. This study was intended to examine the influence of the ECM components present in the basement membrane that may act as natural barriers to tumor cell invasion. We examined the effect of type I and type IV collagens, fibronectin, laminin, and hyaluronic acid on the migration and invasion of four established glioblastoma cell lines, SNB19, U251, UWR1, and UWR2. Lower concentrations of all the ECM components induced the migration and invasion of all the cell lines. However, in the case of SNB19, laminin inhibited both migration and invasion in a concentration-dependent manner. We have also examined the influence of individual ECM components on the migration of cells from a spheroid to a monolayer on ECM component-coated coverslips. Consistent with the invasion studies using the modified Boyden chamber assays, lower concentrations of ECM components induced the migration of cells from spheroids to monolayer. Again, laminin inhibited the migration of cells from SNB19 spheroids. These results indicate that ECM components induce the invasion of glioma cells, apart from components like laminin, which may act as natural inhibitors.

Keywords: collagen, extracellular matrix, glioma, invasion, migration, spheroid

Introduction

Gliomas, the most common type of human brain tumors, are characterized by their ability to be highly invasive [1]. Gliomas display a wide variety of distinct histological appearances. All of the known glial tumors are highly invasive of the surrounding brain [2,3]. However, little is known about the factors responsible for this invasive behavior. Invasion is a complex process in which the cells initially adhere to the immediate extracellular matrix (ECM), gain migratory capacity, and invade the local environment by remodeling the ECM with the aid of proteolytic enzymes [4,5]. Glial limitans externa and cerebral vasculature contain types I, III, and IV collagens, laminin, fibronectin, and glycosaminoglycans [6–11]. However, normal brain lacks a defined structural ECM and all of these ECM proteins are confined to the basement membrane in the vasculature [12]. It is not known whether the cells utilize constitutive ECM for the invasion or synthesize autologous ECM for invasion. It has been shown that primary brain tumors express ECM proteins and several glioma cell lines have been shown to produce ECM proteins in cell culture [13–16]. The concept exists, however, that glioma cell lines migrate in response to natural anatomical determinations rather than by aggressively remodeling native structures, as most basement membrane structures are preserved, even in the case of the most

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malignant gliomas [17]. In this study, purified individual ECM components, such as type I and type IV collagens, fibronectin, laminin, and hyaluronic acid were used as substrates for the evaluation of invasion of human glioma cell lines *in vitro*. We compared three assays, chemotactic migration of cells, invasion of the cells through a reconstituted basement membrane (Matrigel) and the migration of cells from a spheroid to a monolayer culture system.

Materials and methods

Materials

Type I (human) and type IV collagens (human), laminin (human) and fibronectin (human) were obtained from Life Technologies (Gaithersburg, MD, USA). Hyaluronic acid (umbilical cord) was purchased from Boehringer Mannheim (Germany).

Cell cultures

Established human glioma cell lines SNB19 (kindly provided by Dr Morrison, Houston, TX, USA), U251 (kindly provided by Dr Yung, Houston, TX) [18], UWR1 and UWR2 (kindly provided by Dr Ali-Osman, Houston, TX) [19] were used in this study. SNB19 and U251 cells were cultured in high glucose DMEM/F-12 (1:1 ratio), supplemented with 15% fetal bovine serum, 20 mM HEPES, penicillin (100 U/ml), and streptomycin (100 μg/ml). UWR1 and UWR2 cells were cultured in low-glucose DMEM, supplemented with 25% fetal bovine serum and 50 μg/ml gentamicin. The cells were cultured in 100 mm^2 tissue culture plates in a 5% CO_2 humidified incubator at 37°C.

Migration assay

Cell migration was monitored by a chemotactic assay in Boyden chambers. Each lower compartment of the chamber was filled with 100 μl of varying concentrations of the ECM component, diluted in serum-free medium, and overlaid with 8 μm polyvinyl pyrrolidone-free polycarbonate (PVPFP) membranes (Costar, Cambridge, MA, USA), and the upper compartment was assembled. Cells were trypsinized and counted, and 200 μl of a suspension of 2×10^5 cells/ml in serum-free medium was placed in the upper compartment of the Boyden chamber. Cells were incubated in a 5% CO_2 humidified incubator at 37°C for 24 h, and, at the end of the migration assay, filters were removed, fixed, and stained with Hema-3 stain (CMS, Houston, TX, USA). Filters were washed with distilled water, and cells on top of the filter were removed with a cotton swab. The number of cells that had migrated to the lower side of the filter were counted under a light microscope at × 400. Each experiment was performed in triplicate, and at least 10 fields were counted in each experiment.

Invasion assay

Invasion of the glioma cells *in vitro* was measured by the invasion of cells through Matrigel (Collaborative Research Inc., Boston, MA, USA) in a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, MD, USA). For the invasion assay, 25 × 80 mm PVPFP membranes with a 8-μm pore size were coated with a final concentration of 0.78 mg/ml of Matrigel in cold serum-free DMEM to form a continuous layer on top of the filter. Lower wells of the 48-well chemotaxis chamber were filled with 25 μl of serum-free medium containing different concentrations of individual ECM proteins. The chemotaxis chamber was assembled by placing the Matrigel-coated membrane between the lower and upper chamber according to the manufacturer's instructions. Cells were trypsinized, and 50 μl of a suspension of 2×10^5 cells/ml was placed in individual wells of the upper compartment. The invasion assay was carried out at 37°C in a 5% CO_2 humidified incubator for 24–48 h. At the end of the invasion assay, filters were removed, fixed, and stained with Hema-3 stain. Cells that invaded through the Matrigel to the lower side of the filter were counted as described for the migration assay above. All the experiments were done in triplicate, and at least 10 fields were counted for each experiment.

Adhesion assays

Adhesion assays were performed in 96-well tissue culture plates. Plates were pre-coated with 5 μg/ml of different ECM proteins in phosphate buffered saline (PBS) at room temperature for 2 h. The wells were blocked with 1 mg/ml of bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO, USA) in PBS for 1 h at room temperature. Cells were trypsinized, resuspended in DMEM containing 10% fetal bovine serum, and allowed to recover for 1 h. Cells were centrifuged and resuspended in serum-free DMEM to a final concentration of 2.4×10^5 cells/ml and 100 μl of cell suspension was added to each well. Cells were incubated at 37°C for 2 h. At the end of the assay, wells were washed gently with PBS, fixed and stained with Hema-3 stain solution-II (CMS). The dye was extracted in 10% methanol, 5% acetic acid and the absorbance was read at 650 nm on a ELISA plate reader. A linear and repro-

ducible relationship between cell number and absorbance at 650 nm was observed for all the cell lines used.

Glioma spheroid formation

Glioma spheroids were cultured in 100-mm^2 tissue culture plates (Corning Inc., Corning, NY, USA) pre-coated with 0.75% agar (Difco Laboratories Inc., Detroit, MI, USA) prepared in DMEM/F-12 medium for SNB19 and U251 cells or in low-glucose DMEM for UWR1 and UWR2 cells according to a previously described procedure [20]. Briefly, 3×10^6 cells were suspended in the respective medium and seeded onto 0.75% agar plates and cultured until spheroids were formed. Spheroids of 100–200 μm diameter were selected for use in further experiments.

Migration of cells from spheroids

Glass coverslips (Dynalab, Rochester, NY, USA) were coated with 5 μg/ml of ECM components or uncoated coverslips were used as control. ECM proteins were coated onto the coverslips by placing varying concentrations in PBS (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) in a six-well plate and allowed to air dry. Coverslips were washed several times with sterile PBS, followed by distilled H_2O and air dried. Coated or uncoated coverslips were placed into sterile 24-well culture plates, and 200 μl of serum-free medium was added to each well. Single spheroids were placed into the center of each well of a 24-well plate that had ECM protein-coated or -uncoated coverslips. Another 800 μl of serum-free medium was then added to each well. Spheroids were cultured in a 5% CO_2 humidified incubator at 37°C for 48 h, and a total of six to eight spheroids were plated for each experiment. At the end of the migration assay, spheroids were fixed and stained with Hema-3 stain and mounted onto glass microscope slides. The increase in diameter of the spheroids and the migration of cells from the spheroids were measured under a light microscope calibrated with a stage and ocular micrometer.

Results

In this study we examined the individual basement membrane components, type I collagen, type IV collagen, fibronectin, laminin, and hyaluronic acid, on the migration and invasion of four human glioma cell lines. All the ECM components induced varying patterns of migration of SNB19, U251, UWR1, and UWR2 cells *in vitro*. Chemotactic migration of SNB19 cells was induced by all the ECM components tested except laminin (Figure 1a). Among the ECM proteins, fibronectin induced maximum migration at 10 μg/ml concentration. SNB19 cells migrated in a dose-dependent fashion in response to type I collagen up to 10 μg/ml and thereafter reached a plateau even with increasing concentrations up to

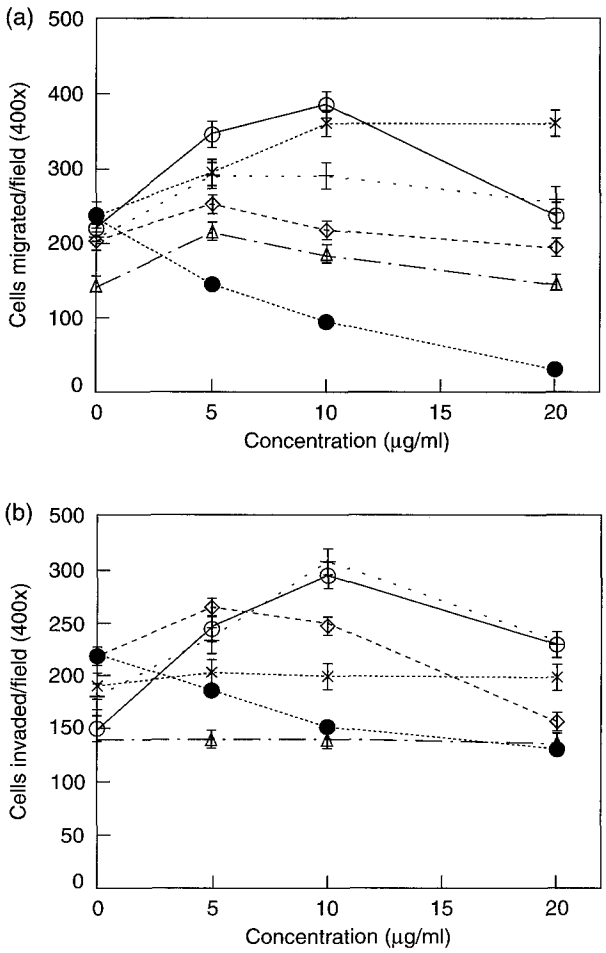

Figure 1. (a) Migration of SNB19 cells in response to fibronectin (○), laminin (●), hyaluronic acid (◇), type I collagen (×), type IV collagen (+) and Matrigel (△). Migration was assayed by the Boyden chamber method. Indicated concentrations of ECM proteins were dispensed into the lower well of the Boyden chamber and 2×10^5 cells/ml of SNB19 cells were added to the upper compartment. Cells were allowed to migrate through 8 μm pore size membrane, fixed, stained and counted. Results are the mean number of cells migrated ± S.D. (b) Invasion of SNB 19 cells in response to fibronectin (○), laminin (●), hyaluronic acid (◇), type I collagen (×), type IV collagen (+) and Matrigel (△). Invasion was measured in a 48-well microchemotaxis chamber. Cells were allowed to invade through 8 μm pore size membranes, precoated with Matrigel, in response to different ECM proteins, fixed, stained and counted. Results are mean number of cells invaded ± S.D.

40 μg/ml (results not shown). In contrast to other ECM components, laminin significantly inhibited migration in a dose-dependent fashion (Figure 1a). Type IV collagen, hyaluronic acid, and reconstituted basement membrane Matrigel induced the migration (over control) at 5 μg/ml concentration.

Invasion of SNB19 cells through Matrigel is shown in Figure 1b. SNB19 cells invaded more in response to fibronectin at 10 μg/ml concentration. The cells showed a similar migration pattern with type IV collagen fibronectin. Type I collagen and Matrigel had no significant effect on the invasion of cells through Matrigel, while hyaluronic acid had a moderate effect. Again, compared to the other ECM proteins, laminin inhibited the invasion of SNB19 cells in a dose-dependent fashion.

Chemotactic migration of U251 cells was induced by all the ECM components at 10 μg/ml concentration (Figure 2a). On the other hand, invasion of U251 cells through Matrigel varied with different ECM proteins (Figure 2b). Type I and type IV collagen showed maximum effect on invasion of cells at 10 μg/ml, while laminin and fibronectin had their highest effect at 5 μg/ml concentration.

In the case of UWR1 cells, the majority of the ECM proteins allowed maximum migration (Figure 3a) and invasion (Figure 3b) at 10 μg/ml concentration, except for laminin, which showed maximum cell migration at 5 μg/ml. However, in UWR2 cells lower concentrations of all ECM components induced migration (Figure 4a). Invasion of UWR2 cells was induced by laminin and type IV collagen at 5 μg/ml, and the rest of the ECM proteins induced maximum invasion at 10 μg/ml (Figure 4b).

In all of the above migration and invasion experiments, no cells were found on the lower side of the filter when individual ECM components were placed in the upper compartment of the Boyden chamber or 48-well chemotaxis chamber (results not shown), confirming the observation that cell movement is not random but is in response to individual ECM components.

We reasoned that the cell's ability to migrate or invade may be dependent on the differential adhesion to individual ECM components. Adhesion assays were performed in which SNB19, U251, UWR1 and UWR2 cells were allowed to attach to 96-well plates coated with ECM components. All the cell lines attached to the ECM components (Figure 5) tested. However we did not observe a significant change in adhesion of cells among different ECM components. Minimal adhesion was found when the plates were pre-coated with BSA, which is used as a negative control in adhesion assays.

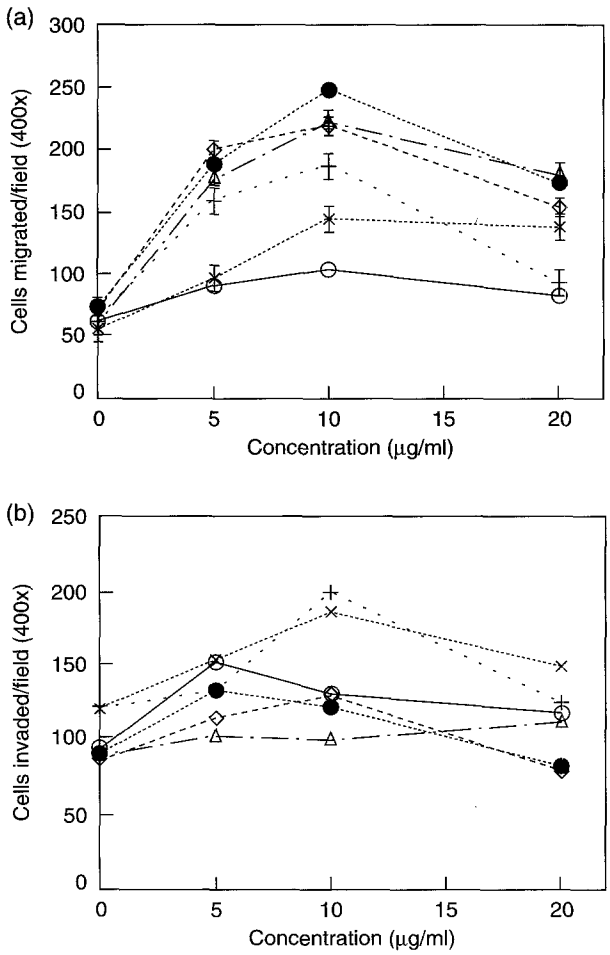

Figure 2. (a) Migration of U251 cells in response to fibronectin (○), laminin (●), hyaluronic acid (◇), type I collagen (×), type IV collagen (+) and Matrigel (△). Migration was assayed by the Boyden chamber method. Indicated concentrations of ECM proteins were dispensed into the lower well of the Boyden chamber and 2×10^5 cells/ml of U251 cells were added to the upper compartment. Cells were allowed to migrate through the 8 μm pore size membrane, fixed, stained and counted. Results are the mean number of cells migrated ± S.D. (b) Invasion of U251 cells in response to fibronectin (○), laminin (●), hyaluronic acid (◇), type I collagen (×), type IV collagen (+) and Matrigel (△). Invasion was measured in a 48-well microchemotaxis chamber. Cells were allowed to invade through 8 μm pore size membrane, precoated with Matrigel, in response to different ECM proteins, fixed, stained and counted. Results are mean number of cells invaded ± S.D.

In the cell migration assay from spheroids, the spheroids were transferred to individual ECM-coated coverslips in a 24-well tissue culture plate, and migration into the monolayer was measured after 24 and 48 h. The spheroids were initially attached to the ECM-coated coverslips as well as to

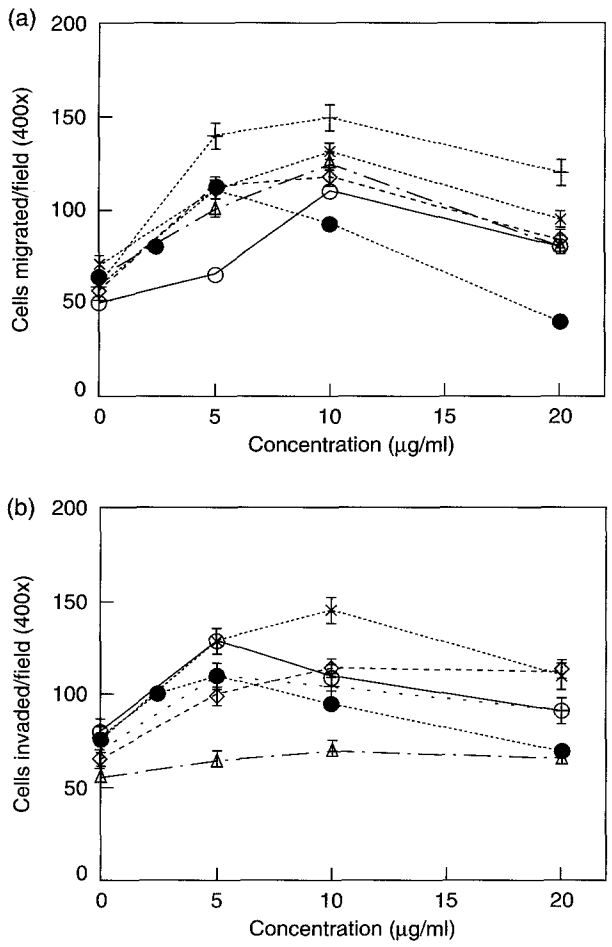

Figure 3. (a) Migration of UWR1 cells in response to fibronectin (○), laminin (●), hyaluronic acid (◇), type I collagen (×), type IV collagen (+) and Matrigel (△). Migration was assayed by the Boyden chamber method. Indicated concentrations of ECM proteins were dispensed into the lower well of the Boyden chamber and 2×10^5 cells/ml of UWR1 cells were added to the upper compartment. Cells were allowed to migrate through the 8 μm pore size membrane, fixed, stained and counted. Results are the mean number of cells migrated ± S.D. (b) Invasion of UWR1 cells in response to fibronectin (○), laminin (●), hyaluronic acid (◇), type I collagen (×), type IV collagen (+) and Matrigel (△). Invasion was measured in a 48-well microchemotaxis chamber. Cells were allowed to invade through 8 μm pore size membrane, precoated with Matrigel, in response to different ECM proteins, fixed, stained and counted. Results are mean number of cells invaded ± S.D.

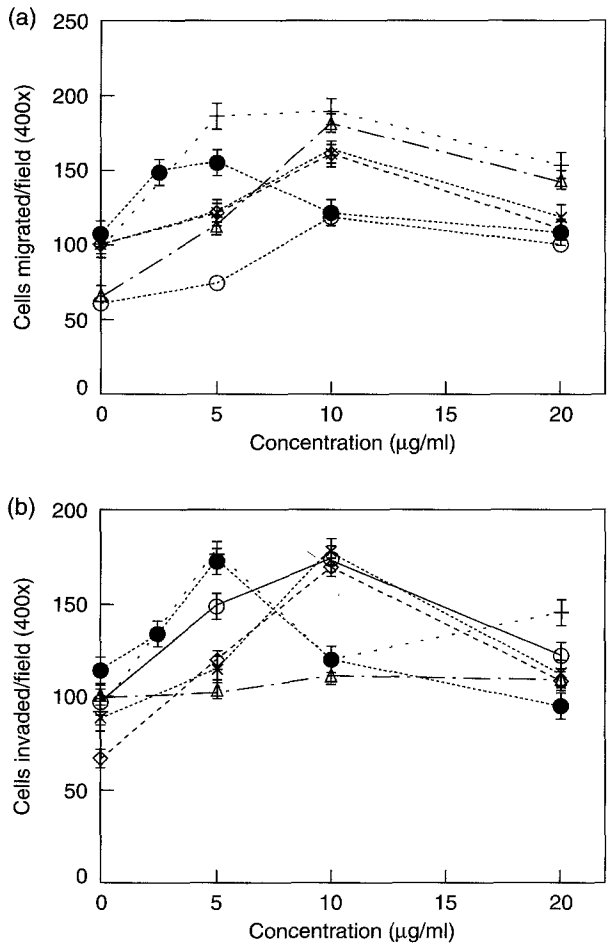

Figure 4. (a) Migration of UWR2 cells in response to fibronectin (○), laminin (●), hyaluronic acid (◇), type I collagen (×), type IV collagen (+) and Matrigel (△). Migration was assayed by the Boyden chamber method. Indicated concentrations of ECM proteins were dispensed into the lower well of the Boyden chamber and 2×10^5 cells/ml of UWR2 cells were added to the upper compartment. Cells were allowed to migrate through the 8 μm pore size membrane, fixed, stained and counted. Results are the mean number of cells migrated ± S.D. (b) Invasion of UWR2 cells in response to fibronectin (○), laminin (●), hyaluronic acid (◇), type I collagen (×), type IV collagen (+) and Matrigel (△). Invasion was measured in a 48-well microchemotaxis chamber. Cells were allowed to invade through 8 μm pore size membrane, precoated with Matrigel, in response to different ECM proteins, fixed, stained and counted. Results are mean number of cells invaded ± S.D.

control uncoated coverslips. Migration of the cells from SNB19 spheroids was induced by type I collagen (Figure 6B), type IV collagen (Figure 6D), and fibronectin (Figure 6E) as compared to control (Figure 6A). In contrast, laminin significantly inhibited the migration of cells from SNB19 spheroids (Figure 6C), consistent with the earlier results shown in Figure 1a. The control spheroids showed few SNB19 cells migrating out even after 48 h. In contrast to SNB19 cells, laminin induced the migration of cells from UWR1 and UWR2 spheroids (Figure 7). The migration pattern of cells from

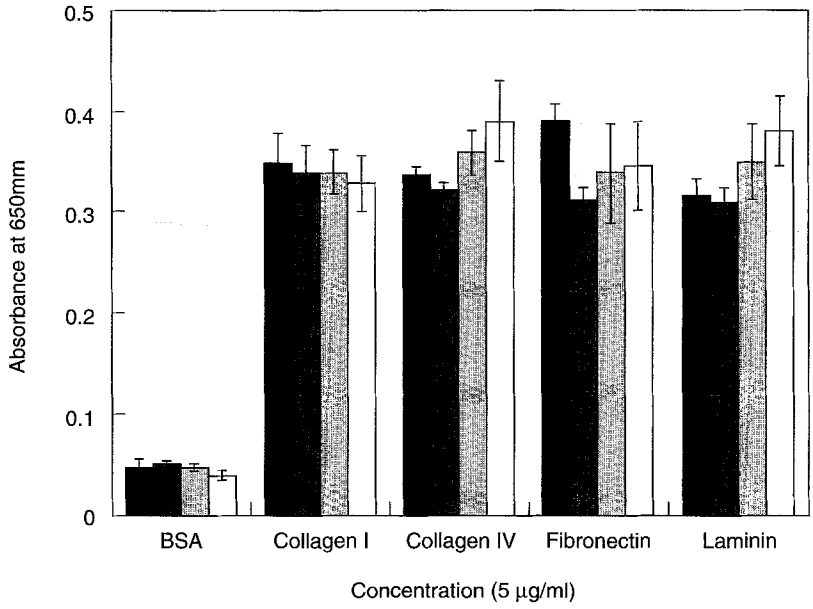

Figure 5. Adhesion of SNB19 (solid bar), U251 (hatched bar), UWR1 (dotted bar) and UWR2 (open bar) cell lines to different ECM proteins. Cells were trypsinized, allowed to recover for 1 h in serum containing DMEM, washed, resuspended in serum-free DMEM, and then incubated in 96-well tissue culture plates pre-coated with 5 μg/ml of each individual ECM protein. After 2 h, unattached cells were removed by washings with PBS and attached cells were measured as described in 'Materials and methods'.

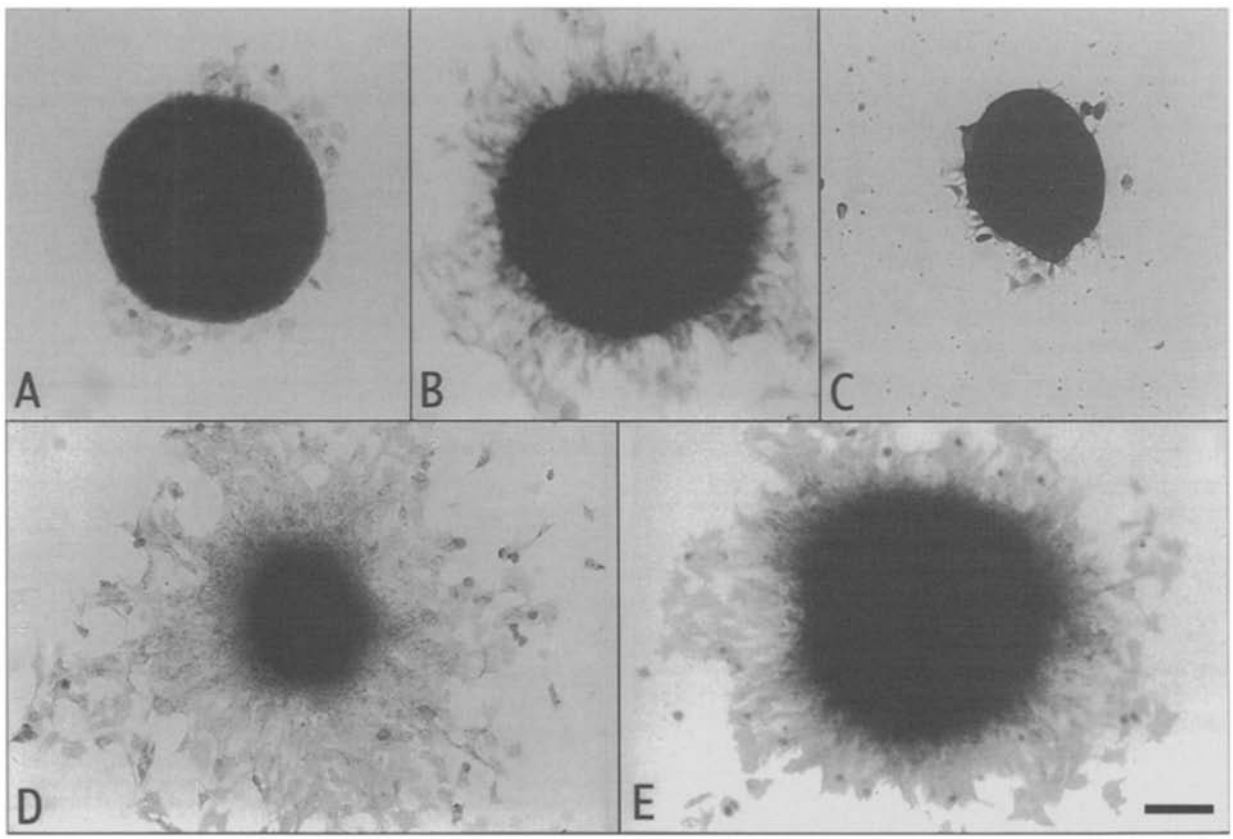

Figure 6. Influence of different ECM components on the migration of SNB19 cells from spheroids to monolayers. Spheroids were transferred to coverslips coated with different ECM proteins and incubated for 48 h, fixed, and stained as described in 'Materials and methods'. The panels show type I collagen (B), laminin (C), type IV collagen (D), fibronectin (E) or without ECM proteins (A). Bar = 800 μm.

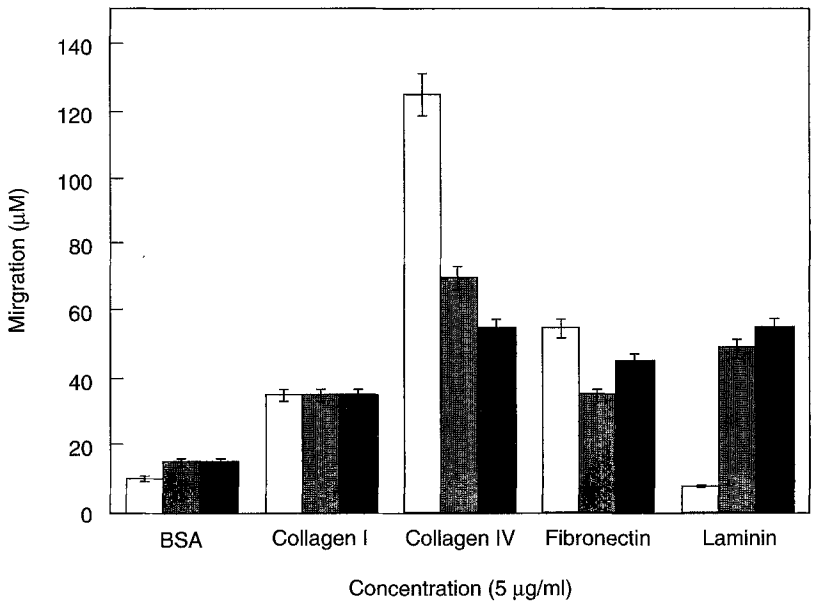

Figure 7. Influence of different ECM proteins on cell migration from SNB19 (open bar), UWR1 (hatched bar) and UWR2 (solid bar) spheroids. Spheroids were transferred to ECM-coated coverslips and cell migration was allowed for 48 h. Cells were fixed and stained and the average cell migration from the spheroids was measured with a microscope calibrated with ocular and stage micrometer.

UWR1 spheroids is different from that of SNB19 and UWR2 spheroids. These cells showed a random streaming migration from the spheroids at lower concentrations of all the ECM components (results not shown).

Discussion

In this study we demonstrated that the ECM proteins type I and type IV collagen, fibronectin, laminin, and hyaluronic acid induced cell migration and invasion in four glioma cell lines. We could not find a significant difference in adhesion properties of the cells to different ECM components studied. These results indicate that the glioma cell lines show a preferential migration on specific ECM proteins and that the substrate preference depends on the cell line used. Most of the cell lines showed preferential migration on type IV collagen and fibronectin, whereas laminin decreased the migration and invasion of the SNB19 cells.

The ECM contains collagens, non-collagenous glycoproteins, and proteoglycans that are unique to different cell types and organs. The ECM proteins type I, III and IV collagen, fibronectin, and laminin are confined to the basal lamina of blood vessels and to the glial limitans externa in the brain [12,16,21]. Immunohistochemical analysis by Bjerkvig *et al.* [15] showed that glioma cells lines express type I and type IV collagen, and fibronectin. Although the developing central and peripheral nervous system contains these ECM proteins, it has not been documented whether the glial cells synthesize the ECM proteins and use them for invasion of the normal brain. It has been reported that the primary brain tumors express ECM proteins both *in vitro* and *in vivo* [22–26], and our own work has shown that primary brain tumors do express laminin, fibronectin, and type IV collagen (Chintala *et al.*, unpublished observation).

In this study we demonstrated that the basement membrane proteins, including the reconstituted basement membrane Matrigel, induced the migration and invasion of human glioma cells, which is consistent with earlier observations [27]. Another study of Merzak *et al.* [28] showed gangliosides increase the invasive behavior of human glioma cells isolated from different grades of gliomas. Among the ECM proteins, fibronectin at lower concentrations induced both the migration and invasion of SNB19 cells (Figure 1a, b). Type I collagen induced migration at lower concentrations; however, invasion was not affected even at higher concentrations. Matrigel, on the other hand, induced moderate migration but did not show any effect on invasion. This could be due to the cells attaching and spreading more on top of the filter where Matrigel was coated instead of invading through the filters toward Matrigel in solution. Type IV collagen induced the invasion of SNB19 cells, similar to fibronectin. In contrast to all other ECM proteins, laminin decreased both the migration and invasion of SNB19 cells. This is similar to an earlier report that laminin inhibits the invasion of rat glioma cells [29]. On the other hand, Koocheckpour *et al.* [27] showed that laminin is the most potent chemoat-

tractant of human glioma cells *in vitro*. However, in our study, laminin at lower concentrations did not inhibit the migration and invasion of the other cell lines, U251, UWR1 and UWR2. This discrepancy in cell behavior could depend on the individual cell lines used, and does not necessarily indicate that laminin inhibits migration in general. The inhibition of cell migration and invasion of SNB19 cells is not due to the toxic effect of laminin; this is ruled out by the observation that laminin-treated SNB19 cells, when placed into the regular medium, grow well and invade well in response to the other ECM components (results not shown).

Adhesion controls like integrins may be involved in the invasion of human glioma cells. Indeed, a recent study by Yamamoto *et al.* [30] showed that glioma cells migrated in response to fibronectin and vitronectin. They also found a positive correlation with the expression of α5 or αV integrins and migration in response to fibronectin and vitronectin, respectively. However, there was no correlation between the cell's ability to adhere to fibronectin or vitronectin and the integrin expression.

High expression of laminin in central and peripheral nervous system neoplasms has been documented [31]. We also found that laminin is expressed in basement membrane of blood vessels in glioma sections and to some extent in normal brain (Chintala *et al.*, unpublished observation). It is possible that the primary glial cells invade in response to the existing laminin in the basement membrane. However, human glial tumor cells also express laminin in cell culture [28]. Along with laminin, fibronectin is expressed in the basement of normal and neoplastic nervous system cells [16,32].

Tumor cell movement can be quantitatively measured by culturing cells to form spheroids [33]. We found that the ECM proteins differentially affected the migration of glioma cells from the spheroids (Figure 6). Cell migration from SNB19 spheroids was induced by collagens I and IV and fibronectin. However, type IV collagen had a more pronounced effect on cell migration compared with type I collagen and fibronectin. Laminin, on the other hand, significantly inhibited the migration of cells from SNB19 spheroids (Figure 6C), consistent with the observation made by Reith *et al.* [29]; from UWR1 and UWR2 spheroids, however, laminin increased migration of the cells. Few cells migrated out from the control spheroids even after 48 h.

In our study, type IV collagen induced a uniform streaming out growth pattern of the cells, whereas type I collagen induced a random outgrowth of cells (in UWR1 spheroids). Type IV collagen induction of migration in all the cell lines suggests that glioma cells invade normal brain in response to the chemotactic attraction of type IV collagen. Moreover, the invasion of glioma cells is often observed as a perivascular migration. Inhibition of cell migration by laminin, in the case of SNB19, is an interesting observation; it may be possible that laminin present in the basement membrane inhibits the metastasis of invading tumor cells [29]. To further investigate the inhibitory effect of laminin, SNB19 spheroids were allowed to adhere to fibronectin or type IV collagen-coated coverslips and laminin was then added at a concentration of 10 μg/ml in serum-free medium and migration experiments were carried out for 48 h. Interestingly, laminin significantly inhibited the migration of cells compared to the cell migration on type IV collagen or fibronectin pre-coated coverslips (results not shown). On the other hand, when type IV collagen or fibronectin (10 μg/ml) were added to spheroids adherent to laminin-coated coverslips, cell migration was induced compared to the cells on laminin pre-coated coverslips alone (results not shown). These observations indicate that laminin inhibits the migration of cells from spheroids in the case of SNB19 cells. However, the precise mechanism by which laminin inhibits migration is not known at this time.

Our results indicate that the ECM components induce both the migration and invasion of glioma cell lines, though with obvious individual differences among the cell lines used. The results are comparable for both migration and invasion through 8-μm PVPFP membranes and cell migration into monolayer from tumor cell spheroids.

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