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Extracellular matrix-induced cell migration from glioblastoma biopsy specimens in vitro

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Abstract The present knowledge about the interaction between the extracellular matrix (ECM) and gliomas is mostly based on studies of permanent cell lines. Since such cultures have undergone an extensive clonal selection in vitro, the experimental results obtained may be quite different from those obtained from studies on true biopsy specimens. The present work demonstrates how different ECM components affect tumor cell migration from human glioblastoma specimens grown as biopsy sample spheroids. Biopsy specimens from 12 glioblastomas and 1 gemistocytic astrocytoma were included in this study. Spheroids were directly initiated from the biopsy specimens, and after 3–4 weeks in culture, they were used in a migration assay. A custom-made filtered medium, where the high molecular weight (> 100 kDa) proteins were removed, was supplemented with the following ECM components: laminin, fibronectin, collagen type IV and vitronectin. The cell migration was negligible when spheroids were propagated in the filtered medium. The ECM components as well as complete DMEM evoked strong stimulatory effects on different biopsy specimens. Opposed to that observed earlier for permanent glioma cell lines, highly variable responses were observed between the different biopsy samples on the various ECM components. In general, correlation analyses revealed that specimens that were strongly stimulated by laminin were also stimulated strongly by fibronectin, collagen type IV and vitronectin. This suggests that the capacity to migrate as a response to ECM was confined more to each biopsy specimen than to any specific ECM component. Since biopsy sample spheroids, as original tumors, consist of different cell types, an immunohistochemical characterization of the migrating cells was also performed. Antiglial fibrillary acidic protein (GFAP) staining revealed both GFAP-positive and -negative migrating cells. Immunostaining for von Willebrand factor and CD11b indicated that the migrating cells were neither endothelial nor microglial cells. This study, therefore, indicates that migratory responses of glioma biopsy specimens to different ECM components is much more heterogeneous than that observed earlier for cell lines. Furthermore, the presented findings support the notion that gliomas may utilize different cell surface receptors for their migration, depending on the cell substrates available.

Key words Extracellular matrix · Biopsy · Glioblastoma · Cell migration · Integrins

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Introduction

Local invasiveness, which profoundly complicates clinical management, is one of the hallmarks of malignant gliomas. The glioma cells infiltrate the surrounding brain tissue and eventually give rise to recurrent tumors despite radical surgery. Invasion is a highly complex process which involves tumor cell attachment to the extracellular matrix (ECM) components via cell surface receptors, degradation of ECM by hydrolytic enzymes and subsequent tumor cell locomotion [9]. It has previously been shown that several ECM components, especially laminin, play an important role during glioma cell migration [1, 4, 11]. In these studies, the interactions between glioma cells and ECM were investigated using permanent cell lines. However, it has been shown that the cellular phenotypes change when the cells adapt to the situation in vitro. For instance, Paulus et al. [17] showed that different culture systems can be characterized by distinct expression patterns of ECM components and corresponding cell surface receptors, and that the mesenchymal features found in cultured glioma cells arise due to a specific transdifferentiation observed during culture. This latter phenomenon

has been termed the mesenchymal drift, and is characterized by phenotypic alterations of numerous antigens expressed by glioma cells [12]. Furthermore, it has been shown that the glioma cells in vitro undergo several biological changes such as alterations in ploidy and growth rate, loss of the expression of glial fibrillary acidic protein (GFAP) and an altered expression of growth factors and their receptors [10, 12, 13, 15, 21–23]. It is well known that human gliomas are karyotypically heterogeneous tumors with numerous cell populations and that glioma cell lines represent the cells that successfully adapt to the tissue culture environment [22]. Consequently, permanent glioma cell lines only vaguely represent the original tumor and many of their biological features do not necessarily reflect the situation in vivo.

To minimize the selection of cellular subpopulations in vitro, gliomas can be propagated as multicellular organotypic spheroids obtained directly from biopsy specimens (biopsy spheroids). Although these biopsy spheroids can possibly undergo certain phenotypic changes, they are much closer to original tumors [7]. These spheroids contain blood vessels, connective tissue elements, ECM components and macrophages, thus reflecting the histiotypic architecture and the microenvironmental conditions found in the original tumor [2, 7]. In contrast to permanent cell lines, cells in biopsy spheroids maintain the same DNA ploidy as the original tumor. Moreover, the glioma heterogeneity, which is a reflection of an underlying genetic instability, is preserved in the biopsy spheroids and they are in this respect fundamentally different from spheroids obtained from permanent cell lines [2].

In the present work the direct interactions between glioblastoma biopsy specimens and ECM components are described. For this purpose, biopsy spheroids were initiated and thereafter stimulated with specific ECM components. The cells response to stimulation was assessed using a cell migration assay. Since such biopsy spheroids contain a variety of different cell types including endothelial cells, microglial and leptomeningeal cells, an immunohistochemical characterization of the cellular subpopulations stimulated by the ECM components was performed. The present study may, therefore, give new insight into how glioma cell migration is stimulated and perhaps regulated by ECM components in vivo.

Materials and methods

Tumor tissue

Tumor fragments, 1 cm or smaller in size, were obtained at surgery from 12 glioblastoma and 1 gemistocytic astrocytoma patient. The collection of tumor tissue was approved by the ethical board at Haukeland Hospital, Bergen, Norway. All patients gave verbal consent to tumor specimen collection for research purposes. The specimens were collected from tumor areas appearing macroscopically viable, corresponding to regions with contrast enhancement on preoperative computerized tomography scans. The specimens obtained at surgery were immediately transferred aseptically to test tubes containing complete growth medium, which consisted of Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Life Technologies, Roskilde, Denmark) supplemented with 10% heat-inactivated newborn calf serum, four times the prescribed concentration of nonessential amino acids, 2% L-glutamine, penicillin (100 IU/ml) and streptomycin (100 IU/ml).

Cell culture

Biopsy spheroids were prepared according to the technique originally described by Bjerkvig et al. [2]. Briefly, the tumor tissue was cut with scalpels into 0.3–0.5 mm pieces which were incubated in 80-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) base-coated with 10 ml of 0.75% agar (Difco, Detroit, Mich., USA) in complete growth medium. The spheroids were maintained in a standard tissue culture incubator with 5% CO_2 in air and 100% relative humidity at 37° C and the medium was changed once a week. After 3–4 weeks in culture, spheroids with diameters between 200 and 300 μm were selected for cell migration experiments using a pasteur pipette and a stereomicroscope (see below).

Light microscopy

After 3–4 weeks of culture, five spheroids from each culture (from biopsy specimens; VI, VII, VIII, X, XI, XII and XIII) were collected for light microscopic examination. The spheroids were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer with 0.1 M sucrose for at least 24 h (pH 7.2; 300 ± 10 mOsm). Using the same buffer, postfixation was performed for 1 h in 1% OsO_4. The specimens were then dehydrated in increasing concentrations of ethanol up to 100%. Embedding in Epon 812 was performed using graded additions of Epon-propylene oxide mixtures. The final polymerization was carried out at 60° C for 48 h. Semithin (1 μm) sections were cut with a ultramicrotome (Reichert Ultracut S, Reichert-Jung, Vienna, Austria) and stained with toluidine blue. For seven biopsy specimens the light microscopic observations of the spheroids were compared to routine histological observations of corresponding operative specimens. These biopsy samples were initially fixed in 4% formaldehyde solution (pH 7.4), embedded in paraffin, and stained with hematoxylin and eosin (H&E).

ECM components

Human laminin (Gibco BRL) was dissolved in the medium at 4° C to avoid agglutination. Human fibronectin (Gibco) and human vitronectin (Collaborative Biomedical products, Bedford, Mass.; cat. no. 40238) were used at a concentration of 10 μg/ml. Human collagen type IV (Sigma, product C 5533) was reconstituted to a concentration of 1 mg/ml in 0.25% acetic acid and then added to the medium. All reagents were used at a final concentration of 10 μg/ml, which has been shown to be optimal for ECM-induced migration of glioma cell lines [11].

Cell migration assay

Biopsy spheroids were placed into 96-well culture dishes (Nunc). Six different culture conditions were chosen, giving six groups with five spheroids in each group. A custom-made medium was prepared where the high molecular weight (>100 kDa) serum protein fraction was removed by ultrafiltration [11]. By this procedure the low molecular mass proteins were kept intact, including growth factors and other cell signaling molecules. The first group of spheroids was grown in a medium with 10% filtered serum; the second to fifth groups were grown in the same medium supplemented with either laminin, fibronectin, collagen type IV or vitronectin, respectively. The last group was grown in complete DMEM. The area covered by the tumor cells migrating out from the spheroid explant was used as an index of cell migration. Two orthogonal diameters of each explant area were measured regularly using a phase contrast microscope over a 9- to 12-day period and

Table 1 Histological information of the biopsy samples used in the study

Biopsy	Patient's sex and age (yrs)	Diagnosis	Histological findings	
			Biopsy specimen (in vivo)	Spheroids (in vitro)
I	M, 56	Glioblastoma multiforme	Variable cellularity and uniform atypical cellular population; areas of calcification; vascular endothelial cell proliferation, necrosis	Not done
II	F, 62	Glioblastoma multiforme	Highly cellular; microvascular proliferation; necrotic areas	Not done
III	F, 72	Glioblastoma multiforme	Variable cellularity, atypical cell and mitotic figures; vascular endothelial cell proliferation; necrotic areas	Not done
IV	M, 60	Glioblastoma multiforme	Highly cellular, atypical oligodendrogliocytes and astrocytes and mitotic figures; vascular endothelial proliferation; necrosis	Not done
V	M, 70	Glioblastoma multiforme	Cellular, pleomorphic nuclei, mitotic and some apoptotic figures; necrosis; vascular endothelial cell proliferation	Not available
VI	M, 62	Glioblastoma multiforme	Variable cellularity with myxoid intercellular substance, small atypical glia cells and mitotic figures; microvascular proliferation; small necrotic areas	Highly cellular; some apoptotic figures; a few vascular elements; loosely connected cells with lightly stained chromatin
VII	M, 57	Glioblastoma multiforme	Highly cellular; atypical cells; necrosis; pathological endothelial cell proliferation	Atypical cells with a dense chromatin structure, mitotic figures; a few areas with necrosis; highly vascularized
VIII	M, 36	Gemistocytic astrocytoma (anaplastic)	Variable cellularity, mostly neoplastic gemistocytic astrocytes and some microglial cells and reactive astroglia cells; some degenerative changes	Spindle-shaped cell and atypical gemitocytic astrocytes; some areas of connective tissue components; numerous vascular elements
IX	M, 61	Glioblastoma multiforme	Cellular and pleomorhic; necrotic areas; pathological endothel proliferation	Not available
X	M, 70	Glioblastoma multiforme	Variable cellularity and atypical cells with pleomorphic nuclei; some areas of collagenous tissue; vascular endothelial cell proliferation; necrosis	Pleomorphic cells with high cellular density; some areas of necrosis and connective tissue
XI	F, 32	Glioblastoma multiforme	Cellular, fusiform and atypical cells, many neoplastic gemistocytic astrocytes; areas of necrosis; atypical vascular cell proliferation	Highly cellular in the periphery of the spheroids, relatively non-cellular in the middle; several vascular elements; areas of connective tissue
XII	F, 69	Glioblastoma multiforme	Highly cellular, pleomorphic; small areas of anaplastic astrocytoma cells; necrosis; enormous endothelial proliferation	Atypical cells scattered in the periphery of the spheroid; conective tissue components; vascular elements
XIII	F, 52	Glioblastoma multiforme	Cellular and atypical, pleomorphic and relatively small astocyte-like cells; microvascular proliferation; small areas of gemistocytic astrocytes; necrotic areas	Fusiform atypical cells, pleomorphic; numerous vascular elements

the mean area covered by tumor cells was calculated [11]. After 9–12 days, the cultures were fixed in ice-cold 90% acetone and prepared for immunohistochemistry as described below. Data from the tumor cell migration assays were analyzed by one-way analysis of variance (ANOVA). Significance was accepted at the 5% level.

Comparative analyses of ECM-stimulated cell migration

To investigate whether there was any correlation between cell responses in the individual groups, regardless of which ECM component was added, we compared the area increments for each group pairwise against each other (for example, biopsy I: laminin vs fibronectin, laminin vs type IV collagen, etc.). The measurements were performed when the cells had migrated for 9 days. The significance of the correlation was analyzed using Student's *t*-test.

Immunocytochemistry

Immunostaining was performed on biopsy spheroids and on acetone-fixed migration cultures. After 3–4 weeks of culture, biopsy spheroids were rinsed in phosphate-buffered saline (PBS), embedded in Tissue-Tec (Miles, Elkshart, Ind.) and frozen in isopentane in liquid N_2. Frozen sections with a thickness of 10 μm were cut on using a Reichart cryomicrotome (Leica Instruments Nussloc, Heidelberg, Germany) and transferred to poly-L-lysine-coated slides. The specimens were fixed in acetone for 3 min before immunostaining. The following antibodies were used: monoclonal anti-GFAP (Sera-lab, Crawley down, UK; diluted 1:5 in PBS), polyclonal anti-von-Willebrand factor (VWF) (DAKO Glostrup, Denmark; 1:100), monoclonal anti-α3 integrin subunit (Becton Dickinson, San José, USA; 1:50), monoclonal anti-β1 integrin subunit (Gibco; 1:100), polyclonal anti-laminin (subtype-1) (Sigma; 1:50),

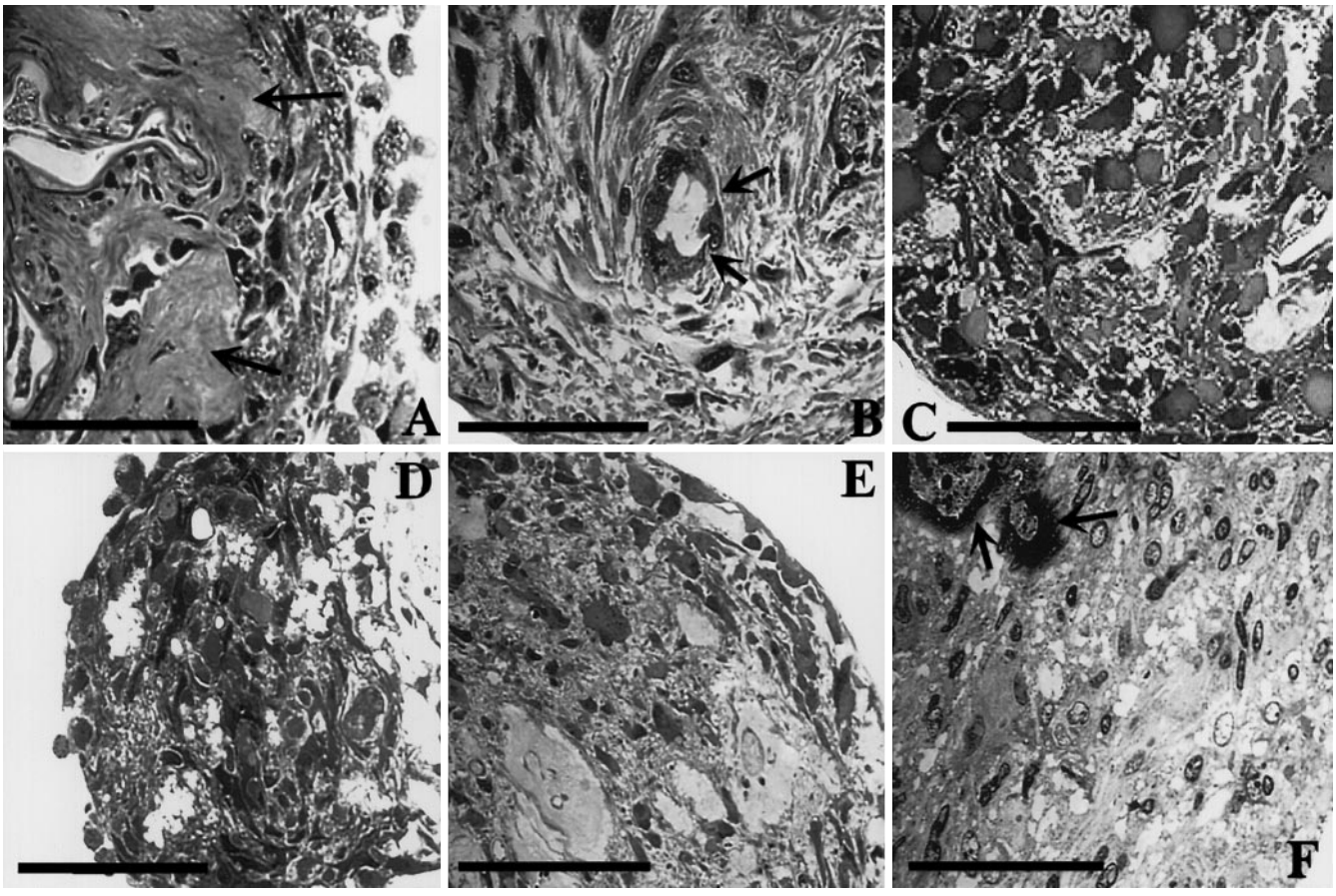

Fig. 1 A–F Photomicrographs of semithin sections of the tumor spheroids after 3 weeks in culture. Note the differences in the morphology. All biopsy spheroids expressed vascular elements as illustrated by *arrowheads* in **B** and **F**. Connective tissue elements were also frequently observed (**A**, *arrowheads*). **A** Biopsy spheroid XII; **B** XIII; **C** VIII; **D** X; **E** VII; and **F** VI. Toluidine blue staining. *Bars* 50 μm

monoclonal anti-fibronectin (Sera-lab; 1:10), monoclonal anti-collagen type IV (Sigma; 1:100) and monoclonal anti-CD11b (clone MRC OX42, cat. no. MCA275G, Serotec, Kidlington, UK; 1:16). The specimens were incubated for 1 h with the primary antibodies, washed three times for 5 min in PBS and further incubated for 1 h with FITC-conjugated swine anti-rabbit IgG (DAKO; 1:30) or FITC-conjugated sheep anti-mouse IgG (Boehringer, Mannheim, Germany; 1:30). Specimens were then washed three times with PBS and treated with RNase (0.5 mg/ml). To visualize the cell nuclei, specimens were counterstained with 10 μg/ml propidium iodide DNA stain, which gives a red nuclear staining in the fluorescence microscope using rhodamine filter optics. The specimens were examined using a Leica TCS NT confocal laser scanning microscope (Leica). As controls, nonspecific IgG and PBS were used. The number of GFAP-positive and -negative cells was determined by differential counts in the migration areas. Furthermore, these numbers were compared to area of cell migration stimulated by the ECM components.

Results

Tumor tissue

An overview of the histological evaluation of the biopsy specimens (12 glioblastomas and 1 gemistocytic astrocytoma) is given in Table 1. The tumor material was obtained from eight males and five females aged, between 32 and 72 years. Most of the tumors diagnosed as glioblastoma multiforme had similar histological characteristics. Briefly, the tumors consisted of atypical cells with mitotic figures; degenerative changes with necrotic areas and pathological endothelial cell proliferation. Of the 13 gliomas studied, 11 formed spheroids after 1 week in culture. The tumor tissue samples from biopsy specimens V and IX were necrotic and did not survive in culture. A light microscopic histological evaluation was done on spheroids from seven biopsies cultured for 3–4 weeks (Table 1). The morphology of each biopsy spheroid was found similar to that observed in the tumor tissue from which they originated. Spheroids of biopsy specimens VIII, XI and XII, however, expressed large amounts of connective tissue elements, which were less prominent in the original tumor (Fig. 1).

Cell migration

The spheroids, which were propagated in growth medium depleted of high molecular weight (> 100 kDa) serum components, showed negligible cell migration (Fig. 2 A). Laminin induced a significant migratory response in 7 out of 11 specimens (III, IV, VI, VII, XI, XII and XIII) (Fig. 2 B). Fibronectin produced a significant stimulatory response in 6 of 11 biopsy spheroids (IV, VI, VIII, X, XII and XIII) (Fig. 2 C). Of the 10 biopsy spheroids, which were stimu-

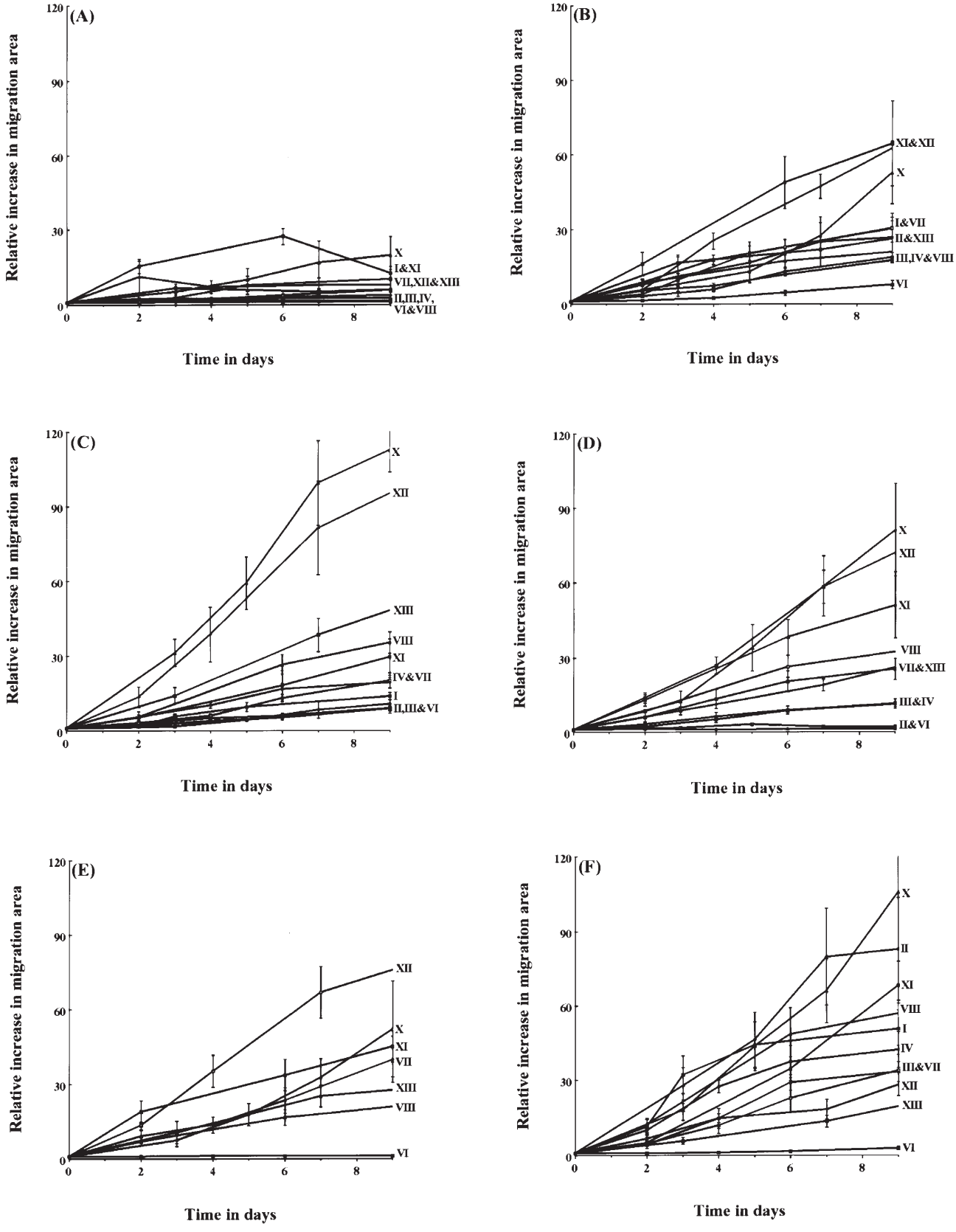

Fig. 2 A–F Cell migration from biopsy spheroids. Migration in: **A** the filtered medium; **B–E** ECM supplemented medium (**B** laminin, **C** fibronectin, **D** collagen type IV, **E** vitronectin); **F** complete growth medium (10% serum supplemented DMEM). Tumor migration was calculated using the formula for the area of a circle. Data expressed as mean ± SEM; five spheroids were studied in each group

Table 2 Correlation of coefficients and corresponding *P* values between stimulatory effects evoked by different ECM components (*ECM* extracellular matrix, *Lam* laminin, *FN* fibronectin, *Coll* collagen type IV, *Vitro* vitronectin)

ECM components	Correlation coefficient *(r)*	*P* value
Lam and FN	0.74	$0.005 < P < 0.01$
Lam and Coll	0.92	$P < 0.001$
Lam and Vitro	0.93	$0.002 < P < 0.005$
FN and Coll	0.91	$P < 0.001$
FN and Vitro	0.74	$0.05 < P < 0.1$
Coll and Vitro	0.91	$0.02 < P < 0.05$

lated by collagen type IV, 7 (III, IV, VII, X, XI, XII and XIII) showed a significant stimulatory response (Fig. 2 D), whereas vitronectin induced significant migratory responses in 4 out of 7 biopsy spheroids (VII, XI, XII and XIII) (Fig. 2 E). The strongest migratory responses were observed in biopsy spheroids X, XI and XII (Fig. 2 B–E). However, as indicated above, the stimulatory responses caused by different ECM components were rather heterogeneous for the different biopsy spheroids studied. Such heterogeneous responses were also observed when the cells were grown in ordinary DMEM with 10% newborn calf serum. As for all the ECM components, the relative increase in the migratory area in response to DMEM stimulation ranged from virtually zero (VI) to 100-fold (X) (Fig. 2 F).

Significant correlation was revealed between the migratory responses evoked by laminin and fibronectin, laminin and collagen type IV, laminin and vitronectin, fibronectin and collagen type IV and collagen type IV and vitronectin (Table 2). For instance, those biopsy spheroids that were strongly stimulated by laminin, also became stimulated in the presence fibronectin ($0.005 < P < 0.01$; Fig. 3).The stimulatory effects between vitronectin and fibronectin did not, however, correlate ($0.05 < P < 0.1$).

Immunostaining

Migration cultures

Immunostaining was carried out to identify the cell types that migrated out from the biopsy spheroids in response to the different ECM components. The GFAP immunostaining revealed that both GFAP-positive and -negative cells migrated in response to ECM stimulation (Fig. 4 A–D). Immunostaining for VWF showed expression within the spheroids but not in the migrating cells (Fig. 4 E). Furthermore, the migrating cells did not express CD11b (Fig. 4 F). Differential counts of GFAP-positive and -negative migrating cells for biopsy samples IV, VI, VII, VIII, X, XI, XII and XIII were performed to analyze if any of the ECM components evoked a stimulatory effect on any specific cell type, but no such relationship was found (data not shown).

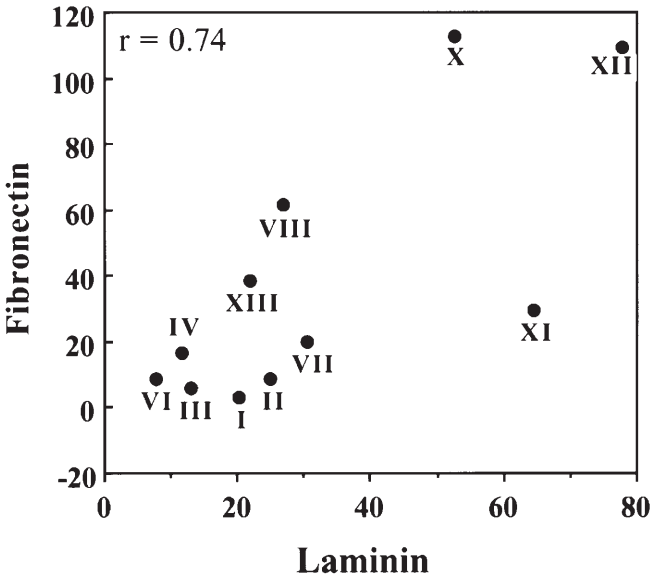

Fig. 3 Scattergram showing the correlation between the migration areas stimulated by laminin *(x axis)* and fibronectin *(y axis)*. Correlation analyses were performed after 9 days of migration

Biopsy spheroids

A summary of the immunostaining results is given in Table 3. An even distribution of β1 integrin subunit and GFAP was observed within most of the spheroids. A variable amount of α3 integrin subunit expression was observed among biopsy spheroids from all groups. Although laminin, fibronectin and collagen type IV were mostly associated with vessel elements, punctate deposits of ECM components were observed in some biopsy spheroids. Six out of ten biopsy spheroids showed a strong VWF expression confined to vessel elements.

Discussion

Glioma growth and invasion occur in a complex cellular microenvironment consisting of host cells, tumor cells and the ECM. It has recently been shown that in animal models and tissue culture, ECM synthesis may be induced in the confrontation zone between glioma cells and brain tissue or in the brain tissue and that the normal tissue is most likely responsible for such neo-production [8, 18]. Several recent reports describe specific interactions between glioma cells and ECM components [1, 4, 11]. This includes the use of glioma cell lines as well as reaggregates of dissociated cells from biopsy samples [3]. However, as briefly mentioned in the introductory section, a selection of tissue culture-adapted cell clones occurs during the establishment of such permanent cell lines [21, 22]. Such a selection may also occur during the establishment of reaggregation cultures. Conclusive data regarding the actual effects of the ECM on glioblastoma biopsy specimens has not been provided. Therefore, the present work represents an attempt to elucidate whether

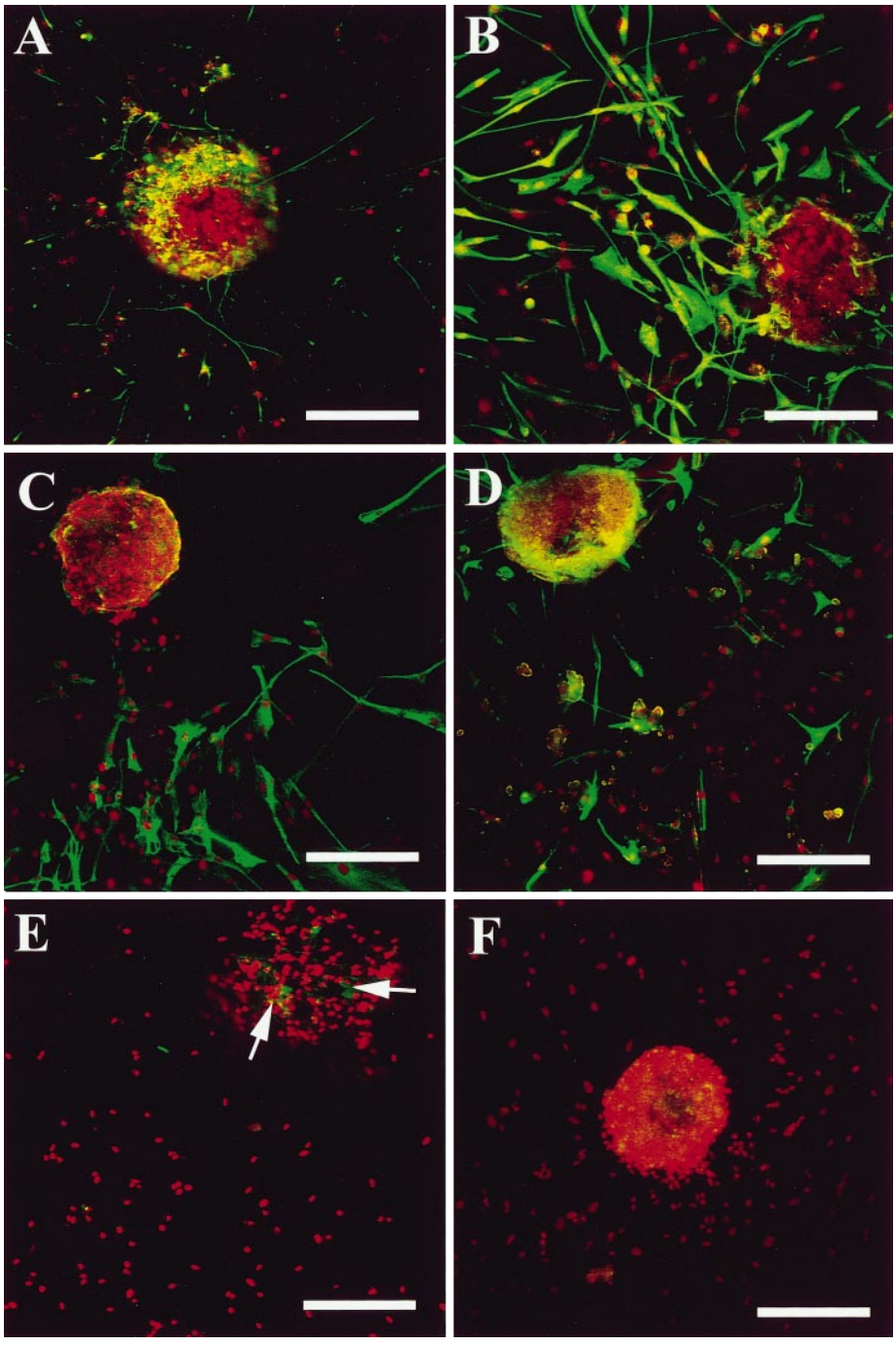

Fig. 4 A–F Immunostaining of cells migrating out from the biopsy spheroids. **A–D** Anti-GFAP staining of cultures from biopsy spheroid X migrating in response to laminin (**A**), fibronectin (**B**), collagen type IV (**C**) and vitronectin (**D**) supplemented medium. **E** Anti-VWF staining of migrating cells stimulated by fibronectin from biopsy spheroid XII. **F** Fibronectin-stimulated migrating cells from biopsy spheroid X stained for anti-CD11b. *Red* fluorescence represents nuclear staining and *green* fluorescence represent respective antibodies (GFAP and VWF) (*GFAP* glial fibrillary acidic protein, *VWF* von Willebrand factor). *Bars* 250 μm

cell migration from glioblastoma biopsy spheroids, which exhibit a lower degree of mesenchymal drift, is affected by defined ECM components. The ECM components studied here, laminin, fibronectin, collagen type IV and vitronectin, have all been suggested to play a major role in glioma cell migration [4, 11].

The work presented here shows that interactions between cells from biopsy specimens and ECM components are rather heterogeneous and complicated. The cells showed a negligible capacity to migrate in filtered medium from which the high molecular weight serum ECM components were removed. However, this response was significantly increased in serum supplemented DMEM and in the filtered medium supplemented with specific ECM components. This implies that cells from biopsy spheroids, as well as cell lines, require serum proteins with a molecular weight of greater than 100 kDa to migrate. As shown in Fig. 2, the cells revealed a wide range of migratory responses to the different ECM components. Earlier studies on permanent glioma cell lines have shown that laminin, fibronectin, collagen type IV and vitronectin stimulated glioma cell migration and that laminin was the

Table 3 Immunostaining of biopsy spheroids (*GFAP* glial fibrillary acidic protein, *VWF* von Willebrand factor)

Biopsy	GFAP	VWF	α 3	β 1	Lam	FN	Coll
I	nd	nd	nd	nd	nd	nd	nd
II	nd	nd	nd	nd	nd	nd	nd
III	+++[a]	–	+++[a]	+++[a]	+++[b, c]	+++[b, c]	+++[b]
IV	+++	–	+++	+++	+++[b]	+++[b, c]	+++[b]
VI	+++	+++[b]	++[c]	+++	+++[b, c]	+++[b]	+++[b]
VII	+++	–	++	++[b]	+++[b, c]	+++[b, c]	+++[b]
VIII	+++	+++[b]	++[c]	+++	+++[b]	+++[b]	+++[b]
X	+++	+++[b]	+	+++	+++[b, c]	+++[b]	+++[b]
XI	+++[d]	+++[b]	++[d]	++[d]	+++[b, c]	+++[b]	+++[b]
XII	+++[a]	+++[b]	+++[a]	+++	+++[b, c]	+++[b, c]	+++[b, c]
XIII	+++	–	+++	+++	+++[b]	+++[b]	+++[b]

[a] Positive in the center of the spheroid
[b] Positive associated with vascular elements
[c] Punctate deposits
[d] Scattered positive in the spheroid

most potent stimulator [1, 4, 11]. The present work shows that not only laminin but also fibronectin, collagen type IV and vitronectin evoked significant stimulatory responses in biopsy spheroids and that the different biopsy specimens showed considerable variation in the magnitude of their response (Fig. 2). This is in line with the fact that human malignant gliomas consist of karyotypically heterogeneous cell populations [21, 22] and that primary gliomas frequently display cell populations with different DNA distributions [13, 15]. The cellular heterogeneity is characterized by variations in cellular morphology, growth rate, chemosensitivity and antigen expression [15, 22, 24], and thus leads to variation in biological activities within and among the gliomas. Spheroids derived directly from biopsy specimens contain many cellular subpopulations with various biological features which are also found in the original tumor. This may explain the large variation in the migratory responses to different ECM components. By evaluating the migratory responses within and between the tumors, it can be concluded that both intertumoral and intratumoral variations exist. However, the statistical analyses indicates that the intertumoral variation is larger than the variation observed between spheroids derived from one tumor biopsy specimen (Fig. 2).

In accordance with tumors in situ, biopsy spheroids contain glioma cells and various normal cell populations. Therefore, to identify the major cell types which responded to stimulation with ECM components, an immunohistochemical characterization of the migrating cells was performed. The most accepted indication of the astroglial lineage is the expression GFAP [20]. Interestingly, the migrating cells expressed GFAP variably. As shown in Fig. 4, for biopsy specimen X, most of the cells stimulated by either fibronectin or collagen type IV expressed GFAP. In contrast, most of the cells that migrated in response to either laminin or vitronectin did not express GFAP. However, such findings varied among the biopsies studied. As indicated in the results, no relation was found between specific ECM-induced stimulation of cell migration and the number of GFAP-positive cells found with in the migration area. This implies that there is a heterogeneous response of GFAP-positive cells to each ECM component studied. However, the differential response of GFAP-positive cells in the same biopsy spheroid to different ECM component may indicate that distinct cellular subpopulations may be stimulated by defined ECM components. Immunostaining for VWF implies that the VWF-positive vascular endothelial cells present in the biopsy spheroids did not migrate in response to the ECM components studied. Furthermore, the cells stimulated by different ECM components did not express CD11b, which is a marker for microglial cells. Thus, the migrating cells that did not express GFAP are most likely GFAP-negative glioma cells, dedifferentiated endothelial cells, leptomeningeal cells which may be found in the perivascular space within the tumor or smooth muscle cells derived from hyperplastic blood vessels.

As shown in Fig. 2, cells from biopsy spheroids that were stimulated strongly by laminin were also stimulated strongly by fibronectin, collagen type IV and vitronectin. As shown in Fig. 3 and Table 2 a positive correlation of cell migration in response to different ECM components was seen in most of the biopsy spheroids studied. From the data presented in Table 2, it is tempting to speculate that there may be a set of common receptor-cell signaling pathways which are, at least partly, responsible for the cell migration on the different ECM components. For instance, it is well known that the α3β1 integrin complex is a common receptor for both laminin, fibronectin and collagen type IV, whereas the αvβ5 integrin is associated with cell attachment to vitronectin [6, 14, 19]. A substantial number of data describe the differential expression of integrins in glioma tissue [4, 5, 16]. It has been shown that neoplastic astrocytes may express an increased level of the α3 and β1 integrin subunits both in vivo and in vitro as compared to normal astrocytes. Furthermore, Paulus et al. [16] have shown that almost all glioma biopsy specimens are positive for α3. However, as shown in Table 3, we observed a heterogeneous expression of α3 in biopsy spheroids, whereas the β1 expression seemed to be homogenous, suggesting that there are more cells expressingβ1 than α3 in the biopsy spheroids. This finding is in concordance with the fact that the β1 subunit also associates with a number of other α subunit to interact with ECM components [6]. Immunopositivity for α3 and β1 subunits in the migration cultures was highly variable, and was found to be independent of the stimulatory ECM component used (data not shown). Using cell lines, Fried-

lander et al. [3] have shown that glioma cell migration in vitro in response to different ECM components can be strikingly inhibited by antibodies to αv and β1 subunits but not to antibodies to α2, α3 and β4. However, using other permanent cell lines, Tysnes et al. [25] found anti-α3 and anti-β1 to have an inhibitory effect on laminin-induced cell migration, a phenomenon which further demonstrates the heterogeneous expression of these integrins.

In conclusion, this study indicates that high molecular weight proteins (>100 kDa) are required for cell migration from glioma biopsy specimens. Furthermore, the migratory responses were heterogeneous independent on the ECM component used. The positive correlation regarding the stimulatory effects of different ECM components suggests that there may be a set of common receptors involved in the interaction between glioma cells and different ECM components. Further studies are, therefore, needed to identify the different cell surface receptors which are responsible for cell migration from biopsy spheroids. This includes studies regarding the variability in cell surface receptor expression and turnover, which probably depends on the ECM components available in the cellular microenvironment. Such studies may in the future lead to modifications of specific receptors and consequent new therapeutic approaches.

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