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Extracellular Matrix Proteins Inhibit Proliferation, Upregulate Migration and Induce Morphological Changes in Human Glioma Cell Lines

S. Koochekpour, A. Merzak and G.J. Pilkington

The influence of an artificial basement membrane (BM), Matrigel, and four individual extracellular matrix proteins, fibronectin, laminin, collagen I and vitronectin, on cell proliferation, morphology and migration was assessed in four glioma cell lines. Matrigel and individual BM proteins differentially inhibited cell proliferation of all cell lines studied. In addition, Matrigel was found to induce extensive morphological changes in glioma cells. Polycarbonate filters, of 8-μm porosity in modified Boyden chambers, were used to assess the chemoattraction activity of Matrigel and the individual proteins on glioma cells. All these components were found to stimulate cell migration, albeit to different extents but laminin proved to be the most effective chemoattractant for glioma cells *in vitro*. These data suggest that basement membrane proteins may inhibit proliferation and stimulate migration in order to facilitate invasion.

Keywords: glioma, proliferation, migration, basement membrane
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INTRODUCTION

GLIOMAS, THE major form of intrinsic brain tumour in man, are characterised by their local invasion of the surrounding brain structures [1]. The factors involved in the control of this particular behaviour are not well documented. To be able to invade, tumour cells must cease proliferation [2], degrade the extracellular matrix (ECM) and then start migration [3–5]. However, the extracellular space of the brain is devoid of a well-defined ECM [6]. Nevertheless, basement membrane (BM) does exist around blood vessels, in the glia limitans externa and underlying choroid plexus epithelial cells [6, 7]. Although BM components have been found to act as chemoattractant and (anti) proliferative factors for various normal and neoplastic non-glial cells [8–16], the role of these molecules in both proliferation and migration of glioma cells is not well documented. In this paper, we report the effect of an artificial basement membrane (Matrigel) as well as fibronectin, collagen I, vitronectin, and laminin on proliferation and migration of four glioma cell lines *in vitro*.

MATERIALS AND METHODS

Cell culture

The cell lines used in this study have recently been extensively characterised [17–25]. Briefly, IPNT-H is a low grade pilocytic astrocytoma-derived cell line; IPSB-18 is an anaplastic astrocytoma cell line; IPRM-5 is a glioblastoma multiforme cell line; and IPNN-8 is a medulloblastoma cell line. Cells were routinely propagated in culture in a standard humidified incubator at 37°C in a 5% carbon dioxide/95% air atmosphere. Plastic tissue culture dishes and plates were obtained from Falcon (London, U.K.). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (GIBCO BRL, Middlesex, U.K.) and a 1% antibiotic/antimycotic solution at a final concentration of 100 IU penicillin, 100 μg streptomycin and 0.25 μg amphotericin per ml (GIBCO-BRL). Human fibronectin, vitronectin, laminin and collagen I were purchased from GIBCO-BRL and resuspended as indicated by the manufacturer. Matrigel was purchased from Collaborative Research (Lexington, Massachusetts, U.S.A.).

Proliferation assay

The effect of Matrigel on the growth of human glioma cell lines was measured as follows: cells were plated at 5×10^4 cells/well in 6-well culture plates and gradually weaned onto serum-free medium (SFM) via 7 and 3% of fetal calf serum over a 48-h period. Then Matrigel was added at 10–100 μg/ml to the cells. After 3 days of incubation, the cell number was determined by trypsinisation and counting in a haemocytometer. The effect of ECM proteins on glioma cell proliferation was measured by plating 2×10^4 cells/well and then treating them as described above for Matrigel. Once the cells were in SFM, ECM proteins were added at 15 μg/ml and the cells counted after 3 days of incubation.

Migration assay

Cell motility was monitored by a chemotaxis assay using 24-well transwell units incorporating 8 μm porosity polycarbonate filters (Costar, Cambridge, U.K.). Each lower compartment of the transwell contained 500 μl of SFM. Cells were incubated for 48 h in SFM and 100 μl of SFM containing 10 000 cells were placed in the upper compartment of the transwell unit. After

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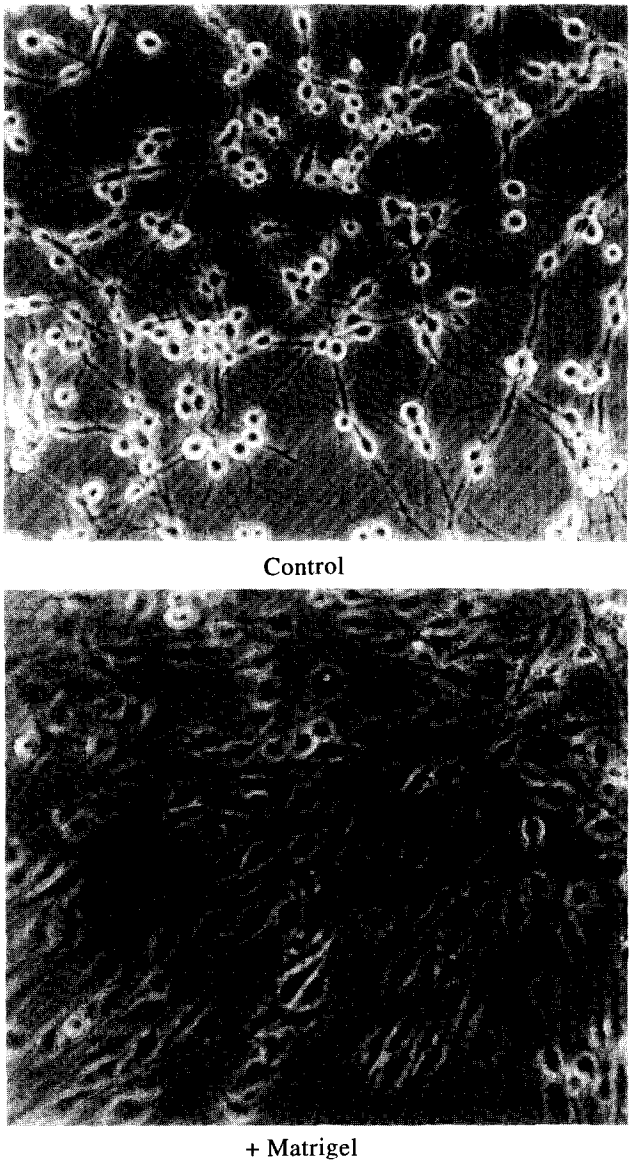

Figure 1. Matrigel treated IPNT-H cells showed larger non-refractile perikaria with elongated thick processes. (Phase contrast micrograph / X 200.) Cells were treated with 25 μg/ml of Matrigel as described in the proliferation assay and the photographs were taken following 3 days incubation.

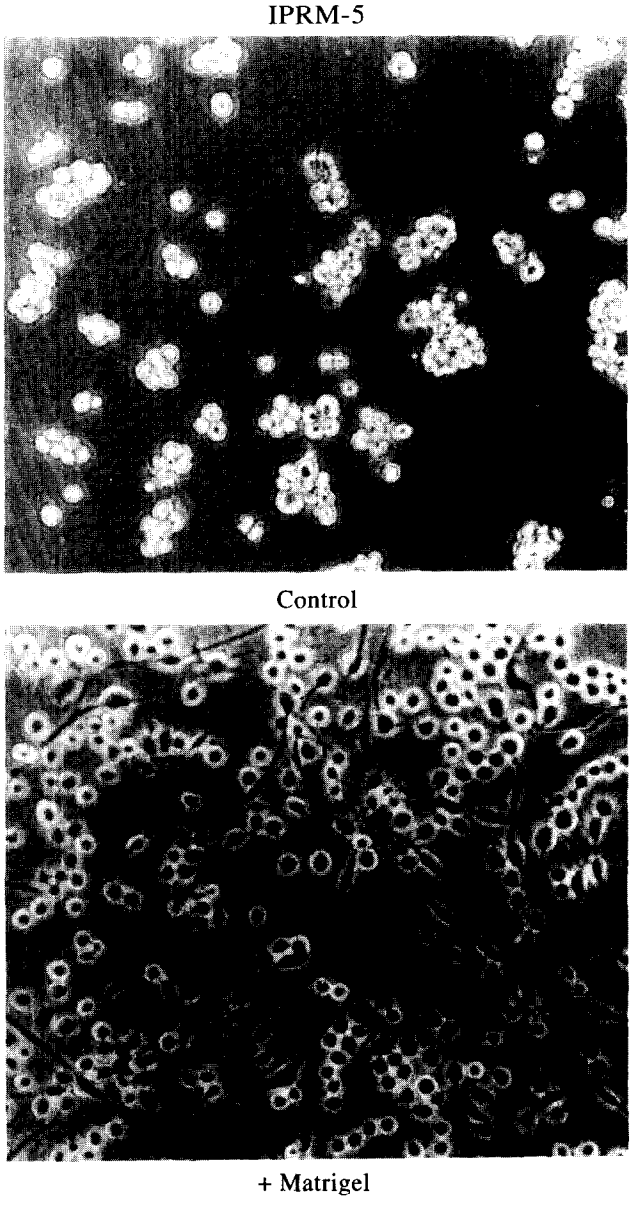

Figure 2. Matrigel treatment of IPRM-5 cells evoked process formation. (Phase contrast micrograph / X 200.) Cells were treated as described in Figure 1.

16 h incubation at 37°C in a humidified 95% air, 5% carbon dioxide atmosphere, cells were fixed with acetic acid/alcohol, and stained with Giemsa. Cells on the upper surface of the filter were removed by wiping with a cotton swab, and motility was determined by counting the cells that had migrated to the lower side of the filter using a phase contrast microscope at 200 X magnification. Of the fields, 20 were counted in each assay. Each sample was assayed in triplicate, and assays were repeated three times.

Statistical analysis

Data were analysed statistically by Student's *t* test and statistical significance was set at $P<0.01$.

RESULTS

Matrigel elicits important morphological changes in human glioma cell lines in vitro

Control IPNT-H cells were, in general, dispersed and showed a bipolar morphology with small nuclei and long thin processes, while Matrigel-treated IPNT-H cells exhibited larger perikarya with elongated thick processes (Figure 1). IPRM-5 control cells showed spheroidal aggregates of cells but when treated with Matrigel, they exhibited dark nuclei and developed long thick processes (Figure 2). Both IPSB-18 and IPNN-8 control cells exhibited a bipolar morphology with extended processes and small nuclei. However, these cells formed a network of streaming cell clusters in the presence of Matrigel (Figure 3).

Growth inhibitory effect of Matrigel on human glioma cell lines

We studied the effect of artificial basement membrane, Matrigel, on proliferation of four cell lines derived from human

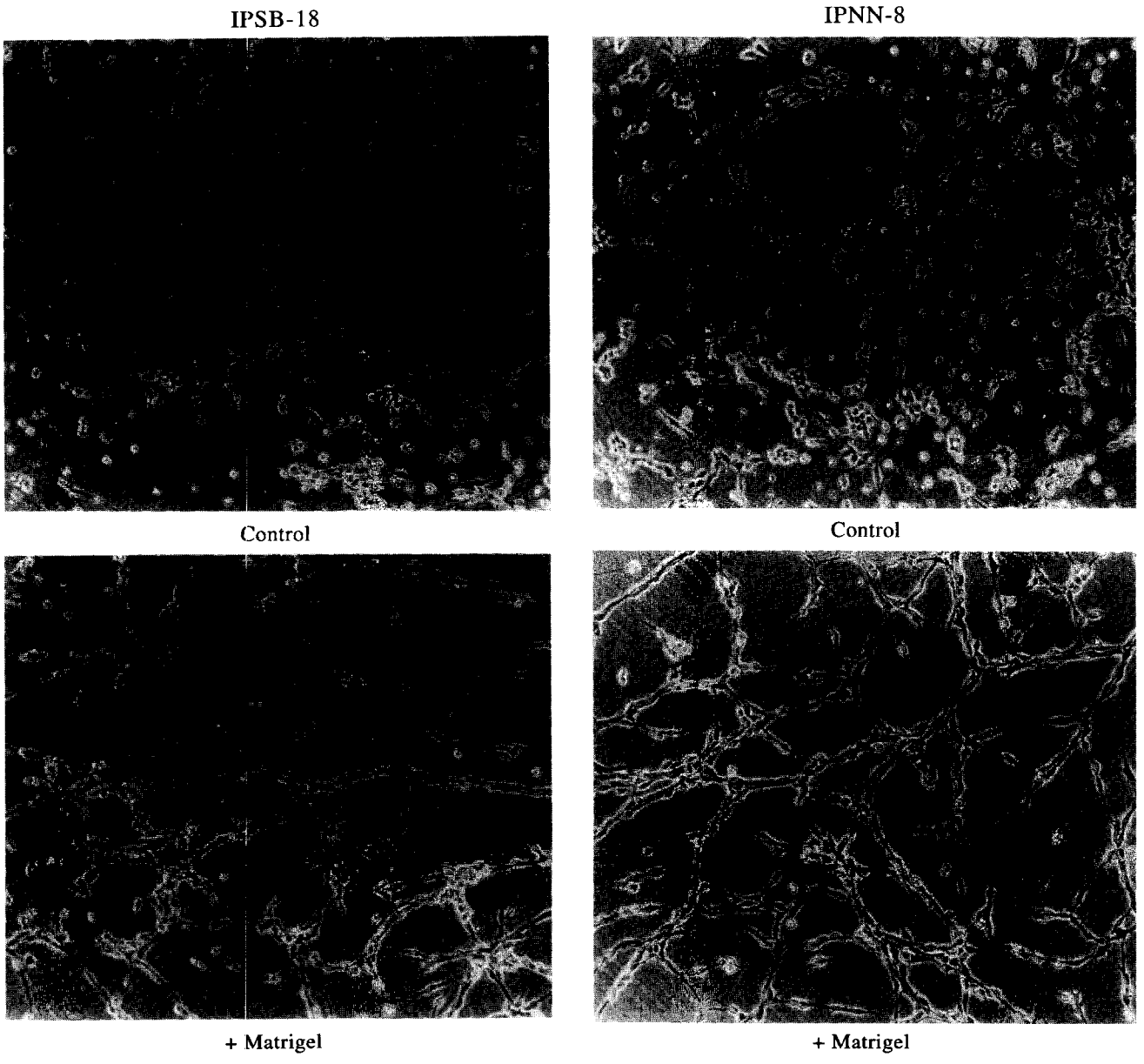

Figure 3. IPSB-18 and IPNN-8 cell lines show an increasing number and thickness of cell processes in the presence of Matrigel. (Phase contrast micrograph / X 200.) Cells were treated as described in Figure 1.

gliomas of different histological types. Proliferation of all cell lines was inhibited by Matrigel to different extents (Figure 4A). In addition, this inhibitory effect proved to be dose dependent. Proliferation of IPNT-H, IPSB-18, IPRM-5, and IPNN-8, was inhibited by 20–73%, 13–81%, 30–78% and 51–80%, respectively.

Growth inhibitory effect of individual basement membrane proteins on human glioma cell lines

In order to identify which BM components were responsible for growth inhibition, the effect of collagen type 1, fibronectin, vitronectin, and laminin on the proliferation of the four glioma cell lines was studied. Since 60% of Matrigel is laminin [13] and the highest growth inhibitory effect of Matrigel was obtained at concentrations around 25 μg/ml, we used laminin as well as the other BM proteins at 15 μg/ml. All four proteins exhibited a growth inhibitory effect on these cells (Figure 5). Collagen I inhibited proliferation of IPNT-H, IPSB-18, IPRM-5, and IPNN-8 by 79, 67, 61 and 75%, respectively. Proliferation of these cell lines was also inhibited by fibronectin by 52–74%, vitronectin by 49–65% and laminin by 47–60%. All these proteins were also found to inhibit glioma cell proliferation at a concentration of 5 μg/ml by 44–89% (data not shown).

Matrigel induces glioma cell migration in vitro

We studied the effect of Matrigel as a chemoattractant, and found that it induced migration of all four glioma cell lines at all the concentrations tested (Figure 4B). A maximal effect was obtained at 25 μg/ml for IPRM-5 and IPNN-8, and at 50 μg/ml for IPNT-H and IPSB-18.

Basement membrane proteins stimulate glioma cell migration in vitro

In order to identify which proteins were relevant to the chemoattraction activity of BM, the chemoattractivity of fibronectin, laminin, vitronectin, and collagen I for glioma cells

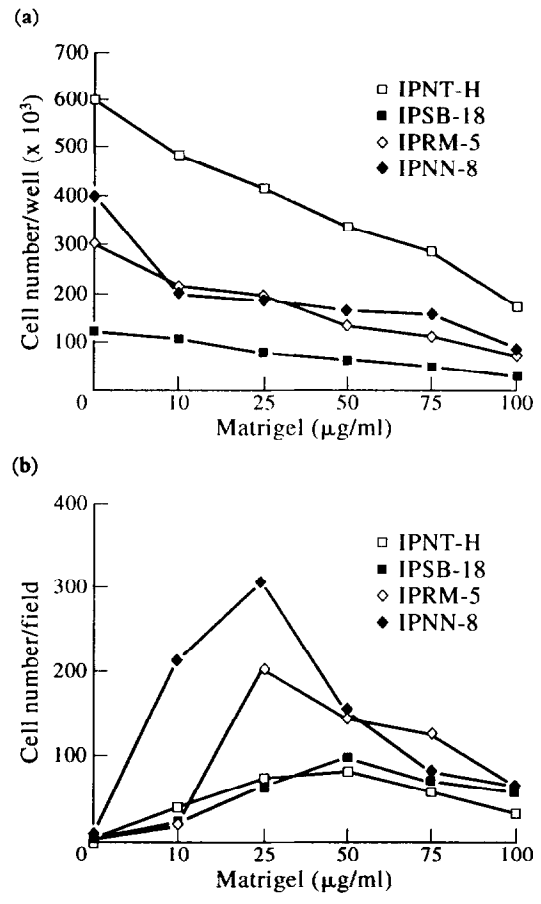

Figure 4. Effect of Matrigel on proliferation (a) and migration (b) of four human glioma cell lines.

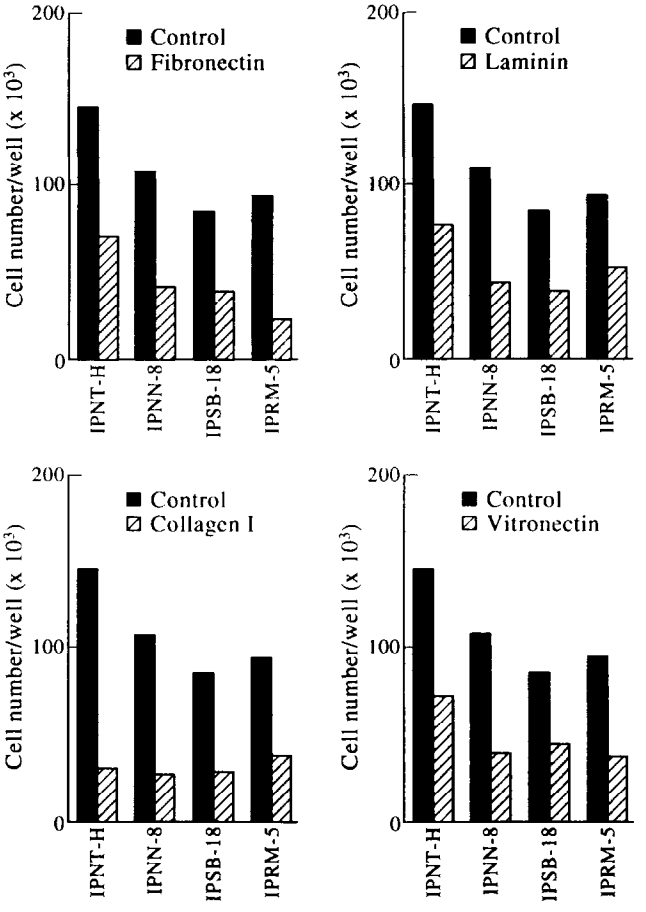

Figure 5. Growth inhibitory effect on glioma cell lines by individual BM proteins at 15 μg/ml after 3 days incubation in serum-free medium.

was assessed. All these proteins were found to significantly induce glioma cell migration to different extents (Figure 6). In addition, this effect was dose dependent. In all cell lines, a maximal migration (53–290 cells/field) was obtained with fibronectin, vitronectin, and collagen I at 10 μg/ml. Interestingly, laminin was found to be the most effective chemoattractant for the two malignant glioma-derived cell lines IPSB-18 and IPRM-5 at almost all concentrations. Furthermore, laminin was also found to increasingly stimulate cell migration in both IPNT-H and IPNN-8 cell lines. No cell line was able to migrate to the lower side of the filter when Matrigel or the other individual components were added in the upper compartment of the transwell chamber (data not shown). This result suggests that the effect observed is due to a chemotactic activity rather than to an increase in cell motility.

DISCUSSION

The artificial basement membrane, Matrigel, as well as individual BM proteins were found to exert a growth inhibitory effect on four cell lines derived from gliomas of different histological types and grades. These components also elicited marked morphological changes in glioma cells. These results are in agreement with the finding of Rutka and associates [26] demonstrating a growth inhibitory effect, as well as important morphological changes by a leptomeningeal basement membrane in one anaplastic glioma-derived cell line, U 343 MG-A. They also reported that collagen I and IV, but not fibronectin and laminin, inhibited the proliferation of this cell line [26]. Here we found that collagen I, fibronectin, laminin, and vitronectin were all able to inhibit cell proliferation of the anaplastic glioma cell line, IPSB-18, as well as three other cell lines derived from a glioblastoma multiforme, a low grade pilocytic astrocytoma, and a medulloblastoma. The neutral effect of fibronectin and laminin on the U343MG-A cell line was probably due to the high concentrations of proteins used for these studies [26]. The cell lines used in our study were not growth inhibited when they were plated on surfaces coated with Matrigel or with the other four proteins at the same concentrations used in the proliferation assays (data not shown). This observation suggests that ECM components, in the soluble form, are able to inhibit glioma cell proliferation, probably by signalling through their cell surface receptors and/or by modulating the action of growth factors released by tumour cells. It is well established that ECM components regulate growth factor activity [27]. There is also increasing evidence that these components signal into the cells via their cell membrane receptors [28]. Glioma cells treated with Matrigel were also found to undergo significant morphological changes. This result is similar to our previous finding that individual basement membrane proteins induce dramatic morphological changes in human glioma cell lines *in vitro* [22].

Matrigel, as well as the other ECM proteins, were all found to exhibit a chemoattraction activity on the four glioma derived cell lines included in this study. To the best of our knowledge, this is the first demonstration that these molecules are involved in chemoattraction of glioma cells *in vitro*. In addition, we demonstrated that, of the four proteins studied, laminin proved

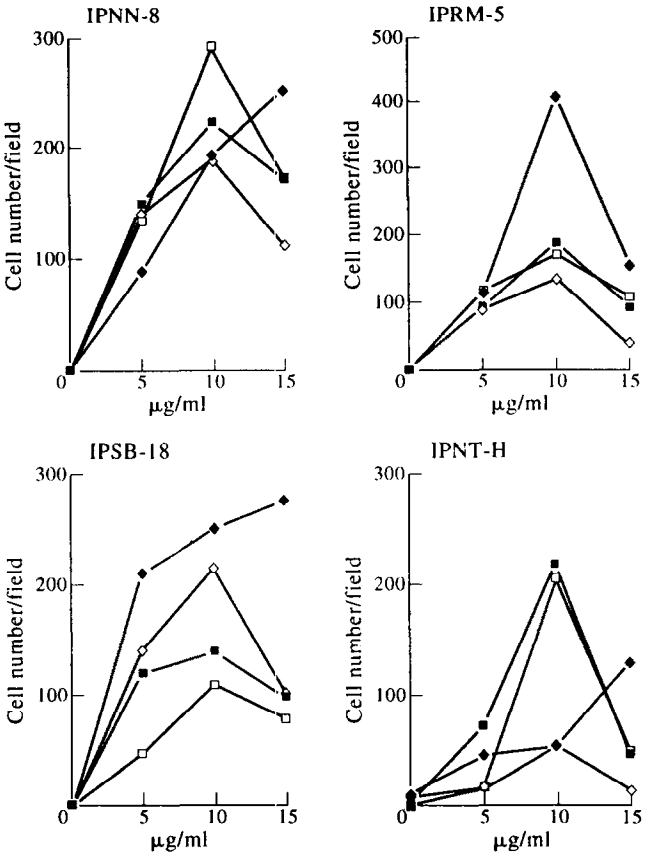

Figure 6. Migration stimulatory effect of individual BM proteins on glioma cells. □ Fibronectin, ■ vitronectin, ◇ collagen I, ◆ laminin.

to be the most potent chemoattractant for malignant glioma cells *in vitro*. Laminin has been demonstrated to be intensely expressed in the basement membranes of blood vessels and leptomeninges in low grade gliomas [29]. Strong expression of laminin has also been found in blood vessels whether located in tumour, adjacent brain tissue or meninges in high grade gliomas [29]. Moreover, the same authors detected an increased expression of laminin in the leptomeningeal membranes and glia limitans of 27 gliomas examined. In accordance with these results, McComb and Bigner [30] reported a high expression of laminin in all 90 neoplasms of the central and peripheral nervous system examined. In addition, they found that in malignant gliomas, laminin was intensely expressed in blood vessels, glomeruloid vascular formations and glial BM. Furthermore, the immunostaining of laminin was found to emphasise the changes occurring in the pial-glial basement membrane during progressive glioma invasion, in that in the early stages of the invasive process, the glial BM remained intact and was found to be destroyed only in the later steps of invasion. This destruction of laminin was found to be associated with direct invasion of malignant glioma into the subarachnoid space [30]. We have recently reported that all the glioma cell lines included in the present study were able to invade and migrate through the artificial basement membrane, Matrigel [17–20], which is known to be composed at 60% of laminin [13]. Furthermore, we found that glioma cell lines attached better to laminin than to the other proteins [22]. Together with these observations, the data presented here suggest that laminin may play an important role in glioma cell proliferation, adhesion, migration, and invasion. It can be speculated that laminin expressed by BM in gliomas may play a central role in attracting neoplastic cells towards blood vessels where angiogenesis occurs. Fibronectin and type 1 collagen are also expressed by BM in the normal and neoplastic nervous system and gliomas [6, 31]. Expression of vitronectin has also been reported in human glioblastomas *in vivo* [32]. These molecules are likely to play an important role in inducing glioma cell migration and invasion. In conclusion, ECM proteins were found to inhibit proliferation and to promote migration of glioma cells *in vitro*, the growth inhibitory effect probably being a prerequisite for cell migration.

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KT-5720 Reverses Multidrug Resistance in Variant S49 Mouse Lymphoma Cells Transduced With the Human *MDR1* cDNA and in Human Multidrug-resistant Carcinoma Cells

H. Galski, P. Lazarovici, M.M. Gottesman, C. Murakata, Y. Matsuda and J. Hochman

T-25-Adh cells, cell variants derived from S49 mouse lymphoma, were transduced with a retrovirus containing the human *MDR1* cDNA. The resultant cells (HU-1) are cross-resistant to colchicine, doxorubicin, vinblastine and actinomycin D, and their resistance to colchicine is reversed by verapamil. HU-1 cells were used to screen several protein kinase modulators for their ability to reverse multidrug resistance. Among the tested indole carbazole (K-252a) family of protein kinase inhibitors, only the antibiotic alkaloid KT-5720 (9-n-hexyl derivative of K-252a) could overcome the multidrug resistance of HU-1 cells and KB-V1 human carcinoma cells. Since other protein kinase A, C and G modulators did not reverse multidrug resistance in the tested multidrug-resistant cells, the chemosensitising activity of KT-5720 on these cells is apparently independent of its kinase inhibitory effects. Since KT-5720 fully reversed multidrug resistance at non-toxic concentrations, it might be a candidate for clinical chemosensitisation in combination chemotherapy.

Key words: chemosensitisers, K-252a derivatives, KT-5720, *MDR1*, multidrug resistance, P-glycoprotein, protein kinases, protein kinase inhibitors
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INTRODUCTION

CLINICAL RESISTANCE to chemotherapeutic drugs is a major obstacle in cancer therapy. One form of drug resistance, termed multidrug resistance (MDR), is defined as the capability of malignant cells subjected to a single cytotoxic agent to develop resistance to many structurally and functionally unrelated drugs