ALIGNANT gliomas have poor prognoses irrespective of treatment modality due to their propensity to migrate into and infiltrate the surrounding tissues, including the normal tissue surrounding the brain. Thus, identification of molecules that promote glioma cell migration is critical and could help in the development of anti-invasion therapies.

Previous studies have shown that a variety of molecules promote glioma cell migration, including growth factors, extracellular matrix proteins, stromal cell derived factor-1α, chemokines, and lysophosphatidic acid-Rho A activating factor. The actual biological functions of these molecules in patients with malignant glioma, however, are not clear because most of the studies have been carried out in vitro or in animal models.

Vascular proliferation is a pathological feature of malignant astrocytoma and GBM, but it also must be recognized that invasion can occur without angiogenesis, such as occurs in diffuse, infiltrative gliomas. This invasiveness could be due to the presence of factors in serum and CSF that promote glioma cell survival and or enhance invasion into the tissue surrounding the brain. These substances may also act as chemoattractants for tumor cells and influence the direction of migration. Indeed, extensive tumor cell movement along blood vessels or myelinated fiber tracts of white matter has been frequently observed in human glioma tissues.

Thus, the purpose of the current study was to identify the substance in human serum and/or cerebrospinal fluid (CSF) that promotes glioma cell migration.

### Materials and Methods

#### Preparation of Human Serum and CSF

Venous blood samples were taken from eight patients with gli...
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omomas, one patient with a pituitary adenoma, and four healthy volunteers. All patients had growing tumors according to magnetic resonance imaging studies, and blood samples were taken preoperatively with written consent from the patients or their relatives. After centrifugation of the blood samples at 1500 G for 5 minutes, supernatants were collected, dialyzed against 25 mM phosphate buffer (pH 7.0) and sterilized using a syringe-driven 0.22-µm filter unit (Millipore). The concentration of protein in serum samples was measured with a protein assay kit (Bio-Rad), and the serum samples were stored at −20°C until use. In two of the four patients with glioma (the patients with anaplastic astrocytoma and gliomatosis cerebri), CSF samples were also taken after surgical removal of the tumor mass and/or invaded brain tissue and after completion of radiation therapy. The CSF samples were stored at −20°C until use.

Cell Culture, Proteins, and Antibodies

A172 human GBM cells (JCRB0228) and T98G human GBM cells (JCRB9041) were obtained from the Health Science Research Resources Bank (Osaka, Japan). The A172 cells were grown in DMEM containing 10% fetal bovine serum and the T98G cells were grown in minimal essential medium containing 10% fetal bovine serum, 1% sodium pyruvate, and 1% nonessential amino acids. Human vitronectin and human plasma fibronectin were purchased from Becton Dickinson. The following neutralizing monoclonal antibodies were purchased from Chemicon: AV1 for human integrin αv subunit, LM609 for integrin αvβ3, P1F6 for integrin αvβ5, and P1D6 for integrin α5 subunit. The murine anti-human vitronectin monoclonal antibodies for immunoblotting were purchased from Chemicon, and sheep anti–human vitronectin antibodies for immunodepletion were purchased from Cedarlane. Control mouse and sheep IgG was purchased from Sigma-Aldrich, Inc., and GRGDSP and GRGESP hexapeptides were purchased from AnaSpec, Inc.

Cell Migration Assay

Cell migration assays were performed in two-well Boyden chambers containing uncoated 8-µm-pore polycarbonate membranes (Becton Dickinson). The upper filter surfaces were coated with 25 µg of Matrigel (BD Biosciences) to facilitate the attachment and spreading of A172 cells because they did not fully attach and spread on uncoated membranes in serum-free conditions even after 20 hours. Trypsinized A172 cells were suspended in DMEM containing human serum (300 µl of DMEM containing human serum (300 µg protein) was added to the filter were fixed with 70% methanol, stained with Giemsa solution, and the cells that migrated to the lower side of the filter were counted using a stereomicroscope (MZ APO, Leica). Each sample was assayed in triplicate and two independent experiments were performed, except for the dose-response assay using CSF samples, which was performed only once because of the limited amount of CSF available. The migration assay using T98G cells was performed without the Matrigel coating because the coating was not needed for the full attachment and spreading of the cells. To examine the effects of integrin-neutralizing antibodies or RGPD peptide, cell suspensions were incubated with antibodies (10 µg/ml) or GRGDSP or GRGESP hexapeptides (50 µg/ml) at room temperature for 15 minutes before they were plated on the filter. The results were expressed as a percentage of the value obtained using control IgG. The statistical significance of differences was determined using a two-tailed Student t-test.

Treatment of Human Serum With Heat or Trypsin

To inactivate serum proteins, normal human serum was heated at 100°C for 10 minutes or incubated with 5 µg trypsin at 37°C for 30 minutes. Treated sera (300 µg protein) were added to the lower compartments of the Boyden chambers, and cell migration assays were performed as described above.

Inhibition of Cell Adhesion

The A172 glioma cells (3 × 104) were incubated with monoclonal antibodies (10 µg/ml) against various types of integrins or with GRGDSP or GRGESP hexapeptide (50 µg/ml) at room temperature for 15 minutes and then seeded on 24-well tissue culture plates coated with purified human vitronectin (10 µg/ml) or human plasma fibronectin (10 µg/ml). After an additional 2 hours’ incubation at 37°C in a humidified atmosphere containing 5% CO2, the cells were viewed with a phase-contrast microscope (IX 70, Olympus) at a magnification of 200 and photographed.

Ultrafiltration of Human Serum

Dialyzed serum taken from a healthy volunteer was filtered through Centricon or Microcon centrifugal concentrators (Amicon) with various molecular weight cutoff membranes (3000, 10,000, 30,000, 50,000, and 100,000) according to the manufacturer’s instructions. The retained material (300 µg protein) was added to the lower compartments of the Boyden chambers, and cell migration assays were performed as described above.

Immunodepletion of Vitronectin

Immunodepletion of vitronectin was performed according to the method described in a previous report.1 Human serum from a normal volunteer was mixed overnight at 4°C with sheep anti–human vitronectin IgG–protein G–Sepharose conjugate or control sheep IgG–protein G–Sepharose conjugate, after which the mixture was centrifuged to sediment the Sepharose beads. This procedure was repeated seven more times, using fresh sheep anti-vitronectin antibody (IgG)–Sepharose conjugate or control sheep IgG–Sepharose conjugate for each step. The vitronectin-depleted serum was separated on a 10% polyacrylamide gel under reducing conditions and electrically transferred to a Trans-Blot nitrocellulose membrane (Bio-Rad), blocked with 3% bovine serum albumin, and subjected to immunoblotting with mouse anti–human vitronectin antibody. The bound antibodies were visualized with anti–mouse IgG–alkaline phosphatase conjugate (Promega) and BCIP/NBT color development substrate (Promega). The immunoblot was scanned with a GT-9500 flat scanner (Epson) and analyzed with National Institutes of Health image software as described previously.34

Measurement of Vitronectin in Human CSF

Vitronectin levels in CSF were measured in duplicate using a Vitronectin ELA Kit (Takara) according to the manufacturer’s instructions.

Results

Promotion of Glioma Cell Migration by Human Sera

We obtained serum samples from eight patients with gliomas, one patient with a pituitary adenoma (as a benign tumor control), and four healthy volunteers and examined the effects of these samples on glioma cell migration using Boyden chambers. We found that approximately 5 to 8% of the cells seeded in the upper chambers had migrated to the lower, serum-containing chambers after 20 hours. In contrast, few glioma cells migrated to the lower chamber when serum was absent (Fig. 1A). The experiments were performed in the presence of 5 mM hydroxyurea, which completely inhibited cell growth (data not shown); thus, cell growth did not contribute to the observed effects. The ability to promote cell migration differed among the various serum samples, but there was not a substantial difference between sera from patients with glioma and the control samples from healthy volunteers.

Migration Promoted by a High–Molecular-Weight Protein

To identify the major factor in human serum that pro-
motes glioma cell migration, we examined the effects of treating the serum by heat inactivation and trypsinization. Heat inactivation and trypsinization inhibited cell migration toward serum by 99% and 55%, respectively (Fig. 1B).

These results suggest that a protein is the major factor in serum that promotes glioma cell migration.

To define the molecular weight of the major factors, we subjected the serum to ultrafiltration through membranes
with various molecular-weight cutoffs. The components
retained following filtration through membranes with mo-
lecular-weight cutoffs of 3000, 10,000, 30,000, or 50,000
had full activity in the Boyden chamber assay (Fig. 1C).
In contrast, the serum fraction retained after ultrafiltration
though a 100,000 molecular-weight cutoff membrane had a
reduced ability to promote glioma cell migration (Fig. 1C).
These results indicate that one of the major factors in serum
that promotes glioma cell migration is a protein with a mo-
lecular weight above 50 kD.

Inhibit Glioma Cell Migration by Antibodies to αv or αvβ5 Integrins and RGD-Containing Peptides

We focused our attention on fibronectin and vitronectin
because they are high–molecular-weight extracellular
matrix proteins abundant in human serum.²³,²⁷ Using flow
cytometry, we previously found that A172 human glioma
cells express integrin subunits α5 and αv.⁸ To determine
which integrin receptors are involved in the interaction
with fibronectin and vitronectin, we examined the effects of
function-blocking monoclonal antibodies against different
integrin subunits or complexes on the adhesion and spreading
of A172 glioma cells (Fig. 2A and B). Cell spreading
on vitronectin-coated substrates was almost completely
blocked by antibodies against the αv subunit or the αvβ5
complex (Fig. 2A) but not by antibodies against the α5 sub-
unit or the αvβ3 complex. In contrast, cell spreading on
fibronectin was selectively inhibited by an antibody against
the α5 subunit (Fig. 2B). The inability of anti-αvβ3 anti-
bodies to inhibit the adhesion and spreading of A172
cells on vitronectin could be due to a very low surface ex-
pression of integrin αvβ3, as indicated by our previous
flow cytometric analysis.⁸ In addition, the RGD-containing
hexapeptide GRGDSP inhibited A172 cell spreading on
vitronectin (Fig. 2A) but did not affect cell spreading on fi-
bronectin (Fig. 2B). These results indicate that adhesion
of A172 glioma cells to vitronectin is mediated by integrin
αvβ5 in an RGD-dependent manner and that adhesion to
fibronectin is mediated by an integrin containing an α5 subunit.

On the basis of these results, we examined the effect of
the RGD peptide and the integrin-neutralizing antibodies
on migration of glioma cells toward normal human serum
(Fig. 2C). Migration of glioma cells towards serum was
substantially inhibited by function-blocking anti-αv (90%)
and anti-αvβ5 (80%) monoclonal antibodies as well as by
the RGD peptide (90%) (Fig. 2C), whereas the anti-αv or
anti-αvβ3 antibodies and the control peptide lacked signif-
icient effects. The effects of the anti-αv and anti-αvβ5 anti-
bodies and the RGD peptide were not due to inhibition of
adhesion to the membranes in the Boyden chambers be-
cause the A172 cells showed full attachment and spreading
and a normal morphology after 20 hours in all samples
examined (Fig. 2D). Human serum–induced migration of
T98G human GBM cells was also completely blocked by
the antibody to integrin αv but not by the antibody to integrin α5 (data not shown). These results indicated that disruption of the interaction between vitronectin and glioma cells inhibits the serum-induced migration of glioma cells.

Effect of Depletion of Serum Vitronectin on Glioma Cell Migration

To confirm that vitronectin is a major serum factor that promotes glioma cell migration, we examined the effect of depleting it from the serum. Immunodepletion of vitronectin from normal human serum was carried out by repeated immunoprecipitation. Immunoblotting confirmed that this treatment reduced the concentration of vitronectin in serum by approximately 50% (Fig. 3A). This 50% reduction in vitronectin caused a substantial reduction (~70%) in the ability of serum to promote A172 cell migration. Furthermore, this inhibition was reversed in a dose-dependent manner by the addition of exogenous vitronectin to the lower chamber (Fig. 3B). Also, human serum–induced migration of T98G human glioma cells was inhibited by the depletion of vitronectin, and this inhibition was similarly reversed by the addition of vitronectin (Fig. 3C). These results confirmed that vitronectin is one of the major factors in human serum that promote the migration of glioma cells.

Measurement of Vitronectin in the CSF of Patients With Glioma and Inhibition of CSF-Induced Cell Migration by Anti-Integrin Antibodies

Because glioma tissues lie adjacent to the CSF, we sus-

<table>
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<tr>
<th>Case No.</th>
<th>Diagnosis</th>
<th>Vitronectin (ng/ml)</th>
<th>Source of CSF</th>
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<tr>
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<td>5</td>
<td>arachnoid cyst</td>
<td>25</td>
<td>lumbar cistern</td>
</tr>
</tbody>
</table>

* Samples were obtained from two sites in Cases 2 and 3.
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![Graph A: Effect of CSF from glioma patients on glioma cell migration.](image)

**A** The A172 cells (2 × 10⁶) were plated on the upper filters and allowed to migrate toward lower chambers that contained CSF taken from three patients with glioma and two patients with non-neoplastic disease (see Table 1 for details). In Case 2 only, the test was performed using three different amounts of CSF from the same site. After 20 hours, the number of cells that migrated to the lower membranes was counted.

**B** Graph showing that cell migration toward CSF was inhibited by the addition of antibodies to α5, β5, or αβ5 integrins. The CSF used in this analysis was obtained from the brain cavity of the patient in Case 2. 100 μl was placed in each lower chamber of the assay apparatus. The results are expressed as the total number of cells that migrated to the lower filters. *p < 0.01 compared with results obtained using control IgG.

The authors of many studies have investigated the factors that promote glioma cell migration to help elucidate the molecular basis of the invasiveness of these tumors. Previous reports on the chemotactic migration of glioma cells have demonstrated that they migrate toward lyosphosphatidic acid-RhoA activating factor (LPA-RAFA) or stromal derived factor-1α, whose specific receptor, CXCR4, is expressed on the surface of glioma cells. In addition, epithelial growth factor has been reported to be the strongest promoter of migration amongst the growth factors present in serum. In this study, we showed that a major migration-promoting factor in serum is a heat-inactivated, trypsin-cleavable protein with a molecular weight greater than 50 kD. These characteristics contrast with those of many growth factors, chemokines, and lipids that can induce migration. Furthermore, using integrin-neutralizing antibodies, RGD-containing peptides, and vitronectin-depleted serum, we found that vitronectin (molecular weight 65–75 kD) is one of the major factors in human serum that promotes the migration of glioma cells.

The ability to promote cell migration differed for the various serum samples tested, but there was no substantial difference among sera from patients with GBM, anaplastic astrocytoma, or pituitary adenoma and normal controls. It has been reported that neoplastic astrocytes in vivo and in vitro showed increased expression of integrin αvβ3 subunit and that benign tumor cells, including cells derived from pituitary adenoma and meningioma, showed the same integrin pattern as their normal counterparts. Therefore, it is possible that in patients with glioma, upregulation of receptors that interact with vitronectin leads to promotion of glioma cell migration by serum.

We also found that the promotion of glioma cell migration by CSF from patients with glioma corresponded to the amount of vitronectin in the sample. Again, blocking the vitronectin-integrin interaction greatly reduced CSF-induced cell migration. These findings suggested that vitronectin is an important promoter of glioma cell migration not only in serum but also in CSF, although our results were valid for only two glioma cell lines and have not been validated with cells from glioma specimens in vivo.

Angiogenesis and high vessel wall permeability are often observed in malignant glioma tissues, and leakage of molecules from blood vessels through the damaged blood–
brain barrier may promote glioma cell invasion. Therefore, it is possible that vitronectin leaking from highly permeable vessels within glioma tissue promotes the migration of glioma cells in vivo.

Both fibronectin and vitronectin are abundant in human serum and enhance the adhesion and migration of various cell types in vitro. Furthermore, several reports indicate that two other factors play important roles in invasion in gliomas: 1) the interaction of integrins on glioma cell surfaces with extracellular matrix molecules, and 2) proteases, including matrix metalloproteinases, which degrade extracellular matrix components. In the current studies, we focused on the role of fibronectin and vitronectin in human serum- or CSF-induced cell migration. Interestingly, blocking the fibronectin–integrin interaction had less of an effect on serum- or CSF-induced migration than blocking the vitronectin–integrin interaction. Immunohistochemical studies have shown that fibronectin or laminins are present in the blood vessels of glioma tumors, whereas vitronectin and tenascin are associated with tumor cells in the tumor parenchyma. Thus, it is possible that molecules surrounding tumor cells, including vitronectin, promote cell motility in the glioma tissue.

We speculate that there are two vitronectin sources that account for the accumulation of this protein in glioma tissue: 1) Vitronectin may be produced by the glioma cell itself, as reported by Gladson et al. and Mahesparan et al., based on immunohistochemical studies and in situ hybridization. 2) It may be leaked through serum from tumor vessels surrounding gliomas, given that serum contains a large amount of vitronectin and vascular permeability in tumor tissues is high.

Our present results indicate that a partial disruption of the interaction between vitronectin and glioma cells can strongly suppress human serum– or CSF-induced glioma cell migration and invasion. Blocking the interaction between vitronectin and integrin on glioma cells could therefore represent a new approach for preventing tumor cell invasion. In vivo experiments using mouse glioma models show that intracranial treatment with an RGD-containing peptide can suppress brain tumor growth by inducing the apoptosis of both brain tumor and brain capillary cells and that this peptide can prolong survival. Vitronectin has only one cell-binding site (RGD sequence), and the current studies confirm that RGD peptides strongly inhibit the interaction of glioma cells with vitronectin.

Conclusions

Our results suggest that vitronectin is an important promoter of glioma cell migration not only in serum but also in CSF.

References

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