Role of galectin-1 in migration and invasion of human glioblastoma multiforme cell lines

Laboratory investigation

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Object. Galectin-1 is highly expressed in motile cell lines. The authors investigated whether galectin-1 actually modulates the migration and invasion of human glioblastoma multiforme (GBM) cell lines, and whether its expression with respect to invasion and prognosis is attributable to certain glioma subgroups.

Methods. In the human GBM cell lines U343MG-A, U87MG, and U87MG-10’, the RNA differential display was evaluated using Genefishing technology. The results were validated by reverse transcription polymerase chain reaction and Northern blot analysis to detect possible genetic changes as the determining factors for the motility of the malignant glioma. The migration and invasion abilities were investigated in human GBM cell lines and galectin-1 transfectant using an in vitro brain slice invasion model and a simple scratch technique. The morphological and cytoskeletal (such as the development of actin and vimentin) changes were examined under light and confocal microscopy. Galectin-1 expression was assessed on immunohistochemical tests and Western blot analysis.

Results. Endogenous galectin-1 expression in the human GBM cell lines was statistically correlated with migratory abilities and invasiveness. The U87-G-AS cells became more round than the U87MG cells and lacked lamellipodia. On immunohistochemical staining, galectin-1 expression was increased in higher-grade glioma subgroups (p = 0.027).

Conclusions. Diffuse gliomas demonstrated higher expression levels than pilocytic astrocytoma in the Western blot. Galectin-1 appears to modulate migration and invasion in human glioma cell lines and may play a role in tumor progression and invasiveness in human gliomas. (DOI: 10.3171/JNS/2008/109/8/0273)

Key Words • galectin-1 • human glioblastoma multiforme cell line • invasion • motility

Current treatments for malignant gliomas, such as surgery, radiotherapy, and chemotherapy, provide only short-term control of the disease.13 The eventual therapeutic failure is primarily due to the invasion of tumor cells into the normal brain.24 For tumor cells to infiltrate adjacent tissue, the invading cells must recognize the barrier matrix, breach it, and then migrate and grow in an ectopic location. Additionally, they must also acquire the ability to control signaling pathways.

It is difficult to directly investigate the specific phenotype of the invasion ability in vivo. However, there is a good correlation between increased invasiveness in vivo and increased motility in vitro. The dysregulation of enhanced motility in tumor cells has been postulated to be an important factor for invasion processes.12,24,29 The identification of the genes related to increased motility is critical to understanding the molecular basis of the biological behavior of invasive malignant gliomas. An improved understanding of this process might lead to the development of new therapeutic strategies that could reduce further disease spread and target invading cells.31

Focusing on the detection of possible genetic changes as factors determining the motility of malignant gliomas, 3 malignant astrocytoma cell lines with different levels of motility were selected. The genes in the high motility group were investigated by DD-PCR using Genefishing technology, known to be a comprehensive screening method for tumor-related genes.26 Galectin-1 was found to be highly expressed in the motile cell lines.

Galectin-1 is a member of the galectin family of...
β-galactosides that bind animal lectin, and is expressed by many different types of normal and neoplastic cells. The pathway for the extracellular release of galectin is different from classic secretory pathways. Galectin-3 and -4 are subject to externalization in a manner similar to that observed in galectin-1. It has been suggested that following externalization, some of the galectin molecules associate with the surface or ECM where the lectin activity is stabilized, while other galectin molecules, free from glycoconjugate ligands, are rapidly oxidized and inactivated in the nonreducing extracellular environment. The ECM components can dramatically modulate the activity is stabilized, while other galectin molecules, free from glycoconjugate ligands, are rapidly oxidized and inactivated in the nonreducing extracellular environment.

Methods

Cell Lines, Cultures, and Antibodies

The human GBM cell lines U87MG and U343MG-A were obtained from the Korean cell line bank (Seoul, Korea) and from the Brain Tumor Research Center, University of California, San Francisco, respectively. To obtain the fastest cell line, we performed the scratch technique to U87MG and selected the cells with the maximal distance. We obtained U87MG-10′ by repetition scratch to the tenth U87MG. All cell lines were routinely grown in DMEM (Gibco BRL) supplemented with 10% FBS at 37°C in a humidified 95% air/5% CO₂ atmosphere.

The antibody against galectin-1 (Santa Cruz Biotechnology) is an affinity purified goat polyclonal antibody raised against a peptide mapping near the amino terminus of galectin-1 of human origin. This antibody is used for the detection of galectin-1 of mouse, rat, and human origin by Western blotting, immunoprecipitation, and immunohistochemistry.

Migration Test

The migration assay of the astrocytoma cell line was performed by the simple scratch technique. To compare the motility of each cell line, the media of the cultured cells was replaced with media containing 5-mM hydroxyurea. A 24-hour treatment with 5-mM hydroxyurea resulted in the complete inhibition of cell proliferation. The cultures were then washed with a single-edged razor blade, washed twice with PBS, and placed in media containing hydroxyurea. After 48 hours of incubation, the cells were again washed twice with PBS, fixed with methanol, and stained with 0.1% toluidine blue. Three microscopic fields were evaluated for each wound injury. The number of cells that traversed the scratched area and the maximum distance traveled were determined in each field and averaged for each injury. These experiments were repeated 3 times.

Isolation of RNA

The total RNA was isolated from the astrocytoma cell lines using TRIzol (Life Technologies) according to the manufacturer’s instructions. The RNA pellets were then frozen and stored at −80°C until use.

Differential Display PCR

Differential display PCR was performed using the Genet elevation kit (Seegene, Inc.) according to the manufacturer’s instructions. For the first strand synthesis, 3 µg of the purified total RNA was incubated with 10 µM dT-ACP for 3 minutes at 80°C and then a buffer solution containing 4 µl of 5 x RT buffer (Promega), 1 µl of dNTP (2 mM each), 2.5 µl of 25 mM MgCl₂, 2 µl of RNase inhibitor (40 U/µl, Promega), and 1 µl of reverse transcriptase (200 U/µl, Promega) was added. The 20-µl reaction mixture was allowed to react for 90 minutes at 42°C and then 2 minutes at 90°C. The PCR protocol for second-strand synthesis was 1 cycle at 94°C for 3 minutes, at 50°C for 3 minutes, and at 72°C for 1 minute. After the second-strand DNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles at 94°C for 40 seconds, 65°C for 40 seconds, and 72°C for 40 seconds, and this was followed by a 5-minute final extension step at 72°C. The PCR products were separated in a 1.2% agarose gel and stained with ethidium bromide. The differentially expressed bands were extracted from the gel using a QiAquick Gel extraction kit (Qiagen) and directly cloned into a pGEM-T Easy vector (Promega) without reamplification of the recovered bands. The products were then sequenced.

Reverse Transcription PCR

The total RNA was isolated from 3 cultured astrocytoma cell lines using TRIzol reagent, and 1 µg was reverse transcribed to synthesize the cDNA. Reverse transcription was followed by PCR. For the first strand synthesis, 1 µg of the purified total RNA was incubated with oligo (dT) (0.5 µg/µl, Promega) for 5 minutes at 70°C, and then in a buffer solution containing 4 µl of 5 x reaction buffer (Promega), 1 µl of deoxyribonucleotide triphosphate (10 mM each), 3.5 µl of 25 mM MgCl₂, 2 µl of RNase inhibitor (40 U/µl, Promega), and 1 µl of RT (200 U/µl, Promega) was added. The reaction volume was 20 µl, and the reaction was performed for 90 minutes at 42°C, and then for 2 minutes at 90°C; 18 genes were identified, and β-actin was used as an internal control. Reactions with varying numbers of PCR cycles were run for each transcription.

Northern Blot Analysis

Northern blot analysis was performed under con-
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vential conditions. The total RNA (20 µg) was separated by electrophoresis on 1.2% agarose-formaldehyde gels, then transferred to a nylon membrane overnight and cross-linked with ultraviolet irradiation. The filters were prehybridized at 65°C for 3 hours and then hybridized to a 32P-labeled probe. The probes were prepared from the clones by digestion with restriction enzymes, and this was followed by gel electrophoresis. The filters were washed in sodium chloride and sodium citrate buffer and then 0.1% sodium dodecyl sulfate for 15 minutes at 65°C, and finally exposed to x-ray film at −70°C.

Preparation of the Plasmid Containing Human Galectin-1 cDNA

Samples of U343MG and U87MG cDNA were used as templates for the PCR reaction. Total RNA was isolated from 2 cultured cell lines using TRIzol reagent. Then 1 µg of RNA was reverse transcribed to synthesize cDNA. The PCR primers were designed to amplify the 408-bp fragment of human galectin-1-S open reading frame; the forward primer, 5′-GGATCCATGCGGTGCTTGTCG-3′ and the reverse primer, 5′-GGCTCAGCTGAGAGGGCTGAGCCACACA-3′. The BamHI and XbaI sites were engineered into the forward PCR primer, and the XbaI (TCTAGA) sites were engineered into the reverse primer. The PCR primers were designed to amplify the 408-bp fragment of antisense human galectin-1 open reading frame; the sense primer, 5′-GGATCCATGCGGTGCTTGTCG-3′ and the antisense primer, 5′-GGCTCAGCTGAGAGGGCTGAGCCACACA-3′. The XbaI (TCTAGA) sites were engineered into the forward PCR primer, and the BamHI (GGATCC) sites were engineered into the reverse primer. The PCR was performed in 20 µl of a mixture solution containing 1 µl of dNTP (10 mM each) mix, 4 µl of 5 × Reaction buffer (Promega), 3.5 µl of 25 mM MgCl2, 0.5 µl of RNase Inhibitor (40 U/µl, Promega), and 1 µl of RT (200 U/µl, Promega). The PCR cycles were performed in a thermal cycler (MJ Research) with the following profile: galectin-1-S denaturation for 5 minutes at 94°C, annealing for 40 seconds at 63.5°C, and a 40-second extension step at 72°C. The PCR products were separated by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining. The differently expressed bands were extracted from the gel using a QIAquick Gel extraction kit (Qiagen). This 408-bp fragment amplified by PCR was subcloned into the pGEM-T Easy Vector (Promega Corp.) according to the manufacturer’s instructions and then the products were sequenced.

Plasmid Construction

A 408-bp fragment of a full-length human galectin-1 cDNA was cloned in the sense and antisense orientation into the BamHI and XbaI sites of expression plasmid vector pcDNA3.1(±) containing the cytomegalovirus promoter and the neomycin-resistance gene. The resulting vectors, pcDNA3.1(±)-galectin-1-S and pcDNA3.1(±)-galectin-1-AS were used to transfect the plasmid DNA.

Transfection Procedure

The U343MG and U87MG cells were maintained under exponential growth conditions in DMEM supplemented with 10% FBS in the absence of antibiotics. The optimum cell density for transfection is normally between 50 and 80% confluence for adherent cells. The pcDNA3.1(±)-galectin-1-S and pcDNA3.1(±)-galectin-1-AS were respectively transfected into the human glioma U343MG and U87MG cells using the GeneJuice transfection reagent (Novagen Corp, 1.33 mg/ml suspension in 80–90% ethanol). These transfectants are referred to herein as U343-G-S and U87-G-AS, respectively. Cells in serum-free DMEM were mixed with 15 µg of plasmid DNA and 45 µl of GeneJuice/serum-free media according to the manufacturer’s protocol. After 5 hours of incubation at 37°C (in 5% CO2), the transfection mixture was replaced with DMEM supplemented with 10% FBS. After 24 hours of incubation, the medium was replaced with DMEM containing 10% FBS and 800 mg/ml G418 and cultured in a CO2 incubator. The G418-resistant clones were isolated, and the expression level of galectin-1 protein induced by 150-µM zinc sulfate was determined by Western blotting analysis. The stable transfectants were maintained in DMEM supplemented with 10% FBS and 400 mg/ml G418.

Western Blotting

Cells were lysed in a lysis buffer (50 mM Tris [pH 8.0], 5 mM ethylenediamine tetraacetic acid, 150 mM NaCl, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate, 1% NP-40, 1 mM phenylmethylsulphonyl fluoride, and 1 mg/ml protease inhibitor cocktail). The protein concentrations were determined using a protein assay kit (Bio-Rad). Then 20 µg of the whole cell lysates were separated by 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Pall Corporation). Subsequently, the membrane was incubated for 2 hours at room temperature in a solution of TBST (10 mM TrisCl [pH 8.0], 150 mM NaCl, and 0.05% Tween 20) supplemented with 5% nonfat dry milk, and probed overnight at 4°C with goat anti-galectin-1 (1:2000 dilution). The bound antibodies were visualized with donkey anti–goat serum (1:80000 dilution, Jackson Immunoresearch) conjugated with horseradish peroxidase using enhanced chemiluminescence reagents (Amersham Biosciences); β-actin was used as an internal control. We used the densitometer software (1-Dmain software) to estimate the expression level of galectin-1.

Cell Morphological Characteristics

The 60-mm dishes containing the cells were fixed with methanol at 4°C for 10 minutes. After washing twice with FBS and staining with toluidine blue, the cells were examined under light microscopy (Nikon) and digitally photographed to evaluate and record the cell population morphological characteristics.

Doubling Time of Stable Transfectants

Transfectants with galectin-1-S and -AS and each parental cell were seeded on 3 × 104 cells in 30-mm culture
dishes. The cells were harvested and counted every 24 hours after 48 hours of serum starvation. The cells were trypsinized and the number of viable cells was counted with a hemocytometer. The doubling-time was calculated from the cell growth curve over 4 days using the equation: doubling time = (final time – initial time) log 2/log (final cell number) – log (initial cell number).

**Immunofluorescence Confocal Microscopy**

For the cytoskeleton analysis, cells were cultured on coverslips in 35-mm dishes until subconfluence, washed with PBS, and fixed with 4% paraformaldehyde for 10 minutes. After washing (3–5 times) in immuno/DNA buffer (Invitrogen Corp.), the cells were treated with Triton X-100 at 0.1% for 5 minutes at room temperature, and washed again 3–5 times. The cells were incubated with antim Vent (1:100 dilution, BD Pharmingen) in a humid chamber for 1 hour, and then with Alexa 488-conjugated goat anti–mouse antibody (1:400 dilution, Molecular Probes) for 40 minutes. For actin staining, rhodamine-conjugated phalloidin (Molecular Probes) was used. The coverslips were mounted on slides with Immuno-mounts (Shandon). Confocal microscopy was performed with an Axiovert 100M confocal microscope (Carl Zeiss Micro-Imaging, Inc.) equipped with a Plan-Apochromat 63 × 1.40 oil objective lens. The confocal images were acquired using LSM 510 2.3 software.

**Immunohistochemical Localization of Galectin-1 in Gliomas**

Immunostaining was performed using the routine avidin–biotin complex method and carried out with a Microprobe Immuno/DNA stainer (Fisher Scientific). Paraffin-embedded blocks of formalin-fixed surgical specimens of astrocytoma, anaplastic astrocytoma, and GBM (obtained from the hospital of Chonnam National University) were cut into 3-µm-thick sections with a microtome and placed on microscope slides. The sections were deparaffinized in xylene and treated with 3% H₂O₂ in methanol for 20 minutes to block endogeneous peroxidase activity. After washing several times with immuno/DNA buffer (Invitrogen Corporation), the sections were incubated with 2% normal serum to block any nonspecific binding, followed by incubation with a monoclonal antibody for galectin-1 (1:100 dilution, Santa Cruz Biotechnology, Inc.) at 4°C overnight. A streptavidin horseradish peroxidase (Dako Cytomation, Inc.) detection system was applied to the capillary channels, followed by 20 minutes of incubation at 37°C. After rinsing, the tissue sections were ready for chromogen reaction with 3-amino-9-ethyl carbazole. The sections were counterstained with hematoxylin and mounted on universal mounts (Shandon, Inc.). Finally, the cover slips were affixed to the slides with mounting solution.

For galectin-1 assessment, reactions in vascular endothelial cells—which were present in all specimens—were used as internal, built-in positive controls. The intensity of galectin-1 staining was scored in each specimen on a 0–3 scale, in which 0 = negative staining, 1 = weakly positive staining, 2 = moderately positive staining, and 3 = strong-ly positive staining. The staining intensity of the internal controls is considered as moderately positive staining and tumor cell expression intensity was compared with internal controls. A tumor with moderate or strong staining was considered to have high galectin-1 expression.

**Brain Slice Invasion Study of Transfectants**

In an attempt to recapitulate the matrix macromolecule representation normally encountered by infiltrating astrocytoma cells, we adapted neural organotypic cultures, as previously described by Jung et al.22,23 We evaluated invasiveness in a brain slice model using the Dil-stained astrocytoma cells (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, 97%; Aldrich Chemical). Briefly, specimens of white matter were cut into 1-mm-thick, 8 × 8–mm² slices using a brain matrix (Harvard Instruments) and placed onto the upper chamber of 24-mm Transwell culture dishes (0.4-µm pore size, Corning Costar Corporation). The brain slices were incubated in medium containing DMEM including 10% FBS, and 5 × 10⁵ astrocytoma cells were placed on a central hole of the brain slice and incubated for 14 days.

**Tissue Preparation and Laser Confocal Microscopy Analysis of Invasion**

After 10 days in culture, the whole brain slice was fixed with 4% paraformaldehyde at 4°C overnight and rinsed carefully with PBS. The 1-mm-thick brain slice was placed on a microscopic slide and covered with mounting medium to maintain its 3D structure. The sample was observed directly using a confocal microscope.

**Data Analysis**

The comparison of the nucleotide sequence homology of the isolated cDNAs with the registered sequence in Gene bank was carried out using the BLAST algorithm. We measured the statistical significance of the cell distance and the cell number using the Mann–Whitney U-test, and the doubling time by repeated measures analysis of variance testing. The relationship between galectin-1 expression and categorical variables was compared with the chi-square test or Fisher exact probability test when appropriate. Probability values < 0.05 were considered significant. Statistical analysis was performed using commercially available software (SPSS version 12.0 for Windows, SPSS, Inc.).

**Results**

**Motility Comparison Among the 3 Astrocytoma Cell Lines**

The motilities of the cell lines were compared using a simple scratch technique. To eliminate any confounding effects on cell proliferation introduced by experimental agents, the media of the culture cells were replaced with medium containing hydroxyurea. The treatment with hydroxyurea resulted in the complete inhibition of cell proliferation. These experiments were repeated 3 times. The U87MG cells were more motile than the U343MG-A cells, and the U87 MG-10⁺ cells demonstrated the fast-
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![Image of cell migration tests](image)

Fig. 1. A–C: Photomicrographs demonstrating the results of the cell-migration tests using the simple scratch technique in U343MG-A (A), U87MG (B), and U87MG-10’ (C) cells. Toluidine blue stain, original magnification × 40.  D: Box plot comparing motility of the cell lines. The U87MG cells were more motile than the U343MG-A cells, and the U87MG-10’ cells were the fastest; these differences were statistically significant.

Identification of Differentially Expressed Genes

To obtain a profile of the genes related to invasion, we estimated the motile abilities of the 3 cell lines that showed different motilities. A comparative analysis of gene expression between low and high motility cell lines revealed only 39 genes that showed differential expression patterns. Twenty genes showed a higher expression level in the motile cell lines; in contrast, there were 8 genes with a higher expression level in the less motile cell lines. The other genes demonstrated insignificant expression pattern differences.

Validation of DD-PCR Data by RT-PCR and Northern Blot

We verified the pattern of gene expression with significant differences by RT-PCR and Northern blot analysis for gene candidates. Of note, 50% of the genes differentially expressed by DD-PCR were not confirmed by the RT-PCR results. Therefore, we preferentially selected genes from the Northern blot and RT-PCR analysis. After repeat analysis, we finally found 5 genes: YWHAB, calpain, galectin-1, metallothionein 1E, and FRJ22905 fis, highly similar to HUMRPL18A Homo sapiens ribosomal protein L18 mRNA. Among these genes, YWHAB, calpain, galectin-1, and metallothionein 1E were increased in the high motility cell lines, while FRJ22905 fis was increased in the lower motility cell line. There were significant differences in expression of galectin-1 between the low and high motility cell lines, and this expression was matched on both the RT-PCR and Northern blot analysis (Fig. 2). We selected the galectin-1 gene for further analysis and are currently investigating the others in other studies.

Endogenous Galectin-1 Content

To assess the relationship between galectin-1 expression and glioma cell proliferation, migration, and invasion, we transfected human glioma U343MG-A cells expressing low levels of galectin-1 with a plasmid DNA that produces sense galectin-1 mRNA under the control of the cytomegalovirus promoter, and in this manner obtained neomycin-resistant–transfected clones. We also
transfected human glioma U87MG cells expressing high levels of galectin-1 with a plasmid DNA that produces antisense galectin-1 mRNA. To determine whether sense galectin-1 mRNA promotes the production of galectin-1 protein and whether antisense galectin-1 mRNA interferes with the production of galectin-1 protein, we selected the respective clones. Monomeric galectin-1 has a molecular weight of 14 kD. Sense U343-G (sense galectin-1 transfected U343MG-A) showed approximately 10-fold galectin-1 level compared with U343MG-A on Western blot analysis with an antigalectin-1 antibody. Antisense U87-G (antisense galectin-1 transfected U87MG) contained only 60% expression of galectin-1 compared with U87MG (Fig. 3).

Effects Mediated by Galectin-1 on Cell Migration In Vitro: Simple Scratch Technique

The least motile cell line tested was U343MG-A. After galectin-1 transfection, U343-G-S became more motile in terms of the mean cell number and maximum migration distance, compared with U343MG-A. The maximum migration distance and mean cell number were 1.43 μm and 37 cells for U343MG-A, and 2.9 μm and 146.3 cells for U343-G-S, respectively (Fig. 4A–C). The difference between the 2 groups was statistically significant for mean cell number (p < 0.034) and maximal distance (p < 0.031).

The most motile cell was U87MG. After transfection, U87-G-AS became less motile in terms of the mean cell number and maximum migration distance compared with U87MG. The maximum migration distance and mean cell number in the parental cell lines were 8.2 μm and 384 cells for U87MG, and 7.1 μm and 157.3 cells for U87-G-AS, respectively (Fig. 4A, D, and E). The difference between the 2 groups was statistically significant for mean cell number (p < 0.014) and maximal distance (p < 0.019).

Cell Doubling Time

We examined the growth of 2 transfected clones compared with the parent cells (Fig. 5). The doubling times were 34.1 and 20.4 hours in the case of U343MG-A and U343-G-S, and 26.2 and 36 hours in the case of U87MG and U87-G-AS, respectively. The difference between U343-G-S and U343MG-A was statistically significant for the doubling time (p < 0.05); there was no significant difference between U87MG and U87-G-AS (p < 0.166).

Morphological and Cytoskeletal Changes After Transfection

The U343MG-A cells had a tightly packed, cobblestone appearance. The structural characteristics of the sense U343-G cells resembled that of the parent U343MG-A cells. The U87-G-AS cells were bipolar in configuration, much like the U87MG cells. However, the shape of the U87-G-AS cells was more rounded.

To determine whether the differences associated
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Fig. 3. Western blot analysis demonstrating galectin-1 expression in human GBM cell lines. A: Western blot analysis showing different expression of galectin-1 in parent and transfectant cells. B: Control protein loading (β-actin) for panel A.

with the cytoskeletal alterations were correlated with tumor cell motility and invasion, we performed immunofluorescence staining of cytoskeletons. The pattern of the cytoskeleton was studied with actin and vimentin. For the actin and vimentin staining, the U343MG-A cells and the U343-G-S cells showed similar expression patterns of lamellipodia and stress fibers. The U87MG cells exhibited extensive lamellipodia in addition to the presence of well-defined stress fibers. The U87-G-AS cells studied lacked lamellipodia, which were indicative of cell motility found in the selected cells, compared with the U87MG cells (Fig. 6A and B).

Fig. 4. A: Box plot graphs demonstrating the correlation between migration ability and galectin-1 expression in parental cells and transfectants found using the simple scratch test. The difference in migrated cells between the U343MG-A and U343-G-S lines was statistically significant for the mean cell number (p < 0.034) and maximal distance (p < 0.031). The difference between the U87MG and U87-G-AS lines was statistically significant in mean number of cells (p < 0.014) and maximal distance traveled (p < 0.019). B–E: Photomicrographs showing results of cell-migration tests with the simple scratch technique. Toluidine blue stain, original magnification × 40.
Expression of Galectin-1 in Human Gliomas

The histological structures were analyzed for the 39 glioma samples (6 astrocytomas, 6 anaplastic astrocytomas, and 27 glioblastomas). The immunohistochemical study revealed variable degrees of galectin-1 expression in the low- and high-grade gliomas (Fig. 7A–C), and galectin-1 was notably localized in the cytoplasm (Fig. 7D). Of the 39 samples obtained, 35 (89.7%) exhibited positive galectin-1 immunoreactivity and 26 (66.7%) exhibited high galectin-1 expression (Table 1). When we correlated galectin-1 expression and tumor histological grade, increased galectin-1 expression was associated with a higher tumor grade ($r = 0.354$, $p = 0.027$).

The protein level of galectin-1 in the diffuse gliomas (astrocytoma, anaplastic astrocytoma, and GBM) and the pilocytic astrocytoma was determined on Western blot analysis (Fig. 7E). Each glioma demonstrated a single band corresponding to 14 kD, suggesting that pilocytic astrocytomas, which do not diffusely invade the surrounding brain parenchyma, express lower levels of galectin-1 than the subgroup of diffuse astrocytic tumors.

Brain Slice Invasion Study of Transfectants

The invasiveness of the astrocytoma cells was evaluated in brain slices by using the organotypic culture technique, described by Jung et al.22,23 The sense U343-G cells, transfected with galectin-1, migrated widely throughout the surrounding normal brain compared with the U343 parent cells (Fig. 8A and B). Most of these cells were found around the implanted site of the brain slice.

Discussion

The complete and thorough removal of gliomas is usually microscopically impossible due to the insidious infiltration of tumor cells into the surrounding normal brain tissue. This is the primary cause of therapeutic failure. We attempted to identify the motility-related genes in vitro—representative of invasion ability in vivo—in malignant astrocytoma cell lines with different motilities using the DD-PCR method. Differential display is a powerful tool for the identification of induced or sup-
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pressed genes in cells or tissues. However, false positive expression frequently complicates the interpretation of results. Therefore, we verified the pattern of gene expression based on significant differences of RT-PCR and Northern blot analysis for the gene candidates identified. In the highly motile cell lines, YWHAB, calpain, galectin-1, and metallothionein 1E were highly expressed; we selected the galectin-1 gene for further analysis.

Galectin-1 is a member of the newly defined family of β-galactoside–specific lectins that share a consensus amino acid sequence corresponding to the carbohydrate binding site. It has been reported that galectin-1 binds to poly-N-acetylactosamine oligosaccharide chains on laminin.

This basement membrane protein has a major influence on cell adhesion, migration, differentiation, and proliferation and has additionally been reported to be involved in development, tumor invasion, and metastasis. Galectin-1 has been proposed to have a variety of functions, such as the mediation of cell–cell interactions and influence on cell growth. Galectin-1 gene expression is developmentally regulated and may vary both in cell lines and mammalian tissues during transformation events. Thus, galectin-1 is a multifunctional galactoside-binding protein expressed by many cell lines and tissue types.

Galectin-1 is normally present in the CNS. Immunochemical studies have shown that galectin-1 is expressed in both neuronal cells and nonneuronal cells, including astrocytes in the rat brain, and is also expressed by many types of neoplastic cells. Galectin-1 mRNA is expressed at higher levels in human gliomas and glioma cell lines than in normal glial cells. In human malignant cell lines derived from various extracranial sites, galectin-1 expression is higher than in benign tumors. In murine melanoma cells, increased galectin-1 expression has been associated with an increased metastatic potential.

Galectin-1 was notably localized in the cytoplasm of cells. The average expression level was only slightly increased in low-grade astrocytoma and anaplastic astrocytoma, but markedly increased in GBM cells. Our data

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<th>Variable</th>
<th>Astrocytoma &amp; Anaplastic Astrocytoma (12 samples)</th>
<th>GBM (27 samples)</th>
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* neg = negative; pos = positive.
showed a positive correlation between galectin-1 expression and tumor grade. On Western blot analysis, the diffuse gliomas demonstrated higher expression levels compared with pilocytic astrocytoma cells. Galectin-1 is expressed in all human glioma types, and quantitative determination of galectin-1 expression enabled the pilocytic astrocytomas to be clearly distinguished from the diffuse astrocytomas. Galectin-1 mRNA expression appears to be important in the development of gliomas and to reflect malignancy. Our findings also suggest that galectin-1 may play a role in invasiveness and tumor progression in gliomas in humans.

We examined the growth of the 2 transfected clones and compared them with parent cells. The doubling-times were 34.1 and 20.4 hours in U343MG-A and U343-G-S, and 26.2 and 36 hours in U87MG and U87-G-AS, respectively. The difference in doubling time between U343-G-S and U343MG-A was statistically significant; however, there was no significant difference between U87MG and U87-G-AS. Our data suggest that galectin-1 might be related with the progression of glioma growth. The cooperation of several growth factors may control the glioma cell proliferation, including insulin-like growth factor I, platelet-derived growth factor, and heparin-binding growth factor. Galectin-1 might function through an autocrine-dependent pathway involving a galectin-1 receptor to regulate the proliferation of glioma cells. The effects of galectin-1 on cell proliferation do not involve its sugar-binding activity. That is, those saccharides that inhibited the sugar-binding activity of galectin-1, such as lactose or thiogalactoside, did not negate its stimulating effect on cell proliferation.

To determine whether the differences associated with the cytoskeletal alterations were correlated with tumor cell motility and invasion, we performed immunofluorescence staining of the actin and vimentin cytoskeletons and found that there were no changes in size and shape of the cells after galectin-1 transfection. For actin and vimentin staining, the U343MG-A cells and the U343-G-S cells showed a similar expression pattern of lamellipodia and stress fibers. The U87-G-AS cells became rounder and lacked lamellipodia compared with the U87MG cells. The induced formation of stress fibers and lamellipodia is indicative of cell motility. Stress fibers, which are characteristic of the filament type, are long cables that can contract, and to exercise tension they form a cellular cortex that can be visualized in the perimeter of the cell. Vimentin filaments appeared to be organized in the form of a net that was distributed throughout the whole cytoplasm; these filaments formed a type of perinuclear ring.

Regardless of its tumor grade, each glioma cell line may exhibit a particular invasiveness. The effect of galectin-1 on the migratory and invasive abilities of human glioma cell lines was evaluated with an in vitro assay, the simple scratch technique, and the brain slice model system. The migratory ability of the U343-G-S cell line was increased compared with the parent cell line, while that of the U87-G-AS cell line was reduced. Our data showed that galectin-1 modulates astrocyte tumor migration in vitro. The simple scratch technique was found to be a rapid and quantitative method and was performed in a confluent cell monolayer. Therefore, its relevance to invasion has occasionally been called into question. A va-
riety of invasion model systems for in vivo and in vitro studies have been introduced for the study of brain tumor invasion. Transplanted tumors in animal models progress principally by expansion rather than diffuse infiltration due to their high rates of proliferation.32 The invasive potential of cancer cell lines has commonly been tested in vitro by using matrigel as a barrier to invasion.19 However, matrigel does not contain significant quantities of elastin and would not permit us to test the specific role of elastin-binding proteins with elastin as a substrate. With regard to the selection of a suitable matrix for the tumor, organotypic coculture model systems have been used to study the invasiveness of brain tumors.22,23 Living brain tissue, especially human brain tissue, is an ideal matrix in which to study malignant glioma cell invasion in vitro. In experimental tumor models and histopathological preparations, single-cell infiltration and micrometastasis have often proven difficult to study because of a lack of suitable and sensitive markers that can discriminate individual tumor cells from the normal cell population. Green fluorescent protein has been used to monitor gene expression and protein localization in living organisms, as well as in the visualization of cancer invasion and metastasis.10,22 The study of brain tumor invasion in brain slice model systems has proven to be more vital and reliable than that of other in vitro models. In the organotypic coculture model, most of the U343MG-A cells maintained their position around the original implantation hole, while the U343-G-S cells migrated widely throughout the brain. These findings presented from in vitro study suggest that galectin-1 may play an important role in the invasiveness and migration of human GBM cells.

Conclusions

Endogenous galectin-1 expression in the human GBM cell lines was directly correlated to their migratory abilities and invasiveness. The U87-G-AS cells became more round and lacked lamellipodia compared with the U87MG cells. The expression of galectin-1 was increased with a higher pathological grade of glioma on immuno-histochemical staining. The diffuse gliomas demonstrated higher expression levels compared with the pilocytic astrocytoma in the Western blot analysis. In conclusion, galectin-1 appears to modulate migration and invasion in human glioma cell lines and may play a role in tumor progression and invasiveness in human gliomas.

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