Increased invasive capacity of connexin43-overexpressing malignant glioma cells

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Object. Malignant glioma cells, similar to astrocytes, express connexin43 (Cx43) universally but at widely varied levels. Data from previous studies have demonstrated that malignant glioma cells form functional gap junction channels among themselves as well as with astrocytes and that such a communication has the potential to modulate the phenotypic characteristics of astrocytes. Recently, gap junctions have been demonstrated to play a role in the invasive phenotype of malignant gliomas. In this study, the authors have further investigated the motility and invasion ability of Cx43-overexpressing and Cx43-deficient malignant glioma cells.

Methods. Using a standard invasion system of a Matrigel transwell invasion chamber, the authors found that the number of Cx43-transfected C6 glioma cells (C6-Cx43 cells) migrating through the Matrigel-coated membrane was similar to that of mock-transfected control cells (C6-mock cells) during the first 24 hours, but increased significantly thereafter. When these cells were cocultured with astrocytes, the number of invading C6-Cx43 cells was more than threefold greater than the number of invading C6-mock cells. Results of an in vitro cell motility assay also demonstrated that C6-Cx43 cells were more motile and scatter-active than C6-mock cells. Furthermore, zymographic analysis of MMPs, an important determinant in glioma invasion, demonstrated that the amounts of MMP-2 and MMP-9 in culture medium collected from C6-Cx43 cells were orders of magnitude higher than those from C6-mock cells. In addition, BB-94, a synthetic MMP inhibitor, significantly inhibited C6-Cx43 cell invasion.

Conclusions. The overexpression of gap junction proteins in glioma cells and the intercellular communication between tumor and nontumor glia cells may play important roles in the facilitation of glioma cell invasion.

Key Words • connexin • glioma • Matrigel • matrix metalloproteinase

Malignant gliomas are the most common primary brain tumors that are predominantly rapidly fatal. One of the key features of these neoplasms is their local, diffusely invasive growth, which has limited all local therapeutic strategies thus far. Malignant glioma cells are exceedingly motile and have extended microscopically through much of the neuraxis at the time of diagnosis. They not only invade normal brain tissue, but also cause the destruction and replacement of normal anatomical structures. Studies of the biological events that take place during the infiltrative process are vital to our understanding of glioma progression in situ.

Glioma invasion is thought to rely on the modification of cell adhesion and proteolysis of ECM components. Recently, it was reported that normal brain tissue has the ability to produce ECM components such as laminin, type IV collagen, and fibronectin, when stimulated by invading glioma cells. The interactions between glioma cells and surrounding host astrocytes may facilitate glioma cell migration and invasion, although the mechanisms remain unclear. We have examined the expression of Cx43 and functional gap junction formation in primary cultures of human malignant glioma cells and nontumor astrocytes. Malignant glioma cells form functional gap junction channels with astrocytes, and such a communication has the potential to modulate the phenotypic characteristics of astrocytes. Gap junctions, a subset of cell membrane channels that directly link the interiors of coupled cells, are expressed widely on normal astrocytes and glioma cells in the brain of adult mammalians. They are permeable to molecules with an atomic mass less than or equal to 1.2 kD, making it possible for second messengers such as cyclic 3’5’-adenosine monophosphate, inositol 1,4,5-trisphosphate, and calcium to affect their cell of origin as well as neighboring cells. Gap junctions are composed of homologous proteins called connexins, which are encoded by a highly conserved multigene family and are expressed in a tissue-specific manner. Astrocytic gap junctions are assembled primarily, if not entirely, with Cx43. In contrast, neurons and oligodendrocytes in the adult brain are poorly coupled and express primarily Cx32. The influence of gap junctions on glioma cell function has been documented. The expression of Cx43 in C6 glioma cells is relatively low in comparison to primary cortical astrocyte cells in culture, and growth retardation was found in Cx43-transfected C6 cells. The role of con-
nexus in glioma regulation is not clear, however. Malignant glioma cells express Cx43 universally, but at widely varied levels. In addition, there are recent data indicating that transfection of Cx43 increases the invasive properties of HeLa cells, and Lin, et al., have recently revealed that Cx43-transfected astrocytic tumor cells demonstrate an increased ability to aggregate and disperse through a greater volume of brain parenchyma than mock-transfected sister cells.

It has been reported that invading tumor cells secrete several proteases such as serine proteases (for example, plasminogen activators) and MMPs. The MMPs are thought to have a role in a number of astrocytic tumors because MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) are significantly elevated at both the messenger RNA and protein levels in malignant gliomas and contribute to the invasive ability of glioma cells in vivo and in vitro. For example, the ability of glioma cells to spread on myelin depends on metalloproteolytic activity. Therefore, it is of interest to know the possible relationship between connexin expression and MMP activation.

In the present study, we show that overexpression of Cx43 promotes malignant glioma cell migration and invasion in vitro, especially when the cells are cocultured with normal astrocytes. Furthermore, Cx43-transfected C6 glioma cells (C6-Cx43 cells) express higher MMP levels than vector control–transfected C6 glioma cells (C6-mock cells), indicating a role for MMPs in this Cx43-promoted glioma cell invasion. Note that Cx32-transfected C6 glioma cells (C6-Cx32) have a lower invasion ability when they are seeded on the top of astrocytes, even though they express higher MMP-2 levels. The interaction between glioma cells and surrounding astrocytes may be an important step in this pathobiological process of glioma cell invasion.

**Materials and Methods**

**Cell Lines, Transfection, and Selection**

Rat C6 glioma cells (American Type Culture Collection, Rockville, MD) were transfected with Cx43 or Cx32. The Cx43 and Cx32 complementary DNAs, which were cloned with the pcDNA1 expression vectors containing the sequence for genetin resistance, were kindly provided by K. Willecke (Bonn University, Bonn, Germany). The C6 cells were transfected with Clontech (Clontech, Palo Alto, CA) according to the manufacturer’s instructions, and stable transfectants were selected with 2 mg/ml genetin. The expression of Cx43 or Cx32 was confirmed by immunostaining and functional dye transfer assays, as described. The control transfectants, like their parental native C6 cells, expressed no detectable levels of Cx43 or Cx32.

**Rat Astrocyte Cultures**

Astrocyte cultures were derived from the brain of prenatal (embryonic Day 17) or newborn (1 day postnatal) rats and were prepared using a standard primary culture procedure in our laboratory, as described. Briefly, forebrains were dissected out and immersed in calcium- and magnesium-free Hanks balanced salt solution at 37°C. An equal volume of 0.25% trypsin (Sigma, St. Louis, MO) was added, and the tissue was triturated through a fire-polished Pasteur pipette, incubated at 37°C for 10 minutes, and triturated to homogeneity. An equal volume of culture medium was added, and the cell suspension was centrifuged for 10 minutes at 1000 rpm. The pellet was diluted in 2 ml of warm medium. A total of 8–10 × 10⁴ cells were plated in cell culture flasks in DMEM/F12, containing 10% FBS, 8 mg/ml d-glucose, 20 U/ml penicillin-G, 20 mg/ml streptomycin, and 50 ng/ml amphotericin (all medium constituents were purchased from Life Technologies, Bethesda, MD). The cultures were incubated at 37°C in 5% CO₂, humidified air, with medium changes every 3rd day. Experiments were performed after 10 to 14 days of incubation.

**Matrix Gel Transwell Invasion Assay**

The invasion of glioma cells was measured in vitro in a Matrigel-coated transwell system (Biocoat Matrigel invasion chamber; Becton Dickinson Labware, Bedford, MA), as described with modification. Briefly, the glioma cells were prelabeled with fluorescent dye (2-chloromethyl)benzoyl)amino)tetramethylrhodamine (red) and harvested by trypsinization. Cell suspensions were prepared in culture medium (serum-free DMEM/F12) and added to the 24-well chambers (5 × 10⁴ cells in 0.2 ml per well). In the lower chamber, 700 μl of regular medium (10% FBS-DMEM/F12) was added. In some assays, these prelabeled glioma cells (5 × 10⁴ cells/well) were mixed with rat astrocytes (2.5 × 10⁶ cells/well) and were seeded into the upper chamber. In another group, rat astrocyte cells were harvested and seeded into the upper chamber at a density of 2.5 × 10⁵ cells per well 3 to 4 days before the glioma cells were added. After incubation, the noninvasive cells with Matrigel matrix were removed from the upper surface of the membrane by scrubbing with a cotton-tipped swab. Invasion was quantified with a confocal microscope (BioRad, Hercules, CA) by counting the total cells before removal of the Matrigel and the cells invading through the Matrigel-coated membrane. The percentage of invasion was calculated according to the following formula: percentage of invasion = (mean invading cells / mean total cells) × 100%. A minimum of 10 fields was evaluated for each well, and all experiments were performed in triplicate. To confirm the results of confocal microscopic observation, we also used a cell invasion assay kit (Chemicon International, Temecula, CA), according to the manufacturer’s instructions. Cells were seeded into the upper chamber as described, and cell migration was checked 48 hours later. The chamber membranes were treated with staining solution after the noninvasive cells as well as the ECM were gently removed using a cotton-tipped swab. Migrating cells were counted with the aid of light microscopy.

**Measurement of MMP Activity**

Cells were seeded in 24-well cell culture plates (5 × 10⁴ cells/well) and incubated in 10% FBS-DMEM/F12 culture medium at 37°C in a humidified atmosphere containing 5% CO₂, for 24 hours. They were then washed twice with serum-free DMEM/F12 medium and incubated in the same type of medium for another 24 hours. The medium was collected and centrifuged at 10,000 G, and the supernatant was mixed with 4 × SDS sample buffer (0.2 M Tris-HCl, pH 6.8; 4% SDS; 40% glycerol; and 0.1% bromophenol blue). The levels of gelatinases (MMP-2 and MMP-9) were measured with the aid of zymography, an in vitro assay that involves the use of gelatin-substrate gel electrophoresis, as described. Gels were stained with Coomassie blue and destained. Gelatinolytic activities were visualized as clear bands against a blue background. Gels were analyzed by performing computerized densitometric scanning of the images with a Hewlett-Packard scanner, Deskscan II software, and Image-1 software. The size and intensity of each band were determined. The expression of MMP-2 and MMP-9 on gelatin zymography was previously confirmed on Western blot analyses and immunoseparation experiments.

**Cell Proliferation**

We measured C6 glioma cell proliferation simply by counting cell numbers. The Cx43-transfected and nontransfected C6 cells were seeded into 24-well culture plates at a density of 5 × 10⁴ cells per well and were incubated in 10% FBS-DMEM/F12 culture medium at 37°C in a humidified atmosphere containing 5% CO₂ for up to 1 week. The cells were harvested by trypsinization and counted using a hemacytometer. Each group of cells was quantified in triplicate, and the assays were repeated for at least three different time points.
Immunocytochemical Analysis

Immunocytochemical staining for Cx43, Cx32, and actin cytoskeleton was performed as described. Cells were plated on 12-mm uncoated coverslips (0.5–1 × 10^5 cells/ml) and fixed 1 to 3 days later with 4% paraformaldehyde for 10 minutes at room temperature. Cultures were permeabilized with 0.1% Triton X-100 and blocked with 10% normal goat serum. Primary antibody was applied for 2 hours at room temperature or overnight at 4˚C. Cultures were washed with PBS three times, and fluorescein isothiocyanate–conjugated goat anti–rabbit or goat anti–mouse immunoglobulin was applied to the cultures for 1 hour at room temperature. The cultures were again washed with PBS several times, and the coverslips were mounted in Slow Fade (Molecular Probes, Eugene, OR). Polyclonal antibodies directed against Cx43 and Cx32 were kindly provided by Dr. Bruce Nicholson (State University of New York, Buffalo, NY). Immunofluorescence was visualized with the aid of a confocal microscope (MRC1000; BioRad) attached to an inverted microscope.

For actin staining, cells were permeabilized with 0.1% Triton X-100 and incubated with Texas Red-phalloidin (Molecular Probes) for 30 minutes. After several washes with PBS, coverslips were mounted and the cells were examined as described earlier.

Cell Motility Assay

The motility of glioma cells was determined using a radial dish assay as described by Chicoine and Silbergeld with some modification. Briefly, C6-Cx43 cells or C6-mock cells were plated in the center of a round Petri dish (100 mm in diameter) at a density of 2 × 10^4 cells in 200 μl of 10% FBS-DMEM/F12. After 6 hours of incubation at 37˚C in a humidified 5% CO₂ atmosphere, the medium was discarded. A circular zone of plated cells in the center of the Petri dish was established. The cells were washed twice with serum-free DMEM/F12, fed with 10 ml of 10% FBS-DMEM/F12, and then incubated for 7 days.

To determine cell motility, the number of cells per random high-power field (×20) at predetermined distances from the perimeter of the central zone was counted daily in triplicate. Finally, the cells were washed with Hanks balanced salt solution, fixed with 4% formalin for 5 minutes, and stained with cresyl violet (Sigma). Glioma cell motility was also determined by using uncoated transwell chambers.

Statistical Analysis

All experiments were performed in triplicate and were repeated at least twice. Data were evaluated by performing an analysis of variance with statistical analysis software (StatView; Abacus Concepts, Inc., Berkeley, CA).

Results

Characteristics of Cx43-Transfected C6 Cells and Their Interaction With Astrocytes

Rat C6 glioma cells were transfected with complementary DNA of Cx43 or Cx32 or vector only. The successfully established subclonal cell lines overexpressed Cx43 (C6-Cx43) or Cx32 (C6-Cx32), whereas C6-mock cells expressed neither Cx43 nor Cx32, similar to their parent C6 cells (Fig. 1A–C, green). A dye transfer assay demonstrated that C6-Cx43 cells, but not C6-mock cells, formed func-

FIG. 1. Overexpression of Cx43 and Cx32 in transfectants and actin cytoskeleton organization. Upper: Immunocytochemical study results demonstrating that C6-Cx43 and C6-Cx32 cells (A and C) strongly express gap junction proteins, whereas C6-mock cells (B) do not express immunoreactive plaques (green). Texas Red-phalloidin staining of stress fibers was combined with outlining of cell–cell boundaries and extended uninterrupted from cell to cell (red). Lower: Dye transfer assay results demonstrating that functional gap junction coupling formed in cocultures of rat astrocytes with C6-Cx43 (D) but not with C6-mock (E) or C6-Cx32 (F) cells. C6-Cx43 cells = connexin43-transfected C6 glioma cells; C6-Cx32 cells = connexin32-transfected C6 glioma cells; C6-mock cells = vector control–transfected C6 glioma cells.
tional gap junctions with normal astrocytes when they were cocultured (Fig. 1D and E). Note that C6-Cx32 cells did not form functional gap junctions with surrounding astrocytes, although gap junction channels formed among themselves (Fig. 1F).

Given that connexin plays an important role in morphological alteration, the localization of actin was visualized with Texas Red-phalloidin staining to analyze the morphological changes of the cells. As shown in Fig. 1A–C, C6-Cx43 cells were larger and flatter than C6-mock cells, and they could form long actin fibers (red).

Increased Invasive Ability of Cx43-Transfected C6 Glioma Cells

Using the in vitro transwell invasion system, we investigated the migration and invasion of C6-Cx43 and C6-mock cells under different conditions. As shown in Fig. 2, C6-Cx43 cells migrated in a manner similar to C6-mock cells when the transwell inserts were precoated with Matrigel during a 24-hour incubation. Nonetheless, C6-Cx43 cells exhibited greater invasion and scatter abilities, as assessed by the number of cells in the lower wells at 48 hours (data not shown). Interestingly, when these cells were mixed with rat astrocytes and were seeded on Matrigel-coated membrane inserts, the C6-Cx43 cells exhibited a greater invasive ability than C6-mock cells. Using a cell invasion assay kit, we confirmed the increased invasion capacity of C6-Cx43 cells, but not the C6-Cx32 or C6-mock cells when they were incubated with normal astrocytes that express Cx43 (Fig. 3).

Increased Concentrations of MMP-2 and MMP-9 Secreted by Cx43-Transfected C6 Glioma Cells

Given that cellular invasiveness is thought to be dependent on the production of MMPs, we determined the levels of MMP-2 and MMP-9 (enzymes responsible for extracellular matrix degradation). After incubation of C6-Cx43, C6-Cx32, and C6-mock cells in serum-free medium for 24 hours, the conditioned media were collected and subjected to gelatin zymography. The levels of MMP-2 and MMP-9 were higher in medium collected from C6-Cx43 cell cultures than that collected from C6-mock control cultures (Fig. 4). The C6-Cx32 cells also exhibited a higher level of MMP-2 but lacked MMP-9.

Attenuation of Glioma Cell Migration In Vitro by an Inhibitor of MMP Activity

Proteases are elementary factors in the glioma invasion process. To address whether the increased invasion ability of C6-Cx43 cells was related to their higher secretion of MMPs, a synthetic MMP inhibitor, BB-94, was used. Invasion was tested in a Matrigel transwell invasion system by using membrane inserts preseeded with or without rat astrocytes. When cells were cultured in the upper chamber, the number of C6-Cx43 cells that had invaded through Matri-
gel or through the confluent monolayer of astrocytes plus Matrigel was counted. As shown in Fig. 5, the percentage of invading C6-Cx43 cells was significantly reduced by BB-94 in both Matrigel and astrocytes/Matrigel chambers, indicating that the increased invasion capacity of C6-Cx43 cells is involved in their increased MMP activity.

Cell Proliferation and Clonogenicity in Cx43-Transfected and Mock-Transfected C6 Glioma Cells

To rule out whether the increased invasive ability and motility of C6-Cx43 cells merely reflected their proliferation rate, cell growth curves were established. The C6-Cx43 cells and C6-mock cells were cultured in 24-well cell culture plates and their numbers were counted daily after trypsinization. The C6-Cx43 cells had a lower proliferation rate than C6-mock cells (Fig. 6 upper) and yet showed greater motility, as was demonstrated on radial dish assay (Fig. 6 lower). Note that the C6-Cx43 cells moved farther from the center of the Petri dish than the C6-mock cells and also exhibited increased clonogenicity after they migrated to their new location.

Discussion

Our findings demonstrate that the overexpression of Cx43 enhances the migration ability of glioma cells in vitro.
MMP-9 in conditioned media from mock-, Cx43, and Cx32-transfected C6 glioma cells. 

Upper: Gelatin zymograph demonstrating MMP levels. Equal amounts of protein were electrophoresed on SDS–polyacrylamide gel electrophoresis gels containing gelatin as a substrate to detect enzyme activity. Samples were added in quadruplicate: conditioned media from C6-Cx43 cells (lanes 1–4), C6-mock cells (lanes 5–8), and C6-Cx32 cells (lanes 9–12). Lower: Bar graph demonstrating results of zymographic analysis. Data are representative of two separate assay sets (relative activity; mean ± SD, n = 4).

These data support the results of Lin, et al., who have recently demonstrated that the expression of Cx43 in C6 cells enhances their capacity to invade the normal brain parenchyma in vivo. Although the C6-Cx43 and C6-mock cells showed similar migration rates during the first 24 hours in the present study, the rate for C6-Cx43 cells increased significantly thereafter, as assessed with the Matrigel transwell invasion chamber assay. Furthermore, when these cells were cocultured with nonmalignant astrocytes, the number of invading C6-Cx43 cells was threefold greater than the number of invading C6-mock cells, indicating that gap junction communication plays an important role in the facilitation of glioma invasion.

In the present study we explore the possible mechanism of this phenomenon further. The reasons that the overexpression of Cx43 enhances glioma cell invasion may be multifold. To gain more insight into the cellular events following Cx43 transfection, its adhesion, cytoskeletal organization, migration, and proliferation as well as MMP expression were investigated. Stimulated cell proliferation or inhibition of cell death may cause an increase in tumor cell mass; for active invasion, however, malignant tumor cells must acquire locomotive properties and release proteolytic enzymes that can digest cell–cell contacts. It has been reported that connexin associated with actin filaments at the tips of cell processes plays an important role in the early stages of adhesion in astrocytes. Our study showed similar results, that is, enhanced immunopositive spots for Cx43 were observed at the tips of cell processes and cell margins, colocalizing with actin filament (Fig. 1). In addition, the overexpression of Cx43 led to membrane ruffling greater than that occurring in mock-transfected cells, which may be associated with cell migration.

Primary malignant brain tumors exhibit invasive diffuse growth. At sites distant from the main tumor, there may be accumulation of tumor cells surrounding neurons and blood vessels. In addition, cells may be found in the subpial marginal zone of the cerebral cortex. In the white matter, tumor cells are found in relation to the intra- and perifascicular concentrations of nerve fiber tracts. As the tumor expands, cells of the host tissue are incorporated into the margins of the tumor. Ultrastructural studies of rat brain tumors show that cells moving along the basal lamina of vessels possess long cellular processes, indicating at least some active cell migration in the perivascular space. The differences in migration on Matrigel could also be due to an upregulation of collagenses in the C6-Cx43 cells, thus degrading type IV collagen and thereby loosening cell–matrix interactions.

The MMPs have been shown to play a potent role in helping cancer cells from metastatic lesions invade through the ECM. Malignant progression in gliomas is correlated with an increased migratory capacity that involves metalloproteolytic activity, and production of MMPs is an important determinant in glioma cell invasion. Among the MMPs, MMP-9 has been shown by Rao, et al., to be most consistently associated with the progression of gliomas. Expression of MMP-9 protein can also be seen to correlate well with messenger RNA levels in gliomas and normal tissues, indicating that this MMP plays an integral role in the infil-
Gap junction and glioma invasion

It was found that there is an upregulation of ECM components and malignant tissues. Immunohistochemical analyses have revealed that there is a strong migratory response in glioma cells. Results of previous studies have demonstrated that functional gap junction communication forms between malignant glioma cells and surrounding nontumor astrocytes in vitro and in vivo. The astrocytes cocultured with Cx43-expressing cells were consistently smaller and expressed a lower level of glial fibrillary acidic protein than astrocytes cocultured with Cx43-deficient control cells. Thus, cellular coupling with glioma cells results in a phenotypic transformation of astrocytes, which may possibly play a role in the permissiveness of surrounding tissue to glioma invasion. It might well be that such a coupling between glioma cells and neighboring nontumor astrocytes leads to a different invasive behavior. The results in our study support this hypothesis. There was a significant increase in invasiveness in the Cx43-overexpressing C6 subline compared with Cx43-deficient controls when the cells were cocultured with normal astrocytes. Interestingly, C6-Cx32 cells did not exhibit increased invasiveness when they were cocultured with normal astrocytes, nor did they form gap junction communication with normal astrocytes that express Cx43. This finding may explain why C6-Cx32 cells did not have an increased invasive capacity when compared with C6-mock cells and reveals that Cx43, but not Cx32, plays an important role in glioma cell invasion. For tumor cell invasion, the type of metabolite that is exchanged between neighboring cells can be significantly influential. In fact, it has been reported that several ECM components known to be involved in cell attachment, differentiation, growth, and migration may be induced in normal brain tissue in response to invading glioma cells. At the same time, tumor cells express specific receptors for these components, which may facilitate tumor cell movement into normal brain tissue.

We acknowledge the limitations of the use of the C6 glioma cell line in drawing conclusions relevant to malignant gliomas in humans in vivo. Grobben, et al., have recently reviewed the merits and limitations of the use of this cell line in their study of glioblastoma growth and invasion. Although it has been thought to originate in random-bred Wistar-Furth rats (nonsyngeneic line), it is associated with high tumor take rates and is invasive in vivo. Notwithstanding their usefulness in neurooncological research, the tumors derived from implanted C6 cells do not share the same heterogeneity, immunocytological features, and invasive capacity as observed in naturally occurring malignant gliomas in humans. Nonetheless, the model used here enables the comparative study of one element (stable transfection and expression of Cx43) on tumor growth and invasion. Such observations should stem other work using similar hypotheses in other tumor models.

Our findings indicate that the overexpression of Cx43 en-

![Graph demonstrating that C6-Cx43 cells exhibit a similar or lower proliferation ability than C6-mock cells, as evidenced by counting cell numbers.](image-url)
hances the motility and invasion capacity of glioma cells, especially when they are cocultured with astrocytes. Furthermore, these results demonstrate that these processes may involve MMP activation and actin cytoskeleton organization as well as the interaction with neighboring astrocytes. A better understanding of the mechanism by which gap junction communication between glioma cells and normal astrocytes might play a role in glioma cell invasion may result in new therapeutic approaches for malignant gliomas.

References


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