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GAP JUNCTION INTERCELLULAR COMMUNICATION IN GLIOMAS IS INVERSELY RELATED TO CELL MOTILITY

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Abstract—Gliomas are lethal because of local invasion into brain parenchyma. Glioma cells were isolated from different regions (white matter, gray matter and tumor core) of a glioma-bearing dog brain. Individual clonal cell lines were established from each area, and characterized for growth, migration and *gap junctions*. The regional clonal cell lines differed in rates and preferred substrate for migration. Cell lines generated from invaded white matter showed stimulated migration on collagen and variable migration on merosin, whereas migration of cell lines derived from invaded gray matter showed the reciprocal responses: stimulation on merosin and inhibition on collagen. Gap junctional communication showed significant degrees of variation between the different clones. A direct inverse relationship between the number of cells demonstrating gap junctional communication and migration rate of cells away from multicellular spheroids was evident. Glioma cells which have a reduced capacity to connect to each other have an accelerated migration rate onto autologous, glioma-derived matrix. These results suggest that invasive glioma cells suppress autologous cell-to-cell cohesion, partly evident as reduced formation of gap junctions. In addition, glioma cells were stimulated to migrate in a dose-dependant manner in response to epidermal growth factor (EGF) coincident with the reduction of Cx43 levels and increased serine phosphorylation. We speculate that in order for glioma cells to invade locally into brain parenchyma they must first detach from neighboring cells (*"let go...let's go"* paradigm of invasion). © 1999 ISDN. Published by Elsevier Science Ltd All rights reserved

INTRODUCTION

Despite heroic surgical and adjunctive therapies developed against malignant glial neoplasms in the recent decades, survival for patients with this disease has remained disappointingly short.[1] Among the biological features of gliomas confounding effective treatments is the tendency of these tumor cells to infiltrate the brain parenchyma, most typically along white matter tracts;[2] dissemination over stromal borders such as the glial limitans or along perivascular structures are also noted.[3] Invasive glioma cells escape surgical resection, and manage to survive radiation and chemotherapy, regenerating the tumor mass.[4] Unique genetic changes may account for the aggressive or invasive behaviors of such recurrent lesions.[5–8]

Since glial tumors are heterogeneous,[9] and the pattern of invasion is nonrandom,[10] and since the invasive margin of these tumors represents a small portion of the entire tumor, we hypothesized that invasive glioma cells would show phenotypic (and possibly genetic) differences when compared with cells isolated from the tumor core. Using a spontaneous canine astrocytoma cell line,[11] we initiated development of an intracranial glioma from which clonal malignant glioma cell lines could be derived from invaded white matter, invaded gray matter, and from the center of the tumor mass. Experiments were conducted to characterize the different clonal populations for monolayer growth, migration on defined extracellular matrices, egress from multicellular spheroids, and the capacity to form gap junctional intercellular communications (GJIC). Additionally, the role of a protein which mediates formation of gap junctions in astrocytes, Connexin-43 (Cx43)[12] was studied in conjunction with induced glioma cell migration. The experiments tested the hypothesis that reduced cell-to-cell interactions are a prerequisite for accelerated migration. We refer to this phenomenon as the "let go–let's go" paradigm.

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MATERIALS AND METHODS

Clonal derivation, cell culture and extracellular matrix

Cell lines were developed from three regions of a canine brain sustaining an allografted canine glioma.[11] The vital brain regions were processed: tumor core, gray matter, and white matter adjacent to the tumor [Fig. 1(a)].. Tumorigenicity was verified by soft agar cloning and cell lineage confirmed by GFAP immunostaining (data not shown). The nomenclature used to distinguish the clones is as follows: J3T, clones from the tumor core; J3Ac, clones from invaded gray matter; and J3Dc, clones from invaded white matter.

Canine astrocytoma cell lines (J3T, J3Ac and J3Dc5) were propagated in monolayer culture in minimal essential medium (MEM, Life Technologies, Gaithersburg, MD) with 10% fetal calf serum (FCS, Hyclone, Logan, UT). Cells were passaged using trypsinization at regular intervals depending on their growth characteristics.

Production of self-derived extracellular matrix (ECM) was achieved by allowing the specific cell lines to persist at confluence 5–10 days.[13] After a thorough rinsing, the cells were lysed from the ECM using treatment with 0.5% Triton X-100, followed by 0.1 M NH_4OH and three rinses with PBS.[14]

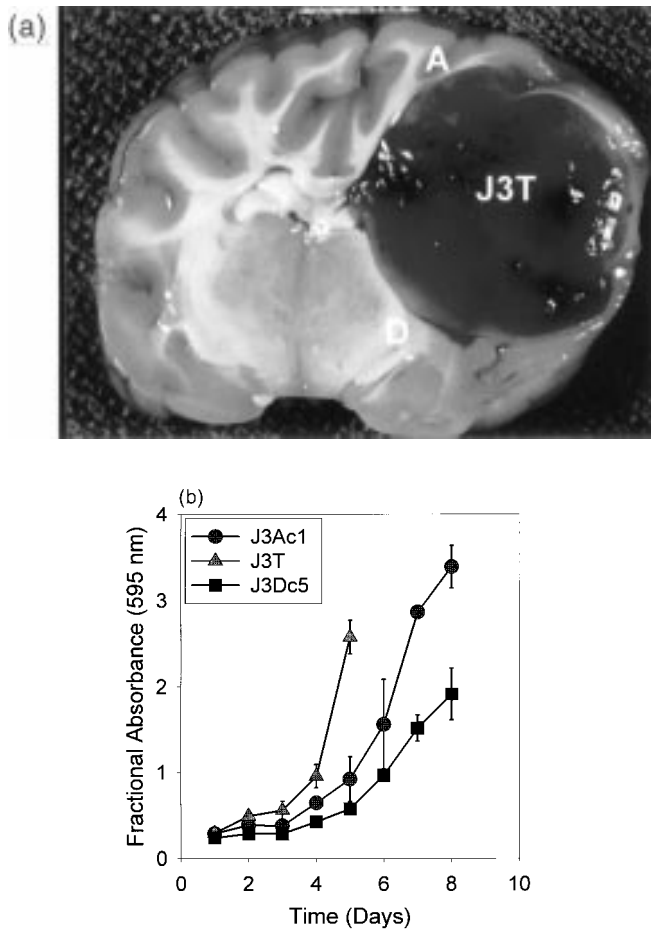

Fig. 1. (a) Brain tumor clone isolation. Regions of a canine malignant glioma from which clones were developed. Cells from the central tumor-J3T, from grey matter adjacent to the tumor–J3A clones, and from a white matter tract–J3D clones were developed into cell lines. (b) Growth curve of three different cell lines on tissue culture plastic. J3T grows the fastest, followed in decreasing order by J3Ac1, and J3Dc5. Points represent the mean of quadruplicate measurements; bars indicate standard deviation of the measurements.

Cell proliferation assay

Cell growth was studied by seeding 1×10^3 cells/well in MEM with 10% FCS into flat bottom 96-well plates (Nunc, Roskilde, Denmark).[15] At daily intervals cells in assigned wells were fixed in 1% glutaraldehyde. After the entire plate had been fixed, cell nuclei were stained with crystal violet (0.1% in ddH_2O), rinsed, and the color was solubilized in 1% sodium dodecyl sulfate (SDS). Cell number was quantified by reading the absorbance at 540 nm (Bio-Tek Instruments, Inc., Winooski, VT). All measurements represent the mean of four determinations.

Monolayer migration of cells on BSA, merosin and collagen

Cell motility was studied using a modification of the technique reported previously.[16] Ten-well, HTC-treated slides (Erie Scientific, Portsmouth, NH) were coated with AES (3-aminopropyltriethoxysilane) (Sigma, St. Louis, MO) to optimize protein and cell adhesion.[17] The surfaces of these slides were coated with purified ECM proteins merosin and collagen as reported by Berens *et al*., 1994.[16] All wells were blocked with BSA (1% w/v in PBS) for 1 h at room temperature to saturate nonspecific protein binding sites. The wells were then thoroughly rinsed with PBS. Cell sedimentation manifolds (Creative Scientific Methods, Inc, Mesa, AZ) were gently placed over the 10-well slides (Erie Scientific, Portsmouth, NH) containing 50 µl of culture media. A 1-µl/cell suspension containing 2000 cells was added to each channel of the manifold. The slide was kept on a cold aluminum plate for 30 min to allow cell sedimentation and then was transferred to a 37°C, 5% CO_2 incubator overnight. The manifolds were removed and fresh media supplemented with 10% FCS was added. In certain experiments, the media was also supplemented with epidermal growth factor (EGF, Sigma, St. Louis, MO).

The cell population area was measured daily using inverted microscopy (Axiovert; Zeiss, Thornwood, NY) and image analysis (VIDAS; Kontron, Eching, Germany). The radius of the cell population increased linearly over time.[13] The migration rate of each cell line was determined by regression analysis of the change in the radius over time beyond the initial radius at time zero. The migration rates of the cells on BSA were subtracted from the rates observed on ECM proteins (specific migration rate). In some instances the migration rate on ECM was slower than the rate on BSA, resulting in a negative specific migration rate. We interpret this to be active and specific migration suppression.

Spheroid migration assay

Spheroids were produced by culturing 4×10^6 cells in Delong flasks on an orbit shaker (70 rpm) for 10–14 days at 37°C in a 5% CO_2 humidified incubator.[18] Spheroids were collected and individually placed in each well of 24-well plates containing previously prepared self-ECM as described above. The migrating cell population area was measured daily as described above. The migration rate of each cell line was determined as described for the monolayer migration assay above.

Gap junctional intercellular communication (GJIC)

GJIC was scored as the number of cells into which calceinAM (Molecular Probes) diffused from previously labeled cells.[19] This score does not measure the number of gap junctions per cell but is a quantative assessment of funtional cell-to-cell communication. Each of the cell lines was propagated in phenol red free media supplemented with 10% FCS. A dual-label dye solution was prepared by adding 10 µl (0.05 µg/µl) calceinAM (Molecular Probes, Eugene, OR) and 1 µl (10 mM in DMSO) DiI (Molecular Probes, Eugene, OR) to 10 ml of isotonic glucose. A 2.5-ml aliquot of trypsinized cells was placed into a 15-ml centrifuge tube containing 2.5 ml of the dual-dye solution. The tubes were incubated for 1 h at 37°C. An unlabeled aliquot of the same cells was held at 4°C for later use. After staining, the labeled cells were centrifuged and rinsed three times with Hanks Balanced Salt Solution (HBSS). The two aliquots of cells were combined in a 1:1000 ratio (labeled:unlabeled) and plated as 40-µl aliquots onto 7-mm diameter, 10-well HTC slides (Erie Scientific, Portsmouth, NH). The total number of cells plated was 30 030, which produced a confluent monolayer in the well. These were incubated for 4 h at 37°C, 5% CO_2 in a humidified incubator. The slides were then rinsed with HBSS, coverslipped,

and observed by epifluorescent microscopy. Cells previously labeled with the membrane restricted dye, DiI, are evident at 530/590 (Ex/Em), while the cytosolic dye, calcein AM fluoresces at 480/530 (Axioplan; Zeiss, Thornwood, NY). The number of cells into which calcein AM had diffused from the initially labeled cells was scored as GJIC.[20]

Flow cytometric analysis for connexin-43

Subconfluent monolayers of J3T, J3Ac1 and J3Dc5 cells were trypsinized to a single cell suspension (5×10^6 cells/tube). After washing twice with PBS and incubating in Superblock (Pierce, Rockford, IL) for 30 min, cells were washed twice with PBS and resuspended in 30% ethanol in PBS. After washing twice with PBS, cells were resuspended in 0.5 ml Superblock containing mouse anti-connexin-43 (Cx43) (1:200; Zymed, San Francisco, CA). For controls, mouse myeloma IgG (Sigma, St. Louis, MO) was added instead of the primary antibody. Cells were incubated with the primary antibody or control antibodies overnight at 4°C. Subsequently, they were washed twice with PBS and incubated with goat-anti-mouse-FITC (1:500; Chemicon, Temecula, CA). Green fluorescence was collected through a 530/30 nm bandpass filter on a FACScan (Beckton–Dickinson, Mountain View, CA) and recorded after logarithmic amplification as a measure of antibody binding. The data were recorded in list mode in combination with forward angle light scatter. Results are presented as histograms of fluorescence intensity versus cell count.

Immunoprecipitation and Western blot analysis for connexin-43 and phosphoserine after EGF treatment

Treatments of 0, 5, 10 and 25 ng/ml EGF were applied to subconfluent monolayers of cells in tissue culture flasks. After treating for a period of 6 h the cells were trypsinized and collected in centrifuge tubes. Cells were lysed with 1.5 ml TBST buffer (20 mM Tris-base, 150 mM NaCl,, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM benzamidine HCl, 1%Triton X-100, 0.05% Tween 20, 0.5 mM PMSF, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin A) per T-75 flask on ice for 30 min. Lysates were collected and spun in microcentrifuge tubes at 11 000*g* for 15 min at 4(C. The supernatants were preabsorbed with nonimmune mouse serum for 30 min, followed by incubation with a 50% suspension of protein G-sepharose (Pierce, Rockford, IL). After centifugation to remove the beads, extracts were incubated overnight a 4°C with the mouse anti-connexin-43 antibody (Zymed, San Francisco, CA). Immunocomplexes were incubated for 1 h with an excess of a 50% suspension of protein G-sepharose. The samples were centrifuged at 11 000*g* for 15 min at 4°C to pellet the beads and immunocomplexes. The supernatants were discarded and the pellets were boiled in SDS-sample buffer (62.5 mM Tris–HCl, 2.3% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) for 5 min and centrifuged at 1 000*g* for 5 min and the Triton-insoluble fraction was discarded. The proteins were separated on a 10% SDS PAGE gel and electroblotted onto nitrocellulose paper. The paper was probed with mouse anti-Cx43 (Zymed, San Francisco, CA) and with goat anti-mouse IgG (H + L) Biotin (Pierce, Rockford, IL). Streptavidin-HRP (Amersham, Arlington Heights, IL) was then added. Chemiluminescence of the proteins was detected using the ECL kit (Amersham, Arlington Heights, IL) and autoradiographic film. Nitrocellulose blots of proteins from the anti-Cx43 immunoprecipitation were also probed with rabbit anti-phosphoserine (Transduction Labs, Lexington, KY) using the same blotting procedure with appropriate antibodies.

Immunofluorescence of connexin-43 following EGF treatment

Slides from the migration assay were fixed with 3% fresh paraformaldehyde in PBS, then cells were permeabilized with 0.1% Triton X-100. The slides were then blocked with 10% normal goat serum. Cells were stained using mouse IgG anti-Cx43 (Zymed; San Francisco, CA 1:200). The secondary antibody was FITC conjugated goat F(ab′)2 anti-mouse IgG (H + L) (Protos Immunoresearch, south San Francisco, CA). Immunofluorescence was evaluated using confocal microscopy (Leica model TCS NT).

RESULTS

Characterization of the selected glioma clones

The proliferation rates of the different glioma clones were determined [Fig. 1(b)]. Cell line J3T grew fastest, followed by J3Ac1 and J3Dc5. The migration rates of the clones on different ECM proteins were obtained [Fig. 2(a)]. The A clones migrated most rapidly on merosin and showed variable migration responses on collagen. Conversely, the D clones were stimulated to migrate on collagen, but were predominantly inhibited on merosin. The migration of J3T cells was strongly stimulated by merosin and marginally by collagen. Basal motility rates of the different clones on the nonspecific substrate, BSA, were similar.

Spheroid migration onto ECM

The ability of the clones to disseminate from a multicellular spheroid on a glioma-derived matrix was assessed as migration. The radial dispersion of cells migrating from the spheroid over time was calculated as the migration rate [Fig. 2(b)]. The J3Dc5 clones exhibited the fastest migration rate followed by J3T and J3Ac1.

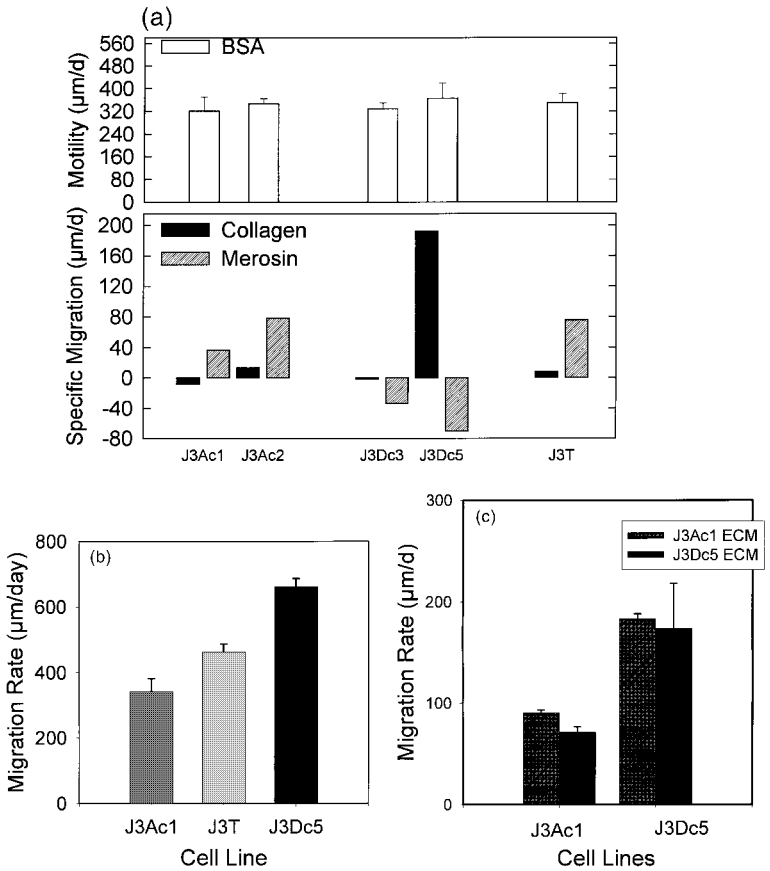

Fig. 2. (a) Effect of different extracellular matrix (ECM) proteins [collagen and merosin] and a neutral, nonspecific protein substrate, bovine serum albumin (BSA) on the monolayer migration rates of five glioma-derived cell lines. The J3A clones were stimulated to migrate on merosin and migration was variable on collagen. Inversely, the J3D clones were stimulated to migrate on collagen, but predominantly inhibited on merosin. The migration rates of J3T were strongly stimulated by merosin and marginally on collagen. Points represent the mean of five measurements; bars indicate standard deviation of the measurements. (b) Migration rates of cell spheroids on self ECM. Dc5 migrated the fastest followed in decreasing order by J3T and J3Ac1. (c) Monolayer migration rates on respective ECMs. The migration rate of Ac1 clones was not stimulated by the presence of Dc5's ECM and the migration rate of Dc5 clones was not inhibited by the presence of Ac1's ECM. Clone J3T was also not affected by either ECM (data not shown).

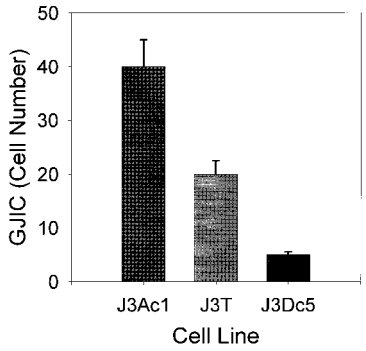

Fig. 3. Gap junction intercellular communication. Fluorescently-labeled cells were cocultured with unlabeled cells. Gap junctional intercellular communication (GJIC) was scored as the number of initially unlabeled cells into which the cytosolic calcein from prelabeled cells diffused. J3Ac1 exhibited the most GJIC followed by J3T and J3Dc5.

In order to assure the migration results were due to the behavior of the clones themselves and not due to possible unique compositions of the different extracellular matrices, the clones were seeded on the different ECMs for assessment of migration [Fig. 2(c)]. The migration rate of Ac1 clones was not stimulated by the presence of Dc5's ECM and the migration rate of Dc5 clones was not inhibited by the presence of Ac1's ECM. Migration of J3T was not affected by either ECM (data not shown). Migration of the glioma cells from multicellular spheroids is an intrinsic trait of glioma cells and not due to migration-inducing or migration-suppressing components in the different glioma-derived matrices.

Gap junctional intercellular communication (GJIC)

Cytosolic dye transfer between cells was assessed for each clone (Fig. 3). Thirty DiI/Calcein AM labeled cells from each cell line were scored for gap junctional intercellular communication by quantifying the number of adjacent cells containing Calcein AM after 4 h. J3Ac1 clones formed the most gap junctions followed by J3T and J3Dc5.

The number of gap junctions formed is inversely related to the migration rate

Comparing GJIC and the migration rates of the different cell lines indicated that these two behaviors are inversely related (Fig. 4).

Flow cytometry of cell surface Cx43 expression in glioma clones

Quantitation of cell surface Cx43 expression in the glioma clones was conducted using flow cytometric analysis (Fig. 5). J3Ac1 showed intense staining, J3T was moderate in levels of surface Cx43 and J3Dc5 showed almost no surface staining.

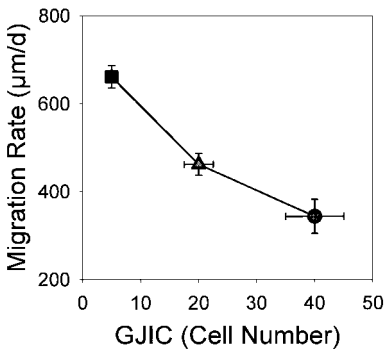

Fig. 4. Relationship between GJIC and cell dispersion from multicellular glioma spheroids (migration rate). Cells demonstrating more abundant intercellular communication did not egress from spheroids as rapidly as cells that exhibited less cell-to-cell communication. J3Dc5 (■), J3T (▲), J3Ac1(●).

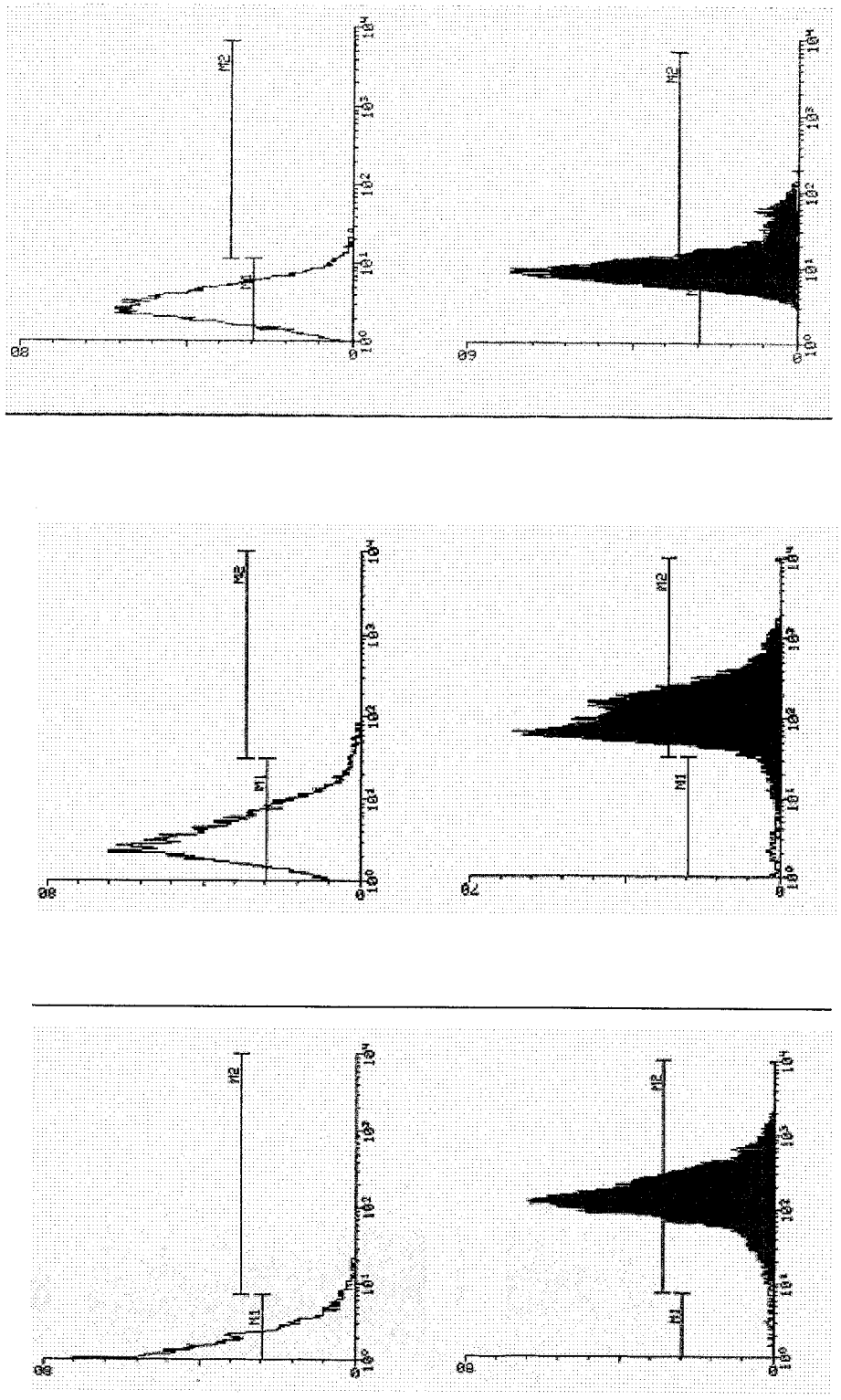

Fig. 5. Flow cytometric analysis of cell surface connexin43 expression of glioma clones. Cells were harvested in log-phase growth, fixed in paraformaldehyde, then stained using anti-Cx43 antibodies. FITC-conjugated anti-mouse antibodies were used as the fluorochrome. Isotype matched non-immune antisera was used for the control staining (top panels). Mean fluorescence intensities of the specific Cx43 stained cells were as follows: J3Ac1, 199.2; J3T, 161.6; J3Dc5, 15.2. Nonspecific values were 1.7, 5.1 and 3.6, respectively.

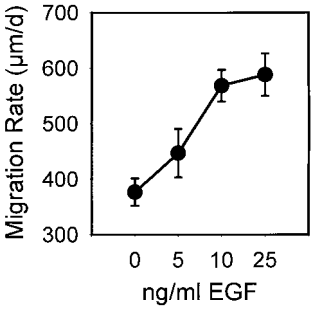

Fig. 6. Migration rates of the J3Ac1 cell line on self-ECM stimulated with EGF. Using a conventional monolayer migration assay as descibed in the materials and methods section J3Ac1 cells treated with varying doses of EGF were stimulated to migrate in a dose-dependent manner. Points represent the mean rate of five independent measurements. Error bars represent the standard deviation of these measurements.

Migration stimulation of J3Acl clones in the presence of EGF

In order to evaluate changes in Cx43 as a component of cell migration, J3Ac1 cells were treated with EGF.[21] Since J3Ac1 was the slowest migrating glioma cell line in this study, and also demonstrated the largest amount of Cx43 expression and functional GJIC formation, it had a high likelihood of revealing an interaction between these two parameters when migration was stimulated. Treatment with EGF stimulated migration of J3Ac1 in a dose-dependent manner (Fig. 6). Confocal microscopy of EGF-treated cells demonstrated a dose-dependent reduction in levels of Cx43 among the cell population, especially a loss of intensely-staining cells (Fig. 7). Additionally, increased intercellular space at the higher EGF treatment concentrations is consistent with the accelerated motility rate seen in the migration assay.

Western blot analysis of Cx43 from J3Ac1 clones after EGF stimulation

The amount of Cx43, identified by a single band at 43 kDa on western blots, decreased with increased concentration of EGF [Fig. 8 (a)]. Furthermore, serine phosphorylation of immunoprecipitable Cx43 increased as the EGF concentration increased [Fig. 8(b)]. The heavily phosphorylated Cx43 also manifests as a higher molecular weight protein in the western blot.

DISCUSSION

Aberrations in cell-to-cell communication have been well documented as being part of the complex process of carcinogenesis.[20] Most normal cells (excluding normally detached cells such as red blood cells or neutrophils and several stem cells) within solid tissue have functional gap junctional intracellular communication (GJIC).[22] However, cancer cells of solid tumors appear to have lost or significantly attenuated their GJIC.[23] Gap junctions in the mammalian CNS are present in the astrocytes.[24] Astrocytes have been shown to form gap junctions predominantly by using the protein Cx43 both *in vivo* and *in vitro*.[25] Variations in gap junction expression have

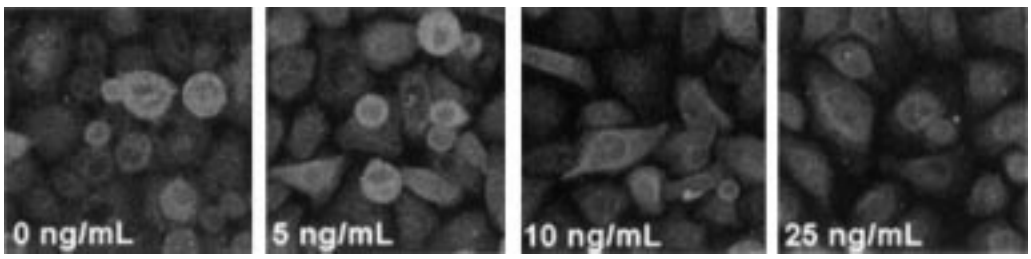

Fig. 7. Confocal microscopy of J3Ac1 cells postmigration on self-ECM following treatment with EGF. Anti-Cx43-FITC immunostaining of EGF migration-stimulated J3Ac1 cells from the experiment in Fig. 6 revealed a dose-dependent reduction in Cx43 among the cell population. A significant increase in intercellular space at the higher EGF treatment concentrations should also be noted.

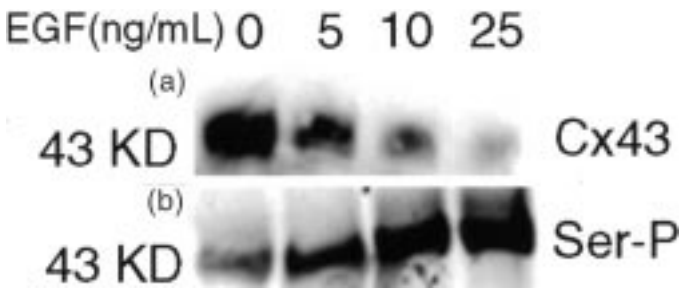

Fig. 8. Western blot analysis of Cx43 immuoprecipitates from the J3Ac1 cell line following EGF stimulation (a) Probed with anti Cx43 antibodies. A single band at 43 kDa can be identified as Cx43. The amount of Cx43 decreased from left to right as the concentration of EGF treatment was increased. (b) Probed with anti phospho-serine antibodies. The intensity of the single band at 43 kDa representing serine-phosphorylated-Cx43 increased from left to right as the concentration of EGF was increased.

been reported in astrocytes derived from different regions of the brain.[26] The level of Cx43 expression and correlated GJIC also differ in astrocytomas with glioblastomas showing the broadest variances.[27]

GJIC can be impacted by endogenous and exogenous chemicals, oncogenes, and tumor suppressor genes. Tumor-promoting chemicals and several oncogenes (i.e., *ras, src, mos, neu*, but not *myc*) reduce GJIC,[28–31] and several growth factors (i.e. EGF, PDGF, TGF-beta, bovine pituitary extract) inhibit gap junction function.[32–35] The molecular basis by which GJIC is regulated is unknown, however, posttranslational phosphorylation of the Cx43 protein has been associated with modulation of gap junctional channels.[36] In addition, Lau *et al.*[37]demonstrated that EGF stimulation of cells leads to disruption of GJIC by serine phosphorylation of the Cx43 protein. The mechanism by which this serine phosphorylation occurs remains unknown, however, it has been shown to be independent of the PKC pathway. Recent studies have confirmed that Cx43 is a MAP kinase substrate *in vivo* and that phosphorylation on Ser255, Ser279, and/or Ser 282 initiates the down-regulation of gap junction communication.[38]

Regulation of GJIC determines whether a cell remains quiescent, proliferates, differentiates, undergoes apoptosis, or migrates.[39,40] Gap junctions play a significant role in glial cell proliferation. Astrocytes from embryonic mice with a null mutation in the Cx43 gene exhibited a reduced proliferation rate compared to the wild-type.[41] Contrastingly, an inverse correlation was found between Cx43 expression and proliferation activity in cultured murine olfactory bulb cells.[42]

Several studies suggest a role for GJIC in the breadth of efficacy from cytotoxic driven gene therapy. It is believed that gap junctions may serve to enhance the bystander effect of gancyclovir metabolites produced by thymidine kinase transduced cells.[43–45] Metabolic cooperation via gap junctions can facilitate the uptake of apoptotic vesicles following glancyclovir mediated cell death.[46] When glioblastoma cells with minimum levels of Cx43 protein were transfected with the gene for Cx43, the transfected cells exhibited a marked increase in the *in vitro* bystander effect from HSV-tk-mediated gene therapy.[27] However, due to the widely varied levels of Cx43 expression in glioblastomas, and supported by our results that the most invasive glioma cells express the least Cx43, expectations that delivery of HSV-tk to target invasive glioma cells are likely to be disappointed.

Our findings indicate that GJIC activity in glioma cells is variable. For the limited number of clones in this study, cells derived from white matter exhibited the least GJIC, expressed the least amount of Cx43 protein, and exhibited the fastest migration rates, while cells derived from gray matter developed greater GJIC, expressed the largest amount of Cx43 protein, and demonstrated the slowest migration rates. Because of the limited number of clones analyzed from the different regions of the tumor it is inappropriate to attempt to draw mechanistic conclusions on the levels of Cx43 expression and anatomical routes of glioma invasion. It remains uncertain whether variable Cx43 expression leads to enhanced invasiveness into selected regions of the brain or whether gap junctions promote local survival of invading cells.

GJIC and the biochemical regulation of Cx43 appears to be intact in the glioma cells used in this investigation. The results using EGF, demonstrate enhanced migration coincident with a decrease in the level of Cx43 expression, raise the possibility for soluble growth factors such as EGF or TGFα to modulate glioma invasion. Since serine phosphorylation of Cx43 retards its function as a conduit of cell-to-cell communication, local growth factor levels may serve as subtle triggers for changes in glioma cell behavior. Because of an emerging understanding of the signal transduction activity of the extracellular matrix receptors (integrins) it is reasonable to anticipate that these pathways may influence CJIC.[47–50]

Our findings demonstrate that decreased expression of the Cx43 gene is associated with accelerated motility of glioma cells. Additionally, invading glioma cells isolated from white matter expressed lower levels of Cx43 and formed fewer cell-to-cell communications than cells from the tumor core or glioma cells isolated from gray matter adjacent to tumor. Since decreased GJIC is associated with proliferation activity and with accelerated migration, we speculate that restoration of the intercellular communication mechanism may retard these malignant behaviors in astrocytomas.

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