Direct Gap Junction Communication between Malignant Glioma Cells and Astrocytes

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ABSTRACT

Gap junctions are intercellular channels that connect the interiors of coupled cells. We sought to determine the extent to which malignant glioma cells form gap junction channels with astrocytes from either adult human brain or rat forebrain. The astrocytic gap junction protein, connexin 43 (Cx43), was identified in immunoreactive plaques at areas of cell-to-cell contact between cocultured glioma cells and astrocytes. These gap junction plaques were composed of functional channels, because extensive dye coupling was evident between the glioma cells and astrocytes from both human and rat brain. Calcium signaling was also readily transmitted from glioma cells to astrocytes and vice versa. In live rat brain, injection of glioma cells prelabelled with the gap junction tracer, dicarboxy-dichlorofluorescein, revealed extensive dye transfer to host cells, demonstrating that malignant glioma cells directly couple with normal brain cells. These observations suggest that intercellular communication via gap junctions may play a role in regulating cellular interactions during tumor invasion. In fact, the presence of gap junctions between astrocytes and glioma cells was sufficient to induce a transformation of astrocytic phenotype. Astrocytes cocultured with C6 glioma cells overexpressing Cx43 were significantly smaller and expressed a lower level of glial fibrillary acidic protein than astrocytes cocultured with otherwise identical mock-transfected, gap junction-deficient C6 cells. Thus, direct cellular coupling with glioma cells result in a phenotypic transformation of astrocytes that may contribute to the susceptibility of surrounding tissue to glioma invasion.

INTRODUCTION

Even in early stages, two hallmarks of malignant gliomas are diffuse infiltration and lack of clear demarcation between tumor and adjacent normal brain tissue. The pattern of glioma cell invasion and spread through host brain differs from that of other tumors. Whereas local infiltration of most types of tumor cells occurs primarily through the blood vessels or lymphoid systems, glioma cells can migrate directly into surrounding brain tissue without the guidance of defined anatomical structures (1). Systemic metastasis by gliomas is exceptionally rare (2). Invasion requires adhesion to the intercellular matrix and subsequent degradation by matrix-degrading proteases. An increased level of metalloproteases has been linked to glioma invasiveness (3). However, interaction of malignant glioma cells with surrounding astrocytes has not been established. It is known that astrocytes in contact with gliomas often show reactive changes, but to what extent this is a result of direct interactions between the two cell types remains poorly understood (4). Also, seizure activity has been linked to phenotypic transformation of astrocytes located in the vicinity of glioma cells (5, 6). We sought to determine whether there are direct cellular interactions mediated by gap junctions between malignant glioma cells and astrocytes.

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3 The abbreviations used are: Cx43, connexin 43; GFAP, glial fibrillary acidic protein; HBSS, calcium- and magnesium-free Hank’s balanced salt solution; DiIC18, 1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; CMTMR, 5-(and-6)-carboxy-3-methyl-4-nitro-2-(3-phenoxyz amino)stilbene-2,2′ disulfonate; CDCF, dicarboxy-dichlorofluorescein diacetate; EGF, epidermal growth factor; MMP, matrix metalloproteinase.
dissected into neocortical samples. Each tissue sample was cut into roughly 0.3 mm³ pieces, trypsinized, and plated on murine laminin-coated culture dishes as described previously (15). When the outgrowth reached confluence, the cultures were replated and processed for assays. Primary cultures of both astrocytes and glioma cells were discarded after five passages.

Rat mixed forebrain cultures were derived from 16-day-gestation embryos and prepared by standard primary culture procedure as described previously (16). Briefly, embryos were removed from pregnant rats anesthetized with pentobarbital (50 mg/kg; Anpro Pharmaceutical) and decapitated. The forebrains were removed and immersed in HBSS at 37°C. The tissue was then trypsinized and triturated to homogeneity. An equal volume of 10% fetal bovine serum-DMEM/F12 medium was added, and the cell
suspension was centrifuged for 10 min at 1000 rpm. A total of $8 \times 10^5$ cells were plated on poly-L-lysine- and gelatin-coated 35-mm dishes (Corning) and incubated at 37°C as described above. After 14 days, >95% of the substrate cells stained positively for GFAP.

**Cell Lines, Transfection and Selection, and Cocultures.** Rat C6 glioma cells and human malignant glioma cell lines, U87-MG and U373-MG, were obtained from American Type Culture Collection (Rockville, MD). The rat gliosarcoma cell line 9L was obtained from the Neurosurgical Tissue Bank, UC San Francisco Brain Tumor Research Center (San Francisco, CA).

Cx43 cDNA in expression vector pcDNA1 containing sequence for genetin resistance was kindly provided by K. Willecke (17). C6 cells were transfected by Clonfectin (Clontech, Palo Alto, CA) according to manufacturer’s instructions, and stable transfectants were selected with 2 mg/ml genetin. Expression of Cx43 was confirmed by immunolabeling and functional dye transfer assays.

**Table 1** Gap junction coupling and calcium signaling in glioma and astrocyte cultures

<table>
<thead>
<tr>
<th>Cell cultures</th>
<th>Cx43 plaques/cell (mean ± SE)</th>
<th>Dye transfer index (mean ± SE)</th>
<th>Calcium wave radius (μm, mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Astrocytes</td>
<td>21.1 ± 1.0</td>
<td>5.96 ± 0.52</td>
<td>148.0 ± 2.3</td>
</tr>
<tr>
<td>U87-MG</td>
<td>5.0 ± 0.6</td>
<td>0.88 ± 0.14</td>
<td>95.0 ± 4.3</td>
</tr>
<tr>
<td>U373-MG</td>
<td>0.2 ± 0.1</td>
<td>0</td>
<td>74.5 ± 11.5</td>
</tr>
<tr>
<td>GBM1</td>
<td>8.7 ± 1.6</td>
<td>1.47 ± 0.26</td>
<td>116.9 ± 9.6</td>
</tr>
<tr>
<td>GBM2</td>
<td>5.3 ± 0.6</td>
<td>2.95 ± 0.07</td>
<td>114.0 ± 11.0</td>
</tr>
<tr>
<td>GBM3</td>
<td>19.3 ± 4.0</td>
<td>3.11 ± 0.14</td>
<td>148.2 ± 12.2</td>
</tr>
<tr>
<td><strong>Rat</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Astrocytes</td>
<td>20.9 ± 1.1</td>
<td>4.55 ± 0.33</td>
<td>229.5 ± 6.8</td>
</tr>
<tr>
<td>9L gliosarcoma</td>
<td>4.0 ± 0.6</td>
<td>3.43 ± 0.71</td>
<td>71.3 ± 7.26</td>
</tr>
<tr>
<td>C6 glioma (Mock)</td>
<td>0.1 ± 0.1</td>
<td>0.21 ± 0.13</td>
<td>59.2 ± 6.8</td>
</tr>
<tr>
<td>C6 glioma (Cx43)</td>
<td>22.3 ± 2.2</td>
<td>4.53 ± 0.65</td>
<td>152.6 ± 5.6</td>
</tr>
</tbody>
</table>

*All values represent mean ± SE of a minimal of three (range, 3–28) experiments.

Fig. 2. Functional gap junction in glioma cell lines, C6 and 9L. Wild-type 9L cells have high endogenous expression of Cx43. Transfection of C6 glioma cells with cDNA for Cx43 result in a dramatic increase in gap junction coupling. **Left panel**, Cx43 expression in C6 cells transfected with Cx43 (C6-Cx43; A), mock-transfected C6 cells (C6-Mock; C), and 9L gliosarcoma cells (wild-type; E). **Right panel**, C6-Cx43 cells and 9L cells are extensively dye-coupled (B and F), whereas C6-mock cells are not (D). Arrows, prelabeled (donor) glioma cells.
For mixed cultures, glioma cells or astrocytes were prelabeled with the fluorescent cell tracer, 10 μM DiIC₁₈ or 2 μM CMTMR (both from Molecular Probes, Eugene, OR), according to the manufacturer’s instructions. Labeled and unlabeled cells were mixed at a ratio of 1:100 just before plating unless otherwise specified. CMTMR contains a thiol-reactive chloromethyl group which, after reaction with intracellular thiols, becomes membrane and gap junction impermeable. Both cell tracers remain detectable for several cell generations and are resistant to fixation and immunohistochemical procedures (18).

**Immunocytochemistry.** A polyclonal antibody against the cytoplasmic COOH-terminal of Cx43 was kindly provided by Dr. Bruce Nicholson (SUNY, Buffalo, NY), and the anti-GFAP polyclonal antibody was from Sigma. Immunocytochemical staining for Cx43 and GFAP was performed as described previously (19). Cells were plated on 12-mm diameter uncoated coverslips put

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**Fig. 3. Cx43 expression and dye coupling in mixed cultures. Left panel, cocultures of malignant glioma cells and astrocytes immunoreacted with an anti-Cx43 antibody. The glioma cells were labeled with DiIC₁₈ and mixed with astrocytes at a ratio of 1:100. Cx43 immunopositive plaques are present at areas of cell-to-cell contact between glioma cells and astrocytes (small arrows). Right panel, gap junction permeability visualized by dye transfer assay in the same set of cocultures as in the left panel. A and B, rat gliosarcoma cell line 9L surrounded by rat astrocytes; C and D, human glioblastoma cell line U87-MG surrounded by human astrocytes; E and F, primary glioblastoma cultures surrounded by human astrocytes.**
into 24-well culture plates (0.5–1 × 10^5 cells/well) and cultured for 1–3 days. After fixation in 4% paraformaldehyde for 10 min at room temperature, cells were permeabilized with 0.1% Triton X-100 and blocked with 10% normal goat serum. Incubation with primary antibodies was for 2 h at room temperature or overnight at 4°C and was for 1 h at room temperature with FITC-conjugated goat anti-rabbit antibodies. After several washes in PBS, the coverslips were mounted in Slow Fade (Molecular Probes). Immunofluorescence was visualized by confocal microscopy (MRC1000; Bio-Rad, Hercules, CA). Cell size and immunofluorescence intensity were quantified using an imaging software, Comos (version 7.0a, Bio-Rad). The level of GFAP expression was measured in a representative cytotopic area.

**Dye Transfer Assay.** The method of Goldberg et al. (20) was followed with minor modifications. Cells were loaded with 5 μM CDCF (excitation, 488 nm) for 5 min, washed, and trypsinized. After centrifugation, the CDCF-loaded cells were labeled in suspension with 10 μM DiIC18 (excitation, 547 nm) for 10 min and mixed with unlabeled cells at a ratio of 1:250. One hour after plating on polylysine-coated dishes, dye transfer from the CDCF/DiIC18-labeled (donor) cells to unlabeled (recipient) cells was evaluated using confocal scanning microscopy. The coupling index was calculated as the fraction of donor cells that transfer dye to their surroundings times the mean number of recipient cells.

**Intercellular Ca^{2+} Signaling.** Calcium signaling was measured according to the method described by Wang et al. (21). Cultured confluent cells were loaded for 1 h with 10 μM Fluo-3 acetoxyxyster (Bio-Rad). All experiments were performed at room temperature. A calcium wave was initiated by mechanical stimulation in an astrocyte in the center of the viewing field with a glass micropipette (tip diameter, <1 μm) mounted on a micromanipulator (MMP-220; Narishige). Excitation was provided by the 488-nm line of the krypton-argon laser of a Bio-Rad confocal microscope. Images were acquired every 3–4 s and recorded on an optical disc (LM-D702W; Panasonic). The radii of calcium waves were measured as the maximum distance traveled by a wave from the point of initiation. Velocity was calculated by dividing the maximum distance of wave propulsion (μm) by time (seconds). A calcium wave was defined as a 50% increase (ΔF/ΔF_0) that propelled for a minimum of 50 μm in at least one direction. Background counts were subtracted from all measurements.

**Surgical Procedure and Injection of Prelabeled Glioma Cells.** Male Wistar rats (7–180 days) were anesthetized with pentobarbital (50 mg/kg body weight i.p.). Supplemental pentobarbital doses of 15 mg/kg were administered hourly. Rectal temperature was kept close to 37°C by means of a thermostat. Male Wistar rats (7–180 days) were anesthetized with pentobarbital (50 mg/kg body weight i.p.). Supplemental pentobarbital doses of 15 mg/kg were administered hourly. Rectal temperature was kept close to 37°C by means of a thermostat. Surgical, and rat brains. All of the primary cultures of human malignant gliomas were coupled, and the extent of coupling was a direct function of Cx43 expression. Both human and rat glioma cell lines varied in the extent of coupling, which was also in direct proportion to Cx43 immunoreactivity (Table 1).

**Direct Gap Junction Coupling between Astrocytes and Malignant Glioma Cells.** To test whether glioma cells and normal astrocytes coupled directly, we prelabeled glioma cells with either the membrane dye, DiIC18, or CMTMR before mixing with astrocytes at a ratio of 1:100. This approach allowed easy identification of cell types in both live cultures and after fixation. Cx43 immunoreactive plaques were readily identified at areas of cell-to-cell contact between astrocytes and glioma cells (Fig. 3). Connexin-expressing glioma cells, either from rats or humans, were extensively coupled with surrounding astrocytes at varied dye transfer rates (Fig. 3; Table 2).

**Gap Junction-dependent Ca^{2+} Signaling between Astrocytes and Malignant Glioma Cells.** One of the functions of the gap junction is transduction of calcium signaling. To study calcium signaling expressed as long-distance calcium waves, we loaded astrocytic and glioma cell cultures with the calcium indicator, Fluo-3. As reported by others, astrocytes from both rat and human brain produced robust calcium waves after mechanical stimulation, although the maximum radius of wave production was larger in rat cultures than in human cultures (Fig. 4; Table 1). By contrast, calcium waves were produced only within short distances in the primary cultures of human gliomas and in the glioma cell lines studied. Furthermore, as shown in Fig. 5, calcium signal can travel from human astrocytes to glioma cells. Of note, intercellular calcium signaling was, in most cases, communicated bidirectionally between glioma and normal astrocytes with the exception of Cx43-negative U373-MG. U373-MG, despite a marked increase in the calcium content of stimulated cells, failed to induce a significant extent of calcium signaling in neighboring astrocytes (data not shown).

**Gap Junction-mediated Transformation of Astrocytic Pheno-type in Glioma Cocultures.** To test whether gap junction-coupled gliomas can change the characteristics of cocultured astrocytes, we measured cell size as well as GFAP expression in rat astrocytes cocultured at a ratio of 1:100 with either mock-transfected C6 cells
(Cx-deficient) or Cx43-transfected C6 cells (C6-Cx43) or 9 L wild-type cells. C6-Cx43 cells and 9 L wild-type cells expressed a high level of Cx43 and were extensively coupled to primary astrocytes (Figs. 2 and 3; Table 2). In three independent sets of experiments performed in triplicates, astrocytic cells were smaller and their GFAP levels lower when they were cocultured with, and thereby coupled by gap junctions to, C6-Cx43 cells or 9 L cells, compared with astrocytes cocultured with Cx-deficient, C6-mock cells (Fig. 6). These observations indicate that glioma cells, by virtue of gap junction coupling, can transform the phenotype of adjacent astrocytes. Transfer of conditioned medium from C6-Cx43 cells to astrocytes cocultured with Cx-deficient C6 cells (mock-transfected) had no significant effect on either cell size or GFAP expression (data not shown).

Injected Glioma Cells Are Coupled to Host Cells in Live Rat Brain. We injected Cx-expressing glioma cells (C6-Cx43 cells or wild-type 9L cells) preloaded with CDCF and DiIC₁₈ into the cortex of live rats (Fig. 7) to determine whether glioma cells can form functional gap junctions in intact brain. Burr holes were performed...
over both hemispheres, and 3 μl of pre-labeled cells in suspension (5 × 10^7 cells/ml) were injected into the cortical tissue. One hour later, the animal was decapitated, and the extent of dye diffusion was assessed by confocal microscopy. We consistently found extensive CDCF diffusion from prelabeled Cx-expressing donor cells to surrounding unlabeled host cells (C6-Cx43, n = 8; 9L, n = 8), but no CDCF diffusion when Cx-deficient C6-mock cells were injected (n = 4, Fig. 8). Similar CDCF transfer was observed when prelabeled C6-Cx43 or 9 L wild-type cells were injected into acutely prepared cortical brain slices (data not shown).

![Fig. 6. Gap junction-mediated transformation of astrocytic phenotype. Rat astrocytes were prelabeled with CMTMR and cocultured with: A and D, mock-transfected, gap junction-deficient C6 glioma cells (C6-mock, -Cx); B and E, Cx43-transfected C6 glioma cells (C6-Cx43, +Cx); or C and F, 9 L gliosarcoma cells (wild-type express high level of Cx43, +Cx). After 2 days in vitro, the cultures were fixed and assayed for immunoreactivity against GFAP. Astrocytes were significantly larger and expressed more intense staining against GFAP in gap junction-deficient cultures (A and D), compared with astrocytes in cocultures connected by gap junctions (B and E; C and F). G, histogram summarizing three independent experiments (each in triplicate). More than 200 astrocytes were evaluated in each set of cocultures; Images are 200 × 200 nm. *, P < 0.01; **, P < 0.001.](image_url)
DISCUSSION

These observations confirm that functional intercellular communication between astrocytes and malignant glioma cells occurs readily via gap junctions, both in cocultures and in live brain (Figs. 3 and 8). Importantly, gap junction coupling with glioma cells have a profound effect upon astrocytic phenotypes in that astrocytes cocultured with Cx-expressing glioma cells were smaller and expressed lower levels of GFAP than in Cx-deficient cocultures (Fig. 6). Gap junctions are a significant means of intercellular exchange of electrolytes, secondary messengers, and other low molecular weight metabolites. During embryogenesis and tissue differentiation, expression of specific gap junction proteins establishes well-defined compartments and thereby produces complex patterns of communication. Within each compartment, groups of cells are joined by gap junctions to each other and not to cells in other compartments (9). Gap junctions provide a pathway of cytoplasmic continuity so that all of the coupled cells have common access to shared pools of small ions and signaling molecules (23).

Considerable evidence suggests that loss of gap junction communication may be essential in neoplastic transformation. Loewenstein and Kanno (24) originally reported that cancer cells lack gap junction communication, and others subsequently found that the down-regulation of gap junction-mediated intercellular communication might lead to uncontrolled growth of malignant cells as well as metastasis (25–29). Communication-deficient fibroblast cell lines isolated from Cx43 knock-out mouse embryos showed characteristics of transformed cells including increased growth rate (30). Transfection with various viral oncogenes leads to reduced gap junction coupling that, in several studies, has been linked to phosphorylation of either serine, tyrosine, or threonine on the gap junction protein. For example, the src oncogene product, pp60v-src, reduced coupling by phosphorylating Cx43 at tyrosine 265. By contrast, transfection with the c-erbB2/neu oncogene (neu+) is most likely associated with phosphorylation of serine/
itself or inositol 1,4,5-trisphosphate has been shown to mediate wave propagation. In our study, calcium waves were elicited in confluent cultures loaded with the calcium-indicator dye fluo-3 by mechanical stimulation. In general, calcium waves propagated over shorter distance in glioma cell cultures compared with astrocytes from either human or rat brain. Nevertheless, calcium signals readily traverse from astrocytes to glioma cells and vice versa, demonstrating that bidirectional communication exists between the two cell types. The extent of such communication was a direct function of the Cx43 level in glioma cells (Table 1). These findings suggest that calcium might be an important signaling molecule involved in the cell-to-cell communication between astrocytes and glioma cells. In fact, Naus et al. (5) found that Cx43 mRNA in peritumor tissue was elevated in patients who have seizures as compared with patients who have no seizures. It is tempting to speculate that calcium signaling between glioma cell and astrocytes plays a role in aberrant electrical activities.

Gap junctions in malignant gliomas have recently been implicated in the “bystander effect.” Proliferating tumor cells expressing herpes thymidine kinase (tk−) die when exposed to acyclovir (40, 41). Several groups have demonstrated that tk− tumor cells die after exposure to acyclovir when connected by gap junctions to the tk+ expressing cells but not when gap junctions are absent. Recently, it has been demonstrated that gap junctions play a role in “natural bystander effect.” In a coculture system containing resistant bcl2-expressing cells (bcl2+) and less resistant bcl2-deficient (bcl2−) cells, gap junctions propagated and amplified injury (18). Bcl2+ cells died after otherwise nonlethal injury when connected by gap junction to bcl2− cells. bcl2+ cells survived similar injury when cocultured with gap junction-deficient bcl2− cells. A significant distinction is that, whereas acyclovir is an exogenous prototoxin, the mediators of bystander death in a later study were generated by the dying cells as intermediates in the apoptotic process.

The recent explosion of information has established that cell-to-cell coupling is a dynamically regulated pathway for intercellular communication and that gap junctions, on the other hand, control basic cellular functions (42). In cancer research, the traditional concept is that the primary function of gap junctions is to regulate proliferation (43–45). We have presented data supporting the notion that tumor cells are directly coupled with normal host cells and that this coupling had profound effects on the phenotypic characteristics of host cells. The significance of these observations awaits further studies, but the lesson from other systems is that gap junction coupling has the potential to both shape and regulate the pattern of cellular interactions that may take place during tumor cell invasion.

Fig. 8. In vivo dye transfer from glioma cells to host cells. Left panel, cell suspension of CDCF (green)- and DiIC18 (red)-labeled glioma cells observed under confocal microscope before injection. All of the cells were double-labeled. Right panel, extensive transfer of CDCF from prelabeled 9 L gliosarcoma cells to surrounding host cells. In contrast, C6-mock (gap junction-deficient) did not transfer CDCF to host cells. The stellate morphology and organization of labeled host cells (recipient cells) are characteristics of cortical astrocytes. All of the cells were double-labeled and appear yellow because of the merging of red and green.
REFERENCES


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