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Communication between malignant glioma cells and vascular endothelial cells through gap junctions

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Object. Extensive invasion and angiogenesis are hallmark features of malignant gliomas. Communication between malignant glioma cells and surrounding astrocytes occurs, resulting in transformation of the astrocytic phenotype. In the present study, the authors examined whether malignant glioma cells and vascular endothelial cells (VECs) communicate through the formation of gap junctions and whether this communication influences angiogenesis.

Methods. Connexin43 (Cx43), a gap junction protein expressed in glioma cells, was identified in human umbilical VECs (HUVECs). Immunocytochemical staining for Cx43 demonstrated immunoreactive plaques at areas of cell–cell contact among HUVECs as well as between HUVECs and Cx43-expressing malignant glioma cells. Dye transfer, performed using the gap junction–permeable dye dicarboxy-dichlorofluorescein diacetate (CDCF), among these cocultures indicated that these were functional communications. Calcium signaling also occurred from malignant glioma cells to HUVECs. Tube formation by HUVECs cocultured with Cx43-transfected T98G malignant glioma cells (T98G-Cx43 cells) or with U87MG malignant glioma cells, which naturally express Cx43, was significantly increased compared with tube formation by HUVECs alone. The difference in tube formation by HUVECs cocultured with empty vector–transfected T98G glioma cells (T98G-mock cells) or with Cx43-deficient U373MG malignant glioma cells and tube formation by HUVECs alone was not statistically significant. Furthermore, the concentration of vascular endothelial growth factor (VEGF), an angiogenic factor important for the induction of angiogenesis and blood vessel formation, was significantly higher in medium harvested from cultures of T98G-Cx43 cells than in that harvested from cultures of control T98G-mock cells. Human malignant glioma U87MG cells also secreted increased concentrations of VEGF as compared with HUVECs alone. Nevertheless, there was no statistically significant difference in tube formation by HUVECs cultured in medium conditioned by either Cx43-expressing or Cx43-deficient glioma cells, suggesting that the direct gap junction communication between glioma cells and HUVECs may play a much more significant role than the increased VEGF secretion in vascular tube formation in this assay.

Conclusions. These results indicate that functional gap junction formation between human malignant glioma cells and VECs occurs. This communication appears to influence tumor angiogenesis. Targeting gap junction signaling may offer a potential mechanism for therapy in patients with these tumors.

KEY WORDS • endothelial gap junction • malignant glioma • endothelial cell • angiogenesis • vascular endothelial growth factor

MALIGNANT gliomas are the most common brain tumors identified in adults. Despite current therapy, the median survival time of patients harboring glioblastoma multiforme remains less than 1 year. Extensive invasion and angiogenesis are critical components of malignant gliomas that contribute to their aggressive behavior and poor prognosis. Invasion requires cellular adhesion, proteolysis of the basement membrane extracellular matrix, and migration. Angiogenesis involves a complex cascade that is thought to begin with growth factor production and release, followed by sprouting of microvessels, basement membrane dissolution, endothelial cell proliferation and migration, vascular tube formation, and arteriovenous differentiation. Once angiogenesis has been initiated, tumors can grow rapidly because their growth is no longer restricted by oxygen and nutrient diffusion. Activation of endothelial cells is associated with the production of angiogenic factors. Although more than 20 factors have been identified, VEGF is one of the major cytokines involved in angiogenesis in gliomas.[10] Direct cellular interaction between malignant glioma cells and endothelial cells may be one mechanism by which angiogenesis is stimulated. The inhibition of intercellular communication prevents endothelial cell migration and the formation of vascular networks.[1] Recently, we demonstrated that gap junctions form readily be-

Abbreviations used in this paper: CDCF = dicarboxy-dichlorofluorescein diacetate; CMFDA = 5-chloromethylfluorescein diacetate; CMTMR = 5-(and 6-)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine; Cx43 = connexin43; $DiIC_{18}$ = 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocardocarbocyanine perchlorate; ELISA = enzyme-linked immunosorbent assay; HUVEC = human umbilical vascular endothelial cell; SD = standard deviation; T98G-Cx43 cells = Cx43-transfected T98G cells; T98G-mock cells = empty vector–transfected T98G cells; VEGF = vascular endothelial growth factor.

tween malignant glioma cells and astrocytes, resulting in a change in the astrocytic phenotype.[22] We sought to determine whether there is direct communication through gap junctions between VECs and glioma cells. We also examined the effect of this gap junction formation by measuring the expression of VEGF protein expression in glioma cells and tube formation in VECs.

Gap junctions are intercellular membrane channels produced between adjacent cells. The structural unit of the gap junction is connexin, a protein belonging to a highly conserved multigene family, which is expressed in a tissue-specific manner.[15] Both glioma cells and astrocytes form gap junctions, with Cx43 being the primary protein that is expressed.[4,16] Connexins on the membrane of one cell dock with connexins in adjacent cells through noncovalent interaction.[5] An aqueous pore or channel forms, allowing for the intercellular exchange of small molecules, ions, and second messengers. Communication between glioma cells and that between glioma cells and astrocytes occurs by intercellular Ca^{++} signaling through gap junctions.[3,22] These junctions can thus play a critical role in regulating cell growth, differentiation, and morphogenesis.

Our present study confirms that Cx43 is expressed in VECs. Gap junction communication between malignant glioma cells and HUVECs as well as between the HUVECs themselves is identified, with Cx43 shown to be necessary for their functional formation. Stimulation of glioma cells is shown to initiate a wave of Ca^{++} that is transmitted to HUVECs. Gap junction coupling appears to induce and potentiate angiogenesis, as demonstrated by increased and more efficient tube formation by HUVECs. The VEGF protein is also increased in medium that has been conditioned by glioma cells that express Cx43.

Materials and Methods

Cell Lines

Human umbilical VECs and the human malignant glioma cell lines U87MG and U373MG were obtained from American Type Culture Collection (ATCC, Rockville, MD). The T98G-Cx43 cells and the T98G-mock cells were kindly provided by Dr. Ruo-Pan Huang (Emory University, Atlanta, GA). The HUVECs were maintained in an endothelial cell growth medium system (Life Technology, Grand Island, NY). The U87MG, U373MG, T98G-Cx43, and T98G-mock cells were cultured in a manner described previously.[22]

Immunofluorescence Study

Immunocytochemical staining was performed as described previously.[22] Briefly, the cells were plated on 12-mm uncoated coverslips ($0.5–1 \times 10^5$ 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. The cultures were washed three times with phosphate-buffered saline, and fluorescein isothiocyanate–conjugated goat anti–rabbit antibody was applied to the cultures for 1 hour at room temperature. The cultures were again washed with phosphate-buffered saline several times and the coverslips were mounted in Slow Fade (Molecular Probes, Eugene, OR). A polyclonal antibody directed against the cytoplasmic C terminus of Cx43 was kindly provided by Dr. Bruce Nicholson (State University of New York, Buffalo, NY).

Dye-Transfer Assay for Determination of Functional Gap Junctions

The dye-transfer method was adapted from that described by Goldberg, et al.[6] Cells were loaded with CDCF, a gap junction–passable tracer, for 5 minutes, and then washed and harvested by trypsinization. The cells were resuspended, labeled with 10 μM of the cell membrane dye $DiIC_{18}$[5] (excitation 648 nm, Molecular Probes) for 10 minutes and mixed with unlabeled cells at a 1:250 ratio. One hour after the cells had been plated on poly-L-lysine–coated dishes, the transfer of dye from the CDCF/$DiIC_{18}$-labeled (donor) cells to the unlabeled (recipient) cells was evaluated using confocal scanning microscopy. The coupling index was calculated according to the following formula: (number of donor cells transferring dye to surrounding cells/total number of donor cells) × (number of recipient cells/number of donor cells transferring dye).

Intercellular Ca^{++} Signaling

Intercellular Ca^{++} signaling was measured in a manner described previously.[19,23] Briefly, the HUVECs were prelabeled with $DiIC_{18}$ and cocultured with malignant glioma cells at a ratio of 1:250. Confluent monolayers of cocultures were loaded for 1 hour with 10 μM fluo-3 acetoxymethylester (Fluo-3 AM; Bio-Rad, Hercules, CA). All experiments were performed at room temperature. To initiate a Ca^{++} wave, a cell in the center of the viewing field was mechanically stimulated by a glass micropipette (tip diameter < 1 μm) mounted on a micromanipulator (model MMO-220; Narishige, Tokyo, Japan). Excitation was provided by the 488-nm line of the krypton–argon laser of a confocal scanning microscope (MRC1000; Bio-Rad) attached to an inverted microscope (Diaphot; Nikon, Tokyo, Japan). Images were acquired every second and recorded on an optical disk (LM-D702W; Panasonic, Osaka, Japan). Calcium waves were quantitated by measuring the maximum distance that the Ca^{++} signal traveled from the point of initiation (radius of the wave).

In Vitro Assay of Tube Formation

The spontaneous formation of capillary-like structures by HUVECs on a basement membrane matrix preparation was measured to assess the angiogenic potential by using an in vitro angiogenesis assay kit (Chemicon International Inc., Temecula, CA). Ninety-six-well plates were coated with Matrigel according to the manufacturer's instructions. The HUVECs were seeded onto the coated wells at a density of 5×10^3 cells/well with or without glioma cells (2.5×10^3 cells/well) and incubated at 37°C for up to 24 hours. Tube formation was observed, photographed, and analyzed with the aid of a confocal microscope. The degree of tube formation was assessed by measuring the total length of the tubular structures formed in three random fields (× 4 magnification) from each well, using commercially available software (SigmaPlot; SPSS, Inc., Chicago, IL) with an IBM-compatible personal computer. Each experiment was performed at least twice.

Enzyme-Linked Immunosorbent Assay

For quantitative evaluation of VEGF concentrations in cell culture supernatants, we performed a sandwich ELISA by using a commercially available assay kit (ELISA kits; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The microplate was precoated with an affinity-purified polyclonal antibody specific for $VEGF_{164}$ (isoform of VEGF). A 50-μl sample of cell culture supernatant was applied. During a 2-hour incubation period, the $VEGF_{164}$ was bound by the immobilized antibody. The plate was washed to remove unbound substances, 100 μl of an enzyme-linked polyclonal antibody specific for $VEGF_{164}$ was added to each well, and the plate was incubated for 2 hours at room temperature. Unbound enzyme–antibody reagent was then removed by rinsing and 100 μl of substrate solution was added. During a 30-minute incubation period, the enzyme reaction yielded a blue product that turned yellow when the stopping solution (100 μl) was applied to the wells. The intensity of the color was measured using a spectrometer (PowerWave200; Bio-Tek Instruments, Winooski, VT) at a wavelength of 450 nm. Optical imperfections were corrected by subtraction of readings at 570 nm. A standard curve was constructed by plotting the mean absorbance for each standard (x axis) against the concentration (y axis). The sample values (in duplicate) were then read off the stan-

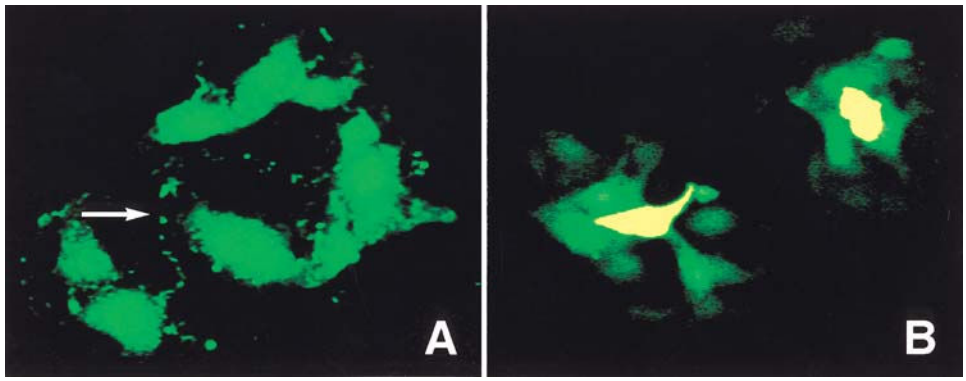

FIG. 1. Immunoreactivity of Cx43 and the results of a dye-transfer assay in HUVEC cultures. A: Expression of Cx43 in HUVECs *(arrow)*. The Cx43 immunoreactivity is widely located in the endothelial cell cytoplasms with some staining concentrated at the sites of endothelial cell–cell attachment. B: Gap junction permeability is visualized by performing a dye-transfer assay in HUVECs. The cells were double labeled with a gap junction–passable tracer, CDCF *(green)*, and a membrane dye, $DiIC_{18}$ (far red dye), and mixed with unlabeled HUVECs (1:100). The extent of CDCF diffusion from double-labeled donor cells (cells appearing *yellow* due to merging of red and green dyes) to unlabeled recipient cells (*green* cells) was evaluated using confocal microscopy.

dard curve and multiplied by the dilution factor. The experiment was performed in triplicate.

Results

Immunoreactivity of Cx43 and Functional Coupling of HUVECs

Gap junction communication among HUVECs was demonstrated by performing immunochemical analysis and dye-transfer assays (Fig. 1). The HUVECs were immunoreactive to Cx43 with staining evident in the cytoplasms of endothelial cells and at the sites of endothelial cell–cell attachment (Fig. 1A). The dye-transfer assay was used to assess the extent of functional gap junction coupling. The HUVECs demonstrated coupling with significant dye transfer (Fig. 1B).

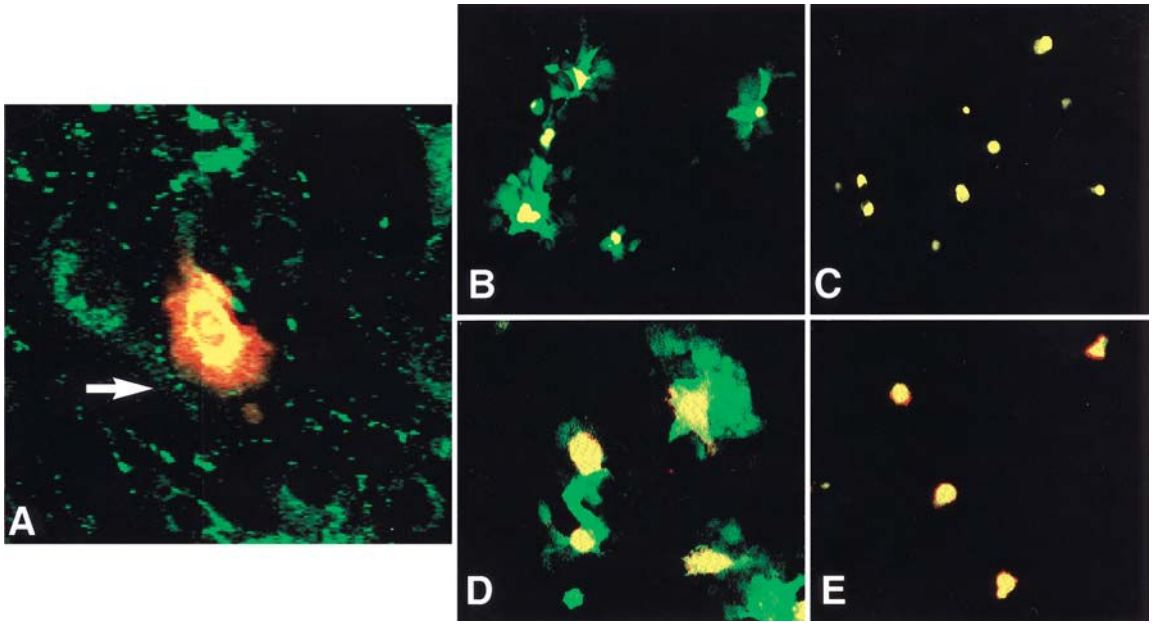

FIG. 2. Immunoreactivity of Cx43 and the results of dye-transfer assay in mixed cultures. A: Cocultures of the human malignant glioma cells U87MG and HUVECs immunoreacted with an anti-Cx43 antibody. The HUVECs were labeled with $DiIC_{18}$ (far red dye) and mixed with glioma cells in a ratio of 1:100. There are Cx43-immunopositive plaques present at areas of cell–cell contact between the HUVECs and glioma cells *(arrow)*. B–E: Gap junction permeability visualized by dye-transfer assay in cocultures of HUVECs with the Cx43-expressing human malignant glioma cells U87MG (B) and T98G-Cx43 (D), and with the Cx43-deficient cells U373MG (C) and T98G-mock (E). Glioma cells were double labeled with a gap junction–passable tracer, CDCF *(green)*, and a membrane dye, $DiIC_{18}$ (far red dye), and mixed with unlabeled HUVECs (ratio 1:100). The extent of CDCF diffusion from labeled donor cells (*yellow* cells) to unlabeled recipient cells (*green* cells) was evaluated with the aid of confocal microscopy.

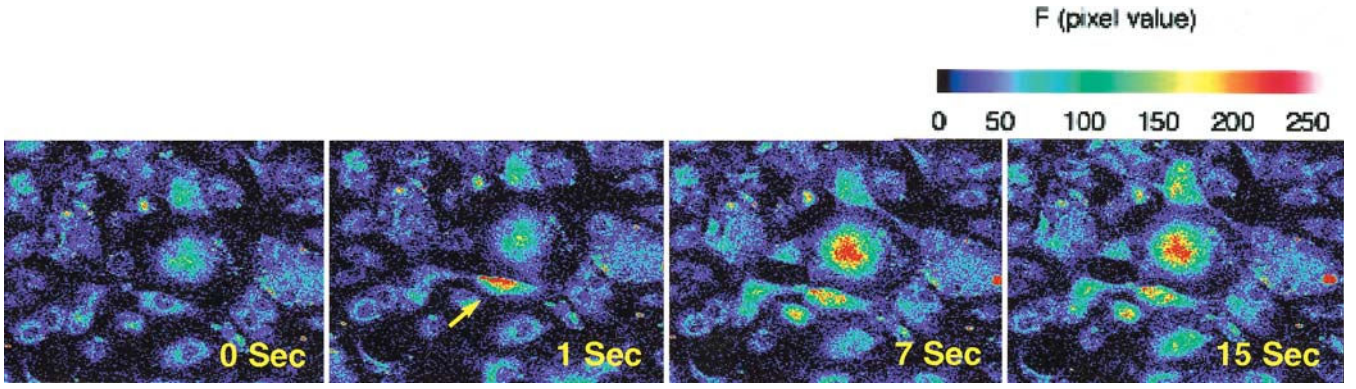

FIG. 3. Intercellular Ca^{++} signaling in HUVECs. Cultures were loaded with Fluo-3 AM and imaged with confocal microscopy. After a cell had been mechanically stimulated *(arrow)*, Ca^{++} signals were transmitted to its surrounding cells, although only within short distances. The panels map the Fluo-3 AM signals in the same field. The images were collected at 0, 1, 7, and 15 seconds after focal mechanical stimulation.

Immunoreactivity of Cx43 and Functional Coupling of HUVECs and Malignant Glioma Cells

To assess whether functional gap junctions form between malignant glioma cells and HUVECs and whether this coupling is dependent on Cx43, we used two human malignant glioma cell lines that express Cx43: U87MG and T98G-Cx43 cells; and two that are Cx43 deficient: U373MG and T98G-mock cells. Cocultures of HUVECs and U87MG cells or T98G-Cx43 cells demonstrated immunoreactivity to Cx43 and immunopositive plaques at areas of cell–cell contact (Fig. 2A), indicating that there is gap junction formation. When the dye-transfer assay was performed to test the function of such Cx43 coupling, these same cells exhibited extensive coupling and dye transfer, whereas the cocultures of HUVECs and U373MG or T98G-mock cells displayed no dye transfer (Fig. 2B–E).

Gap Junction–Dependent Ca^{++} Signaling Between HUVECs and Malignant Glioma Cells

Gap junctions can transmit intercellular signaling through Ca^{++}. To study Ca^{++} signaling between HUVECs and between HUVECs and malignant glioma cells, cell cultures were loaded with the Ca^{++} indicator Fluo-3 AM and imaged using confocal microscopy. The HUVECs transmitted Ca^{++} signals after being mechanically stimulated, as demonstrated by Ca^{++} waves, although only within short distances (Fig. 3). Next, cocultures of HUVECs and malignant glioma cells were prepared and loaded with Fluo-3 AM. Intercellular Ca^{++} signaling between glioma cells and HUVECs was bidirectional. Figure 4 shows Ca^{++} signaling from U87MG cells to HUVECs. The T98G-Cx43 cells also transferred the Ca^{++} signal to HUVECs, whereas U373MG and T98G-mock cells, which lack Cx43 expression, failed to show any significant Ca^{++} signaling.

Tube Formation in HUVECs Cultured With Malignant Glioma Cells

Tube formation by VECs is a determinant of angiogenesis.[11] We examined the extent of tube formation in cultures of HUVECs alone; in cultures of HUVECs with the Cx43-expressing malignant glioma cells T98G-Cx43 and U87-MG; and in cultures of HUVECs with the Cx43-deficient malignant glioma cells T98G-mock and U373MG cells (Figs. 5 and 6). Tube formation was evaluated with the

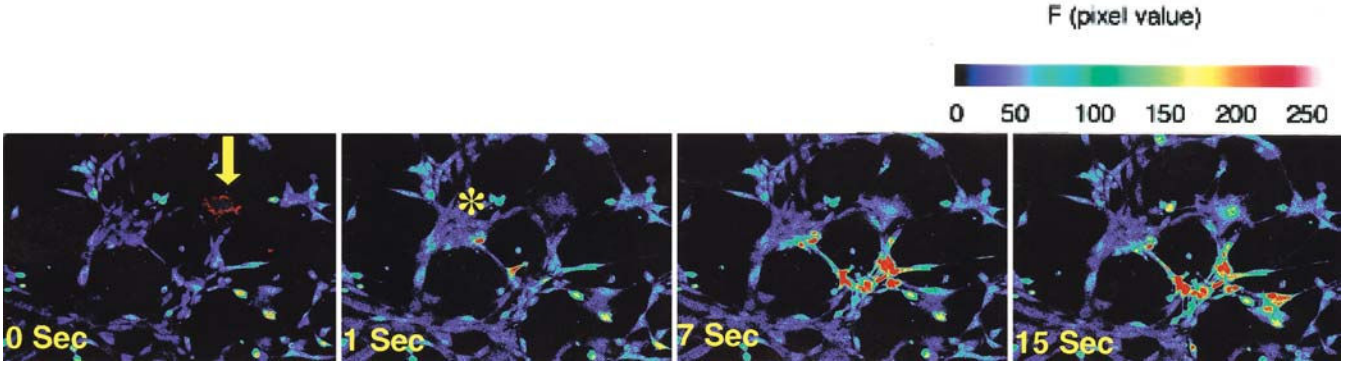

FIG. 4. Intercellular Ca^{++} signaling from human malignant glioma cells to HUVECs. Cocultures of HUVECs (prelabeled with $DiIC_{18}$) and Cx43-expressing U87MG cells mixed in a ratio of 1:100 were loaded with Fluo-3 AM and imaged using confocal microscopy. In the left panel, the $DiIC_{18}$-labeled endothelial cell is visualized in the field of interest (*red area* marked by the *arrow*). The next three panels map the Fluo-3 AM signals in the same field. The images were collected at 0, 1, 7, and 15 seconds after focal mechanical stimulation *(asterisk)*.

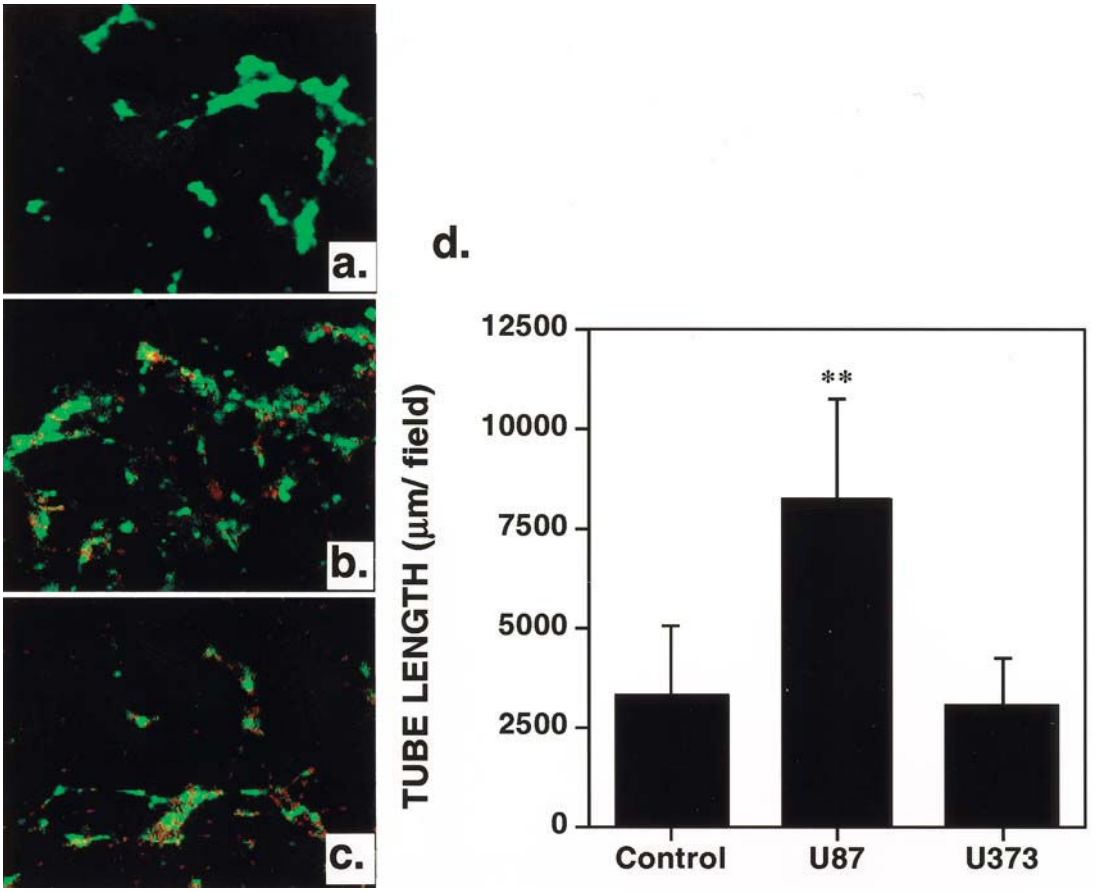

FIG. 5. Tube formation by HUVECs cocultured with U87MG (Cx43-expressing) or U373MG (Cx43-deficient) cells. The HUVECs were prelabeled with a *green* living cell tracer, CMFDA, and were mixed with U87MG or U373MG cells (labeled with the *red* tracer CMTMR). Cells were then seeded onto a Matrigel-coated 96-well plate and incubated at 37°C. Tube formation was observed and photographed with the aid of a confocal microscope. a: The HUVECs alone. b: Some HUVECs cocultured with U87MG cells. c: Some HUVECs cocultured with U373MG cells. d: Bar graph showing the results of a representative experiment performed at the 8-hour incubation time point (**$p < 0.01$).

aid of confocal microscopy after 8 hours. In some cases, HUVECs and malignant glioma cells were prelabeled with the living cell tracers CMFDA (green) and CMTMR (red), respectively. The cultures of HUVECs and Cx43-expressing malignant glioma cells displayed an increased length of tubes and more efficient construction than cultures containing either HUVECs alone or HUVECs mixed with Cx-43-deficient malignant glioma cells. At 8 hours, the mean tube lengths (mean ± SD) were 4671 ± 361 μm/field for HUVECs alone, 5549 ± 736 μm/field for HUVECs and T98G-mock cells ($p = 0.08$), and 6300 ± 310 μm/field for HUVECs and T98G-Cx43 cells ($p < 0.01$). There was no significant difference in tube length between HUVECs cultured in serum-free medium and in those contained in medium conditioned by either T98G-Cx43 or T98G-mock cells (Table 1).

Release of VEGF From Malignant Glioma Cells

To study the effect of Cx43-expressing glioma cells on tube formation by HUVECs, we examined the concentration of VEGF in media harvested from cultures of the Cx-43-expressing glioma cells T98G-Cx43 and U87MG and from media from cultures of Cx43-deficient glioma cells T98G-mock and U373MG by using an ELISA kit (Fig. 7). The concentration of VEGF released from the T98G-Cx43 cells (4500 pg/10^6 cells) was significantly increased, compared with the amount released from the T98G-mock cells (< 1500 pg/10^6cells). Naturally the Cx43-expressing U87-MG cells also produced much more VEGF in the medium within which they were cultured than the Cx43-lacking U373MG cells.

Discussion

We have found that functional communication occurs between VECs and malignant glioma cells similar to that between astrocytes and malignant glioma cells. This communication relies on gap junction formation with Cx43. The coupling of these cells appears to promote the angiogenesis of the VECs, as demonstrated by an increased and more efficient tube formation. In addition, increased VEGF protein was found in media containing human glioma cells transfected with Cx43 compared with media containing empty vector–transfected cells. These results suggest that

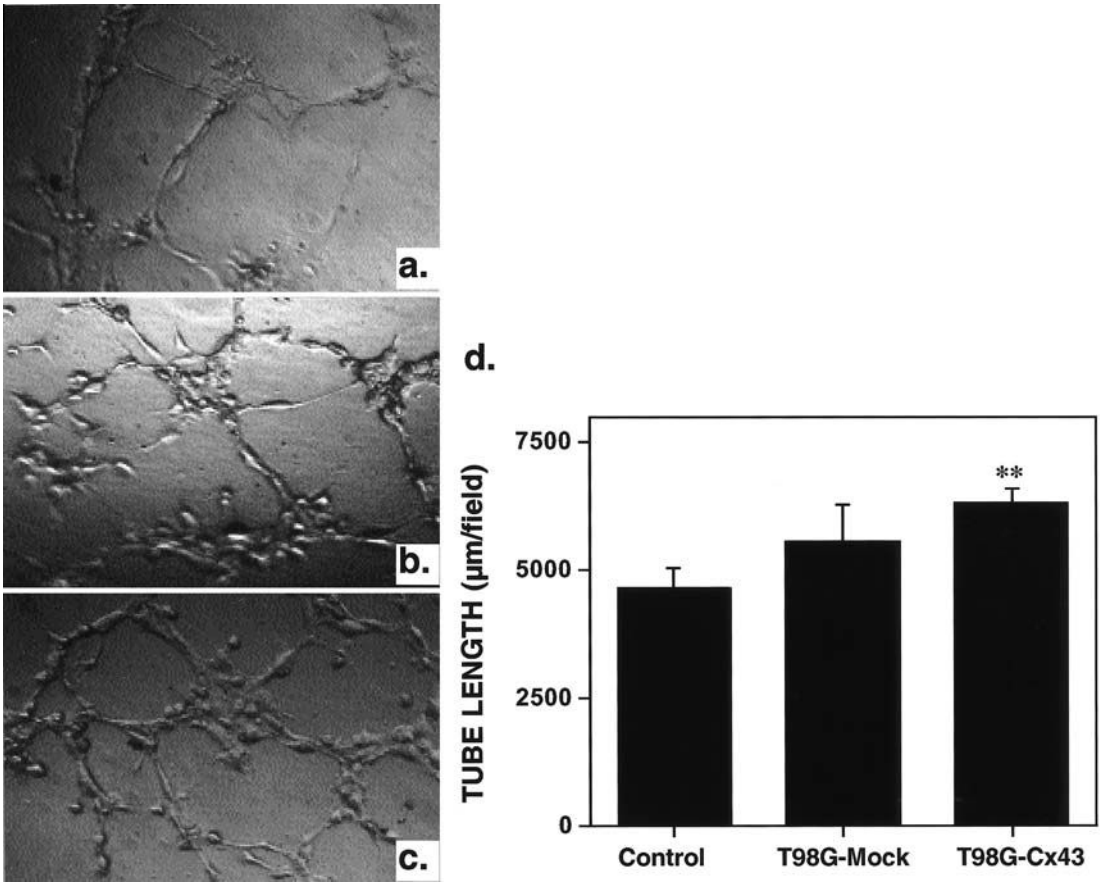

FIG. 6. Tube formation by HUVECs cocultured with T98G-Cx43 (Cx43-expressing) or T98G-mock (Cx43-deficient) cells in the same manner described in the legend to Fig. 5, but without prelabeling the cells. a: The HUVECs alone. b: Some HUVECs cocultured with T98G-Cx43 cells. c: Some HUVECs cocultured with T98G-mock cells. d: Bar graph demonstrating the results of a representative experiment performed at the 8-hour incubation time point (**$p < 0.01$).

communication through gap junction formation between malignant glioma cells and endothelial cells and that between glioma cells has a role in the process of tumor angiogenesis.

An interruption in the gap junction communication between glioma cells is one step in malignant transformation.[7,21] The rates of growth in vitro and in vivo were decreased in rat glioma C6 cells transfected with Cx43.[13,24] In later studies, however, researchers found that high levels of connexin expression may not always be associated with a decreased proliferation rate.[12] High-grade gliomas express Cx43 at widely varied levels.[16]

In mouse melanoma cells, the expression of Cx26 decreased as cells transformed into a more malignant phenotype.[9] In the same study, however, the expression of Cx26 increased in cells metastasizing to lymph nodes. Ito, et al.,[8] demonstrated that functional gap junctions occur between endothelial and melanoma cells, possibly through a Cx26–Cx43 heterologous formation. Connexin26 was markedly increased in melanoma cells in the process of invasion.[8] Thus, the formation of gap junctions between tumor and endothelial cells may contribute to increased susceptibility of the host–tumor invasion.

Connexin43 is the dominant gap junction protein in human endothelial cells.[18,20] Migrating endothelial cells regulate gap junction–mediated intercellular communication.[14] Ashton, et al.,[1] found that inhibition of this communication among endothelial cells, presumably through disruption of Cx43, suppressed endothelial cell migration and tube formation. It is possible that once tumor cells have acquired growth potential, the increased expression of connexins and the formation of gap junctions may promote tumor angiogenesis and invasion.

The angiogenic response requires that endothelial cells alter their phenotype to one capable of proliferation, migration, and tube formation with the development of new vessels. Vascular endothelial growth factor is critical for the earliest stages of vasculogenesis.[2] The concentrations of VEGF in human glioma lines were shown to play an important role in angiogenesis and tumorigenesis. Those lines with higher VEGF expression had larger and denser blood vessels.[10] Previous authors have demonstrated that VEGF expression correlates with glioma growth.[17] The mechanism driving the angiogenic burst in malignant gliomas has not been determined, but VEGF appears to play a pivotal role.

In the present study, Cx43-expressing malignant glioma cells secreted more VEGF than Cx43-deficient cells and promoted increased tube formation by HUVECs when they were cocultured with HUVECs. Nevertheless, there was no significant increase in tube length when HUVECs were

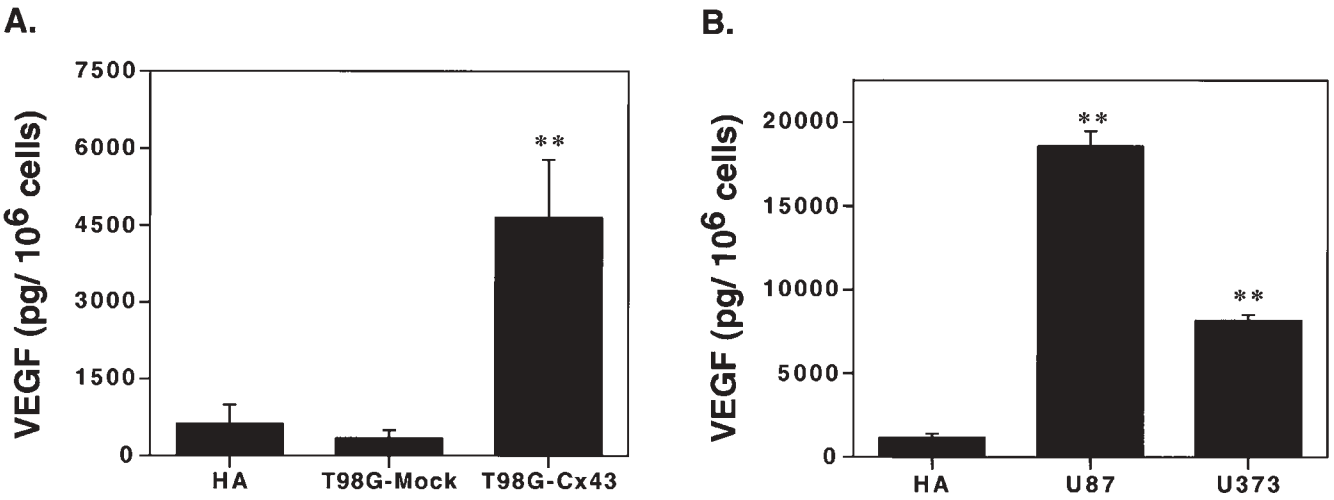

FIG. 7. Concentrations of VEGF in medium conditioned by human astrocyte cultures (HA) and glioma cells. Cells were incubated in a 24-well plate at a density of 5×10^4 cells/well in 10% fetal bovine serum–Dulbecco modified Eagle medium (DMEM)/F12 nutrient mixture overnight and in serum-free DMEM/F12 nutrient mixture for another 24 hours. The concentration of VEGF in the supernatants was determined using a VEGF ELISA kit. The bar graphs show the results of a representative experiment performed in triplicate (**$p < 0.01$). A: Human astrocytes and a Cx43- or empty vector–transfected glioma cell line T98G (T98G-Cx43 and T98G-mock cells, respectively). B: Human astrocytes and the Cx43-expressing U87MG or the Cx43-deficient U373MG cells.

cultured in medium that had been conditioned with either Cx43-expressing or Cx43-deficient malignant glioma cells. Thus, although gap junction communication appears to promote increased VEGF, this alone does not lead to increased tube formation. These findings suggest that this communication between malignant glioma cells and HUVECs plays a role in glioma angiogenesis, in addition to increased VEGF secretion. Identification of the role of gap junctions in angiogenesis poses another avenue for targeting tumor growth with therapies designed to interrupt this signaling mechanism.

Conclusions

This study demonstrates that VECs form functional gap junctions with malignant glioma cells. This cell coupling appears to be dependent on Cx43 and leads to more efficient tube formation. In addition, media containing Cx43-expressing malignant glioma cells were found to have increased concentrations of VEGF. These findings strongly suggest that gap junction communication between malignant glioma cells and VECs plays a potentially important role in glioma angiogenesis.

TABLE 1

*Tube formation by HUVECs**

Culture Conditions	Tube Length (μm/field)	p Value†
HUVECs alone		
serum-free	4671.1 ± 361.2	—
CM (T98G-Cx43)	4956.4 ± 528.5	0.43
CM (T98G-mock)	5196.2 ± 221.9	0.08
HUVEC coculture		
T98G-Cx43	6300.0 ± 309.7	<0.01
T98G-mock	5549.0 ± 736.3	0.08

* Values represent the means ± SDs. The data were obtained from four independent assay sets. Abbreviations: CM = conditioned medium; — = not applicable.

† Student t-test for each group compared with the serum-free HUVEC alone group.

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