Endothelial Cell Barrier Impairment Induced by Glioblastomas and Transforming Growth Factor β2 Involves Matrix Metalloproteinases and Tight Junction Proteins

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Abstract

Gliomas, particularly glioblastoma multiforme, perturb the blood-brain barrier and cause brain edema that contributes to morbidity and mortality. The mechanisms underlying this vasogenic edema are poorly understood. We examined the effects of cocultured primary cultured human glioblastoma cells and glioma-derived growth factors on the endothelial cell tight junction proteins claudin 1, claudin 5, occludin, and zonula occludens 1 of brain-derived microvascular endothelial cells and a human umbilical vein endothelial cell line. Cocultured glioblastoma cells and glioma-derived factors (e.g. transforming growth factor β2) enhanced the paracellular flux of endothelial cell monolayers in conjunction with downregulation of the tight junction proteins. Neutralizing anti-transforming growth factor β2 antibodies partially restored the barrier properties in this in vitro blood-brain barrier model. The involvement of endothelial cell-derived matrix metalloproteinases (MMPs) was demonstrated by quantitative reverse-transcriptase-polymerase chain reaction analysis and by the determination of MMP activities via zymography and fluorometry in the presence or absence of the MMP inhibitor GM6001. Occludin, claudin 1, and claudin 5 were expressed in microvascular endothelial cells in the absence of the MMP inhibitor GM6001. Occludin, claudin 1, and claudin 5 were expressed in microvascular endothelial cells in vitro but not in vivo. These results indicate that glioma-derived factors may induce MMPs and downregulate endothelial tight junction protein and, thus, play a key role in glioma-induced impairment of the blood-brain barrier.

Key Words: Blood-brain barrier, Endothelial cells, Glioblastoma, Matrix metalloproteinases, Permeability, Tight junctions, Tight junction proteins.

INTRODUCTION

The blood-brain barrier (BBB) is essential for the maintenance of brain homeostasis and low permeability. The major components of the BBB are brain microvessel endothelial cells that are closely interconnected via special cell-cell contacts, that is, tight junctions (TJs) (1); along with astrocytes, pericytes, and perivascular macrophages, endothelial cells form the neuromicrovascular unit of the BBB (2). The barrier function of the BBB is regulated dynamically in response to changes in the surrounding environment, including changes in tissue oxygen concentration (3), inflammatory processes (4), and tumors (5). The BBB is disrupted in many pathologic conditions, including infarction, trauma, multiple sclerosis, diabetic retinopathy, and glioma. Disruption of the BBB leads to disturbances of the tissue microenvironment that can subsequently accelerate the progression of disease processes (3, 6).

Glioma is the most frequent type of primary brain tumor; the malignant form glioblastoma multiforme (GBM) is incurable. Despite recent advances in neurosurgical instrumentation and adjuvant therapies, the mean survival time of patients with GBM remains to be less than 1 year. Edema within and in the surrounding tissue due to disruption of the BBB is a prominent feature of GBM and is a major factor contributing to morbidity and mortality. Conversely, induction of a temporally and spatially controlled transient opening of the BBB would be a helpful strategy for delivery of drugs and to introduce gene therapeutic agents. Electron microscopic studies have revealed that brain tumors induce TJ opening that become more pronounced with increasing malignancy (5). The molecular mechanisms of endothelial cell TJ dysfunction in brain tumors, however, remain poorly understood.

The barrier properties of CNS blood vessels are primarily attributable to the presence of complex TJ networks between the endothelial cells (7). The identification of occludin as the first component of TJs represented a turning
point in research on the molecular basis of tight junction proteins (TJPs) (8). Subsequent studies, including gene knockout analyses, demonstrated, however, that occludin is not essential for TJ assembly but, rather, is involved in endothelial cell signal transduction (9). Later, the claudins, a multigene family of more than 20 members, and junctional adhesion molecules were found to be other components of tight junctions (10). These proteins are linked to the cytoskeleton by intracellular TJPs, the membrane-associated guanylate kinase family members zonula occludens (ZO) 1, ZO-2, and ZO-3, and maintain or regulate barrier functions (11). Claudins show an individually restricted expression pattern, and several studies have indicated that they are the key molecules in the assembly of tight junctions contributing to the tissue-specific properties of TJ (12). For example, the claudins expressed in the endothelial cells of neural tissue are claudin 1, claudin 3, claudin 5, and claudin 12, and these molecules have been suggested to be responsible for endothelial barrier function (13).

Transforming growth factor (TGF) β is an effective mediator of cell growth and is indispensable for the normal proliferation and differentiation of cells. On the other hand, TGF-β, particularly the TGF-β2 isoform, is actively secreted by GBM cells (14, 15) and contributes to their increased migration capacity (16, 17) and autocrine tumor cell growth (18). Most importantly, TGF-β suppresses immune reactivity against tumor cells by inactivating T cells and monocytes and by its involvement in the generation of T regulatory cells (14, 19–22). Transforming growth factor β also modulates TJPs in nonneural tissues (23, 24). These observations suggest the possible involvement of TGF-β in blood-tumor barrier and BBB impairment in GBM.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes that are involved in the remodeling of the extracellular matrix in many physiologic and pathologic processes in the CNS (25). Because MMPs can catalyze the degradation of all the protein constituents of the extracellular matrix, it is important for their activities to be tightly regulated to prevent tissue degradation (26). Intracerebral MMP injection leads to extensive leakage of the BBB, T-cell recruitment to the site of the lesion, and demyelination, all of which can be reduced by MMP inhibitors (27). Proinflammatory cytokines, which markedly decrease the barrier function of endothelial cells, have been shown to regulate steps in the secretion and activation of MMPs (28, 29), indicating that MMPs may be critically involved in the mechanisms by which cytokines regulate BBB permeability (30).

The present study was performed to examine the mechanisms of the breakdown of the BBB by gliomas. We used an in vitro BBB model to investigate the effects of ex vivo GBM cells and TGF-β2 on endothelial cell monolayer permeability and the expression and distribution of the TJPs claudin 1, claudin 5, occludin, and ZO-1. The results indicate that TGF-β2 increases endothelial cell monolayer permeability and downregulates claudin 1 expression. The same effects were elicited by cocultured GBM cells or their conditioned media and can be partially neutralized by anti-TGF-β2 antibodies and an MMP inhibitor. These observations suggest that BBB disruption by GBM is partly regulated through TGF-β2 and MMP.

**MATERIALS AND METHODS**

**Reagents**

Rabbit polyclonal antibodies against occludin, ZO-1, claudin 1, and claudin 5 were obtained from Zymed Laboratories (South San Francisco, CA); rabbit polyclonal antibody against Glut 1 was from Chemicon (Chemicon Europe, Hampshire, UK). Secondary antibodies for immunofluorescence on cell cultures were labeled with fluorescein isothiocyanate and Cy3; those for immunoblotting were labeled with horseradish peroxidase (HRP) from Sigma (Fluka AG, Buchs, Switzerland). The following antibodies and reagents were used for immunoenzyme histochemistry:

- goat anti-rabbit (Caltag Laboratories, Burlingame, CA), biotinylated rabbit anti-mouse (Dako Diagnostics AG, Zug, Switzerland), donkey anti-goat HRP-labeled (Jackson Laboratories, West Grove, PA), and HRP-labeled avidin biotin (Dako). For fluorescence immunohistochemistry for confocal microscopy, the following secondary antibodies were used:
  - goat anti-rabbit immunoglobulin G Alexa Fluor 488, goat anti-mouse immunoglobulin G Alexa Fluor 488, and goat anti-rabbit immunoglobulin G Alexa Fluor 568 (Molecular Probes, Eugene, OR).

- Recombinant human TGF-β2, vascular endothelial growth factor (VEGF), polyclonal goat anti-TGF-β2, and isotype control antibodies were obtained from R and D Systems (Abingdon, UK). Recombinant human epidermal growth factor, fibroblast growth factor (FGF) 1, and FGF-2 were from Pepro Tech EC Ltd. (London, UK). Tissue culture materials were from Gibco (Invitrogen AG, Basel, Switzerland). Gel electrophoresis reagents and all standard laboratory chemicals were of the highest grade commercially available and were purchased from Fluka.

**Cell Line and Culture Media**

Human umbilical vascular endothelial cells (HUVECs) (clone CRL-1998; American Type Culture Collection, Manassas, VA) and primary cultured glioblastoma cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 2 mmol/L of L-glutamine, and 20 μg/ml of gentamicin. Human brain-derived microvascular endothelial cells (MVECs) were cultured in DMEM/F12 containing 10% horse serum, 10% fetal calf serum, 10 μg/ml of endothelial cell growth factor supplement, 1.2 μg/ml of bovine brain extract, 0.1 ng/ml of epidermal growth factor, 1 ng/ml of FGF-1, 10 μg/ml of hydrocortisone, 20 μg/ml of gentamicin, and 16 U/ml of heparin.

**Isolation of Human Glioblastoma Cells**

Human brain tumors were obtained from patients who underwent surgery for tumor resection and were diagnosed and classified at the Institute of Neuropathology, University Hospital, Zurich, according to the World Health Organization grading system of brain tumors using standard histologic and immunohistologic methods. All analyses were conducted in...
accordance with the Declaration of Helsinki and approved by the ethics committee of the Canton of Zurich. Informed written consent was obtained from all patients.

The resected tumor tissues were dissociated as described (31). Briefly, the tissues were placed in Petri dishes, minced mechanically, and digested enzymatically using 1 mg/ml of collagenase/dispase (Roche Applied Science, Rotkreuz, Switzerland) for 1 hour at 37°C with stirring using a magnetic bar. Thereafter, the dissociated cells were sequentially filtered through 100- and 70-μm cell strainers (Falcon Plastics, Oxford, CA) to remove any tissue debris. After centrifugation for 5 minutes (380 × g) at room temperature (RT), the erythrocytes were removed by resuspending and incubating the cells in ice-cold ACK buffer for 10 minutes on ice. The cells were washed in Hanks’ balanced salt solution, resuspended in culture medium, and plated in tissue culture flasks at a density of 1 × 10^5/cm².

Production of Tumor Supernatants and FGF-2, VEGF, and TGF-β2 Enzyme-Linked Immunosorbent Assays

Ex vivo cultured tumor cells were seeded in 24-well plates at a density of 5 × 10^4 cells/cm², and the supernatants (SNs) were collected 2 days later, cleared of cellular debris by centrifugation (2,000 × g, 10 minutes, 4°C), and stored at −20°C until the enzyme-linked immunosorbent assays were performed. Commercially available enzyme-linked immunosorbent assay kits (R and D Systems) were used according to the manufacturer’s instructions. The detection limits were found to be 1 pg/ml for FGF-2, 15.125 pg/ml for VEGF, and 31.25 pg/ml for TGF-β2.

Isolation of Human Brain MVECs

Human brain-derived endothelial cells were isolated as described (32). Briefly, cortical tissue samples from epileptic patients undergoing surgical treatment were cleaned of meninges and superficial vessels under a dissecting microscope. The samples were homogenized in DMEM containing 5% fetal calf serum (DMEM/5%) with a 5-ml syringe, digested with 0.25 mg/ml of collagenase A (Roche Applied Science) for 30 minutes at 37°C in a stirring water bath, cooled on ice, and centrifuged (250 × g, 10 minutes, 4°C). The pellet was resuspended in DMEM/5%, triturated with a fire-polished pipette, and then layered on 25% bovine serum albumin in Hanks’ balanced saline (Hanks + 10% FBS). After centrifugation at 2,000 × g for 10 minutes, the supernatant was removed, and the pellet was resuspended in DMEM/5%, and plated on collagen-coated culture dishes in culture medium. After 2 weeks, cells were subcultured and cultured for a further 7 to 10 days before use. Cells were used only at second passage. The purity of the endothelial cultures was checked by staining with polyclonal rabbit anti-human von Willebrand factor and anti-human glial fibrillary acidic protein antibodies (both from Dako). The purity was found to be greater than 90%.

Paracellular Flux

For experimental use, 5 × 10^4 human MVECs or HUVECs were plated on collagen-coated 24-well Transwell culture inserts with 0.4-μm pores (Costar Corp., Cambridge, MA). The medium was changed every third or fourth day, and paracellular flux was analyzed 10 days after plating. After washing of the cells cultured on the Transwell inserts once with PBS, prewarmed fresh culture medium was added to the apical (0.1 ml) and basolateral (0.6 ml) chambers with or without growth factors or 50% of glioblastoma conditioned medium. Neutralization experiments were performed by incubating the conditioned medium with either neutralizing anti-TGF-β2 or isotype control antibodies (both 20 μg/ml) for 2 hours at 37°C before the medium was added to the cultures. As a positive control, recombinant human TGF-β2 was used at a final concentration of 25 ng/ml. For the GBM coculture system, ex vivo cultured GBM cells were plated on 24-well plates at a density of 5 × 10^4 cells/cm² 2 days before stimulation, and inserts were placed in these wells for the experiment. The medium in the apical chamber was supplemented with 1 mCi [3H]-inulin (TRA324; Amersham, Piscataway, NJ) at the indicated time points. After 1-hour incubation, 50-μl samples were taken from the basolateral chamber and added to 1 ml of OptiPhase “HiSafe 3” (PerkinElmer, Turku, Finland). Radioactivity was measured with a Betamatic V liquid scintillation β-counter (Kontron AG, Zurich, Switzerland).

Electrophoresis and Immunoblotting

Western blot analysis was performed as described (33). Briefly, the protein concentrations in the extracts were measured by the Bradford assay (Bio-Rad, Hercules, CA), and equal amounts were loaded on the gel. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). The quality of the transfer was controlled by Ponceau S staining of the membranes. Blots were blocked in 5% nonfat dried milk in 20 mmol/L of Tris-HCl, pH 7.4, and 150 mmol/L of NaCl (Tris-buffered saline [TBS]) containing 0.05% Tween-20 for 45 minutes at 22°C. The filters were then incubated for 1 hour, with the primary antibodies diluted in blocking buffer. After washing 3 times for 5 minutes each time in TBS containing 0.05% Tween-20, the primary antibody was reacted with HRP-conjugated secondary antibody in blocking buffer for 30 minutes. After washing 3 times with TBS plus 0.05% Tween-20 and once with TBS, immunoreactive bands were detected by enhanced chemiluminescence (Amersham) in accordance with the manufacturer’s instructions. The chemiluminescent signals were scanned from autoradiographic films (Fuji RX) and imported into Molecular Analyst (Bio-Rad) for densitometry measurements.

Immunofluorescence Analysis of Cell Cultures

Cells grown on chamber slides were washed once with PBS, fixed with 3% paraformaldehyde for 15 minutes at RT, and permeabilized with 0.5% Triton-X100 (2 minutes at RT). Incubation with the primary antibody was performed in PBS containing 3% bovine serum albumin for 1 hour at RT. The
cells were then washed 3 times for 5 minutes each with PBS and incubated with a 1:200 dilution of fluorescein isothiocyanate-conjugated secondary antibody in PBS containing 3% bovine serum albumin for 1 hour at RT. After washing again three 5-minute washes with PBS, the chamber slides were mounted in 70% glycerol, 5% n-propyl-gallate in 30 mmol/L of Tris-HCl, pH 9.5, and examined by immunofluorescence microscopy using a Leica (Leica Microsystems, Glattbrugg, Switzerland) inverted microscope.

**Immunohistochemistry**

After surgical removal, the unfixed tissue was embedded in TissueTek O.C.T. compound. Cryostat sections 6-µm thick were cut using a Leica Cryostat mounted on SuperFrost slides (Menzel-Glaser, Braunschweig, Germany) and air-dried. Sections were fixed in acetone for 10 minutes at RT and washed in 0.05 mmol/L of TBS (pH 7.6). Sections were stained with anti-occludin (2.5 µg/ml), anti-claudin 1 (10 µg/ml), and anti-claudin 5 (5 µg/ml) antibodies. Controls included the corresponding preimmune antiserum or isotype-matched primary monoclonal antibody. After endogenous peroxidase activity was blocked by use of 0.3% H₂O₂ in methanol, secondary antibodies (goat anti-rabbit, 1:400, or biotinylated rabbit anti-mouse, 1:200) were diluted in TBS containing 10% human serum, followed by HRP-labeled anti-goat (1:80) or antibody-HRP complex. For visualization, 3,3'-diaminobenzidine tetrahydrochloride (Dako) was used. To quantify the intensities of the vessel staining on normal brain tissue and glioma specimens using the anti-claudin 1, anti-claudin 5, and anti-occludin antibodies, 50 high-power fields (magnification: 200 ×) were examined in a double-blinded manner according to the following score: 0, no stain; 1, weakest; 2, low; 3, medium; and 4, strong.

**Immunofluorescence and Confocal Microscopy Analysis**

Samples of the resected tissues were prepared for immunofluorescence confocal analysis by cutting them into halves and fixing them for 3 hours at 4°C in either 2% paraformaldehyde plus 0.2% glutaraldehyde solution or in acetic acid–free Bouin solution. Specimens were then washed in PBS (pH 7.4) and 50% ethyl alcohol, respectively, and stored at 4°C. The blocks of tissue were sectioned at 20 µm using a vibrating microtome (Leica, Milton Keynes, UK), and the sections were collected on Vectabond-treated slides (Vector Laboratories Inc., Burlingame, CA). Single immunolabeling was carried out with anti-occludin (diluted 1:10 in blocking buffer [BB]: PBS, 1% bovine serum albumin, 2% fetal calf serum), anti-claudin 1 (diluted 1:50 in BB), and anti-claudin 5 (diluted 1:50 in BB). Double immunolabeling was performed with either anti-claudin 5 or anti-occludin in combination with anti-GLUT 1 (diluted 1:50 in BB). Sections were incubated as follows: 1) in BB for 5 minutes at RT; 2) in 0.5% Triton X-100 in PBS for 30 minutes at RT; 3) with 1 (single staining) or 2 (double staining) of the primary antibodies suitably diluted in BB overnight at 4°C; and 4) with the appropriate fluorochrome-conjugated secondary antibodies (1:200 dilution in BB) or with a mixture of 2 secondary antibodies (double staining) for 1 hour at RT. Between each step, the sections were washed in PBS 3 times for 10 minutes each time between each step, counterstained with TO-PRO-3 (1:10,000 dilution; Molecular Probes), mounted in Vectashield (Vector Laboratories Inc.), and finally sealed with nail varnish. Negative control sections were prepared by 1) omitting the primary antibodies, 2) preadsorbing the primary antibodies with an excess (20 mmol/L) of the pure antigen when available, and 3) mismatching secondary antibody. In all cases, the results of the negative controls confirmed the specificity of antibody staining. Sections were viewed on a Leica TCS SP2 (Leica, Heidelberg, Germany) confocal laser scanning microscope using a 63 × objective lens with either 1 × or 2 × zoom factors. For double immunolabeling, images were acquired according to a sequential scan procedure. Confocal images were taken at 350-nm intervals through the z axis of the section. Projection images formed by serial optical planes were analyzed, digitally recorded, and stored as Tagged Image File Format files using Adobe Photoshop software (Adobe Systems, San Jose, CA).

**Quantitative Reverse-Transcriptase–Polymerase Chain Reaction**

Aliquots containing 2.5 × 10⁵ cells were suspended in 1 ml of RNAzol. For RNA isolation, the cells were disrupted in FastPrep tubes in RNAzol for 20 seconds in a Savant homogenizer (Bio101 Inc., Buena Vista, CA). Total RNA was isolated according to the manufacturer instructions, and DNase-I treatment (Promega Corporation, Madison, WI) was applied to eliminate interference from genomic DNA. RNA quality was assessed by gel electrophoresis. Total RNA (final concentration, 50 ng/µl) was incubated with 0.5 µg of random hexamers at 70°C (2 minutes) and then reverse-transcribed at 37°C for 1 hour in a reaction mix containing a final concentration of 1 × first-strand buffer, 500 µmol/L of each deoxynucleotide triphosphate, 1 U/µl of Moloney murine leukemia virus reverse transcriptase, and 1 U/µl of ribonuclease inhibitor (RNAsin; all reagents from Promega). cDNA was used as a template for real-time reverse-transcriptase–polymerase chain reaction analysis based on the 5′-nuclease assay (34) with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, PE Europe B.V., Rotkreuz, Switzerland). Polymerase chain reaction primers and TaqMan probe were designed using Primer-Express software (Applied Biosystems). Matrix metalloproteinase 2, MMP-3, MMP-9, MMP-14 (MT1), MMP-16 (MT3), tissue inhibitor of metalloproteinase (TIMP) 1, TIMP-2, TIMP-3, and TIMP-4, and reverse inducing cysteine-rich protein with Kazal motifs expression were analyzed. RNA for the housekeeping gene GAPDH was amplified from all samples on each plate to normalize expression levels of targets between different samples and to monitor assay reproducibility. The relative quantification of all targets was calculated by the comparative cycle threshold method outlined in User Bulletin 2 provided by Applied Biosystems. A nontemplate control was included for each target analyzed.
Gelatin Zymography for MMP-2 and MMP-9

Gelatinase activity in the cell SN was determined by zymography. Briefly, sodium dodecyl sulfate-polyacrylamide gels (10%) were copolymerized with 1% gelatin derived from porcine skin (Sigma). Samples (16 μl of SN + 4 μl of 5× sample buffer) were electrophoresed under nonreducing conditions. As references, recombinant MMP-2 and MMP-9 (Oncogene Research Products, Cambridge, MA) were run in parallel with the samples. After electrophoresis, the gels were incubated in 2% Triton X-100 for 1 hour to remove sodium dodecyl sulfate and then for 15 to 20 hours at 37°C in 150 mmol/L of sodium chloride and 50 mmol/L of Tris-hydrochloric acid, pH 7.6, containing 5 mmol/L of calcium chloride. After staining with Coomassie blue, proteolytic activity was identified as a clear band on a blue background.

MMP Assays of Cell Cultures and SNs

Aliquots of 2.5 × 10⁶ MVECs were cultured in 100 μl of phenol-red-free medium (DMEM/F12; Gibco) supplemented with 1% Nutridoma HU (Roche Applied Science), 2 mmol/L of N-acetyl-l-alanyl-l-glutamine (Biochrom, Berlin, Germany), 1 mmol/L of sodium pyruvate (ICN Biomedicals, Birsfelden, Switzerland), and 20 μg/ml of gentamicin (Gibco) in 96-well F-plates (BD Biosciences, Basel, Switzerland) at 37°C in a humidified atmosphere containing 5% CO₂ for 2 days. After washing once with PBS, 100 μl of medium containing the MMP inhibitor GM6001 (final concentration, 10 μmol/L; Calbiochem, JURO Supply AG, Lucerne, Switzerland), the serine protease inhibitor Pefabloc SC (4-(2-aminomethyl)-benzenesulfonylfluoride, hydrochloride; final concentration, 1 mmol/L; Roche Applied Science), or solvent control (medium containing 0.1% dimethyl sulfoxide) was added and kept for 4 hours at 37°C. Supernatants were transferred to black 96-well plates (Corning, Vitaris AG, Baar, Switzerland), and 10 μl of substrate FS-6 (final concentration, 5 μmol/L; custom synthesized by Bachem AG, Bubendorf, Switzerland) (35) was added to each well. After incubation for 30, 60, 120, and 240 minutes at 37°C, reactions were stopped with 90 μl of 3% (wt/vol) sodium acetate, pH 4.0, and fluorescence of the samples (200 μl) was measured in a SpectraMAX Gemini plate reader (Molecular Devices Corp., Sunnyvale, CA). To measure cell membrane-associated MMP activity, FS-6 was dissolved in fresh medium and added directly to cell cultures. To measure MMP activity, samples of conditioned media (200 μl) were removed from tissue culture 96-well F-plates and, after the addition of 90 μl of 3% (wt/vol) sodium acetate, transferred into 96-well black plates to measure the fluorescence. Medium without cells in the presence or absence of GM6001 or Pefabloc SC was incubated for background correction. Each experiment was performed in triplicate, and the mean value was subtracted by the background fluorescence at each time point.

Statistical Analysis

All data are reported as mean ± standard error of mean. Statistical analyses were performed using StatView software.
glioblastoma SN (Fig. 1C). Interestingly, SN from high-grade gliomas caused greater leakage in HUVECs compared with SN from low-grade astrocytomas (Fig. 1C).

Because glioma cells express and secrete high levels of TGF-β2, VEGF, and FGF-2 (14, 36, 37), we determined the levels of these growth factors in the SN of 3 low-grade and 8 high-grade astrocytoma cultures. Both TGF-β2 and VEGF were found to be present in greater amounts in high-grade than in low-grade astrocytoma SNs (Table). Fibroblast growth factor 2 was detected at very low levels in samples of all grades of astrocytoma (Table). Transforming growth factor A used in the range of 25 pg/ml to 2.5 ng/ml, increased permeability dose-dependently in HUVEC (Fig. 2A) and human MVEC monolayers after 24 hours (data not shown). Half-maximum effects of TGF-β2 were seen for both cell types at concentrations of 0.25 ng/ml for 24 hours of treatment (Fig. 2A and data not shown). In contrast, half-maximum effects of FGF-2 and VEGF were found to be greater than 10 ng/ml (data not shown).

**Permeability Alterations of HUVECs Induced by TGF-β2**

We next pretreated GBM SN with neutralizing anti-TGF-β2 antibody before exposure to HUVECs and found a 50% reduction in permeability increase (p < 0.05; Fig. 2B). This finding suggests that GBM-derived TGF-β2 contributes significantly to the increase in permeability. As a positive control, preincubation of TGF-β2 with neutralizing TGF-β2 antibody almost completely blocked the permeability increase (p < 0.05; Fig. 2B).

**Effects of Glioblastoma-Derived Factors and TGF-β2 on HUVEC TJPs**

To obtain further insight into the mechanisms of the permeability increase induced by TGF-β2 and GBM SN, we examined the production of TJPs by Western blotting analysis. We found a reduction in claudin 1 in HUVECs exposed to GBM SN, whereas the levels of occludin and ZO-1 showed little change (Figs. 3B, C).

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<th>Ex vivo Cultures, WHO grade</th>
<th>FGF-2, pg/ml</th>
<th>VEGF, pg/ml</th>
<th>TGF-β2, pg/ml</th>
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**TABLE. Production of FGF-2, VEGF, and TGF-β2 by Ex Vivo Cultured Astrocytoma Cells**

AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; FGF, fibroblast growth factor; LGA, low-grade astrocytoma; SEM, standard error of mean; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; WHO, World Health Organization.

![FIGURE 3. Glioblastoma multiforme (GBM)-induced changes in tight junction protein expression. Human umbilical vascular endothelial cell (HUVEC) monolayers were incubated for 24 hours with 50% GBM supernatant (SN) and (A) claudin 1, (B) occludin, and (C) zonula occludens 1 expression evaluated by Western blotting. Quantitative analysis revealed a reduction in claudin 1. All results are representative of at least 5 independent experiments.](http://jnen.oxfordjournals.org/.../byguestonOctober8,2016)
Treatment of HUVECs with TGF-β2 resulted in a dose- and time-dependent reduction in claudin 1 (Fig. 4A, B). These experiments were repeated 6 times, and the results were reproducible and statistically significant. The level of occludin was only minimally affected, whereas that of ZO-1 was increased by approximately 50% (data not shown). To determine whether the mechanism underlying claudin 1 reduction by glioblastoma-conditioned medium was related to TGF-β2, we performed neutralization experiments and found that anti-TGF-β2 antibody partly prevented the down-regulation of claudin 1 (Fig. 4C).

To investigate the potential role of MMP in the GBM-induced permeability increase in the in vitro BBB model, we pretreated endothelial cells with the metalloproteinase inhibitor GM6001 for 2 hours before their interaction with GBM SNs or coculture with GBM cells. As shown in Figure 6, GM6001 suppressed the permeability increase by 30% to 70% of the 2 GBM SNs; this effect was statistically significant (p < 0.05). The TGF-β2-induced permeability was also reduced by MMP inhibition (p < 0.05; Fig. 6). Thus,

Involvement of MMPs in GBM and TGF-β2-Induced Permeability Increase in HUVEC Monolayers

To examine endothelial cell structural alterations caused by gliomas and TGF-β2, the distributions of claudin 1 and occludin were analyzed in confluent HUVEC monolayers by immunofluorescence microscopy. In untreated HUVECs, claudin 1 and occludin lined the cell boundaries and appeared as continuous lines (Figs. 5A, D). Treatment with 25 ng/ml of TGF-β2 for 24 hours led to the almost complete disappearance of claudin 1 at the cell boundaries and to accumulation in the cytoplasm as fine dots (Fig. 5B). A similar and even more pronounced effect was observed by 24-hour treatment with GBM-conditioned media (Fig. 5C). In contrast, occludin expression was only slightly affected by the 2 treatments (Figs. 5E, F).

![FIGURE 4](http://jnen.oxfordjournals.org/)

**FIGURE 4.** Transforming growth factor (TGF)-β2-induced alterations in claudin 1 expression. (A) Human umbilical vascular endothelial cell monolayers were incubated for 24 hours with various concentrations of TGF-β2. (B) Claudin 1 expression was analyzed 4, 8, and 24 hours after TGF-β2 (25 ng/ml) treatment. (C) Claudin 1 downregulation by glioblastoma multiforme-derived supernatant (SN) was partly restored by neutralizing anti-TGF-β2 antibody. Human umbilical vascular endothelial cell monolayers were incubated for 24 hours with 50% GBM SN (4) conditioned medium with anti-TGF-β2 or isotype antibody (both 20 μg/ml) as described in Materials and Methods section.

![FIGURE 5](http://jnen.oxfordjournals.org/)

**FIGURE 5.** Effects of glioblastoma multiforme (GBM) supernatant (SN) and transforming growth factor (TGF) β2 on the distribution of claudin 1 and occludin in human umbilical vascular endothelial cell monolayers. Human umbilical vascular endothelial cells were cultured for 7 days to form confluent monolayers and maintained in (A, D) culture medium, (B, E) TGF-β2 (25 ng/ml), or (C, F) 50% GBM SN (SN 4) for 24 hours. Cells were then fixed and stained for (A, C) claudin 1 or (D, F) occludin as described in Materials and Methods section. All micrographs were taken at the same magnification. Bar = 7 μm.
Proteolytic Activity of Human MVECs

Protein expression of pro-MMP-2 and pro-MMP-9 and that of their active forms was evaluated in human MVEC SNs by gelatin zymography. As shown in Figure 7B, unstimulated human MVECs constitutively express high levels of pro-MMP-2 and low levels of pro-MMP-9. Treatment with TGF-β2 (25 ng/ml) for 24 hours increased the production of pro-MMP-2 and led to detectable amounts of active MMP-2, whereas MMP-9 was not noticeably affected (Fig. 7B). This MMP-2 increase was not detected 24 hours after medium exchange in the absence of TGF-β2 (data not shown). From these findings on mRNA levels, we determined the cell membrane-associated and the secreted proteolytic activity of MVECs by FS-6, a metalloprotease-quenched fluorescent substrate (35). This substrate shows increased affinity to metalloproteinases, but it is also susceptible to cleavage by trypsin. The results revealed both cell surface-associated and secreted proteolytic activity of MVECs (Fig. 7C). Similar to earlier observations in glioma cells (35), the cell-associated proteolytic activity (8.3 relative fluorescence units [RFU]/minute) was 34% higher than that of the SN (5.9 RFU/minute). This result is consistent with the previously discussed findings on constitutive mRNA expression of MMP-2, MT1-MMP, and MT3-MMP. The metalloproteinase inhibitor GM6001 inhibited cleavage by 39% (GM6001; 5.0 RFU/minute) and by 35% (GM6001; 3.8 RFU/minute) for proteolytic measurements on the cell surface and in SN, respectively. In contrast, the serine inhibitor 4-(2-aminoethyl)-benzenesulfonylfluoride, hydrochloride showed only a minor effect (reductions of 17% and 13% in cell-associated and SN measurement, respectively). Treatment with TGF-β2 (25 ng/ml) for 24 hours increased membrane-associated and secreted proteolytic activities by 54% and 37%, respectively (data not shown).

Downregulation of TJPs in Microvessels of High-Grade Gliomas

Vessels in GBMs include “glomeruloid vessels” and microvessels (arterioles, capillaries, and postcapillary venules) similar to those in the normal human brain that are considered the anatomic site of the BBB. The expression patterns of the junctional proteins claudin 1, claudin 5, and occludin were investigated in the latter vessel types in 24 cases of low- and
high-grade astrocytomas and 6 nonneoplastic brain tissue specimens by immunohistochemistry. Representative results are shown in Figure 8. Most of the microvessels showed immunoreactivity for all 3 TJPs (Fig. 8A). In high-grade astrocytomas, there was significantly less expression of all 3 TJPs compared with nonneoplastic tissue samples (Figs. 8, 9). There was more reduction of expression in microvessels than in large vessels. A comparison between healthy human brain and tumor vessels was not possible for ethical reasons. Moreover, to avoid autopsy-associated changes, we investigated 6 temporal lobe biopsy specimens obtained during selective amygdalohippocampectomy from patients with pharmaco-resistant temporal lobe epilepsy. High expression levels were found in the temporal lobe samples for all 3 TJPs on both large vessels and microvessels (Fig. 8).

We next examined the microvascular expression of claudin 1, claudin 5, and occludin and that of claudin 5 and occludin in combination with expression of Glut 1, a marker of BBB-endothelial cells, by confocal microscopy in tissue samples from GBM and nonneoplastic brain tissue (Fig. 9). In nonneoplastic brain microvessels, all of the TJPs examined were highly expressed and showed a typical, linear staining pattern that marked distinct endothelial cell borders; the linear junctional labeling was always continuous except that for claudin 1, which was discontinuous labeling formed by a sequence of dots and small plaques (Fig. 9A–D). In double immunolabeling experiments with Glut 1, the nonneoplastic brain microvessels showed high levels of transporter expression together with distinct claudin 5 and occludin labeling (Fig. 9C, D). Overall, the observations carried out on GBM confirmed the previously described findings and indicated greater downregulation of the examined TJP in microvessels compared with large vessels (Fig. 9E–H). In tumor microvessels, claudin 1 was undetectable by confocal microscopy (Fig. 9E). Similarly, claudin 5 was absent in a large part of the tumoral tissue; in some microvessels, however, claudin 5 reactivity showed a diffuse, punctate cytoplasmic expression pattern (Fig. 9F). In GBM microvessels, for claudin 5/Glut 1 and occludin/Glut 1 double immunolabeling, the occludin reactivity was intermediate between the total absence and the punctate staining observed for claudin 1 and claudin 5, respectively; the occludin staining pattern was linear, although highly discontinuous, and was paralleled by a reduction in Glut 1 expression (Fig. 9H). On the other hand, in the absence of junctional labeling for claudin 5, the endothelial cells had virtually no Glut 1 reactivity. The latter was recognizable only on erythrocyte membranes as a positive internal control (Fig. 9G).

**DISCUSSION**

Breakdown of the BBB and the formation of edema are well-characterized hallmarks of brain tumors and are intrinsic to their pathogenesis and clinical effects (38). Although the introduction of corticosteroids has simplified treatment of patients with newly diagnosed tumors, these drugs are associated with marked side effects in the long-term treatment that is often necessary for recurrent malignant gliomas and lesions treated by radiosurgery or radiotherapy, rather than resection. The molecular and cellular mechanisms underlying GBM-induced disruption of the BBB are not clear.

We have demonstrated here that GBM-derived factors increase the permeability of human brain-derived endothelial cells and HUVEC monolayers in vitro. Interestingly, the disruption of the in vitro barrier function was paralleled by downregulation of the TJP claudin 1 but not occludin or ZO-1. Furthermore, by the use of a neutralizing anti-TGF-β2 antibody or a broad-spectrum MMP inhibitor, GM6001, we have shown that the opening of the BBB involves TGF-β2 and the action of MMPs. Moreover, using recombinant GBM-derived TGF-β2, we demonstrated enhanced permeability with similar changes in the TJP expression pattern in...
endothelial cells, and immunofluorescence staining revealed that the continuous pericellular distribution of claudin 1 was disrupted under both sets of conditions. These observations indicate that TGF-β₂ plays an important role in BBB disruption by GBM in this BBB model.

At the transcriptional level, we found an upregulation of MMP-2, MT1-MMP, and MT3-MMP, but not of MMP-9, after stimulation with TGF-β₂ and glioblastoma SN. Previous studies have shown that TGF-β induced MMP-9 activity and increased cell permeability in cultured bovine retinal endothelial cells (24); moreover, a high amount of MMP-2 also increased cell permeability in rodent retinal endothelial cells (39). At present, it is not clear whether these apparently discrepant findings are related to differences in species, cell types, or both. The fact that anti-MMP-9 blocking was not complete, however, suggests that TGF-β₂-induced barrier breakdown involves additional mechanisms independent of MMP-9 production (24); this is consistent with our data showing that the disruption of the barrier function is only partly MMP-dependent. Involvement of MMP-2 and MMP-9 has been demonstrated in early diabetic retinopathy (39) and in focal ischemia in rats (40) in vivo, leading to alterations of the blood-retinal barrier and BBB, respectively. Interestingly, the mechanism involved the proteolytic degradation of the TJP claudin 5 and/or occludin, and this degradation was prevented by an MMP inhibitor. In contrast to the in vivo studies, the mechanism of TGF-β₂-induced disruption of the barrier function in the present study is only partly MMP-dependent because the inhibitor GM6001 could not block the permeability increase completely. Activation of pro–MMP-2 by TGF-β₂ paralleled the upregulation of MT1-MMP mRNA, an activator of MMP-2 (41). This suggests that MMP-2 is the major enzyme involved in the in vitro BBB disruption. In light of the fact that anti-TGF-β₂ antibodies and a broad-spectrum MMP inhibitor only partly restored the BBB function, we suggest that the barrier breakdown involves additional GBM-derived factors such as VEGF, FGF-2, and mechanisms independent of MMP activation.

In contrast to the edema induction role of TGF-β₁ implied in the results of the present study, TGF-β₁ has been shown to inhibit cerebral edema in a rodent glioma model (42). The authors of that study hypothesized that TGF-β₁ may abrogate tumor-associated cerebral edema indirectly by downregulating tumor necrosis factor, a known inducer of VEGF production, or directly by inhibiting the production of VEGF and other mediators of vascular permeability. It will be of interest to examine the role of TGF-β₁ on the induction of MMPs in murine endothelial cells.

In the present study, endothelial cells are shown to be the source of MMP-2, but it should be emphasized that the physiologic and/or pathologic interactions of endothelial cells along with perivascular supporting cells (e.g., pericytes and astrocytes) and tumor cells may play major roles in regulating enzyme expression in vivo. This regulation might be achieved through the release of growth factors, cytokines, MMPs, and MMP inhibitors, as well as by direct cell-to-cell contact. In highly vascularized astrocytomas and GBMs, the expression of FGF-2, VEGF, TGF-β₂, MMPs, and their inhibitors has been demonstrated (14, 36, 37, 43). The role of glioblastoma cells in the angiogenic activation of...
endothelial cells has been described in some experimental models. Regulation of protease activity by heterotypic cell-to-cell contact has also been indicated in experimental models of tumor metastasis, and, in particular, the expression of MMP-9 has been associated with tumor cell invasion. The exact nature of cell-to-cell interactions has not, however, been elucidated. Our endothelial-GBM coculture system provides a powerful model for investigating mechanisms underlying heterologous cell-cell interactions, communication, and induction of intracellular signaling.

The mechanism of the barrier breakdown at the cellular level is not clear. Our observation of reduced immunoreactivity of 2 TJPs, occludin and claudin 1, suggests involvement of a paracellular mechanism. For claudin 5, it was recently shown that the disappearance from the plasma membranes of hypoxic endothelial cells was accompanied by a decrease in the cellular protein level of claudin 5 (44). In contrast, the level of claudin 5 mRNA was almost unchanged regardless of the oxygen concentration. Therefore, the hypoxia-induced changes in the expression of claudin 5 are attributable to posttranscriptional regulation such as the reduced translation and the enhanced degradation of claudin 5 molecules. For the posttranscriptional regulation of the total cellular protein level of TJP, downregulation of claudin 1 by the transcription factor Snail has been reported (45), as has the degradation of occludin in the proteasome pathway through the specific ubiquitination of occludin by the E3 ubiquitin-protein ligase Itch (46). It is possible that the ubiquitin-proteasome pathway is also involved in the changes in our study or through the degradation of the occludin and claudin molecules. The enhanced cleavage of TJP molecules by proteases such as MMP would then further increase the extent of BBB disruption.

To verify our in vitro findings, we assessed TJP alterations in brain tumor microvessels. We found significant losses of occludin, claudin 1, and claudin 5 that correlated with increasing astrocytoma grade. These results are in line with recent data showing that occludin and claudin 3 were lost in most tumor microvessels (47–49) but are in contrast to claudin 5, which was found to be significantly downregulated only in hyperplastic vessels of GBM (48). Previous studies demonstrated that the TJ sealing of the interendothelial clefts is paralleled by the appearance of specific transporters. Among these, Glut 1 has been considered to be an excellent indicator of a normally functioning BBB because changes in its expression are directly related to the loss of both endothelial cell polarity and BBB fence function as a consequence of structural alterations in tight junctions (50–52). Moreover, our results for Glut 1 expression and its endothelial cell membrane localization during human brain development demonstrated that Glut 1 is asymmetrically distributed early at gestational Week 12 (53); at this time, the junctional proteins also begin to be expressed (54). Therefore, the reduction in Glut 1 expression that parallels the TJP alterations is consistent with BBB opening in tumor tissues. The molecular architecture of TJ strands is more complex than expected (55), and different claudins form different freeze-fracture patterns (56). In the liver, claudin 1 seems to be admixed along individual TJ strands, as demonstrated by immunoelectron microscopy on freeze-fracture replicas (57). The observation that claudin 1 is present only in part of the junctional strands suggests a regulatory role for this protein in junctional organization, rather than an “occludentes” role. In contrast to the liver, nonneoplastic brain tissue in our analysis exhibited a junctional distribution of claudin 1, unlike that of claudin 5 and occludin. This discrepancy might explain why claudin 1 is more sensitive than the other TJ proteins to MMP-dependent protein downregulation.

Our in vitro and in vivo data indicate that TJPs of brain microvessels are target molecules of GBM, and that their alterations in expression and distribution affect BBB function. Some evidence suggests that occludin contains a cleavage site for MMP in the first extracellular loop, and, consequently, that MMP-induced occludin degradation might be important for the function of the BBB (40). This is further supported by the findings by several groups that occludin molecules are degraded by secreted MMP (58, 59). Because we showed that endothelial cells in culture express MMP, our data suggest that endothelial cells might be targeted by these proteases in a paracrine or juxtacrine fashion, thus altering TJP and impairing their permeability.

In conclusion, we have shown that TJP expression is altered in situ in anaplastic astrocytomas and GBM and in vitro by treatment with GBM-derived factors, resulting in the disruption of the barrier function of brain-derived endothelial cells and HUVECs. By the use of neutralizing anti–TGF-β2 antibodies and an MMP inhibitor, we demonstrated that the opening of the BBB involves TGF-β2 and the action of MMP. These results point to TGF-β2 as an important player in glioma-induced vascular leakage. Therefore, targeting TGF-β2 or its signaling pathway, as recently shown in a GBM therapy trial by the use of antisense oligonucleotides (60), may also attenuate the tumor edema.

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