Interleukin-8 Differentially Regulates Migration of Tumor-Associated and Normal Human Brain Endothelial Cells

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Abstract

Interleukin-8 (IL-8) is a chemokine involved in angiogenesis, a process vital to tumor growth. Previously, we showed that endothelial cells derived from human tumor tissue have different functional and phenotypic properties compared with normal endothelial cells. This study analyzes the role of IL-8 in regulating angiogenesis of tumor-associated brain endothelial cells (TuBEC). Results show that TuBECs have a higher baseline migration rate compared with normal brain endothelial cells (BEC). TuBECs are unaffected when stimulated with IL-8 whereas BECs are activated. This lack of response of TuBECs to IL-8 is due to the constitutive production of IL-8. Endogenously produced IL-8 activates TuBECs in an autocrine manner as shown by IL-8 receptor inhibition. Blocking either CXCR1 or CXCR2 partially reduces TuBEC migration, whereas blocking both receptors further reduces migration. Treatment with antibody against vascular endothelial growth factor (VEGF) shows that production of IL-8 by TuBECs is dependent on VEGF. Transforming growth factor-β1 (TGF-β1), shown to down-regulate IL-8 production in BECs, does not inhibit IL-8 production in TuBECs. In summary, these studies show that TuBECs constitutively secrete IL-8 and autocrine activation by IL-8 is the result of VEGF stimulation. Furthermore, TuBECs do not respond to the feedback inhibition normally induced by TGF-β1. These data emphasize the functional uniqueness of TuBECs. Understanding the functions and regulatory processes of tumor-associated endothelial cells is critical for developing appropriate antiangiogenic therapies. (Cancer Res 2005; 65(22): 10347-54)

Introduction

Angiogenesis is the multistep process that generates new blood vessels from preexisting ones. This process occurs during tumor growth as well as wound healing (1, 2). Angiogenesis involves activation of endothelial cells, which includes such activities as proliferation, migration, extracellular matrix remodeling, and tubule formation (2–4). Interleukin-8 (IL-8), a member of the ELR (glutamic acid-leucine-arginine) CXC chemokine family, is reported to be a potent angiogenic factor, specifically enhancing endothelial cell migration (3, 4). IL-8 is produced by activated endothelial cells and a variety of tumors, including gliomas (5). In fact, it was shown that in human gliomas, two pathways control angiogenesis in a paracrine manner, one mediated by vascular endothelial growth factor (VEGF) and/or fibroblast growth factor and the other through IL-8 (5). In addition, because both VEGF and endothelin-1 (Et-1), angiogenic factors produced in gliomas, stimulate the expression of IL-8 in endothelial cells (6, 7), the role of IL-8 in the migration of normal and tumor-derived endothelial cells is critical. Consequently, IL-8 is a highly significant mediator of angiogenesis and its role in tumor development is of interest (5, 8, 9).

IL-8 binds to two related seven-transmembrane domain G-protein–coupled receptors on target cells (9, 10). CXCR1 binds specifically to IL-8, whereas CXCR2 binds promiscuously to several CXC chemokines, including IL-8, growth-regulated oncogenes α, β, and γ, neutrophil activating peptide-2, and epithelial neutrophil-activating peptide-78 (10). Ligand binding to the two receptors leads to sequential activation of multiple signaling pathways that lead to activation of enzymes, such as phospholipase D, p-21 activated kinase, focal adhesion kinase, and the mitogen-activated protein kinase cascade (10–12). Microvessels commonly express CXCR1 and CXCR2 mRNA and protein constitutively (3, 13). Both of these receptors are involved in endothelial cell migration (13).

Most reports examining the role of IL-8 in tumor angiogenesis have been done on normal endothelial cells (3–5), with few studies investigating endothelial cells derived from tumor tissue (14). Therefore, it is not clear whether tumor-associated brain endothelial cells (TuBEC) respond the same or differently to angiogenic factors compared with normal brain endothelial cells (BEC). Because the ultimate goal of this study is to determine the mechanism by which brain tumors regulate neovascularization, endothelial cells used in these studies are human BECs obtained from tumor tissue.

In normal human brain, the cerebral endothelial cells, which comprise the blood-brain barrier, differ from systemic endothelial cells by decreased pinocytotic activity, increased expression of tight junction proteins, and the presence of unique transport proteins (15). In glioblastoma multiforme, the most malignant form of glioma, tumor-associated blood vessels are characterized by microvessel hypertrophy and proliferation in both the intratumoral, perinecrotic regions of the glioblastoma, and in the peritumoral brain tissue (16). Microvessels of brain tumors undergo active angiogenesis, resulting in "leaky" vessels, increased expression of αvβ5 integrins, and a breakdown of the blood-brain barrier (17, 18). Cerebral endothelial cells within tumors show increased pinocytosis, decreased tight junction proteins, such as claudin-1 and occludin, and increased permeability (18). Thus, it is evident from our studies and others, in human and animal brain tumor models, that cerebral blood vessels in tumors function differently from normal vessels (19).

In our studies, we analyzed the response and function of isolated, purified, and characterized human BECs and TuBECs with respect to cell migration and chemokine production and regulation.

The experiments show that TuBECs constitutively secrete IL-8, which acts in an autocrine manner to induce migration. This autocrine stimulation is not down-regulated by transforming growth factor (TGF-β1) as in normal endothelial cells. Furthermore, these data suggest that TuBECs contribute growth factors to the proangiogenic microenvironment produced by tumors and emphasize the functional differences between TuBECs and normal BECs.

Materials and Methods

Cell culture and reagents. Human BECs and TuBECs were isolated from normal and glioblastoma brain tissue, respectively, as previously described (19). TuBECs were derived from untreated patients diagnosed with stage IV glioblastoma multiforme. On the other hand, BECs were derived from brain tissue obtained following trauma. Institutional Review Board approval was obtained for discarded brain tissue from trauma surgeries and glioblastoma specimens. BECs and TuBECs were characterized as 99% pure using the following specific markers: von Willebrand factor (DAKO, Carpinteria, CA) for endothelial cells, glial fibrillary acidic protein (DAKO) for astrocytes and glioma cells, CD11b (Immunotech, Villepinte, France) for macrophages and microglia, and α-smooth muscle actin (DAKO). Cell viability was determined to be >99% as assessed by trypan blue exclusion. BECs and TuBECs were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 100 ng/mL endothelial cell growth factor (Endogro, Upstate Biotechnologies, NY), 2 mmol/L L-glutamine (Life Technologies), 10 mmol/L HEPES (Life Technologies), 24 mmol/L sodium bicarbonate (Life Technologies), 300 units heparin USP (Sigma-Aldrich, St. Louis, MO), 1% penicillin/streptomycin (Life Technologies), and 10% FCS (Omega Scientific, Tarzana, CA). Cells were used in passage 4 or 5 only, unless otherwise noted. The experiments presented in this study were done using six different cases of normal BEC and four different cases of TuBEC.

Recombinant human IL-8 (R&D Systems, Minneapolis, MN), tumor necrosis factor-α (TNF-α; Roche Diagnostics, Indianapolis, IN), VEGF (Upstate Biotechnologies), anti-VEGF antibody (Chemicon, Temecula, CA), TGF-β (R&D Systems), and Et-1 (Bachem, Torrance, CA) were purchased. IL-8 receptor CXCR1 and CXCR2 neutralizing antibodies and mouse isotype IgG control sera were purchased (BD PharMingen, San Diego, CA). The anti-IL-8 neutralizing antibody was purchased from R&D Systems. The avidin biotin complex (ABC) and aminoethyl carbazol (AEC) kits used for immunohistochemistry were purchased (Vector Laboratories, Burlingame, CA). Saponin used for permeabilization studies was purchased (Sigma-Aldrich).

Endothelial cell migration assay. Migration assays were done using transwell chambers as described previously (1). Briefly, 8 μm pore, 6.5-mm-diameter polyethylene terephthalate filters (BD Biocoat, Bedford, MA) were coated on top and bottom by immersion in 0.5% gelatin for at least 2 hours and air-dried overnight. Subconfluent cultures of BEC were harvested using trypsin/EDTA (0.05%) solution (Life Technologies); 5×10^4 cells were then resuspended in RPMI medium supplemented with 1% FCS and placed on the filter membrane in the top chamber (100 μL per well). Chemoattractants were placed in the lower compartment (600 μL per well). For receptor blocking studies, IL-8 receptor anti-CXCR1 and anti-CXCR2 antibodies (4 μg/mL each) were added to the lower chamber 30 minutes before the addition of IL-8 (1 ng/mL). Mouse isotype IgG served as the negative control for the CXCR1 and CXCR2 antibodies (BD PharMingen). The isotype control IgG was also added to the cells 30 minutes before the addition of IL-8 at the same concentration as the IL-8 receptor neutralizing antibodies. Transwell plates were then incubated at 37°C in 5% CO_2 for 6 hours. At the termination of the experiment, medium was removed from inserts and the remaining cells on the upper side of the chamber were removed with a wet Q-tip. The filters were then stained with Diff-Quick staining solution (Dade Behring, Inc., Newark, DE). Cells that migrated through to the underside of the filter membrane were counted under high-power magnification; 10 fields per filter were counted using the ×40 objective. Groups were plated in triplicate; experiments were repeated at least twice.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay. BECs and TuBECs (2.5×10^3 per well) were seeded in 96-well plates (100 μL per well) in quadruplicate. Medium was changed to RPMI containing 1% FCS 24 hours before treatment with reagents. Cells were treated with IL-8 (1 ng/mL) and TNF-α (10 ng/mL) for 6, 24, 48, or 72 hours. At the end of treatment, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added (1:10 dilution) to the cultures for 4 hours according to the instructions of the manufacturer (Sigma-Aldrich). The medium was then removed, DMSO was added (150 μL per well), and color intensity, which is proportional to the number of living cells, was quantified using the 570 nm filter wavelength.

Immunocytochemistry. Cells were immunostained using a previously described method (20). Briefly, cells (2.5×10^4 per well) were plated on glass coverslips (Fisherbrand Microscope Cover Glass, Fisher Scientific, Tustin, CA) and coated with 1% gelatin. The cells were fixed in acetone, blocked with 5% normal horse serum in PBS, and incubated overnight with primary anti-IL-8 antibody (5 μg/mL; R&D Systems) or anti-CXCR1 and anti-CXCR2 antibodies (Chemicon). Samples were then incubated with biotinylated horse anti-goat IgG (1:100 dilution; Vector Laboratories) or biotinylated horse anti-mouse IgG (1:200 dilution; 0.01 mg/mL; Vector Laboratories) for 45 minutes. Subsequently, slides were treated with the ABC elite (Vector Laboratories) for 30 minutes followed by AEC substrate kit (Vector Laboratories) according to the instructions of the manufacturer. Slides were counterstained with hematoxylin. A red precipitate identified positive staining. Pictures were taken using an Olympus BH2 microscope (Fluor × 10 objective; numerical aperture 0.25) and a Nikon Coolpix 990 digital camera.

Flow cytometry. Subconfluent cell cultures were prepared as single cell preparations. Total IL-8 receptor expression in cells was determined by fixing cells in 2% paraformaldehyde for 15 minutes followed by permeabilization with 0.1% saponin for 15 minutes. For cell surface receptor expression detection, cells were harvested using Hanks-based dissociation buffer (Life Technologies) and were not fixed or permeabilized. Cells were then incubated first with primary mouse anti-human monoclonal antibody to either CXCR1 or CXCR2 for 30 minutes, followed by a 30-minutes incubation with secondary goat anti-mouse FITC-conjugated antibody. Negative controls included the use of secondary FITC-labeled antibody only. Cells were then analyzed using the FACScan machine with appropriate software (BD PharMingen). Data are expressed as percentage positive cells.

Interleukin-8 ELISA. To determine whether there were differences in the production of IL-8 by BECs and TuBECs, endothelial cells were plated in triplicate in equal numbers in six-well plates. Cells were cultured in RPMI containing 1% FCS medium for 48 hours. Supernatants from cells were then collected and analyzed for IL-8 levels using the commercially available ELISA kit as per instructions of the manufacturer (R&D Systems). BECs cultured in medium containing 10% FCS exhibited similar baseline levels of IL-8 in the ELISA assay with cells cultured in medium containing 1% FCS.[5]

To determine the potential effect of VEGF on IL-8 production, TuBECs were plated in triplicate in equal numbers in six-well plates. Medium was changed to RPMI containing 1% FCS overnight, and cells were then incubated with rabbit anti-human VEGF antibody (10 μg/mL). Cells treated with rabbit serum (Vector Laboratories) served as an isotype control for the rabbit-anti human VEGF antibody. Supernatants from cells were collected after 48 hours and analyzed for IL-8 levels using the commercially available IL-8 ELISA kit.

To determine the potential effects of TGF-β1 on IL-8 production, BECs and TuBECs were plated in triplicate in equal numbers in six-well plates. Medium was changed to RPMI containing 1% FCS overnight and cells were treated with TGF-β1 (10 ng/mL) or Et-1 (100 nmol/L). Supernatants from cells cultured for 72 hours in medium containing 1% FCS were collected and analyzed for IL-8 levels. The concentrations of secreted IL-8 were determined using the commercially available IL-8 ELISA kit as per instructions of the manufacturer.

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[5] F.M. Hofman, unpublished observations.

Reverse transcription-PCR. IL-8 mRNA was detected in the following manner: Equal numbers of BECs and TuBECs (5×10^4) were lysed in cold cell lysis buffer (100 µL) and treated with DNase I according to the instructions of the manufacturer (Cells to cDNA II kit, Ambion, Austin, TX). The lysate RNA was reverse-transcribed directly into cDNA according to the instructions of the manufacturer (Cells to cDNA II kit, Ambion). PCR was preformed using specific *IL-8* gene primers according to the instructions of the manufacturer [IL-8 Gene-Specific Relative Reverse Transcription-PCR (RT-PCR) kit, Ambion] using a PTC-100 thermocycler with an initial denaturation step at 94°C (2 minutes), then 35 cycles at 94°C (30 seconds), followed by 57°C (30 seconds) and 72°C (30 seconds). Final extension of cDNA was performed at 72°C (5 minutes). For negative control, cDNA was replaced with nuclease-free water in the PCR; for positive control, IL-8 DNA was amplified. The PCR mixture was heated to 94°C (2 minutes), then 35 cycles at 94°C (30 seconds), followed by 57°C (30 seconds) and 72°C (30 seconds). Further extension of cDNA was done at 72°C for 5 minutes. The cDNA samples were analyzed using gel electrophoresis in 2% agarose gel (Cambrex, Rockland, ME) containing 0.05 µg/mL ethidium bromide (Sigma-Aldrich). Bands were visualized under UV light and photographed using a Bio-Rad Fluor-S Multi-imager (Hercules, CA). Equal loading was confirmed by amplification of 18S rRNA.

IL-8 receptor mRNA was detected using the following protocol: Neutrophils were isolated by Ficoll-Hypaque (Amersham Pharmacia Biotech, Arlington Heights, IL) density centrifugation from peripheral blood. Total RNA was isolated from subconfluent BECs, TuBECs, and neutrophils by a single-step guanidium thiocyanate/phenol-chloroform extraction using TRIzol reagent (Invitrogen, Carlsbad, CA). Genomic DNA was removed by DNase I treatment (Ambion). For reverse transcription, cDNA was generated using 2.5 µg of RNA in a total volume of 20 µL according to the protocol of the manufacturer (Gene-Specific Relative RT-PCR kit, Ambion). PCR was done using primers synthesized by USC Microchemical Core facility (Los Angeles, CA). The primer sequences used were as follows: CXCR1 sense 5′-GGGGCCACACCAACCTTC-3′ and antisense 5′-AGTGCCTGCCTCAATGTCTCC-3′ (product size 363 bp); CXCR2 sense 5′-GGGCAACAATACAGCAAACT-3′ and antisense 5′-GCACT-TAGGCAGGAGGTCTT-3′ (499 bp); β-actin sense 5′-CCAGAGCAAGAGAGG-CATCC-3′ and antisense 5′-CTGTGGTGGTGAAGCTGTAG-3′ (436 bp). For each reaction, 5 µL of cDNA generated by RT reaction was amplified using specific primers (5 µmol/L) in a final reaction volume of 50 µL. For CXCR1, PCR was done for 40 cycles of 94°C (45 seconds), 57°C (30 seconds), and 72°C (60 seconds) followed by a final 72°C step (6 minutes). For CXCR2 and β-actin, PCR samples were subjected to 40 cycles of 94°C (60 seconds), 57°C (150 seconds), and 72°C (60 seconds) followed by a final 72°C step (6 minutes). PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining (0.05 µg/mL). cDNA-minus PCR was used as a negative control, whereas cDNA from neutrophils served as a positive control. Equal loading of cDNA was confirmed by amplification of β-actin.

Statistical analysis. Values are presented as the mean ± SE. Statistical significance was evaluated using Student's *t* test for paired comparison. $P < 0.05$ was considered statistically significant.

Results

Tumor-associated brain endothelial cells are unresponsive to interleukin-8 induced migration. Studies were done to determine whether normal BECs or TuBECs responded to IL-8 by migration. Cells were plated in the upper chamber of transwell plates and IL-8 (1 ng/mL) was added to the lower chamber; cells were then allowed to migrate for 6 hours. All experiments were done in triplicate cultures. The results (Fig. 1*A*) show that in untreated cultures, TuBEC migration is greater than control BECs ($P = 0.02$). Furthermore, upon activation with IL-8, normal BEC migration increases ($P = 0.003$), whereas TuBECs do not respond to IL-8 ($P = 0.5$). To determine whether this difference in response to IL-8 is dose-dependent, BECs and TuBECs were treated with a range of IL-8 concentrations (0.001-10 ng/mL). The results
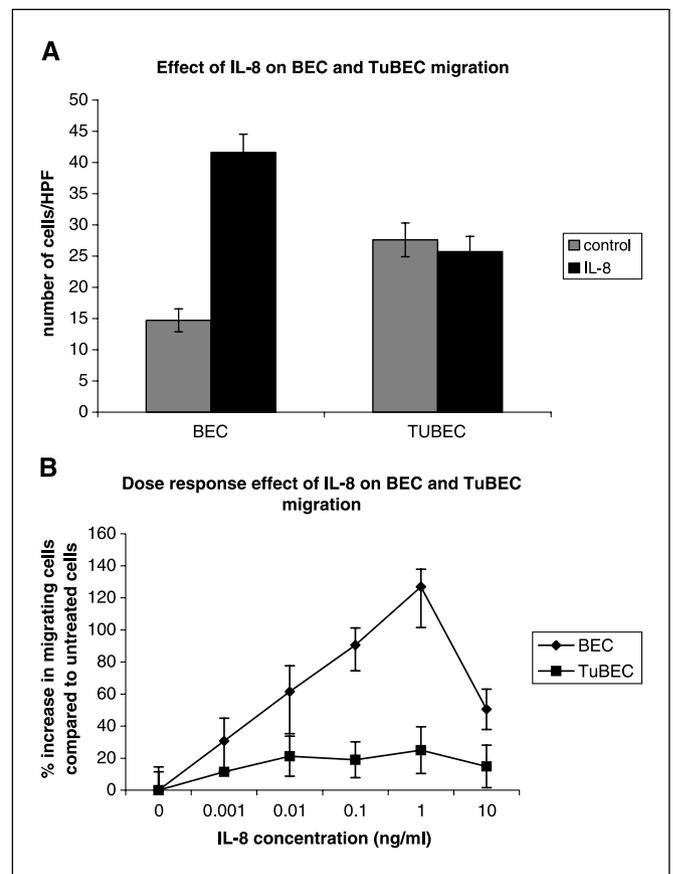

Figure 1. *A*, comparison of migration response to IL-8 in normal and tumor-associated BECs. Equal numbers of BECs or TuBECs (5×10^5/mL) were plated in the upper chamber of transwell plates. After 6 hours, cells that migrated through the filter membrane were counted. Data are expressed as the number of cells in 10 high power fields (*HPF*) under a ×40 objective. *Columns*, mean from each experimental group done in triplicate; *bars*, SE. Student's *t* test values correspond to $P_{(BEC + IL8/BEC)} = 0.003$, $P_{(TuBEC/BEC)} = 0.02$, and $P_{(TuBEC + IL-8/TuBEC)} = 0.5$. This experiment is representative of 10 experiments. *B*, titration of IL-8 activity in BEC and TuBEC migration assay. Equal numbers of BECs and TuBECs were plated in the upper chamber of transwell plates. After 6 hours, cells that migrated through the filter membrane were counted. Data are expressed as the percentage increase in migration in response to different IL-8 concentrations. *Points*, triplicate values; *bars*, SE. Data are representative of two experiments.

show that the optimal dose of IL-8 for BEC is 1 ng/mL, whereas there is no significant response of TuBEC to IL-8 within this range (Fig. 1*B*). Based on these results, we used 1 ng/mL IL-8 for all the experiments presented in this study. Because previous studies by others (4) have shown that IL-8 induces endothelial cell proliferation, the question was raised whether increased migration of IL-8–treated BECs and TuBECs was a result of increased proliferation rather than migration. To test this, TuBECs and BECs, either stimulated with IL-8 or untreated, were analyzed using the MTT assay. MTT was added for the 4 hours after the completion of IL-8 treatment (6 or 24 hours) and cell cultures were then evaluated for proliferation. The results show that IL-8 does not induce proliferation of either BECs or TuBECs within 6 or 24 hours of treatment; additional experiments show that IL-8 does not induce proliferation after 48 and 72 hours either.[6] TNF-α stimulated
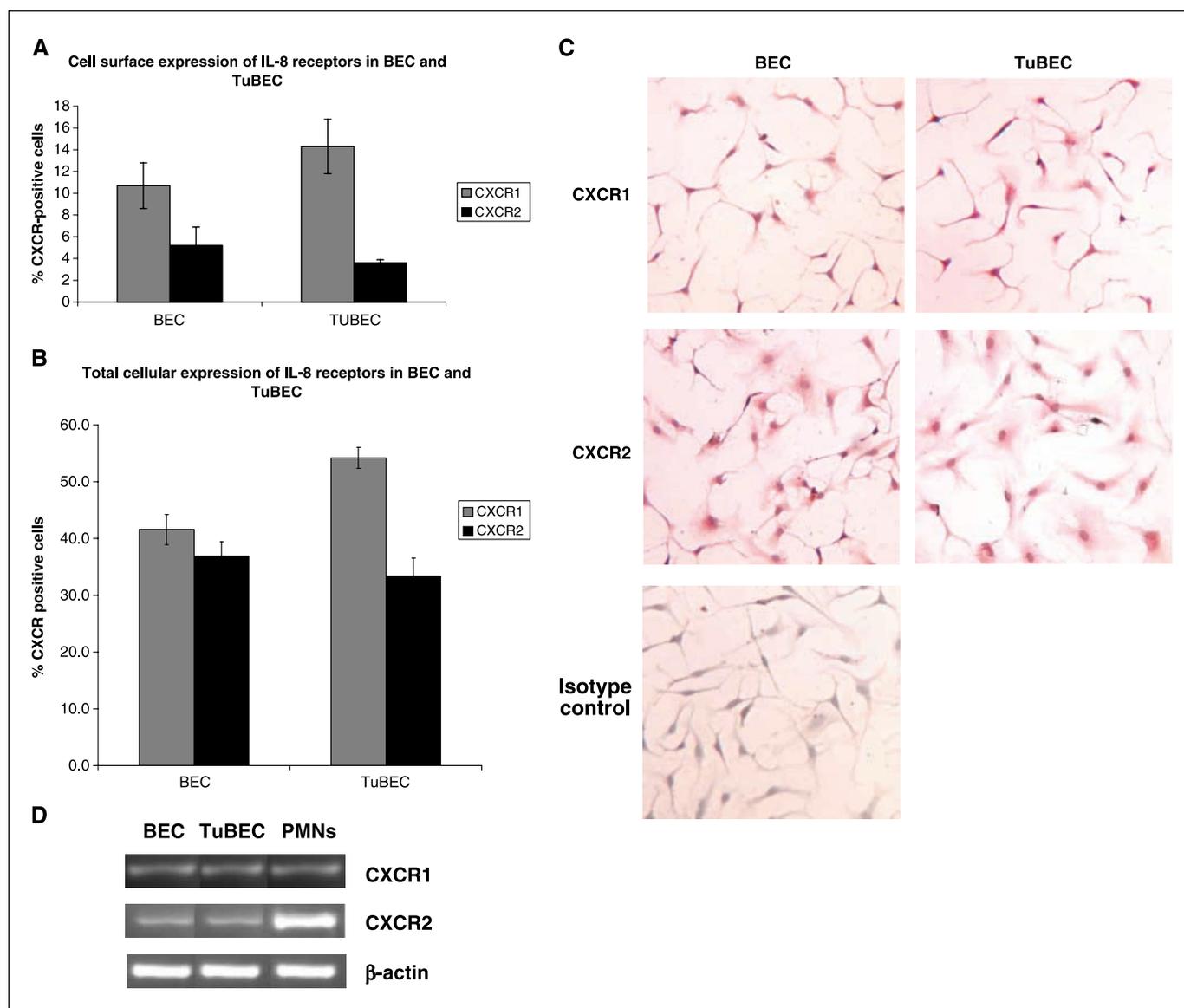
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[6] C. Charalambous, unpublished observations.

Figure 2. *A,* cell surface expression of IL-8 receptors on normal and tumor-derived BECs. Cells were labeled with anti-CXCR1 or anti-CXCR2 antibody and analyzed using flow cytometry. Data are expressed as percentage of receptor-positive cells. *Columns,* means of results from each experimental group done in triplicate. Data are representative of four experiments. *Bars,* SE. *B,* intracellular expression of IL-8 receptors in BECs and TuBECs. Cells were fixed, permeabilized, labeled with anti-CXCR1 or anti-CXCR2 antibody, and analyzed using flow cytometry. Data are expressed as percentage of receptor positive cells. *Columns,* means of results from each experimental group done in triplicate. Data are representative of four experiments. *Bars,* SE. *C,* CXCR1 and CXCR2 immunostaining in BECs and TuBECs. BECs and TuBECs were grown on glass coverslips and immunostained with anti-CXCR1 and anti-CXCR2 antibodies. Positive cells were identified by the presence of a red precipitate (×100 magnification). *D,* CXCR1 and CXCR2 mRNA detection in BECs and TuBECs using RT-PCR. Polymorphonuclear granulocytes (*PMN*) served as the positive control for the expression of the receptor mRNA. β-actin served as the internal control.

endothelial cell proliferation within 24 hours, serving as a positive control. Thus, the constitutively high migration rate of TuBECs and the increased BEC migration in response to IL-8 are not a result of an increase in cell number. These data show that TuBECs and BECs respond differently to IL-8 in migration.

Brain endothelial cell and tumor-associated brain endothelial cells express both CXCR1 and CXCR2 receptors. There is considerable controversy over whether both or only one IL-8 receptor is expressed in human microvessel endothelial cells (4). To address this question, flow cytometry experiments were done on BECs and TuBECs using specific monoclonal antibodies to the IL-8 receptors, CXCR1 and CXCR2. In the first set of experiments, endothelial cells were not permeabilized so that only

the surface receptors were examined. The results show that both receptors are expressed on BECs and TuBECs in a quantitatively similar pattern (Fig. 2*A*). We next permeabilized the cells, which resulted in staining of surface and cytoplasmic receptor determinants. These data exhibit higher receptor expression (Fig. 2*B*); however, the proportion of cells expressing both receptors is similar in both TuBECs and BECs. Flow cytometric analysis of IL-8 receptors expression was done on TuBECs and BECs at an earlier passage (p-3); the results were similar to those obtained with passage 5 cells (data not shown). To confirm flow cytometry studies, TuBECs and BECs were immunostained on cover glass slides, using anti-CXCR1 and anti-CXCR2 antibodies (Fig. 2*C*). The results show that differences in the responses of BECs and TuBECs

to IL-8 are not likely due to the absence or reduced expression of IL-8 receptors in TuBECs. To confirm these findings at the mRNA level, CXCR1 and CXCR2 mRNA expression was measured using semiquantitative RT-PCR. The data show that both BECs and TuBECs have similar levels of CXCR1 and CXCR2 mRNA (Fig. 2*D*). Polymorphonuclear granulocytes served as the positive control; experimental bands were evaluated based on β-actin levels.

Tumor-associated brain endothelial cells constitutively produce interleukin-8. Experiments were done to determine whether the lack of responsiveness to IL-8 by TuBECs was a result of endogenous IL-8 production. To test this, unstimulated TuBECs were cultured for 48 hours. Subsequently, culture supernatants were collected and analyzed for the presence of secreted IL-8 using the ELISA technique. Results show that TuBECs constitutively produce ∼3-fold higher amounts of IL-8 compared with BECs (*P* = 0.004; Fig. 3*A*). In three different experiments, TuBECs consistently secrete two to six times higher levels of IL-8 compared with BECs. To determine whether the increased production of IL-8 was due to a few cells secreting large quantities of IL-8, or the general population of TuBECs producing higher levels of IL-8, endothelial cells were cultured on coverslips and directly immunostained for IL-8. The results show that TuBECs are significantly more positive for IL-8 than BECs (Fig. 3*B*). The intensity of IL-8 staining is greater in TuBECs compared with BECs (Fig. 3*B*) and is relatively homogeneous. Based on the number of positive cells and intensity of immunostaining, TuBECs exhibit higher IL-8 production compared with BECs.

To determine whether increased production of IL-8 in TuBECs is a result of up-regulation of mRNA expression, semiquantitative RT-PCR for IL-8 mRNA was done on untreated TuBECs and BECs. IL-8 bands were normalized to 18S rRNA bands. The data (Fig. 3*C*) show a 4-fold increase in IL-8 mRNA in TuBECs compared with BECs. To determine whether TuBECs could be stimulated to further secrete IL-8, these cells were treated with TNF-α and Et-1, reagents known to induce IL-8 production. Both factors stimulate IL-8 production in TuBECs as well as in BECs (data not shown). Thus, TuBECs constitutively produced IL-8 but can be further stimulated with appropriate mediators.

Role of transforming growth factor-β1 and vascular endothelial growth factor in the constitutive production of interleukin-8 in tumor-associated brain endothelial cells. A potential mechanism causing the constitutive production of IL-8 in TuBECs may be the lack of negative feedback regulation by inhibitory cytokines. One such suppressive growth factor, TGF-β1, has previously been shown to inhibit Et-1–induced IL-8 production in BECs (7). To test this hypothesis, we treated TuBECs and BECs with TGF-β1 (10 ng/mL) for 72 hours, harvested the supernatants, and quantified the IL-8 content using an IL-8 ELISA. Optimal concentrations of Et-1 and TGF-β1 were determined in previous studies carried out in this laboratory (7). The results in Fig. 4*A* showed, as expected (7), that TGF-β1 inhibits Et-1–induced IL-8 production (*P* = 0.004). By contrast, TGF-β1 did not inhibit the production of IL-8 in TuBECs (Fig. 4*B*). In fact, TGF-β1 stimulated IL-8 production in TuBECs (*P* = 0.0006).

We previously showed that TuBECs constitutively secrete VEGF (19) and this growth factor induces IL-8 production in BECs (6). Thus, another potential mechanism for constitutive IL-8 production in TuBECs may be the secretion and autocrine activation of VEGF. To test this, TuBECs were treated with anti-VEGF neutralizing antibody (10 μg/mL) for 48 hours. Supernatants were then harvested and IL-8 content was quantified as described. The data show

that anti-VEGF–treated TuBECs exhibit a significant decrease in IL-8 production (*P* = 0.00002; Fig. 4*C*). Thus, endogenous VEGF produced by TuBECs can induce IL-8 production in these cells. Therefore, both TGF-β1 and VEGF may be responsible, at least in part, for the observed constitutive production of IL-8 in TuBECs.
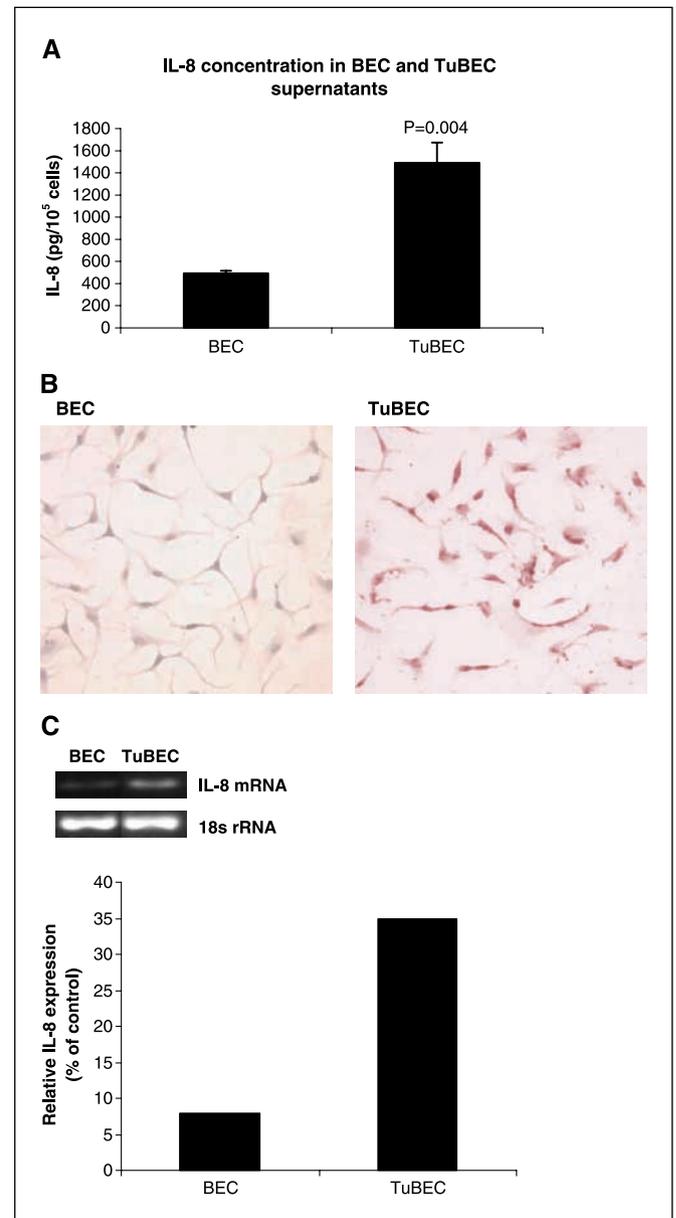
Figure 3. *A,* IL-8 secretion by TuBECs and BECs. Equal numbers of normal and tumor-associated endothelial cells (5 × 10⁴ per well) were plated in six-well plates. After 48 hours, the IL-8 concentration was measured in culture supernatants; triplicate samples were assayed. *Columns,* picograms of IL-8 per 10⁵ cells; *bars,* SE. Student's *t* test value comparing untreated BECs and TuBECs corresponds to $P_{(TuBEC/BEC)} = 0.004$. *B,* IL-8 immunostaining of BECs and TuBECs. Untreated cells were stained with primary mouse anti-human IL-8 antibody (5 μg/mL). Positive cells were identified by the red precipitate (×100 magnification). *C,* detection of IL-8 mRNA expression in BECs and TuBECs using RT-PCR. mRNA in cell lysates from equal numbers of BECs and TuBECs (5 × 10⁴ cells) was converted directly into cDNA, which was then amplified using IL-8 primers (0.4 μmol/L). 18S rRNA cDNA was also amplified as the internal control. The graph represents the relative ratio of the IL-8 band intensity compared with the 18S internal control. *Columns,* percentage intensity of IL-8 bands in BECs and TuBECs compared with their corresponding 18S rRNA bands.
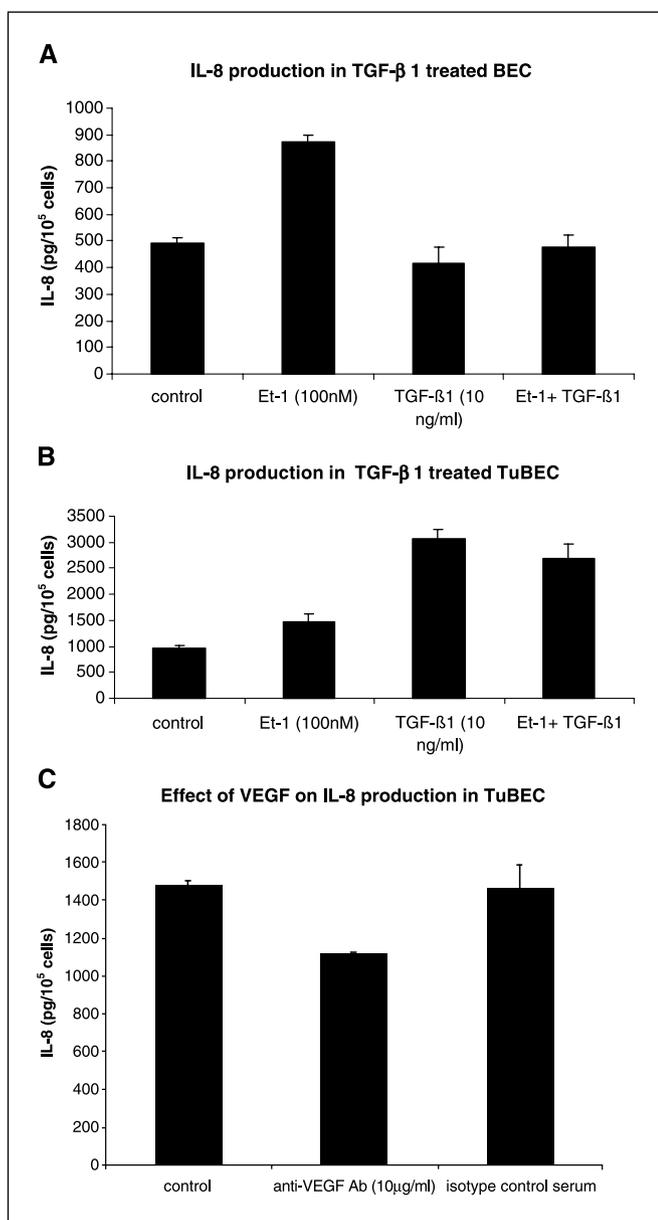
A

IL-8 production in TGF-β 1 treated BEC

B

IL-8 production in TGF-β 1 treated TuBEC

C

Effect of VEGF on IL-8 production in TuBEC

Figure 4. *A,* TGF-β1 inhibits Et-1–induced IL-8 production in BECs. Equal numbers of BECs (5×10^4 per well) were plated in six-well plates. The medium was changed to RPMI containing 1% FCS 24 hours before initiation of the experiments. Cells were then treated with TGF-β1 (10 ng/mL) and Et-1 (100 nmol/L) for 72 hours. All groups were plated in triplicate. After treatment, supernatant was collected from triplicate cultures and measured for IL-8 concentration levels. *Columns,* picograms of IL-8 per 10^5 cells. Student's *t* test values compared with the untreated control group correspond to $P_{(Et-1)} = 0.002$, $P_{(TGF-β1)} = 0.1$, and $P_{(TGF-β1+Et-1)} = 0.33$. *Bars,* SE. *B,* TGF-β1 does not inhibit IL-8 production by TuBECs. Equal numbers of TuBECs (5×10^4 per well) were plated in six-well plates. The medium was changed to RPMI containing 1% FCS 24 hours before initiation of the experiments. Cells were then treated with TGF-β1 (10 ng/mL) and Et-1 (100 nmol/L) for 72 hours. After treatment, supernatant was harvested from triplicate cultures and measured for IL-8 concentration. *Columns,* picograms of IL-8 per 10^5 cells. Student's *t* test values compared with the untreated control group correspond to $P_{(Et-1)} = 0.04$, $P_{(TGF-β1)} = 0.0006$, and $P_{(TGF-β1+Et-1)} = 0.001$. *Bars,* SE. *C,* anti-VEGF antibody treatment inhibits IL-8 production in TuBECs. Equal numbers of TuBECs (5×10^4 per well) were plated in six-well plates. The medium was changed to RPMI containing 1% FCS 24 hours before initiation of experiments. Cells were then treated with anti-VEGF neutralizing antibody (10 μg/mL) for 48 hours. As an isotype control, cells were treated with polyclonal rabbit serum (1:1,000 dilution). After treatment, supernatants were harvested from the triplicate cultures and measured for IL-8 concentration values. *Columns,* picograms of IL-8 per 10^5 cells. *Bars,* SE. Student's *t* test values compared with the untreated control group correspond to $P_{(VEGF)} = 0.00002$ and $P_{(isotype\ control\ serum)} = 0.8$.

Both CXCR1 and CXCR2 are responsible for interleukin-8–induced migration. TuBECs and BECs were analyzed for potential functional differences in IL-8 receptor expression with respect to cell migration. To identify the IL-8 receptor subtype responsible for IL-8–induced BEC migration, BECs were pretreated with neutralizing anti-CXCR1 (4 μg/mL) or anti-CXCR2 antibody (4 μg/mL), or both antibodies in transwell plates. The results show that after 6 hours, either anti-CXCR1 or anti-CXCR2 antibodies completely block IL-8–induced migration ($P = 0.00006$ and 0.0003, respectively; Fig. 5A). To confirm the specificity of these neutralizing antibodies, BECs were stimulated with VEGF and treated with both IL-8 receptor antibodies; these neutralizing antibodies do not block VEGF-induced migration (data not shown). As an additional control, isotype-matched IgG does not block IL-8–induced migration ($P = 0.3$; Fig. 5A). To determine which IL-8 receptor is responsible for TuBEC migration, TuBECs were treated with anti-CXCR1 or anti-CXCR2 or both reagents as described above. The results show that, in contrast to BECs, each antibody partially blocks TuBEC migration, whereas both antibodies have an additive effect in blocking TuBEC migration ($P < 0.05$; Fig. 5B). Experiments were done to determine whether IL-8–neutralizing antibody is able to block TuBEC migration. The results show that anti-IL-8 antibody significantly reduces TuBEC migration ($P = 0.02$; Fig. 5C). These data suggest that both TuBECs and BECs respond to IL-8 through both IL-8 receptors; however, the mechanism of this response may differ. In addition, the data suggest that IL-8 acts in an autocrine manner to induce TuBEC migration.

Discussion

Glioblastomas are angiogenesis-dependent tumors, exhibiting a significant increase in blood vessel density during progression from low- to high-grade tumor (21). To provide the increasing blood supply as the tumor grows, these cancer cells secrete a number of proangiogenic growth factors, such as IL-8, VEGF, basic fibroblast growth factor, and TGF-α (22–24). IL-8 has been reported to regulate angiogenesis and metastasis in several different tumor types, as well as differentially affect endothelial cell migration and proliferation (3, 4). Data presented here show that untreated TuBECs have a higher baseline migration rate than BECs. This is likely the result of the constitutive production of angiogenic factors. Our studies show that TuBECs constitutively produce IL-8. These results are consistent with previous studies showing that endothelial cells derived from ovarian and kidney tumors constitutively produce high levels of IL-8 (25). To eliminate the possibility that contaminating glioma cells are responsible for this IL-8 production (5, 26, 27), extensive analyses were done to confirm that TuBECs were exclusively endothelial cells (19).

Recent studies have shown that normal human brain microvascular endothelial cells produce IL-8 when stimulated with VEGF (6). Tumor cells as well as renal tumor-derived endothelial cells express high levels of endogenous VEGF compared with negligible levels of VEGF in their normal endothelial cell counterparts (14). Similarly, our previously published work showed that TuBECs derived from glioma tissue secreted high amounts of VEGF (19). Therefore, we examined the role of VEGF as a potential trigger for the observed constitutive IL-8 production by TuBECs. Our results show that treatment of TuBECs with anti-VEGF neutralizing antibody decreases IL-8 production, suggesting that VEGF plays a vital role in the ability of TuBECs to produce IL-8. These studies imply that TuBEC-derived VEGF is, at least in part, responsible for the production of IL-8 in these cells.
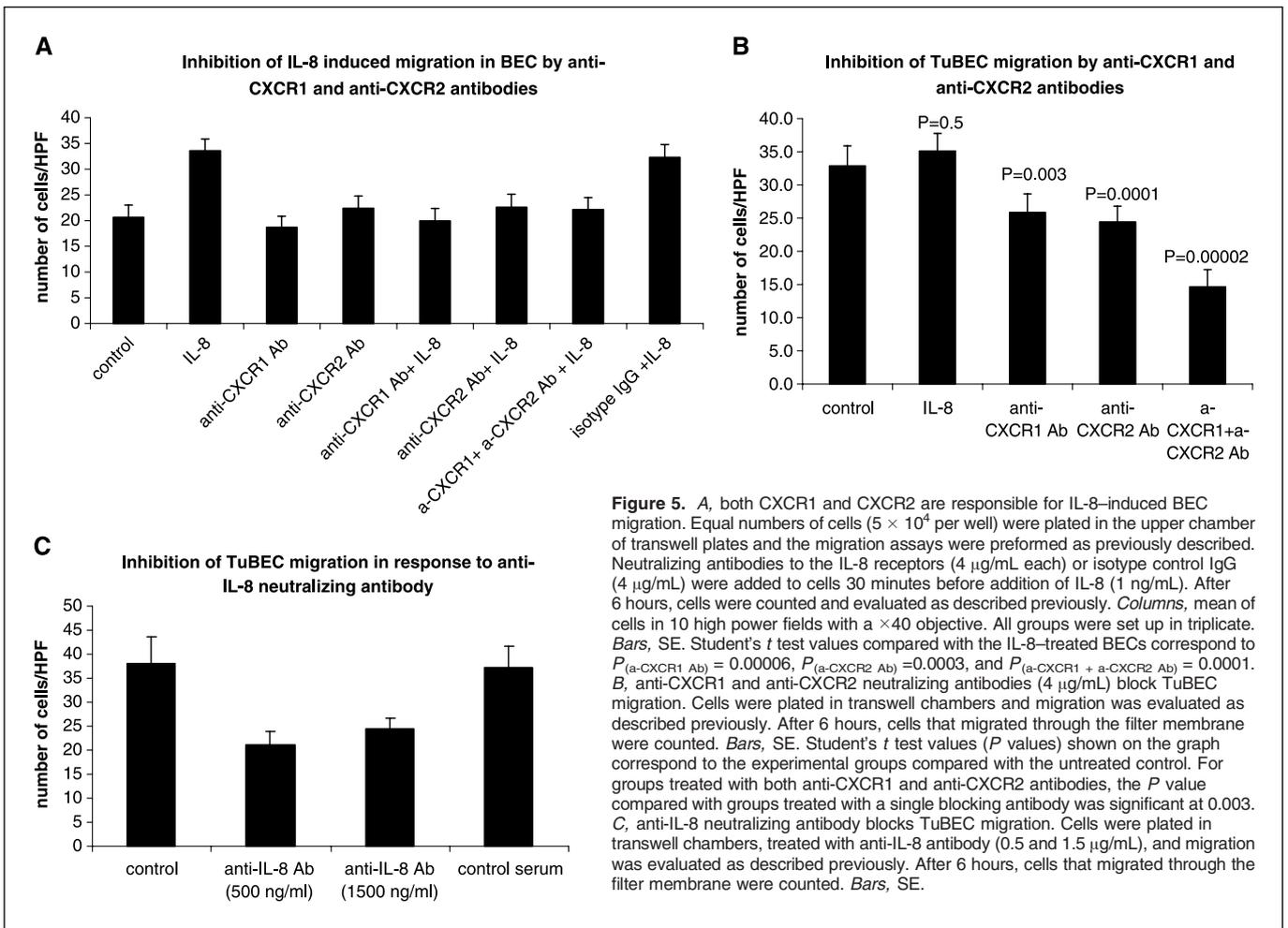
Figure 5. *A,* both CXCR1 and CXCR2 are responsible for IL-8–induced BEC migration. Equal numbers of cells (5×10^4 per well) were plated in the upper chamber of transwell plates and the migration assays were preformed as previously described. Neutralizing antibodies to the IL-8 receptors (4 μg/mL each) or isotype control IgG (4 μg/mL) were added to cells 30 minutes before addition of IL-8 (1 ng/mL). After 6 hours, cells were counted and evaluated as described previously. *Columns,* mean of cells in 10 high power fields with a ×40 objective. All groups were set up in triplicate. *Bars,* SE. Student's *t* test values compared with the IL-8–treated BECs correspond to $P_{(a\text{-}CXCR1\ Ab)} = 0.00006$, $P_{(a\text{-}CXCR2\ Ab)} = 0.0003$, and $P_{(a\text{-}CXCR1\ +\ a\text{-}CXCR2\ Ab)} = 0.0001$. *B,* anti-CXCR1 and anti-CXCR2 neutralizing antibodies (4 μg/mL) block TuBEC migration. Cells were plated in transwell chambers and migration was evaluated as described previously. After 6 hours, cells that migrated through the filter membrane were counted. *Bars,* SE. Student's *t* test values (*P* values) shown on the graph correspond to the experimental groups compared with the untreated control. For groups treated with both anti-CXCR1 and anti-CXCR2 antibodies, the *P* value compared with groups treated with a single blocking antibody was significant at 0.003. *C,* anti-IL-8 neutralizing antibody blocks TuBEC migration. Cells were plated in transwell chambers, treated with anti-IL-8 antibody (0.5 and 1.5 μg/mL), and migration was evaluated as described previously. After 6 hours, cells that migrated through the filter membrane were counted. *Bars,* SE.

The potential action of IL-8 on VEGF production is not clear. There is little evidence in the literature referring to the effects of IL-8 on VEGF production in BECs. Our studies show that IL-8 does not affect VEGF receptor expression in brain microvessels.[7] Thus, the reciprocal effect of IL-8 on VEGF production seems unlikely.

Differences between TuBEC and BEC responsiveness to IL-8 are not likely due to variations in IL-8 receptor expression, as shown by flow cytometry and receptor neutralization data. In the case of IL-8, high levels of this chemokine are associated with receptor desensitization due to receptor internalization; once the ligand is removed, the receptor is recycled to the cell surface (28, 29). Because TuBECs do not seem to be responsive to IL-8, the possibility exists that there may be aberrant recycling of the IL-8 receptor. However, both ligand bound and unbound receptors are recycling from the cell membrane (13, 30). Therefore, it is not surprising that intracellular expression of IL-8 receptors is similar in TuBECs and BECs. The data in Fig. 2*A* and *B* indicate that the differences in function between TuBECs and BECs were not likely due to differences in numbers of receptors, either on the cell surface or those internalized within the cell. These studies, however, do not determine whether the receptor affinities of these cells are similar. Expression levels of IL-8 receptors on all endo-

thelial cells are not homogeneous but organ and tissue dependent. Human intestinal microvascular endothelial cells only express CXCR2 (4), whereas both CXCR1 and CXCR2 were detected on human dermal microvascular endothelial cells and human umbilical vein endothelial cells (3). The results here show that, quantitatively, IL-8 receptor expression on endothelial cells derived from normal brain and tumor microenvironment is similar. However, our inhibition studies suggest that IL-8 receptor function in TuBECs may differ from BECs. In TuBECs, inhibition of migration is achieved by blocking both receptors, whereas in normal BECs either receptor antibody will block IL-8–induced migration. These differences in responses between BECs and TuBECs may represent potential differences in IL-8 signaling pathways. Inhibition of TuBEC migration by IL-8 receptor antibodies and IL-8 antibody suggests that endogenous IL-8 produced by TuBECs acts in an autocrine manner to induce their migration.

Normal BECs secrete IL-8 upon activation and respond to TGF-β1 by down-regulating IL-8 (7). Extensive studies were done by this and other laboratories, showing that TGF-β1 acts as an inhibitory growth factor on normal endothelial cells, smooth muscle cells, and fibroblasts (7, 31, 32). However, in different pathologic conditions, such as rheumatoid arthritis and athero-sclerosis, cells become resistant to the suppressive activity of TGF-β1 (33, 34). These studies correlate resistance to TGF-β1 to a decrease in type II TGF-β1 receptor expression (34). Our data

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[7] C. Charalambous et al., unpublished observations.

show that TuBECs do not respond to TGF-β1 as BECs. In fact, TGF-β1 seems to have a stimulatory effect on IL-8 production in TuBECs. In various normal cell types, IL-8 expression was shown to be differentially down-regulated by TGF-β1, IL-4, or IL-10 (35). However, several reports show that TGF-β1 can also have a stimulatory effect on various cell types. TGF-β1 has been shown to stimulate IL-8 production in smooth muscle cells (36). TGF-β1 also induces stromal derived factor-1 signaling in human macrophages by increasing CXCR-4 (stromal derived factor-1 receptor) expression (37). In addition, TGF-β1 induces VEGF production from conjuctival fibroblasts (38). Gliomas have been shown to produce high levels of both TGF-β1 and TGF-β2 but not IL-10 and IL-4 (39, 40), whereas normal human astrocytes do not produce either IL-4 or IL-10 and only produce TGF-β in its latent form (41). Therefore, in TuBECs, TGF-β1 may function in a stimulatory manner on endothelial cells rather than inhibitory. This growth factor has indeed been shown to be proangiogenic in specific environments (42–45). Therefore, the stimulation of IL-8 production by TGF-β1 in TuBECs may contribute to the observed constitutive production of IL-8 by these cells.

In summary, these studies show that primary cultures of human tumor-associated BECs constitutively produce IL-8 that acts in an autocrine manner to induce TuBEC migration. The production of this chemokine is stimulated by VEGF and is not inhibited by TGF-β1. Understanding the functions and regulatory processes of tumor-associated endothelial cells is critical for developing appropriate antiangiogenic therapies.

Acknowledgments

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