Glial Cell-induced Endothelial Morphogenesis Is Inhibited by Interfering with Extracellular Signal-regulated Kinase Signaling


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ABSTRACT

Purpose: Tumor vasculature provides the infrastructure by which malignant tissue can be nourished; therefore, targeting angiogenesis may be an effective means of treating cancer. We showed previously that SNB19 glioblastoma cells modulate bovine retinal endothelial cells in cocultures to form capillary-like network structures, that matrix metalloproteinase-9 (MMP-9) expression is critical for endothelial morphogenesis, and that MMP-9 expression in glioblastoma cells is regulated by extracellular signal-regulated kinase-1 (ERK-1). In the present study, we investigated whether interfering with the activation of this mitogen-activated protein (MAP) kinase would repress MMP-9 synthesis and inhibit capillary formation.

Experimental Design: Cocultures of bovine retinal endothelial and SNB19 cells were analyzed for MMP-9 secretion, and phospho- and total ERK levels. These cocultures were treated with PD98059, a specific inhibitor of MAP/ERK kinase 1, or transfected with dominant-negative ERK-1 mutant containing expression vector. Alterations in capillary-like structure formation, and actin cytoskeleton and secretion of vascular endothelial growth factor (VEGF), MMP-9, and tissue inhibitor of metalloproteinase-1 were determined by immunofluorescence, gelatin zymography, and Western blotting.

Results: We found that inhibition of the ERK-1/2 pathway with PD98059 abrogated glial cell-mediated capillary formation by the endothelial cells and reduced the levels of MMP-9 in the coculture. Strikingly, the abrogation of MAP kinase by a dominant-negative ERK-1 mutant inhibited glial-induced capillary network formation by reducing VEGF levels and MMP-9 activity and increasing the levels of tissue inhibitor of metalloproteinase-1. Inhibition of ERK activity also disrupted the formation of the actin cytoskeleton, a prerequisite for endothelial cell migration.

Conclusion: The mechanism underlying activation of ERK is involved in reorganization of the actin cytoskeleton, and induction of VEGF and MMP-9, thereby stimulating endothelial cell morphogenesis. These studies clearly provide experimental evidence that ERK inhibition diminishes glial-induced endothelial-cell morphogenesis; therefore, interfering with ERK signaling may be a viable approach to target angiogenesis.

INTRODUCTION

Brain tumor growth, especially that of glioblastoma multiforme, is critically dependent on angiogenesis, the sprouting of new vessels from pre-existing vasculature. Angiogenesis involves a complex interplay among tumor cells, endothelial cells, and their surrounding basement membranes. Growth factors and extracellular matrix components are two major groups of angiogenesis mediators. Host growth factors are likely to influence glioma angiogenesis by stimulating the proliferation of endothelial cells and by inducing the expression of key proteases on endothelial cells necessary for angiogenesis.

Proteolytic enzymes such as plasminogen activators and MMPs(2, 3) are involved in the early steps of endothelial morphogenesis, namely the cleavage of basement membrane and the migration of endothelial cells out of the existing blood vessels. MMPs are considered to be important in the process of matrix remodeling and endothelial cell migration during angiogenesis. Two members of the MMP family, gelatinase A (MMP-2) and gelatinase B (MMP-9), are the MMPs capable of degrading native collagen type IV, the major constituent of basement membranes; both MMP-2 and MMP-9 are involved in vascular cell migration and invasion. In a recent analysis, homozygous mice with a null mutation in the MMP-9 gene exhibited a defect in growth plate angiogenesis, establishing a role for MMP-9 in controlling angiogenesis. MMP-9 is secreted as a proenzyme that is subsequently acti-
CAMs are secreted proteins that also participate in the regulation of MMP activity in vivo (14). Of the four known TIMPs, TIMP-1 is the physiological inhibitor of MMP-9 (15).

The induction of MMP-9 synthesis is thought to involve the sequential activation of the serine threonine kinase c-Raf, MAPK kinase (MEK-1), and the ERKs (16). The classical Raf/MEK/ERK mitogenic cascade is strongly activated when cells are stimulated with growth factors. On activation, ERK/MAPK translocates into the nucleus, where it phosphorylates transcription factors, thereby altering gene transcription patterns (17, 18). ERK has been shown to be a prerequisite for endothelial survival (19) and also to play a pivotal role in angiogenesis (17, 18). ERK has been shown to be a prerequisite for endothelial survival (19) and also to play a pivotal role in angiogenesis (17, 18). ERK-1/2 is involved in the regulation of MMP-9 expression in tumor cells (21–23). Gensch et al. (24) have demonstrated that ERK-1/2 is involved in endothelial regulation of MMP-9 in response to tumor necrosis factor α as well as phosphol-2-myristate-13-acetate.

Our previous studies have shown that cell contact between SNB19 and BRE cells resulted in induction of capillary-like structures and MMP-9 activity (25). It has become apparent that MMP-9 is vital for angiogenesis, and the ability to selectively inhibit MMP-9 would be valuable for controlling angiogenesis. Considering that MMP-9 expression can be regulated by ERK-dependent signaling cascade, we undertook here to study the role of ERK-dependent signaling in the regulation of MMP-9 production during glioblastoma cell interactions.

MATERIALS AND METHODS

Homologous Cell Cultures. BRE cells were isolated and cultured as described previously (26). The cells were grown in humidified 5% CO₂/95% air at 37°C in MEM containing 20% FCS. The human glioblastoma cell line SNB19 was maintained in DMEM/F12 medium supplemented with 10% FCS in a humidified atmosphere containing 5% CO₂ at 37°C.

Cocultures. SNB19 cells were trypsinized, resuspended in culture medium, and plated at 2 x 10⁴ cells on tissue culture chamber slides pretreated with 20 μg/ml fibronectin (Sigma, St. Louis, MO). After an overnight incubation, the medium was removed, and the cocultures were rinsed in PBS, and serum-free medium was added. After 6–7 h of culture, the cultures were rinsed in PBS, and serum-free medium was added. After 48 h of incubation, the medium and cell lysates were collected and analyzed as described below.

Actin Cytoskeleton Staining. Cocultures were washed with PBS, fixed with 3.7% formaldehyde, and permeabilized with 0.2% Triton X-100 for 5 min. Then F-actin was labeled by adding tetramethylrhodamine isothiocyanate-phalloidin (200 ng/ml, Sigma) in PBS for 30 min, and immunofluorescence was recorded photographically on an Olympus microscope fitted with the appropriate filters.

Labeling Cells with the Fluorescent Marker Dil-acyl-LDL. Cocultures were incubated for 4 h at 37°C in MEM containing Dil-acyl-LDL (Biomedical Technologies, Stoughton, MA), which specifically stains endothelial cells. The medium was then removed, and the cocultures were fixed in 3.7% formaldehyde and visualized with Olympus fluorescence microscope fitted with standard rhodamine excitation/emission filters.

Immunofluorescence Staining for MMP-9. Cocultures were grown in eight-well chamber slides as described above, and then the cells were washed with PBS, fixed in cold methanol for 5 min, blocked with 1% BSA for 1 h, and then incubated with anti-MMP-9 monoclonal antibody diluted in blocking buffer for 1 h at room temperature. Cells were rinsed and stained with FITC-conjugated secondary antibody for 30 min at room temperature. After three washes with PBS, immunofluorescence was recorded photographically on an Olympus microscope fitted with the appropriate filters.

Gelatin Zymography. To identify the various collagenolytic enzymes in serum-free medium and cell extracts, gelatin zymography was performed as described earlier (28). Briefly, 20 μg of each sample was subjected to electrophoresis on an SDS-polyacrylamide (8%) gel containing 2 mg/ml gelatin. After electrophoresis, the gels were rinsed twice with 2.5% Triton X-100 and incubated at 37°C for 20 h in 10 mM CaCl₂ and 50 mM Tris-HCl (pH 7.5). The gels were then stained with Coomassie Blue and destained. Gelatinolytic enzymes were detected as transparent bands against the blue background of the
Coomassie stain and scanned using ChemiImager (Alpha Innotech Corp., San Leandro, CA).

Western Blotting Analysis. Cells were lysed in a Tris buffer containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, Aprotinin (10 μg/ml), and 1 mm phenylmethylsulfonyl fluoride (radioimmunoprecipitation assay). Equal concentrations of serum-free medium and cell lysate were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with a solution containing 3.0% BSA and incubated with primary antibody to phospho-ERK, ERK (Santa Cruz Biotechnology, Santa Cruz, CA), MMP-9 (Calbiochem, San Diego, CA), VEGF (Santa Cruz Biotechnology), or TIMP-1 (Calbiochem), or TIMP-1 and then with secondary antibody coupled to horseradish peroxidase. Reactive proteins were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL) according to the manufacturer’s recommendations, and density measurements were done using ChemiImager.

Statistical Assessment. The results were expressed as mean ± SE. The significance of differences between treated and untreated SNB19/BRE cocultures was performed using Student’s t test. The criterion for statistical significance was P < 0.05.

RESULTS

Glial-Endothelial Interaction Induces MMP-9 Activity and Phosphorylation of ERK. Under normal conditions, BRE cells showed moderate pro-MMP-9 activities by gelatin zymography, whereas the pro-MMP-9 activities in SNB19 cells were below detectable levels. When BRE cells were cocultured with SNB19 cells, the activity of MMP-9 in the serum-free medium increased significantly over time (Fig. 1A). Subsequent analysis of phosphorylated isoforms of ERK-1/2 in cell lysates revealed that coculturing the cells resulted in a sustained phosphorylation of ERK-1/2 for several hours (Fig. 1B). There was no significant difference in the total amount of ERK-1/2 protein expressed as determined by probing a duplicate gel with anti-ERK antibody (Fig. 1C). We observed an increase in the phosphorylation of ERK-1/2 (Fig. 1E) that paralleled an increase in MMP-9 activity (Fig. 1D) over time in SNB19-BRE cocultures.

Inhibition of ERK Phosphorylation. SNB19-BRE cocultures and cocultures that had been transfected with empty vector showed similar ERK phosphorylation (Fig. 2, A and B). Conversely, phosphorylated ERK-1/2 levels were decreased significantly in cocultures that had been either treated with PD98059 or transfected with dominant-negative ERK1 knockdown vector, significantly reduced the levels of VEGF in SNB19-BRE cocultures. We also observed that addition of exogenous recombinant pro-MMP-9 abrogated the ERK phosphorylation (data not shown). These findings suggest that the sustained phosphorylation of ERK-1/2 is necessary for increasing MMP-9 activity.

Effect of ERK Inhibition on Induction of VEGF. SNB19 cells secrete appreciable amounts of VEGF compared with BRE cells. Inhibition of ERK, whether by using PD98059 or transfection with a dominant-negative ERK1 mutant cDNA. Treatment of the coculture with PD98059 altered cell morphology and disrupted the actin cytoskeleton (Fig. 3). Untreated SNB19-BRE cocultures showed a marked reorganization of the F-actin microfilament network that was characterized by the formation of stress fibers. To prevent the activation of ERK, we separately transfected BRE and SNB19 cells with a dominant-negative ERK1 mutant and cocultured them; expression of the dominant-negative ERK1 mutant disrupted the actin reorganization as compared with the control vector (Fig. 3).

ERK Inhibition Affects Actin Cytoskeleton Structure. Next, we studied the role of ERK in actin reorganization by inhibiting the phosphorylation of ERK, either by using the Mek1 inhibitor PD98059 or by transfecting the cells with a dominant-negative ERK1 mutant cDNA. Treatment of the coculture with PD98059 altered cell morphology and disrupted the actin cytoskeleton (Fig. 3). Untreated SNB19-BRE cocultures showed a marked reorganization of the F-actin microfilament network that was characterized by the formation of stress fibers. To prevent the activation of ERK, we separately transfected BRE and SNB19 cells with a dominant-negative ERK1 mutant and cocultured them; expression of the dominant-negative ERK1 mutant disrupted the actin reorganization as compared with the control vector (Fig. 3).
Materials and Methods. ERK-1 mutant vector and then cocultured for 48 h as described in "Materials and Methods." Cell lysates were analyzed for VEGF levels by probing Western blots with an anti-VEGF antibody. B, quantitative analysis of VEGF protein levels was estimated by density measurements. Experiments were performed in triplicate and expressed as mean; bars, ±SE. *, P < 0.05.

Fig. 4 A, effect of inhibition of ERK on the levels of VEGF. SNB19-BRE cocultures were either treated with PD98059 (10 μM) or SNB19, and BRE cells were transfected separately with a dominant-negative ERK-1 mutant vector and then cocultured for 48 h as described in “Materials and Methods.” Cell lysates were analyzed for VEGF levels by probing Western blots with an anti-VEGF antibody. B, quantitative analysis of VEGF protein levels was estimated by density measurements. Experiments were performed in triplicate and expressed as mean; bars, ±SE. *, P < 0.05.

Fig. 3 Formation of F-actin is disrupted by inhibition of ERK activity. F-actin was detected with rhodamine-conjugated phalloidin and analyzed by fluorescence microscopy. Bar, 35 μm.

Fig. 5 Endothelial cell morphogenesis induced by glial cells requires ERK-1/2 activation. SNB19-BRE cocultures were either treated with PD98059 for 48 h or SNB19, and BRE cells were transiently transfected with dominant-negative ERK-1 mutant vector and then cocultured for 48 h. The cocultures were then incubated with Dil-acyl-LDL to visualize endothelial cells and photographed under a fluorescence microscope. Bar, 35 μm.

untreated SNB19-BRE cocultures suggesting that ERK signaling does have a role in the induction of VEGF (Fig. 4, A and B).

Inhibition of ERK Activity Impairs Capillary Formation. To test whether capillary-like formation is a direct result of ERK-1/2 activation, we first incubated SBN19-BRE cocultures with PD98059, a MEK-1 inhibitor, for 48 h. Staining the cocultures with Dil-acyl-LDL, which specifically stains endothelial cells, confirmed the inhibition of capillary formation (Fig. 5) in the presence of PD98059. Next, we transfected SNB19 and BRE cells with either mutant ERK-1 or an empty expression vector, cocultured the transfected cells for 48 h, and stained the cocultures with Dil acyl-LDL. Inhibition of capillary formation was apparent in ERK1 mutant-transfected cells as compared with the empty-vector transfecteds (Fig. 5), confirming the participation of ERK-1 in the formation of capillary structures. We also observed that inhibition of glial ERK either by PD 98059 or ERK mutant affected the ability of endothelial cells to form capillary-like structure formation.

Decreased MMP-9 Activity and Protein Levels by Inhibition of ERK. Incubating SNB19-BRE cocultures with the MEK1 inhibitor PD98059 decreased the activity of MMP-9 by >50% in both the secreted form (Fig. 6A) and in the cell lysates (Fig. 6C). The amount of MMP-9 protein produced decreased by >50% was confirmed by Western blotting with MMP-9 antibody (Fig. 6, B and D). Separate transfection of the SNB19 and BRE cells with an ERK mutant followed by coculture also produced the same effect: MMP-9 activity measured by zymography (Fig. 6, A and C) and density measurements (Fig. 6, E and G) was reduced, and MMP-9 protein levels measured by Western blotting with MMP-9 antibody (Fig. 6, B and D) and scanning of bands (Fig. 6, F and H) were reduced as well. As expected, transfecting the cells with empty vector did not affect MMP-9 activity or protein levels. Immunofluorescent labeling confirmed that MMP-9 was present in untreated or empty vector-transfected SNB19-BRE cocultures, but that the amount of MMP-9 was reduced in the cocultures that had been either treated with PD98059 or transfected with the dominant-negative ERK1 mutant (Fig. 7).

Inhibition of ERK Activity Increases the Expression of TIMP-1. Finally, we assessed levels of TIMP-1, the physiological inhibitor of MMP-9, in serum-free conditioned medium and in cell lysates of SNB19-BRE cocultures that were either treated with PD98059 or separately transfected with an ERK-negative mutant and cocultured. When cultured alone, BRE cells secrete significant levels of TIMP-1 in 24 h period, and SNB19 cells secrete relatively less compared with BRE cells. Inhibiting ERK activity by either means (pharmacologically or by transfection) increased the amount of TIMP-1 in both the serum-free medium (Fig. 8, A and C) and the cell lysate (Fig. 8, B and D) fractions.

DISCUSSION

Induction of cerebral angiogenesis is an intricate process that differs greatly from angiogenesis in non-neuronal tissues. In brain, astrocytes and endothelial cells are in close proximity and both contribute to the wall of the brain capillaries. This anatomical relationship suggests that both cell types could play an important role in brain tumor angiogenesis. The cascade of
events in the process of blood vessel formation involves a complex interplay among tumor cells, endothelial cells, and their surrounding basement membrane resulting in the enzymatic degradation of surrounding ground substance, and subsequent endothelial cell migration, proliferation, and tube formation.

Proliferation and migration of endothelial cells have been linked to ERK signaling (29, 30), and cell migration requires actin cytoskeleton reorganization and cell adhesion (31). In this study, we observed that inhibition of ERK disrupts the actin cytoskeleton in SNB19-BRE cocultures and, hence, may affect endothelial migration; in a similar study by others, the MEK1 inhibitor PD98059 decreased ERK-1/2 phosphorylation and blocked actin reorganization in corneal epithelial cells in a dose-dependent manner (32). In another study, endothelial migration and proliferation stimulated by basic fibroblast growth factor were blocked by inhibition of ERK activity by either PD98059 or the overexpression of a dominant-negative mutant of MEK-1 (33). Lyso phosphatidylcholine, which inhibits the Ras/ERK pathway, also reduced the endothelial migration and proliferation in that study (33).

VEGF is a potent angiogenic factor important for cancer neovascularization. Glioblastoma produces large amounts of basic fibroblast growth factor (34) and VEGF (35), which may act to mediate the paracrine control of angiogenesis. In our study, it may be that the glial cells provide the growth factors needed for the endothelial cells to align and form a capillary network. We observed that VEGF expression in the cocultures were decreased by PD98059 treatment and also by dominant-negative ERK-1 mutant transfection, suggesting that ERK par-
ticipates in the regulation of VEGF expression. Another group found that an inhibitor of phosphorylation of ERK-1/2 blocked the increase in VEGF expression and promoter activity induced by serum starvation in colon carcinoma cells (36). The VEGF promoter has four AP-1 binding sites (37); ERK-1/2 has been shown to activate the AP-1 pathway and, thus, may induce VEGF expression (38). Studies of rat fibroblasts have demonstrated that Raf activation of ERK-1/2 leads to induction of VEGF, a direct demonstration that the mitogenic pathway is but one of several pathways that mediate VEGF induction (39). Finally, inhibition of ERK activation by specific inhibitors of MEK-1 has also been shown to block the up-regulation of VEGF by low pH (40).

Previous observations have shown that the duration of ERK-1/2 phosphorylation dictates the biological responses of cells (41). Rapid onset and brief ERK expression underlie cell proliferation, whereas prolonged activated of ERK has been observed during cell differentiation (42). Contact with glia apparently inhibits the growth of endothelial cells, and stimulates their elongation, alignment, and morphogenic differentiation by release of soluble growth factors (43). Recent observations have shown that survival and morphogenesis of human umbilical-vein endothelial cells in a three-dimensional collagen gel are modulated by several signal transduction pathways, one of which is the ERK-1/2 signaling pathway (44). Maru et al. (45) showed that the MAPK/MEK inhibitor PD98059 prevented tubule formation by sinusoidal endothelial cells; another group found that PD98059, used to disrupt angiogenesis, also blocked the phosphorylation of MAPK detected in blood vessels in situ (46). Angiostatin, an endogenous inhibitor of angiogenesis, inhibited human dermal microvascular endothelial cell proliferation by reducing the phosphorylation of ERK-1/2 (29). Taken together, these findings strongly suggest that ERK activity is necessary for endothelial cell morphogenesis, and that ERK-1/2 activation by MEK1 may be an essential step in the signal transduction pathways leading to endothelial cell morphogenesis induced by glial cell interaction.

MMP-9 plays a critical role in angiogenesis (10) by virtue of its ability to degrade type IV collagen in the basement membrane; MMP-9 is known to be regulated by ERK signaling (21). In an earlier study, we found that the induction of MMP-9 by phorbol-2-myristate-13-acetate in cocultures of human brain endothelial cells and SNB19 glioblastoma cells was inhibited by PD98059 (47). We also demonstrated that transient transfection with kinase-deficient dominant-negative ERK-1 vectors decreased MMP-9 promoter activity in glioblastoma, suggesting that ERK-1 participates in the activation of MMP-9 (23). Others have shown that interfering with MEK-1, which is upstream of ERK-1, by using either PD98059 (to prevent the activation of MEK-1) or a dominant-negative expression construct reduced MMP-9 gelatinolysis and MMP-9 promoter activity in squamous cell carcinoma (21). We observed similar results in the present study, finding a decrease in MMP-9 activity and protein in both conditioned medium and cell lysate of cocultures when the ERK signaling pathway was inhibited.

MAPKs are known to be important mediators of signal transduction in the regulation of gene expression, and activation of these enzymes has been implicated in altered phosphorylation of several transcription factors, including c-Jun (48) and ATF2 (49). With respect to the general transcriptional control of the MMP-9 gene, the cis-acting elements AP-1, PEA3, SP1, and nuclear factor kB located between bp −520 and −670 (21) are involved in endothelial MMP-9 regulation. One possible mechanism by which sustained MAPK activation could result in MMP-9 induction is through regulation of essential transcription factor such as c-Fos. Expression of this immediate early gene depends on MAPK activation, and furthermore, phosphorylation of c-Fos by MAPK enhances its activity (50). TIMPs are known to have antiangiogenic effects (7, 51), but their capacity to inhibit vascular regression through blockage of MMP function has not been investigated. We observed decreased TIMP-1 levels in untreated SNB19-BRE cocultures, and in cells transfected with empty vector and increased TIMP-1 levels when ERK activity was inhibited by either PD98059 or transfection with a dominant-negative ERK mutant. Lamoreaux et al. (52) reported that VEGF down-regulates TIMP-1 and TIMP-2 in microvascular endothelial cells. Our findings agree with those of previous reports of increased VEGF levels and decreased TIMP-1 levels in SNB19-BRE cell cocultures.

In summary, the activation of MAPKs is central for angiogenesis, because these kinases regulate growth factors, MMP-9 expression, and endothelial morphogenesis. We propose that during glial-endothelial cell interactions, cell-to-cell contact and the microenvironment may provide the positional or molecular cues necessary for sustained ERK activation. All of these observations point to the complexity of the process involved in angiogenesis, and much more work is needed to understand the process completely.

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