TGF-β Increases Retinal Endothelial Cell Permeability by Increasing MMP-9: Possible Role of Glial Cells in Endothelial Barrier Function

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Purpose. To determine transforming growth factor (TGF) β effects on matrix metalloproteinases (MMPs) as a potential cause of the blood–retinal barrier breakdown at the onset of angiogenesis. Previously, glial cells were shown to play a role in the angiogenesis process and to express the angiogenic regulating factor TGF-β, which becomes active under hypoxia conditions. Here, the authors demonstrate that retinal endothelial cells express MMP-9 when treated with TGF-β or cocultured with glial cells and that both TGF-β and MMP-9 increase endothelial cell permeability.

Methods. Primary cultures of bovine retinal endothelial (BRE) cells grown on porous membranes were treated with TGF-β or purified MMP-9, and permeability changes were assayed. The amount and distribution of the tight junction protein occludin also was analyzed by immunocytochemistry and Western blotting. Cell extracts or conditioned media from TGF-β–treated BRE cells and from glial cell–BRE cocultures were analyzed for MMP-9 content by substrate gel electrophoresis (zymography) or Western blotting.

Results. Both TGF-β and MMP-9 increased the permeability of BRE monolayers and reduced the levels of the junction protein occludin. The effect of MMP-9 on permeability was rapid, but the TGF-β–induced permeability required longer incubation and was blocked by anti–TGF-β and anti–MMP-9 antibodies as well as by TGF-β latency-associated peptide. Zymography showed that MMP-9 activity, which was very low or absent in untreated BRE cultures, was dramatically increased by TGF-β as well as by coculturing with either astrocytes or Müller glial cells. Anti–TGF-β antibody blocked the TGF-β effect, but not the coculture effect on MMP-9 production.

Conclusions. These data indicate a direct correlation between TGF-β–induced MMP-9 activity and increased endothelial cell permeability. Moreover, endothelial cell production of MMP-9 is regulated by glial cells through expression of TGF-β or by direct cell-to-cell contact. During retinal disease, glial cell production of active TGF-β may contribute to breakdown of the blood–retinal barrier by stimulating endothelial cell MMP-9 production. (*Invest Ophthalmol Vis Sci.* 2001;42:853–859)

Extracellular matrix (ECM) turnover is greatly accelerated during angiogenesis. Sprouting of new vessels from preexisting capillaries requires a precisely regulated expression of proteolytic enzymes, including matrix metalloproteinases (MMPs), to allow endothelial cells to penetrate their underlying basement membrane.[1–3] This process generates leaky vessels and also eliminates the contact inhibition phenomenon that otherwise blocks endothelial cell proliferation in quiescent monolayers.[4] Moreover, it has been suggested that the serum components leaking into the perivascular environment provide a medium suitable for endothelial cells' growth, migration, and differentiation to form a new vascular meshwork.

Gelatinases A and B (72-kDa MMP-2 and 92-kDa MMP-9, respectively) comprise a subfamily of MMPs capable of digesting basement membrane proteins collagen type IV, laminin, and fibronectin as well as gelatin (denatured collagen). They are secreted as proenzymes and are subsequently activated by N-terminal cleavage, either through an autocatalytic mechanism or by other proteases such as plasmin. Their activity is further controlled by binding to tissue inhibitor of metalloproteinases (TIMPs). High expression of MMPs has been reported in various pathologic conditions associated with angiogenesis and tumor invasion.[5] MMP-9 and MMP-2 have distinct patterns of regulation.[6,7] Both enzymes are expressed in endothelial cells, but MMP-9 is often produced in conjunction with endothelial cell activation. MMP-9 has been also localized in solid tumors and tumor stromal cells.

In the eye, neovascular disorders such as proliferative diabetic retinopathy[8–10] and age-related choroidal neovascularization[11,12] are accompanied by upregulated MMPs. Inhibition of angiogenesis by TIMPs have been demonstrated both in vivo and in vitro.[13,14] In cultured endothelial cells, it has been shown that angiogenic factors—vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FGF)—upregulate MMPs.[15–17] However, the specific role of MMPs in the angiogenesis process has not been directly addressed.

Our previous observations in rat retina suggested that astrocytes play a role in inducing endothelial cell differentiation and capillary formation.[18,19] This hypothesis is supported by the work of Laterra et al.[20] and Laterra and Goldstein, [21] showing that endothelial cells form capillary-like structures when cocultured with astrocytes. In vitro studies by us and by others have indicated that astrocytes, Müller glia, or other perivascular cells express TGF-β, which is activated under hypoxia or coculture conditions.[22–25] Here, we show that exogenous TGF-β as well as direct contact of astrocytes or Müller cells stimulate release of MMP-9 by retinal capillary endothelial cells. Moreover, TGF-β increases permeability of bovine retinal endothelial (BRE) cells by a mechanism that appears to involve production of MMP-9.

Materials and Methods

Cell Cultures and Preparation of Conditioned Media

Unless indicated otherwise, cells were maintained in media supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml) under an atmosphere of 95% air and 5%

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CO_2 in a humidified 37°C incubator. Primary cultures of microvascular BRE cells were prepared as described previously.[23,26] Cultures were more than 98% pure as tested by acetylated LDL uptake and also by anti–factor VIII immunoreactivity. Rat brain astrocytes and rat retinal Müller cells were prepared according to our established protocols.[22,27] For preparation of conditioned medium, confluent BRE monolayers grown in 12-well plates were rinsed with PBS and incubated overnight in serum-free medium (EBM; Clonetics, San Diego, CA). This medium was then replaced with fresh EBM containing 2 ng/ml TGF-β (recombinant human type-I; R&D Systems, Minneapolis, MN), and cultures were incubated for different times. Conditioned media were then collected and clarified by centrifugation. Samples were directly used for zymography, or aliquots were stored frozen at −20°C and thawed only once for each application. Serum was omitted in media because it contains MMPs, plasminogen activators (which activate MMPs) as well as factors that may stimulate MMP expression.[28] Alternatively, cells were preincubated for 30 minutes with anti–TGF-β antibody or the protease inhibitor, aprotinin, before the addition of TGF-β. Aprotinin has been implicated in blocking TGF-β activation in cocultures.[24] For studying the coculture effects, suspended BRE and glial cells were mixed to the ratio of ~10:1 (3×10^5 BRE and $2–3 \times 10^4$ Müller or astrocytes/well) before plating, or glial cells maintained in serum-free medium were harvested and added to BRE monolayers that had also been preincubated in serum-free medium overnight. In some experiments cell extract and conditioned media were compared on zymograms. After collecting conditioned medium, cells were rinsed two times with PBS, covered with ice-cold lysis buffer, and stored at −20°C. They were thawed and clarified by centrifugation at 15,000*g* for 20 minutes before use. The lysis buffer consisted of 20 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 1% Triton X-100, and 0.1% SDS, to which PMSF at 1 mM was added before use. Human fibrosarcoma HT-1080 (CCL-121; ATCC, Rockville, MD) and HFL-1 cells lines were grown in a mixture of 50:50 vol/vol DMEM/F12 media (Gibco-BRL, Gaithersburg, MD) supplemented with 10% FBS. They were treated with a solution of 2^{-6} M 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in serum-free medium. Conditioned media were used as positive control and for purifying MMP-9.

Characterization and Semiquantitative Assay of MMP-9

MMP activities were assayed by zymography on gelatin gels. SDS-polyacrylamide gels were copolymerized with 0.1% gelatin, and electrophoresis was carried out under nonreducing conditions. Because protein levels in serum-free media were below detectable limits, sample volumes were equated for cell numbers. Gels were rinsed for 1 hour with three changes of 50 mM Tris buffer (pH 7.5) containing 2.5% Triton X-100 to remove SDS and were incubated in reaction buffer at 37°C overnight. They were then stained with brilliant blue G250. Enzyme activities were revealed as clear bands (lysis zone) against the dark blue background of the substrate gel and were quantified by densitometry using an IS-1000 Digital Imaging System (Alpha Innotech). The arbitrary units measured by densitometry were in a linear correlation with the sample volume in a range of 5 to 20 μl (not shown). For control, duplicate samples were applied to gelatin gels. After electrophoresis, gels were cut into halves and were developed in the presence and absence of EDTA.

In electrophoretic gels, SDS facilitates autocatalytic activation of proenzymes by unfolding the inactive molecule[29]; that is, lysis bans are formed on zymograms at positions corresponding to the high-molecular-weight proenzymes. MMP expression assayed by RT-PCR, Northern blot analysis, or immunohistochemistry may not directly reflect the level of enzyme activity in tissue. The actual enzymatic activity is regulated by many factors such as the rate of cellular excretion, extracellular activation, stability of activated enzyme, the molecular site of activation-cleavage, and the presence or absence of TIMPs.[30–32] Although synthetic fluorescein substrates are available for quantitative assay of active MMPs in biological fluids, they do not assess the relative amounts of proenzyme in the sample, and they cannot discriminate MMP-9 from MMP-2.[33,34] Moreover, the commercially available ELISA kits may not be very specific because of possible cross-reactivity of antibodies between MMP-2 and MMP-9. Therefore, for our comparative analysis, we used zymography in the manner commonly used by others.[30,35,36] For Western blot analysis, concentrated conditioned media were subjected to SDS-PAGE under nonreducing conditions, and proteins were transferred to nitrocellulose membranes. Blots were developed with monoclonal anti–MMP-2 (B1 IF3) and anti–MMP-9 (A5 IE3) antibodies prepared in our laboratory (see below). In addition, anti–MMP-9 from Triple Point Biological (Forest Grove, OR) and anti TIMP-1 (Oncogene, Cambridge, MA) were also used. Blots were developed with horseradish peroxidase (HRP)-conjugated secondary antibodies and ECL kit (Amersham, Cleveland, OH).

Purification of MMP-9 and Preparation of Anti-MMP-9 Monoclonal Antibody

Conditioned media from TPA-treated HT-1080 or HFL-1 cells were applied to a gelatin-sepharose column (Pharmacia, Piscataway, NJ) equilibrated with 50 mM HEPES buffer, pH 7.5, containing 0.2 M NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 15 mM NaN_3 (buffer A). The column was washed with 1 M arginine in buffer A to remove fibronectin and then with a solution containing 0.2 M acetic acid, pH 3.0, and 0.2 M NaCl, to remove TIMP. After reequilibration with buffer A, MMP was eluted with 7.5% DMSO and 10% glycerol in buffer A. MMP-9 was separated from MMP-2 by affinity chromatography using Con A sepharose 4B (Sigma). Unglycosylated MMP-2 passed through, and the bound MMP-9 (glycosylated) was eluted with 1 M methyl-α-D-mannoside.[37] Monoclonal antibodies were produced according to previously described methods.[38–40]

Permeability Assay

BRE cells were grown to confluent monolayers on collagen/fibronectin-coated membranes in double-chamber tissue culture plates (Transwell, 12-mm-diameter cups with 0.4-μm pore size membrane; Costar, Cambridge, MA). The cultures were then maintained in serum-free medium for 4 to 5 days before they were treated with designated reagents for various times. For permeability assay, HRP was added as tracer to the upper chambers, aliquots were collected from the lower chamber after 30 minutes, and 1 hour. HRP assay was carried out essentially as described before.[26] A standard curve was prepared from HRP serial dilutions in each experiment, and the samples were diluted such that all readings fell within the linear range of the standard curve. Data in Figures 1 and 2 represent results of quadruplicate assays (±SD), which have been repeated at least two more times with different lots of BRE cells. It should be noted that for all data presented in Figures 1 and 2, the passage of HRP over a constant time (1 hour) has been measured and that the reduction of HRP in the upper chamber is considered negligible (<1/1000) for this duration.

Western Blot Analysis and Microscopic Immunolocalization of Occludin Protein

Endothelial cells were grown in 100-mm-diameter dishes or on microscope slides and were treated in the same manner as for the permeability assay. At the end of treatment, cells were collected from dishes and extracted by Triton/urea buffer as described.[41] Samples were adjusted for protein contents and applied to SDS-PAGE followed by Western blotting. Prestained and unstained molecular weight markers (Bio-Rad) were used, and membranes were immersed in Ponceau's solution (Sigma) to reveal the unstained molecular weight marker bands. Membranes were then developed using anti-occludin antibody (Zymed Laboratory, Inc., San Francisco, CA) and ECL kit. The prominent occludin band migrating at 58 to 60 kDa was quantified by densitometry as described above. Membranes were reprobed with anti–β-actin antibody to correct for variations of protein loading among the lanes.

Cells grown on microscope slides were treated similarly, fixed, and processed for immunocytochemistry as described,[40] using the same anti-occludin antibody used for Western blot analysis. Distribution of

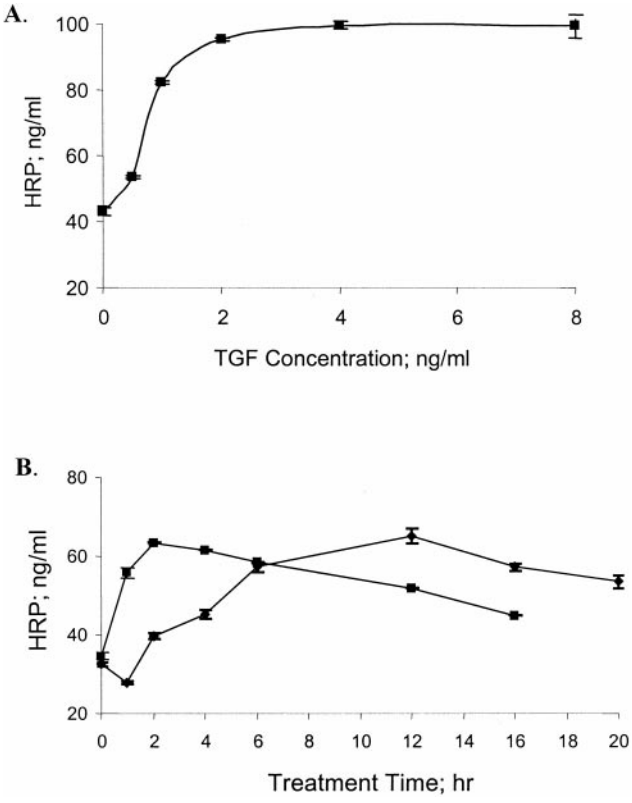

FIGURE 1. TGF-β and MMP-9 induced permeability in endothelial cell monolayers. BRE cells grown to confluent monolayers on porous membranes (Transwell) were incubated overnight with different concentrations of TGF-β (**A**) and passage of the tracer molecule (HRP) from the upper to the lower chamber during the last 1 hour of incubation was measured. (**B**) The same experiment was repeated except that BRE preparations were treated with 2 ng/ml TGF-β (♦) or 0.1 mg/ml purified MMP-9 (■) for the designated times.

occludin was observed by confocal immunofluorescence microscopy using a MultiProbe 2001 confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, CA). The total cellular occludin was quantified by densitometry using Meta Morph Imaging System (v3.6).

RESULTS

TGF-β Effects on Permeability of Endothelial Cell Monolayers

BRE cells form a functional barrier when grown to a confluent monolayer on collagen/fibronectin-coated membranes.[26] When different doses of TGF-β were added to BRE monolayers overnight, the permeability was increased in a dose-dependent manner. A nearly maximum permeability was reached at 2 ng/ml (Fig. 1A). This dose, therefore, was used for all other experiments. Besides, higher concentrations (≥5 ng/ml) resulted in morphology changes over long incubation times, whereas at 2 ng/ml such alteration did not occur. In the presence of 2 ng/ml TGF-β, permeability increased gradually with incubation time, reached a maximum after 8 to 12 hours, and then began to decrease (Fig. 1B). Anti–TGF-β antibody and latency-associated peptide totally blocked TGF-induced permeability (Fig. 2). Western blotting and confocal image analysis indicate that the permeability change is accompanied by alteration in the tight junction protein occludin. Untreated cells demonstrated strong occludin immunoprobe-labeling at the site of cell-to-cell attachment and also within the cytoplasm. By contrast, after 8 hours of TGF-β treatment, occludin labeling of the cell junctions was fragmented, and labeling within the cell was considerably diminished. Densitometric analysis showed a significant reduction in labeling intensity ($P < 0.05$, ANOVA) that was confirmed by Western blot analysis (Fig. 3).

TGF-β and Glial Cell Effects on MMP-9 Production in Endothelial Cells

This series of experiments evaluated the role of MMP-9 in TGF-β–induced permeability of endothelial cells. Because TGF-β is known to regulate ECM turnover,[42] we hypothesized that its effects on permeability involve proteolytic enzymes. Confluent BRE cells grown in serum-free medium were treated with different concentrations of TGF-β for various times, the conditioned media were collected, and cell extracts were prepared. Zymographic analysis showed that MMP-2 is constitutively expressed by BRE cells, and its abundance is not noticeably affected by TGF-β. However, TGF-β treatment induced de novo expression of MMP-9 activity in BRE cells (Fig. 4). This activity, which comigrates with MMP-9 released by HT-1080

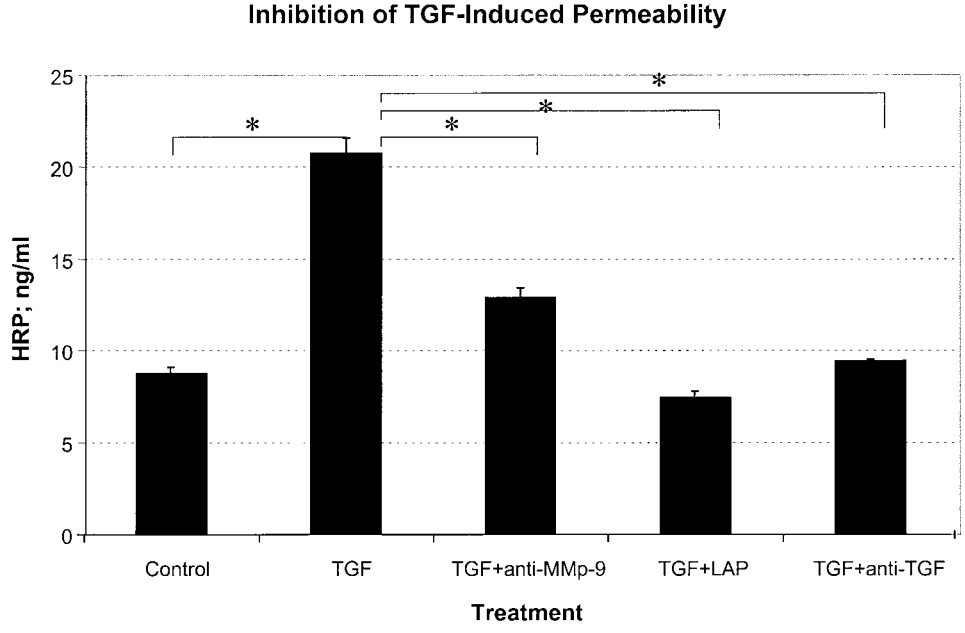

FIGURE 2. TGF-β-induced permeability in BRE cells is mediated by MMP-9. BRE cell monolayers prepared as above were pretreated with designated antibodies and reagents for 30 minutes and with TGF-β overnight. Permeability was measured by HRP assay as explained in Figure 1. *$P < 0.001$.

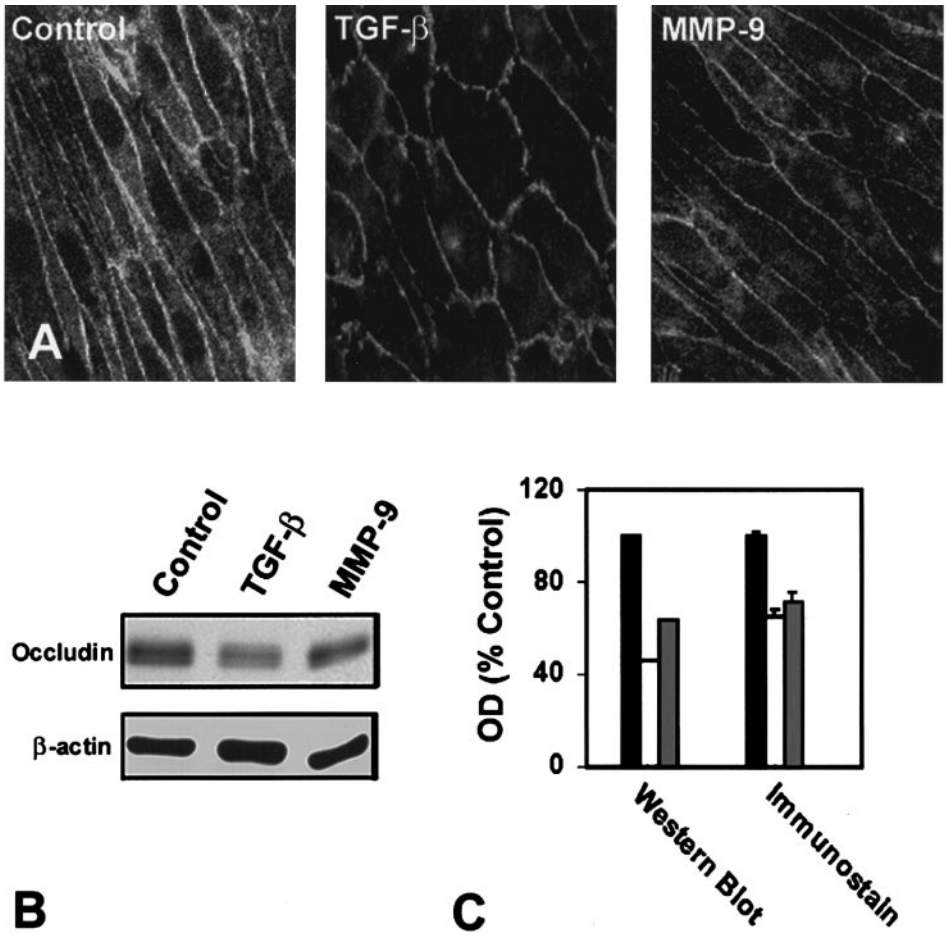

FIGURE 3. Immunoprobe analysis of junction protein occludin. Confluent BRE monolayers grown as described in Materials and Methods were treated with TGF-β at 2 ng/ml or MMP-9 at 0.1 mg/ml for 8 hours and analyzed by immunocytochemistry (**A**) or Western blotting (**B**) followed by densitometry (**C**); ■, control; □, TGF-β; ▨, MMP-9.

cells (Fig. 4A, lane 7), was not detectable or was very weak in untreated control BRE cultures. The same activity was detected in endothelial cells cocultured with astrocytes or Müller glial cells. Figure 4 shows representative zymograms of media conditioned by BRE cells or BRE–glial cell cocultures incubated in the presence or absence of 2 ng/ml TGF-β for 18 hours. Figure 4D demonstrates that TGF-β induction of MMP-9 expression is dose dependent. Our time course analysis indicated that a minimum of 6-hour incubation is required for detectable levels of MMP-9 to accumulate in the culture media (data not shown). Because the same time lag was necessary for enzyme to be detected in cell extract as well, it is conceivable that TGF-induced MMP-9 activity is regulated at the gene expression level. Using Northern blot analysis, Puyraimond et al.[43] have demonstrated that TGF-β upregulates MMP-9 transcription in bovine pulmonary artery endothelial cells. Based on gel mobility, it is evident in Figure 4 that both MMP-2 and MMP-9 are secreted as proenzymes. However, sample handling is crucial in these experiments because the presence of trace amounts of serum in the culture medium as well as repeated freezing/thawing results in appearance of some low-molecular-weight bands (active enzymes) on zymograms.

Astrocytes and Müller cells do not produce MMP-9 even when treated with TGF-β (Fig. 4, lanes 5 and 6), but both of them induce BRE cells' expression of MMP-9 (Fig. 4, lanes 3 and 4). Moreover, as shown by others,[35] conditioned media and cell extracts demonstrated the same pattern of MMP activities (Fig. 4B). Densitometry of lanes 1 to 4 in Figure 4A indicated that the TGF-β–induced MMP-9 is approximately 20% of the MMP-2 activity. Almost the same ratio is also found in coculture, but when TGF-β is added to the coculture (Fig. 4A, lane 4), a further increase of MMP-9 activity is observed. Anti-TGF-β antibody blocked the effect of exogenous TGF-β, but the Müller cell effect was not blocked, even in the presence of high doses of antibody (40–200 μg/ml). Likewise, the protease inhibitor aprotinin, which presumably blocks activation of TGF-β in coculture, [24] showed no significant effect on MMP-9 expression (data not shown).

The high-molecular-weight (~92 kDa) gelatinase produced by BRE is identical with type-B gelatinase or MMP-9 by the following criteria: first, it comigrates on zymograms with the 92-kDa gelatinase secreted by HT-1080 cells.[7,35,44] Second, the lysis band was absent when the zymogram was developed in the presence of EDTA, an inhibitor of metalloproteinases (data not shown). Third, in the presence of APMA (*p*-aminophenylmercuric acetate), the single-band, 92-kDa "inactive" enzyme is cleaved to generate a higher mobility band (82- to 83-kDa active enzyme) on gelatin gel (Fig. 5D). Finally, the BRE-secreted, 92-kDa enzyme reacts with anti–MMP-9–specific monoclonal antibodies on Western blot (Fig. 5C). Anti–MMP-2 monoclonal antibody clearly indicated that the lower and more intense lysis band on zymograms corresponds to MMP-2 activity (Fig. 4B). The expression of TIMPs is reportedly regulated by cytokines. In our experiments, however, when Western blot analyses were developed with anti–TIMP-1 antibody, there was no difference in the intensity of immunoreactive bands in TGF-β–treated cells compared with the control cultures (Fig. 5D). The same membrane was reprobed with anti–MMP-9 antibody to further confirm increased MMP-9 activity in the TGF-β–treated sample. Using large-vessel endothelial cells, Puyraimond et al.[43] have demonstrated an increase in TIMP-1 activity when tested on reverse zymogram; however, the authors did not detect any increase in expression of TIMP-1 gene by Northern blot analysis.

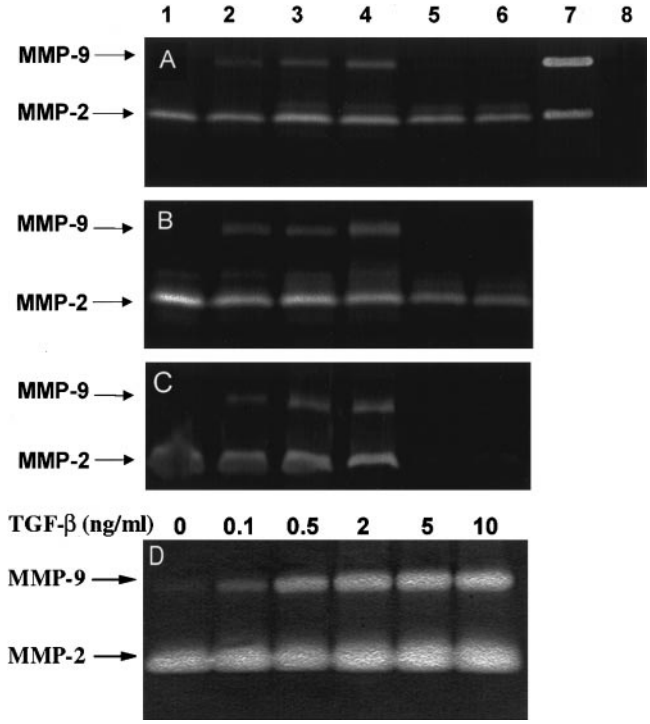

FIGURE 4. Effect of TGF-β and glial cell coculture on the BRE cells' MMP activity. Endothelial cells maintained in serum-free medium were treated as described in Methods. Equal amounts (10 μl each) of conditioned media (**A** and **C**), or cell extracts (**B**), were applied to zymography. Samples were as follows: (**A**) 1, control BRE; 2, TGF-β added at 2 ng/ml; 3, BRE cocultured with Müller glial cells; 4, TGF-β added to coculture; 5, control glial cells; 6, TGF-β added to glial cells; 7, TPA-treated HT-1080 cells (2.5 μl); and 8, fresh serum-free EBM medium. (**B**) The same as (**A**) except that cell extract were used instead of conditioned media. (**C**) The same as (**A**) except that astrocytes were used instead of Müller cells. (**D**) Samples (80 μl each) of the BRE conditioned media treated with different concentrations of TGF-β for 24 hours were applied to gelatin gel and zymogram was developed as described in Materials and Methods section.

Role of MMP-9 in TGF-β–Induced Permeability Increase

To test directly whether or not the TGF-β–induced permeability increase involves induction of MMP-9 activity, BRE cell permeability was assayed after MMP-9 treatment. Purified MMP-9 increased permeability of BRE cells within 1 hour. This effect was maximum at 2 hours and gradually diminished with prolonged incubation, perhaps because of instability of the purified enzyme (Fig. 1). Kinetics of the permeability changes are consistent with the hypothesis that the TGF-β effect involved de novo MMP-9 production. Permeability increased very rapidly with exogenous MMP-9, whereas TGF-β effects required 6 hours of incubation. It is also shown in Figure 2 that TGF-induced permeability is blocked significantly by anti–MMP-9 antibody. Permeability was not affected by addition of unimmunized mouse IgG as control. Morphologic and Western blot analysis of the MMP-9–treated cultures showed reduction in occludin levels similar to those seen after TGF-β treatment (Fig. 3). Neutralization of TGF-β–induced permeability by anti–MMP-9 antibody together with the fact that TGF-β induces MMP-9 production in BRE cultures strongly suggest that TGF-β–induced barrier breakdown is mediated by MMP-9.

DISCUSSION

We have demonstrated here that exogenous TGF-β increases permeability of retinal capillary endothelial cells grown to confluent monolayers on porous membranes. We have further shown that endothelial cells treated with TGF-β or cocultured with glial cells produce a high-molecular-weight (~92 kDa) gelatinase that is identical with gelatinase type-B or MMP-9. Purified MMP-9 also increased permeability of BRE monolayers. TGF-β effects on permeability and also on MMP-9 expression required long incubation times, whereas permeability increased by exogenous MMP-9 appeared soon after the treatment started. On the other hand, TGF-β–induced permeability was blocked by anti–MMP-9 antibody. Therefore, we conclude that the barrier breakdown by TGF-β is mediated by MMP-9. Although the mechanism of the barrier breakdown at the cellular level is not yet clear, our observation of redistribution and reduced immunoreactivity of tight junction protein, occludin, suggests that increased permeability involves a paracellular mechanism.

The observation that TGF-β increases the permeability of retinal endothelial cells by stimulating production/release of MMP-9 has important implications for understanding of the blood–retinal barrier dysfunction in disease or injury. Glial cells have long been considered as regulator of blood–brain barrier function in vivo. The role of glial cells in control of vascular development in retina is also well established. However, there is little evidence of how their alteration contributes to the endothelial barrier dysfunction and pathologic angiogenesis in

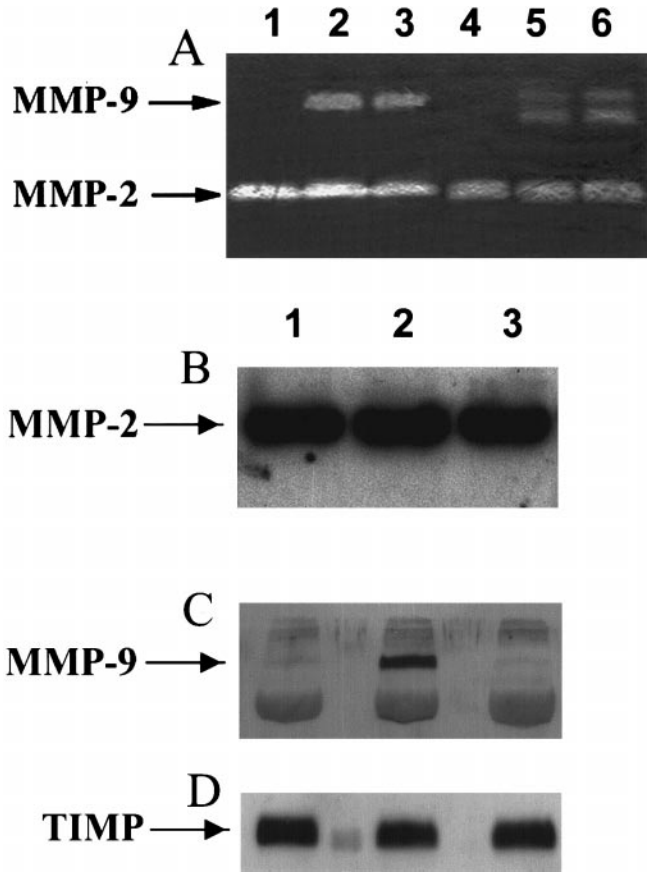

FIGURE 5. Characterization of MMPs and TIMP-1 by control zymogram and Western blot analysis. (**A**) Conditioned media from TGF-β–treated (*lanes 2, 3, 5* and *6*) and control BRE cells (*lanes 1* and *4*) were activated by incubating with APMA (*lanes 4* through *6*) and were subjected to gelatin gel; note the appearance of double bands in *lanes 5* and *6*. (**B** through **D**) Concentrated BRE conditioned media prepared in the presence (*lane 2*) or absence (*lanes 1* and *3*) of TGF-β were subjected to SDS PAGE under nonreducing conditions followed by Western blotting. Membranes were developed with anti-MMP-2 (**B**), anti-MMP-9 (**C**), and anti-TIMP-1 antibodies (**D**).

disease conditions. We have previously shown that in addition to VEGF, Müller cells express latent TGF-β, which is activated under hypoxic conditions. We show here that endothelial cells cocultured with either Müller cells or astrocytes also express MMP-9. This is consistent with previous findings that heterologous cell-to-cell contact activates TGF-β.[24,25] Also our data suggest that glial cell contact would increase permeability of endothelial cells by stimulating TGF-β activation and MMP-9 formation. However, whether this interaction contributes to angiogenesis needs to be verified. Evidently, at least under some in vitro conditions, TGF-β acts as an angiogenic-stimulating factor.[45]

Although it is shown here that endothelial cells themselves are the source of MMP-9, it needs to be emphasized that the physiologic and/or pathologic interactions of endothelial cells with peri-vascular supporting cells (pericytes, smooth muscle, and glial cells) play a major role in regulating enzyme expression, probably through releasing growth factors and cytokines or by direct cell-to-cell contact.[20–25,46,47] Tumors of glial-cell origin (astrocytoma and glioblastoma), which are dramatically vascularized, express very high levels of b-FGF, VEGF, and TGF-β.[48–50] Moreover, expression of MMPs and their inhibitors in human fetal astrocytes and a number of glioma cell lines have been demonstrated.[51,52] Nevertheless, the direct role of glial cells in regulating endothelial protease expression has not been addressed so far.

MMPs are secreted as proenzymes and are activated by autocatalytic cleavage or through cleavage by other proteases such as plasmin. In vitro, autocatalytic activation of MMP-9 is induced by organomercuric compounds such as APMA, which unfold and expose the cleavage site of the molecule.[5,37,53,54] Activation involves two consecutive steps generating 82- to 83-kDa and then 68-kDa molecules. The 68-kDa molecule is very unstable. The 82- to 83-kDa molecule, however, may bind TIMP to form a complex of slightly higher than 92 kDa, which is inactive, stable, and resists further cleavage. In our purification protocol, the arginine/acid washing steps remove TIMP before MMP-9 is eluted from the column. Incubation of purified MMP-9 or conditioned media with APMA rapidly generated both the 82- and 68-kDa enzymes. Total activation of MMP-9 was not possible in the purified preparation because upon long incubation, a large proportion of activated enzyme was degraded. Nevertheless, when added to BRE monolayer, both activated and the inactive forms of purified (TIMP-free) MMP-9 increased BRE permeability. This suggests that the enzyme is activated during incubation with endothelial cells.[55] Based on gel migration, it appears that the BRE-secreted MMPs are also in proenzyme forms. It has been suggested that MMP-9 is activated on the cell surface where it binds CD44 receptors.[55] These authors suggest that the presence of cell-bound enzyme accompanied by the activation of TGF-β promotes tumor angiogenesis and invasion.

In our permeability assay, both anti–TGF-β and anti–MMP-9 blocked TGF effects. The anti–MMP-9 blocking was not complete, probably because of incompetent neutralizing effects of antienzyme antibody. It is also possible that TGF-β–induced barrier breakdown involves additional mechanisms independent of MMP-9 production. Both glial and endothelial cells release latent TGF-β and coculturing, somehow activates TGF-β, [24,25] which in turn induces MMP-9 expression/release in BRE cells. It is also possible that glial cells induce MMP-9 expression by a direct cell-to-cell contact mechanism with no TGF-β involvement. Several lines of evidence support this hypothesis. First, anti–TGF-β–neutralizing antibody, which readily blocked the effect of exogenous TGF-β, did not abrogate the Müller cell–induced MMP-9 expression of BRE cells. Next, as shown in Figure 4, the effects of Müller cells and exogenous TGF-β are additive. Finally, Müller cells seem to have an active biological function in coculture because addition of Müller cell extract to BRE did not induce any MMP-9 activity (not shown).

The role of glial cell contact in angiogenic activation of endothelial cells has been described in some experimental models.[20,21] Regulation of protease activity by heterotypic cell-to-cell contact has also been indicated in experimental models of tumor metastasis, and the expression of MMP-9 in particular, has been associated with tumor cell invasion.[51,52] However, the exact nature of cell-to-cell interactions has not been explained in detail. Our BRE–glial coculture provides a powerful model to investigate mechanism of heterologous cell-to-cell interaction, communication, and induction of intracellular signaling. The role of cell adhesion molecules in this event needs further investigation.

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