Original Article

Proteolytic Degradation of VE-Cadherin Alters the Blood-Retinal Barrier in Diabetes

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OBJECTIVE—Increased vascular permeability due to alteration of the blood-retinal barrier (BRB) is one of the major complications in early diabetes. The aim of the present study was to determine whether diabetes alters the cellular expression and distribution of the adherens junction protein vascular endothelial (VE)-cadherin in retinal endothelial cells and if this alteration is mediated by proteinase activity.

RESEARCH DESIGN AND METHODS—Diabetes was induced in Brown Norway rats using streptozotocin, and retinal vascular permeability was measured by the Evans blue technique. The expression of matrix metalloproteinases (MMPs) and VE-cadherin was examined in isolated retinal vessels or cultured endothelial cells in response to diabetes and advanced glycation end products (AGEs). The cleavage of VE-cadherin from the endothelial cell surface was monitored by Western blotting following MMP or AGE treatment.

RESULTS—Retinal vascular permeability was significantly increased in rats following 2 weeks of diabetes coincident with a decrease of VE-cadherin expression. This increased vascular permeability could be inhibited with an MMP inhibitor. Treatment of endothelial cells with AGE-BSA led to a reduction of VE-cadherin staining on the cell surface and increased permeability, which was MMP mediated. Treatment of cells with specific MMPs or AGEs resulted in cleavage of VE-cadherin from the cell surface.

CONCLUSIONS—These observations suggest a possible mechanism by which diabetes contributes to BRB breakdown through proteolytic degradation of VE-cadherin. This may indicate a role for extracellular proteinases in alteration of the BRB seen in diabetic retinopathy. ***Diabetes*** **56:2380–2387, 2007**

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AGE, advanced glycation end product; BRB, blood-retinal barrier; BRM-VEC, bovine retinal microvessel endothelial cell; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; MMP, matrix metalloproteinase; RAGE, receptor for AGE; VE, vascular endothelial.

The normal permeability of the retinal microvasculature is tightly controlled by a series of complex interactions between adjacent endothelial cells and by additional interactions between the endothelial cells and other cell types in the retina, including vascular pericytes, astrocytes, and Müller glial cells (1). These interactions form the structural basis of the inner blood-retinal barrier (BRB), which facilitates normal functioning of the neural retina. Disruption of these interactions is anticipated to lead to a compromise of the barrier function, resulting in leakage of plasma constituents into the surrounding retinal tissues and retinal edema. The alteration of the BRB followed by retinal edema are key early events in the pathogenesis of diabetic retinopathy, and macular edema is the leading cause of vision loss in diabetic patients.

Endothelial cells of the retinal microvasculature contribute to the function of the BRB through the formation of specialized intercellular junctions including adherens and tight junctions. The cadherin family of proteins, of which there are more than 80 members, is a primary component of the adherens junctions (2). The vascular endothelial (VE)-cadherin mediates calcium-dependent homophilic adhesion between endothelial cells. In addition, VE-cadherin functions as a plasma membrane attachment site for the cytoskeleton through its interactions with the cytoplasmic proteins β- and γ-catenin (3–5). In addition to contributing to the mechanical attachment of cells, VE-cadherin also plays a role in the regulation of a variety of endothelial cell behaviors including motility, morphogenesis, responsiveness to growth factors, and cell survival (6,7). The integrity of the adherens junctions between adjacent endothelial cells is also thought to be critical to normal barrier function (8). The maintenance and regulation of this barrier is likely to involve interactions between VE-cadherin and the tight junction proteins occludin and claudin-5.

Increased endothelial permeability may involve the production and activation of specific extracellular proteinases (9–12). The role of matrix metalloproteinases (MMPs) in the maintenance of systemic vessel integrity and remodeling has been well documented in animal models of vascular morphogenesis and angiogenesis (13–15). The MMPs are zinc-dependent proteinases that are capable of degrading numerous structural components of the ECM (extracellular matrix) and a variety of non-ECM proteins (16). We have previously reported on the upregulation of MMPs in the diabetic retina and their possible role in the proteolytic breakdown of the tight junction protein occludin (17). The present study extends this hypothesis by

examining the expression of MMPs in response to diabetes and the role of these enzymes in the proteolytic turnover of other important components of the BRB.

Changes in the function of the retinal vasculature and the progression of diabetic retinopathy may be mediated by the presence of specific reactive metabolites including sorbitol, reactive oxygen species, and advanced glycation end products (AGEs). AGEs are a heterogeneous group of highly glycosylated proteins that are strongly implicated in the pathogenesis of diabetic retinopathy (18–22). The response of cells to stimulation with AGEs is mediated by the receptor for AGEs (RAGEs) found on the surface of endothelial cells (23,24). However, a better understanding of the downstream mechanisms mediated by AGE and RAGE leading to the pathophysiological phenomena seen in retinopathy warrants further study.

The objectives of this study were to determine a potential role for AGE in the structural modification of the BRB during the early stages of diabetes and to determine whether this effect was mediated through the production and activation of specific MMPs. Results suggest a novel role for MMPs in altering BRB function by modifying the endothelial cell surface presentation of VE-cadherin following AGE stimulation.

RESEARCH DESIGN AND METHODS

Reagents. Brown Norway rats were purchased from Harlan Laboratories. The glycohemoglobin assay kit was purchased from Stanbio. Bovine retinal microvascular endothelial cells and MCDB 131 complete media were purchased from VEC Technologies. Fibronectin-coated dishes were obtained from Becton Dickinson. Glycoaldehyde-modified AGE-BSA was purchased from Calbiochem. Rabbit polyclonal antibody to VE-cadherin was purchased from Alexis. Purified MMP-2 and MMP-9 were purchased from R&D Systems. The metalloproteinase inhibitor BB-94 was provided by British Biotech. All other reagents were purchased from Sigma-Aldrich, unless otherwise noted.

Diabetic animal model. Brown Norway rats were injected with a single intraperitoneal injection of streptozotocin (60 mg/kg i.p.) in 10 mmol/l citrate buffer, pH 4.5. Control nondiabetic rats received injections of an equal volume of citrate buffer only. Animals with plasma glucose concentrations >250 mg/dl, 24–48 h after streptozotocin injection, were considered diabetic and included in these studies. Body weight was measured twice each week, and insulin (0.5 IU) was given based on the stability of body weight. The tolerated maximum difference in weight between diabetic and nondiabetic rats was 40%. Total glycated hemoglobin (GHb) was measured in each animal at the end of the 2- or 8-week period. All experiments were consistent with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were done in accordance with institutional animal care and use guidelines.

Cell culture. Experiments were performed using bovine retinal microvessel endothelial cells (BRMVECs). The endothelial cells were grown in MCDB-131 media containing 10% fetal bovine serum, endothelial cell growth supplement, and antibiotics. Passages 3–8 were used for all experiments.

Quantitative assessment of BRB permeability. Vascular permeability in the retina was measured following 2 and 8 weeks of diabetes using an Evans blue quantitation technique (25). Briefly, rats were anesthetized and received an intravenous injection of 45 mg/kg Evans blue dye. Two hours later, the animal was reanesthetized, a 0.3-cc blood sample was obtained, and the animal was perfused via the left ventricle with PBS followed by 1% paraformaldehyde. The retinas were collected in PBS, dried for 2 h, and weighed. The Evans blue dye was extracted from the dried retinas with formamide at 70°C for 18 h and centrifuged through a 30,000-MW filter (Millipore, Bedford, MA). The absorbance of the retinal extract and a 1:1,000 dilution of plasma was measured by spectrophotometry at 620 and 740 nm. The concentration of dye in the extract and plasma was calculated from a standard curve of Evans blue dye. The BRB permeability was calculated as follows, and data were expressed as $\mu l \cdot mg^{-1} \cdot h^{-1}$: Evans blue retina (ng)/Evans blue plasma (ng/μl) × circulation time × retina dry weight (mg).

In some experiments, normal nondiabetic rats were anesthetized and microinjected intraocularly with 5 μg VE-cadherin antibody or PBS in 1 μl (three eyes each from three different animals). Sham animals received a needle puncture only (four eyes from two different animals). Permeability was assessed 2 h later.

Semiquantitative real-time PCR. Retinal vasculature was selectively isolated from other components of the retina using a procedure previously described (26). Briefly, retinas were incubated in ice-cold sterile water for 1 h at 4°C followed by incubation with RQ DNase for 5 min. Microvascular networks were transferred to ice-cold water and cleaned of debris by gentle pipetting with a wide-bore pasteur pipette. Total mRNA was extracted from the microvascular networks using Trizol reagent (Invitrogen, Carlsbad, CA), and the integrity was confirmed by agarose gel electrophoresis. cDNA was synthesized using the TaqMan Reverse-Transcriptase reagents (Applied Biosystems, Foster City, CA). The following TaqMan assays for real-time RT-PCR were obtained from Applied Biosystems: VE-cadherin, RatCad5-RC50; MMP-2, Rn02532334; MMP-9, Rn00579162; and 18S, Hs99999901. Amplification and detection was performed using the Applied Biosystems 7500 Fast system. Data were derived using the comparative C_t method for triplicate reactions (27).

Western blot analysis. Endothelial cells were incubated in serum-free media for 24 h with AGE-BSA (50 μg/ml) with or without the metalloproteinase inhibitor BB-94 (2 μmol/l). Additional cultures were incubated for 2 h in serum-free media with either purified MMP-2 (0.1 μg/ml) or MMP-9 (0.1 μg/ml). The conditioned media from the various treatments were concentrated using centricon filters (30 kDa cutoff) and the total protein quantitated using the MicroBCA assay (Pierce). Equal amounts of total protein were loaded onto a 10% SDS-PAGE.

Proteins were transferred onto polyvinylidine fluoride membrane and probed with an antibody to VE-cadherin. The bands were visualized using a chemiluminescent detection kit (Pierce, Rockford, IL) and the GNOME-GENESNAP advanced image acquisition software (Syngene, Frederick, MD).

Proteinase assays. Levels and degree of activation of MMP-2 and MMP-9 were examined in cells by zymography. The conditioned media from cells cultured in serum-free media were collected after 48 h, and aliquots were removed for total protein determinations. Conditioned medium was subjected to electrophoresis in 10% polyacrylamide mini-gels, into which gelatin (1 mg/ml) was cross-linked. Zones of clearing corresponding to the presence of proteinases in the samples were quantitated using the Alpha Innotech Image analysis system.

In vitro permeability assays. BRMVECs were seeded onto fibronectin-coated Transwell inserts (0.4-μm pore size) and grown in complete media for 5 days. The media was changed every alternate day in the upper chamber until confluency was reached. The functional confluency of the cells was determined by measuring the TER (transendothelial resistance). Confluent BRMVECs exhibited a TER reading of 220 ± 10 ohms, which reflected a functional barrier in these cells. The cells were changed to serum-free media, and 5 μl of 40 mg/ml fluorescein isothiocyanate (FITC)-dextran (40,000 MW) was added to the top chamber. Different wells received AGE-BSA (50 μg/ml) with or without BB-94 (2 μmol/l). Fifty-microliter aliquots of media were removed from the bottom chamber at 0, 4, 24, and 48 h and analyzed by quantitative fluorimetry at 485 nm. In some experiments, cells were incubated with purified MMP-9 (0.1 μg/ml) and the media collected at 0, 2, and 4 h. Rates of permeability were calculated and the data expressed as fluorescence units per hour.

Immunofluorescence microscopy. BRMVECs were passed onto glass coverslips coated with fibronectin and allowed to reach confluence. Cells were stimulated for 48 h with AGE-BSA (50 μg/ml) with or without BB-94 inhibitor (2 μmol/l). The cells were fixed with 4% paraformaldehyde for 5 min and stained with an anti–VE-cadherin antibody. The cells were examined using a fluorescence microscope, and images were captured and pseudo-colored using the MetaMorph Image Analysis program.

Microvessels in the retinas of 2- or 8-week-old diabetic and control animals were stained for VE-cadherin in retinal whole mounts. Briefly, retinas were isolated and fixed in 100% ethanol, delipidated in 100% ice-cold acetone, and permeabilized with 0.3% Triton X-100. Samples were incubated with an antibody to VE-cadherin at 4°C and washed extensively. VE-cadherin was localized using an FITC-labeled secondary antibody and *Z*-stack images obtained using a confocal microscope (LSM 510 META; Carl Zeiss Meditec, Gottingen, Germany) and analyzed qualitatively for differences in VE-cadherin staining. Analysis was limited to the mid-peripheral region of the retina and up to 12 separate areas per retina photographed.

Cellular enzyme-linked immunosorbent assay. The expression of VE-cadherin on the cell surface was determined by a cellular enzyme-linked immunosorbent assay (ELISA). Confluent monolayers of BRMVEC on 24-well plates were untreated or treated with purified MMP-2 (0.1 μg/ml for 2 h), MMP-9 (0.1 μg/ml for 2 h), AGE-BSA (50 μg/ml for 24 h), or AGE-BSA plus BB-94 inhibitor (2 μmol/l for 24 h). After treatment, the cells were fixed with 0.025% glutaraldehyde for 5 min and washed extensively with PBS. Cells were incubated overnight at 4°C with a VE-cadherin antibody (2.5 μg/ml). Cells were washed extensively and incubated for 1 h at room temperature with a horseradish peroxidase–labeled anti-rabbit antibody. The cells were washed, and the level of VE-cadherin on the cell surface was colorimetrically estimated

following the addition of *o*-phenylenediamine dihydrochloride substrate and measuring the absorbance at 492 nm. Controls included cells incubated with secondary antibody only and cells incubated with the colorimetric substrate only.

Statistical methods. For all quantitative experiments, statistical analyses of data were performed using either an unpaired *t* test or a one-way ANOVA using the Prism4 software (GraphPad Software).

RESULTS

Rats were injected with streptozotocin to induce diabetes and were used 2 or 8 weeks later for these studies. Animals with blood glucose levels >250 mg/dl were considered diabetic and were included in the study. The blood glucose levels for the nondiabetic animals averaged 167 ± 17 mg/dl and animals with diabetes 450 ± 44 mg/dl. The ratio of glycated hemoglobin to total hemoglobin in blood samples was elevated almost twofold in diabetic animals compared with nondiabetic controls at the end of both 2 and 8 weeks.

The degree of retinal vascular permeability was determined using Evans blue dye, a technique that effectively measures the movement of albumin from the plasma into the surrounding tissues. Retinal vascular permeability was significantly increased in the diabetic animals (0.055 ± 0.0034 $\mu l \cdot mg^{-1} \cdot h^{-1}$) compared with the nondiabetic controls (0.030 ± 0.0035 $\mu l \cdot mg^{-1} \cdot h^{-1}$) following 2 weeks of hyperglycemia ($P = 0.0002$). Similarly, at 8 weeks of diabetes, the permeability of the retinal vasculature in the diabetic animals (0.067 ± 0.0031 $\mu l \cdot mg^{-1} \cdot h^{-1}$) was significantly increased compared with the nondiabetic controls (0.037 ± 0.0027 $\mu l \cdot mg^{-1} \cdot h^{-1}$) ($P = 0.0013$).

The presence and distribution of VE-cadherin in the retinal microvasculature of control and diabetic rats were evaluated by immunohistochemistry and confocal microscopy. In nondiabetic rat retinas, the small (8- to 10-μm diameter) retinal vessels were positive for VE-cadherin staining (Fig. 1*A* and *B*). VE-cadherin was distributed along the endothelial intercellular junctions with no obvious areas of discontinuity. In contrast, after only 2 weeks of diabetes, the small vessels of the diabetic retinas demonstrated a substantial loss of VE-cadherin staining (Fig. 1*C*). The loss of VE-cadherin in the microvessels persisted following 8 weeks of diabetes (Fig. 1*D*). The decreased expression of VE-cadherin protein in the diabetic retina at 2 weeks was also associated with a significant downregulation in the level of VE-cadherin mRNA in isolated retinal microvessels (Fig. 1*E* and *F*).

To determine whether disruption of VE-cadherin alone alters retinal vascular permeability, normal nondiabetic rats received a single intravitreal injection of anti–VE-cadherin antibody (5 μg/μl) and were analyzed 2 h later for changes in vascular permeability using the Evans blue dye technique. The antibody recognizes the VE-cadherin ectodomain (AA1–258) and causes a disruption of VE-cadherin engagement in a confluent monolayer of bovine retinal endothelial cells, resulting in cell-cell separation (Fig. 2*A* and *B*). A nearly fivefold increase in retinal vascular permeability was detected in the antibody-injected eyes compared with the vehicle-treated or sham-injected eyes, suggesting a crucial role for VE-cadherin in maintaining BRB integrity (Fig. 2*C*).

We next examined the expression of MMPs in the retinal vasculature to determine whether the loss of VE-cadherin protein and increased retinal vascular permeability might, in part, be due to proteolytic turnover. We have previously shown that MMPs are upregulated in the retinas of animals after 12 weeks of diabetes (17). In animals with only 2 weeks of diabetes, the levels of MMP-2 and MMP-9 mRNA in isolated retinal vessels were also significantly increased compared with nondiabetic controls (Fig. 3).

To confirm a role for MMPs in disruption of BRB function, we assessed the ability of a MMP inhibitor to block the increases in retinal vascular permeability after 2 weeks of diabetes. The broad spectrum inhibitor BB-94 has been shown to effectively inhibit the MMP-induced retinal neovascularization in an animal model of oxygen-induced retinopathy (15). After daily intraperitoneal administration of BB-94 (1 mg/kg) to diabetic animals, the degree of retinal vascular permeability was nearly twofold lower compared with diabetic animals receiving PBS injections only (Fig. 4*A*). This functional change in permeability, in response to MMP inhibition, coincided with a structural change in the interendothelial junctions of the microvessels. The inhibition of MMPs prevented the loss of VE-cadherin from the cell surface of adjacent endothelial cells. VE-cadherin was present in a continuous uninterrupted pattern in the inhibitor-treated diabetic retinas similar to that seen in the nondiabetic animals (compare Fig. 4*B* with Fig. 1*A* and *B*).

The previous results suggest that during the development of diabetic retinopathy, hyperglycemia creates an environment that facilitates increased microvascular permeability associated with the upregulation of MMP expression and the loss of VE-cadherin. Several biochemical mechanisms have been proposed to explain the changes seen in the diabetic retina, including the formation and activity of AGE. The role of AGE in the regulation of endothelial barrier function was assessed using isolated bovine retinal microvascular endothelial cells. Cultured endothelial cells incubated with 50 μg/ml AGE-BSA demonstrated a significant increase in the rate of monolayer permeability compared with untreated cells (Fig. 5). This effect was found to be mediated by MMPs, as simultaneous treatment of cells with AGE-BSA and an inhibitor for MMPs resulted in a reduction of permeability toward control untreated levels. AGE stimulation could also account for the alterations in proteinase expression seen in the vasculature of the diabetic retina. The conditioned media of BRMVECs treated with AGE-BSA was analyzed by zymography and demonstrated a marked increase in both the pro and active forms of MMP-2 and MMP-9 after 48 h of stimulation (Fig. 6).

We next examined whether alterations in monolayer permeability observed in AGE-treated endothelial cells coincided with a change in VE-cadherin distribution. Control untreated cells displayed a continuous pattern of VE-cadherin staining associated with the lateral cell borders (Fig. 7*A*). Treatment with AGE-BSA caused a disruption in the pattern of VE-cadherin staining on the cell surface (Fig. 7*B*). The VE-cadherin staining was found to be punctate as opposed to continuous and was absent from regions where cell-cell contact was lost. Cells treated simultaneously with AGE-BSA and the MMP inhibitor demonstrated cellular morphology and VE-cadherin staining similar to untreated cells (Fig. 7*C*). This result suggests a possible mechanism by which AGE-BSA might alter adherens junctions in endothelial cells through the upregulation and activation of MMPs, leading to increased vascular permeability.

Retinal endothelial cells treated with AGE-BSA or purified MMPs release the VE-cadherin ectodomain while undergoing cell-cell separation. The ectodomain fragment of VE-cadherin was found in the conditioned media with a

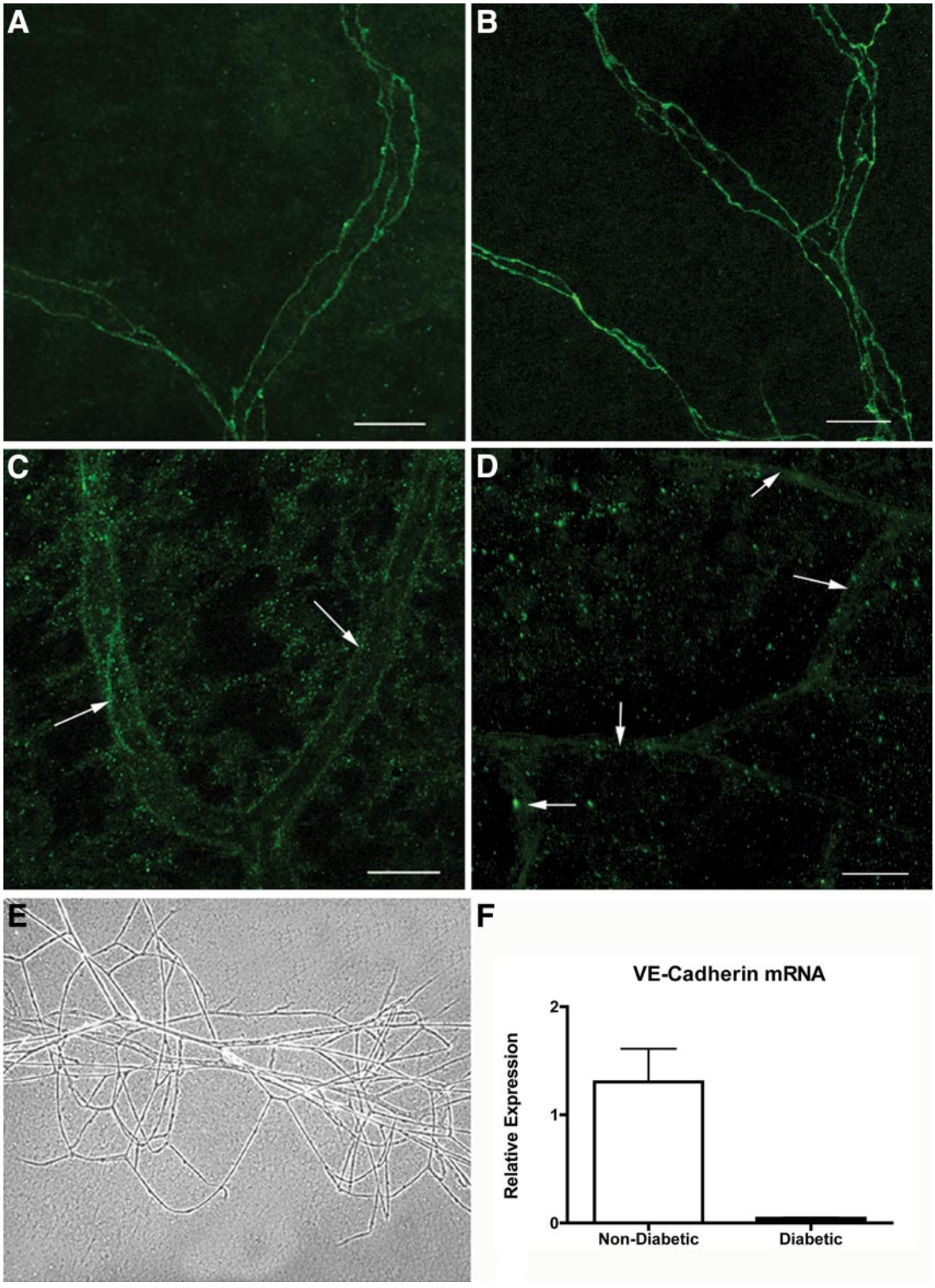

FIG. 1. VE-cadherin protein and mRNA are decreased in the retinal vessels of diabetic rats. Representative retinal whole mounts from control and diabetic rats stained with anti–VE-cadherin antibody and examined by confocal microscopy. VE-cadherin expression was evident at the interfaces between adjacent endothelial cells in control nondiabetic animals (*A* and *B*), while it was mostly obliterated from the microvessels in the diabetic retina at 2 weeks (*C*) and 8 weeks (*D*) of diabetes. Images shown are reconstructions of a series of *Z*-stacks of retinal blood vessel segments. Vascular networks were isolated from whole retinas of diabetic and nondiabetic animals as described in RESEARCH DESIGN AND METHODS. A phase-contrast image of such a network is shown in *E*. Total RNA was isolated and semiquantitative real-time RT-PCR performed. Vessels from diabetic animals demonstrated less VE-cadherin mRNA than vessels from nondiabetic controls (*F*). *Significantly less than nondiabetic controls ($P = 0.0002$). $n = 3$ animals for staining and PCR analysis. Bar = 25 μm. (Please see http://dx.doi.org/10.2337/db06-1694 for a high-quality digital representation of this figure.)

molecular weight of ~75 kDa. The shedding of this portion of the protein appears to be a normal part of cadherin turnover in cells. This was demonstrated by the appearance of the fragment in media collected from cells with no treatment (Fig. 8*A*). Cells treated with purified MMP-9 or AGE-BSA demonstrated a significant increase in the

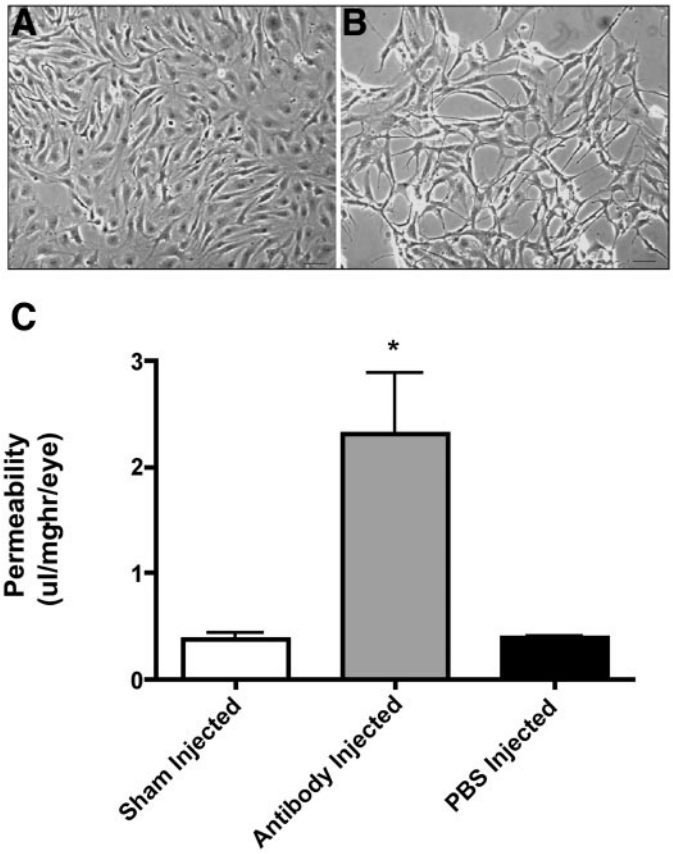

FIG. 2. Disruption of VE-cadherin causes endothelial cell separation and an increase in retinal permeability. Representative figures of confluent BRMVEC monolayers treated with an antibody directed to the VE-cadherin ectodomain (100 ng/ml). The antibody disrupts VE-cadherin in cells and causes cell-cell separation within 2 h (*A*, untreated; *B*, antibody treated; duplicates of $n = 3$ separate cultures). *C*: Normal nondiabetic rats received a 1-μl intravitreal injection of anti–VE-cadherin antibody (5 μg) in the left eye and an equal volume of PBS in the right. The permeability of the retinal vasculature as measured by Evans blue dye was nearly fivefold higher (129.6 ± 4.17; $n = 3$) in antibody-injected eyes compared with PBS (27.67 ± 2.8; $n = 3$)- or sham (25.71 ± 2.6; $n = 4$)-injected eyes. Values are the means ± SD for each group. Differences among means were tested by ANOVA and corrected using the Bonferroni post test. Values for $P < 0.05$ were considered significant. Antibody injected vs. PBS injected or sham injected, $P < 0.001$; sham injected vs. PBS injected, $P > 0.05$.

amount of 75 kDa fragment released into the media. Cells treated with purified MMP-2 did not show the same degree of shedding, suggesting a degree of specificity to the process. The addition of BB-94 (2 μmol/l) to the AGE-BSA–treated cells resulted in a significant decrease in the level of shedding comparable with control conditions. The release of the VE-cadherin ectodomain into the media was consistent with a loss of VE-cadherin from the cell surface as determined by cellular ELISA (Fig. 8*B*). These results suggest that AGE causes a significant shedding of the VE-cadherin ectodomain through a specific MMP-dependent mechanism, which may lead to increased vascular permeability.

DISCUSSION

Diabetic retinopathy is one of the most common and most severe complications of diabetes, affecting ~50% of all patients with diabetes (28,29). Breakdown of the BRB is an early feature of diabetic retinopathy and results in vascular leakage and the development of macular edema, which can cause distortion and loss of central vision (30–32). The molecular mechanisms that regulate BRB remodeling during diabetes remain unclear. The data presented here suggest that AGE stimulation results in the upregulation of proteinases that cleave VE-cadherin from the retinal endothelial cell surface, leading to increased vascular permeability.

In the present study, we have observed a significant decrease in the degree of VE-cadherin staining of the retinal vasculature coincident with an increase in vascular permeability. This was associated with a significant downregulation of VE-cadherin mRNA in isolated microvessels from the diabetic animals. A similar finding of decreased VE-cadherin has been reported in the retina of a human patient with nonproliferative diabetic retinopathy. Interestingly, this study also demonstrated that other junctional proteins involved in the formation and function of the BRB, including occludin and ZO-1, showed normal patterns of distribution (33). The present study is the first report demonstrating the loss of VE-cadherin and altered BRB function during early stages of diabetic retinopathy. Decreases in occludin levels have been reported in the diabetic rat retina following 3 or 4 months of diabetes (34,35). Whether the distribution of occludin and other tight junction proteins are altered at 2 weeks of diabetes is unknown.

The importance of VE-cadherin in maintenance of the BRB was confirmed in this study as vascular leakage was increased nearly fivefold 2 h following a single intravitreal injection of a VE-cadherin disrupting antibody. This dramatic increase in permeability upon disrupting VE-cadherin alone involves a major alteration of the BRB including the apparent disruption of tight junction proteins. Cadherins, including VE-cadherin, may interact with occludin and/or claudin proteins through specific intracellular signaling molecules to regulate the function of these proteins and the interendothelial barrier (36). Previous

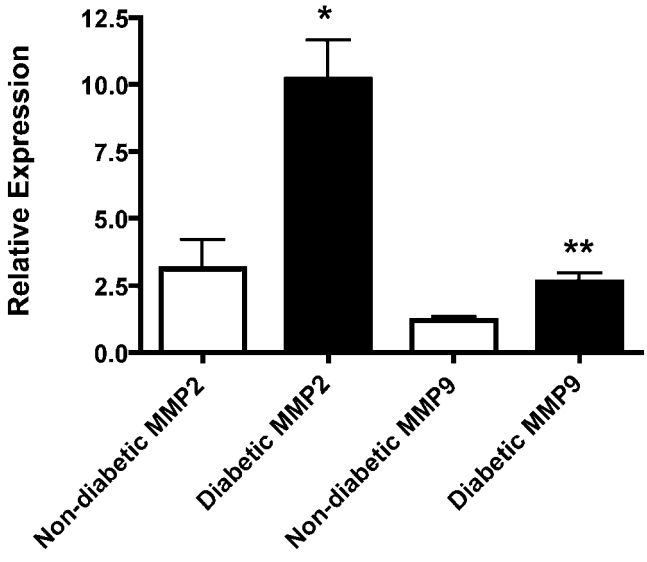

FIG. 3. MMP2 and MMP9 mRNA levels are increased in the retinal vasculature following 2 weeks of diabetes. Vascular networks were isolated from whole retinas of diabetic ($n = 4$) and nondiabetic ($n = 6$) animals as described in RESEARCH DESIGN AND METHODS. Total RNA was isolated and semiquantitative real-time RT-PCR performed using the comparative C_t method of quantitation. Vessels from diabetic animals demonstrated significantly more MMP2 and MMP9 mRNA than vessels from nondiabetic controls. *Significantly greater than nondiabetic controls ($P = 0.0168$). **Significantly greater than nondiabetic controls ($P = 0.0237$).

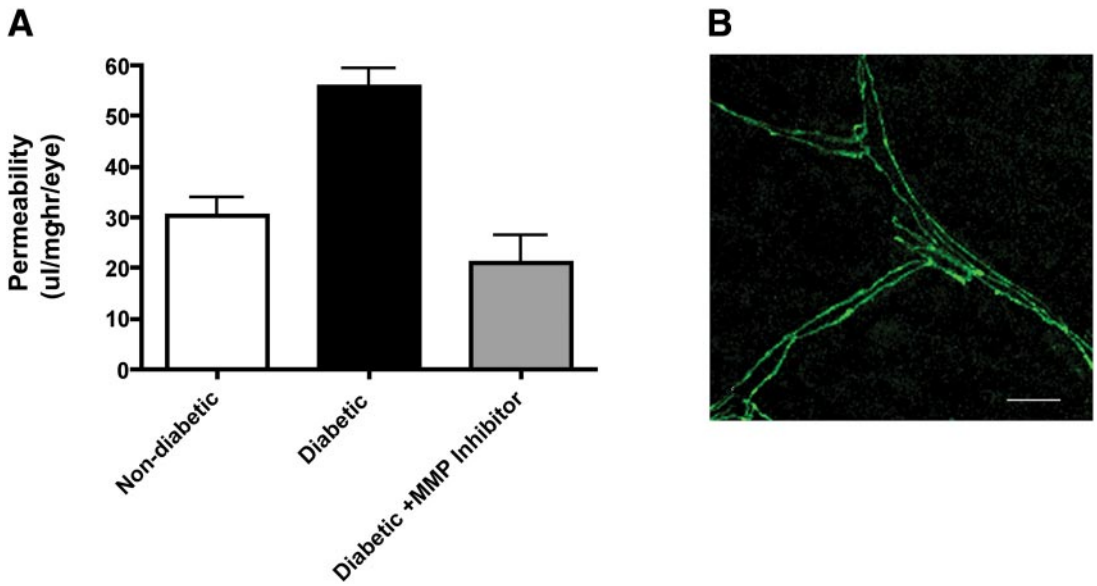

FIG. 4. MMP inhibitor blocks retinal vascular permeability in the diabetic rat. Diabetic rats received an intraperitoneal injection of the MMP inhibitor BB-94 (1 mg/kg) daily for 2 weeks. Retinal vascular permeability was quantitated using the Evans blue assay (*A*). The permeability of the retinal vasculature was nearly twofold lower in BB-94–injected diabetic rats (21.11 ± 5.394; $n = 4$) compared with vehicle-injected diabetic rats (55.98 ± 3.479; $n = 9$). Values are the means ± SD for each group. Differences among means were tested by ANOVA and corrected using the Bonferroni post test. Values for $P < 0.05$ were considered significant. Diabetic vs. nondiabetic, $P < 0.01$; diabetic + MMP inhibitor vs. diabetic, $P < 0.01$; nondiabetic vs. diabetic + MMP inhibitor, $P > 0.05$. *B*: Representative image of retinal whole mount demonstrating VE-cadherin staining in the microvessels of a diabetic rat treated with BB-94 for 2 weeks. Bar = 25 μm. (Please see http://dx.doi.org/10.2337/db06-1694 for a high-quality digital representation of this figure.)

studies have also reported that VE-cadherin is not only important for regulating interendothelial cell permeability but also for cell survival and angiogenesis, which may involve similar mechanisms (6,37,38).

The turnover or remodeling of VE-cadherin in interendothelial junctions may involve different mechanisms depending on the particular stimulus. A recent study describes a mechanism by which vascular endothelial growth factor and its receptor induces the rapid internalization and recycling of VE-cadherin (39). This mechanism may predominate in situations requiring a rapid and reversible alteration of vascular permeability. Proteolytic modification of VE-cadherin on the cell surface, as seen in the retina during diabetes, may represent an alternative mechanism initiated in response to a sustained pathological stimulus. Previous studies have demonstrated that E- and N-cadherin can be cleaved by MMPs in a variety of circumstances involving pathological vascular remodeling (40–44). In the present study, we have reported that the incubation of cultured retinal microvascular endothelial cells with purified MMPs results in an MMP-specific shedding of the VE-cadherin ectodomain and loss of cell-cell contact.

The VE-cadherin molecule has four highly conserved cadherin repeat domains that are used to negotiate cell-cell adhesion. MMP-9 is known to cleave specific peptide bonds such as Gly-Val, Gly-Leu, Gly-Asn, and Gly-Ser (45). The VE-cadherin extracellular repeats have one Gly-Leu

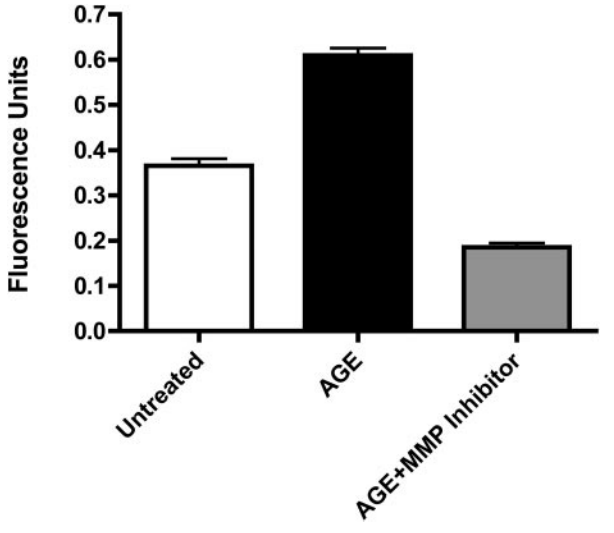

FIG. 5. AGE stimulation increases endothelial cell monolayer permeability that is reduced by an MMP2/MMP9 inhibitor. The permeability of confluent BRMVEC monolayers was determined using FITC-dextran as described in RESEARCH DESIGN AND METHODS. Cells were incubated with or without 50 μg/ml AGE-BSA in the presence or absence of the MMP inhibitor BB-94 (2 μmol/l). Values represent means ± SD from triplicate wells. Differences among means were tested by ANOVA and corrected using the Bonferroni post test. Values for $P < 0.05$ were considered significant. Untreated vs. AGE, $P < 0.01$; untreated vs. AGE + MMP inhibitor, $P > 0.05$; AGE vs. AGE + MMP inhibitor, $P < 0.001$.

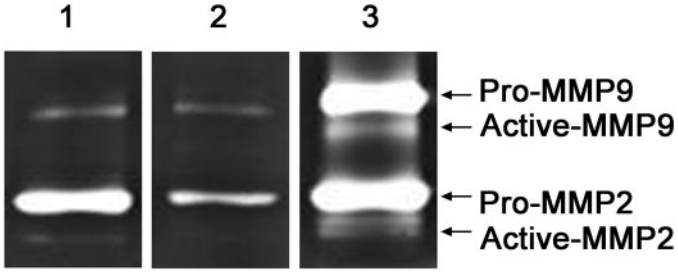

FIG. 6. AGE stimulation increases MMP2 and MMP9 secretion and activation in cultured retinal endothelial cells. Representative gelatin zymogram of the conditioned media collected from untreated cells (*lane 1*), cells treated with 50 μg/ml BSA (*lane 2*), and cells treated with 50 μg/ml AGE-BSA (Calbiochem) (*lane 3*). Following 48 h of AGE treatment, an increase in production of both the pro and activated forms of the enzymes was seen. Image represents different parts of the same gel.

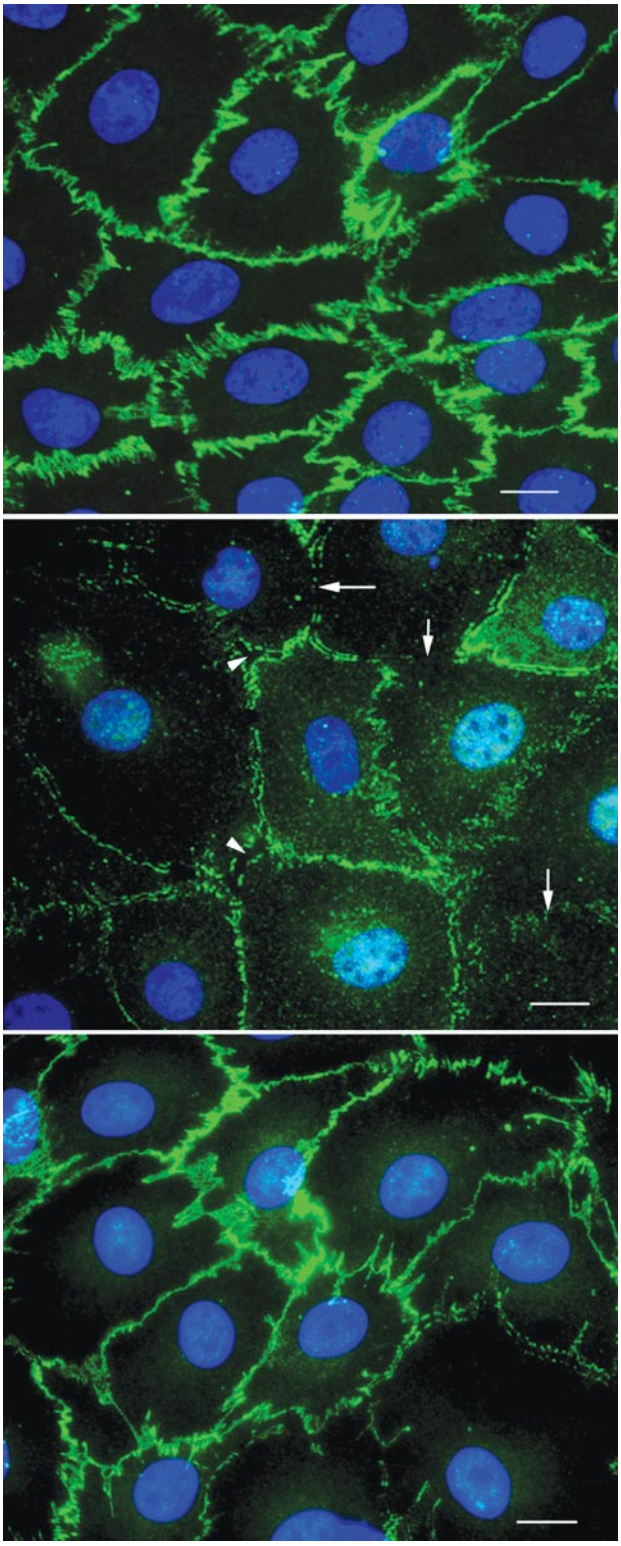

FIG. 7. AGE stimulation alters endothelial cell morphology and cell surface expression of VE-cadherin. Representative images of bovine retinal endothelial cells cultured in serum-free media for 48 h under various treatment conditions, fixed and stained for VE-cadherin. The treatment conditions were serum-free media alone (*A*), AGE-BSA (50 μg/ml) (*B*), and AGE-BSA (50 μg/ml) with BB-94 (2 μmol/l) (*C*). Disruption of the continuous pattern of VE-cadherin staining (arrows) and the presence of open gaps between adjacent cells (arrow heads) are seen in cultures treated with AGE-BSA. $n = 3$ cultures from two separate experiments. Bar = 10 μm. (Please see http://dx.doi.org/10.2337/db06-1694 for a high-quality digital representation of this figure.)

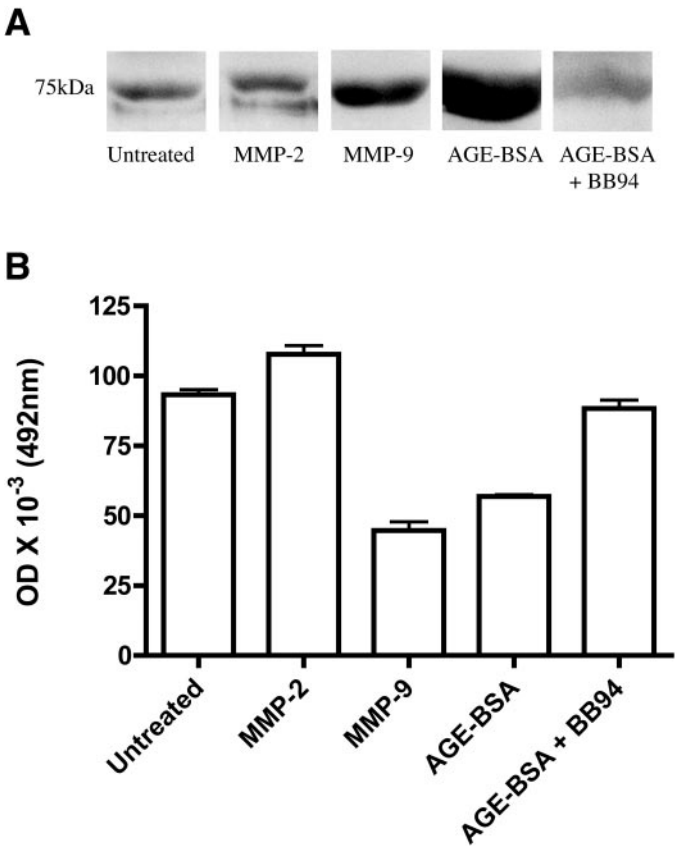

FIG. 8. The VE-cadherin ectodomain is released from cells in response to AGE-BSA stimulation and treatment with purified MMPs. *A*: The proteolytic degradation product of VE-cadherin (~75 kDa ectodomain) was detected by Western blotting of the conditioned media from untreated cells or cells treated with MMP-2 (0.1 μg/ml, 2 h), MMP-9 (0.1 μg/ml, 2 h), AGE-BSA (50 μg/ml, 24 h), or AGE-BSA in the presence of BB-94 (2 μmol/l, 24 h). Equal amounts of total protein were loaded in each lane. Representative blot of three replicate experiments. Image represents different parts of the same blot. *B*: Cell surface VE-cadherin content was measured by cellular ELISA. Bar graph shows the effect of the various treatments on VE-cadherin expression. All data points represent the means ± SD of three wells. Differences among means were tested by ANOVA and corrected using the Bonferroni post test. Values for $P < 0.05$ were considered significant. Cells treated with MMP9 and AGE showed significantly less cell surface VE-cadherin compared with all other treatments ($P < 0.001$). No other significant differences were found.

residue and five Gly-Ser residues within amino acids 51–576. It is possible that MMP-9 can cleave VE-cadherin at one or more of these sites, resulting in shedding of parts of or the entire ectodomain. A detailed mapping of the proteolytic fragments using purified VE-cadherin is necessary to confirm the exact sites of cleavage.

Hyperglycemia and other diabetic metabolites, including AGEs, are known to have negative effects on the retinal microvasculature. Numerous studies have localized AGEs to the retinal microvessels and basement membrane in both humans and rats (46). These factors can also affect MMP expression and activity (17,47,48). In this study, we examined the role of AGE in MMP upregulation by vascular endothelial cells and in alteration of VE-cadherin expression. Incubation of cells with AGE-BSA resulted in the upregulation of MMPs, caused the shedding of VE-cadherin in an MMP-dependent manner, and increased monolayer permeability. The mechanism by which AGE upregulates MMPs is currently unknown. One hypothesis is that AGE interacts with its receptor RAGE on the endothelial cell surface and directly induces the expres-

sion of MMPs through the activation of nuclear factor-kB or other signaling molecules. Another possibility is the indirect upregulation of proteinases by AGE through increased growth factor synthesis and activity (49).

Data from this study confirm an important role for specific extracellular proteinases in alteration of the BRB during the development of diabetic retinopathy. The ability of a broad-spectrum MMP inhibitor to block retinal permeability changes substantiates the direct involvement of MMPs in the breakdown of the BRB. This result suggests a potentially useful alternative therapeutic approach to the treatment of macular edema in diabetic retinopathy.

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