Characterization of Azurocidin as a Permeability Factor in the Retina: Involvement in VEGF-Induced and Early Diabetic Blood-Retinal Barrier Breakdown

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PURPOSE. Azurocidin, released by neutrophils during leukocyte-endothelial interaction, is a main cause of neutrophil-evoked vascular leakage. Its role in the retina, however, is unknown.

METHODS. Brown Norway rats received intravitreal injections of azurocidin and vehicle control. Blood-retinal barrier (BRB) breakdown was quantified using the Evans blue (EB) dye technique 1, 3, and 24 hours after intravitreal injection. To block azurocidin, aprotinin was injected intravenously before the intravitreal injections. To investigate whether azurocidin plays a role in vascular endothelial growth factor (VEGF)-induced BRB breakdown, rats were treated intravenously with aprotinin, followed by intravitreal injection of VEGF164. BRB breakdown was quantified 24 hours later. To investigate whether azurocidin may mediate BRB breakdown in early diabetes, aprotinin or vehicle was injected intravenously each day for 2 weeks to streptozotocin-induced diabetic rats, and BRB breakdown was quantified.

RESULTS. Intravitreal injection of azurocidin (20 μg) induced a 6.8-fold increase in vascular permeability compared with control at 1–3 hours (P < 0.05), a 2.7-fold increase at 3 to 5 hours (P < 0.01), and a 1.7-fold increase at 24 hours (P < 0.05). Aprotinin inhibited azurocidin-induced BRB breakdown by more than 95% (P < 0.05). Furthermore, treatment with aprotinin significantly suppressed VEGF-induced BRB breakdown by 93% (P < 0.05) and BRB breakdown in early experimental diabetes by 40.6% (P < 0.05).

CONCLUSIONS. Azurocidin increases retinal vascular permeability and is effectively blocked by aprotinin. The inhibition of VEGF-induced and early diabetic BRB breakdown with aprotinin indicates that azurocidin may be an important mediator of leukocyte-dependent BRB breakdown secondary to VEGF. Azurocidin may become a new therapeutic target in the treatment of retinal vascular leakage, such as during diabetic retinopathy. (Invest Ophthalmol Vis Sci. 2008;49:726–731) DOI:10.1167/iovs.07-0405

Firm adhesion of neutrophils to the inflamed endothelium is a cause of vascular leakage.1–3 Vascular endothelial growth factor (VEGF) is a hypoxia-induced angiogenic factor,4,5 and a major vasopermeability factor.6–7 VEGF is causally linked to the pathogenesis of diabetic retinopathy, playing an important role in leukocyte-mediated breakdown of the blood-retinal barrier (BRB) and retinal neovascularization.8 Within the first 2 weeks of experimental diabetes in rats, retinal VEGF levels increase with associated upregulation of intracellular adhesion molecule (ICAM)-1 in retinal endothelial cells and its ligands, the β2-integrins, on the surfaces of peripheral blood neutrophils.9–11 These molecular events result in an increased adhesion of leukocytes, predominantly neutrophils, and a concomitant increase in retinal vascular permeability.9–11

Intravitreal injection of VEGF induces the retinal vascular changes seen in experimental diabetes, including retinal leukostasis and concomitant BRB breakdown, whereas blockade of VEGF abolishes retinal leukostasis and vascular leakage in experimentally induced diabetes.8,9,12 Furthermore, when leukocyte adhesion is inhibited, through the blockade of ICAM-1 or β2-integrins, VEGF-induced and diabetic BRB breakdown is suppressed, indicating a link between leukocyte adhesion and increased retinal vascular leakage.11,13 However, the molecular pathways involved in BRB breakdown downstream of leukocyte adhesion are not well understood.

Azurocidin (heparin-binding protein or CAP37) is a 28-kDa inactive serine protease stored in the azurophilic granules of neutrophils.14,15 It is a multifunctional protein with antimicrobial and chemotactic properties, especially for monocytes.16 Recently, azurocidin has been shown to be the missing link in neutrophil-induced endothelial permeability in non–central nervous system (CNS) vessels.17 During firm adhesion of neutrophils to activated endothelium, β2-integrin ligation with endothelial intercellular adhesion molecule-1 (ICAM-1) causes azurocidin release.18 Azurocidin induces Ca2+–dependent cytoskeletal rearrangement and intercellular gap formation in endothelial cell monolayers in vitro and increases macromolecular permeability in peripheral non-CNS vessels in vivo.17 Moreover, azurocidin blockade prevents neutrophil-induced endothelial hyperpermeability, emphasizing the crucial role of azurocidin in vascular responses during inflammation.17

However, whether azurocidin may have an effect on vessels of the CNS is unknown. A unique property of these vessels is their neurovascular barrier function, known as the blood-brain barrier (BBB) or, in the case of the retina, the BRB. The BBB and the BRB act as regulatory interfaces between the blood and the nervous system and are essential for the protection of neu-
rons from blood-borne molecules and cells. In diabetic retinopathy, BRB breakdown leads to macular edema, which results in visual loss. Therefore, elucidating the factors that compromise the BRB may improve our therapeutic approach to macular edema, the main cause of visual loss in patients with diabetes.

Given the important role of azurocidin in mediating neutrophil-induced endothelial permeability in vascular systems outside the CNS and given that neutrophil adhesion occurs in VEGF-induced and diabetic BRB breakdown, we hypothesize that azurocidin may also be an important mediator of BRB breakdown in these two scenarios.

Materials and Methods

Animals

All animal experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and protocols were approved by the Animal Care Committee of the Massachusetts Eye and Ear Infirmary. Male Brown Norway and Long-Evans rats, weighing 200 to 300 g each, were used for the experiments. Animals were fed standard laboratory chow and allowed free access to water in an air-conditioned room with a 12-hour light/12-hour dark cycle until they were used for the experiments.

Injections

Animals were anesthetized with intramuscular injection of xylazine hydrochloride (6 mg/kg; Phoenix Pharmaceutical, St. Joseph, MO) and ketamine hydrochloride (40 mg/kg; Parke-Davis, Morris Plains, NJ). To perform the intravitreal injections, a 31-gauge needle (Hamilton) was inserted into the vitreous 1 mm posterior to the corneal limbus. Insertion and infusion were directly viewed through an operating microscope to prevent injury to the lens and retina. Eyes that exhibited signs of damage to these structures were excluded from the experiments. Intravenous injections were performed through the tail vein with a 27-gauge butterfly needle under anesthesia.

Induction of Diabetes

Male Long-Evans rats weighing approximately 200 g each were used for these experiments. To induce diabetes, each animal received a single 60 mg/kg intraperitoneal injection of streptozotocin (Sigma, St. Louis, MO) in 10 mM sodium citrate buffer, pH 4.5, after an overnight fast. Control nondiabetic animals received citrate buffer alone. Animals with blood glucose levels higher than 250 mg/dl 24 hours after injections were considered diabetic. All experiments were performed 2 weeks after the induction of diabetes.

Administration of Azurocidin and Aprotinin

Rats received intravitreal injections of 5 μL sterile phosphate-buffered saline (PBS) containing 1 or 20 μg human neutrophil azurocidin (Athens Research and Technology, Atlanta, GA) in one eye and 5 μL of sterile PBS in the contralateral eye. Retinas were analyzed for BRB breakdown quantification by the Evans blue (EB) technique. Intravitreal injection for BRB breakdown quantification.

In a second group, rats also received the broad protease inhibitor aprotinin (50,000 KIU; equivalent to 5 mL Trasylol) by intravenous injection. Aprotinin was administered through the tail vein 1 hour before and 8 and 16 hours after intravitreal injection of VEGF or PBS. Retinas were analyzed 24 hours after VEGF administration for BRB breakdown.

Blood-Retinal Barrier Breakdown Measurement with the Evans Blue Technique

Retinal vascular permeability was quantified as previously described. After the animals were deeply anesthetized, EB dye (30 mg/mL in saline; Sigma) was injected through the tail vein over 10 seconds at a dosage of 45 mg/kg. Blood samples were obtained from the left ventricle, just before perfusion, to obtain the time-averaged EB plasma concentration. Blood samples were centrifuged at 12,000 rpm for 15 minutes to separate the plasma from the cellular components. Plasma samples were diluted to 1/10,000 of their initial concentration in formamide (Sigma). Absorbance was measured with a spectrophotometer at 620 nm and 740 nm. After the dye had circulated for 2 hours, the chest cavity was opened, and the rats were perfused through the left ventricle with paraformaldehyde 1% in citrate buffer (0.05 M, pH 3.5) at a constant pressure of approximately 120 mm Hg. Retinas were then carefully dissected under an operating microscope. After measurement of the retinal weight, EB was extracted by incubation of each retina in 180 μL formamide for 18 hours at 70°C. The extract was ultracentrifuged at a speed of 14,000 rpm for 60 minutes at 25°C. Sixty microliters of the supernatant was used for spectrophotometric measurement at 620 nm and 740 nm. Background-subtracted absorbance was determined by measuring each sample at 620 nm (absorbance maximum for EB in formamide) and 740 nm (absorbance minimum). BRB breakdown was calculated as previously described, and values were expressed as plasma (μL) × retinal weight (g) × time (hours)⁻¹.

Qualitative Evaluation and Visualization of Retinal Vascular Permeability

Retinal vascular permeability was also demonstrated in a histologic manner by intravenous injection of 20 kDa FITC-conjugated dextran (50 mg/kg; Sigma). Rats were killed 30 minutes later and perfused with 4% paraformaldehyde to fix the dextran conjugate in the tissues. Retinas were carefully dissected and flat mounted in an antifading medium (Vector Laboratories, Burlingame, CA). Flat-mounted retinas were examined by fluorescence microscopy. Digital color enhancement (green) was equally applied to all images to improve visualization of the fluorescence.

Results

Azurocidin Increases Retinal Vascular Leakage In Vivo

To investigate whether exogenous azurocidin causes leakage in vessels with neurovascular barrier properties, we assessed the effect of intravitreal administration of azurocidin on retinal vascular leakage by the Evans blue technique. Azurocidin increased retinal vascular leakage in vivo in a time-dependent manner with a peak 1–3 hours after administration. Intravitreal injection of 20 μg azurocidin induced a 6.8-fold increase in the leakage of EB from retinal vessels compared with vehicle-injected control eyes, 1–3 hours after injection (31 ± 11 vs. 4.5 ± 1 μL/g/h, n = 12, P = 0.02), a 2.7-fold increase 3 to 5 hours after injection (21 ± 2.3 vs. 8.3 ± 1.4 μL/g/h, n = 11, P = 0.0006), and a 1.7-fold increase 24 hours after injection (16.5 ± 2.8 vs. 8.7 ± 1.8 μL/g/h, n = 6, P = 0.04; Fig. 1A).

Role of Azurocidin in Diabetic BRB Breakdown

PBS in the contralateral eye. Retinas were analyzed 24 hours after VEGF injection for BRB breakdown quantification.

In a second group, rats also received the broad protease inhibitor aprotinin (50,000 KIU; equivalent to 5 mL Trasylol) by intravenous injection. Aprotinin was administered through the tail vein 1 hour before and 8 and 16 hours after intravitreal injection of VEGF or PBS. Retinas were analyzed 24 hours after VEGF administration for BRB breakdown.
To investigate the dose-response of azurocidin on BRB leakage, 1/20 g of purified protein was injected intravitreally, and ocular leakage was compared with the vehicle-injected eyes. At the lower dose of 1/20 g, azurocidin induced a 3.4-fold increase in BRB leakage compared with the vehicle-injected eyes of the same animals (25.1 ± 6.4 vs. 7.3 ± 5.5 μL/g/h, n = 3, P = 0.1). Three to five hours after the injection of 1 μg azurocidin, the difference in leakage between the injected eyes and the control eyes further decreased and remained statistically nonsignificant. Our results suggest a concentration and time-dependent waning of the impact of azurocidin on BRB leakage in vivo (Fig. 1B).

Aprotinin Suppresses Azurocidin-Induced BRB Breakdown

To investigate whether the protease inhibitor, aprotinin, is effective in inhibiting azurocidin-induced BRB breakdown, rats were treated with a single intravenous injection of 1 or 3 mL aprotinin (10,000 KIU or 30,000 KIU) 1 hour before intravitreal injection of azurocidin or vehicle. BRB breakdown was quantified with Evans blue technique 1 hour later. Data represent mean ± SEM. *P < 0.05.

Aprotinin Suppresses VEGF-Induced BRB Breakdown

Intravitreal injection of 50 ng VEGF induced a significant 3.2 ± 0.7-fold increase in retinal vascular leakage after 24 hours compared with vehicle (PBS)-injected eyes (29.7 ± 7.5 vs. 9.6 ± 1.4 μL/g/h, n = 8, P = 0.02; Fig. 3). To assess whether the protease inhibitor, aprotinin, reduces VEGF-induced BRB breakdown, rats were treated in one eye with intravenous injections of aprotinin 1 hour before and 8 and 16 hours after
intravitreal injection of VEGF or vehicle control in the contralateral eye. BRB breakdown was quantified with the EB technique 24 hours after the intravitreal injections. VEGF-induced BRB breakdown was suppressed by 93% with intravenous administration of aprotinin (29.7 ± 7.5 vs. 6.2 ± 1.6 μL/g/h, n = 8 and n = 6, respectively, P = 0.02; Fig. 3).

To visualize the differences in leakage, the retinal distribution of intravenously injected 20 kDa FITC-conjugated dextran was performed. Fluorescence microscopy of flat-mounted retinas from VEGF-injected eyes showed diffusely distributed fluorescence throughout the retinal tissues in intraluminal and extravascular locations. However, when animals were additionally treated with aprotinin, fluorescence was mainly confined to the intraluminal space of the retinal vessels with very little extravascular fluorescence (Fig. 4).

Aprotinin Reduces BRB breakdown in the Diabetic Retina

To assess whether aprotinin reduces BRB breakdown in the STZ-induced model, diabetic rats were treated with aprotinin (1.5 mL/d bolus tail vein injection) for 10 days, and BRB breakdown was measured at 14 days using the Evans blue leakage assay. BRB breakdown was increased 2.4-fold in the diabetic animals compared with age-matched nondiabetic animals (18.2 ± 3.3 vs. 7.2 ± 1.6 μL/g/h, n = 8 and n = 9, respectively, P = 0.01; Fig. 5). Treatment with aprotinin resulted in a significant reduction of diabetic BRB breakdown by 40.6% (18.9 ± 2.97 vs. 11.2 ± 1.43 μL/g/h, n = 9 in each group, P = 0.03; Fig. 5).

**DISCUSSION**

This study investigated the role of the inflammatory mediator, azurocidin, in BRB leakage. We show that azurocidin increased retinal vascular leakage in vivo and that aprotinin was an effective inhibitor of the azurocidin-induced leakage in the retina. Furthermore, aprotinin significantly decreased BRB breakdown after intravitreal VEGF administration and in a model of experimentally induced diabetes.

Azurocidin is an important mediator of neutrophil-induced leakage during firm adhesion to the endothelium. However, its effect as a permeability factor on vessels with neurovascular barrier properties is unknown. In our study, intravitreal administration of azurocidin rapidly increased albumin leakage in the retinal vessels, suggesting that azurocidin may be a key mediator of BRB breakdown. Our results are in line with those of previous reports showing that the administration of azurocidin in vitro decreases transendothelial electrical resistance in aortic endothelial cell monolayers within 30 minutes of application. The slightly different response time between our experiments and the previous reports may be attributed to different experimental models. In our experiments, azurocidin was administered into the vitreous cavity but required diffusion into the inner retina to exert its effects on retinal vessels. In contrast, in the in vitro studies, azurocidin was applied directly to the endothelial cells, allowing immediate contact of azurocidin with its putative endothelial receptors. Furthermore, the focus of our study was barrier-privileged retinal vessels, which may have different dose-response and response-time to azurocidin than non-CNS endothelial cells. Our results indicate that exogenous azurocidin was a potent cause of leakage in retinal vessels in vivo.

However, it is important to note that intravitreal azurocidin administration may induce the expression of VEGF or other cytokines, which can also compromise BRB function. Therefore, indirect effects of azurocidin on BRB in vivo cannot be excluded. In addition, even though our results indicate intravitreal azurocidin causes retinal vascular leakage, the mechanistic details, such as changes in tight endothelial junctions or paracellular transport, remain to be investigated.

Our results show that aprotinin is an effective inhibitor of the azurocidin-induced leakage in retinal vessels in vivo. Aprotinin treatment also significantly decreases VEGF-induced leakage and BRB breakdown in experimentally induced diabetes, suggesting a possible role for azurocidin in these events. The role of azurocidin in diabetic retinopathy in humans remains to be investigated. As a broad serine
protease inhibitor, aprotinin also blocks other serine proteases, such as neutrophil-derived elastase, cathepsin G, proteinase 3, and some proteases in coagulation and fibrinolysis pathways, including plasmin and kallikrein. Because aprotinin does not exclusively block proteolysis pathways, including plasmin and kallikrein, proteinase 3, and some proteases in coagulation and fibrinolysis, such as neutrophil-derived elastase, cathepsin G, protease inhibitor, aprotinin also blocks other serine proteases.

Aprotinin is in clinical use for patients undergoing extensive cardiothoracic and orthopedic surgery, who often experience neutrophil sequestration in organs and massive leakage of fluid from the vasculature. Aprotinin can help to reduce blood loss and blood transfusion requirements after surgery. Gautam et al. proposed the inhibition of azurocidin as a possible mechanism of action of aprotinin for these patients given the crucial role of azurocidin in neutrophil-evoked permeability. Two recently published articles report increased risk for renal and cardiovascular toxicity, including myocardial infarction and stroke, after aprotinin administration in major surgeries. These systemic side effects of aprotinin may be due in part to its limited specificity in vivo. However, if used in the treatment of diabetic retinopathy and ocular inflammatory conditions, aprotinin could be delivered intravitreally with low risk of systemic side effects.

In summary, our results suggest that azurocidin plays a role in BRB breakdown induced by VEGF or in experimental diabetes. Azurocidin release from neutrophils may be the final common pathway for a variety of upstream factors, which during diabetic retinopathy promote neutrophil adhesion and cause BRB breakdown. These findings indicate that targeting azurocidin may prove beneficial in the treatment of retinal vascular leakage caused by ocular diseases such as diabetic retinopathy.

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References

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