Retinal Neovascularization Is Suppressed With a Matrix Metalloproteinase Inhibitor

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Objectives: To determine the role of extracellular proteinases in ischemia-induced retinal neovascularization in an animal model and to examine the effect of proteinase inhibitors on retinal neovascularization.

Methods: Retinal neovascularization was induced in newborn mice exposed to 75% oxygen for 5 days, followed by room air. Retinal extracts underwent zymographic analysis to measure the activity of urokinase and matrix metalloproteinases (MMPs). Some animals under the same conditions also received intraperitoneal injections of an MMP inhibitor. Histological analysis was done to quantitate the neovascular response in these animals.

Results: Levels of urokinase and MMPs (MMP-2 and MMP-9) in retinas were significantly increased in animals with induced retinal neovascularization. Neovascularization was significantly inhibited with intraperitoneal administration of an MMP inhibitor.

Conclusion: Systemic inhibition of MMPs may have therapeutic potential in preventing retinopathy associated with retinal neovascularization.

Clinical Relevance: Because up-regulation and activation of proteinases represents a final common pathway in the process of retinal neovascularization, pharmacological intervention of this pathway may be an alternative therapeutic approach to proliferative retinopathy.


Retinal neovascularization is a leading cause of blindness in a variety of clinical conditions, including diabetic retinopathy, retinopathy of prematurity, and retinal vein occlusion. Left untreated, these conditions can result in intraocular hemorrhage and retinal detachment leading to severe visual loss. Current laser treatment for these diseases, although successful in slowing the growth of new vessels, is not optimal. This treatment may result in the loss of peripheral and night vision, and the disease may progress despite treatment. It is well accepted that hypoxia occurs in these clinical conditions and leads to an initiation of the angiogenic process in the retina.1,2 Numerous angiogenic factors are present during the development of retinal neovascularization,3,4 among which vascular endothelial growth factor (VEGF) is currently thought to be the major mediator of neovascularization.5,7

One phase of the angiogenic process is the invasion and migration of microvascular endothelial cells through the capillary basement membrane and into the adjacent extracellular matrix. This invasive process is tightly coupled to the production and activity of specific extracellular proteinases, including the serine proteinase urokinase and specific members of the matrix metalloproteinase (MMP) family.8,9 A balanced interplay of proteinases and proteinase inhibitors has been implicated in the process of angiogenesis and has been extensively studied during the development of tumor angiogenesis.10,11

The objective of this study was to determine the role of proteinases in ischemia-induced retinal neovascularization in an animal model, namely, newborn mice exposed to the variable oxygen cycle. This model system closely resembles retinopathy of prematurity and some of the characteristics seen in proliferative diabetic retinopathy, such as capillary dropout and neovascularization of the optic disc.12 We also investigated the effect of a proteinase inhibitor on retinal neovascularization in this model.
MATERIALS AND METHODS

ANIMAL MODEL OF RETINAL NEOVASCULARIZATION

Retinal neovascularization was induced in newborn mice per the protocol of Smith et al. This model has been used to test the efficacy of different drugs on ischemia-induced retinal neovascularization. C57Bl/6j mice were exposed to 75% oxygen on postnatal day 7 for 5 days. Mice (n = 12) were then brought to room air on day 12. A condition of relative hypoxia resulted, and retinal neovascularization developed. By day 17, new retinal vessels grew in 100% of animals. Animals were killed on postnatal day 17. Newborn mice kept in room air only for 17 days served as control subjects (n = 12).

Some experimental animals (n = 12) initially exposed to the oxygen cycle mentioned above received a synthetic MMP inhibitor (BB-94; British Biotech Pharmaceuticals Ltd, Oxford, England) intraperitoneally. The low molecular weight (molecular weight 46768) compound BB-94 inhibits a broad spectrum of MMPs and contains both a peptide backbone that binds it to MMPs and a hydroxamic acid group that binds it to the catalytic zinc atom.16 Under physiologic conditions, BB-94 inhibits the activity of gelatinase A and B, interstitial collag enase, and stromelysin with 50% inhibitory concentration (IC50) values of 4, 10, 3, and 20 nmol, respectively.16-18 Intraperitoneal injection of BB-94 has been shown to inhibit the growth of human ovarian carcinoma xenographs and murine melanoma metastasis.19 It is now under clinical trial in cases of pancreatic and ovarian cancers as an antiangiogenic agent.20 BB-94 was suspended with brief sonication in phosphate-buffered saline solution containing 0.01% Tween 20. A stock solution of 1.25 mg/mL was diluted to administer an intraperitoneal injection (0.1 cm3) of either 1 mg/kg on postnatal days 12, 14, and 16 or 15 mg/kg on postnatal day 12. Animals exposed to the oxygen cycle but receiving intraperitoneal injections of isotonic sodium chloride (0.1 cm3) as a placebo on days 12, 14, and 16 served as control subjects. Because weight loss or a slower rate of weight gain, secondary to toxic effects of using the drug, can give rise to a nonspecific decrease in retinal neovascularization, we weighed animals on days 12 and 17. All experiments were consistent with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

HISTOLOGICAL ANALYSIS

Mice were killed using carbon dioxide, and the eyes were enucleated and fixed overnight in 0.1 mol/L of phosphate-buffered saline solution containing 4% paraformaldehyde at 4°C. Intact eyes were embedded in paraffin, and 6-µm serial axial sections were cut parallel to the optic nerve. Nuclei were stained in sections using a mounting medium with 4’-6 diamidinophenylindole (Vector Laboratories, Burlingame, Calif) and were examined with a fluorescence microscope. Nuclei on the vitreous side of the inner limiting membrane of the retina, representing microvascular cells, were counted in each section using a masked protocol. Sections containing the optic nerve were excluded because of epiretinal vasculature that may have been mistaken for neovascular nuclei. Sections obtained from the immediate medial and lateral portions of the eye did not contain the lens and were excluded as well. Neovascular nuclei from every third section per eye were counted to avoid counting individual nuclei more than once. The average number of neovascular nuclei per section per eye was calculated, and a comparison was done between the 3 groups of animals.

Results of zymographic analyses of retinal extracts from animals on day 17 (the active angiogenic phase) revealed significant increases in both the high (54-kd) and low (32-kd) molecular weight forms of urokinase compared with controls (Table 2). Significant increases were also found in the levels of proenzyme (72-kd) and active (62-kd) forms of MMP-2 in the retinas of animals with neovascularization (Table 2). Similar increases in the levels of proenzyme (92-kd) and active (84-kd) forms of MMP-9 were also found in experimental animals. Other members of the MMP family, specifically MMP-3 and MMP-7, were not detected in retinas by zymography. In the preangiogenic phase on day 12—before the initiation of new vessel formation and growth—the levels of MMP-2 and MMP-9 were no different from controls (data not shown). Therefore, the increases observed in the expression and activation of MMP-2 and MMP-9 on day 17 are associated with the active phase of the angiogenic process.

Continued on next page
ies within the retina in the drug-treated group was similar to that of control animals. The number of capillaries per section was 8.0 ± 2.3 in the drug-treated group vs 6.6 ± 2.8 in the control group (P = .05). However, some animals died after receiving a single injection of the high dose (15 mg/kg) of BB-94. These effects may have been caused by an inhibition of MMP that potentially play an important role in neuronal maturation occurring in these animals during this time.

Oxygen-exposed animals without any treatment gained an average of 1.5 g in weight (27.3%) from day 12 to day 17, whereas those treated with BB-94 gained an average of 1.3 g in weight (21.3%) during the same time. Oxygen-exposed animals injected with isotonic sodium chloride had an average weight gain of 1.2 g (22.5%), which was not significantly different from the latter groups. Thus, the possibility of any nonspecific effect of the drug on retinal neovascularization, secondary to weight loss or slower rate of weight gain from toxic effects of using the drug, was ruled out in this experiment.

Significant decreases in the level of active species of MMP-2 and MMP-9 were seen in response to BB-94 treatment when retinal extracts from drug-treated animals and controls were compared (Table 2). In addition to its ability to inhibit the function of the active forms of MMP-2 and MMP-9, BB-94 may also prevent the activation of their proenzyme forms. For MMP-2 and MMP-9, conversion of the proenzyme form of the protein has been shown to be partially dependent on the activity of MMPs.²¹

Our results show that the expression of proteinases is increased during the retinal neovascularization process. The production of urokinase and specific members of the MMP family are significantly elevated in retinal extracts from mice with active neovascularization. These results correlate well with data obtained from examining proteinases in epiretinal neovascular membranes that were surgically removed from humans with proliferative diabetic retinopathy.²²

A common mechanism in mouse retinal neovascularization and human proliferative diabetic retinopathy may be an initiating hypoxic event followed by an increased expression of angiogenic proteins, including VEGF. Either or both of these mechanisms (hypoxia and VEGF) may affect the subsequent expression of proteinases by microvascular cells. Results of a previous study²³ demonstrate that isolated retinal capillary endothelial cells selectively up-regulate and activate the MMP-2 enzyme under hypoxic conditions and in response to VEGF stimulation. This is interesting in light of the present findings and suggests that interactions occur in intact retina.

ZYMOGRAPHIC ANALYSIS

Retinas underwent zymographic analysis to measure the activity of urokinase and MMPs. Immediately after enucleation, the retinas were removed and extracted overnight at 4°C in 0.1-mol/L phosphate-buffered saline containing 0.1% Triton X-100, pH 8.0. Aliquots were removed for DNA determination using a fluorometer (TK100; Hoefer Scientific Instruments, San Francisco, Calif) as an estimate of cell number. Retinal extracts were then subjected to electrophoresis in 10% polyacrylamide minigels into which casein (1 mg/mL), casein and plasminogen (0.04 U/mL), or gelatin (1 mg/mL) was cross linked. After electrophoresis, gels were soaked for 15 minutes in 2.5% Triton-X-100 and rinsed with water. For detection of urokinase, the casein and plasminogen gels were incubated for 24 hours at 37°C in 100 mmol Tris buffer, pH 8.0, containing 0.02% sodium azide. Matrix metalloproteinases were detected by incubating either gelatin gels (for MMP-2 and MMP-9) or casein gels (for MMP-3 and MMP-7) at 37°C in LSCB buffer (50 mmol of Tris, 0.2 mol/L of sodium chloride, 5 mmol of calcium chloride, 0.02% poloxoyxyleneglycol dodexy-lith (Brij 35; Sigma-Aldrich Corp, St. Louis, Mo), and 0.02% sodium azide. Matrix metalloproteinases were detected by incubating either gelatin gels (for MMP-2 and MMP-9) or casein gels (for MMP-3 and MMP-7) at 37°C in LSCB buffer (50 mmol of Tris, 0.2 mol/L of sodium chloride, 5 mmol of calcium chloride, 0.02% poloxoyxyleneglycol dodexy-lith (Brij 35; Sigma-Aldrich Corp, St. Louis, Mo), and 0.02% sodium azide, pH 7.6 for 48 hours. After incubation, gels were stained for 1 hour with 0.1% Coomassie brilliant blue R-250 and destained with 10% acetic acid. Zones of clearing that corresponded to the presence of proteinases in the gel were quantitated using image analysis software (National Institutes of Health, Bethesda, Md), and the data were expressed as pixels per microgram of DNA.
result in proteinase expression, other than MMP-2, by vascular cells. Alternatively, retinal capillaries may not be the sole source of these enzymes in the retina. In addition, it is not known whether proteinase expression by microvascular endothelial cells occurs as a direct response to hypoxic conditions or whether it is mediated through the production of VEGF or other factors.

Extracellular proteinases are essential during the invasive stage of the angiogenic process in facilitating the degradation of the capillary basement membrane and the subsequent invasion of activated endothelial cells into surrounding tissues. In addition to its role in matrix degradation through the production of plasmin, urokinase has been shown to affect the motile behavior of cells by regulating cell-matrix interactions. Of significance in this study was the finding of an increase in the high and low molecular weight forms of urokinase in retinas exhibiting neovascularization. The increased low molecular weight form suggests the presence of an amino terminal fragment of urokinase in this tissue. This protein fragment contains a growth factor–like domain and has been suggested to play a role in the stimulation of cell proliferation, another important event in the angiogenic process.

MMP-2 has also been shown to play an important role in the interactions, which take place between a motile cell and its substratum. In angiogenic blood vessels in particular, MMP-2 has been shown to interact with the αvβ3 integrin on the endothelial cell surface to create localized areas of high proteolytic activity. This interaction has been postulated to facilitate the generation of extracellular matrix protein fragments that serve as substrates for the αvβ3 integrin and lead to increased cell survival and matrix invasion. Results of these studies and our own lead us to speculate that the decrease in angiogenic response seen in the retina after administra-

Table 1. Histological Analysis of Ischemia-Induced Neovascularization of the Mouse Retinaa

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug Treatment</th>
<th>Eyes, No.</th>
<th>Neovascular Nuclei per Section per Eye, Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>None</td>
<td>12</td>
<td>0.37 ± 0.07</td>
</tr>
<tr>
<td>Experimental</td>
<td>None</td>
<td>12</td>
<td>43.50 ± 3.50†</td>
</tr>
<tr>
<td>Experimental</td>
<td>BB-94, 1 mg/kg</td>
<td>12</td>
<td>12.40 ± 0.79‡</td>
</tr>
<tr>
<td>Experimental</td>
<td>BB-94, 15 mg/kg</td>
<td>4</td>
<td>4.00 ± 0.52‡</td>
</tr>
</tbody>
</table>

* Retinal neovascularization was induced in C57Bl/6J mice as previously described, and sections of the eye stained with diamidino-phenylindole were evaluated for signs of retinal neovascularization. Some mice were injected intraperitoneally with either 1 or 15 mg/kg of the matrix metalloproteinase inhibitor BB-94 before or during the angiogenic period.

†Significantly different from controls at the P < .001 level.

‡Significantly different from experimental (non–drug-treated mice) at the P < .001 level.
of potential action of an MMP inhibitor may be caused by a disruption of the matrix metalloproteinases and urokinase. Some mice were injected with either 1 or 15 mg/kg of the matrix metalloproteinase inhibitor BB-94 before or during the angiogenic period. ND indicates not done.

Reverse transcription polymerase chain reaction analysis of matrix metalloproteinases in the retina. A, First-strand complementary DNA was synthesized from total RNA extracted from the retinas of control (lanes 1-6) and experimental (lanes 7-12) mice (retinas pooled from 5 animals on postnatal day 17). Complementary DNA was used in standard polymerase chain reactions with primers specific for MMP-2 (lanes 1 and 7), MMP-3 (lanes 2 and 8), MMP-7 (lanes 3 and 9), MMP-9 (lanes 4 and 10), MT-MMP (lanes 5 and 11), and the 18s ribosomal RNA as an internal control (lanes 6 and 12). The identity of the polymerase chain reaction products was confirmed by cloning into the pCR2.1 vector followed by sequencing. B, Relative reverse transcription polymerase chain reaction analysis of MMP-2, MMP-9, and MT-MMP in retinas from control and experimental mice (n = 3). Significant increases were seen in the relative amount of messenger RNA for each of these proteinases in animals with retinal neovascularization compared with control animals. As seen in (A), neither control nor experimental animals showed expression of MMP-3 or MMP-7 in the retina. Asterisk indicates significantly different at P<.01.

Table 2. Zymographic Analysis of Proteinases Present in the Retina of Mice Exhibiting Ischemia-Induced Neovascularization

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug Treatment</th>
<th>Eyes, No.</th>
<th>MMP-2 72 kd</th>
<th>MMP-2 62 kd</th>
<th>MMP-9 92 kd</th>
<th>MMP-9 84 kd</th>
<th>Urokinase 54 kd</th>
<th>Urokinase 32 kd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>None</td>
<td>12</td>
<td>0.25 ± 0.04</td>
<td>0</td>
<td>1.2 ± 0.34</td>
<td>0</td>
<td>10.4 ± 1.1</td>
<td>3.9 ± 0.55</td>
</tr>
<tr>
<td>Experimental</td>
<td>None</td>
<td>12</td>
<td>0.94 ± 0.19†</td>
<td>0.53 ± 0.11†</td>
<td>5.3 ± 0.89†</td>
<td>1.6 ± 0.33†</td>
<td>29.8 ± 2.5†</td>
<td>17.2 ± 1.6†</td>
</tr>
<tr>
<td>Experimental</td>
<td>BB-94, 1 mg/kg</td>
<td>12</td>
<td>0.73 ± 0.09</td>
<td>0.24 ± 0.06‡</td>
<td>1.3 ± 0.19‡</td>
<td>0‡</td>
<td>21.7 ± 3.4</td>
<td>12.5 ± 1.8</td>
</tr>
<tr>
<td>Experimental</td>
<td>BB-94, 15 mg/kg</td>
<td>4</td>
<td>0.28 ± 0.02‡</td>
<td>0.13 ± 0.02‡</td>
<td>1.2 ± 0.54‡</td>
<td>0‡</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are given as pixels per microgram of DNA, mean ± SEM. Retinal neovascularization was induced in C57Bl/6J mice as described, and the retinas were collected and analyzed for the presence of matrix metalloproteinases and urokinase. Some mice were injected with either 1 or 15 mg/kg of the matrix metalloproteinase inhibitor BB-94 before or during the angiogenic period. ND indicates not done.

†Significantly different from controls at the P<.001 level.
‡Significantly different from experimental (non–drug-treated) mice at the P<.001 level.

Accepted for publication December 18, 1998.

Supported by research grants P20 RR11830-02 and RO 1 EY12604-01 from the National Institutes of Health, Bethesda, Md (Dr Das).


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REFERENCES


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From the Archives of the Archives

A look at the past...

Ngelucci examined bacteriologically twelve eyes enucleated after having caused sympathetic ophthalmia, and also portions of the iris removed by iridectomy from the eyes sympathetically inflamed. From the detailed reported results of his inoculations, it is particularly worthy of note that the cocci and diplococci from the iris of the two eyes sympathetically inflamed caused similar inflammatory changes when inoculated into the eyes of rabbits.