Regulation of Matrix Metalloproteinase Expression by Tumor Necrosis Factor in a Murine Model of Retinal Neovascularization

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PURPOSE. Hypoxia and growth factors are postulated to be involved in the development of retinal neovascularization through the regulation of extracellular proteinase production. It has been shown that matrix metalloproteinases (MMPs) are elevated in the retina during the neovascularization process. However, the factors and mechanisms that regulate the expression of these enzymes are not well characterized. The present study examines the potential role of tumor necrosis factor (TNF)-α as a regulator of MMPs in the retinal neovascularization process.

METHODS. C57/Bl6 mice were treated with 75% oxygen (experimental) or room air (control) from postnatal days (P)7 through P12, followed by room air until P17. Retinas were collected at P13, P15, or P17 and total RNA analyzed for the relative level of TNFα, TNF receptor (p55), and TNFα-converting enzyme (TACE). Immunostaining was used to identify changes in TNF protein expression as well as to localize TNFα within specific retinal cell types. The role of TNFα in stimulating retinal microvascular endothelial cell (RMVEC) proteinase production was evaluated using isolated murine RMVECs grown in normoxic or hypoxic conditions. Message expression was analyzed by RT-PCR and protein expression by zymographic analysis.

RESULTS. TNFα mRNA was increased in the retinas of experimental animals on P13 and P15, during the early stages of retinal neovascularization. In addition to being expressed by Müller glial cells and the inner nuclear layer, additional expression was noted in the outer nuclear layer of experimental animals. No significant level of apoptosis was detected in the retina of experimental animals with retinal neovascularization. Isolated RMVECs did not significantly increase MMP production directly in response to a hypoxic stimulus, but required the presence of exogenous TNFα. TNFα increased the expression of MT1-MMP, MMP-3, and MMP-9 in these cells. The levels of TACE and p55, proteins important in mediating the response of cells to TNFα, were found to be increased by the angiogenic protein, vascular endothelial growth factor (VEGF), which was also elevated in the experimental retinas.

CONCLUSIONS. TNFα levels increase in experimental mouse retinas exposed to hypoxic stimuli. Increased production of MMPs by RMVECs does not occur directly in response to a hypoxic stimulus. These cells are responsive, however, to stimulation by TNFα, which enhances the production of specific members of the MMP family. VEGF also plays a role in this process through its regulation of TACE and p55 mRNA in the vascular endothelial cells. These findings support the hypothesis that these two growth factors have a role in the regulation of extracellular proteinase expression during retinal neovascularization. (Invest Ophthalmol Vis Sci. 2002;43:260–266)

NEovascularization of the retina is one of the leading causes of vision loss worldwide. An important step in the formation of new vessels is the degradation and penetration of the capillary basement membrane by activated endothelial cells to form new capillary sprouts.1,2 This process is facilitated by the expression of extracellular proteinases, including members of the matrix metalloproteinase (MMP) family of enzymes. The expression of MMPs by retinal microvascular endothelial cells (RMVECs) may occur in response to local changes in the environment that may include an initiating hypoxic event followed by the increased expression of specific angiogenic proteins by cells of the retina.3-7 An example is the rapid response shown by Müller cells to conditions of local ischemia. These cells produce the potent angiogenic protein, vascular endothelial growth factor (VEGF).4,8

In addition to VEGF, other proteins, including tumor necrosis factor (TNF)-α, have been found to be expressed in the retinas of humans with proliferative eye diseases9-11 and in animal models of retinal neovascularization.12 TNFα is a 26-kDa transmembrane protein that is processed by TNFα-converting enzyme (TACE), to yield a 17-kDa soluble protein.13,14 TNF functions through its binding to two receptors: p55, implicated in apoptosis and NFκB activation, and p75, involved with lymphocyte proliferation.15,16

The objective of the present study was to determine the role of TNFα in the neovascular response of the mouse retina to a hypoxic stimulus and the role of this factor in the regulation of RMVEC behavior.

METHODS

Mouse Model of Proliferative Retinopathy

Specific pathogen-free C57Bl/6j mice were bred at the University of New Mexico Animal Research Facility. All experiments were consistent with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Litters of 7-day-old C57Bl/6j mice were placed with their nursing mothers into an oxygen incubator maintained at 75% oxygen until postnatal day (P)12, as described previously.17 Mice were removed from the incubator at P12 and maintained in room air until P15 or P17. By P17 retinal neovascularization was present in 100% of the experimental animals. Newborn mice exposed only to room air served as control subjects.

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Submitted for publication February 6, 2001; revised September 12, 2001; accepted October 2, 2001.

Commercial relationships policy: N.

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RNA Isolation RT-PCR Analysis

RNA was isolated from murine retinas (n = 3 at each time point) or RMVECs (n = 6–8) using extraction reagent (Trizol; Gibco, Rockville, MD). First-strand cDNA was prepared from 0.5 µg total RNA using an oligo-dT primer and reverse transcriptase (Superscript; GibcoBRL, Gaithersburg, MD). For semiquantitative PCR, 1 µL of each first-strand reaction was then amplified using MMP-, TACE-, p55-, and 18S-specific oligonucleotide primers. Standard PCR amplification was performed at 94°C, 1 minute; 60°C, 1 minute; and 72°C, 1 minute for 27 cycles, which was determined to be within the linear range of product amplification. After completion, 10 µL of the reactions were analyzed by agarose gel electrophoresis and ethidium bromide staining to determine the presence or absence of specific transcripts in the cells or tissue, as well as the levels of transcript relative to the control transcript 18S RNA. Quantitation of band density was performed with image analysis software (Alpha Imager; Alpha Innotech, San Leandro, CA). The following specific primer pairs were used for these analyses: MMP-2: 5'-CTATTCTGTCAGCATTGG-3' and 5'-CAGACTTTTGTTCTCCAACCT-3; MMP-9: 5'-AAATGTGGGTGTACCA-GGGC-3' and 5'-TTCACCCGTTGTGGAACACT-3; MT1-MMP: 5'-TGGA-TAGCCATGAAGTCTGC-3' and 5'-AGTAAAGCAATGCGTIGG-3'; TACE: 5'-CTCTTAAAACCTGATTATCC-3' and 5'-GGGAGATATAACAATGA-GG-3'; p55: 5'-ACATGGAAGTCTGCATCTC-3' and 5'-ATCCTGCTGACCAAGTGCC-3'; and 18S: 5'-GGGCGGCTTGTGTTTGGTGTT-3', and 5'-TACCCTGGTGATCTGGCACG-3'.

Analysis of Activity Levels of MMP-2 and -9

Zymographic analysis was performed using RMVECs solubilized in 0.2% Triton X-100, 0.1 M phosphate (pH 7.3). Samples were equilibrated

**In Vitro Cell Culture System**

Mouse RMVECs were obtained from the laboratory of Jeff Gidday (Washington University, St. Louis, MO). Briefly, retinal tissue was collected and homogenized, followed by digestion with a solution of collagenase, dispase, DNAse I, and N(a)-prolyl-l-lysyl chloromethyl ketone (TLCK). The microvessels were separated from other cells by density-dependent centrifugation in a 50% density gradient (Percoll, Pharmacia & Upjohn, Uppswala, Sweden). The RMVEC band was collected, washed, and cultured on collagen-coated plates in DMEM containing 10% fetal calf serum and 30 µg/mL endothelial cell growth supplement (Sigma Chemical Co., St. Louis, MO). Endothelial cells were characterized by immunostaining with factor VIII and were incubated in culture medium containing either TNFα (1 ng/mL) or VEGF165 (10 ng/mL; R & D Systems, Minneapolis, MN) for 24 hours at 37°C, and the RNA was extracted as described in the following section. Three cultures were used for each treatment.
to total DNA content and electrophoresed in 10% polyacrylamide minigels, into which gelatin was cross-linked. After electrophoresis, the enzymes were renatured by soaking the gels in a solution of 2.5% Triton X-100 followed by incubation for 24 to 48 hours in LSCB buffer (50 mM Tris, 0.2 M sodium chloride, 5 mM calcium chloride, 0.02% polyoxyethyleneglycol dodecylether (Brij 35; Sigma), and 0.02% sodium azide [pH 7.6]). The zones of proteolysis corresponding to the enzymes were renatured by soaking the gels in a solution of 2.5% Triton X-100 followed by incubation for 24 to 48 hours in LSCB buffer (50 mM Tris, 0.2 M sodium chloride, 5 mM calcium chloride, 0.02% polyoxyethyleneglycol dodecylether (Brij 35; Sigma), and 0.02% sodium azide [pH 7.6]). The zones of proteolysis corresponding to the presence of MMP-2 and -9 were visualized by staining the gel with specific antibodies and then reacted with a secondary antibody. Detection was achieved using the avidin biotin system (Vector Laboratories, Burlingame, CA).

For immunohistochemical localization of TNFα, murine eyes from experimental and control animals were collected at P15 or P17 and fixed in 2% paraformaldehyde in 0.1 M phosphate buffer. Eyes were embedded in optimal cutting temperature (OCT) compound (Tissue Tek; Miles Laboratories, Elkhart, IN) and frozen. Tissue sections were incubated in 10% goat serum for 1 hour at room temperature followed by 1 μg/mL TNFα antibody. The sections were washed with TBST and incubated with a secondary antibody coupled to biotin (Vector Laboratories) for 1 hour. Detection was achieved using the avidin biotin system (Vector Laboratories). Slides were coverslipped (Permount; Fisher Scientific, Fairlawn, NJ) and examined by light microscopy.

Detection of Apoptosis

Apoptosis detection was performed with a kit, according to instructions (In Situ Death Detection kit; Roche Molecular Biochemicals, Indianapolis, IN). Briefly, paraffin-embedded tissue sections were processed for TUNEL staining. The TUNEL reaction was prepared using the recommended 9:1 buffer-to-enzyme ratio. The sections were placed in a dark, humidified chamber at 37°C for 1 hour. Sections were rinsed once for 5 minutes with PBS and mounted using diamidino-phenylindole (DAPI) mounting medium (Vector Laboratories). Apoptotic nuclei were visualized by FITC and counted (n = 25 sections).

RESULTS

Components of the TNF system were analyzed in the retinal tissues from mice with developing neovascularization. The message for TNFα was significantly increased in experimental animals during the early stages of retinal neovascularization at P13 and P15 compared with the levels seen in control animals (Fig. 1). The TNF processing enzyme, TACE, was also significantly elevated early in the process and returned to control levels as the neovascularization developed (Fig. 1). Increased expression of the TNF receptor I (p55) was also seen in the experimental animals, but this occurred after the increased expression of the growth factor and TACE (Fig. 1). In addition to the mRNA levels, the levels of both the soluble and membrane-bound forms of TNF protein were significantly increased in the retina of experimental animals at both P15 and P17 (Fig. 2). No difference was seen in the levels of soluble and membrane-bound TNF proteins on P13 (data not shown). TNF was localized by immunostaining to the inner nuclear layer in both control and experimental retinas at P17. Because the stained cell processes were seen to extend from the inner nuclear layer toward the ganglion cell layer similar to the foot processes of the Müller cells, we speculate that these cells are Müller cells. Additional staining was detected in the outer nuclear layer of experimental animals only (possibly photoreceptors; Fig. 3A, 3B). Sections incubated without the primary antibody showed no significant background staining (Fig. 3C).

We next examined the retinas of control and experimental animals for the degree of apoptosis, because one role of the TNFα protein is the induction of this process. Examination of TUNEL-stained retinas revealed a small number of apoptotic cells throughout the retina at P13, P15, and P17 and no significant difference between experimental and control animals (Fig. 4). This result suggests that TNFα may have other roles in

**FIGURE 3.** Localization of TNFα in the murine retina. Immunohistochemical analysis of TNFα protein expression and localization in frozen sections of control (A) and oxygen-treated experimental (B) murine retinas on P17. Müller glial cell bodies and processes are positive for TNFα in both control and experimental retinas. Experimental retinas showed, in addition, significantly increased intensity of staining in the outer nuclear layer (arrows). Retinas of experimental animals without any primary antibody treatment served as control samples for the specificity of staining (C). V, vitreous side.
the retina during neovascularization, one of which may be in the regulation of extracellular proteinase production.

We have previously shown that specific members of the MMP family of enzymes are upregulated in the retina of the mouse undergoing a neovascular response. These include MMP-2, MMP-9, and MT1-MMP. The effect of TNFα on the regulation of these specific MMPs was subsequently examined in cultures of isolated RMVECs.

Cells were treated with TNFα for 24 hours followed by analysis of mRNA or protein levels. In some cases, cells were treated with VEGF as a positive angiogenic control. The effects of VEGF on endothelial cell behavior and MMP expression have been well characterized.

Treatment of cells with TNFα significantly and selectively altered the expression of MMPs at the levels of both mRNA and protein. RT-PCR analysis demonstrated that TNFα increased the expression of MT1-MMP and MMP-9 in the cultured cells, but had little effect on the expression of MMP-2 (Fig. 5). In comparison, these cells responded to VEGF treatment with a significant increase in the expression of MT1-MMP and smaller increases in MMP-2 mRNA levels. VEGF did not appear to affect the expression of MMP-9 (Fig. 5). Results from the zymographic analysis paralleled that of the message levels of MMP-2 and -9 in cells treated with TNFα (Fig. 6). There were increases in both the pro and active forms of MMP-9, with no change in the levels of MMP-2 when compared with untreated control cells. The effect of VEGF was seen primarily in the expression of the pro form of MMP-2. In addition, cells treated with VEGF showed increased expression of TACE and a slight although nonsignificant increase in the expression of p55 (Fig. 7).

DISCUSSION

The formation of new vessels in the retina and other tissues requires the initial stimulation of existing capillaries by specific angiogenic growth factors. These factors may act individually or in combination to regulate the various steps of the angiogenic process.

TNFα binds to the p55 receptor on responsive cell types and elicits apoptosis or other events that require the activity of the NFκB transcription factor. The level of TNFα message was increased significantly at P13 and less dramatically at P15 in the model of retinal neovascularization. TNFα expression was found to be significantly increased, along with its processing enzyme TACE during the early stages of angiogenesis after a hypoxic stimulus. Immunohistochemical analysis localized the TNFα protein to Müller glial cells and their processes in the inner nuclear layer. Additional staining was evident in a population of cells (possibly photoreceptors) present in the basilar portion of the outer nuclear layer of experimental animals. The staining pattern suggests that either these cells are being stimulated themselves by TNFα or that they are the source of the growth factor for action on other cell types, including the capillary endothelial cells. It is possible that the Müller cells secrete TNFα that may be released through the foot processes onto the capillary endothelial cells located in the nerve fiber layer of the retina. Further studies using in situ hybridization may resolve this question to some extent.

The absence of significant apoptosis in the experimental retina suggests an alternative function for TNFα in this system. The cytoplasmic domain of the TNFα receptor, p55, has an 80-amino-acid residue “death domain” that can regulate the apoptotic pathway. This is not the only outcome of p55 ligation, however. NF-κB is also activated by p55 stimulation, although the mechanism that determines the choice of pathways is not completely clear. Stimulation of NF-κB activity by the alternative pathway may result in a variety of cellular responses, including the transcriptional regulation of expres-
sion of select members of the MMP family of extracellular proteinases.

Previous studies have reported an early and rapid increase in VEGF expression in the model used for these experiments. The VEGF expression precedes the formation of new vessels that becomes maximal on P17. The overlapping temporal expression of VEGF and TNF may suggest an interactive role for these two proteins during the angiogenic period. Indeed, the expression of the MMPs detected in the retinal tissues of this model appears to require both TNFα and VEGF activity for increased expression, as shown in the isolated RMVECs. The TNFα increased the expression of MT1-MMP and MMP-9, but had no effect on MMP-2. VEGF, in contrast, was seen to increase the expression of MT-MMP-1 and -2, but had little effect on MMP-9 mRNA levels. It was interesting to note that VEGF also significantly affected the expression of TACE in the cultured cells. This early change in TACE production, in response to VEGF stimulation, may result in the release of membrane-bound TNFα from cells, such as Müller glial cells, yielding a soluble form of the protein, which has been shown to be a more potent stimulator and may have more distant effects on other cell types.

We have previously shown that a synthetic MMP inhibitor, BB-94, can significantly inhibit the development of retinal neovascularization in the mouse model. In addition to inhibiting the active forms of MT1-MMP and MMP-2 and -9, BB-94 also appeared to significantly reduce the levels of the pro form of MMP-9 in the retinal tissues of animals treated with this compound. This can now be explained by results from the present study, in which TNFα and TACE were expressed in the mouse retina and TNFα had a major effect on the expression of MMP-9 in isolated microvascular endothelial cells. The solubilization and activity of TNFα is dependent on the activity of TACE, an integral membrane metalloproteinase that causes the secretion of the active form of TNFα from its plasma membrane precursor. In addition to inhibiting the members of the MMP family, BB-94 has been shown to inhibit the activity of TACE and other α-disintegrin and metalloproteinase domains (ADAMs). Inhibition of TACE activity and therefore TNFα activity would thus be expected to lower the expression of MMP-9 in the retinal tissues of BB-94–treated animals.

In conclusion, the results of this study suggest a major role for TNFα in the initiation of retinal neovascularization and provide a more complete understanding of how retinal neovascularization in general may be regulated. This information may be critical to the identification of potentially new targets for therapeutic intervention in the treatment of this disease process, thereby alleviating some of the negative side effects of the current laser treatment.
The authors thank Jeff Gidday, Jennifer Reiger, and Aarti Shah (Washington University, St. Louis, Missouri) for their expertise in cell isolation and technical assistance.

FIGURE 7. TACE and p55 expression in TNFα- and VEGF-treated RMVECs. RT-PCR analysis of TACE and p55 (TNF receptor 1) mRNA in isolated RMVECs after treatment with either TNF (1 ng/mL) or VEGF (10 ng/mL). (A) The levels of specific mRNA normalized to 18S mRNA are expressed as a percentage of untreated control levels. Data are the mean ± SEM for n = 3 cultures for each treatment. *Significantly different from control untreated cells (P < 0.005). (B) Representative RT-PCR gels. Lane 1: TNF treatment; lane 2: VEGF treatment; lane 3: no treatment.

Acknowledgments

The authors thank Jeff Gidday, Jennifer Reiger, and Aarti Shah (Washington University, St. Louis, Missouri) for their expertise in cell isolation, and E. Sage Colombo (University of New Mexico, Albuquerque, New Mexico) for technical assistance.

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