Matrix Metalloproteinases and Tumor Necrosis Factor α in Glaucomatous Optic Nerve Head

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Objective: To study expression and location of matrix metalloproteinases (MMPs) and tumor necrosis factor α (TNF-α) in glaucomatous optic nerve heads, which are known to be secreted in response to a variety of neuronal injury.

Methods: Four postmortem eyes from patients with primary open-angle glaucoma, 7 eyes from patients with normal-pressure glaucoma, and 4 eyes from age-matched normal donors were studied by immunohistochemistry. The sections of the optic nerve heads were examined after immunostaining with antibodies to MMPs (MMP-1, MMP-2, and MMP-3), TNF-α, or TNF-α receptor 1.

Results: The intensity of the immunostaining and the number of stained cells for MMPs, TNF-α, or TNF-α receptor 1 were greater in the glaucomatous optic nerve heads, particularly in eyes with normal-pressure glaucoma compared with age-matched controls. Positive immunostaining was observed in all regions of the glaucomatous optic nerve heads, but most prominently in the postlaminar region. Immunostaining was observed mainly in glial cells and their processes around the axons and blood vessels and in pial septae.

Conclusion: There is increased immunostaining for MMPs, TNF-α and TNF-α receptor 1 in the glaucomatous optic nerve head, which suggests increased expression of these proteins in glaucoma and thereby implies a role in the tissue remodeling and degenerative changes seen in glaucomatous optic nerve heads.

Clinical Relevance: The MMPs and TNF-α may be components of astroglial activation that occurs in glaucomatous optic nerve heads. The biological alterations in the expression of these proteins may play a role in the progression of glaucomatous optic neuropathy.

Arch Ophthalmol. 2000;118:666-673

Histopathologic studies of the glaucomatous optic nerve head in primary open-angle glaucoma reveal astroglial activation and tissue remodeling, which accompanies neuronal damage. As a part of tissue remodeling, backward bowing and disorganization of the laminar cribriform plates are common characteristics of glaucomatous eyes with either normal or high intraocular pressure. These histologic changes are accompanied by the up-regulation of extracellular matrix components, including collagen and proteoglycan, and adhesion molecules by optic nerve head astrocytes in glaucomatous eyes. The astroglial activation seen in glaucomatous optic nerve heads likely represents an attempt to limit the extent of the injury and promote the tissue repair process. However, despite the astroglial activation, there is limited deposition of extracellular matrix in glaucomatous optic nerve atrophy, which does not retain characteristics of scar tissue formation. This suggests that there are diverse cellular responses to the initial event or subsequent tissue injury, which preferentially results in tissue degradation.

Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade components of extracellular matrix. Increased secretion of MMPs by activated glial cells have been implicated in various extracellular matrix remodeling events that occur during normal development and in a number of pathologic processes, including atherosclerosis, arthritis, tumor growth, and metastasis. In addition, reactive astrocytes after neuronal injury produce various neurotrophic factors and cytokines, including tumor necrosis factor α (TNF-α), which play a critical role in the regulation of the synthesis of MMPs. Furthermore, the release of TNF-α from its membrane-bound precursor is a MMPs-dependent process.
PATIENTS AND METHODS

PATIENTS

Four postmortem human eyes with a diagnosis of POAG and 7 human eyes with a diagnosis of NPG were obtained. The age of patients ranged from 68 to 84 years. Table 1 outlines the clinical findings that were available from glaucomatous eyes. Four human donor eyes with no history of eye disease were used as age-matched controls (age range, 61-81 years). The death-to-fixation time for the specimens ranged between 6 and 9 hours.

IMMUNOHISTOCHEMISTRY

After enucleation, all eyes were fixed in 10% neutral buffered formalin for 24 hours, dehydrated in graded alcohol, and embedded in paraffin. Since some of the specimens contained only the optic nerve head and small portions of the peripapillary retina, retinal distribution of immunostaining was not studied. After deparaffinization, 5-µm-thick longitudinal sections of optic nerve heads were incubated with monoclonal antibodies against MMP-1, MMP-2, or MMP-3 (2.5 µg/ml) (Oncogene Science, Cambridge, Mass) or polyclonal antibodies against TNF-α or TNF-α receptor 1 (2 µg/ml) (R &D Systems, Minneapolis, Minn) overnight at 4°C, after endogenous peroxidase was blocked with 2% hydrogen peroxide in methanol and followed by several washes in phosphate-buffered saline solution. The 3 anti-MMP antibodies recognized both latent as well as active forms of MMPs. Prior to incubation with primary antibodies, the sections were incubated with either mouse skin extract (during MMP staining) or 20% nonimmune donkey serum (during TNF-α and TNF-α receptor 1 staining) for 20 minutes to block background staining. Biotinylated secondary antibody (anti-mouse or anti-goat IgG) (Dako Corp, Carpinteria, Calif) was applied to the sections for 30 minutes at room temperature. The slides were then incubated with horseradish peroxidase–labeled streptavidin solution (Dako Corp) for 30 minutes, and the reaction was visualized by incubation in a solution of 0.02% 3,3′-diaminobenzidine tetrahydrochloride and 0.006% hydrogen peroxide in 0.05M Tris-HCl (pH, 7.6). The slides were lightly counterstained with Mayer hematoxylin. Sections incubated with mouse serum or phosphate-buffered saline solution in place of the primary antibody served as negative controls. Sections from biopsy specimens of infiltrating ductal breast carcinoma served as a positive control for all antibodies used in this study.

Three to 5 sections from each optic nerve head were examined by immunohistochemistry for each protein including MMPs, TNF-α, and TNF-α receptor 1. To obtain comprehensive semiquantitative evaluation of the immunostaining, the intensity of immunostaining for MMPs and TNF-α and its receptor in the prelaminar, laminar, and postlaminar regions of the optic nerve head was graded using an arbitrary score in which each region was graded from − to +++. A semiquantitative score (indicated in parentheses) was then calculated for each optic nerve head (−, absent [0]; ±, ranging from absent to weak [0.5]; +, weak staining [1]; ++, moderate staining [2]; ++++, strong staining [3]). The grading of the immunostaining was performed in a masked fashion by an observer who was skilled in grading immunohistochemical staining but was not familiar with the pathologic changes in the optic nerve head. The observer graded the intensity of immunostaining in optic nerve head regions (prelaminar, laminar, and postlaminar) that were pointed out by one of the authors (X.Y.). Both the scored results and the photographs of representative sections from each group are presented.

RESULTS

Recently, we reported the histopathologic features of a pair of eyes with normal-pressure glaucoma (NPG) that demonstrated Schnabel optic atrophy.1 In addition to the eyes presented in the report, our histopathologic examinations in an additional 5 postmortem eyes from patients with NPG revealed Schnabel cavernous atrophy. Like eyes with POAG, the eyes with NPG exhibited bowing of the lamina cribrosa, areas of glial activation, and axonal atrophy. However, the remarkable histopathologic distinctions between the eyes with NPG or POAG were the large cavernous spaces in the optic nerve head, extensive loss of pial septae, and the absence of a fibroglial response. These findings suggested that one possible mechanism for the cavernous degeneration might be an exaggerated astrogial response to degrade extracellular matrix by proteolytic enzymes such as the MMPs.

In this immunohistochemical study, antibodies against MMPs (MMP-1, MMP-2, and MMP-3), TNF-α, and TNF-α receptor 1 were used to label optic nerve head sections from postmortem eyes with POAG or NPG and from age-matched normal donors. Our observations suggest that there is increased expression of MMPs, TNF-α, and TNF-α receptor 1 in the glaucomatous optic nerve head, particularly with NPG, which likely contributes to the tissue remodeling seen in glaucomatous optic neuropathy.

The normal eyes exhibited glial columns and nerve bundles in the prelaminar region when observed by light microscopy. In the lamina cribrosa, there were glial cells lining the collagenous laminar beams; in the postlaminar region, the glial cells were mainly distributed along the pial septae and were also scattered among the axonal bundles.

The glaucomatous eyes either with POAG or NPG demonstrated backward bowing of the lamina cribrosa and axonal atrophy. The degree of the laminar bowing was comparable in the eyes with POAG or NPG. The degree of axonal atrophy was mild to moderate in the eyes with POAG and was especially noted in the postlaminar region. In the eyes with NPG, the axonal atrophy was moderate in most eyes and characterized with focal loss in the areas of cavernous degeneration. In 1 eye with NPG, severe axonal loss was noted through the optic disc cup, with axonal preservation in more peripheral areas. The postlaminar region of the optic nerve head in the eyes with POAG demonstrated mild disorganization of the pial septae without tissue destruction. These changes were uniformly consistent in all eyes with POAG. In contrast, the eyes from the patients with NPG exhibited varying stages of Schnabel cav-
In normal eyes, faint immunostaining for MMP-1 was observed in the cytoplasm of a few glial cells, located mostly in the laminar and postlaminar regions of the optic nerve head. Faint staining was also noted around the axons or in the pial septae.

Although the intensity of immunostaining for MMP-1 was similar in all regions of the optic nerve head of glaucomatous eyes and in control eyes, the number of positively stained glial cells was greater in glaucomatous eyes, either with NPG or POAG. In addition, in glaucomatous eyes, immunostaining was positive around the axons in the postlaminar region (Figure 1).

MMP-2

In normal eyes, the prelaminar, laminar, and postlaminar regions of the optic nerve head exhibited faint immunostaining for MMP-2 in a few glial cells and around the axons.

However, both the intensity of the immunostaining and the number of stained glial cells were moderately increased in the prelaminar region of the optic nerve head, as well as along the laminar beams, in the glaucomatous eyes with either NPG or POAG. In the postlaminar region, positive immunostaining was seen around the axons and around the pial blood vessels in the glaucomatous eyes, which was more prominent in the eyes with NPG compared with those with POAG. The MMP-2 labeling in the eyes with NPG was also noted along the degenerating laminar plates and pial septae lining the cavernous spaces as well as within the astrocytes in these structures (Figure 2). In the postlaminar region of some eyes with NPG, areas of axonal preservation were seen adjacent to areas of severe axonal atrophy. In the areas of preserved axons, intracytoplasmic MMP immunolabeling was more intense than seen in the glial cells located in the areas of severe atrophy (Figure 3, A).

MMP-3

In the normal eyes, faint immunostaining for MMP-3 was observed in rare glial cells and around the axons in the all regions of the optic nerve head.

In the glaucomatous eyes, immunostaining of glial cells and their processes around the axons for MMP-3 demonstrated an increase in the all regions of the optic nerve head compared with controls. The increase in the labeling intensity was particularly evident in the glial cells and along the axons and pial septae in the eyes with NPG (Figure 4). Perivascular anti–MMP-3 immunostaining was also noted.

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Table 1. Clinical Data of Postmortem Glaucomatous Eyes

<table>
<thead>
<tr>
<th>Patient No./Age, y/Sex</th>
<th>Diagnosis</th>
<th>IOP Range, mm Hg</th>
<th>Vertical C/D Ratio</th>
<th>Visual Field Damage</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/82/M</td>
<td>POAG</td>
<td>21-25</td>
<td>NA</td>
<td>NA</td>
<td>Topical β-blocker</td>
</tr>
<tr>
<td>2/78/M</td>
<td>POAG</td>
<td>12-28</td>
<td>NA</td>
<td>NA</td>
<td>Medical</td>
</tr>
<tr>
<td>3/74/F</td>
<td>POAG</td>
<td>16-28</td>
<td>0.7</td>
<td>Moderate</td>
<td>Medical</td>
</tr>
<tr>
<td>4/82/F</td>
<td>POAG</td>
<td>15-31</td>
<td>0.8</td>
<td>Moderate</td>
<td>Surgical + medical</td>
</tr>
<tr>
<td>5/84/F</td>
<td>NPG</td>
<td>11-14</td>
<td>0.95</td>
<td>Advanced</td>
<td>Topical β-blocker</td>
</tr>
<tr>
<td>6/84/F</td>
<td>NPG</td>
<td>11-14</td>
<td>0.95</td>
<td>Advanced</td>
<td>Topical β-blocker</td>
</tr>
<tr>
<td>7/88/F</td>
<td>NPG</td>
<td>14-18</td>
<td>0.8</td>
<td>Moderate</td>
<td>Topical β-blocker</td>
</tr>
<tr>
<td>8/82/F</td>
<td>NPG</td>
<td>13-17</td>
<td>0.8</td>
<td>Moderate</td>
<td>Topical β-blocker</td>
</tr>
<tr>
<td>9/82/F</td>
<td>NPG</td>
<td>13-17</td>
<td>0.8</td>
<td>Moderate</td>
<td>Topical β-blocker</td>
</tr>
<tr>
<td>10/74/F</td>
<td>NPG</td>
<td>13-18</td>
<td>0.8</td>
<td>Moderate</td>
<td>Topical β-blocker</td>
</tr>
<tr>
<td>11/74/F</td>
<td>NPG</td>
<td>12-19</td>
<td>0.9</td>
<td>Advanced</td>
<td>Topical β-blocker</td>
</tr>
</tbody>
</table>

Table 2. Semiquantitative Evaluation of the Intensity of Immunostaining in Optic Nerve Head

<table>
<thead>
<tr>
<th>MMP-1</th>
<th>MMP-2</th>
<th>MMP-3</th>
<th>TNF-α</th>
<th>Receptor 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 4)</td>
<td>0.50</td>
<td>0.56</td>
<td>0.28</td>
<td>0.38</td>
</tr>
<tr>
<td>POAG (n = 4)</td>
<td>0.29</td>
<td>0.96</td>
<td>0.71</td>
<td>0.72</td>
</tr>
<tr>
<td>NPG (n = 7)</td>
<td>0.67</td>
<td>1.86</td>
<td>1.19</td>
<td>0.94</td>
</tr>
</tbody>
</table>

* IOP indicates intraocular pressure; C/D, cup-disc; M, male; POAG, primary open-angle glaucoma; NA, not applicable; F, female; and NPG, normal-pressure glaucoma.
along the pial septae. In the eyes with NPG, glial cells located in the areas of preserved axons demonstrated more intensive immunostaining compared with the cells located in the areas of cavernous atrophy (Figure 3, B).

**TNF-α**

In control eyes, there was faint immunostaining for TNF-α and TNF-α receptor 1 in the processes of a few glial cells and around the nerve bundles and blood vessels of the optic nerve head.

In glaucomatous optic nerve heads, both the intensity of immunostaining and the number of stained cells for TNF-α or TNF-α receptor 1 were increased in all regions of the glaucomatous optic nerve head compared with controls. Immunostaining was positive in glial cells around the axons and vessels in the prelaminar and laminar regions of the optic nerve head in the glaucomatous eyes. In the postlaminar region, the glial cells distrib-
uted along the pial septae and scattered among the nerve bundles exhibited immunostaining. Although immunostaining for TNF-α was mostly associated with glial cells, an increased immunostaining for TNF-α receptor 1 was also observed in the nerve bundles, which was prominent in the prelaminar region of the glaucomatous optic nerve heads (Figures 5 and 6).

COMMENT

The integrity and turnover of the extracellular matrix are influenced by many factors, including MMPs. The MMPs are a family of proteolytic enzymes secreted by glial cells, and are capable of degrading almost all components of the extracellular matrix. The MMPs have been divided into the following 3 broad families based on their domain structure and substrate specificity. (1) Interstitial collagenase (MMP-1) and neutrophil collagenase (MMP-8) belong to the collagenase family; their major substrates are fibrillar collagen types I, II, and III. (2) The enzymes MMP-2 and MMP-9 are members of the gelatinase family; their substrates include types IV and V collagen, and fibronectin, proteoglycans, and gelatin. (3) Members of the stromelysin family include MMP-3 (stromelysin, transin) and MMP-7 (matrilysin); they act on a wide range of substrates, including proteoglycans, laminin, fibronectin, gelatin, and procollagen precursor peptides.15,19-23

Although they are implicated in several diseases of the central nervous system,11-13 little is known about the role of MMPs in either normal or glaucomatous human optic nerves. The localization of MMP-3 and MMP-2 and tissue inhibitor of metalloproteinases (TIMP-1) have been shown to be present in the normal primate optic nerve head and retina.24 In addition, increased gelatinase ac-
Activity has been found in glaucomatous monkey eyes.\(^{25,26}\) Our observation of the mild MMP immunolabeling of the glial cells in normal optic nerve heads and increased immunolabeling of MMPs in glaucomatous eyes is consistent with these limited studies.

Our observations revealed that the intensity of immunostaining for MMPs, TNF-α, and TNF-α receptor 1 was greater in glaucomatous optic nerve heads compared with controls. In addition, differential immunostaining patterns for these proteins were noted in the prelaminar, laminar, and postlaminar regions of the optic nerve head. Some of these differential patterns included the most prominent labeling of MMPs in the postlaminar region and the most prominent labeling of TNF-α and TNF-α receptor 1 in the prelaminar region of the glaucomatous optic nerve heads. One possible explanation of these findings may be based on the recently described regional and functional heterogeneity of glial cells in the optic nerve head. For ex-
ample, the size and the density of type 1B astrocytes in the prelaminar and laminar regions, and the type 1A astrocytes in the postlaminar region, are greater in glaucomatous eyes than in normal tissue.\textsuperscript{77-20}

Increased immunostaining of MMPs was noted in the cytoplasm of astroglial cells and their processes as well as in the extracellular matrix of optic nerve head in the eyes with POAG or NPG. The distribution of increased immunostaining for MMPs in the different regions of optic nerve head was comparable in the eyes with POAG or NPG. However, the intensity of immunostaining for MMPs, especially for MMP-2, was greater in the eyes with NPG compared with the eyes with POAG. In the eyes with NPG, immunostaining along the pial septae was moderately increased in the region of cavernous degeneration.

Cells secrete MMPs in an inactive form and the proenzyme can be activated in the extracellular space by various molecules. The antibodies used to recognize MMPs in this study identify both MMP precursors and the proteolytically processed active forms. Therefore, immunohistochemistry cannot distinguish the functional state in which the MMPs are present within the tissue. The abundance of immunoreactivity in the astrocytes suggests the presence of a large pool of intracellular MMPs that might function, under normal conditions, at relatively low levels in the extracellular space. Such pools could possibly be rapidly activated to act on substrates in the extracellular matrix under pathologic conditions.\textsuperscript{23}

The generalized increase in the expression of MMPs in the glaucomatous optic nerve head may have various consequences. Since MMPs are responsible for the degradation of the extracellular matrix components, their increased expression in the glaucomatous optic nerve head may represent a physiological response to counteract the increased extracellular matrix deposition that occurs in glaucomatous optic nerve head.\textsuperscript{30} This may explain the absence of glial scar tissue in glaucomatous optic nerves despite astroglial activation. It is tempting to speculate that tissue degeneration resulting from increased MMP activity may in part account for the excavated appearance of optic disc cupping that accompanies glaucomatous optic neuropathy, regardless of other factors such as intraocular pressure.

Matrix metalloproteinases have been proposed to play a role in axonal growth by preventing scar tissue formation in vivo.\textsuperscript{35,32} which is thought to be a barrier to trophic substances necessary for neuronal regeneration.\textsuperscript{33} Therefore, our observation of prominent immunostaining for MMPs in the areas of preserved axons may signify that activated glial cells increase secretion of MMPs for the dual purposes of preventing scar tissue formation while simultaneously promoting neuronal growth.

The pial septae of the normal optic nerve contains collagen types III and IV and fibronectin mainly around the blood vessels.\textsuperscript{34} These are the major substrates of MMP-2 and MMP-3. The increased immunostaining of MMP-2 and MMP-3 in the astrocytes and along the pial septae in the glaucomatous optic nerve head suggests that these MMPs may play a role in the disruption of pial septa seen in the areas of cavernous degeneration.

In addition, we observed increased expression of MMP-2 in the astrocytic processes enveloping blood vessels in the glaucomatous optic nerve head, particularly in the eyes with NPG. Since MMP-2 causes a thinning of the basal lamina and an increase in the capillary permeability,\textsuperscript{35} it seems possible that increased expression of MMPs in the perivascular area may influence the blood-brain barrier in this area.

Another finding we observed was increased immunostaining of TNF-α and TNF-α receptor 1 in the glaucomatous optic nerve heads either with POAG or NPG. Tumor necrosis factor α is a potent immunomediator and proinflammatory cytokine that is rapidly up-regulated in the brain after injury.\textsuperscript{36,37} It is also known as an inducer of apoptotic cell death via TNF-α receptor 1 occupancy.\textsuperscript{38} Tumor necrosis factor α has been implicated in the pathogenesis of several diseases of the nervous system, such as multiple sclerosis and autoimmune encephalomyelitis; it has also been thought to account for axonal degeneration and glial changes observed in the optic nerves of patients with acquired immunodeficiency syndrome.\textsuperscript{39} Although our studies demonstrated that the TNF-α immunostaining was mostly positive in the glial cells of the optic nerve head, TNF-α receptor 1 immunostaining was more prominently positive in nerve bundles located in the prelamellar section of the optic nerve head, which was increased in the glaucomatous eyes. This observation suggests that neuronal tissue is an important target for the effects of TNF-α. Our findings that the expression of TNF-α and MMPs are both increased in the glaucomatous optic nerve head is not surprising, since it is well known that there are interactions between TNF-α and MMPs for the regulation of their secretion and function.\textsuperscript{14-18} Therefore, increased expression of TNF-α in the glaucomatous optic nerve head suggests that this cytokine may play a role in tissue remodeling as a part of the astroglial activation process and/or may participate in tissue injury.

In addition to its potential to directly activate the cell death cascade in retinal ganglion cells and to facilitate remodeling of the optic nerve head in glaucoma, TNF-α may also contribute to the pathogenesis of glaucomatous neuropathy, as it is a potent stimulator of nitric oxide synthase,\textsuperscript{40-42} Recent evidence suggests that up-regulation of nitric oxide synthase occurs in human and experimental glaucomatous eyes.\textsuperscript{43} Furthermore, pharmacological inhibition of nitric oxide synthase–related ganglion cell death, which may be an important causal factor in glaucoma.

CONCLUSIONS

Increased expression of MMPs, TNF-α, and TNF-α receptor 1 may be collective components of the astroglial activation process that occurs in the glaucomatous optic nerve head. They may serve to prevent scar tissue formation and thus facilitate neuronal viability and repair. However, their increased expression may also have a role in the degenerative process of glaucomatous optic neuropathy as a result of facilitating formation of cavernous spaces, cupping, and the progression of neuronal damage.
Accepted for publication December 8, 1999.

This study was supported in part by the Otsuka Research Fellowship from the American Glaucoma Society, San Francisco, Calif, and gifts from the Laura K. Binder Fund and Pondill Glaucoma Research Fund, Chicago, Ill (Dr Edward), institutional core grants EY001792 (Dr Edward) and EY012314 (Dr Wex) from the National Eye Institute, Bethesda, Md, a grant from the Glaucoma Foundation, New York, NY (Dr Tezel), and an unrestricted grant to Washington University School of Medicine, Department of Ophthalmology and Visual Sciences, St Louis, Mo, from Research to Prevent Blindness Inc, New York, NY.

We thank David Brocato, ASCP, and Belinda McMahen for preparing the histopathologic sections.

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REFERENCES


