Characterization of the Gelatinase System of the Lamina Human Optic Nerve, and Surrounding Annulus of Bruch's Membrane, Choroid, and Sclera

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PURPOSE. We determined the presence and levels of gelatinase matrix metalloproteinases (MMPs) in the optic nerve and surrounding rim region of the human fundus.

METHODS. Samples of optic nerve, rim region, and Bruch's membrane-choroid from macular and peripheral regions were isolated from 9 pairs of human donor eyes. The MMPs were extracted and separated by gelatin zymography. Individual gelatinase species were identified by their respective molecular weights and levels quantified by standard densitometric techniques. Ratios of active/latent MMPs were calculated as representative indicators of the degree of proteolytic activity at each of the locations examined.

RESULTS. All of the gelatinase species normally found in Bruch's membrane also were present in the optic nerve region. The presence of the high molecular weight MMP species (HMW1 and HMW2) was indicative of the age-related accumulation of polymerized MMPs 2 and 9. Level of activated MMPs was considerably raised in comparison with their latent forms at the optic nerve and surrounding region indicative of greater ongoing turnover of the matrix ($P < 0.005$).

CONCLUSIONS. The components of the gelatinase pathway mediating matrix turnover in Bruch's membrane also were present in the optic nerve region. The presence of high levels of active MMPs 2 and 9 in comparison with the latent forms in the optic nerve and rim area is indicative of a high rate of matrix remodeling in these regions. Enhanced matrix turnover within the optic nerve region may represent an important mechanism for maintaining the plasticity of the lamina cribrosa.

Keywords: optic nerve, matrix metalloproteinases, glaucoma

The optic nerve head (ONH) is the convergence point for nearly two million retinal ganglion cell axons that, having arrived, must turn 90° to enter the neural canal, passing through an opening in Bruch's membrane, choroid, and sclera.¹ Within the neural canal, bundles of axons pass through holes in a perforated series of connective tissue beams known as the lamina cribrosa.^{2,3} These circular plate-like beams are attached to a circumferential ring of collagen and elastin fibers on the wall of the neural canal, and function as the load-bearing structures of the optic disc.^{4,5} Also present within the lamina cribrosa are astrocytes and laminar capillaries that together sustain the environment of the extracellular matrix (ECM), and provide nutritional support to cellular elements and traversing axonal segments.⁶⁻⁸

Ageing is associated with increased deposition of collagen and other ECM proteins within the lamina cribrosa.⁹⁻¹¹ Collagens I, III, IV, V, and elastin are increased within the cores of the cribriform plates, leading to an increase in laminar beam thickness.^{9,12} This is accompanied by an increase in the thickness of the laminar astrocyte basement membrane.^{9,13}

Decreased collagen solubility is a good indicator of the amount of abnormal collagen present. In donors under the age of 1 year, collagen solubility in the lamina cribrosa was observed to be 100%; that is, the presence of very little

damaged collagen. By the seventh decade of life, this solubility had diminished to 40%, indicative of the accumulative presence of oxidatively damaged and cross-linked collagen molecules.¹¹ Some of these cross-links arise from the nonenzymic glycosylation of the collagen molecule leading to the formation of advanced glycation end-products (AGEs).¹⁴ The pentosidine-based AGE cross-link has been shown to increase with age in the lamina cribrosa.^{11,14,15} The AGEs bind to their receptors (RAGE) to induce release of profibrotic cytokines, such as TGF-β, and proinflammatory cytokines, such as TNF-α and IL-6, leading to increased expression of ECM proteins.^{14,15}

These compositional and molecular alterations of ageing lamina cribrosa lead to increased thickness and rigidity of the laminar beams, restricted nutritional diffusion across the thickened basement membrane of laminar astrocytes, and a compromised nutritional delivery pathway to axonal segments.^{9,10,13,16-19} Similar changes also occur in the peripapillary sclera and the greater rigidity of the scleral canal also contributes to the pathophysiologic changes at the ONH.²⁰⁻²² The aged ONH, therefore, is more susceptible to damage from increased IOP or non-IOP-mediated insults.^{18,23-27}

More advanced changes in the composition of the lamina cribrosa^{7,28,29} and its elasticity^{18,30} have been noted in glaucomatous samples, leading to the hypothesis that damage

to ganglion axons within this region may underlie the primary pathophysiology leading to visual loss in this disease.^{4,31-37}

Most ECMs are maintained by coupled processes of synthesis and degradation to renew damaged components of the matrix. The degradation pathway is mediated by a family of Zn^{2+} -containing, Ca^{2+} -dependent enzymes referred to as the matrix metalloproteinases (MMPs),^{38,39} together with their tissue inhibitors (TIMPs).⁴⁰ The presence of MMPs 1, 2, and 3 has been demonstrated in ONH with levels considerably increased in primary open-angle and normal-pressure glaucoma patients.^{7,8,28,41}

Therefore, determining the level of activity of MMPs is important in understanding the role of matrix turnover at the ONH in normal ageing and disease processes. Most MMPs are secreted into the ECM as inactive zymogens (proenzymes). These proenzymes do not possess catalytic activity, but on exposure to SDS during the process of zymography, they are partially activated and, hence, their presence can be identified on zymographic gels. Physiologic activation involves the catalytic removal of a small inhibitory peptide from the pro-MMP molecule.⁴²⁻⁴⁴

Immunohistochemical studies undertaken so far cannot distinguish between active and inactive species, and the overall increase in degree of staining of glaucomatous samples has been assumed to reflect increased MMP activity. This could be an erroneous interpretation, since in human Bruch's membrane from AMD donors, active levels of MMP9 represented only 0.35% of the activity of pro-MMP9.⁴⁵

Recent work has shown the MMP system to be far more complicated than previously envisioned. In addition to the monomeric pro and active forms, high molecular weight polymeric forms, termed HMW1 and 2, also have been characterized.⁴⁶ Furthermore, large macromolecular MMP complexes, termed LMMC, and comprising pro-MMP9, and HMW1 and 2, and traces of proMMP2, also have been identified.⁴⁷ These species are interlinked to form the MMP Pathway (Fig. 1). The functional importance of this pathway lies in its ability to regulate ageing and regeneration of the extracellular matrix. In Bruch's for example, a shift of the pathway to the left leads to accumulation of high molecular weight species reducing the regeneration potential of the membrane.^{46,47} A greater shift to the left is apparent in age-related macular degeneration (AMD), compromising the level of active MMPs, thereby contributing to the structural and functional demise of Bruch's membrane.⁴⁵

Does the MMP pathway also operate at the ONH to maintain the structural and functional integrity of the extracellular

FIGURE 1. The MMP pathway in human RPE-Bruch's-choroid. Members of the pathway: pro- and active forms of MMPs 2 and 9, high molecular weight MMPs HMW1 and HMW2, and the LMMC comprising pro-MMPs 2 and 9, and HMW1 and 2. Apart from LMMC, all other components have been shown to exist in bound and free forms. Ageing shifts the pathway to the left with accumulation of high molecular weight species, whereas regeneration of Bruch's requires a shift of the pathway toward the right, with elevated levels of active MMPs 2 and 9. (Reprinted from Hussain AA, Lee Y, Zhang JJ, Marshall J. Disturbed matrix metalloproteinase activity of Bruch's membrane in age-related macular degeneration (AMD). *Invest Ophthalmol Vis Sci.* 2011;52:4459-4466.)

matrix? In this preliminary work, we have undertaken to screen human optic nerve and rim regions for the presence and relative distribution of the various gelatinase species comprising the MMP pathway. Furthermore, the relative level of active to proenzymes has been used to assess the degree of degradative activity in comparison with activities in the ECM of Bruch's membrane from macular and peripheral regions.

METHODS

Tissue Preparation

Human donor eyes, with consent granted for research (9 pairs; age range, 60-72 years and postmortem times, 24-48 hours) were obtained from the Bristol Eye Bank, UK, and processed for the analysis of MMPs. A further 5 pairs (age range, 56-75 years) were used for estimation of protein content at the various fundal locations examined. The corneas were removed for use in transplantation surgery and the remaining globes were transported to the laboratory on saline moistened pads in an icebox. Human donor tissue was procured from National Eye Banks following the principles of the Declaration of Helsinki.

Using a pair of curved scissors, the postscleral portion of the optic nerve was removed from the scleral shell. A circumferential incision then was made 5 mm posterior to the scleral sulcus, and the remaining anterior segment, lens, and vitreous discarded. The globe then was "opened" in the shape of a Maltese cross and laid flat. Regions to be sampled included macular and peripheral Bruch's choroid, optic nerve, optic nerve plus a 1-mm border area, a 1-mm wide annular rim adjacent to the border area, and the nerve fiber layer (NFL) overlying the optic disc (Fig. 2A). Obviously, all these regions could not be obtained from the same eye because of the overlapping of macular and rim regions, but the following number of samples were obtained from the nine pairs of donated eyes: optic nerve (9), optic nerve plus border area (5), rim (8), NFL (6), and 9 samples each for macular and peripheral regions.

Macular sample was obtained using a 6-mm diameter full thickness trephine centered on the fovea. This macular trephine was placed in PBS (Sigma-Aldrich Company Ltd., Dorset, UK) and the retina removed. The RPE cells then were brushed away with the aid of a camel-hair brush and the underlying Bruch's-choroid preparation isolated by blunt dissection. An identical procedure was followed to obtain a peripheral sample of Bruch's-choroid from the fundal periphery with the center of the trephine located on an imaginary line joining the optic disc, fovea, and the cut edge of the globe.

The remaining globe was immersed in PBS and the entire retina gently removed by cutting the attachments at the optic disc. In eyes where macular regions were not sampled, a 6-mm full thickness trephine centered on the optic disc initially was removed. This sample was processed further with a central 4-mm trephine to obtain the outer rim region. An incision then was made from the periphery of the 4-mm sample to the edge of the optic disc to allow access to the optic nerve. Then, using a fine pair of curved scissors, the entire optic nerve region was isolated. Optic nerve plus border regions were isolated in eyes where macular regions were sampled using a central 4-mm trephine. In a few samples ($n = 6$ eyes), the retina was removed by cutting around the edges of the optic disc with a fine pair of curved scissors. The remaining NFL overlying the optic disc then was isolated to determine its content of MMPs. A photograph of the optic nerve region and corresponding histologic section depicting the various regions isolated is shown in Figure 2B.

FIGURE 2. (A) Schematic representation to show the topographical relationship of the various samples used in the zymographic studies. (B) The *top photograph* shows a hemi-sectioned 8-mm trephine (retina to sclera) delineating the source of the full thickness samples obtained in the study. Retina and RPE would be removed prior to the sampling of tissue. The corresponding tissue sampled is shown in the histologic section at the *bottom* (stained with toluidine blue). The border area (BA) also would include the wall of the neural canal.

Sample preparation for zymography was performed according to the method of Hussain et al.,⁴⁵ by adding 100 μ L nonreducing SDS sample buffer (0.06 M Tris-HCl, 10% glycerol, 2% SDS, and 0.05% bromophenol blue, pH 6.8). The tubes were vortexed five times for periods of one minute each. Samples then were centrifuged for 5 minutes at 12,500g and 30 μ L of the supernatant were loaded onto the zymographic gels.

Zymography

For zymography, 10% SDS-PAGE gels (1.0 mm thick) were prepared containing a 4% stacking layer and 0.1% gelatin in the separating layer. Samples for analysis were loaded into lanes together with prestained protein molecular weight markers, spanning a molecular weight range of 6 to 500 kDa (Invitrogen, Paisley, Scotland, UK) and 20% fetal calf serum (FCS; Sigma-Aldrich Company, Ltd.) as an internal standard to correct for gel-to-gel variation in staining intensity. Electrophoresis was performed using the X Cell SureLock Mini-Cell system (Invitrogen).

After electrophoresis (150 V, 1 hour), the gels were removed from their cassettes, rinsed in distilled water and incubated for two half-hour periods in 2.5% Triton X-100 to remove SDS and renature the proteins. They then were transferred to reaction buffer (50 mM Tris-HCl, 10 mM CaCl_2 ,

75 mM NaCl, and 0.02% NaN_3 , pH 7.4) and incubated at 37°C for 18 hours to allow proteolytic digestion of the gelatin substrate. Gels were rinsed again in distilled water and stained with SimplyBlue SafeStain (Invitrogen) containing Coomassie G-250 for a period of three hours. Destaining was carried out with distilled water for 1.5 hours.

Gelatinase activity was observed as clear bands on a blue background. These gels were scanned at a resolution of 2400 dpi (Epson 3490 scanner, Epson, Hertfordshire, UK) and uploaded into the Quantiscan gel analysis software package (Quantiscan Version 3.0; Biosoft, Cambridge, UK) in grey-scale format and colors inverted so that MMPs were now visualized as dark bands against a whitish background. Densitometric scans were obtained providing the area under each individual gelatinase bands.⁴⁸ For normalization to control for gel-to-gel variation in staining intensity, the proMMP-9 band of the FCS sample was chosen as a reference since the alternative proMMP-2 band often showed distortion and skewing effects. These areas were corrected further to take into account the amount of sample loaded and, finally, the MMP activity of a given region expressed per microgram of protein.

Protein Estimation

Tissue samples from the various fundal locations were solubilized for 24 hours in 0.5 N NaOH and proteins estimated by the method of Lowry et al.,⁴⁹ using BSA as standard.

Statistical Analysis

Data are presented as mean $\pm$ 1 SD. The Mann-Whitney test was employed to assess significant differences ($P < 0.05$, $P < 0.005$) between optic disc and surrounding regions, and macular regions of Bruch's membrane using the add-in XLSTAT statistical analysis software for Microsoft Excel (Addinsoft, New York, NY, USA).

RESULTS

Representative zymograms of samples incorporating optic nerve region, rim region, macular and peripheral Bruch's membrane, and the NFL from four donors are shown in Figure 3. All the gelatinase species normally encountered in Bruch's also were found to be present in the optic nerve region. These included HMW1 and 2, pro- and active-MMP9, and pro- and active MMP2. The molecular weights of the MMP species in the optic nerve region were similar to those found in Bruch's membrane as judged by the appropriate migratory distances on the gels. The MMPs in the NFL (6 eyes) were barely detectable and, hence, any residual NFL tissue at the nerve head is unlikely to have generated a significant variation in the matrix pool of the optic nerve region.

It would have been ideal to base MMP levels on the basis of tissue volume so as to depict the actual concentration differences between the regions. Measurement of tissue volumes is extremely difficult, but protein estimation provides a good approximation, since protein levels should reflect tissue content. Protein content of the various regions sampled is expressed as mean $\pm$ SD (n), where n represents the number of eyes examined. The values are expressed as micrograms of protein per isolated sample: optic nerve region, 186 ± 47 (5) per sample; optic nerve + border area, 818 ± 71 (4) per sample; rim, 581 ± 104 (5) per sample; macular Bruch's-choroid, 229 ± 80 (5) per 6-mm sample; peripheral Bruch's-choroid, 178 ± 13 (5) per 6-mm sample.

Semiquantitative estimation of the level of individual gelatinase species found in the various regions is shown as

FIGURE 3. Representative zymograms showing the presence and relative distribution of individual gelatinase species. The blue colored gelatin zymographs were converted to a grey scale and color inverted so that MMP activities are represented as *dark bands* against a *greyish* background. All the gelatinase species normally encountered in macular and peripheral Bruch's membrane also were present in the optic nerve region. Levels of active MMPs 2 and 9 were much elevated in the optic nerve region compared to macular and peripheral locations. (A) Samples where rim regions were not obtained. (B) Samples where rim regions were obtained. ON, optic nerve region; Mac, macular; Per, peripheral.

the activity per microgram of protein (Fig. 4). There was no statistical difference in the level of gelatinase species between macular and peripheral Bruch's-choroid preparations. Activities in the various regions were compared to activities at the macula. Thus, gelatinase activities of the following MMP species, HMW1, HMW2, pro-MMP9, and pro-MMP2, were all significantly reduced in the optic nerve, optic nerve + border area, and rim region ($P < 0.005$ for all except $P < 0.05$ for HMW1 in the optic nerve).

The reduction in gelatinase activity in the optic nerve + border area compared to the optic nerve alone may be due to reduced activity in the border region and the high level of proteins in this compartment.

Despite the reduction in levels of pro-MMP9 in the optic nerve and surrounding region, levels of active-MMP9 were not different from those in macular Bruch's-choroid. Similarly, levels of active-MMP2 were similar in the optic nerve and rim region to macular Bruch's, but a significant reduction was observed when the combined optic nerve and border region was examined ($P < 0.005$).

Ratios of active to inactive MMPs also were calculated and are shown in Figure 5. These ratios were significantly elevated in the optic nerve and surrounding regions, indicative of greater matrix turnover in these regions.

DISCUSSION

The collagen molecule forming the backbone of most ECMs is a long-lived species, and it inevitably undergoes oxidative damage and must be replaced.^{11,50} Despite the presence of the MMP system capable of renewing ECMs, the cribriform plates show an age-related increase in thickening and rigidity.^{9,10,19} In aged eyes, compromised distensibility of the plates means that as the beams bend (however little) they risk shearing of the axonal segments and this may explain the

FIGURE 4. Semiquantitative analysis of the levels of individual gelatinase species at various locations in the human fundus. The MMP activities are expressed as gel band area per microgram protein and compared to activities found in the macular region. Levels of HMW1 and 2, and pro-MMPs 2 and 9 were all lower in the optic nerve and surrounding region. Active-MMP9 levels were similar to those in Bruch's membrane. Active-MMP2 levels in the optic nerve and rim region were similar to Bruch's, but the combined ON + BA sample showed a significant reduction. Data are represented as mean $\pm$ SD with $n = 9$ eyes for optic nerve, macular, and peripheral Bruch's; $n = 8$ for rim; $n = 5$ for ON + BA samples. * $P < 0.05$, ** $P < 0.005$.

greater susceptibility of the ageing optic nerve head to damage.^{24,51-53}

The underlying mechanisms leading to the ECM changes in the lamina cribrosa appear to be complex. In the present study, all the gelatinase species normally encountered in the ECM of Bruch's also were found to be present at the ONH (Fig. 3). One group of workers (Agapova et al.⁸) have failed to identify pro-MMP9 in optic nerve tissue or cultured astrocytes, but we observed pro- and active-MMP9 in all the optic nerves examined. The same group also was unable to detect MMP3, but its presence in the optic nerve region has been

FIGURE 5. Ratio of active to inactive MMPs 2 and 9 at various locations in the human fundus. Ratios of active MMPs were significantly elevated at the optic nerve and surrounding rim region. Data are represented as mean $\pm$ SD. ** $P < 0.005$, * $P < 0.05$.

confirmed.⁴¹ This discrepancy may lie in the sensitivity of the zymography technique used, the amount of tissue sampled on the gels, and the method of extraction. In the present study, MMPs were extracted directly into SDS sample buffer, and this solubilizes free and bound forms of the enzymes.^{45,46}

Identification of HMW1 and HMW2 species at the optic nerve region demonstrates the presence of the MMP pathway (Fig. 1). This pathway determines the rate of turnover of a matrix and, therefore, is important for maintaining the functional status of an ECM. In Bruch's membrane, it has been shown that the operation of the MMP pathway leads to an age-related increase in the level of high molecular weight complexes.^{46,47} These complexes are formed by the homo- and/or heteropolymerization of the monomeric MMP 2 and 9 species. High molecular weight MMP complex formation effectively sequesters the monomeric forms from the activation process.

Levels of the high molecular weight species (HMW1 and HMW2) were lower in the optic nerve area compared to Bruch's-choroid, but this may be due to correspondingly lower levels of their precursors, namely pro-MMPs 2 and 9 (Fig. 4). Despite the decrease in precursors, levels of active MMPs were similar to those in Bruch's membrane. Thus, the ratio of active to inactive MMPs was significantly elevated (Fig. 5). Provided that these active MMPs were accessible to their respective substrates, the data suggested a much higher level of degradative activity in the optic nerve region compared to Bruch's membrane. Such a high rate of turnover of the ECM may be very important in maintaining the plasticity of the cribriform beams.

Interestingly, the ratio of active to pro-MMPs 2 and 9 also was much elevated in the border annulus and rim region surrounding the optic nerve region. The border annulus region houses the circular ring of collagen that anchors the lamina beams to the side of the neural canal.^{5,54} High MMP activity in this region would be expected to maintain the flexibility of the attachment site, allowing it to bear some of the loading stress experienced in the optic nerve region and thereby reducing the degree of bending of the lamina beams. This may explain the need for maintaining a high level of matrix turnover in this region. The rim area also exhibited high MMP activity and this may be related to high rod photoreceptor density.⁵⁵

Although the level of active MMPs 2 and 9 was found to be considerably high compared to level of the proenzymes, ageing changes leading to the stiffness of the lamina beams have been widely reported as indicated earlier. This would suggest an inefficiency of the degradation process. The age-related increase in the amount of insoluble (and chemically altered) collagen extracted from the lamina cribrosa may make it less susceptible to enzymatic hydrolysis. Similarly, the age-related increase in levels of AGE also may contribute to reduced enzymatic breakdown, since AGEs are known to be powerful inhibitors of the MMP system.^{56–59} The presence of HMW1 and HMW2 in the optic nerve region provides another possible reason for compromised MMP activity, since it may remove proenzymes from the activation process. In Bruch's membrane, it has been shown that the operation of the MMP pathway leads to an age-related increase in the level of high molecular weight complexes^{46,47} and concomitant reduction in active MMP enzymes. Whether age-related changes in the optic nerve region also shift the MMP pathway toward the left has yet to be determined.

In glaucomatous tissue, additional factors appear to be involved in modulating the MMP system. Increased IOP is suggested to activate astrocytes, the major glial type in the optic nerve region, leading to the release of MMPs.^{8,60,61} The enhanced proteolytic activity then may degrade the extracellular matrix contributing to optic nerve head excavation.^{24,62} A

previous in vitro study has shown a reduction in the stiffness of the lamina cribrosa and peripapillary sclera on incubation with MMP2.⁶³ Thus, astrocyte activation and increased release of MMPs not only would assist in greater bending of the lamellar beams, but the sustained high IOP also would be expected to compress the lamina beams. Pressure-independent activation of astrocytes would be expected to lead to a similar outcome.

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