A Prospective Study of Matrix Metalloproteinases in Proliferative Vitreoretinopathy

Chee Hing Kon,1 Nicholas Laurence Osclestone,2 David Charteris,1 Julie Daniels,1 George William Aylward,1 and Peng Tee Kbhaw1

PURPOSE. The migration, proliferation, differentiation, and adhesion of cells and other cellular functions are influenced by the surrounding extracellular matrix, in normal and wound-healing conditions. The matrix metalloproteinases (MMPs) are a family of enzymes that degrade and remodel the extracellular matrix and, thus, play a central role in the wound-healing process. Proliferative vitreoretinopathy (PVR), a wound-healing process in the retina, is a major cause of the failure of retinal detachment surgery. The role of MMPs in the pathobiology of PVR is unknown. We have investigated the presence of MMPs in the vitreous of patients with retinal detachment and the predictive value of MMPs for the future development of PVR.

METHODS. A prospective study was conducted on 140 consecutive patients with a rhegmatogenous retinal detachment in whom vitrectomy was considered necessary because of a giant retinal tear and the presence of preoperative PVR, among other reasons. Vitreous samples were obtained and analyzed by zymography for the presence of MMPs. The patients were then followed up for the development of postoperative PVR (mild and severe).

RESULTS. Two species of MMPs were detected in the vitreous: MMP-2 and MMP-9. MMP-2 was detected in all of the vitreous samples obtained, whereas MMP-9 was found in only 64 (47%) of 136 samples. The levels of MMPs detected were not significantly associated with the presence of preoperative PVR (P > 0.05), but they were significantly associated (P < 0.05) with the development of postoperative PVR (mild and severe).

CONCLUSIONS. The results from this prospective study suggest that MMPs may be an important predictor and may also play a role in the development of postoperative PVR.

atic, and the patient, therefore, requested a vitrectomy. The second patient had undergone a cataract operation but had experienced a tear in the posterior capsule with vitreous prolapse, which required a limited vitrectomy.

**Matrix Metalloproteinase Profile Analysis by Gelatin Zymography**

The stored vitreous samples were defrosted and were centrifuged at 200g for 10 minutes, and the supernatant obtained (15 μL) was used for the gelatin zymography analysis. The samples first were denatured with an equal volume of dissociating buffer (65 mM Tris-HCl, pH 6.8, 10% [vol/vol] glycerol, 2% [wt/vol] sodium dodecyl sulfate, 0.0025% [vol/vol] b-mercaptoethanol [Novex R & D Systems, Oxon, UK]) for 10 minutes at room temperature. The samples then were resolved in conjunction with prestained, broad-range molecular weight standards (28,300–208,000 Da; Bio-Rad, Hertsfordshire, UK) on a 10% (vol/vol) Tris-glycine polyacrylamide gel containing 0.1% (wt/vol) gelatin (Novex) for 90 minutes at a constant voltage (125 V) and current (40 mA) in running buffer (25 mM Tris base, 192 mM glycine, 0.1% [wt/vol] sodium dodecyl sulfate, pH 8.3; Novex). The gels were carefully removed and incubated in renaturing buffer (2.5% [vol/vol] Triton X-100; Novex) for 30 minutes at room temperature. The renaturing buffer then was removed and was replaced with developing buffer (10 mM Tris base, 40 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl₂, 0.02% [vol/vol] Brij35; Novex) for a further 30 minutes. Fresh developing buffer was added, and the gels were incubated at 37°C for 20 hours before staining with 0.5% (wt/vol) Coomassie blue (Bio-Rad) in a solution of 45% (vol/vol) methanol, 45% (vol/vol) distilled water, and 10% (vol/vol) glacial acetic acid on an orbital shaker for 2 hours. Gelatinolytic activity appeared as clear bands on a blue background. To enhance band contrast, the gels were further destained in 45% (vol/vol) methanol, 45% (vol/vol) distilled water, and 10% (vol/vol) glacial acetic acid.

**Calculation of the Molecular Weight of Bands of Activity**

The molecular weights of a number of MMPs have been determined in previous studies. The molecular weight of each band in the sample was determined using prestained protein standards of known molecular weights. For each band of a standard, a value for relative distance travelled (R) was calculated and was used to plot a standard graph of log(molecular weight) against R.

\[
R = \frac{\text{distance moved by band}}{\text{distance moved by furthest standard band}}
\]

An R value for each band in the sample was calculated using the same formula as described earlier, and, using the graph plotted from the standards, the molecular weight of the each band was determined.

**Quantification of Matrix Metalloproteinase Activity**

To quantify the MMP activities using zymography, four twofold dilutions, starting at 2.5 ng (in 15 μL) of a commercially available, pure 92-kDa human MMP-9 proenzyme (Biogenesis, Bournemouth, UK) were run concurrently with the vitreous samples on each gel run. The pure MMP-9 served as a standard, whereby the MMP activity bands on the same zymogram gel of the vitreous samples could be compared. This was done as follows. The images of the gel first were photographed using a CCD video camera (model XC-77CE, Sony, Japan), and the image was captured into the personal computer using a video capture card and computer software (MicroScale TC/TM version 2, DigiHurst Limited, Hertfordshire, UK). The images then were analyzed using a computer software (Optimas; Data Cell, Maidenhead, UK) running under Microsoft Windows, version 3.1 (Microsoft, Redmon, WA). The area and average density of each of the four dilution bands of pure MMP-9 was measured. A standard curve then was constructed using the product of the area and density (y-axis) and the amount of pure MMP-9 used (x-axis). The relative level of MMP activities in the vitreous samples then could be determined by measuring the area and density of each band and comparing them to the standard curve. The level of MMP activity was classified into five groups, from 0 to ++ + + +. Each dilution band of the pure MMP-9 in the standard curve served as a cutoff point for the grouping.

**Inhibition of Gelatinolytic Activity**

To determine that the bands of gelatinolytic activity were in fact a result of MMPs, zymography was repeated in the presence of one of two broad-spectrum MMP inhibitors, Galardin-MPI (a gift from Glycomed, Alameda, CA) and 1,10-phenanthroline, which were prepared in dimethyl sulfoxide. These compounds are specific inhibitors of MMPs. Numerous vitreous samples that express the two gelatinolytic bands were chosen for this experiment. The concentrations of Galardin-MPI that were used were 10 nM, 100 nM, 1 μM, and 100 μM, and the concentrations of 1,10-phenanthroline were 2 nM, 20 nM, 200 nM, and 20 μM. Phosphate-buffered saline served as the control.

**Activation of Matrix Metalloproteinases**

To provide further evidence for the identity of the MMPs in the vitreous samples, full MMP profiles (active forms and inactive proenzymes) for the vitreous samples were obtained with and without prior activation. MMPs are initially produced as high-molecular weight, inactive proenzymes (pro-MMPs), which are then activated to give rise to the active, smaller molecular weight forms of the enzyme. When using standard zymography, the molecular weight bands correspond to the proenzymes. However, the proenzymes can be activated to their active molecular weight components before electrophoresis by incubation with 2 mM aminophenylmercuric acetate (APMA; Sigma, Poole, UK) for 2 hours at 37°C. The molecular weights of proenzyme and active MMP types were calculated using the standard plot, as described earlier. The results were compared with those obtained from pure human MMP-2 and MMP-9 (Biogenesis) treated in a similar manner.

**Statistics**

The chi-square test (statistics program SPSS for Windows Release 6; SPSS, Chicago, IL) was used to test for significant association, and the Mantel-Haenszel test was used for any linear association between vitreous MMP activity and the development of PVR.
RESULTS

Patient Profile
Data were available for 136 of 140 patients. Four patients were lost to follow-up because of the transfer of care to other hospitals and nonattendance at follow-up clinics. Of the 136 patients, 94 were male and 42 were female. The mean age was 59.0 years (range 16–86 years). The patients were followed up for at least 3 months with a mean follow-up time of 8.3 months.

Clinical Results
Thirty-nine (28.7%) and 14 (10.3%) of the 136 patients had mild and severe PVR before surgery, respectively. After surgery, 29 (21.3%) developed mild PVR, whereas 11 (8.1%) developed severe PVR.

Matrix Metalloproteinase Profile
Zymography runs conducted on the vitreous samples (N = 136) revealed two bands of activity corresponding to molecular weights of approximately 71 and 92 kDa (Fig. 1). The bands comigrated with the pure MMP-2 and MMP-9 standards. The 71-kDa band occurred in all samples at varying intensities. However, the 92-kDa band was detected in only 64 of the 136 vitreous samples (47%).

Inhibition of Gelatinolytic Activity by 1,10-Phenanthroline and Galardin-MPI
The gelatinolytic activity of the bands in the vitreous samples was progressively inhibited by increasing concentrations of Galardin-MPI (concentrations of 10 nM, 100 nM, 1 μM, and 100 μM) and 1,10-phenanthroline (concentrations of 2 nM, 200 nM, and 20 μM). This suggests that the activity of the bands is as a result of MMPs.

Activation of Matrix Metalloproteinase with Aminophenylmercuric Acetate
MMP-2 and MMP-9 proenzymes were activated by APMA with corresponding changes in molecular weight from 71 to 65 kDa and from 92 to 79 kDa, respectively. MMP-2 and MMP-9 proenzyme standards were activated to similar molecular weights (Fig. 2). This suggests that the two bands of MMP activity in the vitreous (molecular weights 71 kDa and 92 kDa) were proenzymes of MMP-2 and MMP-9, which were then activated to their respective active components.3,8

“Normal” Vitreous
The two “normal” vitreous samples expressed low MMP-2 activity (+++) and did not express any (0) MMP-9 activity.

Relationship between Matrix Metalloproteinase Activity and Preoperative Proliferative Vitreoretinopathy
Table 1 shows MMP-2 and MMP-9 activity in the vitreous of patients who had preoperative PVR and in that of patients who did not. There was no statistically significant (P > 0.05) association between either MMP-2 or MMP-9 activity and the presence of preoperative PVR.

Relationship between Matrix Metalloproteinase Activity and Postoperative Proliferative Vitreoretinopathy
Table 2 shows MMP-2 and MMP-9 activity in the vitreous of patients who developed postoperative PVR (mild and severe) and in that of patients who did not. There was a statistically significant (P < 0.05) association between the development of postoperative PVR and MMP-2 and MMP-9 activity. The Mantel-Haenszel test showed a significant (P < 0.05) linear asso-
FIGURE 2. Activation of matrix metalloproteinases (MMPs). Zymogram of unactivated and activated (with aminophenylmercuric acetate [APMA]) vitreous samples and samples of pure MMP-2 and pure MMP-9. On activation, lower molecular weight active MMPs were produced (71 to 65 kDa and 92 to 79 kDa). The two bands of MMP present in the vitreous were activated to similar molecular weights as pure MMP-2 and pure MMP-9. This suggests that the two bands in the vitreous were MMP-2 and MMP-9. A, Vitreous sample (unactivated); A', vitreous sample (activated with APMA); B, pure MMP-2 (unactivated); B', pure MMP-2 (activated with APMA); C, pure MMP-9 (unactivated); and C', pure MMP-9 (activated with APMA).

DISCUSSION

The formation of PVR membranes is part of the wound-healing response in which MMPs may play a part in the remodeling and contractile phase. Occleston et al. found increased levels of MMP expression during the contraction of three-dimensional collagen lattices containing anterior segment fibroblasts, and they also found that the inhibition of MMP activity abolished contraction. Similar results were obtained using retinal pigment epithelial cells in collagen lattices (Sheridan C., personal communication, July 1996). It is possible that secreted MMPs

**TABLE 1. MMP-2 and MMP-9 Activity and Preoperative PVR**

<table>
<thead>
<tr>
<th>Preoperative PVR No. of Patients (%)</th>
<th>None</th>
<th>Mild</th>
<th>Severe</th>
</tr>
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<tr>
<td>MMP-2*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>++</td>
<td>27 (32.5)</td>
<td>12 (30.8)</td>
<td>3 (21.4)</td>
</tr>
<tr>
<td>+++</td>
<td>20 (24.1)</td>
<td>10 (25.6)</td>
<td>2 (14.3)</td>
</tr>
<tr>
<td>++++</td>
<td>36 (43.4)</td>
<td>17 (43.6)</td>
<td>9 (64.3)</td>
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<tr>
<td>Total</td>
<td>85 (100.0)</td>
<td>39 (100.0)</td>
<td>14 (100.0)</td>
</tr>
<tr>
<td>MMP-9†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>47 (56.6)</td>
<td>20 (51.3)</td>
<td>5 (35.7)</td>
</tr>
<tr>
<td>+</td>
<td>19 (22.9)</td>
<td>8 (20.5)</td>
<td>1 (7.1)</td>
</tr>
<tr>
<td>++</td>
<td>17 (20.5)</td>
<td>11 (28.2)</td>
<td>8 (57.2)</td>
</tr>
<tr>
<td>Total</td>
<td>85 (100.0)</td>
<td>39 (100.0)</td>
<td>14 (100.0)</td>
</tr>
</tbody>
</table>

MMP, matrix metalloproteinase; PVR, proliferative vitreoretinopathy.

* $\chi^2 = 2.269; P > 0.05$ (not significant).
† $\chi^2 = 8.606; P > 0.05$ (not significant).

**TABLE 2. MMP-2 and MMP-9 Activity and Postoperative PVR**

<table>
<thead>
<tr>
<th>Postoperative PVR No. of Patients (%)</th>
<th>None</th>
<th>Mild</th>
<th>Severe</th>
</tr>
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<tr>
<td>MMP-2*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>++</td>
<td>36 (37.5)</td>
<td>6 (20.7)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>+++</td>
<td>25 (24.0)</td>
<td>8 (27.6)</td>
<td>1 (9.1)</td>
</tr>
<tr>
<td>++++</td>
<td>37 (38.5)</td>
<td>15 (51.7)</td>
<td>10 (30.9)</td>
</tr>
<tr>
<td>Total</td>
<td>96 (100.0)</td>
<td>29 (100.0)</td>
<td>11 (100.0)</td>
</tr>
<tr>
<td>MMP-9†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>60 (62.5)</td>
<td>10 (34.5)</td>
<td>2 (18.2)</td>
</tr>
<tr>
<td>+</td>
<td>19 (19.8)</td>
<td>7 (24.1)</td>
<td>2 (18.2)</td>
</tr>
<tr>
<td>++</td>
<td>17 (17.7)</td>
<td>12 (41.4)</td>
<td>7 (63.6)</td>
</tr>
<tr>
<td>Total</td>
<td>96 (100.0)</td>
<td>29 (100.0)</td>
<td>11 (100.0)</td>
</tr>
</tbody>
</table>

MMP, matrix metalloproteinase; PVR, proliferative vitreoretinopathy.

* $\chi^2 = 13.160; P < 0.05$.
† $\chi^2 = 17.231; P < 0.05$. 
degrade the matrix and create a pathway for the migration of cells within the matrix. In the in vivo PVR membrane, it is likely that retinal pigment epithelial cells produce MMPs, which then may play an important role in the contraction of the membrane. PVR occurs mainly in the inferior vitreous and retina where the retinal pigment epithelial cells settle, and, although membrane formation in this region is of little consequence because it is away from the macula, the problem arises when the membrane contracts and causes a tractional retinal detachment, or a break in the retina, or both.

The detection and quantification of MMPs can be performed by numerous methods. In this study, we chose to use zymography rather than methods such as enzyme-linked immunosorbent assay primarily because the quantity of vitreous that can be safely collected from patients is small. Enzyme-linked immunosorbent assay detects substances individually, and a larger quantity of vitreous would, therefore, be required to study each of the MMPs in turn. Zymography, however, is a sensitive test that requires only a small volume of vitreous from which the MMP profiles, active and inactive, can be detected.

From the study, two bands of gelatinolytic activity were found in the vitreous of patients with retinal detachment. There was good evidence that the bands of gelatinolytic activity were the proenzyme forms of MMP-2 and MMP-9. First, Galardin-MPI and 1,10-phenanthroline, two known inhibitors of MMPs, were shown to abolish these two gelatinolytic bands in a concentration-related manner in repeated zymography of the vitreous samples. Second, using molecular weight standards, the two bands were found to have molecular weights of approximately 71 kDa and 92 kDa, which correspond to the proenzyme forms of MMP-2 and MMP-9 respectively. Third, when the zymography was repeated using vitreous samples and commercially available pure MMP-2 and MMP-9 proenzyme standards, the two bands were found to comigrate to the same position in the gel as the known MMP standards. Last, on activation with APMA, two bands of similar lower molecular weights were exhibited by the vitreous samples and the known MMP standards.

In both “normal” vitreous samples, MMP-2, but not MMP-9, was detected. This is consistent with findings by other authors that MMP-2, but not MMP-9, is present in normal tissue and has a possible “surveillance” function for collagen remodeling in the event of occasional damage. Brown et al. found that MMP-2 was an endogenous vitreous MMP and was the major gelatinase in the human vitreous. Because the normal human vitreous contains collagen Types II, V, and IX and glycoproteins, some of which are substrates for MMP-2, Brown et al. have speculated that MMPs may be involved in the aging process of vitreous degeneration. Unfortunately, because of the small sample size of “normal” vitreous (n = 2), statistical comparison between vitreous from normal eyes and vitreous from eyes with retinal detachment cannot be made.

The finding of MMP-9 in the vitreous of eyes with retinal detachment is interesting. Studies have shown increased expression of MMP-2 and MMP-9 in wound healing of the cornea and skin, and Brown et al. have detected gelatinolytic activity in diabetic vitreous, which corresponds to a molecular weight of 92 kDa, which is similar to MMP-9. It has been suggested that the appearance of locally produced MMP-9 in ocular tissues is associated with trauma, inflammation, and wound healing, although its exact role is still unclear. Matsubara et al. suggested that MMP-9 may play a part in controlling resynthesis of the epithelial basement membrane in corneal wound healing. During retinal detachment and PVR, retinal pigment epithelial cells are dislodged from their basement membrane (the inner cuticular layer of Bruch’s membrane), proliferate, and secrete an extracellular matrix around themselves that contains molecules similar to the original basement membrane. It remains speculative whether, in PVR, MMP-9 performs a function similar to the one it performs in the cornea: the reorganization of the extracellular matrix.

In our study population of patients with retinal detachment, there was no significant difference in vitreous MMP-2 and MMP-9 activities between those with preoperative PVR (mild and severe) and those without. If we assume that PVR is an exaggerated form of wound healing in retinal detachment, this finding is unexpected, particularly concerning MMP-9. The results suggest that the expression of MMPs in wound healing may be unrelated to the intensity of the process. Although this could be true, it is more likely that an alternative explanation exists. The duration of retinal detachment and PVR processes vary considerably between patients, and it is possible that the levels of MMP activity reach a peak and decline with time. Therefore, the timing of collection of the vitreous samples is crucial. If the collections occurred after the stage of peak activity, any differences in the MMP activity between patients with uncomplicated retinal detachment and those with PVR would be undetectable.

To date, there have been no studies investigating the relationship between MMPs and the development of postoperative PVR. In this study, although no relationship between MMP activity and the presence of preoperative PVR was found, this was not the case for the development of postoperative PVR. Higher vitreous levels of MMP-2 and MMP-9 were found in a significantly greater proportion (P < 0.05) of those who developed postoperative PVR (mild and severe) compared with those who did not. There also was a significant association between the degree of MMP activity and the severity of postoperative PVR. This finding suggests that, regardless of its relationship to PVR before surgery, MMP activity may be a risk factor for the development of postoperative PVR. A possible explanation for this finding is that, in the presence of MMPs, any trauma or surgical intervention results in an exaggerated wound-healing response.

The findings in this study of the presence of MMP-2 and MMP-9 in the vitreous and of the relationship between their activity and the development of postoperative PVR have important implications for prediction for the future development of PVR and its treatment. At present, the measurement of MMP concentrations in the vitreous in our laboratory requires several hours. In the future, if this duration is shortened, it would enable the measurement of vitreous MMP concentrations during the operation and would allow the identification of those at risk of developing PVR who may benefit most from intravitreal pharmacologic treatment. Further studies are required to elucidate the exact role of MMPs and methods of inhibiting their activity in vivo to enable us to pursue possible new therapeutic avenues in PVR.

Acknowledgment

The authors thank Gillian Murphy for her helpful comments.
References

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