A Prospective Study of Matrix Metalloproteinases in Proliferative Vitreoretinopathy

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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. Vitreous samples were obtained and analyzed by zymography for the presence of MMPs. 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. (Invest Ophthalmol Vis Sci. 1998;39:1524-1529)

Proliferative vitreoretinopathy (PVR) is a major cause of failure in retinal detachment surgery. The pathobiology of PVR is complex, but, essentially, it is a wound-healing process that leads to the formation of epiretinal membranes, which subsequently contract and detach the retina. Wound healing, in general, involves inflammation, extracellular matrix deposition, and contraction, and tissue remodeling. A ubiquitous family of enzymes, the matrix metalloproteinases (MMPs), may play a role in the degradation of the extracellular matrix, which also occurs. The activity of MMPs is regulated by the tissue inhibitors of MMPs (TIMPs), of which four forms have been reported (TIMP 1, 2, 3, and 4). The degree of degradation of the extracellular matrix is dependent on the balance between MMPs and TIMPs.

We have found recently that MMPs also seem to be an important component of cell-mediated collagen contraction and are likely to play a part in the pathobiology of retinal detachment and PVR. To date, there have been no prospective studies assessing the prognostic value of vitreous MMP levels in PVR. Therefore, this study was carried out to determine whether MMPs were present in the vitreous of eyes undergoing surgery for retinal detachment, and whether this was associated with existing PVR or would be predictive of the future development of PVR.

Material and Methods

All subjects were treated in accordance with the tenets of the Declaration of Helsinki, and informed consent was obtained from patients after the nature of the study was explained to them.

Patient Recruitment

One hundred forty consecutive patients with rhegmatogenous retinal detachment who were to undergo a primary vitrectomy were enrolled between January 1995 and February 1996. Eyes with the following conditions were excluded: penetrating eye injury; history of blunt trauma to eye of less than 6 months; concurrent eye conditions, such as, infection; history of intraocular eye surgery of less than 6 months; and topical or systemic steroid treatment. The presence of PVR was defined as at least Grade C PVR in the updated version of the Retinal Society classification. This classification was further divided into mild and severe groups, which were defined as Grade C with up to 6 clock hours of involvement, and Grade C with greater than 6 clock hours involvement, respectively.

Collection of Vitreous Sample

At the beginning of a routine three-port pars plana vitrectomy, a neat vitreous sample was collected using the vitreous cutter without an infusion to prevent dilution of the sample. Because repeated freezing and defrosting could affect the composition and activity of any biologic factors, the samples were divided into aliquots and were placed in siliconized Eppendorfs. They were kept at $-70^\circ$C and were defrosted only in small quantities for each analysis.

Although fresh "normal" vitreous is, in general, rarely available for study, samples were obtained from two patients. The first patient had a large vitreous floater that was symptom-
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] β-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] Brom; 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] methylol, 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, two 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, Dighihurst Limited, Hertsfordshire, UK). The images then were analyzed using a computer software (Optimas; Data Cell, Maidenhead, UK) running under Microsoft Windows, version 3.1 (Microsoft, Redmond, 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. Therefore, the level of activity in a band was classified as 0 if it was below that of the fourth (highest) dilution, and as + if it was between the fourth and third dilution, and so on.

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.3,4

“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 (22.5)</td>
<td>2 (14.3)</td>
</tr>
<tr>
<td>++++</td>
<td>36 (43.4)</td>
<td>17 (45.0)</td>
<td>9 (64.3)</td>
</tr>
<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>
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<tr>
<td>++</td>
<td>17 (20.5)</td>
<td>11 (28.2)</td>
<td>8 (57.2)</td>
</tr>
<tr>
<td>Total</td>
<td>83 (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).
† $\chi^2 = 8.566; P > 0.05$ (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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<tbody>
<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>23 (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.
* $\chi^2 = 13.160; P < 0.05$.
† $\chi^2 = 17.231; P < 0.05$. 

X2 = 2.269; $P > 0.05$ (not significant).
$\chi^2 = 8.566; P > 0.05$ (not significant).
formed by numerous methods. In this study, we chose to use 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 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.8 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.8 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 cornea9 and skin10, and Brown et al.8 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.9 Matsubara et al.9 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.9 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

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References

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