Transplantation of Amniotic Membrane in Murine Herpes Stromal Keratitis Modulates Matrix Metalloproteinases in the Cornea

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PURPOSE. To study matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP) in the corneas of mice with ulcerative herpes stromal keratitis (HSK) treated with amniotic membrane transplantation (AMT).

METHODS. The corneas from BALB/c mice were infected with HSV-1. Mice with ulcerative HSK on postinfection (PI) day 14 were used for the experiments. In one group of mice, the corneas were treated with transplantation of amniotic membrane (AMT) that was secured with a tarsorrhaphy, and a control group underwent tarsorrhaphy alone. After 2 days, the appearance of corneal ulcers and stromal inflammation was judged clinically. Corneal sections were studied by immunohistochemistry for the expression of MMP-2, -8, and -9 and TIMP-1 and -2. MMP activity in the corneas was investigated by zymography, and the expression of the enzymes was measured by the Western blot technique.

RESULTS. At day 14 PI, the ulcers stained intensely positive for MMP-2, -8, and -9 and TIMP-1 and -2. Ulceration (P < 0.001), stromal inflammation (P < 0.01) and inflammatory cell infiltration (P < 0.001) markedly improved by day 2 after AMT. This was associated with reduced expression (P < 0.01) and activity of MMP-8, and -9 and increased localization of TIMP-1 (P < 0.01), whereas TIMP-2 was not affected. In contrast, high levels of expression of MMP-8 and -9 remained in the cornea after tarsorrhaphy, and the TIMP-1 expression was only slightly upregulated.

CONCLUSIONS. Rapid improvement of HSV-1-induced ulcerative keratitis is noted after amniotic membrane transplantation. This may be caused by reduced expression and activity of MMP-8 and -9, increased expression of TIMP-1, and sustained expression of TIMP-2. (Invest Ophthalmol Vis Sci. 2005;46: 4079–4085) DOI:10.1167/iovs.05-0192

An HSV-1 infection of the cornea may induce a severe inflammatory disease that is termed herpetic stromal keratitis (HSK). The corneal lesion is not caused primarily by viral replication, but by immune responses directed against the viral components. CD4+ Th1 lymphocytes play a key role in the initiation of the disease.1,2 Polymorphonuclear cells (PMNs) are the most prominent cells during development of the inflammation and are involved in the destruction of the corneal architecture.3,4 Recent experiments suggest that the proinflammatory cytokines interleukin-1 and TNF-α play major roles in the pathogenesis of HSK.5–7

The amniotic membrane (AM) is the innermost layer of the fetal membrane, comprising a thick, continuous basement membrane and an avascular stroma.8 AMs suitable for transplantation can be obtained shortly after elective cesarean delivery.9 In recent years, several anti-inflammatory effects from AMs have been described (Bültmann S, et al. IOVS 1999;40: ARVO Abstract 3044).12–16 Most recently, it has been shown that ulcerating necrotizing HSK rapidly improves after amniotic membrane transplantation (AMT).

Metalloproteinases (MMPs) are a family of proteolytic enzymes that are capable of degrading the extracellular matrix and basement membrane components. In general, MMPs are released as proenzymes and are activated by proteolytic cleavage of the N-terminal region. Tissue inhibitors of metalloproteinases (TIMPs) are the most important endogenous regulators of MMP activity.19–21 MMPs participate in tissue remodeling and also play an important role in corneal scarring. Previously, it has been shown that MMP-9, a considerable amount of which is produced by neutrophils, participates in the development of HSK and corneal ulcer.22,25

Natural inhibitors of various MMPs have been found in AMs.1,2,4 We therefore investigated whether AMT in experimental HSK would change the expression and activity of MMPs and their inhibitors in the HSV-1-infected cornea. We found that the AMT induced a strong improvement of the HSK and that the improvement was associated with reduced expression and activity of MMP-2, -8, and -9. The expression of TIMP-1 was increased, whereas TIMP-2 remained at the same expression level.

MATERIALS AND METHODS

Animals

Female BALB/c mice 6 to 8 weeks of age were used in the studies. The animals were maintained according to the guidelines of the protocols approved by the Institutional Animal Care and Use Committee. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Corneal HSV-1 Infection and Clinical Observation

The virus was propagated on Vero cells. Cells were harvested when viral cytopathic effects were present on all cells. The cells were freeze thawed three times, homogenized, and centrifuged at 5000g. The supernatants were then collected, divided into aliquots, and stored at −80°C until use. The titer of HSV-1 was evaluated by a standard plaque assay, as described elsewhere.25

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The BALB/c mice were infected as described previously.26 Briefly, mice were anesthetized intraperitoneally with ketamine-hydrochloride (2 mg) and mepivacaine-hydrochloride (400 ng). The corneas of the right eyes were scratched, and 5 μL of a suspension with 10³ plaque-forming units (PFU) of HSV-1 (KOS strain) was dropped on the cornea. Animals were examined daily with a surgical microscope (Carl Zeiss Meditec, Göttingen, Germany) for the course of HSK. The extent of stromal keratitis was graded on a scale of 0 to +, depending on the area of corneal opacity with corneal neovascularization, edema, and thinning: 0, clear eye; +1, <25%; +2, <50%; +3, <75%; and +4, 75% to 100%.26

Experimental Design
Mice with + ulcerating HSK were used in all experiments. Briefly, the cornea and conjunctiva of the right eye were covered with AM with the epithelium facing up and secured by tarsorrhaphy. In our experiments, the entire cornea and bulbar conjunctiva were covered with AM, with the epithelium facing up as a temporary patch and secured by tarsorrhaphy with three interrupted 10-0 nylon sutures. Tarsorrhaphy only was performed in the control group. After 2 days, the clinical signs of HSK were assessed. The mice were then killed, and the corneas or whole eyes were removed and fixed in formalin or snap frozen in liquid nitrogen. Whole-eye paraffin-embedded sections were used for histopathologic analysis. Corneal sections were studied by immunohistochemistry for the distribution of MMPs and TIMPs,23 by ELISA for the cytokine content and by zymography for the MMP enzymatic activity. MMP and TIMP expression was confirmed by Western blot analysis.

Preparation of Human AMs
The human placenta was obtained shortly after elective cesarean delivery. Under sterile conditions, the AM was removed by blunt dissection and washed three times in phosphate-buffered saline (PBS) containing antibiotics (50 μg/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL neomycin, and 2.5 μg/mL amphotericin B). The AM was flattened onto nitrocellulose paper (Hybond N+; GE Healthcare, Amersham, UK), with the epithelium facing away from the paper. The membranes were then stored at −80°C in 50% glycerol/50% DMEM.9,17

Histologic and Immunohistochemical Staining
Paraffin-embedded eyes were cut into 5-μm sections with a microtome (model RM 2135; Leica, Wetzlar, Germany). Sections were fixed on APES (3-aminopropyltriethoxysilane) covered slides and stained with a standard hematoxylin and eosin, as described previously.26 The central cornea was analyzed for the number of infiltrating PMNs and other mononuclear cells (lymphocytes, plasma cells, natural killer cells, and macrophages).

Alternatively, paraffin-embedded sections were stained with anti-MMP or anti-TIMP antibodies and visualized by the alkaline phosphate-anti-alkaline phosphatase (APAAP) detection system before counterstaining with Gill No.3 hematoxylin. The primary antibodies (all purchased from Santa Cruz Biotechnology, Santa Cruz, CA) used were goat anti-mouse MMP-2 (dilution, 1:200), goat anti-mouse MMP-8 (dilution, 1:100), goat anti-mouse MMP-9 (dilution, 1:200), rabbit antibody to TIMP-1 (dilution, 1:100), and goat anti-mouse TIMP-2 (dilution, 1:100). Secondary antibodies applied were biotin-conjugated rabbit anti-goat (dilution, 1:100; block, 5% mouse serum) or biotin-conjugated swine anti-rabbit (dilution, 1:100, block, 5% mouse serum). Negative controls were performed by staining without primary antibodies. The number of total infiltrating inflammatory cells, PMNs, the number of positively stained cells, and the distribution of the positively stained tissue areas were investigated in high-power fields (×450). The analysis was performed by two observers in a masked fashion.23

Western Blot Analysis
The amounts of soluble MMPs and TIMPs in the corneal specimens were compared with the Western blot technique.25 Briefly, the membranes gained after electrophoresis and blot transfer were incubated with the primary antibodies (all diluted 1:1000). Primary antibodies were directed against MMP-2, -8, and -9 and TIMP-1 and -2. Secondary antibodies were bovine anti-goat or bovine anti-rabbit antibody (diluted 1:1000 in the blocking buffer). β-Actin served as a standard. Membranes were placed in a chemiluminescent reagent (Santa Cruz Biotechnology) and then exposed to an x-ray film. The immunoreactive bands for the antigens were identified with prestained molecular weight standards. The protein concentrations were measured by the Bradford method (Bio-Rad, Munich, Germany). At each time point, three corneas were pooled from each of the experimental groups. From each solution, the same quantity of protein (15 μg) was used for each lane.

Zymography
Zymography was performed according to a published method.23 Briefly, gelatin and casein substrate zymography of HSV-1-infected corneas after tarsorrhaphy or AMT were analyzed to detect activities of MMP-2, -8 and -9 on 10% acrylamide minigels containing 0.1% gelatin or on 12% acrylamide gels containing 0.1% casein. Soluble proteins were extracted from each cornea by homogenizing the tissue in 2% SDS solution, and an equal amount of protein per group was analyzed. Activities of metalloproteinases were visible as clear bands against a dark-blue background.

Quantitation of Corneal Cytokines by ELISA
To investigate the effect of AMT on corneal cytokine production, the corneas were excised from the globes, and the conjunctiva and iris were removed. Samples were stored at −80°C until assayed. The corneas were thawed, minced, and sonicated for 30 seconds, and the supernatant was clarified by centrifugation at 10,000g for 10 minutes. The tissue homogenates were assayed for IL-1α, and TNF-α with the use of commercially available enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, Heidelberg, Germany).

Statistical Analysis
Data are presented as the mean ± SEM. Student’s t-test was applied to the cell counts. Fisher’s exact test was used to compare the incidence of HSK and a nonparametric test (Mann-Whitney) was applied to the clinical scores (all analyses were performed with SPSS software; Chicago, IL). P < 0.05 was considered statistically significant.

RESULTS
Clinical and Histopathologic Course of HSK after AMT
BALB/c mice were infected with 10⁵ PFU HSV-1 (KOS). The corneas of mice with severe stromal inflammatory infiltration, edema, neovascularization, and ulceration on day 14 PI were covered with an AM; control mice underwent a tarsorrhaphy (T). In agreement with a previous publication,17 the stromal inflammation only mildly improved after a 2-day treatment with a tarsorrhaphy, and the ulcerations were still present in most of the mice (Table 1). In contrast, rapid and marked regression of stromal keratitis was noted in all mice in the AMT group, and the ulcerations had already healed in nearly all the mice (Table 1).

The epithelial defects in the tarsorrhaphy group were still present in four of five specimens studied histologically. Edema, necrosis, neovascularization, and anterior chamber inflammation only mildly regressed in these mice (Fig. 1A). The numbers of total inflammatory cells and of PMNs enumerated in the central cornea were slightly reduced (Table 1), in contrast to the morphologic appearance in the AMT group. Two days after AM application, the epithelial defects had healed, and the
various signs of inflammation were also reduced (Fig. 1B). The number of inflammatory cells was markedly reduced after AMT (Table 1).

Localization of MMP-2, -8, and -9 and TIMP-1 and -2 in Tarsorrhaphy- and AMT-Treated Corneas with Ulcerative HSK

At day 14 PI, the entire epithelium and keratocytes expressed MMP-2. The area of the necrosis and at the ulceration had the highest MMP-2 staining intensity, in support of previous findings. In addition, a large proportion of the total infiltrating inflammatory cells, mainly consisting of PMNs, stained positive (Table 2). After 2 days of treatment with the tarsorrhaphy technique, MMP-2 was detected in the epithelial cells and keratocytes and, especially, close to the ulceration. The number of MMP-2-positive stained inflammatory cells in the cornea was slightly decreased (Table 2). Two days after AMT, the epithelium was stained less intensely. Compared with the tarsorrhaphy group, the staining for MMP-2 in the stroma was moderately reduced in the AMT mice. The total number of MMP-2-positive inflammatory cells, primarily of the PMNs, was slightly reduced (Table 2).

Table 2. Number of Infiltrating Inflammatory Cells in the Central Cornea with Positive Staining for MMPs and TIMPs

<table>
<thead>
<tr>
<th>Group</th>
<th>Positively Stained PMNs</th>
<th>Positively Stained Inflammatory Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>Day 14: 135.4 ± 25.9</td>
<td>102.1 ± 39.8</td>
</tr>
<tr>
<td>T</td>
<td>110.8 ± 24.6</td>
<td>91.3 ± 37.2</td>
</tr>
<tr>
<td>AMT</td>
<td>75.7 ± 28.4*</td>
<td>62.1 ± 20.7*</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Day 14: 247.3 ± 46.1</td>
<td>199.8 ± 24.7</td>
</tr>
<tr>
<td>T</td>
<td>229.4 ± 29.1</td>
<td>175.4 ± 12.2</td>
</tr>
<tr>
<td>AMT</td>
<td>92.6 ± 21.2†</td>
<td>83.3 ± 19.8†</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Day 14: 245.7 ± 49.5</td>
<td>225.3 ± 51.6</td>
</tr>
<tr>
<td>T</td>
<td>225.0 ± 30.9</td>
<td>194.6 ± 31.0</td>
</tr>
<tr>
<td>AMT</td>
<td>80.4 ± 15.1†</td>
<td>68.2 ± 12.9†</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Day 14: 89 ± 39.0</td>
<td>58.8 ± 38.4</td>
</tr>
<tr>
<td>T</td>
<td>68.0 ± 31.4</td>
<td>51.2 ± 24.1</td>
</tr>
<tr>
<td>AMT</td>
<td>106.0 ± 20.4†</td>
<td>92.6 ± 12.9†</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>Day 14: 239.2 ± 23.1</td>
<td>188.8 ± 12.9</td>
</tr>
<tr>
<td>T</td>
<td>225 ± 15.3</td>
<td>194.6 ± 17.3</td>
</tr>
<tr>
<td>AMT</td>
<td>80.4 ± 15.9</td>
<td>68.2 ± 14.5</td>
</tr>
</tbody>
</table>

BALB/c mice were infected with 10⁵ PFU HSV-1 (KOS). Mice with ulcerating + disease on day 14 PI underwent AMT or a tarsorrhaphy (T) Two days after treatment, the eyes were removed. Paraffin-embedded sections were stained with monoclonal antibodies directed against MMP-2, -8, -9, or -2 and against TIMP-1 and -2 and visualized by the alkaline phosphatase-anti alkaline phosphatase (APAAP) method. Cell-counting data are expressed as the mean ± SEM (n = 5 corneas per group).

*p < 0.05. † P < 0.01. Difference in the cell counts between T and AMT group (Student’s t-test).
At day 14 PI, intense MMP-8 staining was noted in the epithelium, stroma, and especially at the site of the ulceration. Many of the inflammatory cells, particularly the PMNs, stained positively for MMP-8 (Tables 1, 2). After 2 days of treatment with the tarsorrhaphy, the frequency of MMP-8-positive cells in the cornea was not altered (Table 2). In contrast, after 2 days of AMT, MMP-8-positive staining was markedly more reduced in the epithelium and stroma than in the tarsorrhaphy group. Correspondingly, the number of MMP-8-positive inflammatory cells was profoundly reduced (Table 2).

At day 14 PI, intense MMP-9 staining was present in the epithelial cells and keratocytes. A high number of positively stained cells were close to the ulcer. The inflammatory cells stained highly positive for MMP-9. Compared with these findings, the MMP-9 staining pattern of the cornea (Fig. 2A) in the tarsorrhaphy-treated group was not altered (Table 2). In contrast, after 2 days of treatment with AMT, the corneal sections showed less MMP-9 staining in the stroma than in the other groups, whereas some of the epithelial cells remained faintly stained (Fig. 2B). The number of positively stained inflammatory cells was markedly decreased (Table 2).

In the corneas of untreated mice at day 14 PI, the epithelium and keratocytes at the edges of the ulcer were faintly positive for TIMP-1. Compared with the untreated HSV-1-infected corneas, the TIMP-1 staining pattern in the corneas was not altered profoundly after a 2-day treatment with tarsorrhaphy (Fig. 2C). Only a small number of positively stained infiltrating inflammatory cells was detected in the cornea (Table 2). In contrast, strongly positive TIMP-1 staining was found in the inflammatory cells and keratocyte-like cells 2 days after AMT. The staining was also present in the epithelium and superficial stroma of these mice (Fig. 2D). The number of TIMP-1-positive inflammatory cells increased significantly (Table 2).

At day 14 PI, strong TIMP-2 staining was found in the epithelium, stroma, and inflammatory cells, especially close to ulcerative regions. In the corneas of mice treated with a tarsorrhaphy, a similar staining pattern and intensity for TIMP-2 were found. After 2-day treatment with AMT, brightly positive TIMP-2 staining was seen in the epithelium, stroma, and inflammatory cells of the cornea (Table 2).

Expression of MMPs and TIMPs after AMT or Tarsorrhaphy and in Corneas with Severe HSK

To confirm MMP and TIMP expression on the protein level in the different experimental groups, we performed a Western blot analysis. Corneal extracts were taken from mice in the three groups. A 63-kDa (MMP-2), 75-kDa (MMP-8), and 92-kDa (MMP-9) band was found in all the specimens from all groups of mice (Fig. 3). The bands identified as MMP-2, -8, and -9 showed a high level of expression in the corneas.
harvested on day 14 after the corneal HSV-1 infection and in those mice that had been treated with a tarsorrhaphy for 2 days. In contrast, MMP-8 and -9 expression was markedly reduced in corneal specimens obtained from mice in the AMT group, whereas MMP-2 expression was reduced slightly (Fig. 3).

A band corresponding with TIMP-2 (21 kDa), but not TIMP-1, (28 kDa) was detected in the corneal specimens of mice 14 days after infection. Only a small band of TIMP-1 was noted in the tarsorrhaphy specimens, whereas a strong TIMP-1 band was typically detected in the corneas harvested from the mice 2 days after AMT (Fig. 3). A strong TIMP-2 band was detected in all three groups. In contrast to the TIMP-1 findings, the protein level detected for TIMP-2 did not differ profoundly between the mice that were only HSV-1 infected and the mice that had undergone either tarsorrhaphy or AMT (Fig. 3).

The bands detected had molecular masses similar to the MMP and TIMP standards applied. No obvious nonspecific bands were detected during the Western blot process.

Gelatinolytic and Caseinolytic Activities of Soluble Corneal Proteins after AMT or Tarsorrhaphy

In the gelatin zymogram, bands corresponding with the proenzyme and active forms of MMP-9 and -2 were detected. A band of the proenzyme form of MMP-9 (92 kDa) was found in the corneas from all groups of infected mice. Two bands corresponding to the active forms of MMP-9 (83 and 65 kDa) were present in each group. In the corneas of mice at 14 days after HSV corneal infection, both of these bands were slightly more intense than those in the mice that had been treated for 2 days with tarsorrhaphy. However, both of these bands were more intense than in the corneas that were collected from mice 2 days after AMT treatment (Fig. 4A). The band that corresponded to the proenzyme form of MMP-2 (63 kDa) was present to a similar extent in the corneal specimens of all mice (Fig. 4A). In addition, one band that is typical of the active form of MMP-2 (57 kDa) was also found in all three of the experimental groups, and the enzymatic activity appeared to be similar in the different groups of mice (Fig. 4A).

In the casein zymograms, the bands corresponding with the proenzyme (75 kDa) and active form of MMP-8 (40 kDa) were detected in all specimens of the three groups. Strong caseinolytic activities were found in samples of mice on day 14 PI, and the samples of mice treated with tarsorrhaphy had slightly reduced enzymatic activity. In contrast, in samples of the AMT group, the activity identified for the proenzyme and active form of MMP-8 was reduced in comparison to that in the tarsorrhaphy group (Fig. 4B).

Levels of IL-1α and TNF-α in the HSV-1-Infected Corneas 2 Days after AMT or Tarsorrhaphy

A set of experiments was performed to elucidate the content of IL-1α and tumor necrosis factor (TNF)-α in the HSV-1-infected cornea after AMT, as these cytokines are well-known inducers of the expression and activation of MMP-2, -8 and -9. The level of cytokine expression in the corneal tissues was compared between the tarsorrhaphy- and AMT-treated mice. The results showed that the content of IL-1α and TNF-α was strongly reduced in the AMT-treated mice compared with the tarsorrhaphy group (Fig. 5).

![Figure 4](image-url) Gelatinolytic and caseinolytic activities in corneas with 4+ HSK on day 14, and 2 days after treatment with AMT or tarsorrhaphy (T). Soluble proteins were extracted from single homogenized corneas. Samples of 10-μg protein were then loaded on the gels and were electrophoresed under identical conditions. Shown are (A) minigels containing 10% polyacrylamide copolymerized with 0.1% gelatin for the detection of MMP-2 and -9 or (B) 12% polyacrylamide gel copolymerized with 0.1% casein for the detection of MMP-8. Molecular size standards are indicated in kilodaltons.

![Figure 5](image-url) Effect of human AMT on the expression of IL-1α and TNF-α in the cornea. Mice with necrotizing keratitis at day 14 after corneal infection were treated with AMT as a patch, or underwent tarsorrhaphy (T). Lysates from individual corneas (n = 10, each group) were prepared and measured by ELISA 2 days later. Compared with the control group, the AMT group showed reduced secretion of IL-1α and TNF-α. *P < 0.05.
**DISCUSSION**

Destruction of the extracellular matrix in the cornea is closely related to the increased activity of metalloproteinases.\(^{30}\) MMPs are expressed by the corneal epithelial cells and keratocytes, but also by infiltrating PMNs and macrophages. Activated PMNs are able to secrete MMPs from their granules.\(^{31,32}\) We have shown\(^{33}\) that the expression and activity of gelatinases (MMP-2 and -9) and collagenase (MMP-8) are upregulated in the corneas of mice with ulcerating necrotizing keratitis.

Recently, it has been shown that the severity of ulcerating HSK and the number of inflammatory cells in the cornea significantly decrease after AMT in HSV-1-infected BALB/c mice.\(^{17}\) The underlying T-cell-mediated immune disease may recur during the subsequent follow-up after removal of the membrane or after its dissolution. Local effects were suggested to be primarily responsible for the improvement.\(^{18}\)

Our immunohistochemical studies of HSV-infected corneas treated with AMT for 2 days revealed that the expression of MMP-8 and -9 was markedly reduced in corneal epithelium and keratocytes. It was also evident that the total number of inflammatory cells and the PMNs that stained positively with the respective MMP antibodies were significantly reduced after AMT, compared with the control group of mice that underwent a tarsorrhaphy. The reduction of the MMP-2 expression in the cornea after AMT was less pronounced.

These findings are supported by the Western blot analysis, showing that AMT was followed by a decrease in MMP-8 and -9 expression in the corneas. Furthermore, the zymographic investigation of the corneas disclosed that the enzymatic activities of MMP-8 and -9 were reduced after AMT. In contrast, the MMP-2 expression and gelatinolytic activity was only slightly reduced in the corneas at 2 days after AMT, but was apparent at 7 days after AMT (data not shown). Taken together, these findings suggest that the improvement of inflammation in the HSV-1 infected cornea after AMT may result in part from reduced expression and activity of MMP-8 and -9, especially in the infiltrating inflammatory cells.

There are several physiological mechanisms that control MMP expression and activity. MMP activity is critically regulated by TIMP.\(^{35}\) The proenzyme of MMP is bound and inactivated by the TIMP that is present in tissue. The balance between proteases and TIMP is critical for enzymatic activity.

AM itself has some components that are capable of inhibiting proteinases. Recently, TIMP-1 and -2 were identified in AM.\(^{31,34}\) The expression of TIMP-1 to -4 was presented on the freshly prepared and the cryopreserved membranes\(^{35}\) used in our experiments.

In our experiments, the immunohistochemical and Western blot techniques detected that TIMP-1 expression was increased in the HSV-1-infected corneas after AMT. The level of TIMP-2, which is expressed constitutively in tissue,\(^{22,35,36}\) was not altered after the AM treatment. The decreased MMP activity found in corneal tissue after AMT may have resulted from TIMP-1 and -2. Amniotic membrane patches also inhibits proteinase activity in a rabbit model of acute corneal alkali burn.\(^{24}\) It has also been shown that MMP-2 and -9 are suppressed in human corneal epithelium and fibroblasts cocultured with an AM extract (Kim JC, et al. \textit{IOVS} 2000;41:ARVO Abstract 1393; Park GS, et al. \textit{IOVS} 2001;44:ARVO Abstract 3083).\(^{24}\)

Recently, it has been shown that MMP-9 is involved in the corneal angiogenesis caused by HSV-1 infection.\(^{22}\) The development of stromal keratitis was greatly improved when MMP-9 activity was inhibited by topical administration of TIMP-1 plasmid DNA or when the MMP-9 gene was genetically knocked out in the mice. Of note, the regression of corneal neovascularization we noted in HSV-1-infected mice after AMT\(^{17}\) also correlated with downregulation of MMP-9 expression and activity and upregulation of TIMP-1 expression.

The expression and secretion of many MMPs are regulated by various proinflammatory cytokines (e.g., IL-1 and TNF-\(\alpha\))\(^{37–39}\). IL-1 and TNF-\(\alpha\) are upregulated in corneas from mice with recurrent HSK, whereas treatment with anti-cytokine antibodies abrogates the keratitis.\(^{5}\) The TNF-\(\alpha\) is essentially released from PMNs and macrophages.\(^{37}\) Transgenic mice that overexpressed the IL-1 receptor antagonist (IL-1ra) protein were more resistant to HSK than were IL-1ra knockout mice, and mice receiving IL-1ra had less severe HSK and corneal inflammatory cell infiltration.\(^{6,53}\)

In our results, high levels of TNF-\(\alpha\) and IL-1 were detected in the corneas of HSV-1-infected mice 2 days after tarsorrhaphy, whereas the levels of IL-1 and TNF-\(\alpha\) were significantly reduced 2 days after AMT. It has been shown that IL-1 and TNF-\(\alpha\) increase the expression of some of the MMP, including MMP-9, in a dose-dependent manner.\(^{38,40}\) It has also been shown that the expression of IL-1 in corneal epithelial cells is markedly suppressed by AM.\(^{15}\) It may be speculated that the decreased MMP-9 expression and activity in the HSV-1-infected cornea after AMT is caused by the reduced expression of TNF-\(\alpha\) and IL-1.

Taken together, the results show that the improvement in HSK after AMT may be attributable to the reduced expression of IL-1 or TNF-\(\alpha\) or indirectly to the cytokine-regulated down-regulation of MMP expression. As the IL-1 receptor antagonist was recently detected in both epithelial and mesenchymal cells of AM,\(^{10}\) this may be another reason for the reduced MMP expression observed in HSV-1-infected corneas after AMT.

Interleukin-10, which is capable of reducing the severity of HSK in mice,\(^{30,41}\) was also found in AM.\(^{15}\) This cytokine has been reported to promote the production of TIMP-1 and suppress the expression of MMP.\(^{42}\) However, no studies are available yet to define the action mechanism of IL-10 in the course of HSK after AMT.

In conclusion, our observations show that AMT modulates the expression and activity of collagenses (especially MMP-9) and of a caseinase (MMP-8) in the HSV-1-infected mouse cornea. This correlates with the upregulation of TIMP-1 and the sustained high expression of TIMP-2, two well-known inactivators of MMPs. Our findings suggest that proinflammatory cytokines are involved in the differential regulation of MMP expression and activity after AMT. The action mechanisms by which MMP and TIMP are influenced after AMT remain to be defined.

**References**


