

Active Form of Gelatinases in Tear Fluid in Patients with Corneal Ulcer or Ocular Burn

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Purpose: To investigate the expression of the active form of gelatinases (gelatinase A: matrix metalloproteinase-2 [MMP-2] and gelatinase B: matrix metalloproteinase-9 [MMP-9]) in ocular surface disorders, we determined the presence of the active form of these gelatinases in the tear fluid of patients with corneal ulcer or ocular burn.

Methods: The subjects consisted of the ulcer group comprising 13 eyes, the burn group comprising 6 eyes, and the normal group comprising 10 eyes. Tear samples were taken by the method of the Schirmer test I. The tears were eluted using extraction buffer containing 0.01 M phosphate buffer solution (pH 7.2), 1% Tween 20 and 1 M NaCl, and analyzed by gelatin zymography.

Results: Only the proforms of MMP-2, and MMP-9 were detected in the normal group and in uncomplicated herpetic keratitis or herpetic keratitis cases complicated by mixed infection in the ulcer group. Active MMP-9 was detected in mild corneal ulcer cases and mild ocular burn cases. Both active MMP-2 and active MMP-9 were detected in endogenous corneal ulcer cases and severe burn cases, including perforation cases.

Conclusions: Both active gelatinases were detected in tears of severe corneal ulcer or severe ocular burn cases. The active form of gelatinase expression may be related to the severity of ulceration. **Jpn J Ophthalmol 2003;47:423–426** © 2003 Japanese Ophthalmological Society

Key Words: Basement membrane, gelatinase, matrix metalloproteinase, ulceration, zymography.

Introduction

The matrix metalloproteinase (MMP) family is made up of neutrality enzymes and each of its substrates shares a part of the structure of the extracellular matrix (ECM). In normal conditions, they play a role in maintaining the turnover of the ECM by its degrading and remodeling. However, in pathological conditions, they take part in the process of tumor infiltration and metastasis,^{1–4} angiogenesis,¹ and other inflammatory reactions.⁵

Gelatinase is one of the MMPs that causes the degradation of basement membrane and is comprised of MMP-2 (gelatinase A) and MMP-9 (gelatinase B). In general, MMPs are secreted by cells initially as proform enzymes, and then they turn into the active form. Most MMPs are activated by other MMPs or proteases in extracellular

milieu. Only MMP-2 is activated by membrane type 1-MMP, which has a functional transmembrane domain on its surface.^{6,7} The activation of MMPs is an essential step to exhibit their functions.

Both MMP-2 and MMP-9 were reported to be the indispensable factors to cause a corneal ulcer in an animal model.⁸ In the human eye, it was reported that the tears in patients with peripheral keratitis and other corneal diseases contained a higher concentration of proform MMP-2 and MMP-9.^{9,10} However, the expression and the role of the active form of gelatinases are still unknown in various ocular surface disorders. We determined their concentration in the tears of patients with corneal ulcer or ocular burn to further investigate the expression of this type of gelatinase.

Materials and Methods

This study was approved by the Ethics Committee in the Nihon University School of Medicine and we followed the tenets of the Declaration of Helsinki. Informed

Received: January 22, 2003

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consent was obtained from the subjects. The subjects comprised patients with corneal ulcer, ulcer group (13 eyes); patients with ocular burn, burn group (6 eyes); and normal subjects, normal group (10 eyes). The ulcer group comprised 3 herpetic keratitis cases, 5 bacterial keratitis cases, 4 endogenous corneal ulcer cases, and 1 trophic keratitis case.

Tear samples were taken by the method of the Schirmer test I. The sampled test strip was stored at -20°C . After the stored strip was thawed at room temperature, it was soaked in 100 μL extraction buffer (0.01 M phosphate-buffered saline (pH 7.2), 1% Tween 20 and 1 M NaCl), and incubated overnight at room temperature. After elution, 20 μL of the elute with 20 μL loading buffer was analyzed by electrophoresis using gelatin zymography (Gelatin Zymo Electrophoresis Kit; Yagai Research Center, Yamagata) under the conditions of 20 mA for 20 minutes and 40 mA for 80 minutes. After washing with a washing buffer solution included in the zymography kit for 30 minutes, two times, the gel was incubated for 30 hours at 37°C in the reaction buffer solution, also included in the zymography kit, and the gel was stained in Coomassie brilliant blue and destained in methanol/acetic acid.

Results

A representative result of zymography is shown for the normal group in Figure 1. The proforms of MMP-2 and MMP-9 were detected at 72 and 92 kDa, respectively, but no active form of these gelatinases was detected.

Table 1 summarizes the zymographic results in the ulcer and burn groups. In the ulcer group, three types of expression were categorized based on the detected gelatinases. Eyes of patients with herpetic keratitis or herpetic keratitis complicated by subepithelial abscess due to suspected bacterial infection showed proform gelatinases in

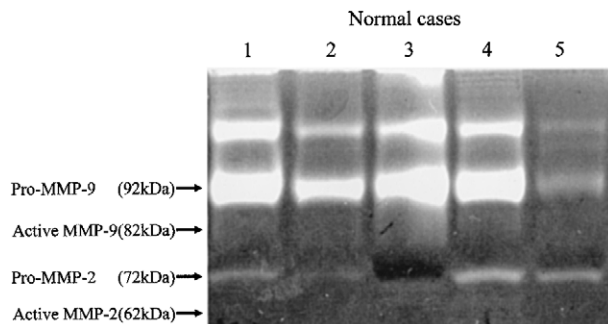


Figure 1. Representative results of gelatin zymography in the normal group. Proforms of matrix metalloproteinase (MMP)-2 and MMP-9 were present in all cases, but the active form was not detectable.

Table 1. Results of Gelatin Zymography in Ulcer Group and Burn Group

	No. of Samples Used for Gelatin Zymography	No. of Positive Cases*	
		Active MMP-2	Active MMP-9
Herpetic keratitis (Mixed infection, 2 cases)	3	0 (0)	0 (0)
Bacterial keratitis (Caused by <i>Pseudomonas aeruginosa</i> , 3 cases)	5	2 (0)	5 (3)
Endogenous corneal ulcer	4	4	4
Trophic keratitis	1	0	1
Alkali burn Grade I	3	0	3
Grade II	2	2	2
Grade III	1	1	1

* MMP: matrix metalloproteinase.

a pattern similar to that in the normal group. Eyes of patients with trophic ulcer or bacterial keratitis due to *Pseudomonas aeruginosa* expressed active MMP-9 (82 kDa) and both proform types. Eyes of endogenous corneal ulcer patients, including the perforation case or severe bacterial keratitis cases, expressed both active MMP-2 (62 kDa) and active MMP-9 (82 kDa), as shown in Figure 2.

In the burn group, mild alkali burn cases graded as Grade I by Roper-Hall classification expressed active MMP-9 and both proform gelatinases.¹¹ However, burn cases graded as Grade II or III expressed not only active MMP-9 but also active MMP-2, as shown in Figure 3.

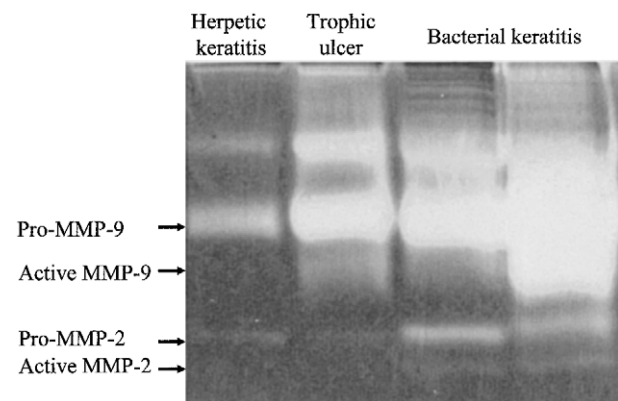


Figure 2. Representative results of gelatin zymography in the corneal ulcer group. No active form of gelatinase appeared in patients with herpetic keratitis. In patients with trophic ulcer, only active matrix metalloproteinase (MMP)-9 was detected. In bacterial keratitis cases, both active MMP-2 and active MMP-9 appeared.

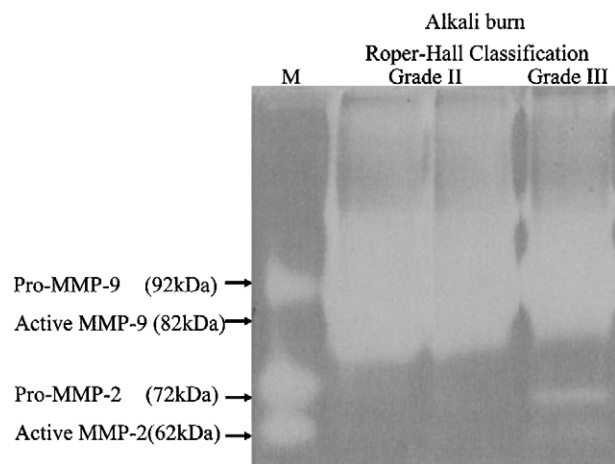


Figure 3. The results of gelatin zymography in alkali burn cases. The active form of both gelatinases appeared in 2 cases of Grade II and 1 case of Grade III by Roper-Hall classification.

Discussion

The basement membrane of the corneal epithelium consists mainly of type IV collagen and laminin.¹² The primary substrates of gelatinase are type IV collagen and laminin, and MMP-2 has been reported to exert a specific cleavage effect on laminin.^{13,14} Therefore, the gelatinase on the ocular surface has effects on corneal integrity.

Matsubara, Fini, and colleagues reported in their studies using animal injury models that MMP-2 and MMP-9 played an indispensable role in the degradation of the basement membrane of the corneal epithelium and in the inhibition of re-epithelization.^{8,14} In their studies, MMP-9 appeared within a day after the injury and disappeared in a few weeks. However, the activity of MMP-2, as well as of collagenase and stromelysin, maintained an increased level over several weeks. They concluded that MMP-9 was suspected to participate in the degradation of basement membrane in the early phase after the corneal injury, and that MMP-2 participated in the remodeling of the ECM and basement membrane after wound healing. So far, many studies on the relationship between MMP-9 and corneal wound healing have been performed.^{9,10,15,16} However, previous *in vivo* studies have mainly reported on the proform gelatinases alone, not the active form.^{9,10,14}

In our study, neither active MMP-2 nor MMP-9 could be detected in cases with herpetic keratitis or herpetic keratitis complicated by mixed infection. This suggests that in herpetic keratitis, not gelatinase but a toxic substance produced by microorganisms might have more potency in tissue destruction. Further investigation is needed with a larger number of cases to clarify this point. In bacterial keratitis caused by *Pseudomonas aeruginosa*

infection, though the ulceration was not so mild, active MMP-2 was not detected. This might indicate the presence of pseudomonal proteinases, but further investigation would be needed.¹⁷ In endogenous corneal ulcer or severe bacterial keratitis cases, including the perforation case, in the ulcer group and in the severe burn cases in the burn group, both active MMP-2 and MMP-9 were detected, suggesting the pivotal role of gelatinases, especially MMP-2 rather than MMP-9, in severe ulcer formation.

Because there seems to be a closer relationship between the expression of active MMP-2 and pathogenicity, compared with other MMPs, active MMP-2 expression has been thought to be more potent, not only in ulcer formation but also in tumor cell infiltration or metastasis, in terms of pathophysiological significance.⁴ Many studies have suggested that MMP-2 activity would be a good marker of cell infiltration or metastasis of malignant tumors. This phenomenon was considered to be a degradation activity of basement membrane and an angiogenic activity of MMP-2.¹ It was also reported that both active MMP-2 and active MMP-9 were detected in the tears of vernal keratoconjunctivitis patients and were thought to be a causative factor of shield ulcer.¹⁸

It has been reported that MMP-2 could be a remodeling factor after the corneal wound heals. Therefore, the activity of MMP-2 in tears may be coordinated with the wound-healing process. However, the subjects of our present study were eyes with corneal ulcer and we examined the MMPs in tears only once. Accordingly, our results could not show whether there was a dynamic change in the activity of the MMPs or not. In addition, the origin of MMPs in the tears is still unknown. Smith et al proposed in an *in vitro* experiment that MMP-2 and MMP-9 in the tear fluid were produced by granulocytes.⁹ Further investigation would be needed to determine whether the corneal cells produce the disease-causative MMPs. It could be confirmed that active MMPs are present in the tears of severe ulceration cases. The active form of gelatinases, especially MMP-2, may be closely related with corneal ulceration.

References

1. Fang J, Shing Y, Wiederschain D, et al. Matrix metalloproteinase-2 is required for the switch to the angiogenic phenotype in a tumor model. *Proc Natl Acad Sci USA* 2000;97:3884-3889.
2. Hrabec E, Strek M, Nowak D, et al. Activity of type IV collagenases (MMP-2 and MMP-9) in primary pulmonary carcinomas. *J Cancer Res Clin Oncol* 2002;128:197-204.
3. Onisto M, Garbisa S, Caenazzo C, et al. Reverse transcription-polymerase chain reaction phenotyping of metalloproteinase and inhibitors involved in tumor matrix invasion. *Diagn Mol Pathol* 1993;2:74-80.

4. Polette M, Nawrocki B, Gilles C, et al. MT-MMP expression and localization in human lung and breast cancers. *Virchows Arch* 1996;428:29–35.
5. Hasty KA, Reife RA, Kang AH, et al. The role of stromelysin in the cartilage destruction that accompanies inflammatory arthritis. *Arthritis Rheum* 1990;33:388–397.
6. Nagase H. Activation mechanisms of matrix metalloproteinases. *Biol Chem* 1997;378:151–160.
7. Atkinson SJ, Crabbe T, Cowell S, et al. Intermolecular autolytic cleavage can contribute to the activation of progelatinase A by cell membranes. *J Biol Chem* 1995;270:30479–30485.
8. Matsubara M, Zieske JD, Fini ME. Mechanism of basement membrane dissolution preceding corneal ulceration. *Invest Ophthalmol Vis Sci* 1991;32:3221–3237.
9. Smith VA, Hoh HB, Easty DL. Role of ocular matrix metalloproteinases in peripheral ulcerative keratitis. *Br J Ophthalmol* 1999;83:1376–1383.
10. Smith VA, Rishmawi H, Hussein H, Easty DL. Tear film MMP accumulation and corneal disease. *Br J Ophthalmol* 2001;85:147–153.
11. Roper-Hall MJ. Thermal and chemical burns. *Trans Ophthalmol Soc UK* 1965;85:631–653.
12. Klyce SD, Beuerman RW. Structure and function of the cornea. 2nd ed. In: Kaufman HE, ed. *The cornea*. Boston: Butterworth-Heinemann, 1998:3–50.
13. Giannelli G, Falk-Marzillier J, Schiraldi O, et al. Induction of cell migration by matrix metalloproteinase-2 cleavage of laminin-5. *Science* 1997;277:225–228.
14. Fini ME, Parks WC, Rinehart WB, et al. Role of matrix metalloproteinases in failure to re-epithelialize after corneal injury. *Am J Pathol* 1996;149:1287–1302.
15. Azar DT, Hahn TW, Jain S, et al. Matrix metalloproteinases are expressed during wound healing after excimer laser keratectomy. *Cornea* 1996;15:18–24.
16. Sobrin L, Liu Z, Monroy DC, et al. Regulation of MMP-9 activity in human tear fluid and corneal epithelial culture supernatant. *Invest Ophthalmol Vis Sci* 2000;41:1703–1709.
17. Kosaku K, Maeda N, Yokoyama T, et al. Effect of matrix metalloproteinase inhibitor on experimental pseudomonal corneal ulceration. *Nippon Ganka Gakkai Zashi (J Jpn Ophthalmol Soc)* 1998;102:717–723.
18. Kumagai N, Yamamoto K, Fukuda K, et al. Active matrix metalloproteinases in the tear fluid of individuals with vernal keratoconjunctivitis. *J Allergy Clin Immunol* 2002;110:489–491.