The Effect of Sodium Hyaluronate on the Expression of Gelatinases in Rabbit Corneal Epithelial Wound Healing

Makoto Sakuma, Satoshi Miyachi and Takashi Sakamoto
Tokyo Research Institute, Seikagaku Corporation, Tokyo, Japan

Purpose: We investigated the effect of sodium hyaluronate (Na-HA) on the expression of gelatinases in a rabbit model with corneal epithelial defects.

Methods: Topical administration of Na-HA or phosphate-buffered saline (PBS) was carried out in the experimental eyes after surgical removal of the corneal epithelium. At 0, 6, 24, 48, and 72 hours after wounding, mRNA expression of 72 kDa type gelatinase (MMP-2), 92 kDa type gelatinase (MMP-9), and tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) were analyzed by reverse transcription polymerase chain reaction in those corneas. In addition, gelatinolytic activities were investigated using gelatin zymography.

Results: The levels of constitutive expression of MMP-2 and TIMP-1 mRNA persisted, whereas MMP-9 mRNA in the PBS-treated side was expressed temporarily after surgical removal. In the Na-HA-treated side, at 6 hours after wounding, a much higher expression of MMP-9 mRNA was reproducibly observed compared with that in the PBS-treated side. In zymography, the levels of gelatinolytic activity corresponding to proMMP-9 were significantly higher in the Na-HA-treated side than in the PBS-treated side at 6 hours after wounding.

Conclusions: These results suggest a novel participation of Na-HA in the expression of MMP-9 in rabbit corneal epithelial wound healing.

Key Words: Cornea, epithelial wound healing, hyaluroma, MMP-9, rabbit.

Introduction
Hyaluronan is an important component of the extracellular matrix and is thought to participate in migration, proliferation, and differentiation of cells in connective tissues. Hyaluronan is also involved in the physiological function of cells in the cornea. It has been reported that sodium hyaluronate (Na-HA) enhanced epithelium migration, the proliferation of epithelial cells, and the corneal epithelial wound healing in vivo.

Recently, it has been suggested that matrix metalloproteinases (MMPs) also play an important role during corneal wound healing. The MMPs from an enzyme family that shares some highly conserved domains and that has the activity of degrading various components of the extracellular matrix. The proteolytic activity of the family is inhibited by members of the tissue inhibitor of matrix metalloproteinases (TIMP) family. Moreover, the MMP family can be divided into three main subfamilies: collagenases, stromelysins, and gelatinases. Gelatinases consist of 72 kDa type gelatinase (MMP-2) and 92 kDa type gelatinase (MMP-9), which can cleave gelatin and type IV, V, and VII collagens. There are reports of gelatinases in remodeling rabbit cornea; that is, MMP-2 was increased in the stroma and MMP-9 was detected in both the epithelium and the stroma. Consequently, it was assumed that MMP-2 participates in the prolonged process of collagen remodeling in the corneal stroma and the MMP-9 functions in controlling resynthesis of the subepithelial basement membrane. We expected participation of these gelatinases not only in such a remodeling but also in corneal epithelial cell migration.

Therefore, in order to understand the mechanism of the enhancement of corneal epithelial wound healing by Na-HA, we investigated the effect of Na-
HA on the expression of gelatinases, MMP-2 and MMP-9, and their inhibitor, TIMP-1, in rabbit corneal epithelial wound healing.

**Materials and Methods**

**Animals**

Twenty-seven female Japanese white rabbits, weighing about 3.0 kg, were used in our experiments.

**Test Substances**

Sodium hyaluronate with a molecular weight of $64 \times 10^4$ was obtained from Seikagaku Corporation (Tokyo); it was purified from chicken combs and free from pyrogen. Sodium hyaluronate was dissolved with phosphate-buffered saline (PBS) at a concentration of 0.2% (w/v), and the solution was used as a test solution. Phosphate-buffered saline, a solvent of Na-HA, was used as a negative control solution.

**Corneal Organ Culture**

The organ culture conditions described previously were modified as follows: a cornea was excised along the corneo-scleral border and cultured for 24 hours at 37°C in a humidified 5% CO$_2$ atmosphere in 3 mL of 199 medium (The Research Foundation for Microbial Diseases of Osaka University, Osaka) containing phorbol 12-myristate 13-acetate (100 ng/mL) to induce gelatinase production. The cultured cornea was used as the origin of gelatinase standards.

**Surgical Removal of Corneal Epithelium**

The corneal epithelium of the central region was removed using a trephine (8 mm inside diameter), a 23-gauge needle and microscissors after general anesthesia with an intravenous injection of ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (20 mg/kg), and with a topical application of 0.4% oxybuprocaine hydrochloride eyedrops. The corneal epithelium, basement membrane, and a part of the stroma were removed.

**Topical Administration**

At 1 and 4 hours after surgical removal of the corneal epithelium, 150 μL of the 0.2% Na-HA solution was administered topically to the left eyes, and the same volume of PBS was administered to the right eyes. In addition, on the first and second days after the operation, the Na-HA solution and PBS were applied four times per day, and on the third day twice a day at an interval of 3 hours.

**RNA Extraction**

At 0, 6, 24, 48, and 72 hours after the operation, rabbits were sacrificed by intravenous injection of a lethal dose of sodium pentobarbital. The corneas were dissected along with the scleral rim and were immediately frozen in liquid nitrogen. The frozen corneas were crushed using a Freeze-Smasher (Cryo-Press; Microtec, Chiba), and the resultant frozen corneal powder was homogenized in phenol reagent (Isogen; Nippon Gene, Toyama). The homogenized samples were centrifuged in order to remove insoluble material.

Total RNA was isolated from the resulting supernatant by the method of acid guanidinium thiocyanate-phenol-chloroform extraction modified as follows: the supernatant was extracted with phenolchloroform, and then RNA was precipitated with isopropanol. The RNA pellet dissolved in water was digested with proteinase K (Sigma Chemical, St. Louis, MO, USA) and chondroitinase ABC (Seikagaku) to eliminate the contaminating impurities. The RNA treated with these enzymes was further extracted with phenol-chloroform and precipitated with ethanol. The concentration of RNA was determined by absorbance at 260 nm.

**Reverse Transcription Polymerase Chain Reaction (RT-PCR)**

The first-strand cDNA was synthesized from 1 μg of the extracted total RNA using 200 units of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL, Gaithersburg, MD, USA) with 15 units of RNase inhibitor (Promega, Madison, WI, USA) and oligo(dT) primers in a 10-μL of reaction solution at 37°C for 1 hour.

The PCR primers were designed from the published sequences for human MMP-2, rabbit MMP-9, rabbit TIMP-1, and, as an internal standard, rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1). The primers for MMP-2 were chosen from the sequences encoding some highly

<table>
<thead>
<tr>
<th>Species</th>
<th>5' Primers</th>
<th>3' Primers</th>
<th>Size (Base Pairs)</th>
<th>Probes</th>
</tr>
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<tr>
<td>MMP-2</td>
<td>1352–1369</td>
<td>1841–1858</td>
<td>507</td>
<td>1781–1807</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>160–177</td>
<td>547–564</td>
<td>405</td>
<td>457–486</td>
</tr>
<tr>
<td>GAPDH</td>
<td>678–695</td>
<td>954–971</td>
<td>294</td>
<td>807–836</td>
</tr>
</tbody>
</table>

MMP-2: 72 kDa type gelatinase; MMP-9: 92 kDa type gelatinase; TIMP-1: tissue inhibitor of matrix metalloproteinases-1 (TIMP-1); GAPDH: glyceraldehyde-3-phosphate dehydrogenase.
conserved parts from species between human and rat. The expected sizes of each PCR product are presented in Table 1.

The PCR reaction was performed with 1/10 volume of reverse transcription reaction product, adding 1.25 units of Taq DNA polymerase (Promega), and 20 pmols each of 5' and 3' primer in a 50-μL final volume. The PCR reaction was carried out in a DNA Thermal Cycler model P 2000 (Perkin Elmer Cetus, Norwalk, CT, USA) with the following cycle protocol: initial denaturation at 94°C for 3 minutes, followed by 30 or 35 cycles each of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 1 minutes. Five microliters (1/10 volume) of the PCR products were electrophoresed on a 2% agarose gel. The gel was stained with ethidium bromide, and the PCR products were sized by comparison to DNA size markers (Promega). In order to confirm the identity of the PCR products, the PCR fragments were transferred to a nylon membrane and hybridized with 32P-labeled internal oligonucleotide probes (Table 1). Moreover, to confirm the fidelity of the PCR products which were expected to be derived from MMP-9 mRNA, these products were subcloned using a pGEM-T Vector System (Promega) and sequenced. The number of PCR cycles was 35 times except 30 times for GAPDH, chosen to avoid a saturation of the reverse transcription reaction product, adding 1.25 units of reverse transcriptase (Promega) and 20 pmols each of 5' and 3' primer in a 50-μL final volume. The obtained values were normalized with each standard in the gels and statistically analyzed by the paired t-test between two samples.

Results

RT-PCR

The MMP-2 and TIMP-1 mRNA were expressed constitutively in normal cornea and expressed stably at all measured times after corneal epithelial wounding in both eyes (Figures 1A,C). In order to confirm the reproducibility, the same experiments were independently carried out using the corneas derived from rabbits of a different group, and the results were the same. Equal intensities were observed in the PCR product for GAPDH at all measured times (Figure 1D), so that similar amounts of cDNA would be amplified in each of the different samples. Thus, it was concluded that Na-HA had no effect on the expression of MMP-2 and TIMP-1 mRNA.

In contrast to those messengers, it was observed that MMP-9 mRNA was expressed temporarily after corneal epithelial wounding (Figure 1B). The MMP-9 mRNA was not expressed in the normal cornea. In the PBS-treated side, at 6 hours after wounding, MMP-9 mRNA was first detected, followed by an obvious increase at 24 hours, a great decrease at 48 hours and a second increase at 72 hours. Similar results of the kinetics of the expression were obtained in other corneas derived from rabbits of a different group. In the Na-HA-treated side, the level of the initial expression at 6 hours was much higher than that in the PBS-treated side. In addition, the obvious increase from 6 to 24 hours shown in the PBS-treated side was not observed, and little MMP-9 mRNA was detected at 48 hours (Figure 1B).

We focused our attention on the higher expression of MMP-9 mRNA in the Na-HA-treated side at 6 hours after wounding; an additional experiment was carried out to confirm that tendency. More PCR products for MMP-9 were consistently detected in the Na-HA-treated side compared with the amount of products in

Extraction of Gelatinases from Corneas

Six different rabbits were used for each of the three groups, ie, normal group, 6-hours-later group and 24-hours-later group. At 6 and 24 hours after surgical removal, rabbits treated with topical administration of the Na-HA solution and PBS were sacrificed by intravenous injection of sodium pentobarbital. The corneas of these rabbits and the cultured corneas as the origin of gelatinase standards were frozen and centrifuged in order to remove insoluble material.

Zymography

Zymography was performed with 7.5% SDS-polyacrylamide gel containing 0.1% gelatin according to the method previously reported. An aliquot (8 μL) of corneal extract was subjected to SDS-polyacrylamide gel electrophoresis under nonreducing conditions. The gel was then incubated in 25 mL of the incubation buffer (50 mM Tris-HCl, 5 mM CaCl2, 1 μM ZnCl2, 0.02% NaN3, pH 7.5) for 22 hours at 37°C, followed by rinsing with the same buffer to which Triton X-100 (2.5%) was added, in order to remove the SDS in the gel. Thereafter, the gel was stained with Coomassie Brilliant Blue R-250 and gelatinolytic bands were visualized. After staining, the gel was dried and analyzed with a spectrophotometer (DU-640; Beckman Instruments, CA, USA) using a gel scan accessory. The gelatinolytic bands corresponding to proMMP-9 were quantified with respect to the corneas of the PBS-treated side (right eye) and the Na-HA-treated side (left eye).
the PBS-treated side from three different rabbits (Figure 2). In lane 6 of Figure 2B, relatively weak MMP-9 mRNA was induced. This would reflect differences of sensitivity to Na-HA among rabbits. Moreover, PCR products of unexpected size were shown. In confirming the identity of the PCR products by southern blot analysis and sequencing (Materials and Methods), we found that these were nonspecific products.

Zymography

In order to examine whether gelatinase activity corresponding to MMP-9 production was affected by administration of Na-HA, we prepared an extract from normal corneas, or wounded corneas after administration of PBS or Na-HA, and then subjected it to gelatin zymography.

The extract from cornea cultured with PMA was used as the gelatinase standard for the cornea

Figure 1. Polymerase chain reaction (PCR) analysis of effect of sodium hyaluronate (Na-HA) on expression of 72 kDa type gelatinase-2 (A), 92 kDa type gelatinase (B), and tissue inhibitor of matrix metalloproteinases-1 (C) mRNA in wounded corneas. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (D) was used as internal standard. Expected size of each PCR product is indicated by arrow. Total RNA samples from phosphate-buffered saline-treated (lanes 3, 5, 7, 9) and Na-HA-treated (lanes 4, 6, 8, 10) corneas were extracted at 6 (lanes 3, 4), 24 (lanes 5, 6), 48 (lanes 7, 8), and 72 (lanes 9, 10) hours after wounding. Amplifications were performed for 35 cycles with exception of 30 cycles for GAPDH using primers shown in Table 1. Lane M: DNA size markers; lane C: no RNA template for negative control of PCR reaction; lane 1: normal cornea; lane 2: cornea immediately after wounding.

Figure 2. Reproductive polymerase chain reaction (PCR) analysis of effect of sodium hyaluronate (Na-HA) on expression of 72 kDa type gelatinase (A), 92 kDa type gelatinase (B) and tissue inhibitor of metalloproteinases-1 (C) mRNA in corneas at 6 hours after wounding. Glyceraldehyde-3-phosphate dehydrogenase (D) was used as an internal standard. Expected size of each PCR product is indicated by arrow. Three different rabbits were used in this experiment, so that lanes 1 and 4 or lanes 2 and 5 or lanes 3 and 6 were from same rabbit. Lane M: DNA size markers; lane C: no RNA template for negative control of PCR reaction; lanes 1, 2, 3: phosphate-buffered saline-treated side; lanes 4, 5, 6: Na-HA-treated side.
wounded corneas bilaterally. At 6 hours after wounding, the levels of proMMP-9 in the Na-HA-treated side were higher than those in the PBS-treated side (Figure 3B,C). At 24 hours after wounding, these levels were considerably increased in the PBS-treated side, while those in the Na-HA-treated side were almost the same as those at 6 hours (Figure 3D,E).

In order to quantify the activity of proMMP-9, the bands corresponding to proMMP-9 were analyzed by gel scanning. The activity of proMMP-9 correlated well with the level of its mRNA expression; the activity in the Na-HA-treated side was significantly higher than that in the PBS-treated side at 6 hours after wounding (Figure 4).

**Discussion**

The present study demonstrates the stable expression of MMP-2 and TIMP-1 mRNA and the temporal expression of MMP-9 mRNA during corneal epithelial wound healing. As shown in Figure 1, the level of MMP-9 mRNA expression showed a first peak at 6 to 24 hours and a second increase at 72 hours after the wounding. In the rabbit corneal epithelial defective model in this study, the healing rate of the corneal epithelium is almost constant and most of the wounded area is re-epithelialized for 72 hours after surgical removal. In addition, it has been reported that proMMP-9 production is increased in both the epithelium as well as the stroma of the cornea in a penetrating keratotomy rabbit corneal wound model and that the expression of MMP-9 is localized in the migrating epithelium during human or rat skin wound healing. Therefore, the first peak of the MMP-9 expression in this study would be related to the epithelial migration during epithelial wound healing. Although the meaning of the second increase in the MMP-9 expression in the wound healing is unclear, the increase may be involved in a remodeling of the subepithelial basement membrane after the re-epithelialization.

**Figure 3.** Zymographic analysis of effect of sodium hyaluronate (Na-HA) on gelatinase activities in corneas after wounding. Extract from cornea cultured with phorbol 12-myristate 13-acetate was used as gelatinase standard (S). (A) Normal corneas. (B) Phosphate-buffered saline (PBS)-treated sides at 6 hours after wounding. (C) Na-HA-treated sides at 6 hours after wounding. (D) PBS-treated sides at 24 hours after wounding. (E) Na-HA-treated sides at 24 hours after wounding. Molecular weights from markers are indicated to left of zymograms. M-2; proMMP-2; M-9; proMMP-9.

**Figure 4.** Quantification of levels of gelatinolytic activity corresponding to proMMP-9 in corneas of phosphate-buffered saline (PBS)-treated side and sodium hyaluronate (Na-HA)-treated side (Figure 3). Weight of frozen corneas used in this experiment was almost identical in each group. Na-HA-treated side (plain bar) showed significantly higher activity compared to PBS-treated side (stippled bar) at 6 hours after wounding. Mean = SE (n = 6), *P < .05 (vs. PBS-treated side).
The administration of Na-HA advanced the kinetics of MMP-9 mRNA expression earlier after wounding, whereas it had no effect on the expression of MMP-2 and TIMP-1 mRNA (Figure 1, Table 2). These results were confirmed by a repeated experiment using other corneas derived from another group of rabbits. In addition, the activity of MMP-9 was also detected using zymographic analysis. As shown in Figure 4, significantly higher activity of proMMP-9 was observed in the Na-HA-treated side at 6 hours after wounding, compared to the PBS-treated side. Thus, the levels of activity of proMMP-9 were consistent with those of the gene expression of MMP-9. These results suggest that Na-HA accelerates corneal epithelial wound healing by advancing the healing process through the expression of MMP-9 mRNA and the activity corresponding to the production of proMMP-9. Whereas the correlation with fibronectin function or the epithelial cell proliferation has already been presented as the mechanism of the accelerating effect of Na-HA on corneal epithelial wound healing, the effect of advancing the expression of MMP-9 should also be considered as a novel participation in this mechanism.

In this study, whole corneas were employed, so that we cannot find whether MMP-9 was produced mainly in epithelial cells or stromal cells. We should further investigate using the in situ hybridization method to detect the localization of MMP-9 expression, so that the functions of MMP-9 during corneal wound healing would become clearer. Interestingly, Na-HA showed an effect on the MMP-9 expression as early as 6 hours after wounding in vivo, while Na-HA had no effect on the epithelial healing area event at 24 hours after wounding in vivo. This early response would result in significant acceleration of epithelial healing in a later stage during wound healing. Binding of hyaluronan to its cell surface receptors triggers signal transduction events. The signals are transmitted by the activation of protein phosphorylation cascades that involve mitogen-activated protein kinase (MAPK). The MAPK also regulates the expression of MMP-9 in breast epithelial cells. Na-HA might show an effect on the MMP-9 expression in this study, mediated by MAPK regulation through its receptors. CD44 is the principal cell surface receptor for hyaluronan. It has been reported that the CD44 isoform is closely associated with MMP-9 in metastatic breast cancer cells. Association with Na-HA, CD44, and MMP-9 may be important for corneal epithelial wound healing. In fact, in our previous studies, CD44 was observed in a condition of colocalization with endogenous hyaluronan during corneal epithelial wound healing, and it was suggested that CD44 was involved in the effect of Na-HA on the proliferation of corneal epithelial cells in vitro. Also a subject for future study is the investigation of the role of hyaluronan receptors as a factor connecting exogenous Na-HA with the expression of MMP-9 mRNA.

### Table 2

<table>
<thead>
<tr>
<th>Side</th>
<th>0</th>
<th>6</th>
<th>24</th>
<th>48</th>
<th>72 (Hours)</th>
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<tr>
<td>PBS</td>
<td>nd</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Na-HA</td>
<td>nd</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>nd</td>
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PBS: Phosphate-buffered saline; Na-HA: sodium hyaluronate; Hours: hours after wounding; nd: not detected.

References