Bioluminescence Imaging of Matrix Metalloproteinases-2 and -9 Activities in Ethanol-injured Cornea of Mice

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Abstract. *Background/Aim:* This study aimed to investigate the usefulness of in vivo bioluminescence imaging (BLI) to examine the role of matrix metalloproteinases (MMP)-2 and MMP-9 activation in the development and healing of ethanol-induced damage in the cornea of mice. *Materials and Methods:* Mouse corneal injury was induced by topical treatment with 20% ethanol. BLI was obtained from the ocular region of mice intravenously injected with an active-MMP-2/9 probe. In vivo results were validated in primary corneal epithelial cells. *Results:* BLI indicated that treatment of the eye with 20% ethanol elevated MMP-2/9 activity, which was inhibited by the application of eye drops (hyaluronic acid and serum). Treatment of corneal epithelial cells with 20% ethanol increased the activities of MMP-2 and MMP-9, which were also inhibited by eye drops. *Conclusion:* BLI can be applied in vivo in mice with corneal injury to examine the activity of MMPs and clarify the efficacy of eye drops.

Injuries caused by chemicals including alkali and ethanol cause serious clinical ophthalmological problems, such as

limbal stem cell damage, corneal melt and corneal scarring (1). Ethanol is being used in ocular surface surgeries such as laser-assisted subepithelial keratectomy and pterygium excision to remove the epithelium during procedures of surface ablation (2). It is well known that topical treatment of the eye surface with ethanol can induce morphological and functional changes and damage the cornea (3). These lesions of the cornea by application of ethanol are due to a variety of mechanisms including cell death and apoptosis or severe inflammatory reactions of corneal epithelial cells (4).

Matrix metalloproteinases (MMPs) are zinc-dependent peptidases that play a fundamental role in inflammation and apoptotic processes when the extracellular matrix (ECM) is excessively damaged (5). MMPs are expressed in the cornea of animals and human (6). Multi-isoforms of MMPs are up-regulated in corneal epithelial cells during injury and healing (5, 7). MMP-2 and MMP-9 (gelatinases A and B, respectively) are also involved in the process of degradation of corneal epithelium in response to a variety of agents (8). The levels of MMP-9 transcripts produced by human corneal limbal epithelial cells is elevated in ethanol-induced injury (9, 10). In addition, 70% ethanol can induce corneal epithelium damages and up-regulate the expression of MMP-2 and MMP-9 (11). Eye drops containing hyaluronic acid (HA) can protect the ocular surface epithelium by facilitating corneal epithelial healing and by regulating the expression of MMP-2 and MMP-9 (12). Moreover, platelet-activation factor, a bioactive lipid, can elevate the expression of MMPs. Its antagonist can protect against the development of epithelial damages by reducing the capacity of MMPs to degrade gelatin (13). Elevated activities of MMP-2 and MMP-9 are associated with every step of ocular surface injury and can lead to delayed corneal wound healing (14).

Bioluminescence imaging (BLI) is an optical imaging technique that visualizes biological processes using luciferase-catalyzed reactions with a target substrate (15). It has been documented that BLI data can be used for non-

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invasive visualization of a variety of biological processes, including retinoblastoma growth and metastasis *in vivo* (15-17). BLI can be used to obtain a repeated and longitudinal image to analyze localization and quantify drug efficacy in experiment animals (17, 18). Therefore, BLI has been used in the diagnosis and initial management of eye injury (19, 20). Previously, a representative BLI study has shown that an annexin V probe can be used on ethanol-treated corneal epithelium to detect apoptosis (21). Moreover, a longitudinal analysis using BLI has been performed to study tumorigenesis in mice and determine the expression of MMP-2 (22). As mentioned above, MMP-2 and MMP-9 expression levels are correlated with corneal functions including the growth of corneal epithelial cells (14). However, no study has examined corneal epithelial MMP-2 and MMP-9 activities *in vivo* during ethanol-induced injury or application of protective eye drops. Therefore, the objective of this study was to investigate the usefulness of BLI for examining MMP-2/9 activity in corneal pathophysiology *in vivo*. BLI changes in cornea were assessed using an MMP-2/9 probe after ethanol-induced injury and treatment with protective eye drops. The results obtained from BLI analysis were validated by analyzing the activities of MMP-2 and MMP-9 in primary corneal epithelial cells.

Materials and Methods

Materials. Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were purchased from Hyclone (Logan, UT, USA). Sodium hyaluronate (HA) was obtained from Taejoon Pharmaceuticals (Seoul, Republic of Korea). Penicillin/streptomycin and trypsin-ethylene diamine tetraacetic acid (EDTA) were purchased from Fisher Scientific (Pittsburgh, PA, USA). NpFlamma® MMP-2/9 probe was purchased from BioActs (Incheon, Republic of Korea). Antibodies used in this study included anti-MMP-2, anti- β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-MMP-9 (Abcam Plc, Cambridge, UK), and anti-active MMP-9 (Merck KGaA, Darmstadt, Germany).

Animals and BLI measurement. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). They were approved by Animal Subjects Committee of Konkuk University, Korea (Approval number: KU19118). ICR (Institute of Cancer Research) mice (8-week-old, male; Nara Biotech, Seoul, Republic of Korea) were given free access to water and food pellets.

Before BLI and application of alcohol to eyes, mice were anesthetized by intraperitoneal injection of Zoletil® (40 mg/kg body weight; Virbac Laboratories, Carros, France) and Rompun® (10 mg/kg body weight; Bayer Korea, Seoul, Republic of Korea). Adequacy of anesthesia was determined based on the lack of reflex response to foot pinching. A 6-mm-diameter filter paper disk (Advantec, Tokyo, Japan) soaked in 20% ethanol was placed on the right eye surface of the anesthetized mouse for 30 s to induce corneal injury. The ethanol-treated eyes were washed with 20 μ l of normal saline immediately after

removing the paper disk, and then wiped the saline water with sterile cotton balls. NpFlamma® MMP-2/9 probe solution (1.2 μ g/ μ l) was prepared by diluting it with PBS. It was then injected intravenously at 100 μ l/mouse *via* the tail vein. BLI intensity was measured and detected at various time points (*i.e.*, 3 h, 6 h, 12 h, 24 h, and 48 h after injection of the probe). BLI was performed using an Ami HTX (Spectral Instruments Imaging, Tucson, AZ, USA) at excitation wavelength of 683 nm and emission wavelength of 694 nm. Non-injury and non-treated control images were obtained for the left eye of mice administered 100 μ l/mouse saline. High concentration serum and HA were diluted in PBS to obtain 50% and 0.15% solutions, respectively, for topical treatment. Mice received 10 μ l/mouse of eye drop. Regions of interest (ROI) were selected from the ocular region of the image. Photon intensity of BLI was quantified automatically using a dedicated software (Spectral Instruments Imaging) as photon counts per second (p/s) in background-subtracted ROI.

Cell culture and viability assay. Primary corneal epithelial cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were cultured in DMEM/F-12 supplemented with 10% FBS, 1% penicillin/streptomycin, and epidermal growth factor (10 ng/ml) and incubated at 37°C in a humidified incubator with 95% air and 5% CO₂.

Cell viability was analyzed using 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxamide inner salt (XTT) assay with a WelCount™ cell proliferation assay kit (Welgene, Daegu, Republic of Korea). Briefly, corneal epithelial cells (1 $\times$ 10⁴ cells/well) were seeded into a 96-well plate and incubated at 37°C for 48 h. After adding XTT (200 μ g/ml) to each well, the plate was incubated for 2 h to allow the formation of the formazan dye. Absorbance was then measured at 450 nm using an Absorbance Microplate Reader (SpectraMAX®, Molecular Devices, CA, USA). To analyze effects of protective agents, corneal epithelial cells were incubated in a 60-mm dish with 4% serum containing DMEM/F-12 and grown to 80-90% confluence before treatment. Cells were treated with 20% ethanol for 30 s in the absence or presence of test agents.

Immunoblotting. Corneal epithelial cells were lysed with a buffer containing 1% NP40, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% sodium deoxycholate, 1 mM Na₂EDTA, 1 mM EGTA, 1 mM β -glycerophosphate, 2.5 mM sodium pyrophosphate, 1 mM Na₃VO₄, and 1 μ g/ml leupeptin. Cell lysates containing proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The membranes were blocked with PBS containing 5% bovine serum albumin, incubated overnight at 4°C with primary antibodies (1:1,000), followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The bands were then visualized using enhanced chemiluminescence kits (Amersham Pharmacia, Piscataway, NJ, USA) and a luminescent image analyzer LAS-4000 (Fujifilm, Tokyo, Japan). Band intensities were quantified using ImageJ software (NIH, Bethesda, MD, USA).

Statistical analysis. Data were processed and analyzed using GraphPad Prism 7.04 software (GraphPad Software, San Diego, CA, USA). The mean and standard deviation (SD) were computed for all variables. Normality of data distribution was assessed by using Shapiro-Wilk test. Sphericity was examined by using Mauchley's test. Statistical analysis was performed using RM-

Figure 1. In vivo bioluminescence images of MMP-2/9 activity in response to ethanol. (A) Bioluminescence images of ethanol-treated right eye and saline-treated left eye of mice. (B) ROI analysis of bioluminescence signal was compared between right eye (20% ethanol) and left eye (saline) ($n=6$). Data were analyzed by two-way RM ANOVA [interaction $p<0.001$ $F(4, 40)=11.43$; time $p<0.001$ $F(4, 40)=73.37$; treatment $p<0.001$ $F(1, 10)=22.28$; subjects $p<0.001$ $F(10, 40)=9.059$] and post-hoc Sidak's multiple comparisons test (right eye vs. left eye, 12 h, $p<0.01$; 24 h, $p<0.001$; 48 h, $p<0.01$). ** $p<0.01$; *** $p<0.001$.

ANOVA, Friedman test, and Kruskal–Wallis test. Post-hoc Dunn's multiple comparisons test was performed to determine statistically significant effects. Statistical significance was set at $p<0.05$.

Results

In vivo MMP-2/9 bioluminescence image in ethanol-injured cornea. Changes of MMP-2/9-associated intensities were first determined by BLI, and compared between 20% ethanol-injured and saline-treated control eyes. BLI of MMP-2/9 activity was observed over time (3–48 h) in ethanol-injured right eye (Figure 1A). As shown in Figure 1B, the MMP-2/9-associated intensity in BLI at 3 h was $1.52 \times 10^8 \pm 3.54 \times 10^7$ p/s. It was elevated gradually, reaching the maximum at 24 h after injury ($3.84 \times 10^8 \pm 5.03 \times 10^7$ p/s). The intensity showed a slight diminution at 48 h ($2.32 \times 10^8 \pm 6.34 \times 10^7$ p/s). The right eye

had a higher intensity signal in BLI than the saline-treated left eye (Figure 1B). In addition, there were significant differences in the intensity of the signal of MMP-2/9 activity in BLI between the right and left eyes at 12, 24, and 48 h (Figure 1B).

Effects of agents on MMP-2/9 bioluminescence in ethanol-injured eyes. We next investigated changes in MMP-2/9 signal intensity in BLI after administration of protective eye drops on cornea injured by ethanol. Injured eyes received 10 μ l/mouse of eye drop containing HA (0.15%) or high concentration serum (50%) and examined at 3 h, 6 h, 12 h, 24 h, and 48 h after the injury (Figure 2A). As shown in Figure 2B, MMP-2/9 signal intensity in BLI in response to 20% ethanol at 24 h was $4.35 \times 10^8 \pm 1.09 \times 10^8$ p/s. It was

Figure 2. Effects of protective eye drops on bioluminescence images showing MMP-2/9 activity in ethanol-injured right eyes. (A) In vivo bioluminescence images showing MMP-2/9 activity at 24 h after treatment with hyaluronic acid (HA) and serum. (B) ROI analysis of bioluminescence signal was compared between each group for right eyes (control, n=5; HA, n=5; serum, n=5). Data were analyzed with Friedman test ($p < 0.001$) and then post-hoc Dunn's multiple comparisons test after Kruskal–Wallis test (*control vs. serum at 6 h, $p < 0.05$; **control vs. serum at 12 h, $p < 0.01$; #control vs. HA at 24 h, $p < 0.05$; **control vs. serum at 24 h, $p < 0.01$; #control vs. HA at 24 h, $p < 0.05$; **control vs. serum at 48 h, $p < 0.01$).

inhibited by HA and serum to $2.12 \times 10^8 \pm 3.38 \times 10^7$ p/s and $1.8 \times 10^8 \pm 3.94 \times 10^7$ p/s, respectively. Their BLI intensities at 6 h, 12 h, 24 h, and 48 h in the serum treated-group and those in the HA-treated group at 12 h and 24 h were significantly different from those in ethanol-injured control group (Figure 2B).

Effects of ethanol and agents on MMP activation in corneal epithelial cells. To validate the results obtained by BLI analysis using MMP-2/9 activity probe, the activities of MMP-2 and MMP-9 were examined using primary corneal epithelial cells treated with HA and high concentration serum. We first determined the effects of ethanol and protective eye drops on cell viability. Treatment with ethanol (20–100%) for 30 s inhibited the viability of corneal epithelial cells in a concentration-dependent manner (Figure 3A). HA (0.01–0.3%) significantly reversed the decrease in cell viability in response to treatment with ethanol 20% for 30 s (Figure 3B). Similar to HA, serum (20–50%) also reversed the decrease in viability of cells treated with

ethanol, which was significant at concentrations of 40% and 50% (Figure 3C).

As shown in Figure 4, treatment of corneal epithelial cells with 20% ethanol increased cleavage and activation of MMP-2 as well as activation of MMP-9. However, treatment with 20% ethanol did not change the expression levels of MMP-2 or MMP-9 (Figure 4A and D). Elevated activities of MMP-2 and MMP-9 at 0.5 h, 1 h, and 3 h after treatment with 20% ethanol were attenuated upon treatment with HA (0.1%) (Figure 4A–C) and serum (50%) (Figure 4D–F). However, treatment of cells with ethanol, HA, or serum did not affect the expression of β -actin that was used as a housekeeping protein (Figure 4A and D).

Discussion

In the present study, we showed using BLI that ethanol treatment elevated MMP activity in the cornea of mice injected with MMP-2/9 probe. In vivo BLI is a powerful technique that plays a crucial role in diagnosis and initial

Figure 3. Effects of ethanol and protective eye drops on the viability of corneal epithelial cells. (A) Effects of ethanol on viability of corneal epithelial cells. Corneal epithelial cells were treated with ethanol (20-100%) for 30 s. (B and C) Effects of protective agents on the viability of corneal epithelial cells injured with ethanol. Hyaluronic acid (HA; 0.01-0.3%) (B) and high concentration serum (20-50%) (C) were used to treat corneal epithelial cells injured with 20% ethanol for 30 s. The levels of viability were determined using an XTT assay as described in the Methods section. Cell viability of the untreated-treated group (control) was expressed as 100% (n=8). *** $p < 0.001$ vs. control (A). ** $p < 0.01$, *** $p < 0.001$ vs. ethanol alone, respectively (B and C).

management of a variety of disease processes at the molecular level (22). The luminescence probe (NpFlamma[®] MMP-2/9 probe, BioActs) used in this study comprised a Flamma[®] Fluor 675 dye that was linked to a quencher through MMP-2,9 cleavable peptide. It can be used for monitoring MMP-related diseases and evaluating the potential therapeutic efficacy of drugs targeting these diseases (23). However, the use of BLI to investigate ocular surface injury for MMP-2 and MMP-9 has not been reported yet. Furthermore, it is known that mRNA expression levels of MMP isoforms are up-regulated in corneal epithelial cells at 18 h after corneal abrasion injury (24). In the present study, MMP-2/9 signal intensity in BLI was elevated gradually up to 24 h after ethanol-induced injury. The intensity of the signal was slightly reduced at 48 h. Moreover, protective effects of eye drops with HA and high serum were obtained from 6 h to 48 h. These results imply that ethanol-induced injury results in long-term activation of MMP-2 and MMP-9 and that eye drops with HA and serum may have long-term effects on MMP-2/9 activity during corneal wound healing. From these results, it can be assumed that changes of MMP-2/9 activity based on its intensity in BLI are tightly associated with altered MMP-2 and MMP-9 activation during the development and repair of corneal injury in response to treatments with ethanol and protective eye drops.

In the present study, ethanol decreased the viability of corneal cells in a dose-dependent manner. Moreover, this study confirmed that the activities of MMP-2 and MMP-9 were elevated in corneal epithelial cells treated with 20% ethanol. A similar pattern was found by ocular BLI in response to ethanol treatment. These results imply that a high concentration of ethanol can induce corneal cell injury *via* a pathway involving MMPs, thus influencing corneal cell viability. Previously, it has been shown that ethanol and alkali solution application can increase MMP-9 expression in corneal epithelial cells (10, 25). It has been demonstrated that both MMP-2 and MMP-9 may disrupt corneal epithelial integrity by proteolytic cleavage of occludin, a component of intracellular tight junction (26-28). The activities of MMP-2 and MMP-9 can be inhibited by GM6001, an MMP inhibitor, in corneal cells (27). These results confirmed that activation of MMPs, especially MMP-2 and MMP-9, may play important roles in the development and repair of corneal injury induced by ethanol treatment.

Moreover, in the present study, HA eye drop significantly decreased MMP-2/9 activity based on BLI signal intensity that was elevated by ethanol treatment. HA also inhibited the activity of MMP-2 and MMP-9 in corneal epithelial cells. HA has high binding affinity for water. Thus, it can act as a reservoir to slowly release water molecules (29). HA can accelerate epithelial cell migration and protect the ocular surface by increasing the expression of MMP-2 and MMP-9 (30). Thus, HA eye drops are commonly used to treat dry eye

Figure 4. Effects of protective eye drops on the activation of MMP-2 and MMP-9 in corneal epithelial cells. (A and D) Corneal epithelial cells were treated with 20% ethanol for 30 s and then incubated in the absence or presence of hyaluronic acid (HA; 0.1%) (A) and high concentration serum (50%) for the indicated times (D). Cell lysates were immunoblotted with anti-MMP-2 and anti-MMP-9. β -actin was used as a housekeeping protein for each test protein. (B and C) Quantification of the data obtained from panel A. (E and F) Quantification of the data obtained from panel D. Enzyme activation was determined by the expression of cleaved MMP-2 and active MMP-9 on blots. Expression of each protein in untreated group was expressed as 100% ($n=4$). * $p<0.05$, ** $p<0.01$ vs. ethanol alone at indicated time point, respectively.

disease. From these results, it can be hypothesized that MMP activity plays a central role in the process of development of alcohol-induced corneal injury and HA facilitates healing. Furthermore, accumulated results have shown that autologous serum is beneficial in promoting corneal epithelial migration and providing accelerated epithelial healing (31, 32). Serum may include several proteins that can act as antioxidant and free radical scavengers. It is known that corneal cell injury is accompanied by imbalanced antioxidant protection systems (33). Furthermore, it has been shown that serum can diminish the expression and activity of MMP in corneal cells (34). A similar result was obtained using corneal epithelial cells in the present study. The present

study also revealed that eye drop with a high concentration of serum could potentially diminish MMP-2/9 activity. Although the mechanisms through which serum and HA promote corneal wound healing are not fully understood yet, these results imply that serum and HA can act as inhibitors of MMP activity. These results suggest that HA and serum can promote corneal wound healing by inhibiting the activities of MMP-2 and MMP-9. Thus, regulating these enzymes would be a new strategy to develop drugs for topical eye application with major clinical effects.

In conclusion, the present study showed using BLI that treatment with 20% ethanol increased MMP-2/9 activity. The application of protective eye drops (serum and HA)

significantly inhibited MMP-2/9 activity as assayed by BLI after ethanol-induced injury. These results indicate that MMP-2 and MMP-9 activities may be tightly involved in the development of corneal damage and healing by eye drops containing protective agents. Results of this study also suggest that BLI is a useful tool for determining *in vivo* the activities of MMPs and monitoring the efficacy and mechanism of drug action in corneal epithelial disorders.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

S.B.P., S.H.J. B.K., H.J.S., and K.-J.W. conceived and designed the work. S.B.P., S.H.J., H.J., S.J.K., and K.-J.L. carried out experiments and data acquisition. B.K., H.J.S., and K.-J.W. wrote the manuscript. S.B.P. and Y.R. verified the analytical methods. All Authors discussed the results and contributed to the final manuscript.

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