Hydrogen and N-Acetyl-1-Cysteine Rescue Oxidative Stress-Induced Angiogenesis in a Mouse Corneal Alkali-Burn Model

Miyuki Kubota,1 Shigeto Shimmura,1 Shunsuke Kubota,1 Hideyuki Miyashita,1 Naoko Kato,1 Kousuke Noda,2 Yoko Ozawa,1 Tomobiko Usui,3 Susumu Ibida,2 Kazuo Umezawa,4 Toshibide Kuribara,1 and Kazuo Tsubota1

PURPOSE. To investigate the role of reactive oxygen species (ROS) as the prime initiators of the angiogenic response after alkali injury of the cornea and observe the effects of antioxidants in preventing angiogenesis.

METHODS. The corneal epithelia of SOD-1–deficient mice or wild-type (WT) mice were removed after application of 0.15 N NaOH to establish the animal model of alkali burn. ROS production was semiquantitatively measured by dihydroethidium (DHE) fluorescence. Angiogenesis was visualized by CD31 immunohistochemistry. The effects of the specific NF-κB inhibitor DHMEQ, the antioxidant N-acetyl-L-cysteine (NAC), and hydrogen (H2) solution were observed.

RESULTS. ROS production in the cornea was enhanced immediately after alkali injury, as shown by increased DHE fluorescence (P < 0.01). NF-κB activation and the upregulation of vascular endothelial growth factor (VEGF) and monocyte chemotactrant protein-1 (MCP-1) were significantly enhanced (P < 0.01), leading to a significantly larger area of angiogenesis. Angiogenesis in SOD-1−/− mice corneas were significantly higher in WT mice (P < 0.01), confirming the role of ROS. Pretreatment with the specific NF-κB inhibitor DHMEQ or the antioxidant NAC significantly reduced corneal angiogenesis by downregulating the NF-κB pathway (P < 0.01) in both WT and SOD-1−/− mice. Furthermore, we showed that irrigation of the cornea with hydrogen (H2) solution significantly reduced angiogenesis after alkali-burn injury (P < 0.01).


Alkali Burn Model

Male ICR mice at the age of 6 to 7 weeks were purchased from CLEA Japan, Inc. (Tokyo, Japan), and SOD-1–deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the study was approved by the institutional board on the use of animals. CNV was induced by alkali injury. Briefly, after general anesthesia with pentobarbital (Nembutal, 50 mg/kg; Abbott Pharmaceutical, Abbott Park, IL), 2 μL of 0.15 M NaOH was applied to

From the 1Department of Ophthalmology, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan; 2Department of Ophthalmology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; 3Department of Ophthalmology, University of Tokyo, Graduate School of Medicine, Tokyo, Japan; 4Department of Applied Chemistry, Keio University Faculty of Science and Technology, Yokohama, Japan. Supported by a Grant-in-Aid for Scientific Research (C) by the Ministry of Education, Culture, Sports, Science and Technology, Japan. Submitted for publication July 6, 2010; revised August 19, 2010; accepted August 19, 2010.

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Corresponding author: Shigeto Shimmura, Department of Ophthalmology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan; shige@sc.itc.keio.ac.jp.

the corneal surface. Subsequently, total corneal limbus and epithelium were scraped off with a surgical blade under a microscope. Oftoloxin ophthalmic ointment was instilled immediately after the operation.

Treatment with NAC or DHMEQ

From 3 days before the alkali burn, WT and SOD-1−/− mice were treated with an N-acetyl-L-cysteine (NAC; Nakarai Tesque, Kyoto, Japan) or a specific NF-κB inhibitor (dehydroxymethylepoxyquinomicin [DHMEQ]) or vehicle (0.083% dimethyl sulfoxide [DMSO]) in phosphate-buffered saline (PBS) daily for 6 days. NAC and DHMEQ were injected into mice intraperitoneally. DHMEQ is a novel NF-κB inhibitor, based on the structure of epoxyquinomicin C, originally isolated from Amycolatopsis. DHMEQ has been shown to inhibit nuclear translocation of NF-κB without affecting the phosphorylation and degradation of IκBα. Mice received NAC at a dose of 200 mg/kg or DHMEQ at 5 mg/kg body weight.

Treatment with H2 Water

To observe the prophylactic effects of H2 water, a potent antioxidant found effective in the prevention of ischemic brain injury, 10 eyes after concentrated PBS 1:20 before irrigation; the final H2 concentration was rendered isotonic by diluting 20% Triton 0.5 mL) in 0.1% Triton for 2 hours, whole corneas were stained overnight at 4°C with purified rat anti–mouse CD31 (PECAM-1) (1:300, Becton Dickinson, Franklin Lakes, NJ), washed, and further incubated with AlexaFluor 488 goat anti–mouse CD31 (PECAM-1) (1:300, Becton Dickinson, Franklin Lakes, NJ), washed, and further incubated with AlexaFluor 488 goat anti–rat IgG (1:300; Life Technologies, Carlsbad, CA) for 1 hour at room temperature. The flat mounts were imaged with a fluorescence microscope (Biorevo BZ-9000; Keyence, Osaka, Japan) and quantified by ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). The area of CNV was calculated by the following equation in which the Total Area is the area within the limbal vessel arcade and the Avascular Area is the area of the remaining clear cornea: Area of Neovascularization (%) = (Total Area − Avascular Area)/Total Area × 100.

Measurement of ROS

Eyes were enucleated and immediately frozen in OCT compound (Sakura Finetek, Torrance, CA) as soon as 0.15 N NaOH dropped and washed out. Unfixed cryosections (10 μm) were incubated with 5 μM DHE (Molecular Probes, Eugene, OR) for 15 minutes at 37°C, as previously reported.11,12 Sections were examined using a microscope equipped with a digital camera (Carl Zeiss, Oberkochen, Germany), and the intensity of the staining was measured using the ImageJ program. To facilitate the detection of ROS by image analysis, the epithelium was left intact for this assay.

Immunochemical Staining

Eyes were enucleated and fixed in 4% PFA overnight at 4°C. After fixation, tissues were processed and embedded in an OCT compound, frozen in liquid nitrogen, and stored at −80°C until sectioning. Frozen 6-μm to 8-μm-thick sections were cut with a cryostat and mounted on slides. We used the anti–mouse CD31 antibody (rat monoclonal, clone MEC 13.3, 550274; BD PharMingen, San Diego, CA) for detecting blood vessels in the cornea, or anti–mouse F4/80 antibody (rat monoclonal, clone CI: A3–1, MCA497R; AbD Serotec, Raleigh, NC) for macrophages. AlexaFluor 488- or 546-conjugated goat anti–rat antibodies were used as secondary antibody. Nuclei were counterstained with Hoechst 33342. The increase in the number of F4/80-positive cells per field was calculated at days 0, 3, and 6 after injury.

Enzyme-Linked Immunosorbent Assay

Three days after alkali burn, mice were killed with an overdose of anesthesia, and the eyes were immediately enucleated. The whole cornea, including the limbus, epithelium, stroma, and endothelium, was isolated and placed into 100 μL lysis buffer (0.02 M HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid], 10% glycerol, 10 mM NaP, 1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0), supplemented with protease inhibitors (2 mg/L aprotinin, 100 μM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 2.5 μM pepstatin A), and sonicated. The lysate was centrifuged at 15,000 rpm for 15 minutes at 4°C. VEGF and MCP-1 levels in the supernatant were determined with the mouse VEGF and MCP-1 ELISA kits (R&D Systems, Minneapolis, MN), respectively, according to the manufacturer’s protocols. Similarly, phosphorylated NF-κB p65 levels...
were measured with the phosphorylated NF-κB p65 ELISA kit (Cell Signaling Technology, Danvers, MA) according to the manufacturer's instructions. The tissue sample concentration was calculated from a standard curve and corrected for protein concentration evaluated with a spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific, Waltham, MA).

Quantitative RT-PCR

Animals were killed with an overdose of anesthesia. The eyes of each were immediately enucleated, and the cornea (including epithelium, stroma, endothelium, and limbus) was carefully isolated. Total RNA was extracted from the cornea using an extraction reagent (TRizol; Life Technologies, Grand Island, NY), and cDNA was synthesized (First-Strand cDNA Synthesis Kit; GE Health Care, Piscataway, NJ). For the RT-PCR reaction, mRNA transcripts were detected using the TaqMan real-time quantitative RT-PCR procedure (TaqMan Fast Universal PCR Master Mix Reagents Kit; Applied Biosystems, Foster City, CA). The RT-PCR assay was performed with a PCR system (7500 Fast Real-Time PCR; Applied Biosystems). The threshold cycle (Ct) was calculated by the instrument's software (7500 Fast; Applied Biosystems). A quantitative PCR assay for mRNA of f4/80 and mcp-1 was performed using gene expression master mix (TaqMan Gene Expression Assay Mix; Mm00802530_m1 and Mm00441242_m1, respectively; Applied Biosystems).

Statistical Analysis

All results were expressed as mean ± SD. Values were processed for statistical analyses (Mann-Whitney U test) or unpaired Student's t-test, and differences were considered statistically significant at P < 0.05.

RESULTS

Alkali Burn Enhances Oxidative Stress in the Cornea

To elucidate the contribution of ROS in alkali-burned injury, we examined ROS induction by the administration of NaOH using the DHE assay, where DHE reacts with O2− to become fluorescent. Application of 0.15 N NaOH to the cornea induced a significant increase of ROS in the epithelium (2.31 ± 0.51-fold of untreated control; P < 0.01; Figs. 1A, 1B). Alkali burn induced angiogenesis (PECAM-1) and recruited macrophages (F4/80), which were not observed in untreated controls (Fig. 1C). F4/80-positive macrophages significantly increased by day 3 and day 6 (P < 0.05).

Alkali Burn-Induced CNV Was Inhibited by the Antioxidant NAC or the NF-κB Inhibitor DHMEQ

Increased oxidative stress in the alkali-burned cornea suggested that oxidative stress induced by alkali injury was the

![Figure 2](https://i.imgur.com/1234567.png)

**Figure 2.** CNV caused by alkali burns is suppressed by NAC or DHMEQ. (A, B) Alkali burn-induced CNV in mice cornea. Neovascularization area was significantly (***P < 0.01 each) lower in NAC- or DHMEQ-treated mice (18.31 ± 4.25, 17.06 ± 5.99; n = 8) than in vehicle-treated controls (35.98 ± 6.35; n = 6; day 6). Scale bar, 500 μm. (C) NF-κB activity was significantly higher in alkali-burned mice, which was significantly suppressed by treatment with NAC or DHMEQ. n = 8–12 (day 3). **P < 0.01. (D) Similarly, VEGF protein levels were significantly elevated in alkali-burned mice, which were significantly suppressed by treatment with NAC or DHMEQ. n = 8–11 (***P < 0.01 each; day 3). f4/80 (E) and mcp-1 (F) mRNA in alkali-burned mice was significantly elevated compared with controls, which was significantly suppressed by treatment with NAC or DHMEQ. n = 7–8 and 8–9 for f4/80 (**P < 0.05 each) and mcp-1 (*P < 0.05 each), respectively. Error bars indicate SD.
main trigger for corneal angiogenesis. Therefore, we examined
CNV in mice treated with the antioxidant NAC. As expected,
antioxidant treatment significantly suppressed alkali-burn in-
duced CNV ($P < 0.01$). Furthermore, the inhibition of NF-κB,
a transcription factor downstream of oxidative stress, sup-
pressed CNV as well ($P < 0.01$; Figs. 2A, 2B).

** FIGURE 3. ** CNV is enhanced in SOD-1−/− mice. (A, B) CNV area was sig-
nificantly (**) higher in SOD-1−/− mice (43.63 ± 13.42) than in SOD-1+/− mice (30.11 ± 7.16; $n = 19$ each; day 6). Scale bar, 500 μm. (C) $f4/80$ mRNA levels in SOD-1−/− mice were significantly elevated compared with those in SOD-1+/− mice ($n = 4$ each; day 6) **$P < 0.01$. (D) VEGF protein levels in SOD-1−/− mice were significantly higher than those in SOD-1+/− mice ($n = 5–6$; *$P < 0.05$; day 3). Error bars indicate SD.

** FIGURE 4. ** CNV in SOD-1−/− mice is suppressed by NAC and DHMEQ. (A, B) CNV area in SOD-1−/− mice was significantly lower than in control (45.85 ± 8.67; $n = 11$) when pre-
treated with NAC (38.53 ± 7.19; $n = 15$) starting 3 days before injury (*$P < 0.05$). (C, D) A similar reduction in neovascularization area was observed in DHMEQ-treated mice (26.78 ± 7.81; $n = 13$) compared with vehicle-
treated controls (43.626 ± 13.427; $n = 19$; **$P < 0.01$; day 6). Error bars indicate SD. Scale bar, 500 μm.
To investigate the molecular mechanisms involved, we examined NF-κB phosphorylation and VEGF expression in alkali-burned corneas. A statistically significant increase in NF-κB phosphorylation (P < 0.01; Fig. 2C) and VEGF expression (P < 0.01) was induced by alkali injury, which was inhibited by the administration of NAC or DHMEQ (Fig. 2D). CNV induced by various kinds of stimuli, including alkali burn, is accompanied with and accelerated by infiltrating macrophages.13 To quantify macrophage infiltration to the cornea, we measured mRNA levels of the macrophage marker f4/80. We found that f4/80 mRNA in alkali-burned corneas 6 days after injury decreased with NAC (P < 0.05) or DHMEQ (P < 0.05) treatment (Fig. 2E). Similarly, alkali burn-induced increase of mcp-1, a chemotactant for macrophage infiltration,14 was also suppressed by NAC (P < 0.05) or DHMEQ (P < 0.05; Fig. 2F). These results indicated antioxidant treatment or inhibition of NF-κB suppressed alkali-burned CNV by the downregulation of VEGF and the suppression of macrophage infiltration.

**SOD-1−/− Mice Show Enhanced CNV**

To confirm the role of ROS in corneal angiogenesis induced by alkali burn, we compared neovascularization areas in SOD-1−/− mice with littermate WT controls. The neovascularization area in SOD-1−/− mice (43.63% ± 13.42%, n = 6) was significantly larger than in SOD-1+/+ control (30.11% ± 7.16%; n = 6; P < 0.01; Figs. 3A, 3B). This shows that the loss of SOD-1 function enhanced alkali burn-induced CNV. The expression of f4/80 was significantly increased in SOD-1−/− compared with SOD-1+/+ (P < 0.01; Fig. 3C). Furthermore, VEGF protein was significantly increased in SOD-1−/− compared with SOD-1+/+ (P < 0.05; Fig. 3D). These results suggest that the antioxidant effect of SOD-1 suppresses corneal angiogenesis by inhibiting macrophage infiltration and VEGF upregulation after alkali burns.

**NAC and DHMEQ Rescues Increased CNV in SOD-1−/− Mice**

Because the loss of SOD-1 expression caused an increase in macrophage infiltration, VEGF expression, and neovascularization, we examined whether NAC can rescue the phenotype and whether NF-κB was involved. As expected, we found that both NAC (Figs. 4A, 4B; P < 0.05) and DHMEQ (Figs. 4C, 4D) significantly decreased the neovascularization area (P < 0.01), indicating that the NF-κB pathway was activated by the increase in ROS associated with the loss of SOD-1 protein.

**Post-Trauma Irrigation with H2 Water Suppresses CNV**

We found that pretreatment with NAC can prevent neovascularization formation in the cornea. Given that pretreatment is not possible in the clinical setting, we further sought to find a rescue protocol that is effective after alkali injury. The most important emergency procedure after corneal chemical burns is meticulous irrigation of the ocular surface using saline solutions to dilute residual chemicals in the tissue. We therefore hypothesized that irrigating with H2 water, a recently recognized antioxidant, instead of saline will enhance the prophylactic effects against ensuing inflammation and CNV. NAC was not used in the irrigation experiment because of its acidic pH. As shown in Figure 5, irrigating with isotonic H2 water significantly reduced ROS-associated DHE fluorescence (P < 0.05; Figs. 5A, 5B) and NF-κB phosphorylation (P < 0.05; Fig. 5C). More important, eyes irrigated with H2 water also had significantly smaller neovascularization areas (P < 0.01; Figs. 6A, 6B) that were associated with lower VEGF (P < 0.01; Fig. 6C) and MCP-1 (P < 0.01; Fig. 6D) protein levels compared with PBS alone.

**DISCUSSION**

In the present study, we showed that oxidative stress triggered angiogenesis through activation of the NF-κB pathway using a corneal alkali burn model. The role of ROS was confirmed using SOD-1−/− mice, which showed increased VEGF protein levels and accumulation of macrophages that further accelerated CNV (Fig. 3). Although angiogenesis induced by alkali burn is not recognized as an oxidative stress model, we also examined CNV by ultraviolet B radiation (UVB), a more common source of oxidative stress. SOD-1−/− mice had significant corneal ROS formation is suppressed with H2. To examine oxidative stress as a potential stimulus for angiogenic and molecular inflammatory events, corneal ROS expression was analyzed by DHE fluorescence. (A, B) ROS-induced DHE fluorescence in the corneal epithelium was significantly higher in vehicle-treated alkali-burned mice (1.6 ± 0.2 ratio of control; *P < 0.05) than in untreated controls. DHE intensity was significantly suppressed in H2-treated mice (0.6 ± 0.3 ratio of control; *P < 0.05) in vehicle-treated alkali-burned mice (1.6 ± 0.2 ratio of control; n = 4–5). Scale bar, 100 μm. (C) Phosphorylated NF-κB p65 levels were significantly (*P < 0.05) higher in vehicle-treated alkali-burned mice (311% ± 381% of control) than in untreated controls (100% ± 176% of control). Administration of H2 to alkali-burned animals significantly (*P < 0.05) reduced phosphorylated NF-κB p65 levels (140% ± 158% of control; n = 28–29). Error bars indicate SD.

**FIGURE 5.** Corneal ROS formation is suppressed with H2. To examine oxidative stress as a potential stimulus for angiogenic and molecular inflammatory events, corneal ROS expression was analyzed by DHE fluorescence. (A, B) ROS-induced DHE fluorescence in the corneal epithelium was significantly higher in vehicle-treated alkali-burned mice (1.6 ± 0.2 ratio of control; *P < 0.05) than in untreated controls. DHE intensity was significantly suppressed in H2-treated mice (0.6 ± 0.3 ratio of control; *P < 0.05) in vehicle-treated alkali-burned mice (1.6 ± 0.2 ratio of control; n = 4–5). Scale bar, 100 μm. (C) Phosphorylated NF-κB p65 levels were significantly (*P < 0.05) higher in vehicle-treated alkali-burned mice (311% ± 381% of control) than in untreated controls (100% ± 176% of control). Administration of H2 to alkali-burned animals significantly (*P < 0.05) reduced phosphorylated NF-κB p65 levels (140% ± 158% of control; n = 28–29). Error bars indicate SD.
the effects of the specific NF-κB inhibitors reported in the literature, DHMEQ is unique in that it blocks the translocation of NF-κB p65 into the nucleus and is therefore highly specific to this pathway. However, the most important finding of our study is the fact that direct ROS formation by alkali injury precedes the inflammatory response. ROS-related DHE fluorescence was enhanced immediately after alkali burn (Fig. 2A). Given that oxidative stress was upstream of the cascade leading to CNV, we hypothesized that supplementing exogenous antioxidants such as NAC may protect the cornea from alkali burn-induced angiogenesis. As expected, pretreatment with NAC significantly reduced the angiogenic response after alkali burns (Fig. 2). Nuclear translocation of NF-κB upregulates mcp-1 and vegf, leading to increased macrophage infiltration and angiogenesis, respectively. Compared with other NF-κB inhibitors reported in the literature, DHMEQ is unique in that it blocks the translocation of NF-κB p65 into the nucleus and is therefore highly specific to this pathway.

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deficiency and corneal angiogenesis. In conclusion, our study showed that oxidative stress is a direct result of alkali injury and that immediate action to quench ROS can inhibit pathologic angiogenesis. Furthermore, compared to using expensive reagents such as antibodies or antimetabolites, H2 water is inexpensive and safe to use in any clinical setting. We hope our results will shed light on the importance of antioxidant therapy in alkali burns.

References