Cytoprotective Effects of Epidermal Growth Factor (EGF) Ointment Containing Nafamostat, a Protease Inhibitor, on Tissue Damage at Burn Sites in Rats

Yoshifumi Kiyohara, Kohshi Nishiguchi, Fusao Komada, Seigo Iwakawa, Midori Hirai, and Katsuhiko Okumura

Department of Hospital Pharmacy, School of Medicine, Kobe University, Chuo-ku, Kobe 650, Japan and Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan.

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When epidermal growth factor (EGF) ointment containing a protease inhibitor, nafamostat (NM), was applied to burn sites in rats, the superoxide dismutase (SOD) enzyme activity and protein content increased 45% and 60%, respectively, at these sites 1 d after the burns compared with the control ointment. Following treatment with EGF plus NM (EGF+NM) ointment, messenger RNA for SOD also increased, to about 1.6 times that of the control at 1 d after the burn, indicating that this ointment stimulates SOD synthesis at burn sites in vivo. In contrast, following treatment with EGF+NM ointment, the content of heat shock protein (HSP 70) in the burned tissue decreased to about 70% of the control value 1 d after the burn. These findings suggest that EGF+NM ointment alleviated tissue damage at burn sites at an early stage, and that this was related to the stimulation of SOD synthesis and reduced HSP 70 levels. We also examined the effects of SOD ointment on wound healing at burn sites. A dose-dependent increase in the dry weight of granulation tissue at wound sites 3 d after the burn was observed following the application of this ointment. These results suggest that SOD may play an important role in wound healing after burns.

Keywords epidermal growth factor (EGF); nafamostat mesilate; protease inhibitor; burn; superoxide dismutase (SOD); cytoprotective effect

Epidermal growth factor (EGF) accelerates wound healing. In a previous study, in a second degree burn model in rats, we did not observe any accelerated wound healing following the application of EGF ointment without an EGF stabilizing agent. However, we found a marked improvement in burn healing following treatment with EGF ointment containing nafamostat (NM), a potent protease inhibitor; this suggested the importance of EGF stabilization at the administration site, since we had previously demonstrated insulin degradation at subcutaneous injection sites. To date, there have been few reports regarding the EGF-induced biochemical healing process following injury.

Superoxide anion radicals cause inflammation and cytotoxicity at wound sites after injury. Superoxide dismutases (SOD) scavenge these radicals and prevent radical induced inflammation. Decreases in topical SOD activity subsequent to burns have recently been reported. Heat shock protein (HSP) 70, a 72 kilo daltons (72 kDa) protein, is induced when cells or tissues are exposed to stressful conditions such as heat shock, and it has been shown by Burdon that HSP 70 synthesis is related to the free radical level. The HSP 70 content at burn sites may thus change according to changes in topical SOD content. In this study, to clarify the cytoprotective action of EGF+NM ointment, we investigated topical SOD and HSP 70 levels following treatment with this ointment after burns. We also applied SOD ointment at burn sites to determine whether topical SOD accelerated wound healing and alleviated the systemic effects of burns.

Materials and Methods

Chemicals Human EGF and NM were kindly supplied by Wakanaga Pharmaceutical Co. (Hiroshima, Japan) and Torii & Co. (Osaka, Japan), respectively. Human Cu,Zn-SOD, rabbit anti-human Cu,Zn-SOD antiserum, and human Cu,Zn-SOD-cDNA were kindly provided by Ashi Chemical Industries (Tokyo, Japan). Mouse monoclonal anti-72 kDa HSP antibody (IgG) and an ECL direct nucleic acid labelling and detection system were purchased from Amersham (Amersham, U.K.).

Preparation of Burn Model in the Rat A second-degree burn model was prepared according to a previously described method. Briefly, male Wistar rats (170–250 g) were anesthetized with pentobarbital (Nembutal; 50 mg/kg, i.p.), the hair from their backs was carefully removed, and two burns (8 mm in diameter; 200 °C, 10 s) were made on the hairless area. Body weight was measured every day while the animals were under pentobarbital anesthesia (50 mg/kg, i.p.). On the 7th day, ointments (0.2 g for each site) were applied once daily with a graduated syringe. After the ointment was applied, the wound site was covered with surgical tape (Benefix; Nippon Sigmax, Tokyo, Japan) as soon as possible. During the experimental period the animals received a normal diet (MF food; Oriental Yeast Co., Tokyo, Japan) and water ad libitum, and they were housed individually in stainless steel cages.

Preparation of EGF and SOD Ointment EGF ointment, containing NM as an EGF stabilizing agent, was prepared according to a previously described method. This EGF ointment contained 50 μg of EGF and 3.0 mg of NM per g. SOD ointment was prepared in three strengths: SOD 50, 500 μg/g and 5 mg/g. A control group of rats was treated with ointment base only (white petrolatum; purified lanolin + 4:1).

Determination of SOD Enzyme Activity Tissue samples for the determination of SOD activity in the skin were prepared by the method of Niwa et al. with some modifications. Normal skin and burn tissues were separated and homogenized with 6 ml of physiological saline. The homogenate was centrifuged at 7000 g for 10 min at 4 °C. SOD activity in the supernatant was measured by Oyaguni’s method.

Immunoblotting of SOD Protein The supernatants (3 μg protein for normal skin, and 6 μg protein for the control or EGF+NM-treated burn skin, respectively) used for the assay of SOD enzyme activity were subjected to 15% SDS-PAGE. After electrophoresis, the proteins were transferred to a PVDF membrane (Millipore) and immunoblotted by the avidin–biotin-peroxidase complex (ABC) technique, using rabbit anti-human Cu,Zn-SOD antiserum as the first antibody, and donkey anti-rabbit IgG antiserum as the second antibody. More than 80% homology has been reported for the amino acid sequence of rat and human SOD.

Recombinant human Cu,Zn-SOD was used as the standard. SOD content in the tissue samples obtained from each group was determined with a densitometer (Shimadzu double beam chromatoscaner CS-930; Shimadzu Co., Kyoto, Japan). When normal skin samples (6 μg protein) were
analyzed, the SOD content was estimated to be greater than the linear region of the standard curve. We therefore reduced the content of the normal skin sample to 3 μg protein.

**Northern Blotting of SOD-mRNA** Normal and burn tissues were separated and homogenized with 4 ml of 5.3 M guanidine thiocyanate. Total RNA was prepared by CsCl centrifugation, after which SOD mRNA was measured by hybridizing human Cu,Zn-SOD cDNA, according to the procedure described by the manufacturers of the ECL direct nucleic acid labelling and detection system (Amersham). SOD-mRNA content was measured with a densitometer.

**Measurement of 72kDa Protein** The supernatants (3 μg protein) used for the assay of the 72kDa protein were subjected to 1.5% SDS-PAGE. After electrophoresis, the protein was stained with Coomassie Blue and the content of 72kDa protein in each group was determined with a densitometer.

**Immunoblotting of HSP 70** The supernatants (3 μg protein) used for the assay of HSP 70 protein were subjected to 7.5% SDS-PAGE. After electrophoresis, the proteins were transferred to a PVDF membrane (Millipore) and immunoblotted by the ABC technique, using mouse monoclonal anti-HSP 70 antibody as the first antibody and sheep anti-mouse IgG antiserum as the second antibody. HSP 70 content was determined with a densitometer.

**Measurement of Dry Weight of Granulation Tissue** Three days after the burn, the back skin of the rat was removed and the granulation tissue was carefully dissected from the wound site; it was then stored at under −20°C until analyzed. The dry weight of the granulation tissues was measured after lyophilization.

**Statistical Analysis** The data were expressed as means ± S.E. The results were compared using the Student’s t-test.

**Results and Discussion**

Figure 1 shows that SOD activity in the burn sites at 1 d after the burn had decreased dramatically compared with that in normal skin. This result was consistent with those in previous studies.1,5,16 Following treatment with EGF ointment containing NM, SOD activity increased 45% at these sites 1 d after the burns compared with the control ointment. However, 3 d after the burn, there was little difference in SOD activity between the EGF + NM ointment-treated sample and the control ointment-treated sample.

On immunoblotting of the samples 1 d after the burn (Figs. 2A, B), the content of SOD protein in the burn tissue treated with EGF + NM ointment was also observed to have increased to about 1.6 times that of the control. Treatment with EGF ointment alone or NM ointment alone had no statistically significant effect on SOD activity when compared with treatment with the ointment base (data not shown). These results suggest that the acceleration of SOD synthesis induced by EGF + NM ointment takes place in an early period (Fig. 1). We therefore examined the effect of EGF + NM ointment on SOD-mRNA levels in the wound tissue to clarify whether its effect was due to the inhibition of SOD degradation or to the induction of SOD synthesis.

Following treatment with EGF + NM ointment, SOD-mRNA at the burn sites 1 d after the burn also increased, to about 1.6 times that of the control-treated tissue (Fig. 3). Following treatment with EGF ointment alone and NM ointment alone, the changes in SOD-mRNA were insignificant. Accordingly, these results suggested that the
increased synthesis of SOD induced by treatment with EGF + NM ointment may contribute to ameliorating inflammation and may accelerate wound healing.

Figure 4A shows that the 72 kDa protein content at the wound sites 1d after the burn increased remarkably. Following treatment with EGF + NM ointment, however, the 72 kDa protein content decreased by about 30% compared with the control. It is known that HSP 70 is induced in cultured cells by thermal treatment. We therefore measured HSP 70 content by immunoblotting. After thermal treatment, HSP 70 levels also increased, to 3.5-fold those in normal skin; however, following treatment with EGF + NM ointment, the HSP 70 content at the wound sites 1d after the burn decreased to about 70% of the control value (Fig. 4B).

Burdon has reported that HSP 70 synthesis may be stimulated by the activation of heat shock transcription factor (HSF), which is induced by free radicals. In this context, it would be reasonable to assume that the suppression of free radicals by the induction of SOD following treatment with EGF + NM ointment may inhibit HSP 70 synthesis. In a previous study, we found that treatment with EGF + NM ointment alleviated systemic damage 1d after burns. In this present study, we investigated changes in SOD and HSP 70 at wound sites 1d after thermal injury. Our findings indicate that the alleviation of systemic damage may be due to changes in topical factors such as SOD occurring during the early period (1d after burn).

If increases in topical SOD induced by treatment with EGF + NM ointment accelerate wound healing, then direct topical treatment with SOD may increase the rate of wound repair. Indeed, in clinical studies, Mizushima et al. found that SOD cream was an effective treatment for burns and skin ulceration in humans. To confirm that SOD does increase the rate of wound repair, we applied ointment containing various concentrations of SOD to the burn sites in the rats 3d after thermal injury (Fig. 5). Following treatment with SOD, the dry weight of granulation tissue 3d after the burn increased in a dose-dependent manner. These findings suggest that wound healing was accelerated by the SOD treatment.

Our previous studies and those of other investigators had indicated that the body weight of animals in the control groups subjected to burns decreased. We investigated changes in body weight in the rats (Fig. 6).

Following treatment with the SOD ointments, there was no loss of body weight 1d after the burn. However, alleviation of body weight loss was not observed from 2d after the burn. Our previous studies showed that the loss of body weight observed after burns was inhibited by the application of EGF + NM ointment at an early period (from 1 to 3d),
suggesting that EGF + NM ointment has systemic alleviatory effects on hormone and body fluid disturbances. The effects of EGF + NM ointment in producing alleviation of systemic damage may thus be different from those of SOD ointment. Till et al.²⁷ described the generation of toxic oxygen metabolites induced by increases in plasma xanthine oxidase in thermally injured skin after burns, and they observed improvement in thermal injury following treatment with the antioxidant SOD. These findings indicate that the topical cytoprotection and systemic alleviation produced by treatment with EGF + NM ointment may be related to the scavenging of toxic oxygen metabolites due to the increased synthesis of SOD.

In conclusion, we investigated the effects of EGF + NM ointment on changes in SOD and HSP 70 at wound sites. There were increases in SOD due to wound repair at burn sites following treatment with this ointment, whereas the HSP 70 content was reduced by this treatment. We found, further, that SOD ointment accelerated wound healing in a dose-dependent manner. These findings suggest that the regulation of topical SOD and HSP by EGF may contribute to the wound healing effect produced by this agent. We are now investigating the effects of SOD ointment on two other injury models (open wounds and burn ulcers).²⁸ The present findings support our previous results from a biochemical point of view and suggest that the stabilization of EGF at the wound site is important for the expression of its alleviatory action.

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References


