Improvement in Wound Healing by Epidermal Growth Factor (EGF) Ointment. II. Effect of Protease Inhibitor, Nafamostat, on Stabilization and Efficacy of EGF in Burn*

Yoshifumi KIYOHARA,** Fusao KOMADA,** Seigo IWAKAWA,** Midori HIRAI,** Tohru FUWA,*** and Katsuhiko OKUMURA**,**

Department of Hospital Pharmacy, School of Medicine, Kobe University,** Chuo-ku, Kobe 650, Japan and Central Research Lab., Wakunaga Pharmaceutical Co.,*** 1624 Shimokoutachi, Hiroshima 729-64, Japan

(Received August 20, 1990)

The healing effect of human epidermal growth factor (hEGF) on second degree burn was studied in rats. No improvement in wound healing was found on topical application of EGF alone to burn sites, but an ointment containing EGF and nafamostat mesilate (NM), a protease inhibitor, accelerated the healing rate of burns. The dry weight of the granulation tissue on the wound site in the group treated with EGF plus NM ointment did not change, although that in other groups decreased. After treatment with EGF ointment containing NM, the content of uralonic acid, as an index of acid mucopolysaccharide, at 3 d after burn rapidly increased and had recovered to nearly normal levels at 7 d after burn. However, the uronic acid content in the other groups (control, EGF alone, and NM alone) showed a higher value at 7 d than at 3 d. When compared with the control values significant increases in hydroxyproline, as an index of collagen, in the wound site were observed at 7 d after treatment with EGF ointment containing NM. The degradation of [125I]EGF in burned tissue homogenate decreased significantly in a concentration-dependent manner in the presence of NM. Body weights did not change after treatment with EGF plus NM ointment, although the body weights of other treatment groups decreased after burn, suggesting that EGF ointment containing the protease inhibitor, NM, alleviated the effects of burn shock. These findings indicate that the stabilization of EGF at the wound site is an important factor for the expression of its healing effects.

**Keywords** — epidermal growth factor (EGF); nafamostat mesilate; protease inhibitor; burn; wound healing; shock

Introduction

Epidermal growth factor (EGF), a polypeptide of 53 amino acid residues, has wound healing effects.2,3 Nafamostat mesilate (NM), 4-{(aminoiminomethyl) amino} benzoic acid 6-(aminoiminomethyl)-2-naphthalenyl ester, is a new potent serine-protease inhibitor.4,5 This drug has been used for the treatment of acute pancreatitis, disseminated intravascular coagulation (DIC), and as an anticoagulation agent in extracorporeal dialysis in Japan.6 In our previous study7 we demonstrated that EGF ointment containing a protease inhibitor, such as NM or gabexate mesilate,8 or gelatin, accelerated wound repair when it was applied to open wound sites in the rat. However ointment containing EGF alone or protease inhibitor alone did not accelerate wound healing. The stabilization of EGF in the wound tissue was indicated to be important for its growth activity.

It is well-known that burn wounds and open wounds have different repairing processes.6,7 Burn shock caused by burn toxin after burns and hemorrhagic shock which occurs after open (traumatic) wounds belong to different categories of shock.8 Repairing agents should be clinically useful for different injuries. Therefore, in this study we have investigated the effect of EGF plus NM ointment on wound healing at burn sites in rats as another injury model. EGF plus NM ointment also accelerated wound healing for thermal injury. Furthermore, we have indicated that EGF plus NM ointment have alleviated effects on some damage in this paper.

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* Preceding paper, Part I: Ref. 1.
** To whom correspondence should be addressed.
Materials and Methods

Materials — Human EGF was provided by Wakunaga Pharmaceutical Co. (Hiroshima, Japan). Radioiodinated hEGF was purchased from Amersham (Amersham, U.K.). The specific activity of $[^{125}]$EGF was 1.3 Ci/$\mu$mol. NM and egg white lysozyme were supplied by Torii & Co. (Osaka, Japan) and Wako Pure Chemicals (Osaka, Japan), respectively. Hanks buffer (Na$^+$, 140 mM; K$^+$, 6.0 mM; Ca$^{2+}$, 1.0 mM; Mg$^{2+}$, 1.0 mM; Cl$^-$, 145 mM; HCO$_3^-$, 4.0 mM; PO$_4^{3-}$, 1.0 mM; pH 7.2) was obtained from Whittaker M. A. Bioproducts (Walkersville, Maryland). White petrolatum, liquid paraffin and purified lanolin were JP XI grade. All other chemicals were obtained from commercial sources and were reagent grade.

Preparation of Ointments — Ointments were prepared according to previously described methods.$^{10}$ The components of the ointments are shown in Table I. The composition of the ointment base was white petrolatum: purified lanolin (80: 20). The ointment was prepared weekly and stored at 4 °C. For the prevention of infection, lysozyme was added at a concentration of 50 μg/g.

Preparation of Burn Model — Under pentobarbital (Nembutal; 50 mg/kg, i.p.) anesthesia, the hair from the back of male Wistar rats (170—250 g) was carefully removed with an animal clipper and a shaver, and two burns (8 mm in diameter) were prepared on the right and left sides of the back, using a thermo-controlled device (Asahi Medical Instruments, Tokyo, Japan) at 200 °C for 10 s. The ointments (0.2 g for each site) were applied by the use of graduated syringes every day after measurement of body weight. The wound site was covered with surgical tape (Benefix, Nippon Sigmax, Tokyo, Japan). During application of the ointments, the rats were under pentobarbital anesthesia. During the experiments the animals received a normal diet (MF food, Oriental Yeast Co., Tokyo, Japan) and water ad libitum. They were housed individually in stainless steel cages under the following conditions: light, from 06:00 to 18:00; temperature, 22 ± 2 °C.

Measurement of Dry Weight of Granulation Tissue, Uronic Acid, and Hydroxyproline — After removing the back skin from the rat the granulation tissue was carefully dissected from the wound site and was stored at under —20 °C until analyzed. The dry weight of the granulation tissue was measured after lyophilization. As an index of acid mucopolysaccharide, uronic acid was measured by the method of Masamune et al.$^{10}$ The hydroxyproline content of the wound tissue was also measured, as an index of collagen, by the method of Inayama et al.$^{11}$

Degradation of EGF in Burn Tissue Homogenate — One day after wound induction the burn tissue was homogenized with 10 volumes of Hanks buffer (pH 7.2) using a glass homogenizer. The homogenate (0.2 mg of protein per tube) and $[^{125}]$EGF (1 ng per tube) were incubated, with or without NM, at 37 °C for 15 min (total volume 0.6 ml). After incubation, 0.6 ml of 20% (w/v) trichloroacetic acid was added to the incubation mixture and the mixture was then centrifuged at 2000 g for 10 min. The radioactivities in the supernatant and in the precipitate were measured by a gamma-counter (1260 Multigamma II, LKB Wallac, Finland) and the degradation rate of EGF was calculated. The protein concentration was measured by Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA).

Statistical Analysis — The data were expressed as the mean ± S.E. The results were compared using Student’s (unpaired) t-test.

Results and Discussion

Table I. Contents of EGF Ointments

<table>
<thead>
<tr>
<th>EGF (μg/g)</th>
<th>Nafamostat (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>EGF alone</td>
<td>50</td>
</tr>
<tr>
<td>NM alone</td>
<td>0</td>
</tr>
<tr>
<td>EGF + NM</td>
<td>50</td>
</tr>
</tbody>
</table>

Figure 1 shows the histological findings before and after burn induction. This burn model was a second degree deep dermal burn according to Arzt’s classification.$^{12}$ The dry weight of the granulation tissue 3 and
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7 d after burn was measured (Fig. 2). Following treatment with the control ointment and ointments containing EGF alone or NM alone, the dry weight of granulation tissue was decreased 3 and 7 d after burn, compared with the dry weight of normal skin (the dry weight of normal skin was 48.7 ± 0.9 mg/site). However, 3 and 7 d after burn, following treatment with EGF plus

Fig. 1. Histological Photograph before, (1) and after, (2) Burn in the Wound Tissue
EP, epidermis; D, dermis (s, superficial; d, deep); HD, hypodermis.

Fig. 2. Changes in Dry Weight of Granulation Tissue at 3 and 7 d after Burn
Animals were treated daily with the following ointments: control, EGF alone, NM alone or EGF plus NM. The dotted line is dry weight of normal skin. Results are expressed as the mean ± S.E. (n = 9—14). Significant differences from control group: a) p < 0.01, b) p < 0.001.
□, control; △, EGF alone; ▽, NM alone; ⊳, EGF + NM.

Fig. 3. Effects of EGF Plus NM Ointment on Uronic Acid Content at 3 and 7 d after Burn
The dotted line is uronic acid content of normal skin. Results are expressed as the mean ± S.E. (n = 8—11). Significant differences from control group: a) p < 0.02, b) p < 0.001.
□, control; △, EGF alone; ▽, NM alone; ⊳, EGF + NM.
NM ointment the dry weight of granulation tissue was not decreased. Similarly, in the open wound model, the formation of granulation tissue after treatment with EGF plus NM ointment was increased significantly when compared with granulation tissue formed after treatment with control ointment. Figure 3 shows uronic acid content, as an index of acid mucopolysaccharide synthesis in the burn site, 3 and 7 d after burn. The production of uronic acid was increased after treatment with EGF plus NM ointment 3 d after burn, and then decreased to normal levels 7 d after burn. On the other hand, uronic acid content after treatment with the control, EGF alone, or NM alone showed a higher value 7 d than 3 d after burn, indicating that rapid synthesis of acid mucopolysaccharide had been induced by EGF plus NM ointment. In the open wound model, uronic acid content also increased at 3 d after injury following treatment with EGF plus NM ointment.11

Figure 4 shows hydroxyproline content, as an index of collagen synthesis, 3 and 7 d after burn. Hydroxyproline levels at the burn sites were decreased 7 d after burn following treatment in all 4 groups, which is consistent with the results of Isobe et al.13 Then, 14 d after burn, hydroxyproline levels at the burn sites returned to nearly normal levels in all 4 groups (data not shown). Presumably, denatured collagen, formed as a scab after the burn, might be removed by released lysosomal proteases up to 7 d after the burn14; the burned necrotic tissue may be replaced by granulation tissue. However, in the open wound, formation of granulation tissue is a major process of wound repair and this removal process for necrotic tissue does not occur. Three days after burn, the hydroxyproline content in the tissue of the treated groups was not significantly different from that in the control group, but at 7 d after burn the hydroxyproline content after treatment with EGF plus NM ointment showed a significantly high level. In general, normal healing is considered to consist of two healing processes.15 Firstly, formation of acid mucopolysaccharides is accelerated at the wound site (productive or substrate phase), and then collagen is formed rapidly at the wound site, while the synthesis of acid mucopolysaccharides is reduced (collagen phase). In our preparation of the burn model, 3 d after burn collagen was not formed, while 7 d after burn, collagen syn-

Fig. 4. Effects of EGF Plus NM Ointment on Hydroxyproline Content at 3 and 7 d after Burn

The dotted line is hydroxyproline content of normal skin. Results are expressed as the mean ± S.E. (n = 9–11). Significant differences from control group: a) p < 0.05.

□, control; △, EGF alone; ■, NM alone; ⊚, EGF + NM.

Fig. 5. Effect of Nafamostat on the Degradation Rate of [125I]EGF in Burn Tissue Homogenate

Burn tissue was obtained at 1 d after burn preparation. Tissue homogenate (0.33 mg/ml of protein) was incubated with [125I]EGF (0.28 nm) for 15 min at 37 °C. The degradation of [125I]EGF was determined using 10% trichloroacetic acid. The degradation rate of normal tissue homogenate was 20.6 ± 1.2 pg/min per mg of protein. Results are expressed as the mean ± S.E. (n = 3). Significant differences from burn tissue without NM: a) p < 0.001.

□, normal; △, burn; ■, 0.5 mM NM; ⊚, 1.0 mM NM.
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![Graph showing percentage change in body weight over days after burn](image)

Fig. 6. Effects of EGF Plus NM Ointment on Percentage Change in Body Weight in the Burn Rat

Animals' body weights were measured daily before treatment with control (○); EGF alone (●); NM alone (□); and EGF + NM (■). Results are expressed as the mean ± S.E. (n = 5—12). Significant differences from control group: a) p < 0.01, b) p < 0.001.

thesis was accelerated by treatment with EGF plus NM ointment. Therefore, in thermal injury the healing process achieved with EGF plus NM ointment was considered to be normal but accelerated healing; it was also confirmed histologically to be normal healing.

In our previous papers, we have found that bioavailability and efficacy of subcutaneously injected insulin are increased by the cotreatment with small peptide, collagen or protease inhibitors, which indicates that stabilization of peptide drugs at the application site is an important factor to show its action properly. We also found the inhibitory effect of NM, gabexate or collagen on the degradation of EGF in the homogenate of open wound tissue. Therefore, we hypothesized that the addition of a protease inhibitor, such as NM, might also stabilize EGF and might increase the efficacy of EGF at the burn site. Next, we investigated the effect of NM on EGF degradation in burn tissue homogenate (Fig. 5). The rate of [125I]EGF degradation in burn tissue homogenate was increased more than in normal skin (p < 0.05). These data are consistent with previous findings that proteases in burn tissue are induced through a mechanism of protein synthesis and that proteases are released from lysosomes by thermal injury. The rate of [125I]EGF degradation was decreased, in a concentration-dependent manner, by the addition of NM. Accordingly, it was considered that wound healing with EGF plus NM ointment would be important for the inhibition of EGF degradation by NM in burn tissue as well as in open wound tissue.

Loss of body weight and imbalance in body fluids are major characteristics of shock after wound formation. Figure 6 shows percentage change in body weight after burn. At 1 day after burn formation the body weights of three groups (control, EGF alone, and NM alone) had decreased, by a factor of between 3 and 5% of initial body weight. However, after combined treatment with EGF and NM the body weight did not change. This finding suggests that EGF plus NM ointment alleviated the effects of shock induced by burn. This burn shock is caused by burn-induced lysosomal proteases. Recently, application of protease inhibitors such as NM or gabexate to inhibit protease activity has been performed clinically for the treatment of burn shock. However alleviation of shock after treatment with EGF alone or the protease inhibitor, NM alone, was not observed in this study. Therefore, we conclude that the effects of EGF plus NM ointment on body weight change may be caused by a synergistic mechanisms between EGF and protease inhibitors.

In this study we have demonstrated that EGF ointment, containing a protease inhibitor such as NM, accelerates normal healing in burn wounds and alleviates shock after the wound.

Acknowledgement We thank Dr. O. Imaida and Dr. S. Kasuga at Wakunaga Pharmaceutical Co. for preparing histological samples.

References


3) B. L. Brown, L. Curtisinger, J. R. Brightwell, D. M.


