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*Report*

# Improvement in Wound Healing by Epidermal Growth Factor (EGF) Ointment. I. Effect of Nafamostat, Gabexate, or Gelatin on Stabilization and Efficacy of EGF[1]

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The healing effect of human epidermal growth factor (hEGF) on open wounds was studied in rats. No improvement in wound healing was found by topical application of EGF alone to open wound sites. We found an ointment containing EGF and a protease inhibitor, nafamostat mesilate or gabexate mesilate, or gelatin accelerated the healing rate of open wounds. Significant increases in the dry weight of the wound site granulation tissue, uronic acid (as an index of acid mucopolysaccharide) and hydroxyproline (as an index of collagen) were observed by treatment with EGF ointment containing nafamostat compared with the controls. The effects of the protease inhibitor on wound healing were dose dependent. Nafamostat was more efficient than gabexate or gelatin on wound healing. The degradation of $^{125}$I-EGF in wound tissue homogenate was significantly decreased in the presence of a protease inhibitor, such as nafamostat or gabexate, or gelatin. These findings indicate that the stabilization of EGF at the wound site is an important factor in permitting the expression of its healing effects and suggest that the ointment containing EGF and a stabilizing agent would be a suitable dosage form for acceleration of wound repair.

KEY WORDS: epidermal growth factor (EGF); nafamostat mesilate; gabexate mesilate; gelatin; protease inhibitor; open wound; wound healing.

## INTRODUCTION

Epidermal growth factor (EGF), a polypeptide of 53 amino acid residues, has wound healing effects (1–5). Application of EGF ointment every 12 hr produced faster and better healing of wounds in rabbit ears (1). EGF also increased the rate of epidermal regeneration of split-thickness wounds and partial-thickness burns of miniature pigs (2,3). The process of wound repair in rats was accelerated by a local sustained release of EGF in a polyvinyl alcohol sponge implanted subcutaneously (4). A double-blind clinical trial revealed that the healing rate of skin graft-donor sites was accelerated by EGF cream (5). However, several animal and clinical studies did not show significant EGF healing effects (6,7). A recent study using six growth factors and a partial-thickness wound model in pigs also indicated that EGF alone produced no significant changes in the healing parameters measured (8). The fate (degradation and absorption) of EGF at the application site has not been investigated, though local inactivation of EGF by epidermal peptidase has been presumed (7).

Our previous studies (9,10) demonstrated rapid degradation of peptide drugs such as insulin at the application site and indicated that the coadminstration of a protease inhibitor, gabexate, 4-[[6-[(aminoiminomethyl)amino]-1-oxohexyl]oxy]benzoic acid ethyl ester, which is a potent serine-protease inhibitor (11–15), or collagen could increase the stability and bioavailability of insulin after subcutaneous injection. Nafamostat, 4-[(aminoiminomethyl)amino]benzoic acid 6-(aminoiminomethyl)-2-naphthalenyl ester, is a new potent serine-protease inhibitor (11–16). This drug has been used for the treatment of acute pancreatitis or disseminated intravascular coagulation (DIC) and as an anticoagulation agent in extracorporeal dialysis in Japan (13,14). The present study was undertaken to develop an EGF ointment containing protease inhibitors or gelatin, heat-denatured collagen, for wound healing.

## MATERIALS AND METHODS

### Materials

Highly purified (more than 99%) human EGF was pro-

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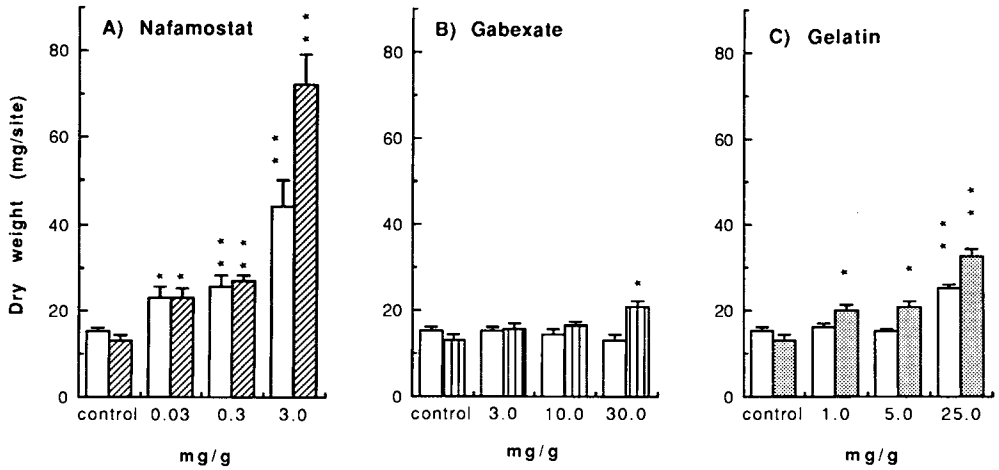


**Fig. 1.** Changes in dry weight of granulation tissue at 3 days after wounding. Animals were treated daily with the following ointments: control, EGF + nafamostat, EGF + gabexate, and EGF + gelatin. Results are expressed as the mean ± SE ($n$ = 7–15). Significant differences from the control group: (*)$P < 0.05$; (**)$P < 0.01$. A, with nafamostat; B, with gabexate; C, with gelatin; open columns, without EGF; closed columns, with EGF.

vided by Wakunaga Pharmaceutical Co. (Hiroshima, Japan). Radioiodinated hEGF was purchased from Amersham (Amersham, U.K.). The specific activity of $^{125}$I-EGF was 1.3 Ci/μmol. Nafamostat mesilate, gabexate mesilate, and gelatin were supplied from Torii & Co. (Osaka, Japan), Ono Pharmaceutical Co. (Osaka, Japan), and Upjon Co. (Kalamazoo, MI), respectively. White petrolatum, liquid paraffin, and purified lanolin were JP XI grade. Egg white lysozyme was purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals were obtained from commercial sources and were reagent grade.

### Preparation of Ointments

One milligram of EGF and nafamostat mesilate (0.6, 6, 60 mg), gabexate mesilate (60, 200, 600 mg), or gelatin (20, 100, 500 mg) were mixed with 4 ml of liquid paraffin and then added to the ointment base and mixed (total, 20g). Control ointment was prepared without EGF and protease inhibitor. 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.

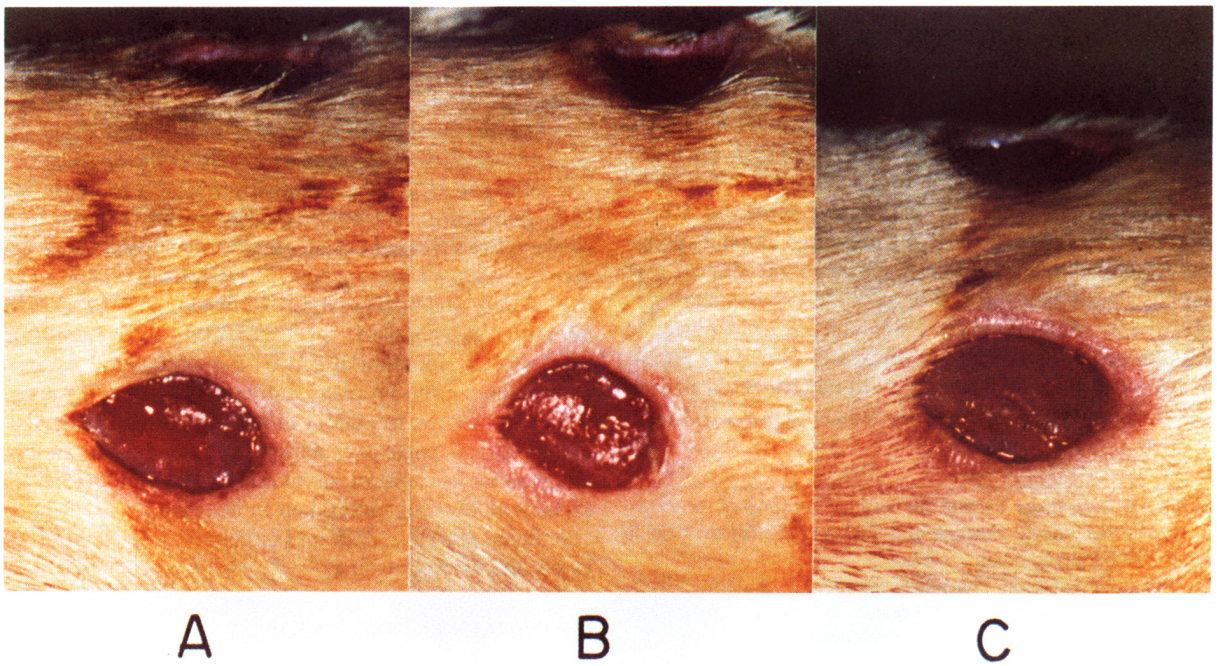


**Fig. 2.** Effect of EGF ointment on open wound healing at 3 days after wounding. A, control; B, EGF alone; C, EGF + nafamostat (3.0 mg/g).

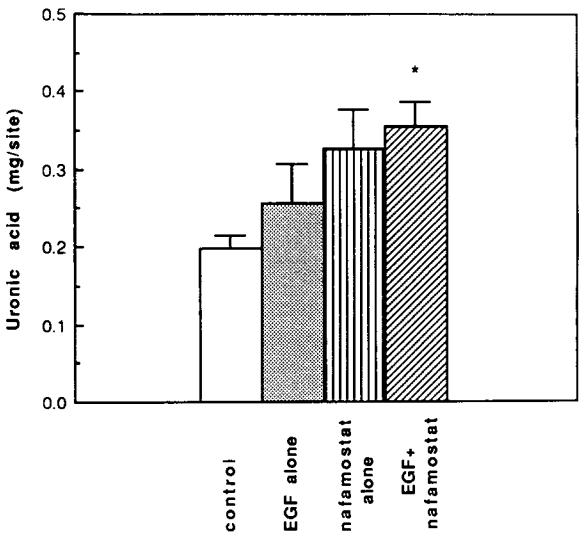


Fig. 3. Effects of EGF + nafamostat ointment on uronic acid content at 3 days after wounding. Open column, control; stippled column, EGF alone; vertical column, nafamostat (3.0 mg/g) alone; slashed column, EGF + nafamostat (3.0 mg/g). Results are expressed as mean ± SE ($n$ = 6–15). Significant differences from the control group: (*)$P < 0.05$.

### Preparation of Open Wounds in Rats

Under pentobarbital (Nembutal; 50 mg/kg, i.p.) anesthesia, the hair from the back of male Wistar rats (170–250 g) was removed and two open wounds (10 mm in diameter) were made symmetrically on the back using a punch. Ointments (0.2 g for each site) were applied by the use of graduated syringe under pentobarbital anesthesia every day to separate groups of rats after the measurement of body weight. The wound site was covered with surgical tape (Benefix, Nippon Sigmax, Tokyo). During the experiments the animals received a normal diet and water *ad libitum* and were housed individually in stainless steel cages.

### Measurements of Dry Weight of Granulation Tissue, Uronic Acid, and Hydroxyproline

The granulation tissue was separated from the wound site after removing the back skin from the rat and was stored 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 Dische (17) with the modification of Masamune *et al.* (18). The hydroxyproline content in the wound tissue was also measured as an index of collagen by the method of Kivirikko *et al.* (19) with the modification of Inayama *et al.* (20).

### Degradation of EGF in Wound Tissue Homogenate

The excised whole wound tissue at 1 day after wound preparation was homogenized with 10 vol of Hanks buffer (pH 7.2) using a glass homogenizer. The homogenate (0.8 mg of protein per tube) and $^{125}$I-EGF (2.5 ng per tube) were incubated with or without nafamostat, gabexate, or gelatin 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 centrifuged at 2000$g$ for 10 min. The radioactivities in the supernatant and in the precipitate were measured and the degradation rate of EGF was calculated. The protein concentration was measured by Bio-Rad protein assay kit.

### Statistical Analysis

The data were expressed as the mean ± SE. The results

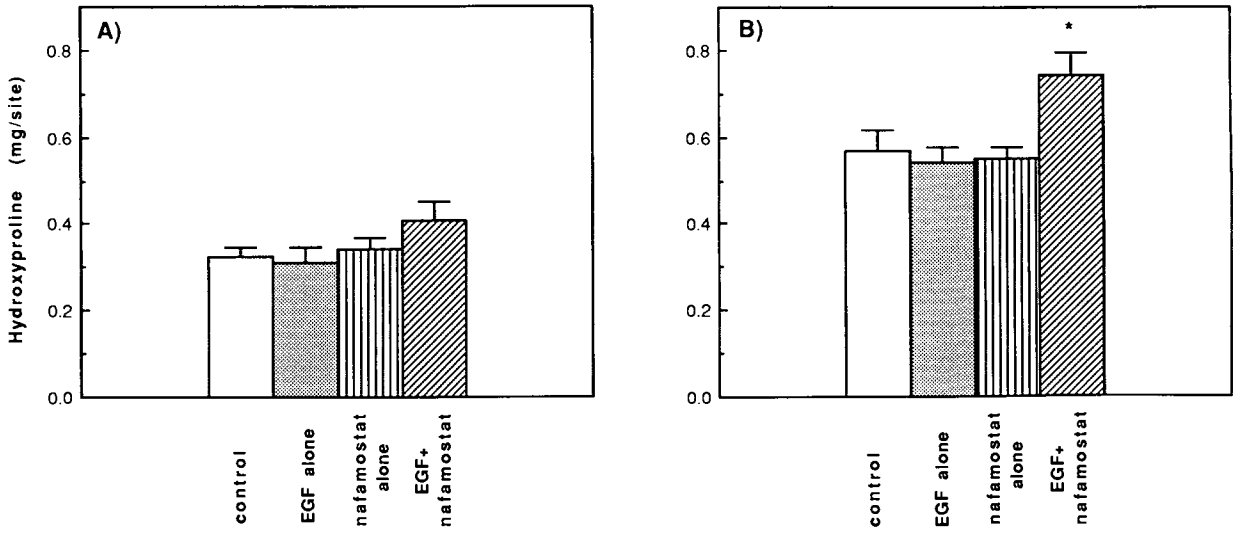


Fig. 4. Effects of EGF + nafamostat ointment on hydroxyproline content at 3 and 7 days after wounding. A, at 3 days after wounding; B, at 7 days after wounding; open column, control; stippled column, EGF alone; vertical column, nafamostat (3.0 mg/g) alone; slashed column, EGF + nafamostat (3.0 mg/g). Results are expressed as the mean ± SE ($n$ = 9–15). Significant differences from the control group: (*)$P < 0.05$.

were compared using Student's *t* test with Bonferroni's correction.

## RESULTS

Figure 1 shows the changes in dry weight of the granulation tissue after open wounds were made in rats. There were no significant differences in the dry weight of the granulation tissue between EGF alone and control ointment administration. The dry weight of the granulation tissue was increased over the controls and EGF alone at 3 days by cotreatment with nafamostat, gabexate, or gelatin in a dose-dependent manner. Nafamostat was more potent than gabexate or gelatin in increasing wound healing. Coadministration of nafamostat (3.0 mg/g) with EGF caused a threefold ($P < 0.01$) increase in the dry weight of the granulation tissue compared with the control ointment at 3 days after wounding.

The wound sites at 3 days after preparing the open wound are shown in Fig. 2. Treatment with EGF alone did not improve the wound healing. The formation of granulation tissue at the wound sites was visually confirmed to be accelerated by cotreatment with EGF and nafamostat (3.0 mg/g).

The content of uronic acid in the open wound granulation tissue, as an indicator of acid mucopolysaccharides, was significantly increased at 3 days during treatment with EGF plus nafamostat (3.0 mg/g) compared with control (Fig. 3). The uronic acid content in the granulation tissue after treatment with EGF alone or nafamostat (3.0 mg/g) alone was not significantly different from the controls. Hydroxyproline is one of the major amino acids of the collagen molecule. An increased content of hydroxyproline in the wound tissue was observed during the combined treatment of EGF and nafamostat (3.0 mg/g) at 7 days after open wounding, while the hydroxyproline content at 3 days after wounding in all groups was not significantly different from control (Fig. 4). Hydroxyproline levels for other treatments were not different from those of the control group.

The degradation rate of $^{125}$I-EGF in the wound homogenate is shown in Table I. The degradation rate was significantly inhibited in the presence of nafamostat, gabexate, or gelatin. The rate of $^{125}$I-EGF degradation was decreased by nafamostat in a concentration-dependent manner ($r = 0.953$) (Fig. 5). A linear relationship between the degradation rate of EGF and the dry weight of the granulation tissue treated with EGF ointment containing different amounts of nafamostat ($r = 0.984$) was observed (Fig. 6).

Table I. Effect of Nafamostat, Gabexate, or Gelatin on Degradation of $^{125}$I-EGF in Wounding Tissue Homogenate[a]

| | Degradation rate (pg/min per mg of protein) |
|---|---|
| Control | 16.40 ± 0.43 |
| + Nafamostat (1 m*M*) | 7.10 ± 0.92** |
| + Gabexate (1 m*M*) | 8.62 ± 0.59** |
| + Gelatin (2.5 mg/ml) | 9.89 ± 1.84* |

[a] Results are expressed as the mean ± SE ($n$ = 4–5). Tissue homogenate (0.8 mg/ml of protein) was incubated with $^{125}$I-EGF (0.7 n*M*) for 15 min at 37°C. The degradation of $^{125}$I-EGF was determined by using 10% trichloroacetic acid.

* Significant differences from the control group: $P < 0.05$.

** $P < 0.01$.

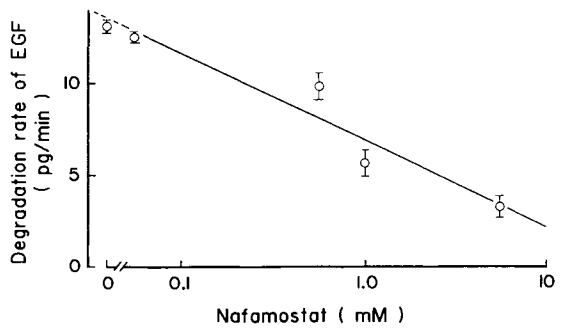


Fig. 5. Effect of nafamostat on the degradation rate of $^{125}$I-EGF in wound tissue homogenate. Repair tissue was obtained at 1 day after open wound preparation. Tissue homogenate (0.8 mg/ml of protein) was incubated with $^{125}$I-EGF (0.7 n*M*) for 15 min at 37°C. The degradation of $^{125}$I-EGF was determined using 10% trichloroacetic acid. Results are expressed as the mean ± SE ($n$ = 4–5).

## DISCUSSION

Some differences have been noted in the efficiency of EGF in promoting wound healing in previous studies (1–8). One of the possible explanations for the ineffectiveness of EGF may be the dosage form administered to the wound site. The optimal frequencies of dosing and the dosing level of EGF have yet to be determined. Buckley *et al.* (4) demonstrated that sustained release of EGF accelerates wound repair. When EGF was administered as an aqueous solution (6) or as a mist (7), insignificant wound healing was observed. The present study also revealed that ointment containing EGF alone did not improve wound healing. Proteolytic activity in cutaneous lesions is significantly increased over normal tissue (21). Therefore, stabilizing of EGF with a protease inhibitor may be important to demonstrating wound healing by EGF.

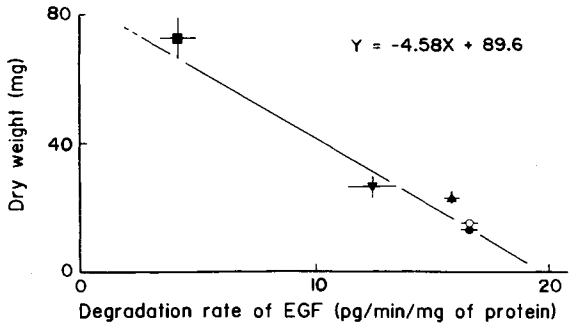


Fig. 6. Relationship between the degradation rate of EGF with different contents of nafamostat in wound tissue homogenate and the dry weight of the granulation tissue with EGF ointment containing different nafamostat contents. Tissue homogenate (0.8 mg/ml of protein) at 1 day after wounding was incubated with $^{125}$I-EGF (0.5 n*M*) for 15 min at 37°C. The degradation of $^{125}$I-EGF was determined using 10% trichloroacetic acid. The granulation tissue was removed from the wound site and was lyophilized. Results are expressed as the mean ± SE ($n$ = 4–18). Control, ○; EGF alone, ●; 0.03 mg/g nafamostat, ▲; 0.3 mg/g nafamostat, ▼; 3.0 mg/g nafamostat, ■.

In this study, the dry weight of the granulation tissue as an index of healing rate was increased with cotreatment by EGF and the protease inhibitors, nafamostat and gabexate, or gelatin in a dose-dependent manner. The EGF-nafamostat ointment showed a more potent effect than the EGF-gabexate or EGF-gelatin. This difference in wound repair may result from the difference in inhibitory potency to many proteases (22). Since nafamostat is known to have a lower $IC_{50}$ for many proteases than gabexate, the granulation tissue at the wound site might be remarkably increased by treating with EGF plus nafamostat ointment.

The content of acid mucopolysaccharides at 3 days after wounding, measured as uronic acid (23) in the tissue, was increased by the administration of the ointment containing EGF and nafamostat. The collagen content at 7 days after wounding measured as hydroxyproline (23) in the tissue was increased by the administration of the ointment containing EGF and nafamostat, although the collagen content at 3 days after wounding was not significantly increased compared with the controls. Normal wound repair is considered to be composed of two healing processes (24). Formation of acid mucopolysaccharides is accelerated at the wound site within 4 days after wounding (productive or substrate phase). Five days after the injury, collagen is rapidly formed at the wound site (collagen phase). Therefore, the healing process with EGF plus nafamostat ointment may be considered to be normal but accelerated healing; this was also confirmed histologically. Therefore, the addition of protease inhibitor such as nafamostat may stabilize EGF and therefore increase its efficacy.

EGF's degradation rate was significantly inhibited in the presence of nafamostat, gabexate or gelatin. There was a linear relationship between the degradation rate of EGF and the dry weight of the granulation tissue, which indicates the importance of stabilization of EGF on wound healing. The instability of EGF in wound tissue may have contributed to the ineffectiveness of EGF on wound healing in some previous studies (6–8).

This study indicates the importance of the stabilization of peptide drugs for topical formulations. An ointment containing EGF and a protease inhibitor may constitute a dosage form useful to accelerating wound healing.

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