Systemic Effects of Epidermal Growth Factor (EGF) Ointment Containing Protease Inhibitor or Gelatin in Rats with Burns or Open Wounds

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The systemic effects of epidermal growth factor (EGF) ointment containing nafamostat (NM), gabexate, or gelatin was studied in rats with burns or open wounds. At 1 d after burn, plasma epinephrine, cortisol, and glutamic–oxaloacetic transaminase (GOT) levels were elevated, but treatment with EGF plus NM (EGF+NM) ointment significantly suppressed the increase in these levels. Further, there was no loss of body weight in the open wound model following treatment with EGF+NM ointment, while loss of body weight occurred in animals in which EGF ointments without NM were applied. Increases in plasma epinephrine 1 d after open wound formation were also suppressed by the application of EGF+NM ointment. Treatment with EGF ointment containing gabexate (GX) or gelatin (GL) ameliorated changes in body weight that occurred after open wound formation, while loss of body weight in animals with open wounds occurred following the application of ointment base, EGF ointment, GX ointment, or GL ointment. The present study thus indicates that the topical application of EGF ointment containing a protease inhibitor has ameliorative systemic effects.

Keywords epidermal growth factor (EGF); burn; wound; nafamostat; gabexate; gelatin

Introduction

Epidermal growth factor (EGF), which is composed of 53 amino acid residues, accelerates wound healing. However, as we have indicated previously, EGF ointment without a stabilizing agent did not accelerate the healing of open wound or burn sites, and the importance of EGF stabilization at the application site was demonstrated.

In other previous studies, we also found that the bioavailability and efficacy of subcutaneously injected insulin were increased by cotreatment with small peptide, collagen, or protease inhibitors, indicating that the stabilization of these peptide drugs at the administration site is an important factor in allowing the full exhibition of their action.

There is a difference in repairing processes between burn wounds and open wounds. The healing of an open wound is a regeneration of tissue at the trauma site which is lack of denatured tissue, while the repairment of a burn wound is the removal of denatured protein (scab) along with a regeneration of tissue at the injury site. And burn shock, which is caused by burn toxin after burns, and hemorrhagic shock, which occurs after open (traumatic) wounds, belong to different categories of shock.

Several reports have described significant loss of body weight after injury. In our previous study with rats, we found that after the production of a deep dermal burn, body weight decreased following treatment with the control, EGF alone or nafamostat (NM) alone ointment, although loss of body weight was not observed following treatment with EGF plus NM (EGF+NM) ointment. This result suggested that the EGF+NM ointment which we have developed has ameliorative effects on some systemic damage. In this study we investigated the efficacy of EGF ointment containing a protease inhibitor, NM, or gabexate (GX), or gelatin (GL) in ameliorating damage occurring after injuries in rats.

Materials and Methods

Materials Human EGF was provided by Wakunaga Pharmaceutical Co. (Hiroshima, Japan). NM, GX, and GL were supplied by Torii & Co. (Osaka, Japan), Ono Pharmaceutical Co. (Osaka, Japan), and the Upjohn Co. (Kalamazoo, MI), respectively. 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 EGF (50 µg/g) ointments containing NM (0.05 mg/g, 0.3 mg/g, 3.0 mg/g), GX (30 mg/g), or GL (5 mg/g), were prepared according to a previously described method. A control ointment (white petrolatum: purified lanolin = 80:20) was prepared without EGF or protease inhibitors. The ointments were prepared weekly and stored at 4°C. For the prevention of infection, lysozyme was added, at a concentration of 50 µg/g.

Preparation of Burns and Open Wounds in Rats The procedure used to prepare burn and open wound models has been described previously. Male Wistar rats (170-250 g; Clea Japan, Tokyo, Japan) were given two burns (6 mm in diameter) at 200°C for 10 s or two open wounds (10 mm in diameter) on the back. Body weight was measured everyday under pentobarbital anesthesia (30 min) and then ointments (0.2 g for each site) were applied once a day with a graduated syringe. After the application of ointment the wound site was covered with surgical tape (Benefix, Nippon Sigmax, Tokyo, Japan) as soon as possible. During the experiments the animals received a normal diet (MF Food, Oriental Yeast Co., Tokyo, Japan) and water ad libitum and were housed individually in stainless steel cages.

Measurements of Plasma Epinephrine, Cortisol, and Glutamic–Oxaloacetic Transaminase (GOT) Level The rats were anesthetized with pentobarbital and arterial blood (5 ml) was collected from the abdominal artery. The concentration of epinephrine in plasma was measured by the trihydroxy indole method, using HPLC. Plasma cortisol and glucose were measured by a fluorescence polarization immunoassay, using a TDX analyzer (Abbott Laboratories, MI, U.S.A.). Plasma GOT level was measured with a GOT assay kit (Wako Pure Chemicals, Osaka, Japan), using the Karmen method. Plasma pyruvate and lactate were measured with kits of Sigma Chemicals Co. (St. Louis, MO, U.S.A.).

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

Results and Discussion

Effects of EGF Ointment on Burn Wounds Figure 1 shows the effects of EGF+NM (3.0 mg/g) ointment on changes in body weight, plasma epinephrine, cortisol, and GOT 1 d after the burn was produced. Body weight 1 d after burn was reduced when animals were treated with the control ointment (ointment base only), but no loss of body
weight was observed when the animals were treated with EGF + NM ointment (Fig. 1A). Plasma epinephrine and cortisol level were increased 1 d after burn when animals were treated with control ointment, but, following treatment with EGF + NM ointment, the elevation of plasma epinephrine and cortisol levels was suppressed (Fig. 1B, C). In the control ointment-treated group of animals, the plasma GOT level was increased 1 d after the burn, while animals treated with EGF + NM ointment did not exhibit increased plasma GOT levels (Fig. 1D). Changes in body weight and in plasma epinephrine, cortisol, and GOT following treatment with ointments containing EGF alone or NM alone were not significantly different from the control (data not shown). There were no significant differences in other body fluid components, such as plasma pyruvate, lactate, or glucose, following treatment with EGF + NM ointment compared to levels of these components in the control group (data not shown).

From an early period, loss of body weight, imbalance of body fluids and such plasma hormones as epinephrine and cortisol are major characteristics of shock after wounds are inflicted.9–11,16–18 However, following treatment with EGF + NM ointment, loss of body weight and elevation of plasma epinephrine and cortisol were not observed, suggesting ameliorative effects of this preparation on burn-induced damage. Ischemia in the liver after injury causes an elevation of plasma transaminase level.19 We found an elevation of plasma GOT levels in control animals after burns, while animals treated with EGF + NM ointment did not display a significant increase in these levels. These results suggest that the systemic circulation may be improved by treatment with EGF + NM ointment.

**Effects of EGF Ointment on an Open Wound** Next, we investigated the ameliorative effect of EGF ointment on an open wound model. Loss of body weight was not observed following treatment with EGF + NM (3.0 mg/g) in the open wound model, while the body weight was decreased in this model following treatment with other ointment (control, NM (3.0 mg/g) alone and EGF alone) (Fig. 2). These findings were similar to those in the burn model and suggested that the EGF + NM ointment had ameliorative effects in the open wound model also.

We studied the relationship between improvements in body weight and plasma epinephrine 1 d after the open wound was produced. Application of EGF ointment containing a low concentration (0.03 mg/g) of NM did not ameliorate the loss of body weight or the elevation of plasma epinephrine, whereas following the treatment with EGF containing more than 0.3 mg/g of NM, improvements in body weight and plasma epinephrine were observed (Fig. 3A, B). The efficacy of EGF ointment containing 3.0 mg/g NM was similar to that of the EGF ointment containing 0.3 mg/g NM with regard to changes in body weight and plasma epinephrine. This result in the open wound model

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**Fig. 1. Effects of EGF plus NM Ointment on Percentage Change in Body Weight (A), Plasma Epinephrine (B), Plasma Cortisol (C), and Plasma G0T (D) 1 d after Burn**

The dotted line represents the value for the normal animal. Open column, control; slashed column, EGF + NM (3.0 mg/g). EGF content was 50 μg/g. Results are expressed as the mean ± S.E. (n = 6–11). Significantly different from control group: a) p < 0.05, b) p < 0.02, c) p < 0.01, d) p < 0.001.

**Fig. 2. Effects of EGF plus NM Ointment on Percentage Change in Body Weight in Rats with an Open Wound**

Animals' body weights were measured daily before treatment with control ointment, ○; EGF alone, ●; NM alone (3.0 mg/g), □; and EGF + NM (3.0 mg/g). Results are expressed as the mean ± S.E. (n = 6–15). Significant differences from control group: a) p < 0.05, b) p < 0.01.

**Fig. 3. Effects of NM on Body Weight Change (A) and on Epinephrine Concentration in Plasma (B) 1 d after Preparation of Open Wound**

The dotted line represents the value for the normal animal. Open column, control; vertical column, EGF + NM (0.03 mg/g); dotted column, EGF + NM (0.3 mg/kg); slashed column, EGF + NM (3.0 mg/g). Results are expressed as the mean ± S.E. (n = 5–8). Significant differences from control group: a) p < 0.05, b) p < 0.02, c) p < 0.001.
Fig. 4. Effects of EGF plus GX (A) and EGF plus GL (B) Ointment on Percentage Change in Body Weight in Rats with an Open Wound

Animals' body weights were measured daily before treatment with control ointment, ◆: EGF alone, ◆: GX alone (30 mg/g), ◆: EGF + GX (30 mg/g), ▲: GL alone (5.0 mg/g), ▲: and EGF + GL (5.0 mg/g). Results are expressed as the mean ± S.E. (n = 6—11). Significant differences from control group: a) p < 0.01, b) p < 0.001.

Fig. 5. Effects of EGF plus GX or EGF plus GL Ointment on Percentage Change in Body Weight (A) and on Plasma Epinephrine (B) 1 d after Preparation of Open Wound.

The dotted line represents the value for the normal animal. Open column, control; vertical column, EGF + GX (30 mg/g); dotted column, EGF + GL (5.0 mg/g). Results are expressed as the mean ± S.E. (n = 5—8). Significantly different from control group: a) p < 0.01.

was consistent with that in the burn model. These findings suggest that the amelioration of the hormone imbalance that was manifest in increases in epinephrine and cortisol levels may have led to the improvement in body weight. In a previous paper, we reported that wound repair was more rapid with EGF ointment containing 3.0 mg/g NM than with EGF ointment containing 0.3 mg/g NM. The ameliorative effects on body weight and plasma epinephrine, however, were observed with a lower content of NM (0.3 mg/g) than that which ameliorated the effects of topical injuries.

Figure 4 shows changes in body weight following treatment with EGF plus GX (EGF + GX) and EGF plus GL (EGF + GL) ointment. In a previous paper, we reported that EGF ointment containing GX or GL, as EGF stabilizers, had an accelerative effect on wound healing. In this study, we found a significant improvement in body weight also in rats treated with EGF + GX (Fig. 4A) or EGF + GL (Fig. 4B) ointment, whereas no improvement in body weight was observed following treatment with the other ointments (control, EGF alone, GX alone, and GL alone). There was no improvement in body weight with EGF ointment containing less than 30 mg/g of GX or 5 mg/g of GL (data not shown).

Next, we studied the relationship between body weight changes and plasma epinephrine in the open wound model during the treatment with EGF + GX or EGF + GL ointment. The ameliorative effects of EGF ointment containing GX or GL are also shown in terms of those changes in body weight in Fig. 5A, and the ameliorative effects on plasma epinephrine that were also observed following treatment with EGF + GL ointment (but not following treatment with EGF + GX) are shown in Fig. 5B.

Protease inhibitors such as NM or GX have been used clinically for the treatment of shock. However, in this study, treatment with the protease inhibitor, NM or GX, or GL alone did not have any effects on plasma hormones or on body weight (data not shown). Thus, the effects of EGF ointment plus the protease inhibitor, NM or GX, or GL on body weight change and plasma hormones may be due to synergism between EGF and these compounds.

In conclusion, improvements in the systemic condition were observed after the topical application of EGF ointment containing a protease inhibitor in burn and open wound rat models in this study. Several systems, such as cytokines, kallikrein-kinin, and prostaglandins may be involved in this repair process. We are now examining the influence of EGF ointment on topical biochemical factors during wound repair to elucidate the mechanism underlying the action of this agent on wound repair. The present study suggests that the systemic amelioration brought about by the activity of EGF ointment containing a protease inhibitor may be due to synergism between these two agents.

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References and Notes