Therapeutic Effects of Recombinant Human Epidermal Growth Factor (rhEGF) in a Murine Model of Concurrent Chemo- and Radiotherapy-Induced Oral Mucositis

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INTRODUCTION

Head and neck cancers have historically been treated with radiotherapy (RT). In recent years, a number of therapeutic regimens have been developed that combine RT with chemotherapy (chemoradiotherapy or CCRT) in the treatment of advanced stage of head and neck cancer patients.1) However, CCRT is associated with several complications including mucositis, dermatitis, stomatitis, etc. This study was conducted to evaluate the therapeutic effect of systematically administrated recombinant human epidermal growth factor (rhEGF) in CCRT-induced oral mucositis in a mouse model. Oral mucositis was induced in male BALB/c mice through combination treatment with cisplatin (11 mg/kg, i.p.) and irradiation (17 Gy) of the head and neck area. rhEGF (1.0 mg/kg/day for consecutive 3 days) was administered systemically, and the therapeutic effect was determined by histological evaluation of the oral mucosa. To elucidate optimal dose of rhEGF on CCRT-induced mucositis, various concentrations (0.04–3 mg/kg) of rhEGF were injected for 3 days. Systemic rhEGF administration accelerated the recovery of body weight. Histologically, rhEGF-treated mice showed significantly increased epithelial cell layer thickness, basal cell number, and expression of Ki-67 compared to control mice. Most effective dose was 1 mg/kg among other doses tested. Systemic administration of 1 mg/kg of rhEGF reduces the severity of oral mucositis induced by CCRT in a mouse model, suggesting that rhEGF can be used for treating CCRT-induced mucositis during the cancer treatment.

Several growth factors mediate mucosal wound healing.4–8) Keratinocyte growth factor (KGF; commercial name, Palifermin) has been shown to repair mucosal damage induced by RT in both animal models9) and in hematologic cancer patients who received intensive chemotherapy and radiotherapy in a prospective randomized phase III study.8) Transforming growth factor-β3 (TGF-β3) has similarly been found to reduce radiation-induced cell damage both in animal4) and in human studies.7) Granulocyte macrophage colony-stimulating factor (GM-CSF) enhances the proliferation of hematopoietic stem cells as well as keratinocytes10) and shows the therapeutic effect on the treatment of severe and anorexia. Mucositis affecting the oral cavity and pharynx is the most common dose-limiting factor in this treatment.2) In addition, this complication may significantly increase medical costs and the duration of hospital stay,2) leading to growing concern regarding the toxicity of combined CRT in head and neck cancer treatment.3) Furthermore, treatments typically employed for CCRT-induced mucositis only palliate symptoms without addressing the underlying cause, and therefore more effective treatment methods are needed.

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mucositis in patients receiving bone marrow transplant. However, it was not effective in trials seeking to reduce sequelae of radiation-induced mucositis.

Growth factors are thought to stimulate wound healing, in part, by stimulating cellular proliferation. Epidermal growth factor (EGF), which is produced by platelets, macrophages, and monocytes, interacts with its receptor on epidermal cells and fibroblasts. During wound healing, EGF acts by stimulating epithelial cell growth across the wound, but it can also act on fibroblasts and smooth muscle cells. Our group previously reported that topical or systemic application of EGF accelerates the wound healing process after RT along with the accelerating proliferation of basal cell layers. It was reported that KGF stimulated the cell growth in epithelium-derived cancer cell lines. In contrast, available evidences suggest that exogenous EGF does not carry the same risk. In this study we tested whether the systemic administration of exogenous rhEGF to mice can ameliorate mucosal damage in oral cavity after CCRT and searched the optimal dose of rhEGF for treatment of CCRT-induced mucositis. To test the therapeutic effect of EGF, we monitored body weight and examined histological changes in the tongue and buccal mucosa after combined cisplatin and RT in mice with and without EGF treatment.

MATERIALS AND METHODS

Recombinant human epidermal growth factor (rhEGF)

rhEGF was provided from the Daewoong® Pharmaceutical Company (Seoul, Republic of Korea) and was dissolved in PBS (pH6.5) shortly before use.

Mice and housing

Male BALB/c stain mice (6 weeks, average weight 22–25 g) were purchased from Central Lab Animal Inc. (Seoul, Republic of Korea) and maintained under pathogen-free conditions with free access to standard chow and water. All the animal experiments were approved by Animal Institutional Review Board of Asan Life Science Research Center in Seoul, Republic of Korea.

Establishment of concurrent chemo- and radiotherapy (CCRT)-induced mucositis mice model

Oral mucositis was induced by the combination of cisplatin and external RT on the head and neck area. Mice were injected with a single dose of intraperitoneal (i.p.) cisplatin (11 mg/kg; Boryung® Pharmaceutical Co., Seoul, Republic of Korea) 4 hours before RT to enhance the effects of radiation.

For immobilization during the irradiation, mice were anesthetized by i.p. injection with a mixture of ketamine (80 mg/kg) and xylazine (16 mg/kg). Then mice were irradiated once in the head and neck with 17 Gy, using a 6-MV therapeutic linear accelerator (CLINAC EX, Varian CP, Palo Alto, CA, USA) at a dose rate of 2 Gy/min. The distance from a source center to oral cavity was 100 cm and 1.5 cm bolus was used to build up the radiation dose on the oral mucosa. Medical physicists in the Department of Radiation Oncology regularly checked radiation output with an ionizing chamber.

Therapeutic effect of rhEGF on CCRT-induced mucositis

Mice were divided into two groups, CCRT-control and CCRT-rhEGF treated (n = 40 in each group). rhEGF-treated group received rhEGF (1 mg/kg/day for consecutive 3 days) subcutaneously after CCRT and control group received the vehicle only for the 3 days following CCRT. Body weight tracked daily until the termination to evaluate the therapeutic effect of rhEGF. The other set of animal study was conducted to evaluate the histological changes after rhEGF treatment on the oral mucosa and tongue. Seven mice in each group were sacrificed on day 7 after CCRT and the dissected tissues were fixed in 10% neutral buffered formalin and embedded in paraffin block. H&E staining was carried out using conventional methods, and the epithelial thickness was randomly measured at 20 sites on the tissue slides. Ki-67 staining kit (Dako Cytomation, Denmark) was used to evaluate the basal cell proliferation following the manufacture’s protocol.

Fig. 1. Effect of rhEGF on body weight after CCRT. Mice were injected with a single dose of intraperitoneal cisplatin (11 mg/kg) 4 hr before RT (17 Gy) to induce oral mucositis. CCRT mice were treated with either vehicle or 1 mg/kg rhEGF in the post-CCRT period for consecutive 3 days. Data showed mean ± SE (n = 40 in the each group). Asterisks indicate significant differences compared to vehicle treated- control group (* p < 0.05, ** p < 0.01 respectively).
**Determination of effective dose of rhEGF on CCRT-induced mucosal damage**

Mice were divided into five groups (n = 12 in each group) and oral mucositis was induced by the combination with cisplatin and radiation as described above. Various concentrations of rhEGF (0.04, 0.2, 1, and 3 mg/kg/day) were injected subcutaneously for 3 consecutive days after CCRT. Body weight was measured during 17 days. For the histological evaluation, six mice per each group were sacrificed on day 7 after CCRT and oral mucosa was fixed with same protocol described previously. H&E staining and Ki-67 staining were examined.

**Statistical analysis**

All values were expressed as means ± standard error (S.E.) or standard deviation (S.D.). Student’s t-test was used

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**Fig. 2.** Histological changes in BALB/c mice after CCRT with rhEGF treatment. (A) H&E staining images of mouse tongue on day 7 after CCRT. The cellularity of both the entire epithelium and basal layer was significantly reduced in the control mice (a) compared to that of rhEGF-treated mice (b). Ki-67 positive cells in the basal cell layer was relatively rare in control group (c), whereas rhEGF-treated mice showed more frequent Ki-67 reactivity (d). (B) Mucosal epithelial thickness in vehicle- and rhEGF treated oral mucosal tissue. Mucosal epithelial thickness was randomly measured at 20 sites in tissue slides. Data represent mean ± SD (n = 7, p = 0.0027). (C) Ki-67 positive cell numbers in tongue epithelium. Ki-67 positive cells were counted at three random sites (200 × magnification). Data represent mean ± SD (n = 7, p = 0.00017).
RESULTS

**Body weight changes after chemoradiotherapy**

A loss of body weight was observed for 6 days after CCRT and begun to recover on day 7 (Fig. 1). This losing weight was observed in both the control and rhEGF treatment groups, although it was significantly less in the rhEGF-treated group especially on day 4 and 6 (p < 0.05). When the body weight started to recover on day 7, the difference between control and rhEGF treated group until the end of experiment was much greater than in early time point (p < 0.05 or p < 0.01). Weight loss can be induced by results of decreased dietary intake and poor absorption due to the mucosal damages from chemoradiotherapy. Thus body weight changes can be used as an evaluation item even though it was considered as an indirect method. During the experimental period, 4 mice in the control group (n = 40) died on early days after CCRT. In contrast, all mice survived in the EGF treated group until the end of observation period for 14 days. Data suggested that rhEGF may have a reparative effect on CCRT-induced oral mucositis with significantly increased body weight compared to control group.

**Histological evaluations**

To evaluate the therapeutic effect of rhEGF on CCRT-induced oral mucosal damage, the morphological changes examined in tissues on day 7. H&E staining revealed that severe atrophy was developed in the tongue and buccal mucosa in the control group (Fig. 2A(a)). Moreover, decreased cellularity of the mucosal layer and destruction of the normal epithelial layer were evident in those mice samples. In contrast, rhEGF-treated mice had a relatively intact epithelium with preservation of glandular structure compared to irradiated control mice (Fig. 2A(b)). The cellularity of the epithelial and basal cell layer were preserved normal features compared to CCRT control mice. We also measured epithelium thickness of oral mucosa, representing the

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**Fig. 3.** Dose effect of rhEGF on body weight after CCRT. CCRT mice were treated with either vehicle or different concentration of rhEGF (0.04, 0.2, 1, 3 mg/kg/day) in the post-CCRT period for consecutive 3 days. Data showed mean ± SE (n = 12 in each group). Asterisks indicate significant differences compared to vehicle treated-control group (*p < 0.05, **p < 0.01 respectively).

**Fig. 4.** Histological changes in BALB/c mice after CCRT depending on the concentration of rhEGF treatment. (A) Mucosal epithelial thickness in vehicle- and various concentration of rhEGF treated- oral mucosal tissue. Mucosal epithelial thickness was randomly measured at 20 sites in tissue slides. Data represent mean ± SD (n = 6). Asterisks indicate significant differences compared to vehicle treated-control group (*p < 0.05, **p < 0.01, ***p < 0.001 respectively). (B) Ki-67 positive cell numbers in mucosal epithelium. Ki-67 positive cells were counted at three random sites (200 × magnification). Data represent mean ± SD (n = 6). Asterisks indicate significant differences compared to vehicle treated-control group (**p < 0.01).
impairment of cellular structures (Fig. 2B). Decreased mucosal epithelial thickness in the CCRT-control mice was significantly restored in rhEGF-treated mice. That result indicated that rhEGF accelerates cell proliferation even in the drastic remedy such as the combination of cisplatin and irradiation. The Ki-67 labeling index in the basal cell layer was also significantly increased in rhEGF-treated mice as shown in Fig. 2A(d) and in Fig. 2C. Overall, the treatment of rhEGF on oral mucositis induced by CCRT can reduce mucosal damages by the preservation or reconstruction of normal epithelium. Thus the application of rhEGF may helpful to shorten the duration of treatment after cancer therapy as well as relieve patient’s pain.

**Optimal dose of rhEGF on CCRT-induced mucosal damage**

To define the optimal dose of rhEGF on CCRT-induced mucositis, various concentrations of rhEGF were tested after the CCRT. As shown in Fig. 3, the treatment of 1 mg/kg/day rhEGF for 3 consecutive days showed dramatically increased body weight compared to the control or other concentrations tested (p < 0.01).

Histological evaluation supported that epithelium thickness was significantly increased in 1 mg/kg (p < 0.001) and 3 mg/kg rhEGF-treated group (p < 0.05) (Fig. 4A). Similarly, Ki-67 positive cell numbers which indicate the accelerated proliferation of basal cells were significantly increased in both 1 mg/kg and 3 mg/kg group (p < 0.01) (Fig. 4B). Data suggest that the application of proper dosage of rhEGF has much better advantage than of lower or higher dose to palliate the symptoms induced by CCRT.

**DISCUSSION**

Although RT combined with chemotherapy have improved outcomes in head and neck cancer patients, significant sequelae including mucosal damages occur. Oral mucositis is a major complication apparent clinically with symptoms such as oral pain, dysphagia, loss of taste, nausea, and decreased appetite. Management of mucositis may require hospitalization, feeding tube placement, and intensive supportive care. Even though there are several therapeutic methods to treat oral mucositis, their efficacy is limited. Clinicians have used cryotherapy or low level laser therapy, as well as various pharmacologic agents including benzydamine, chlorhexidine, amifostine, pentoxifyllin, glutamine or hematologic growth factors such as granulocyte colony stimulating factor (G-CSF) or GM-CSF. However, outcomes have been generally unsatisfactory. Other growth factor, KGF has shown promising results in oral mucositis in various conditions, however it has been approved only in hematologic malignancies due to the possibility of tumour cell proliferation. Thus it is still necessary to investigate the novel agent with both efficacy and safety for wound repair during the cancer treatment.

Therapeutic effect of rhEGF in a radiation-induced oral mucositis has been previously reported in rat model. In that study, rhEGF was topically sprayed to the oral cavity of rats. However topical application carries the limitation of poor delivery to deeper body sites such as pharynx, larynx, and upper esophagus frequently developing mucositis after RT on head and neck cancer. Thus rhEGF was systemically applied by subcutaneous injection to overcome the topical delivery limit in this study. In addition to the alteration of delivery system, we extended our experimental design to reflect more aggressive treatment regimens such as concurrent chemoradiotherapy (CCRT) according to the clinical applications. Cisplatin, a chemotherapeutic agent often employed in the treatment of head and neck cancers, enhances radiation-induced mucosal damages. Thus cisplatin and radiation were administrated on the same day which is comparable to clinical CCRT regimen in this study.

We first measured the changes of body weight which indicate the loss of appetite, pain, inflammation, impaired absorption, and dysphagia. As we expected, body weight was decreased dramatically during the several days after CCRT and then started to recover on day 7. Mucosal damages detected as shown in the histological examination on day 7 along with weight loss. Significant increase in both mucosal epithelium thickness and Ki-67 labelling index were observed in rhEGF-treated mice, as well as increased body weight compared to vehicle-treated mice. It is uncertain whether rhEGF has a protective effect or stimulates cell proliferation after some cells undergo a necrotic and/or apoptotic cell death. However histological data clearly show that a systemically applied rhEGF stimulates epithelial cell proliferation, especially in basal cell layer as detected by Ki-67 staining. Thus we assumed that the therapeutic effect of rhEGF is in part contributed from its cell proliferating activity. It raises concern about tumour cell proliferation when growth factors used to palliate the complications induced by chemo- or radiotherapy in cancer patients. Recently it has been reported that exogenous EGF didn’t stimulate cancer cell proliferation as well as the tumour size in tumour-bearing xenograft mice. Interestingly, EGF treatment resulted in significant decreases in tumor burdens compared to PBS-treated control group in most cases. Moreover EGF did not block the anti-tumoural effect of radiotherapy or cisplatin in A431 xenograft model (unpublished data). Although further investigations are necessary, treatment of rhEGF on the mucosal damages induced by chemo- and radiotherapy may have a beneficial effect on the quality of patients’ life.

Dose effect of rhEGF on CCRT-induced mucositis was tested using the same CCRT-animal model. In the present study, we measured both epithelium thickness and Ki-67 positive cell numbers to quantitate the therapeutic effect in various concentrations of rhEGF, as well as body weight.
Low concentrations of rhEGF have less or no benefit for palliating mucosal damages. The 3 mg/kg of rhEGF didn’t show any increase in body weight, nevertheless, the extent of epithelium recovery and cell proliferation was significantly stimulated. However the most effective dose is 1 mg/kg on CCRT-induced oral mucositis. It is the same effective dose for small intestinal mucosal damages induced by radiation.17)

In conclusion, systemic administration of rhEGF is promising treatment of oral mucositis induced by the combination of cisplatin and radiation and further studies are necessary to define optimal treatment schedule and mechanisms.

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