Recombinant Human Epidermal Growth Factor Accelerates the Proliferation of Irradiated Human Fibroblasts and Keratinocytes in vitro and in vivo

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Recombinant human epidermal growth factor (rhEGF)/Irradiation/Fibroblast/Keratinocyte/BALB/c mice
Irradiation causes the impaired proliferation of cells lining mucosal membranes. Epidermal growth factor (EGF) facilitates proliferation of various skin cells; however, the wound healing effects of EGF on radiation-damaged cells is less well known. To evaluate the effects of recombinant human EGF (rhEGF) on the proliferation of cells following irradiation, we tested two types of fibroblast cell lines and one keratinocyte cell line. The viable cell numbers were significantly increased by rhEGF treatment for 24 h immediately after 8 Gy of irradiation. The most effective dose of rhEGF was 10 nM in all cell lines used in this study. The percentage of BrdU-labeled cells was also significantly increased by rhEGF treatment. To evaluate the effects of rhEGF on radiation-induced oral mucosal damage in BALB/c mice, we systematically injected 1 mg/kg/day EGF for three days after 17 Gy of irradiation. Administered rhEGF ameliorated radiation-induced mucosal damage in vivo. rhEGF significantly increased the epithelial cell layer thickness and the proliferation of basal layer cells as detected by Ki-67 staining. Our results suggest that rhEGF can be a therapeutic treatment for radiation-induced wounds by stimulating the proliferation of fibroblasts and keratinocytes following irradiation.

INTRODUCTION
Radiotherapy is a powerful treatment for cancer, especially of the head and neck. However, side effects such as skin damage, oral dryness (xerostomia), and radiation-induced dermatitis or mucositis frequently occur and become dose-limiting factors.1–4) Tissues with rapid turnover rates are easily damaged by irradiation or chemotherapy agents because dividing cells are sensitive to genotoxic agents.5) Normal tissue responses after radiation are commonly divided into two categories, early and late, according to the type of damage occurring in these periods.6) The early response results from the death of large numbers of cells and occurs within several days of irradiation. Marked impairments in proliferation and migration of irradiated fibroblasts lead to serial tissue damage, including impairment of the epithelial barrier, denudation, and ulceration.7,8) Mucosal tissue is representative of tissues with a pronounced early response.9) The turnover time of oral mucosal cells is five days in mice and humans, making these cells susceptible to treatment-induced injury.10–14)

The fibroblast, which plays a key role in healing, becomes functionally abnormal after radiation. In normal skin, fibroblasts are stimulated to lay down extracellular matrix, including collagen, by growth factors such as platelet-derived growth factor, EGF, fibroblast growth factor, and transforming growth factor β.15) Among these, human EGF facilitates proliferation of various skin cells; however, the mechanism of EGF on radiation-damaged cells is less well known. In the present study, we therefore evaluated the effects of recombinant human EGF (rhEGF) on irradiated fibroblasts and keratinocytes.

EGF was first discovered by Cohen in 1962 and purified from the mouse submaxillary gland.16,17) Its molecular weight is 6,045 Da, and it is a single-chain polypeptide of 53 amino acids.18,19) The strong mitogenic effect of EGF is
rhEGF

rhEGF was a kind gift from the Daewoong® Pharmaceutical Company (Seoul, Korea) and was dissolved in PBS shortly before use. Cells were treated with rhEGF-containing medium, and this culture medium was replaced with fresh rhEGF-free medium 24 h later.

Cell lines and culture conditions

We used two human fibroblast cell lines and one keratinocyte cell line. One fibroblast cell line was established by primary culture from human skin, and the other (WI38, purchased from the ATCC) originated from human lung tissue. The HaCaT line (keratinocyte) was purchased from the ATCC. Cell lines were cultured in Dulbecco’s Modified Eagle’s Medium or Minimum Essential Media with Earle’s salts supplemented with 10% (v/v) fetal bovine serum at 37°C in a humidified atmosphere under 95% air/5% CO2. The culture medium was changed twice per week. Cell morphology was observed using light microscopy, and all cells were used after less than 10 passages, and during exponential growth.

Irradiation

Cells were prepared in 6-well or 24-well plates and irradiated with single doses of 5 Gy or 8 Gy. Irradiation was performed with a 6-MV photon beam generated by a linear accelerator (CLINAC 600C; Varian, Palo Alto, CA) at a dose rate of 2 Gy/min.

Trypan blue assay

Live cells were measured using trypan blue assay. Suspended cells were seeded into 6-well plates. Cells were irradiated when they became attached to the plates. rhEGF at concentrations of 1~1000 nM was added, and 24 h later, the medium was changed to rhEGF-free medium. Numbers of living cells were counted from days 1–6 as assessed using trypan blue assay.

BrdU assay

To confirm the effects of EGF on proliferation of normal fibroblasts and keratinocytes, we detected proliferating cells using the 5-Bromo-2’-deoxy-uridine (BrdU) incorporation assay. We seeded 2 × 10^4 cell/ml WI38 cells and 1 × 10^4 cell/ml HaCaT cells on a cover glass in 24-well plates. In the case of irradiated cells, initial cell numbers were increased to 5 × 10^4 cells for WI38 and 2 × 10^4 cells for HaCaT, and 5 Gy of radiation was applied to the cells. After radiation, 10 or 100 nM EGF was added to the well for 24 h and then changed to EGF-free complete media for 48 h. As described in manufacturer’s manual (Roche Applied Science, USA), BrdU labeling media were added to the cells for 50 min in a 37°C incubator, and the cells were fixed with acidic ethanol for 30 min at –20°C. After washing the cells with PBS three times, we incubated cells with anti-BrdU antibody in incubation buffer for 30 min at 37°C, followed by incubation with anti-mouse IgG-FITC antibody for 30 min at 37°C. DAPI was used for counterstaining. Images were captured with a fluorescence microscope, and the percentage of BrdU-labeled cells was calculated using the ImageJ program from NIH.

Animal study

Male BALB/c strain mice (eight weeks, average weight 22–25 g) were obtained from Central Lab Animals Inc. (Seoul, South Korea) and divided into two groups, control and rhEGF treated (n = 7 in each group). Mice were intraperitoneally injected with a mixture of ketamine (80 mg/kg) and xylazine (16 mg/kg) to anesthetize them during irradiation. Mice were irradiated once on the head and neck area with 17 Gy of radiation using a 6-MV therapeutic linear accelerator (CLINAC EX, Varian CP., Palo Alto, CA, USA). Dose rate was 2 Gy/min, and a 1.0-cm bolus was used to build up the radiation dose. In the rhEGF group, mice received 1 mg/kg/day rhEGF subcutaneously for three days after radiation. The control group received the vehicle in the same way. After seven days of irradiation, mice were euthanized, and the dissected tongue was submerged in 10% neutral buffered formalin and embedded in paraffin. H&E staining was carried out using conventional methods, and the epithelial thickness was randomly measured on tissue slides. To measure cell proliferation we used a Ki-67 staining kit (Dako Cytomation, Denmark) following the manufacturer’s protocol. This animal study was approved by the Animal Institutional Review Board of Asan Life Science Research Center in Seoul, South Korea.

Statistical analysis

All values are expressed as the average ± standard devia-
tion. Student’s t-test was used for comparisons between control and treatment groups.

RESULTS

Increased proliferation of fibroblast by rhEGF in normal or irradiated conditions

In this study, two normal fibroblast cell lines and one normal keratinocyte cell line were used to measure the proliferative effects of rhEGF. Fibroblast cells were treated with various rhEGF concentrations (0 nM, 1 nM, 10 nM, 100 nM, 1000 nM), and the culture medium was changed to rhEGF-free medium 24 h later. Surviving cells were measured three days later using trypan blue assay. Optimal rhEGF concentrations for primary cultured skin fibroblasts and WI38 cells were 10 nM. With these rhEGF levels, the numbers of surviving primary cultured skin fibroblasts increased 1.7-fold compared to rhEGF-free control, whereas a 1.2-fold increase was seen with WI38 cells. If the rhEGF concentrations were increased to above the optimum, cell survival decreased (Fig. 1). In addition, we evaluated the proliferative effect of rhEGF on irradiated (8 Gy) fibroblast cell lines. Fibroblasts were seeded into 6-well plates and irradiated with a single dose of 8 Gy. Cells were then divided into five subgroups and incubated with various concentrations of rhEGF (0 nM, 1.0 nM, 10 nM, 100 nM, 1000 nM). Surviving cells were measured three days after irradiation using trypan blue assay. The results showed significantly increased numbers of fibroblasts after rhEGF treatment compared to the radiation (RT) control without rhEGF. In primary cultured skin fibroblasts, maximal stimulation was seen with rhEGF at 10 nM (Fig. 2A). A similar tendency was seen in non-irradiated cells (see above), where 10 nM rhEGF also showed the greatest increase. The stimulatory effect of rhEGF on irradiated WI38 cells was similar to that seen with primary cultured fibroblasts. Whereas the proliferation ratio was 1.2 for non-irradiated WI38, it was 1.7 for irradiated WI38 cells treated with rhEGF (Fig. 2B). The differences in cell survival after rhEGF treatment were more pronounced with irradiated cells than with non-irradiated cultures.

Effect of treatment schedule of rhEGF in irradiated fibroblasts

Irradiated fibroblasts were treated with rhEGF, and cell survival increased. We suggest two possible cell -proliferat-
ing mechanisms afforded by rhEGF in irradiated cells. One is a radioprotective effect, and the other is simply proliferation of surviving cells. The experiment was designed to explore the timing of rhEGF treatment. Primary cultured fibroblasts were irradiated with 8 Gy, and then the cells were divided into four groups with varied timing of rhEGF treatments. Treatment with rhEGF (10 nM) was not given (control cells), was administered immediately, or was applied after 2 or 4 h. Although rhEGF-treated cells increased more in number than did control cells, cell survival levels were similar in each of the rhEGF-treated groups (Fig. 3).

Increased proliferation of keratinocyte by rhEGF in normal or irradiated conditions

Keratinocyte cells were treated with various rhEGF concentrations (0 nM, 1 nM, 10 nM, 50 nM, 100 nM, 1000 nM), and the culture medium was changed to fresh rhEGF-free medium 24 h later. Surviving cells were measured using trypan blue assay (Fig. 4A). Keratinocytes were stimulated by rhEGF in a concentration range of 10–100 nM. When keratinocytes were cultured with rhEGF at concentrations outside this range, proliferation was not observed. After irradiation, however, keratinocyte proliferation was accelerated by all rhEGF concentrations tested. Keratinocytes were more radiosensitive than were normal fibroblasts. After 8 Gy of irradiation, most cells were dead four days later. On day 3 after irradiation, cells treated with rhEGF (10–100 nM) survived much better than did control irradiated cells (Fig. 4B). The survival difference was > 50-fold.

Measurement of proliferating cells using BrdU assay

Effects of rhEGF on cell proliferation of normal fibro-

Fig. 3. Treatment timing of rhEGF on irradiated fibroblasts. Irradiated fibroblasts (8 Gy) were treated immediately, 2 h later, and 4 h later, with rhEGF (10 nM) for 24 h, and the medium was then changed to rhEGF-free medium. Survival of fibroblasts was assessed using trypan blue assay. The number of surviving fibroblasts did not differ with the timing of rhEGF treatment.

Fig. 4. Stimulating effect of rhEGF on proliferation of normal and irradiated keratinocytes. (A) Effect of various concentrations of rhEGF on proliferation of normal keratinocytes. HaCaT cells were cultured with various concentrations (0 nM, 1 nM, 10 nM, 50 nM, 100 nM, 1000 nM) of rhEGF for 24 h, and the media were then changed to rhEGF-free media. Surviving cells were counted daily using trypan blue assay. Concentrations ranging between 10 and 100 nM rhEGF accelerated the proliferation of keratinocytes. (B) Stimulating effect of rhEGF on proliferation of irradiated keratinocytes. A total of 8 Gy of radiation was applied to the cells using a 6-MV photon beam generated by a linear accelerator. Irradiated fibroblasts were immediately treated with rhEGF (1.0 nM, 10 nM, 50 nM, 100 nM, 1000 nM) for 24 h, and then the rhEGF-containing media were replaced with rhEGF-free media. Surviving fibroblasts were counted on day 3 using trypan blue assay. The bar indicates the average ± SD. Statistical analysis was performed with Student’s t test. ***p < 0.001 was considered as statistically significant compared to radiation alone.
blasts and keratinocytes were evaluated using BrdU incorporation assay (Fig. 5). WI38 cells with or without irradiation (5 Gy) were treated with 10 nM or 100 nM of rhEGF for 24 h and then were changed to rhEGF-free medium for 48 h. In the case of the WI38 cells, 30.43 ± 2.25% of the BrdU positive cells were detected in the no radiation treatment control (Fig. 5A). The percentage of positive cells was significantly increased by 38.50 ± 1.53% and 41.63 ± 3.51% in cells with 10 or 100 nM EGF, respectively. As we expected, BrdU positive cells decreased by 14.70 ± 1.80% after 5 Gy of radiation. However, 10 or 100 nM EGF significantly increased the percentage of proliferating cells by 22.37 ± 2.02% and 26.43 ± 2.80%, respectively. BrdU positive cells much decreased by 10.21 ± 1.91% after 8 Gy of radiation and 10nM and 100 nM of EGF treatment also increased the positive cells by 21.21 ± 4.68% and 17.29 ± 6.29% (Data not shown). EGF had a much greater effect on HaCaT cells in both the no RT group and the RT group (Fig. 5B). The 37.91 ± 2.70% of BrdU positive cells in the control group were increased by 62.28% and 65.03% after 10 and 100 nM of EGF treatment, respectively. This pattern was preserved even when cells were irradiated at a total dose of 5 Gy. The 30.96% of BrdU positive cells in the RT only group were increased more than 2-fold by 63.36% and 68.25% in EGF -treated cells. These results are consistent with previous data showing that EGF helps to increase the proliferation of fibroblasts and keratinocyte with or without RT and that keratinocytes are more sensitive to EGF treatment than are WI38 cells.

**Fig. 5.** BrdU incorporation assay on EGF-treated cells with or without radiation. WI38 and HaCaT cells seeded on cover slides were irradiated or not irradiated with a single dose of 5 Gy. A total of 10 nM or 100 nM EGF was added to the cells for 24 h, and then the media were replaced with rhEGF-free media for 48 h. The BrdU assay was conducted as described in Methods. Graph showing the percentage of BrdU positive cells in (A) WI38 and (B) HaCaT. The bar indicates the average ± SD. Statistical analysis was performed with Student’s t test. *p < 0.05, **p < 0.01, and ***p < 0.001 were considered as statistically significant compared to the control or radiation alone respectively.

**Fig. 6.** Protective effect of EGF on radiation-induced oral mucosal damage in BALB/c mice. (A) Mucosal thickness in vehicle- and rhEGF (1 mg/kg × 3 times)—treated mice at day 7 after 17 Gy of radiation. Mucosal epithelial thickness was randomly measured at 20 sites on the tissue slides. The bar indicates the average ± SD (n = 7 in each group, p = 0.0000). (B) Ki-67 staining of mucosal epithelium under the same conditions as described above. Ki-67 positive cells were counted at five random sites (200× magnification). The bar indicates the average ± SD (p = 0.00024).

**Therapeutic effect of rhEGF on irradiated mice**

The therapeutic effect of rhEGF on irradiated BALB/c mice was determined in tongue mucosal epithelium on day 7 after 17 Gy of radiation. A total of 3 mg/kg rhEGF was administered to the rhEGF -treated mice, and a histological examination was conducted. Epithelial thickness was significantly increased in the rhEGF -treated group compared to the vehicle -treated group (p < 0.000, Fig. 6A). Ki-67 positive cell numbers were significantly increased in the basal layers of rhEGF -treated mice compared to the vehicle -treated, irradiated mice (p < 0.001, Fig. 6B). This result clearly shows that rhEGF has a therapeutic effect on radiation-induced damage in an in vivo system.

**DISCUSSION**

In the treatment of cancer patients, the intensity of early mucosal damage is a major limiting factor of radiotherapy. The purpose of this study was to explore the wound healing effects of rhEGF on cells of tissues injured by radiation. Facilitating the proliferation of fibroblasts and keratinocytes is an important route of promoting wound healing. This study demonstrates that rhEGF stimulates proliferation of fibroblasts and keratinocytes under both normal and irradiated conditions.

Each cell type requires an optimal rhEGF dose for stimulation. Kamata et al. found that 10 nM rhEGF inhibited cancer cell proliferation but stimulated the growth of epidermal keratinocytes. Thus we suggest that the proliferation of cells treated with rhEGF depends on the rhEGF dose and on the type of cells examined. If the rhEGF dose is above the optimal dose, the number of cells will be decreased. Although the mechanism of this phenomenon is not clear, stimulation of an EGF receptor signal yields a biphasic
response. Either growth stimulation or growth inhibition may follow. According to Buss et al.\textsuperscript{33} if tyrosine-specific protein kinase activity and phosphorytoseine content is increased by EGF, cell growth will be inhibited. Another suggested mechanism is that an excessive tyrosine kinase signal may inhibit a cell growth signal that is normally quiescent.\textsuperscript{33,31} Also, the optimal stimulating dose of rhEGF is affected by the amount of fetal calf serum in the culture medium. A similar result was found in a study using keratinocyte growth factor.\textsuperscript{30} If growth factors are removed from the culture medium, the optimal stimulating dose of rhEGF will be increased. The optimal stimulating dose of rhEGF is thus dependent on the levels of growth factors in the culture medium.\textsuperscript{32} Although the exact mechanism is unknown, cells excessively stimulated by EGF ligands through the EGF receptor paradoxically show decreased proliferation. The optimal cell growth-stimulating dose of rhEGF varies by cell type, and the optimal growth-stimulating levels of rhEGF are much higher for normal cells than for cancer cells. Our idea was to exploit this difference.

Radiation is a well-known genotoxic agent, especially for rapidly proliferating cells. DNA is the principal target of radiation.\textsuperscript{60} In response to DNA damage, irradiated cells activate DNA damage-induced cell cycle checkpoints that help to prevent proliferation of potentially genetically unstable cells and support DNA repair.\textsuperscript{30} Irradiated cells that pass the checkpoints after DNA repair are thus able to maintain normal growth and division. The second experiment in this study was designed to determine if irradiated cells show the same rhEGF-induced proliferative effects as non-irradiated cells. We found that irradiated cells were also stimulated by rhEGF and that the optimal dose of rhEGF was the same as that for non-irradiated cells. Furthermore, the number of surviving irradiated WI38 cells after rhEGF treatment was greater than the increase in cell number seen after rhEGF treatment of non-irradiated cells.

Sublethal damage repair was complete within 2 h.\textsuperscript{30} Elkind et al.\textsuperscript{30} showed that increased cell survival is observed if a given radiation dose is split into two fractions separated by a time interval. When the time interval is less than 2 h, the damage of the first irradiation accumulates with that of the second irradiation, but if the time interval is longer, the cumulative effect disappears. This means that radiation-induced damage is almost completely repaired within 2 h. In this study, variations in the timing of rhEGF treatment after irradiation did not make any difference to cell survival. The rhEGF mechanism of action on irradiated fibroblasts thus remains unknown. rhEGF may show a radiation protection effect or simply mediate a proliferation effect on surviving fibroblasts. Regardless, however, it is clear from the present results that rhEGF facilitates the proliferation of surviving cells. This result could potentially be used as a basis for in vitro work and clinical trials. It is relevant to note that EGF enhances the cytotoxic effect of ionizing radiation on cancer cells.\textsuperscript{37}

The keratinocyte (HaCaT) cell line was more radiation sensitive than the fibroblasts. Although most irradiated (8 Gy) keratinocytes died, a dramatically higher number of rhEGF-treated keratinocytes survived. RhEGF may therefore have a wound healing effect in irradiated tissue.

The proportion of BrDU positive cells was increased by treatment with rhEGF, which means that DNA replication was accelerated by EGF in both cell lines. D-type cyclins act as growth factor sensors, and S-phase entry and duration are controlled by cyclin dependent kinases that are sequentially regulated by cyclins D, E, and A.\textsuperscript{30} EGF activates cyclin D1 expression by phosphatidylinositol 3-kinase in NIH 3T3 fibroblasts.\textsuperscript{30} Further work is needed, however, to identify exactly which molecules are affected by EGF. In this study, we couldn’t explain whether proliferating cells after EGF treatment are normal or aberrant cells. Chromosome aberrations can be observed in metaphase result from the radiation-induced chromosome damage.\textsuperscript{39} Cell cycle checkpoint tightly regulate to prevent G2 cells entering mitosis before DNA repair has been completed.\textsuperscript{40} It is evident that severely damage cells show arrest or delay in the cell cycle progression and further undergo necrosis or apoptosis.\textsuperscript{30} 8 Gy of irradiation used in this study is considered as high dose to normal cell lines. Thus we assumed that those proliferating cells after treatment of EGF are repairable normal cells, whereas most damaged cells after irradiation may undergo necrotic cell death.

Since wound healing is a complex process involving multiple cells, inflammatory mediators, cytokines, and growth factors, in vitro data cannot explain whole process of radiation-induced wound healing. Thus it is necessary to demonstrate the protective effect of EGF in an in vitro system which represents the complexity of wound healing process. For that reason, we tested the therapeutic effects of rhEGF on irradiated mice to prove our hypothesis. Previously we published several relevant papers that showed that rhEGF could ameliorate radiation-induced mucositis or intestinal mucosal damage in rats and mice.\textsuperscript{15,41} In the present study, we measured epithelium thickness and Ki-67 positive cell numbers to quantify the extent of damage repair or the stimulation of proliferating cells. These values are distinctly correlated to morphological changes after irradiation with or without rhEGF treatment. Even though further studies are necessary to clarify the exact mechanism of action of rhEGF, it is clear that systemically administrated rhEGF can help treat radiation-induced oral mucosal damage in an in vivo system by stimulating Ki-67. Albeit the limited cases, it has been reported the clinical usage of EGF on treatment of radiation induced-normal tissue damages. Recently our group has reported that EGF stimulates epithelialisation for chronic radiation ulcer of patient who had history of mastectomy and radiation therapy long times ago.\textsuperscript{42} In addition, topicaly applied rhEGF showed therapeutic effect on mucositis in.
head and neck cancer patients received radiotherapy in a double-blind placebo-controlled prospective phase 2 multi-institutional clinical trial.43) Those clinical trials support that rhEGF can be used for treatment of radiation-induced damage. However, further accumulating studies are needed for clinical use.

In summary, rhEGF facilitates the proliferation of normal and irradiated fibroblasts and keratinocytes. Each cell line has an optimal stimulating dose of rhEGF. The cell proliferation effect is decreased at doses of rhEGF less than, or more than, the optimal dose. Most important, systemically administered rhEGF can stimulate the proliferation of the mucosal cell layer in radiation-damaged mice. These results suggest that rhEGF has a therapeutic effect on normal cells damaged by radiation and thus can help to palliate symptoms for cancer patients during radiotherapy.

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REFERENCES

28. Carpenter, G. (1980) Epidermal growth factor is a major


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