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Recombinant Human Epidermal Growth Factor (rhEGF) Protects Radiation-Induced Intestine Injury in Murine System

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rhEGF/Radiation/Small intestine/Apoptosis.

This study was to investigate whether rhEGF protects radiation induced intestine injury without compromising antitumor effect of radiation in murine system. A radiation induced intestinal injury model was established in mice by whole body irradiation. Using this model, 4 groups were set; control, rhEGF (100 μg/kg intraperitoneally), radiation (10 Gy), and a combination (rhEGF and radiation). The level of apoptosis and proliferation were analyzed by TUNEL assay and proliferation cell nuclear antigen (PCNA) immunohistochemical staining, respectively, as well as observation of survival and body weight change. A tumor growth delay assay was performed using murine syngeneic tumors; one radioresistant tumor, HCa-I and one radiosensitive tumor, MCa-K. In the radiation induced intestinal injury model, the 10 Gy group had significantly more weight loss with less number of crypt cells and higher apoptosis than the 8 Gy group. Using 10 Gy model, radioprotective effect of rhEGF was tested. Addition of rhEGF improved not only the body weight loss but also survival following radiation. It also induced suppression of apoptosis as well as increase of PCNA expression and recovery of villi. rhEGF did not enhance the tumor growth after radiation exposure in the tested tumors. These findings suggest that combination of exogenous rhEGF and radiation can be a new anticancer strategy by protecting radiation-induced intestinal injury without alleviating antitumor effect of radiation.

INTRODUCTION

Gastrointestinal (GI) cancer is a major malignancy, particularly in Asia. Although radiotherapy is a potent anticancer modality, its applicability is limited due to radiation-induced toxicity in GI organs. Following ionizing radiation, a diverse spectrum of clinical syndromes appears from acute symptoms of diarrhea and malabsorption to the later appearing chronic illness.[1–3] The mechanisms underlying the effect of radiation on the small intestine have been reported; apoptosis and necrosis have been identified as two main forms of cell death after radiation. However, mucosal injury has been primarily attributed to apoptosis of epithelial cells, as evidenced by a mouse model of a GI syndrome following whole body irradiation.[4,5]

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A variety of approaches using growth factors have been assessed in preclinical models for their efficacy in alleviating radiation-induced mucosal injury. Keratinocyte growth factor (KGF) induces a variety of responses in the epithelia that involve stimulation of epithelial proliferation, modification of migration, and differentiation processes. KGF plays a key role in wound healing, with a substantial increase in dermal transcription activity. Topical application stimulates wound healing in animal models.[6,7]

Epidermal growth factor (EGF) is a polypeptide hormone that binds specifically to the epidermal growth factor receptor (EGFR) located ubiquitously in epithelial cells of the gastrointestinal tract and other organs. The ligand-receptor interaction between EGF and EGFR results in intestinal cell migration and proliferation to repair and restore mucosal continuity.[8,9] EGF is present in relevant concentrations in the saliva and other biological fluids; it is also in the milk of breast-feeding mothers, in which it can reach about 300 times the normal serum concentration.[10,11] EGF plays a critical role in wound repair and healing. EGF has also been reported to have mucoprotective potential.[12–15] It has been reported that the EGF receptor and EGF-producing cells increase around gastric ulcers in rats.[16] EGF administration has also been shown to regulate the healing of ulcers in rats

and humans.[17,18] In a radiation-induced oral mucosal injury model, EGF has been shown to enhance recovery.[19] These results suggest a potential that EGF might play an important role in the repair of small intestinal mucosal injury by radiation.

However, it is questionable whether protective entity of EGF might work on tumors, resulting in favor of tumor growth. It also needs to be tested whether antitumor effect of radiation might be suppressed by EGF. Therefore, it is critical to study on how EGF could influence either on tumor growth or on antitumor effect of radiation. The purpose of this study was to investigate whether rhEGF protects radiation induced intestine injury without compromising antitumor effect of radiation.

MATERIALS AND METHODS

Animals

This study was reviewed and approved by the committee that oversees the ethics of research involving the use and welfare of animals. The study involved 8-10-week-old male C3H/HeJ mice that were bred in a specific pathogen-free mouse colony at the Division of Laboratory Animal Medicine, College of Medicine, Yonsei University. Temperature (22°C) and humidity (55%) were controlled constantly. The water (RO water) and diet (PMI) were supplied *ad libitum*. The care and use of laboratory animals in these experiments were based on the Guidelines and Regulations for Use and Care of Animals in Yonsei University.

Radiation and rhEGF administration

To establish a model of radiation-induced intestinal injury, C3H/HeJ mice were given two different whole-body radiation doses (8 Gy or 10 Gy) as a single dose using a clinical linear accelerator (Varian Co. Milpitas, CA, USA).

Using this mouse model, the therapeutic effect of rhEGF on radiation-induced intestinal injury was tested. The mouse model was injected with rhEGF (100 μg/kg/day) i.p. on days 1 to 4 after 10 Gy irradiation. rhEGF (Daewoong Pharmeceutic Co., Seoul, Republic of Korea) was administered i.p. on radiation-treated mice. The mice were divided into four groups: control, radiation alone, rhEGF alone, and a combination of radiation plus rhEGF. Ten mice were allocated to each group. The radiation-alone group received a single dose of whole-body radiation. The combination group received a single dose of radiation plus rhEGF (100 μg /kg/day).

Analysis of body weight

Mice were weighed daily to measure changes in body weight over time. The mice were monitored closely for any signs of morbidity during the experiments.

Crypt survival

Intestinal damage was assessed using the jejunal microcolony assay of Withers and Elkind.[20] For the jejunal microcolony assay, the mice were sacrificed 1 to 7 days after irradiation, and 2.5 cm segments of jejunum were removed and fixed in neutral-buffered formalin. After paraffin embedding, 4 μm transverse sections were cut and stained with hematoxylin and eosin (H&E). The number of surviving crypts per transverse histological section was counted (using the criterion that at least 10 surviving cells must be present to define a surviving crypt). Crypts in five to eight circumferences per mouse were counted and average values were calculated.

Assessment of proliferating cell nuclear antigen (PCNA) expression and apoptosis index (A.I.)

Small intestine tissue was analyzed for morphological changes. Immunohistochemical staining was performed according to the method previously described using antibodies targeting proliferating cell nuclear antigen (PCNA) (PC 10; Dako A/S, Glostrup, Denmark).[21] Antibodies were used at the dilution recommended by the manufacturer. The PCNA count was scored on coded slides at 400X magnification. Ten fields of non-necrotic areas were then selected randomly across each jejunum section. In each field, the fraction of proliferating cells, PCNA index, was expressed as a percentage of 1000 nuclei. The number and position of labeled cells in the crypts of the small intestine were recorded. Only strongly labeled cells were counted. The proliferation count was calculated as 100 times the number of labeled cells per crypt.

Assessment of apoptosis was performed according to a previously described method.[21] Briefly, tumor samples were collected and apoptosis was assessed in tissue sections. The tumors were immediately excised and placed in neutral-buffered formalin. The tissues were embedded in paraffin blocks, and 4 μm sections were cut and stained with the Apoptag staining kit (Chemicone, Temecula, CA, USA). Apoptotic cells were scored on coded slides at 400X magnification using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method. TUNEL-positive cells were considered apoptotic only when associated with apoptotic morphology as previously described.[21] Ten fields of non-necrotic areas were selected randomly across each jejunum section. In each field, the fraction of apoptotic bodies was expressed as a percentage of 1000 nuclei.

Treatment and tumor growth delay analysis

A tumor growth delay assay was performed using murine syngeneic tumors. Two kinds of murine tumors were used; a radioresistant tumor, HCa-I hepatocarcinoma with tumor cure dose of 50% of animal (TCD 50) greater than 80 Gy, and a radiosensitive tumor, MCa-K mammary carcinoma with TCD 50 around 42.9 Gy).[22] Also, for tumor growth delay analysis, four experimental groups were formed with

10 mice per group: a control group, radiation alone group, rhEGF alone group, and radiation plus rhEGF group. rhEGF was obtained from Daewoong Pharmaceutic Co (Seoul, Republic of Korea). The mice in radiation alone group were irradiated when the tumor growth reached to a mean diameter of 7.5–8 mm. The tumor-bearing legs were treated with a single dose of 25 Gy using a linear accelerator (Varian Co., Milpitas, CA, USA). The rhEGF alone group was given 100 μg/kg rhEGF once daily intraperitoneally for 4 days when the tumors had grown to a mean diameter of 7.5–8 mm. For the combination group, radiation was given first and then daily 100 μg/kg rhEGF was administered intraperitoneally for 4 day consecutive days. Tumors were measured regularly for tumor growth delay after treatment. The effect of radiation on tumor growth was determined by measuring three orthogonal tumor diameters with calipers at 2-day intervals until the tumors grew to at least 12 mm in diameter.

The effect of treatment on tumor growth delay (AGD) was defined as the time in days for the tumors to reach 12 mm in the treated group minus the mean time to reach 12 mm in the untreated control group. The enhancement factor of tumor radioresponse was obtained by dividing the normalized tumor growth delay (NGD) by the AGD caused by radiation. The NGD was defined as the time in days for tumors to reach 12 mm in mice treated with the combination treatment minus the time in days for tumors to reach 12 mm in the group treated with rhEGF only. Animals were closely observed for any occurrence of toxicity until the last observation day.

Statistical analysis

All values were expressed as mean ± SE. Statistical analysis was performed by analysis of variance and the t-test. A *p*-value of less than 0.05 indicated statistical significance.

RESULTS

RADIATION-INDUCED SMALL INTESTINAL INJURY

Survival rate and change in body weight

All mice survived to day 10 and then started to die. Eventually all mice were dead within 20 days. The 15-day survival was 20% and 0%, in 8 Gy and 10 Gy group, respectively (Fig. 1). Also, the 8 Gy and 10 Gy groups showed a statistically significant difference in body weight after whole body irradiation ($p < 0.05$) (data not shown). The 8 Gy group gained weight until day 2, then began to show a sudden decrease at day 6. In contrast, the 10 Gy group showed considerable weight loss until day 7.

Number of crypts per circumference

In the 8 Gy and 10 Gy groups, crypt survival decreased on day 1. However, in the 8 Gy group, crypt survival increased continuously. On the other hand, crypt survival decreased until day 3 and then increased until day 7 in the 10 Gy group. As a result, the number of crypts per circumference was significantly lower in the 10 Gy group than in the 8 Gy group (Fig. 2a) ($p < 0.05$).

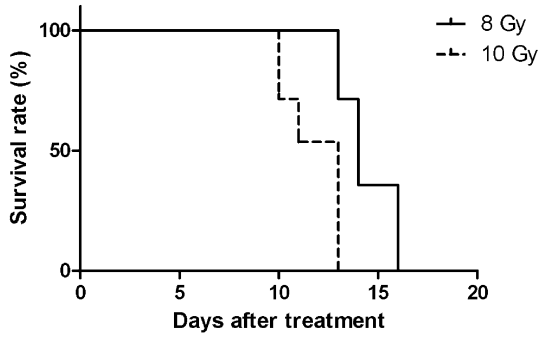

Fig. 1. Survival studies with different doses of 8 or 10 Gy whole body radiation in C3H/HeJ mice.

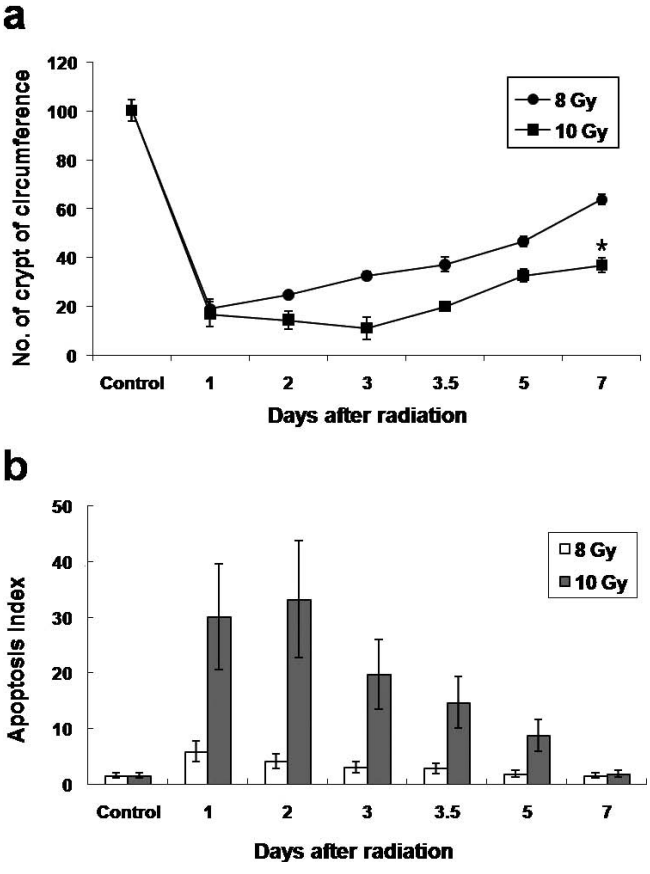

Fig. 2. Jejunal crypt count (a) in transverse tissue sections of the full jejunal circumference after 8 Gy (●) and 10 Gy (■) whole body radiation. Change in apoptosis level (b) in jejunal crypt is shown after 8 Gy (white bar) and 10 Gy (grey bar) whole body irradiation. The apoptosis index is the number of apoptosis body per 1,000 nuclei.

Apoptosis index (A.I.) in the irradiated jejunum crypt

The apoptosis index was determined in sections of the small intestine jejunum. The effect of the dose was detected in the segments of the intestine in mice (Fig. 2b). In the 8 Gy and 10 Gy groups, A.I. began to increase on day 1, but then decreased gradually until day 7. In addition, apoptosis dramatically increased in the 10 Gy group compared with the 8 Gy group. A greater than 6-fold more apoptosis was seen in the 10 Gy group compared with the 8 Gy group.

THERAPEUTIC EFFECT OF rhEGF ON RADIATION-INDUCED SMALL INTTESTINAL INJURY

Survival rate and Change in body weight

Mice survival after radiation or combination was observed from the day of treatment for 2 weeks. While all the mice given 10 Gy radiation were dead within 15 days, 15-day survival rate in the mice given 10 Gy and rhEGF combination treatment was 40% ($p < 0.05$) (Fig. 3a). In body weight analysis, the 10 Gy group showed weight decrease from day 1. In the 10 Gy plus rhEGF (combination) group, body weight decreased on day 1 and started to recover on day 3. The difference in body weight between the 10 Gy and combination groups was statistically significant ($p < 0.05$). In addition, the combination group showed weight gain compared with the 10 Gy group beginning on day 1, while the 10 Gy group mice showed a significant decrease in body weight until day 7 (Fig. 3b).

A.I. and PCNA ratio in the irradiated jejunum crypt

The levels of apoptosis and PCNA were measured on days 5 and 7. As shown in Fig. 4, addition of rhEGF significantly decreased the number of TUNEL-positive apoptotic cells following radiation. In PCNA-staining, addition of rhEGF significantly increased proliferative activity following radiation. On day 5, the A.I. and PCNA ratio decreased significantly more in the combination group than in the 10 Gy group ($p < 0.05$) (Fig. 4). This is because the A.I. decreased in combination group compared to the 10 Gy group. These data suggest that rhEGF can suppress apoptosis and accel-

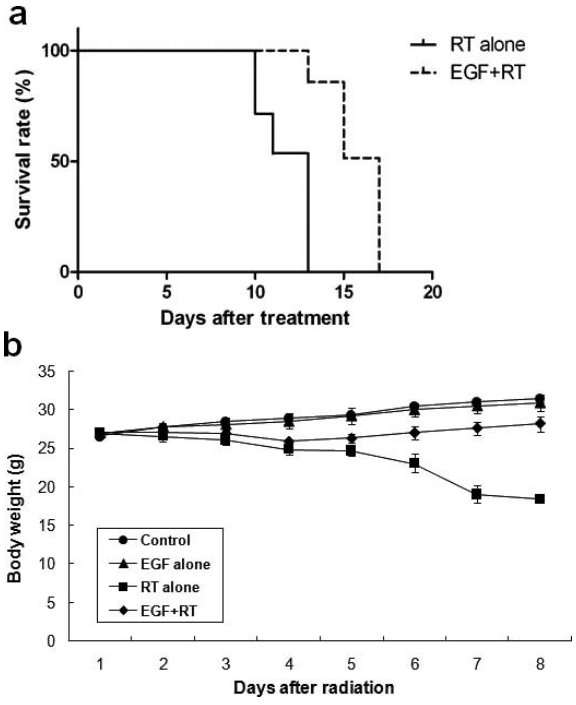

Fig. 3. Effect of rhEGF 100 μg/kg on mice survival (a) and body weight gain (b) after treatment of 10 Gy radiation in C3H/HeJ mice.

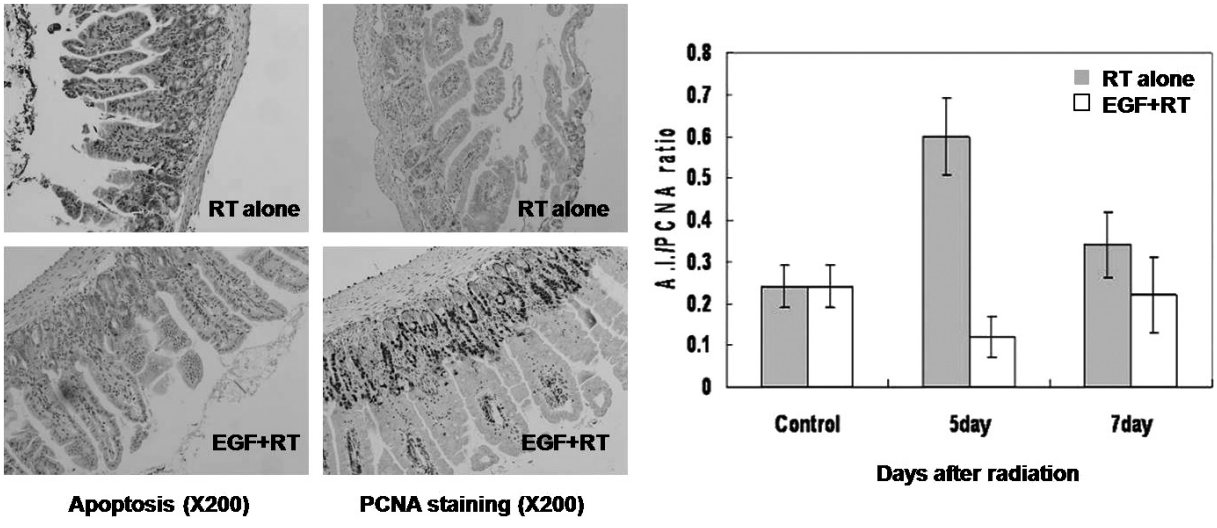

Fig. 4. Effect of rhEGF 100 μg/kg on PCNA and apoptosis after treatment of 10 Gy radiation in C3H/HeJ mice, shown as the ratio of apoptotic index to PCNA index in the right panel. Representative pictures are shown for apoptosis (left panels) and PCNA staining (middle panels) in radiation group (RT) and radiation plus rhEGF group (RT+EGF).

erate proliferation in radiation-induced jejunal crypt injury in C3H/HeJ mice.

Histology

To evaluate the effect of rhEGF on irradiated jejunal mucosa, histological samples of small intestine mucosa were examined on days 3.5 and 7. Histological findings in the jejunal mucosa on day 3.5 revealed a difference between the radiation group and the combination group (Fig. 5). The most obvious changes in intestinal histology induced by radiation were an increase in the number of apoptotic bodies, destruction of crypt cells, necrosis of the gastrointestinal epithelium, and architectural changes consisting of shortened villi. With addition of rhEGF, repaired villi were observed more frequently. As shown in Fig. 5, villous height decreased in radiation group (131 ± 28 μm) and restored by addition of rhEGF (314 ± 37 μm), which showed statistical significance ($p < 0.05$).

Tumor growth delay in the rhEGF-treated group

In radioresistant HCa-I, the time for tumor growth from 8 to 12 mm was 11.6 days and 8.8 days in the radiation alone and the rhEGF alone groups, respectively, which corresponds to AGD values of 2 days (rhEGF alone) and 4.8 days (radiation alone). When radiation was combined with rhEGF, the time for growth from 8 to 12 mm was 11.6 days and the NGD was 2.8 days. The enhancement factor was calculated as 0.6 (Fig. 6a). In radiosensitive MCa-K, the time for tumor growth from 8 to 12 mm was 11.5 days and 9.2 days in the radiation-alone and rhEGF-alone groups, respectively, which corresponds with the AGD values of 1.6 days (rhEGF alone) and 3.9 days (radiation alone). When radiation was combined with rhEGF, the time for growth from 8 to 12 mm was 11.6 days and the NGD was 3.2 days. The enhancement factor was calculated as 0.8 (Fig. 6b). These data showed that rhEGF did not alleviate the antitumor effect of the radiation.

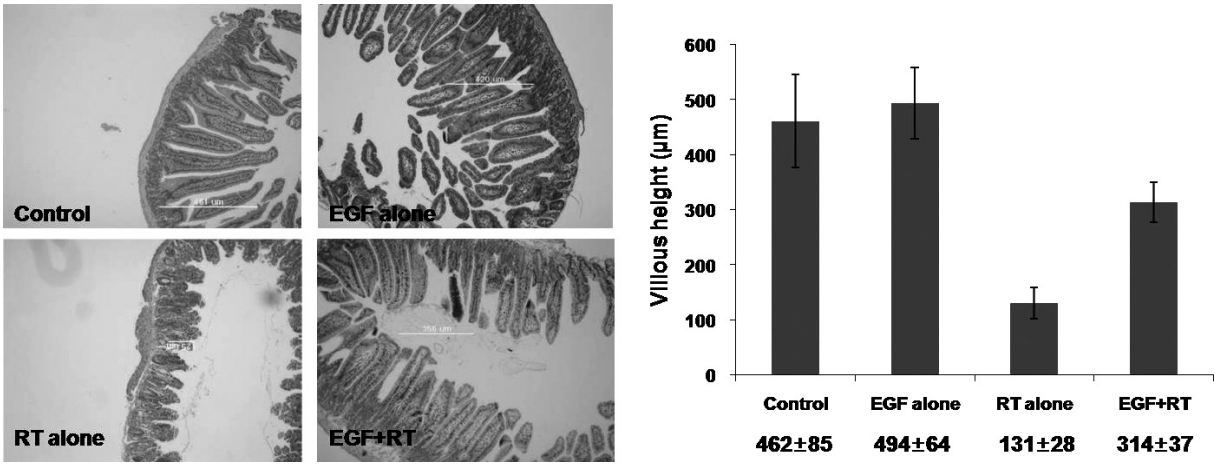

Fig. 5. Comparison of villous height of jejunal mucosa on day 3.5, shown in the right panel. Representative pictures are shown for each group (left and middle panels), control, EGF, radiation (RT), and radiation plus EGF group (RT+EGF) (hematoxylin & eosin stain 200X).

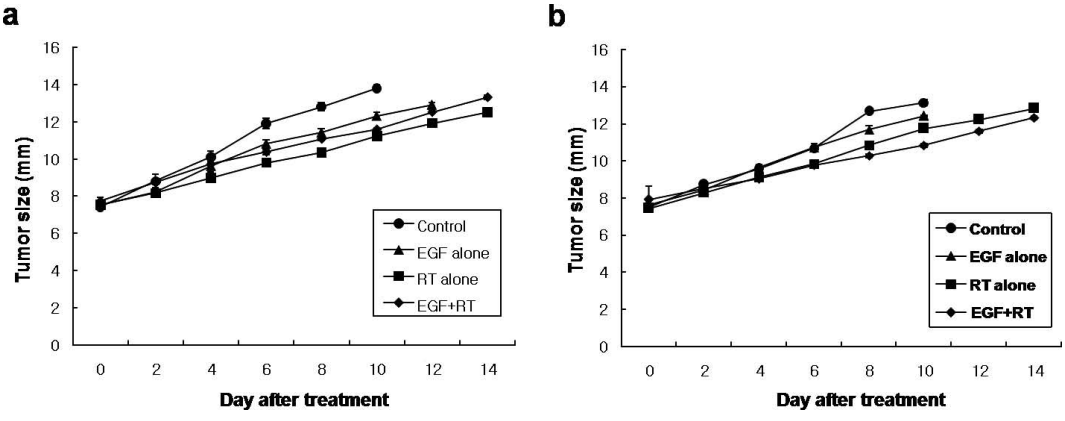

Fig. 6. Tumor growth delay assay after 25 Gy radiation in syngeneic hepatocarcinoma (HCa-I, a) and mammary carcinoma (MCa-K, b), showing each group of control (●), rhEGF alone (▲), radiation alone (RT, ■), and combination (RT+EGF, ◆).

DISCUSSION

Epidermal growth factor (EGF), a ~6-kDa polypeptide, induces cell proliferation, differentiation, and migration. EGF is present in various body fluids and tissues and is continuously secreted into the gastrointestinal lumen by submandibular glands, mucous neck cells of the stomach, Brunner's glands of the duodenum, Paneth cells of the small intestine, and the ulcer-associated cell lineage (a recently identified glandular structure induced at the site of injury). In particular, EGF and the EGF family of related peptides are involved as key components in the maintenance and repair of the mucosa. EGF binds to both low- and high-affinity sites on cells expressing the EGF receptor (EGFR). Ligand binding to EGFR activates RTK activity, leading to DNA synthesis and cell growth.[23,24)]

Following radiation, the damaged gastrointestinal mucosa shows a compensatory response in the form of increased proliferation. Saxena *et al*.[25)] showed that rate of crypt cell proliferation increased following radiation, which was closely associated with endogenous EGF/urogastrone. Other authors attempted to enhance such recovery process by administering exogenous EGF. McKenna *et al*.[26)] reported that EGF treatment (10 μg/kg every 8 hours for 2 days prior to and 3 days after radiation) significantly increased mitogenecity of intestines and decreased the acute clinical manifestation following 10 Gy radiation. The opposite result has also been reported. Lindegaard *et al*.[27)] showed that EGF (5–10 μg/day either before or after radiation) did neither reduce the median skin score nor increased LD50 following total body irradiation. The contradictory results shown in those studies seem to be related with different EGF doses or schedules and different end point as well. It also suggests a strong need to test the radioprotective effect of EGF.

Potten *et al*. has described the sequence of events after whole-body exposure to radiation.[28)] At doses < 15 Gy, surviving progenitors, even a single surviving clonogen per crypt, lead to crypt recovery, which can be identified 3.5 days after irradiation as typical regenerative crypts. In addition, extensive depletion of crypt-villus units leads to mucosal denudation and animal death from the GI syndrome at doses ≥ 15 Gy. Therefore, 8- and 10 Gy doses were used in establishing the radiation-induced intestinal mucosal damage model. In this study, the 10 Gy model was shown to be suitable since the severity of damage and its repair was dose-dependent more noticeably in the 10 Gy than in the 8 Gy model. In our 10 Gy model, mucosal damage was maximal on day 2 and began to recover from day 3.5, ultimately approaching control levels on day 5 (Fig. 2. a, b).

Mucosal recovering agents have been primarily investigated on oral mucosal damage. Promising results have been reported for KGF as well as for EGF in preventing or improving oral mucosal injury by irradiation.[29,19)] In the present study, systemic rhEGF administration alleviated the loss of body weight; this effect seemed to be due to the recovery of mucosa from radiation-induced injury (Fig. 5). We observed rhEGF to be associated with morphologic changes. In the radiation-alone group, crypts were diminished and villi lengths were shortened on day 3.5. However, the mucosa in rhEGF-treated mice showed relatively more crypt cells and significantly longer villi height after radiation. The apoptosis index (A.I.) significantly decreased and PCNA index (P.I) significantly increased in the combination group compared with the radiation alone group on Day 5 (Fig. 4). This result suggests that rhEGF protects the mucosa from radiation-induced apoptosis and accelerates proliferation, suggesting the possibility of rhEGF as a mucoprotectant in irradiated intestines. We also tested a higher dose (200 μg/kg) of rhEGF to determine whether radiation-injured mucosa shows dose-dependent responses to rhEGF. From 50 to 100 μg/kg, the response was dose-dependent, but no additional effects were seen at a dose of 200 μg/kg (data not shown).

With systemically administrated growth factors, there is a concern regarding stimulation of tumor growth *in vivo* or protection of tumor cells from radiotherapy.[30)] In this study, EGF alone showed a low level of antitumor effect in both radioresistant tumor (HCa-I) and radiosensitive tumor (MCa-K). It has been reported that high concentration of exogenous EGF induces cell death in EGFR-elevated cell lines.[31,32)] The underlying mechanisms have been suggested to involve activation of STAT1[33)] and p38 MAPK[34)] Another recent study showed that the exogenous EGF treatment leads to growth inhibition rather than inducing tumor cell proliferation even in the absence of EGFR overexpression.[35)] This study reassured the antitumor effect of exogenous rhEGF in syngeneic tumor system with different radiosensitivity. The mechanism underlying the differential effect of rhEGF, protecting intestinal mucosa but not tumor, hasn't been fully investigated. Further study is required to solve this issue.

This study showed that exogenous EGF treatment did not alleviate the antitumor effect of radiation. These finding suggest that combination of exogenous rhEGF and radiation can be a new anticancer strategy by protecting radiation-induced intestinal injury without alleviating antitumor effect of radiation.

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