Keratinocyte Growth Factor Protects Mice from Chemotherapy and Radiation-induced Gastrointestinal Injury and Mortality


ABSTRACT

Keratinocyte growth factor (KGF) stimulates the proliferation and differentiation of epithelial cells including those of the gastrointestinal tract. Although chemotherapeutics and radiation exposure kill rapidly proliferating tumor cells, rapidly dividing normal cells of the host’s gastrointestinal tract are also frequently damaged, leading to the clinical condition broadly termed “mucositis.” In this report, recombinant human KGF used as a pretreatment in several mouse models of chemotherapy and/or radiation-induced gastrointestinal injury significantly improved mouse survival. Using multiple-dose 5-fluorouracil, methotrexate, and radiation in combination and total body radiation alone models, KGF increased survival by 55% or greater. In the models that used chemotherapy with or without radiation, KGF significantly ameliorated weight loss after injury and accelerated weight gain during recovery. The basis of these systemic benefits appears to be due in part to the trophic effects of the growth factor on the intestinal epithelium because KGF pretreatment caused an increase in measures of mucosal thickness (villus height and crypt depth) that persisted during the course of 5-fluorouracil chemotherapy. Treatment with KGF also afforded a 3.5-fold improvement in crypt depth that was sustained by continuous proliferation of cells in crypts, which are ultimately dependent on continuous regeneration. Hence, patients undergoing these therapies frequently develop enteric mucositis and diarrhea, which can be lethal complications. KGF was evaluated in several murine models of single and combination cytotoxic therapy. We determined that short-term systemic administration of KGF before treatment with cytotoxics significantly ameliorated mortality and morbidity in these models of cytotoxic gastrointestinal injury in mice and propose that KGF may be therapeutically useful to lessen the intestinal side effects of current cancer therapy regimens.

INTRODUCTION

KGF4 was initially identified as a soluble factor that stimulated the proliferation of epithelial cells in vitro (1, 2). Administration of KGF to normal animals stimulated proliferation and differentiation of a variety of epithelia (3–8), notably in the gastrointestinal tract (9). The gastrointestinal effects of this growth factor are probably direct in that the transcript for the receptor, a splice variant of fibroblast growth factor receptor 2 (10) is expressed in the epithelium at all levels of the gastrointestinal tract (9). Chemotherapeutics and radiation kill rapidly dividing cancer cells but exhibit dose-limiting toxicities in normal, rapidly proliferating tissues such as the bone marrow and gastrointestinal mucosa. The epithelial lining of the intestinal mucosa is maintained by continuous proliferation of cells in crypts, which are ultimately dependent on crypt stem cells. Proliferation is followed by cellular differentiation and migration up villi, where they replace the mature enterocytes that are shed from the tips. Cytotoxic doses of chemotherapy or radiotherapy compromise the absorptive and barrier function of the mucosa by killing the crypt stem cells, thereby impairing normal regeneration. Hence, patients undergoing these therapies frequently develop enteric mucositis and diarrhea, which can be lethal complications (11). KGF was evaluated in several murine models of single and combination cytotoxic therapies. We determined that short-term systemic administration of KGF before treatment with cytotoxics significantly ameliorated mortality and morbidity in these models of cytotoxic gastrointestinal injury in mice and propose that KGF may be therapeutically useful to lessen the intestinal side effects of current cancer therapy regimens.

MATERIALS AND METHODS

KGF Production. Recombinant human KGF was produced in Escherichia coli, refolded, purified to homogeneity by conventional chromatography techniques, and tested to be endotoxin free. KGF was assayed in the BALB/MK keratinocyte cell line (American Type Culture Collection, Rockville, MD) as described previously (1).

Mucositis Models. In all of the mucositis model experiments using chemotherapy and/or radiation, female BDF mice were randomized into groups for 3 days of treatment with 5 mg/kg/day of KGF or vehicle sc on days −2, −1, and 0 prior to cytotoxic injury with chemotherapy or radiation from a cesium source beginning on day 1. In some experiments, KGF was administered as a continuous or posttreatment. Control mice were injected appropriately with vehicle.

To model multiple-dose chemotherapy, mice were injected with 5-fluorouracil (50 mg/kg) for 4 consecutive days. This regimen of 5-fluorouracil typically induced rapid weight loss, which was followed by modest weight recovery. Thereafter, only 20–50% of mice survive beyond 2 weeks. To model combination chemoradiotherapy, mice were injected with a single dose of 150 or 300 mg/kg of methotrexate on day 1 and irradiated (6 Gy) 1 h later. Carboplatin was used as a model of nonlethal chemotherapy and was administered as a single dose of 125 mg/kg on day 1. In experiments modeling radiotherapy alone, mice were subjected to total body irradiation at 8:00 a.m. by placing groups of five mice in polycarbonate cages and exposing them to radiation from a cesium source with the exposure time adjusted to provide the a dose of 12 Gy. In some experiments where bone marrow was transplanted, the mice were given 2 × 10⁶ syngeneic cells the day after irradiation. In all experiments, the mice were weighed daily and monitored closely for signs of morbidity over the course of the following weeks. The experimental protocols executed at the Amgen site were performed at an Association for the Assessment and Accreditation of Lab Animal Care International accredited facility.

Tumor Growth. CD1 nu/nu mice were injected s.c. on the left flank with 1.5 × 10⁶ HT29 human colon carcinoma cells on day 0, followed by dosing with 3 or 5 mg/kg/day KGF or vehicle sc for 3 days and subsequently with 5-fluorouracil given i.p. at 50 mg/kg/day for 5 consecutive days. The mice were weighed daily, and the progression of tumor growth was monitored by caliper measurements at the indicated time points.

Histology and Crypt and Villus Morphometry. Morphometry was performed on mice in experiments where they were pretreated with 5 mg/kg KGF or vehicle, followed by a course of 5-fluorouracil chemotherapy as described above. The mice were euthanized, intestines were removed, and a segment of the duodenum was fixed and processed and blocked into paraffin to produce four cross-sections per mouse. The slides were stained with H&E and used for image analysis to obtain 20–40 separate measures of villus length and crypt.
depth per mouse. These measures were used to calculate the mean length and depth for each mouse.

**Crypt Survival.** Male BDF1 mice were dosed with 0.5 mg/kg/day KGF or vehicle sc for 3 days, inclusive of the day of irradiation, and subsequently irradiated at a dose rate of 3.8 Gy/min, or dosed similarly with KGF 3 days after irradiation, or given pre and post dosing. This dose of KGF was chosen based on results of pilot experiments. The mice were euthanized 4 days after irradiation, and the terminal ileum fixed in Carnoy’s fixative and prepared as gut bundles (12). Paraffin sections were stained with H&E, and micro colonies were recorded as described elsewhere (12). Each mouse provided 10 cross-sections of the ileum, and the number of crypts in the unit of length presented by the circumference was recorded. Thus, for each experimental group, 60 transverse sections of the intestine were scored for surviving crypts. Fifteen representative longitudinal sections of crypts from each mouse had the width measured at the midpoint along the crypt to enable the data to be corrected for any variations in the size of the crypts between experimental groups (13). Crypt survival curves were obtained, and the parameters describing these curves, the $\Delta^*$ value (reciprocal of the slope of the exponential portion of the line, a measure of radiosensitivity) and $V$ (the extrapolation number obtained by extrapolating the straight line portion of the curve to the survival axis at zero dose) were determined using the DRIFT program (14).

**BrdUrd Labeling of Cells in Surviving Crypts.** Female BDF1 mice were injected with 50 mg/kg BrdUrd 4 days after irradiation with 12 Gy. After 1 h, the mice were euthanized. The small intestine was removed, flushed, and divided into duodenum, jejunum, and ileum, which were fixed, processed into paraffin, sectioned, and stained with antibodies to BrdUrd (Accurate Chemical & Scientific Corporation, Westbury, NY). Surviving crypts were scored in cross-sections based on the presence of three or more BrdUrd-labeled cells/crypt in at least four sections/segment of duodenum, jejunum, and ileum per mouse.

**RESULTS**

**Protection in Mucositis Models.** KGF was protective in this model when administered as a pretreatment for 3 days before the 5-fluorouracil injections. In this experiment where the mice were monitored for 27 days to allow hematopoietic recovery, the majority of the KGF-treated mice recovered, significantly increasing survival from 27% in the control group to 87% ($P < 0.006$; Fig. 1A). The KGF-treated mice lost significantly less weight than the vehicle-treated mice ($P < 0.0001$; Fig. 2A). In KGF dose-response and schedule experiments, pretreatment with KGF led to significant improvements in survival at doses as low as 0.5 mg/kg/day, although the effects were best at doses above 1 mg/kg, and treatment with KGF following the four daily doses of 5-fluorouracil was ineffective (data not shown).

To exclude the possibility that the beneficial effects of KGF specifically counteracted only 5-fluorouracil toxicity, mice were treated with a nonlethal dose of carboplatin. As with the multidose 5-fluorouracil model, KGF pretreatment significantly ameliorated weight loss ($P < 0.0001$) after carboplatin exposure (Fig. 2B). In chemotherapy and radiation combination experiments, mice were injected with a single dose of 150 or 300 mg/kg of methotrexate and irradiated (6 Gy) 1 h later. KGF pretreatment significantly improved survival at both low ($P < 0.0001$) and high ($P < 0.003$) doses of methotrexate (Fig. 1B) and significantly improved the nadir of weight loss ($P < 0.01$; Fig. 2C).

In whole-body irradiation models, we found that single doses of radiation over 9 Gy were lethal, even when the animals were pretreated with KGF, likely due to the overlap in the lethal intestinal and marrow toxicities of high-dose radiation. Therefore, mice exposed to 12 Gy also received a syngeneic bone marrow transplant to eliminate marrow toxicity as a cause of radiation-related mortality. In this paradigm, the group of mice that received KGF prior to irradiation had a dramatic and significant increase in survival to 90% compared with 0% in the control group, which had also received a transplant ($P < 0.0001$; Fig. 1C). The vehicle-treated mice lost weight rapidly and became moribund within 8 days (data not shown).

**Tumor Growth Studies.** To determine whether KGF could potentially exert a protective effect on tumors, nude mice were s.c. implanted with the KGF receptor-positive (by RNase protection assay) HT29 human adenocarcinoma cell line and treated with KGF for 3 days prior to chemotherapy with 5-fluorouracil for 5 days. The use of KGF did not alter the growth rate of the tumors or the tumor growth-inhibiting effect of the chemotherapy (Fig. 3). In these experiments, the KGF pretreatment did protect the mice from the loss of weight that...
EFFECT OF KGF ON MUCOSITIS MODELS IN MICE

Fig. 2. The effect of KGF on body weight in mice treated with chemotherapy and/or irradiation. BDF; mice treated with 50 mg/kg/day 5-fluorouracil (5-FU) i.p. for 4 days (days 1–4; n = 15/group; A); 125 mg/kg carboplatin i.p. (day 1; n = 10/group; B), and 150 or 300 mg/kg of methotrexate (MTX) i.p. 1 h before receiving 6 Gy radiation from a cesium source (day 1; n = 20/group; C), were pretreated with KGF or vehicle for 3 days. The changes in weight in A and C were analyzed by calculation of the percentage of change from baseline where a linear mixed model for repeated measures was used. It includes the fixed effects of treatments, days of measurements, and the interaction treatment by day. Bars, SE. In B, the mean weights at the nadir were compared using a Student’s t test. The data are expressed as the mean ± SE. In all of these experiments, the pretreatment with KGF significantly reduced weight loss induced by the cytotoxic injuries. Bars, SE.

was observed to occur during the course of 5-fluorouracil treatment in the vehicle-treated mice (data not shown).

Crypt and Villus Morphometry. To confirm that the therapeutic effects of KGF in these injury settings were due, at least in part, to a trophic action on intestinal epithelium, we measured the lengths of the crypts and villi in the mice. After three doses of KGF, the intestines from KGF-treated mice appeared histologically normal, but crypt depth and villus length were significantly larger than normal vehicle-treated controls (Fig. 4). Crypts and villi were diminished in size by the subsequent 5-fluorouracil treatments, but in the KGF-pretreated mice, these measures were not significantly different from those of animals that had not received either KGF or chemotherapy (i.e., normal vehicle-treated mice on day 1), even in the presence of histological evidence of cellular injury (Fig. 5).

In the radiation model of mucositis, the mean villus length was also normalized by KGF pretreatment. Four days after irradiation, we observed a rebound hyperplasia in the crypts (131 ± 5 and 141 ± 7

Fig. 3. Effect of KGF on tumor growth in chemotherapy-treated mice. The sizes of tumors growing in the flanks of nude mice inoculated with HT29 colon adenocarcinoma cells were measured, and the data were analyzed by an ANOVA. Each point represents the mean tumor volume, where n = 10 mice/group; bars, SE. KGF administered for 3 days prior to 5 days of 5-fluorouracil chemotherapy neither stimulated HT29 tumor growth nor protected the tumors against the growth-inhibitory effects of 5-fluorouracil.

Fig. 4. Effect of KGF on villus length and crypt depth in the duodenum of chemotherapy-treated mice. BDF; mice were given 50 mg/kg/day of 5-fluorouracil i.p. for 4 days after a 3-day pretreatment with KGF or vehicle. A two-way ANOVA to evaluate the effect of pretreatment and time on the group means revealed an overall effect of the pretreatment and day and a significant pretreatment by day effect (P < 0.0042 for villus length, P < 0.0002 for crypt depth). The data are expressed as means, where n = 5 mice/group; bars, SD. *, significant differences between means of KGF- or vehicle-pretreated groups at each time point.
Fig. 5. Effect of KGF on the small intestines of mice administered chemotherapy. The intestinal mucosa of BDF$_1$ mice was thinned as a consequence of 4 days of 5-fluorouracil chemotherapy (A). Mice pretreated with KGF for 3 days (C) had thickened mucosa relative to chemotherapy-treated controls due to longer villi and deeper crypts (data presented in Fig. 4) and looked similar to normal (E) at this magnification and in cross-sections (insets). However, at higher magnification, apoptotic cells and cells with swollen nuclei were present in the crypts of both control (B) and KGF (D) groups after 5-fluorouracil chemotherapy. Normal crypts are shown in F. Bar, 50 μm.
Fig. 6. Effect of KGF on crypt survival in irradiated mice. The plot represents the radiation dose-response for crypt survival in the intestines of BDF1 mice. The parameters defining the curves are as follows: for the saline group, $D^* = 1.43 \pm 0.06$ with $n = 886 \pm 294$; for the pretreatment group, $D^* = 1.91 \pm 0.12$ with $n = 173 \pm 65$. Statistical comparisons between data sets performed in DRIFT using variance-ratio F tests demonstrated that the two data sets differ significantly ($P < 0.0001$). Bars, SE.

The mechanism of action for protection in the mucositis models is likely multifactorial. In these studies, several types of cytotoxic therapies were used to induce damage, indicating that the protective effects of KGF on the small intestine are not limited to a single mechanism of cellular injury. Therefore, KGF may protect the intestine from a wide variety of agents and/or conditions with intestinal toxicities other than those examined here.

The trophic effect of KGF on the intestinal mucosa was seen in this study where crypt depth and villus height were increased, confirming earlier findings in the rat (9). Mucosal hypertrophy may result in an increase in functional capacity that would help sustain the mice after cytotoxic therapy, and the lack of effect of KGF when given post-

DISCUSSION

The preceding studies demonstrate that short-term systemic administration of KGF significantly ameliorated mortality and morbidity in various mouse models of chemotherapy and radiation-induced gastrointestinal injury. Numerous experiments performed to optimize the dose and schedule of KGF in these mucositis models have shown that pretreatment, as opposed to posttreatments, yielded the best results. End points such as weight and survival were measured along with others that provided evidence that the protective effects of KGF were likely due to a direct effect of KGF on the intestine.

Fig. 7. Effect of KGF on BrdUrd labeling of surviving crypts. BDF1 mice irradiated with 12 Gy and given a bone marrow transplant have scant numbers of BrdUrd-labeled crypts (A) compared with mice pretreated with KGF for 3 days (B; data presented in Table 1). Bar, 500 μm.
KGF also affects cell differentiation in the intestine. KGF increases the number of goblet cells in the stomach and intestine (9), and their secretory products, including mucins, are thought to form an important physiological barrier between the intestinal mucosa and the lumen environment (28). Goblet cells also produce nonmucinous proteins, called trefoil proteins, that contribute to gut defense and repair (29–31). Elevated expression of these proteins together with mucin before chemotherapy or radiotherapy might enhance barrier function.

Many epithelial tumors express the KGF receptor, and there was a concern that KGF could either stimulate tumor growth in vivo or protect the tumor cells from the cytotoxic effects of therapy. In these experiments, doses of KGF that are clearly protective of epithelia did not enhance tumor growth or interfere with the growth-inhibiting effects of the chemotherapy. Whether this is because the dose and schedule of KGF used does not alter tumor response or because the cells cannot respond to KGF is unclear. In situ hybridization studies have demonstrated that endogenous KGF is expressed in host cells surrounding these tumors (data not shown), so that if the KGF signaling pathways are intact and functional, the tumor cells may already be receiving stimulation. In a comprehensive study performed using several tumor cell lines, Ning et al. (32) showed that KGF did not stimulate proliferation or reduce radiosensitivity of squamous carcinoma cells either in vitro or in vivo. In their in vitro studies of similar tumor lines, Drukan et al. (33) also determined that KGF did not provide a selective growth advantage for malignant oral keratinocytes relative to normal human keratinocytes. These studies provide evidence supporting the concept that therapeutically effective doses of KGF do not stimulate tumor cell growth or protect the tumor cells from cytotoxic therapy.

The effects of KGF are directed toward epithelial cells where the receptor is expressed, but multiple indirect systemic benefits were also apparent in these mucositis models. The maintenance and rapid regeneration of the intestinal mucosa by KGF may allow for better food and water absorption and help to maintain an intact epithelial barrier against invasion by gut microorganisms. The health and survival of the mice were improved by KGF pretreatment in these experiments, and their food and water intake was comparable to that of normal mice (data not shown). Indirect data to support the idea that KGF improves barrier function was obtained by analyzing the survivors of a 5-fluorouracil study on day 18. All of the surviving control mice (8 of 15), but none of the KGF-pretreated animals (15 of 15), had grossly visible barrier function was obtained by analyzing the survivors of a 5-fluorouracil study on day 18. All of the surviving control mice (8 of 15), but none of the KGF-pretreated animals (15 of 15), had grossly visible barrier function was obtained by analyzing the survivors of a 5-fluorouracil study on day 18. All of the surviving control mice (8 of 15), but none of the KGF-pretreated animals (15 of 15), had grossly visible barrier function was obtained by analyzing the survivors of a 5-fluorouracil study on day 18. All of the surviving control mice (8 of 15), but none of the KGF-pretreated animals (15 of 15), had grossly visible barrier function was obtained by analyzing the survivors of a 5-fluorouracil study on day 18. All of the surviving control mice (8 of 15), but none of the KGF-pretreated animals (15 of 15), had grossly visible barrier function was obtained by analyzing the survivors of a 5-fluorouracil study on day 18. All of the surviving control mice (8 of 15), but none of the KGF-pretreated animals (15 of 15), had grossly visible

Table 1 Proliferating crypt counts in mice irradiated with 12 Gy compared with normal, unirradiated mice

<table>
<thead>
<tr>
<th>Injury</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>124 ± 5</td>
<td>112 ± 2</td>
<td>86 ± 7</td>
</tr>
<tr>
<td>Vehicle + 12 Gy</td>
<td>40 ± 5</td>
<td>27 ± 4</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>KGF + 12 Gy</td>
<td>80 ± 3*</td>
<td>67 ± 3*</td>
<td>25 ± 3*</td>
</tr>
<tr>
<td>Vehicle + 12 Gy + BMT</td>
<td>35 ± 3</td>
<td>25 ± 3</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>KGF + 12 Gy ± BMT</td>
<td>67 ± 3*</td>
<td>63 ± 3*</td>
<td>36 ± 3*</td>
</tr>
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* Significantly different from control at P < 0.05 or greater compared to by a t-test.
the development of dose-intensification regimens that are associated with severe mucositis.

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