AMELIORATION OF ACUTE ORAL MUCOSITIS BY KERATINOCYTE GROWTH FACTOR: FRACTIONATED IRRADIATION

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INTRODUCTION

Keratinocyte growth factor (KGF) is a member of the heparin-binding family of fibroblast growth factors (FGF-7) and is exclusively synthesized by mesenchymal cells, particularly fibroblasts. The receptor, a tyrosine kinase, is expressed exclusively on epithelial cells in a variety of tissues. These include epidermis and hair follicles, oral and gastrointestinal epithelium, corneal epithelium, lung epithelium, urothelium, prostate epithelium, etc. (1). KGF represents a paracrine mediator of mesenchymal-epithelial communication.

KGF induces in the epithelia a variety of responses, which include not only stimulation of epithelial proliferation, but also modification of migration and differentiation processes. Systemic administration of exogenous KGF to normal animals results in hyperproliferation of epithelium and growth of various organs (1).

During skin wound healing, KGF plays a predominant role. In experimental models, a marked increase in dermal transcription activity was observed (2–5). Topical application stimulated wound healing in animal models (6).

Epithelia are typical turnover tissues with a hierarchical proliferative organization. Enduring cell loss via differentiation and mechanical stress at the surface is precisely counteracted by continuous cell production in the deeper, germinnal epithelial layers. Radiation exposure in this type of tissue results in an impairment of proliferation in the germinmal layers. The consequence is a reduction in cellular supply to the postmitotic, functional cell layers. On the other hand, cell loss in these tissues, e.g., by mechanical shedding at the surface, is dependent on the natural turnover rate, but is largely independent of radiation exposure. Therefore, cell loss continues at its near-normal rate after...
irradiation (7–9). Cells present at the time of radiation injury undergo near-normal differentiation (8, 10).

The radiation-induced imbalance between cell production and loss eventually results in, after sufficient doses, complete cellular depletion of the functional cell layers, with few surviving (stem) cells in the germinal layer (7, 9, 11). Clinically, in skin and mucosae, this manifests itself in the form of denudation and ulceration, which are associated with a significant impairment of the epithelial barrier.

Of clinical importance is the fact that cells that have lost their unlimited proliferative capacity (“clonogenicity”) as a result of radiation injury can still undergo a limited number of “abortive” divisions (7, 11). This residual but significant cell production is the basis for the latent time to ulceration being markedly longer than the turnover time, even after high radiation doses. In oral mucosa, the turnover time was estimated to be about 5 days in mouse lower tongue surface (7, 12) and also in various intraoral sites in normal human mucosa (13, 14). Confluent mucositis, however, is seen 10–11 days after sufficient doses in the mouse, about 9 days after a threshold dose of 20 Gy in human oral mucosa (15, 16), and somewhat later in supraglottic mucosa (16). In the current investigation, the effect of recombining human keratinocyte growth factor (rHuKGF) on oral mucositis induced by daily fractionated irradiation was quantified. The incidence of mucosal ulceration was used as a clinically relevant end point for radiation dose–response analyses. The residual tissue tolerance after 1 week of fractionated irradiation given in various protocols, with or without rHuKGF, was determined by graded test doses. The effect of rHuKGF was then quantified by comparison of isoeffective test doses.

METHODS AND MATERIALS

Mice and housing
Mice of the inbred C3H/Neu strain, based on the colony of GSF, Neuherberg and bred in the colony of the Medical Faculty Carl Gustav Carus, were used in all experiments. Both genders were included in this investigation, because no gender differences had been found in this mouse strain in radiation response in mouse tongue mucosa (17). The mice were bred and housed under specified pathogen-free conditions. Humidity (30%–50%) and temperature (21–24°C) were controlled. A lighting regime warranted a 12/12-hour light-dark rhythm, with lights on from 06:00 to 18:00.

The mice were housed in size 3 Macrolon cages, ≤10 per cage, on sawdust bedding (Sniff 3/4, Altrogge, Lage, Germany). Free access was provided to standard mouse diet (Altromin 1326, Altrogge) and filtered city tap water from standard Perspex drinking bottles.

Irradiation technique
Radiation damage to the epithelium of the lower tongue was inflicted by a combination of two techniques: percutaneous irradiation of the whole snout, and local top-up treatment of the lower tongue surface. Both setup and technique for snout and local radiation treatment of the lower tongue surface were recently reported in detail (17, 18).

Snout irradiation was performed without anesthesia. An Isovolt 320/20 X-ray device (Seifert Röntgenwerke, Ahrnsburg, Germany) with a beam filter of 0.6 mm Cu and 1 mm Al was used. The device was operated at 200 kV with a tube current of 20 mA, resulting in a dose rate of 1.07 Gy/min at the focus-skin distance of 45.5 cm.

The mice were guided into plastic tubes with an inner diameter of 28 mm, and each snout was positioned in the conical hole of a Perspex block. Subsequently, the back ends of the tubes were closed to prevent the mice from withdrawing. Eight mice were irradiated in parallel: Two opposing rows with four tubes each were arranged on a Perspex plate. Behind a plane from the eyes to the throat, the bodies of the mice were shielded with 6 mm of lead-equivalent MCP-96 (HEK Medizintechnik, Lübeck, Germany). The treatment field encompassed the snout, including the entire tongue.

For dosimetry, the dose rate was regularly checked with an ionization chamber (M23323, PTW Freiburg, Germany) with a volume of 0.1 cm³ in connection with a dosimeter Dosimenter SN4 (PTW). The stability of the dose rate allowed us to define the treatment dose by adjustment of the time for irradiation. Dose homogeneity among the individual snout treatment fields was ±3%.

An irradiation test was given to a local treatment field of the central lower tongue. A DARPAC 150-MC device (Forward Raytech Ltd.) was operated at 25 kV with a tube current of 20 mA. With the beam filter, 0.3-mm Al, the resulting dose rate was 3.78 Gy/min at the focus-to-skin distance of 15 cm. The dose rate was checked regularly by medical physicists in the radiotherapy department and found to be constant.

For immobilization during local irradiation, Pentobarbitone sodium (Narcoren, Rhone Merieux) was administered i.p. at a dose of about 60 mg/kg. The mice were placed in the central bore (diameter: 25 mm) of a prewarmed aluminum block (≈35°C) in a supine position. A hole in the roof of the block (diameter: 3 mm) allowed us to guide the tongue by means of forceps and to fix the upper tongue surface to the outer surface of the block by double adhesive tape. Subsequently, the head was supported by a polystyrene wedge to avoid traction at the base of the tongue, with consequent hypoxia.

A 3 × 3 mm² window in an aluminum plate (thickness: 1 mm) defined the treatment area. The window was positioned centrally over the tongue, shielding the tip, margins, and base.

KGF
rHuKGF, produced in Escherichia coli, was provided by Amgen Inc. (Thousand Oaks, CA). Purification to homogeneity was achieved by conventional chromatography. Endotoxin tests were performed by Amgen Inc.

The lyophilizate was dissolved in the reconstitution solution provided by the company, which consisted of sterile water and Tween 20 at a concentration of 5.21 mg/mL, and the solution was further diluted in sterile phosphate-buffered saline to a final concentration of 1 mg/mL.
The solution was freshly prepared on each day of injection. The final concentration rendered injection volumes 0.1–0.12 mL per mouse for a dose of 5 mg/kg per injection; KGF was administered s.c. Dosage and route of administration were based on previous studies of mice (19, 20).

**Experimental design**

Figure 1 illustrates the individual experimental protocols performed in the present study, as follows:

1. Single-dose irradiation alone: In a control experiment, graded single doses were administered to the lower tongue surface to define the radiation tolerance of normal, untreated mouse tongue. Five dose groups with at least 7 mice each were used.

2. Fractionated irradiation: Fractionated irradiation, 5 daily fractions of 3 Gy, was given over 1 week and followed by local test irradiation with graded doses on Day 7. Test irradiation comprised at least 5 dose groups with at least 8–10 mice each, to generate a full dose–effect curve. In this control experiment, no rHuKGF was administered.

3. rHuKGF treatment protocols: The experimental protocols are illustrated in Fig. 1; the experimental code gives the first and last day of injection; doses were 5 mg/kg/day. The follow-up period (two-way arrow) included daily scoring of mucosal reactions.

**Follow-up, end point, and statistical analysis**

Radiation effects in tongue epithelium were scored daily from the onset of first symptoms until complete reepithelialization. The mice were immobilized by ultrashort anesthesia with Methohexitone (Brevimytal, Lilly) at a dose of ~40 mg/kg i.p. (21). Scoring was performed under a cold light source.

Mucosal ulceration, corresponding to confluent mucositis RTOG/EORTC Grade 3, was used as the quantal end point in all studies. The frequency of responding mice was used for dose–effect analyses. Additional parameters assessed were latent time, defined as time from test irradiation to first diagnosis of ulcer, and individual ulcer duration.

For all statistical procedures, the Statistical Analysis System was used (22). For analysis of dose–effect relationship, probit analysis was performed, assuming a log–normal distribution (logit analysis). This reveals ED50 values, i.e., doses at which a response is expected in 50% of the mice treated, and their standard deviation, σ (23). Also, p values were calculated for the effect of dose on ulcer induction, based on the slope of the regression line of the probit curve. For the comparison of dose–effect relationships, a maximum-likelihood chi-square test, without the assumption of a threshold dose, was applied (23). For analyses of variation, the general linear models procedure of the Statistical Analysis System was used (24).

**RESULTS**

**Single-dose irradiation**

The ED50 value for single-dose irradiation alone, in good agreement with previous results (17, 18, 25–27), was
Fractionated irradiation

Fractionated snout irradiation with 5 × 3 Gy/3 days resulted in an ED50 for test irradiation (Day 7) of 5.6 ± 3.7 Gy (Table 1). Average latent time was 10.8 ± 0.7 days, and mean ulcer duration was 2.3 ± 0.7 days (mean ± standard deviation). Again, the treatment was well tolerated.

KGF treatment protocols

The results of the rHuKGF experiments are summarized in Table 1. Administration of rHuKGF resulted in a significant increase in isoeffective radiation doses in all treatment protocols applied.

rHuKGF applied before radiotherapy resulted in an ED50 of 8.3 ± 1.6 Gy and 7.8 ± 3.3 Gy, respectively (Fig. 2). A more pronounced increase in ED50s was observed when the growth factor was given during radiotherapy (10.8 ± 1.7 Gy, 10.9 ± 1.7 Gy). The maximum effect was seen with rHuKGF administration over the weekend break (13.5 ± 2.3 Gy). The protocols with injections on Days −3 to +1 and +4 to +8 yielded ED50 values of 9.2 ± 0.7 Gy and 12.0 ± 2.4 Gy, respectively.

Replacement of three injections each of 5 mg/kg KGF by only one injection of 5 mg/kg was similarly effective (Fig. 2), with ED50 values of 10.7 ± 1.7 Gy for Day −1 and 11.3 ± 1.3 Gy for Day +4. Application of 1 × 15 mg/kg instead of 1 × 5 mg/kg resulted in higher ED50 values of 14.1 ± 1.5 Gy and 14.1 ± 1.8 Gy for Day −1 and +4, respectively. This suggests a dose effect of KGF.

The time course of the mucosal response was largely independent of rHuKGF administration, and no systematic variations were found with the rHuKGF administration protocols (Table 1).

DISCUSSION

Confluent oral mucositis is a severe side effect of radiotherapy for advanced head-and-neck tumors that frequently

<table>
<thead>
<tr>
<th>Protocol</th>
<th>ED50 ± σ (Gy)</th>
<th>Pe vs. control</th>
<th>Latency [x ± SD (days)]</th>
<th>Ulcer duration [x ± SD (days)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single dose, alone</td>
<td>10.9 ± 0.7</td>
<td>–</td>
<td>9.8 ± 2.3</td>
<td>4.2 ± 1.2</td>
</tr>
<tr>
<td>Fract. + TU</td>
<td>5.6 ± 4.1</td>
<td>0.0008</td>
<td>10.4 ± 1.0</td>
<td>2.4 ± 0.7</td>
</tr>
<tr>
<td>−4/−2</td>
<td>8.3 ± 1.6</td>
<td>0.0047</td>
<td>11.1 ± 0.3</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>−3/−1</td>
<td>7.8 ± 3.3</td>
<td>0.0001</td>
<td>11.4 ± 0.4</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>0/+2</td>
<td>10.8 ± 1.7</td>
<td>0.0001</td>
<td>8.0 ± 1.6</td>
<td>3.3 ± 1.1</td>
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<tr>
<td>0/+4</td>
<td>11.9 ± 1.7</td>
<td>0.0001</td>
<td>8.1 ± 1.2</td>
<td>3.5 ± 1.2</td>
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<tr>
<td>+4/+6</td>
<td>13.5 ± 2.3</td>
<td>0.0103</td>
<td>10.7 ± 0.5</td>
<td>2.3 ± 0.8</td>
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<tr>
<td>+4/+8</td>
<td>12.0 ± 2.4</td>
<td>0.0103</td>
<td>9.2 ± 1.5</td>
<td>2.6 ± 0.9</td>
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<tr>
<td>−3/+1</td>
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<td>0.0007</td>
<td>7.5 ± 1.0</td>
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<tr>
<td>−1 (5 mg/kg)</td>
<td>10.7 ± 0.7</td>
<td>0.0001</td>
<td>8.0 ± 1.1</td>
<td>3.0 ± 0.9</td>
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<tr>
<td>−1 (15 mg/kg)</td>
<td>14.1 ± 1.5</td>
<td>0.0001</td>
<td>9.7 ± 1.0</td>
<td>2.8 ± 0.9</td>
</tr>
<tr>
<td>+4 (5 mg/kg)</td>
<td>11.3 ± 1.3</td>
<td>0.0003</td>
<td>6.8 ± 0.7</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>+4 (15 mg/kg)</td>
<td>14.1 ± 1.8</td>
<td>0.0003</td>
<td>7.0 ± 0.2</td>
<td>2.5 ± 0.5</td>
</tr>
</tbody>
</table>

Abbreviations: SD = standard deviation; TU = top up irradiation.

Notes: The experimental code refers to Fig. 1 and represents the first and last day of keratinocyte growth factor injection, respectively. Dose-effect analyses for test irradiation were done by probit analysis, resulting in ED50 values and their standard deviation, σ. Comparison of dose-effect curves was performed by maximum-likelihood χ² test, with the p values given in the table. Average latencies and ulcer durations (mean ± SD) were calculated from all responders in the respective experiment, independent of dose.
necessitates interruptions in the prescribed treatment course. Prolongation of the overall treatment time for squamous carcinoma, however, bears the risk of decreased tumor control probability (29–31). Moreover, severe acute mucosal changes, associated with substantial impairment of the epithelial barrier and additional trauma to underlying tissues, can result in aggravated late effects in the form of a consequential component (32–41). Therefore, amelioration of the acute mucosal effects of radiotherapy may be of double benefit for the patients experiencing successful therapy of their malignant disease.

A number of measures for the management, both prophylactic and therapeutic, of acute oral mucosal radiation effects have been tested both preclinically and clinically (42–44). Most of them aimed at reduction of secondary infections, by topical antiseptic or antibiotic treatment or by stimulation of the immune system by respective growth factors (granulocyte colony stimulating factor, granulocyte macrophage colony stimulating factor). The approaches also encompassed administration of growth factors, such as interleukin-1 (45), epidermal growth factor (46, 47), tumor necrosis factor-α (46), transforming growth factor-α (46, 48), insulin-like growth factor I and II (46), acidic fibroblast growth factor (46), or transforming growth factor-β (49).

However, up to now—as clearly illustrated by the large variety of approaches that has been, and is currently being, studied—no effective treatment has been identified in clinical studies, and none of the approaches have found entrance into the clinical routine of supportive care.

rHuKGF is known to stimulate proliferation in epithelial cells, including those of the mucosal lining in the upper gastrointestinal tract. Hence, this growth factor could have the mucoprotective potential that has already been demonstrated for epithelia of the oral cavity, esophagus, and intestine (19, 20). The identity between human and rat KGF is very high, with 89.3% at the DNA and 90.3% at the protein level. Mouse and rat KGF are virtually identical. This growth factor is hence well preserved, which explains the effectiveness of the human recombinant form in the mouse.

In mouse tongue mucosa, ulcer frequency was significantly decreased if rHuKGF was administered in combination with single-dose irradiation (18). Dose modification factors ranged between 1.7 and 2.3. The effect was most pronounced when rHuKGF was applied after irradiation.

The current study was initiated to quantify rHuKGF effects in mouse tongue epithelium, using ulceration as the clinically most relevant end point, in a daily fractionated irradiation protocol. In all KGF administration protocols tested, i.e., rHuKGF administration before, during, or after fractionated irradiation, or in a combination, a significant increase was found in isoeffective radiation doses for test irradiation. Conversion into the number of dose fractions compensated by rHuKGF treatment demonstrated that with preirradiation administration, 1–1.5 fractions were counteracted by KGF; in all other protocols, about 3 fractions were counteracted by KGF.

This illustrates a substantial reduction in the mucosal response to fractionated irradiation. In a clinical setting, this should translate into a marked reduction of radiation sequelae at a given dose.

With single injections of rHuKGF at a dose of 5 mg/kg, given on either Day −1 or Day +4, the mucoprotective efficacy was comparable to that of repeated application. The resulting ED50 values were in the range of that for single-dose irradiation alone, indicating compensation of the entire fractionated radiation dose. Single rHuKGF doses of 15 mg/kg were even more effective and resulted in ED50 values that were higher than those for single-dose irradiation, indicating that tissue tolerance was even higher than in previously untreated control tissue. These results suggest that the rHuKGF treatment protocol, both the timing and dose, may be subject to optimization.

The maximum effect of KGF was observed after its administration during irradiation or, even more effective, during the weekend break. This facilitates speculation about the underlying mechanisms. In mouse oral mucosa, repopulation, i.e., the regenerative response of the tissue to radiation injury, sets in within the first week after onset of radiotherapy (7, 11, 25, 50). This process comprises acceleration of stem cell divisions in combination with loss of the division asymmetry, resulting in two stem cell daughters instead of one stem and one differentiating daughter. Another important mechanism is residual proliferation of lethally damaged cells that have lost their clonogenic character, but which obviously undergo 1 to 3 postirradiation divisions before they terminally differentiate. During treatment breaks, the proliferative activity of these sterilized cells seems to be most efficient. Also, during treatment splits even as short as the weekend break, stem cells return to asymmetrical divisions. Hence, during treatment, but most effectively during breaks, cell loss is at least partially compensated. This has been described in detail for mouse mucosa (7–9, 11), but has also been observed in patients (14).

The preirradiation effect, but partially also the efficacy of rHuKGF given during radiotherapy, may be based on stimulation of stem cell proliferation, yielding more stem cells and also more differentiating cells. However, definitely the efficacy after single irradiation, and presumably also the rHuKGF effect during radiotherapy and treatment breaks, may have another biologic basis. rHuKGF seems to stimulate residual proliferation, thus enabling damaged cells to undergo a higher number of abortive divisions. This results in higher overall cell numbers when radiotherapy is continued. The higher number of cells is able to counteract cell loss for a longer time, and hence provides longer intervals for stem cell repopulation to become effective without the development of clinically manifest ulcer. These hypotheses, however, have to be validated in histologic studies, which are currently under way.

One major prerequisite for mucoprotection by rHuKGF in radiotherapy of malignant disease is the safety of the application in human patients. Based on experimental results, a Phase I study was initiated in healthy volunteers; it
was found that three injections of KGF induced a significant increase in oral mucosal proliferation rate (51, 52). Efficacy of rHuKGF was evaluated in patients receiving high-dose chemotherapy in combination with radiotherapy for hematologic malignancies. In this Phase II study, a combined pre- and post-rHuKGF treatment reduced the duration of severe oral mucositis and accompanying sequelae, such as mouth and throat soreness and the use of pain medications (53). The recombinant human growth factor was well tolerated.

Importantly, rHuKGF must not protect or spare tumors, relative to normal tissue, from radiotherapy. In vitro studies in established tumor cell lines have illustrated a minimum, if any, sparing effect by KGF (54). In primary human cell lines, established from tumors of lung or head and neck, an effect of KGF was found on neither clonogenic survival nor proliferation (colony size) (55, 56). In contrast, normal epithelial cells showed an increased survival (54) or a significant increase in colony size (55, 57), again indicating stimulation of nonclonogenic proliferation. Tumor xenografts, transplanted into nude mice and subjected to radiotherapy, also did not show a significant effect of rHuKGF (54).

In conclusion, these results suggest that rHuKGF may provide an effective method for the modification of upper gastrointestinal side effects of radiotherapy without impairment of tumor cure rates.

REFERENCES

35. Horiot JC, Bontemps P, Van den Bogaert W, et al. Accelerated fractionation (AF) compared to conventional fractionation (CF) improves loco-regional control in the radiotherapy of advanced head and neck cancers: Results of the EORTC 22851 randomized trial. Radiother Oncol 1997;44:111–121.
37. Kaanders JH, Van der Kogel AJ, Ang KK. Altered fractionation (AF) compared to conventional fractionation (CF) improves loco-regional control in the radiotherapy of advanced head and neck cancers: Results of the EORTC 22851 randomized trial. Radiother Oncol 1997;44:111–121.