Keratinocyte Growth Factor Prevents Radiation Damage to Salivary Glands by Expansion of the Stem/Progenitor Pool

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ABSTRACT
Irradiation of salivary glands during radiotherapy treatment of patients with head and neck cancer evokes persistent hyposalivation. This results from depletion of stem cells, which renders the gland incapable of replenishing saliva to produce acinar cells. The aim of this study was to investigate whether it is possible to expand the salivary gland stem/progenitor cell population, thereby preventing acinar cell depletion and subsequent gland dysfunction after irradiation. To induce cell proliferation, keratinocyte growth factor (ΔN23-KGF, palifermin) was administered to C57BL/6 mice for 4 days before and/or after local irradiation of salivary glands. Salivary gland vitality was quantified by in vivo saliva flow rates, morphological measurements, and a newly developed in vitro salisphere progenitor/stem cell assay. Irradiation of salivary glands led to a pronounced reduction in the stem cells of the tissues, resulting in severe hyposalivation.

INTRODUCTION
Yearly, head and neck cancer is newly diagnosed in more than 45,000 patients worldwide [1]. Radiotherapy, either alone or in combination with surgery and chemotherapy, is often applied as a treatment for these patients. The radiation dose by which the tumor can be treated is limited by the sensitivity of surrounding normal tissues within the field of radiation. For head and neck cancers, even with the most optimal radiation schedule, salivary glands are tissues at risk. Progressive loss of function may occur within the first weeks of radiotherapy and can persist for life [2]. Radiation-induced salivary gland dysfunction may cause oral dryness, dental caries, hampered speech, and xerostomia (dry mouth syndrome), which collectively severely limit the quality of life of the patients [3, 4].

The delayed loss of gland function after radiation is thought to be due to a loss of stem cells that are no longer able to replenish aged saliva-producing acinar cells [5]. In normal salivary glands, the ductal system that includes excretory, striated, and intercalated ducts biochemically modifies and transports saliva, produced by acinar cells, into the oral cavity. This ductal system also contains the tissue stem/progenitor cells [6–8]. Proliferation and differentiation of these primitive cells within the ducts maintains homeostasis of the acinar cells. Expansion of the salivary gland stem/progenitor cell population may prevent acinar cell depletion and subsequent gland dysfunction after radiation. In line with this, transplantation of the tissue stem cells induced regeneration of irradiated salivary glands [7].

Although the effect of keratinocyte growth factor (ΔN23-KGF, fibroblast growth factor-7 [FGF]-7, palifermin) on the salivary gland has not been studied, ΔN23-KGF has been shown to ameliorate radiation-induced damage in a variety of other tissues, such as lung [9–12], gut [13, 14], tongue [15], and oral mucosa [16]. The mechanism is believed to result from either stimulation of proliferation [14, 17–21], direct radioprotection [22], and/or stimulation of cell motility [23].

In this study, several ΔN23-KGF treatment schedules were evaluated for their efficacy to reduce salivary gland morphology and intercalated ducts biochemically modifies and transports saliva, produced by acinar cells, into the oral cavity. This ductal system also contains the tissue stem/progenitor cells [6–8]. Proliferation and differentiation of these primitive cells within the ducts maintains homeostasis of the acinar cells. Expansion of the salivary gland stem/progenitor cell population may prevent acinar cell depletion and subsequent gland dysfunction after radiation. In line with this, transplantation of the tissue stem cells induced regeneration of irradiated salivary glands [7].

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To determine the effect of ΔN23-KGF on cell proliferation, 5-bromo-2′-deoxyuridine (BrdU) (50 mg/kg i.p.) was administered together with ΔN23-KGF treatment, when appropriate. Twenty-four hours after the last ΔN23-KGF/BrdU injection, mice were sacrificed, and glands were collected for further investigation.

Saliva Collection
At 30, 60, and 90 days after irradiation the saliva flow rate was determined. Animals were placed in a restraining device [24], and saliva was collected for 15 minutes after pilocarpine injection (2 mg/kg s.c.). The saliva volume was determined gravimetrically, assuming a density of 1 g/ml for saliva.

Immunohistochemical Processing
After extirpation, the submandibular glands were weighed and incubated for 30 hours at 4°C in 4% buffered formaldehyde. After dehydration, the tissue was embedded in paraffin. Sections (5 μm) were analyzed for acinar cells using periodic acid-Schiff’s base (PAS) staining. Ductal cells were identified using an anti-CK7 (M0N3007; Monosan, Burlingame, CA, http://www.monosan.com) antibody, the receptor for KGF was detected using an anti-β-catenin antibody (MAB7161; R&D Systems, Minneapolis, http://www.rndbContext.com) after a trypsin pretreatment, and proliferation was assessed by BrdU presence using anti-BrdU antibodies (ab6326; Abcam, Cambridge, U.K., http://www.abcam.com) (1:500 1 hour) after citrate treatment. Secondary anti-rat biotin antibodies (Elite ABC-kit; Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) and diaminobenzidine were used to detect the expression. In addition, nuclear staining was performed (hematoxylin).

Quantification of Acinar and Ductal Cells in Salivary Glands
Tissue sections of submandibular glands were analyzed using bright-field microscopy (CX40; Olympus, Hamburg, Germany, http://www.olympus-global.com) under ×400 magnification, with evaluation of 100 squares of 0.25 mm² each. The percentage of surface area occupied by acinar cells was counted from two different sections (upper middle) of each submandibular gland.

The different duct compartments (excretory, striated, and intercalated duct cells) were quantified by using the analySIS program (Olympus Soft Imaging System; Olympus, Münster, Germany, http://www.olympus-global.com) by measuring the area occupied by the different duct compartments. In further data processing, the percentage of the surface area of the ducts and acinar cells per gland was calculated.

Determination of Stem/Progenitor Cell Number
Submandibular glands were extirpated and processed for cell isolation as described previously [7]. Salivary gland cells were plated in a defined medium of Dulbecco’s modified Eagle’s medium/Ham’s F-12 (catalog nos. 41966-029 and 21765-029, respectively; Gibco, Carlsbad, CA, http://www.invitrogen.com), penicillin, streptomycin, GlutaMAX, epidermal growth factor (20 ng/ml), FGF-2 (20 ng/ml), N2 (1/100), insulin (10 μg/ml), and dexamethasone (1 μM) [25]. All growth factors were purchased from Sigma-Aldrich (St. Louis, http://www.sigmaldrich.com), except for N2 (Gibco). After 3 days of culture, spheres were counted and recalculated as a percentage of plated cells.

For flow cytometric analysis of c-Kit+ cells, salispheres were dissociated by 0.05% trypsin-EDTA (catalog no. 25300; Gibco) with mechanical use of 26-gauge needles. Cells were incubated for 20 minutes with anti-c-Kit fluorescein isothiocyanate (catalog no. 553354; BD Biosciences Pharmingen, San Diego, http://www.bdbiosciences.com/index_us.shtml) antibodies at 4°C. Cells were analyzed on a FACS Calibur Flow Cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www bd.com) after the addition of propidium iodide (2 μg/ml) to select for living cells. For each measurement a minimum of 100,000 events were collected. Data were analyzed

**Materials and Methods**

**Animals**
Female C57BL/6 mice, 8–12 weeks old, were purchased from Harlan (Horst, The Netherlands, NL, http://www.harlan.europe.com). The mice were kept under clean conventional conditions and fed ad libitum with food pellets (RMH-B; Hope Farms B.V., Woerden, The Netherlands, http://www.hopefarms.nl) and acidified tap water (pH = 2.8). All experiments were approved by the ethics committee on animal testing of the University of Groningen.

**Irradiation of the Salivary Glands**
Salivary glands were locally irradiated with a single dose of 10, 12.5, 15, 17.5, or 20 Gy of radiation (CMG 41 X, 200 kV, 10 mA, 5 Gy/min; Philips, Eindhoven, The Netherlands, http://www.philips.nl). Mice were protected from off-target radiation by a lead shield. These radiation doses are known to induce sufficient damage without compromising the general health of the animals.

**ΔN23-KGF and 5-Bromo-2′-Deoxyuridine Administration**
ΔN23-KGF (5 mg/kg per day, Amgen, Thousand Oaks, CA, http://www.amgen.com), the recombinantly produced form of KGF in which the first 23 amino acids of the amino terminus have been deleted from the mature KGF, was used because of its increased stability and was administered s.c. before or/and directly after irradiation according to the scheme in Figure 1. Unirradiated mice received ΔN23-KGF for 4 or 7 consecutive days.
Gland Dysfunction

Salivary glands (supplemental online Fig. 1). As expected, irradiated mice developed severe hyposalivation (Fig. 2A, blue cells, arrowheads) comprising three different cell types; intercalated, striated, and excretory duct cells which are collectively responsible for the modification and transport of the saliva produced by the acinar cells [26].

To investigate which cells are responsible for the protective effect of \( \Delta N23 \)-KGF on acinar cells, the mice were sacrificed at 30 days after irradiation, and the acinar cells were isolated and cultured in vitro. The expression levels of markers specific to each type of cell were analyzed using RT-qPCR. The results showed that the expression levels of intercalated duct cells and acinar cells were significantly increased in the \( \Delta N23 \)-KGF-treated group compared to the control group, indicating that \( \Delta N23 \)-KGF treatment restored the normal morphology and function of salivary glands.

RESULTS

\( \Delta N23 \)-KGF Prevents Radiation-Induced Salivary Gland Dysfunction

To investigate whether administration of \( \Delta N23 \)-KGF could reduce radiation toxicity to salivary glands, mice receiving local salivary gland irradiation (IR) (15 Gy) were treated with \( \Delta N23 \)-KGF either four times before (K-IR) or after irradiation (IR-K) or for a 7-day schedule with irradiation in the middle of the treatment (K-IR-K) (Fig. 1). Ninety days after irradiation, submandibular gland weight decreased to 58% of that in untreated controls. In agreement with the flow rate data, all \( \Delta N23 \)-KGF treatment schedules had significant beneficial effects on submandibular gland weight and gland weight correlated with saliva production, suggesting that \( \Delta N23 \)-KGF exerts protective effects before and after irradiation.

Trophic Effect of \( \Delta N23 \)-KGF on Acinar Cells

To investigate which cells are responsible for the protective effect of \( \Delta N23 \)-KGF, submandibular glands from \( \Delta N23 \)-KGF-treated irradiated and nonirradiated mice were examined (Fig. 3). A normal salivary gland consists of approximately 60% of PAS\(^+\) mucin-containing acinar cells grouped in acini (Fig. 3A, purple cells, arrows) connected to the ductal compartment (Fig. 3A, blue cells, arrowheads) comprising three different cell types; intercalated, striated, and excretory duct cells which are collectively responsible for the modification and transport of the saliva produced by the acinar cells [26].

\( \Delta N23 \)-KGF treatment for 4 or 7 days caused a rapid (24 hours after the last injection) but modest, increase in acinar cell surface area (10%) (data not shown), which almost normalized in time (90 days post-treatment) (Fig. 3B, 3G). Massive acinar cell depletion (Fig. 3C, arrows, 3G) and fibrinoid cell deposition (Fig. 3C, asterisks), hallmarks of late irradiation damage, were clearly visible 90 days after irradiation. In contrast, post-irradiation treatment with \( \Delta N23 \)-KGF (Fig. 3D, 3G) reduced acinar

Figure 2. \( \Delta N23 \)-KGF treatment affects gland function. (A): Saliva flow rates were measured 30, 60, and 90 days after radiation. IR-K, K-IR, and K-IR-K (see Fig. 1) treated mice produced significantly more saliva compared to the untreated irradiated mice (IR). Saliva flow rates of 4D K and 7D K treated mice did not significantly differ from those of normal mice. (B): Saliva flow rates were measured in IR and K-IR-K treated mice 30 days after irradiation. No change in slope could be detected. For all data a minimum of three mice were used. p < .05 *, Error bars represent SEM. Abbreviations: 4D K, treatment with \( \Delta N23 \)-KGF for 4 days; 7D K, treatment with \( \Delta N23 \)-KGF for 7 days; IR, irradiation; IR-K, \( \Delta N23 \)-KGF after irradiation; K-IR, \( \Delta N23 \)-KGF before irradiation; K-IR-K, irradiation in the middle of \( \Delta N23 \)-KGF treatment; KGF, keratinocyte growth factor.
cell loss, whereas pretreatment largely (Fig. 3E, 3G) and preplus post-treatment (Fig. 3F, 3G) almost completely abrogated the net loss of acinar cells.

**ΔN23-KGF Enhances Stem/Progenitor Cell Numbers**

To assess whether the protective effect of ΔN23-KGF is due to a proliferative effect on acinar cells, in vivo BrdU incorporation in submandibular glands from animals treated with ΔN23-KGF for 4 days was investigated. Whereas glands from normal mice hardly showed any proliferating cells, large numbers of BrdU+ acinar cells were observed in salivary glands of ΔN23-KGF-treated animals 24 hours after the last treatment (Fig. 4A). Similar effects of ΔN23-KGF on the proliferation of oral mucosa were shown before by Borges et al. [18]. Additionally, BrdU incorporation was clearly present in intercalated (arrowheads) and excretory duct cells (arrows), indicating that these cell types also were stimulated to proliferate. Remarkably, however, the receptor for ΔN23-KGF, FGFR2IIIb, was exclusively expressed on intercalated (Fig. 4B, arrowheads) and excretory duct cells (Fig. 4B, arrows) and not on acinar cells. In addition to the 10% increase in acinar cells noticed after 24 hours in the 4-day KGF-treated mice, this implies that the increase in proliferation of acinar cells probably originated from a direct stimulatory effect of ΔN23-KGF on intercalated and/or excretory duct cells that subsequently differentiate into acinar cells. To test this, the surface area occupied by these different ductal cell types, as a reflection of cell number, was evaluated after a 4-day exposure to ΔN23-KGF. Twenty-four hours after the last ΔN23-KGF injection, an increase in surface area of excretory (2.8-fold) and intercalated duct cells (1.8-fold) in these glands was observed (Fig. 4C), reflecting their high proliferation rate. After 90 days the surface area occupied by excretory duct cells remained somewhat elevated, whereas that of other cell types was back to baseline values. The transient enhancement of the number of intercalated and excretory duct cells in particular shortly after treatment indeed indicates that the increase in proliferation of acinar cells originates from dividing and subsequently differentiating intercalated duct cells. It also suggests that ΔN23-KGF might expand the number of stem/progenitor cells known to reside in the ducts [6].

To further substantiate the idea of expanding the pool of stem/progenitor cells, we cultured (sali)-spheres from salivary gland stem/progenitor cells [7], similar to mammospheres [27] and neurospheres [28]. These spheres clearly expressed the KGF receptor (KGFR/FGFR2IIIb) (supplemental online Fig. 2A) and responded to KGF treatment with phosphorylation of MAPKs (supplemental online Fig. 2B). Interestingly, significantly more salispheres were formed from submandibular glands of mice treated with ΔN23-KGF for 4 days compared with untreated animals (Fig. 4D, Normal vs. 4D K), demonstrating that KGF indeed induced the expansion of salivary gland salisphere-forming cells. As expected, irradiation (15 Gy) resulted in a pronounced reduction in the number of salispheres formed in culture (Fig. 4D, IR). In the ΔN23-KGF pretreated animals, the remaining number of salisphere-forming cells that were recovered from the glands after 15 Gy was far higher and almost equal to the number recovered from glands from untreated controls (Fig. 4D, K-IR). Salipheres cultured from untreated animals contained ~5% c-Kit+ cells, a percentage that did not change after IR and/or any of the ΔN23-KGF treatments (data not shown). This finding indicates that ΔN23-KGF treatment results in a net increase in salivary gland stem/progenitor cells (expansion), and thus the absolute number or remaining salisphere-forming cells after radiation is increased.

However, it has also been suggested that ΔN23-KGF may be radioprotective for certain types of cells [22, 29–31],
which would imply that the relative loss of salivary gland stem/progenitor cells also may be reduced after \( \text{H9004 N23-KGF} \) pretreatment. In fact, our 15-Gy data (Fig. 4D) indeed suggested that the relative loss of salisphere-forming cells may be reduced after \( \text{H9004 N23-KGF} \) pretreatment. To test whether this effect is indeed significant, glands of mice treated with \( \Delta N23 \)-KGF were subsequently irradiated with graded doses (10, 12.5, 15, 17.5, and 20 Gy), and the number of salispheres that were formed in vitro 24 hours after the last \( \text{H9004 N23-KGF} \) injection was calculated (Fig. 4E). Indeed, a dose-dependent decrease in sphere formation after an \( \Delta N23 \)-KGF pretreatment was equal over all doses, and the slopes of these curves were virtually the same (Fig. 4E). This result suggests that \( \Delta N23 \)-KGF has no effect on the intrinsic radiosensitivity of these primitive cells.

Taken together, these data show that \( \Delta N23 \)-KGF increases resistance of salivary glands to irradiation by increasing the endogenous stem cell compartment, resulting in larger absolute number of surviving stem cells after irradiation.

**Postirradiation \( \Delta N23 \)-KGF Treatment Expands the Number of Radiation-Surviving Stem Cells**

If the protection of \( \Delta N23 \)-KGF pretreatment is due to an increment in the pool of progenitor/stem cells, the question...
arises as to why the post-treatment with ΔN23-KGF enhances gland recovery, especially in glands that were also pretreated with ΔN23-KGF (Figs. 2, 3). To selectively investigate the effects of postradiation ΔN23-KGF, we analyzed the glands of animals 24 hours after the last injection for proliferative cells using BrdU labeling. In glands that were irradiated without ΔN23-KGF, BrdU+ cells were totally absent (not shown), but post-treatment with ΔN23-KGF revealed BrdU+ cells in intercalated (Fig. 5A) and excretory duct cells (not in figure). In parallel, the surface area of these duct cells was higher at 10 days and even at 90 days after irradiation for the animals post-treated with ΔN23-KGF (Fig. 5B). In agreement with this finding, a post-irradiation ΔN23-KGF treatment induced almost a doubling in the number of salispheres that could be isolated from glands 4 days after irradiation compared to untreated glands (Fig. 5C). Similar, albeit more pronounced, results were obtained after a ΔN23-KGF pre- and post-irradiation treatment schedule (data not shown). To summarize, whereas ΔN23-KGF pretreatment enhances absolute salivary gland cell numbers before radiation, post-treatment accelerates the expansion of these surviving progenitor/stem cell pools. The latter effect is obviously more pronounced when there are more remaining progenitor/stem cells, explaining why post-treatment with ΔN23-KGF is specifically effective in sparing gland function in ΔN23-KGF-pretreated animals.

**DISCUSSION**

This study demonstrates that expansion and activation of stem/progenitor cells by administration of ΔN23-KGF before and after irradiation of the salivary glands yielded an almost normal saliva secretion and long-term preservation of all submandibular gland cell types. Several studies have suggested that KGF can increase the radioresistance of epithelial cells by enhancing DNA repair [22], by altering expression of mediators or antagonists of apoptosis [32], or by altering the ability of cells to scavenge free radicals [29–31]. On the other hand, forcing cells into the cell cycle may make them more susceptible to irradiation. However, we were unable to detect any change in radiosensitivity by ΔN23-KGF using our ex vivo salisphere assay. Instead, we suggest that the expansion of the stem cell pool appears to be mainly responsible for the observed amelioration of radiation-induced damage to the submandibular gland. We showed that pretreatment with ΔN23-KGF increases the number of progenitor/stem cells, leading to a higher absolute number after radiation. Post-treatment ΔN23-KGF can accelerate the proliferation/ expansion of the fraction of progenitor/stem cells that survived the radiation and hence further stimulate the effect of pretreatment ΔN23-KGF.

ΔN23-KGF enhanced BrdU labeling in cells of acinar and duct compartments of the submandibular gland, which suggested that all cell types are proliferating. However, acinar cells do not express the FGFR2IIb receptor and are therefore not likely to be stimulated by ΔN23-KGF. Differentiation from intercalated duct cells into acinar cells has been shown to occur in submandibular glands of mice [6], rats [8, 33], and humans [34]. Therefore, the labeling and enhanced number of BrdU+ acinar cells is probably caused by proliferation and subsequent differentiation of intercalated duct cells. After ΔN23-KGF stimulation, excretory and intercalated duct cells, both of which do express the ΔN23-KGF receptors, rapidly increased in number, resulting in elongation of excretory ducts. Interestingly, during normal aging these ducts decrease in length [6]. The current study shows that after irradiation the number of salivary gland stem/progenitor cells surviving irradiation can be doubled by a pretreatment with ΔN23-KGF.

The capacity of surviving stem/progenitor cells to (partly) repopulate the gland after stimulation with ΔN23-KGF provides an interesting opportunity for novel targeted therapy. For future clinical use, a potential issue of concern is the possibility that ΔN23-KGF may stimulate tumor proliferation. However, malignant hematopoietic cells, for example, are unresponsive to ΔN23-KGF [35] and, strikingly, ΔN23-KGF is now used in phase III trials to prevent chemo/ radiotherapy-induced oral mucositis in patients with hematopoietic malignancies [35]. Furthermore, head and neck squamous carcinoma cell lines expressing FGFR2IIb receptors did not show an in vitro growth advantage or alteration in radiation sensitivity relative to normal nasal epithelial cells upon ΔN23-KGF stimulation [36, 37]. This finding indicates that therapeutically effective doses of ΔN23-KGF may not stimulate head and neck tumor cell growth. However, this issue needs to be further explored carefully. Other membrane receptor-stimulating agents that ameliorate salivary gland damage after irradiation such as the muscarinic agonist pilocarpine [38] have been suggested to enhance postirradiation proliferation [39]. However, KGF is by far the most effective but only when administered both before and after irradiation.

Our study provides the first evidence that in vivo induction of expansion and differentiation of stem/progenitor cells by ΔN23-KGF protects salivary glands against radiation damage. Hence, ΔN23-KGF...
is a promising therapeutic modality to prevent radiation-induced gland dysfunction in patients with head and neck cancer.

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