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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 and a reduced number of acinar cells. ΔN23-KGF treatment for 4 days before irradiation indeed induced salivary gland stem/progenitor cell proliferation, increasing the stem and progenitor cell pool. This did not change the relative radiation sensitivity of the stem/progenitor cells, but, as a consequence, an absolute higher number of stem/progenitor cells and acinar cells survived after radiation. Postirradiation treatment with ΔN23-KGF also improved gland function, and this effect was much more pronounced in ΔN23-KGF pretreated animals. Post-treatment with ΔN23-KGF seemed to act through accelerated expansion of the pool of progenitor/stem cells that survived the irradiation treatment. Overall, our data indicate that ΔN23-KGF is a promising drug to enhance the number of salivary gland progenitor/stem cells and consequently prevent radiation-induced hyposalivation. STEM CELLS *2008;26:2595–2601*

Disclosure of potential conflicts of interest is found at the end of this article.

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

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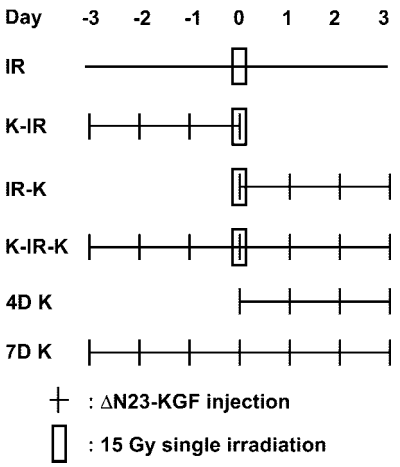

Figure 1. Schematic representation of the experimental setup. Local 15-Gy salivary gland irradiation was given before or after a 4-day ΔN23-KGF treatment (5 mg/kg per day), or on the fourth day of a 7-day ΔN23-KGF treatment. Mice in the 4- or 7-day KGF group only received daily ΔN23-KGF injections for 4 or 7 days, respectively. Abbreviations: 4D K, treatment with ΔN23-KGF for 4 days; 7D K, treatment with ΔN23-KGF for 7 days; IR, irradiation; IR-K, ΔN23-KGF after irradiation; K-IR, ΔN23-KGF before irradiation; K-IR-K, irradiation in the middle of ΔN23-KGF treatment; KGF, keratinocyte growth factor.

and function after irradiation in mice. In addition, effects were evaluated using a recently developed in vitro salivary gland stem/progenitor assay [7]. It was found that ΔN23-KGF administration before and after irradiation of salivary glands induced a long-term expansion of duct stem/progenitor cells, which elicited short- and long-term maintenance of the acinar cell compartment, resulting in permanent preservation of the salivary gland function after irradiation.

Materials and Methods

Animals

Female C57BL/6 mice, 8–12 weeks old, were purchased from Harlan (Horst, The Netherlands, NL, http://www.harlaneurope.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.

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 (MON3007; Monosan, Burlingame, CA, http://www.monosan.com) antibody, the receptor for KGF was detected using an anti-FGFR2IIIbantibody (MAB7161; R&D Systems, Minneapolis, http://www.rndsystems.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 mm^2 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'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.sigmaaldrich.com), except for N_2 (Gibco). After 3 days of culture, spheres were counted and recalculated as a percentage per 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 FACSCalibur 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

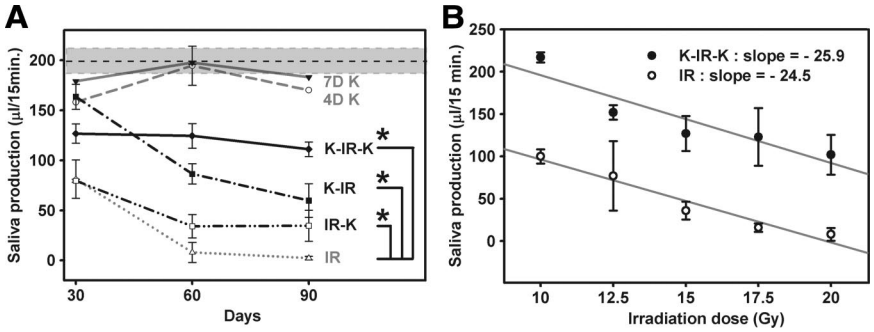

Figure 2. ΔN23-KGF treatment affects gland function. **(A):** Saliva flow rates were measured 30, 60, and 90 days after radiation. IR-K, K-IR, and K-IR-K (see Fig. 1) treated mice produced significantly more saliva compared to the untreated irradiated mice (IR). Saliva flow rates of 4D K and 7D K treated 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 ΔN23-KGF for 4 days; 7D K, treatment with ΔN23-KGF for 7 days; IR, irradiation; IR-K, ΔN23-KGF after irradiation; K-IR, ΔN23-KGF before irradiation; K-IR-K, irradiation in the middle of ΔN23-KGF treatment; KGF, keratinocyte growth factor.

using FlowJo (Tree Star, Ashland, OR, http://www.treestar.com).

Salispheres Are Responsive to ΔN23-KGF

Three-day-old untreated salispheres were stimulated with KGF (100 ng/ml) and collected 10, 30, or 60 minutes hereafter. The spheres were treated on ice with lysis buffer (50 mM Tris-HCl [pH 7.4], 5 mM EDTA, 250 mM NaCl, and 0.1% Triton X-100) supplemented with protease inhibitors (catalog no. 1697498; Roche Diagnostics, Laval, QC, Canada, http://www.roche-applied-science.com) and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 25 mM Na_3VO_4, and 0.5 M NaF). After the protein concentration was determined by the Bradford assay, the extracts were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were electrotransferred to nitrocellulose membranes. Immunodetection was performed using ECL WB substrate (catalog no. 32106; Pierce, Rockford, IL, http://www.piercenet.com) and antibodies against mitogen-activated protein kinase (MAPK) (M5780; Sigma-Aldrich), FGFR2IIIb (MAB7161; R&D Systems), and γ-tubulin (T6557; Sigma-Aldrich).

Statistical Analysis

The results were analyzed using a Mann-Whitney test or Student's *t* test. Statistical significance was defined as $p < .05$ using SPSS (SPSS, Chicago, IL, http://www.spss.com). Numbers represent mean ± SEM.

RESULTS

ΔN23-KGF Prevents Radiation-Induced Salivary Gland Dysfunction

To investigate whether administration of ΔN23-KGF could reduce radiation toxicity to salivary glands, mice receiving local salivary gland irradiation (IR) (15 Gy) were treated with ΔN23-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). As control groups, mice that were only irradiated and mice that received only ΔN23-KGF were included. Salivary gland function measurements (30, 60, and 90 days after irradiation) showed that saliva production in nonirradiated mice that were treated with ΔN23-KGF for 4 or 7 days was not significantly different from that in untreated animals (Fig. 2A) albeit with some gland hypertrophy as indicated by an increase in wet weight of the submandibular glands (supplemental online Fig. 1). As expected, irradiated mice that did not receive ΔN23-KGF developed severe hyposalivation (Fig. 2A). In contrast, ΔN23-KGF treatment, irrespective of the treatment schedule, resulted in significantly more saliva production in irradiated mice than in nontreated animals. ΔN23-KGF treatment before radiation resulted in a delayed onset of radiation-induced reduction in saliva flow rate, whereas ΔN23-KGF treatment after radiation was least effective. However, when ΔN23-KGF was administered both before and after radiation, saliva production was best preserved (Fig. 2A). To establish potential dose dependence of a K-IR-K treatment, saliva flow rate was measured at 30 days after graded doses of irradiation (Fig. 2B). Indeed, a dose-dependent reduction in flow rate was observed, however, with a virtually identical slope as that for irradiation alone, indicating that the effect of ΔN23-KGF treatment before and after irradiation is not radiation dose-dependent.

Significantly higher wet submandibular gland weights were observed 3 months after 4 or 7 days of ΔN23-KGF treatment, compared with those in untreated animals (supplemental online 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 ΔN23-KGF treatment schedules had significant beneficial effects on submandibular gland weight and gland weight correlated with saliva production, suggesting that ΔN23-KGF exerts protective effects before and after irradiation.

Trophic Effect of ΔN23-KGF on Acinar Cells

To investigate which cells are responsible for the protective effect of ΔN23-KGF, submandibular glands from ΔN23-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].

ΔN23-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 fibrotic cell deposition (Fig. 3C, asterisks), hallmarks of late irradiation damage, were clearly visible 90 days after irradiation. In contrast, post-irradiation treatment with ΔN23-KGF (Fig. 3D, 3G) reduced acinar

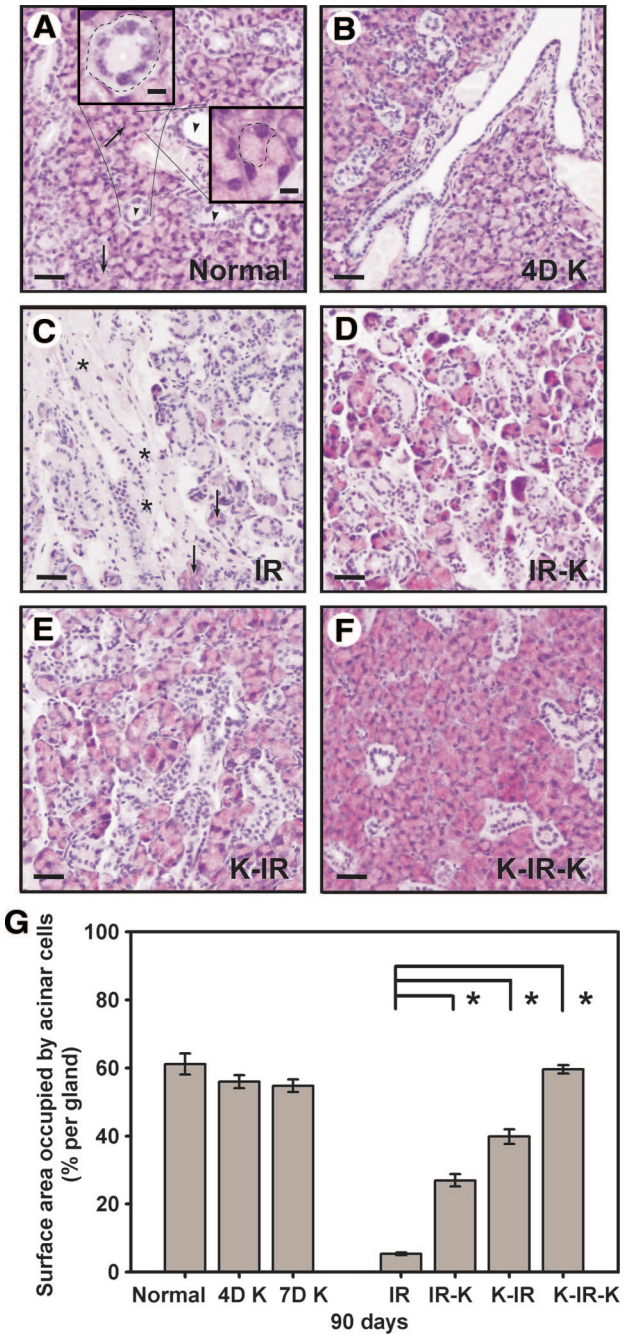

Figure 3. The effect of ΔN23-KGF on acinar cell number. **(A–F):** Salivary glands of different groups (normal, IR, 4D K, K-IR, IR-K, and K-IR-K; see Fig. 1) were evaluated by PAS staining 90 days after irradiation. Arrows represent acinar cells, arrowheads represent duct cells, and asterisks indicate fibrosis. Enlarged pictures represent duct and acinar cells (dotted line in insets). **(G):** 90 days after irradiation, glands of K-IR, IR-K, and K-IR-K treated mice contained significant more acinar cells than those of IR treated mice. *, $p < .05$. Scale bar = 50 μm, inset = 20 μm, $n \geq 3$, depicted as mean ± SEM. Abbreviations: 4D K, treatment with ΔN23-KGF for 4 days; 7D K, treatment with ΔN23-KGF for 7 days; IR, irradiation; IR-K, ΔN23-KGF after irradiation; K-IR, ΔN23-KGF before irradiation; K-IR-K, irradiation in the middle of ΔN23-KGF treatment; KGF, keratinocyte growth factor.

cell loss, whereas pretreatment largely (Fig. 3E, 3G) and pre- plus 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% $c\text{-}Kit^+$ 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],

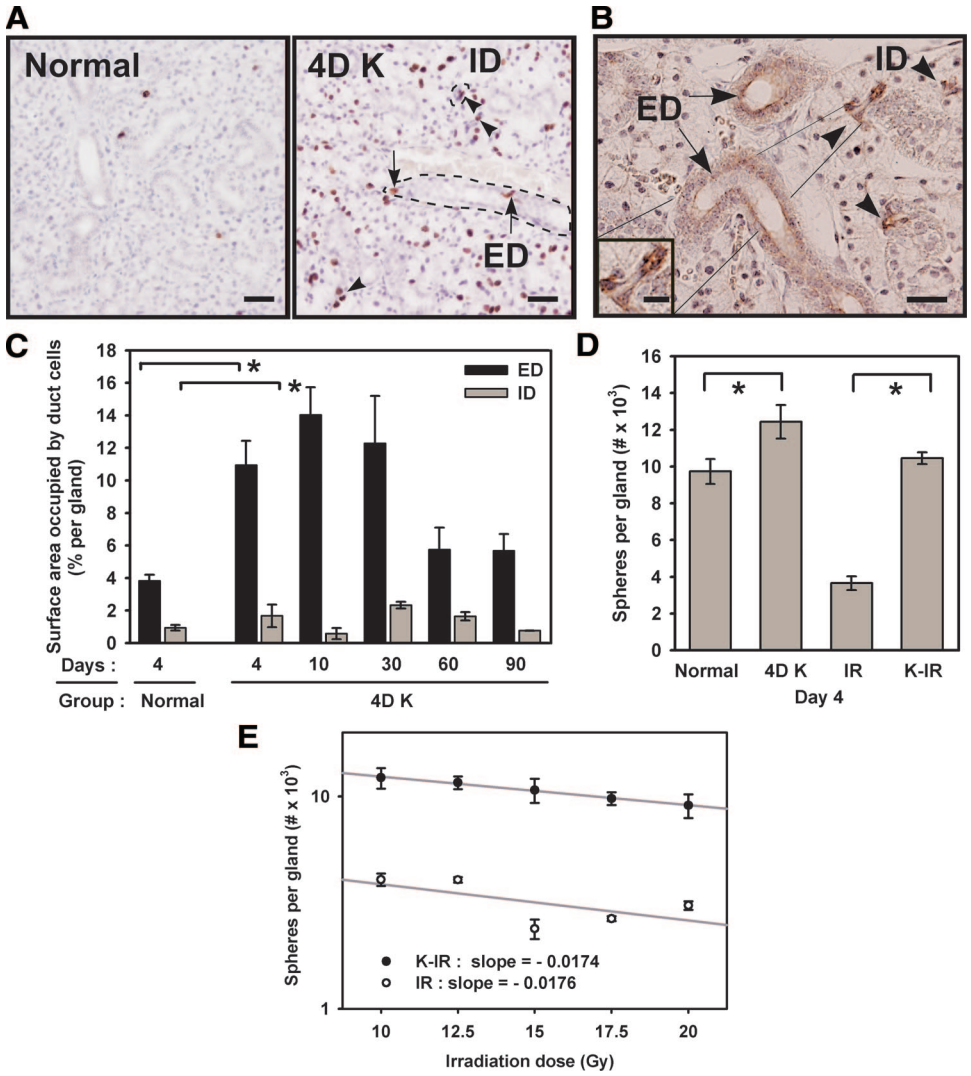

Figure 4. ΔN23-KGF increases progenitor/stem cells after radiation. **(A):** Untreated, normal glands and glands 4D K treated mice were evaluated for the presence of BrdU incorporation 24 hours after the last ΔN23-KGF/BrdU administration. BrdU staining (brown) was present in ED cells (arrows) and ID cells (arrowheads). **(B):** Immunostaining for ΔN23-KGF receptor (brown) revealed its expression by ID (arrowheads, inset) and ED cells (arrows). **(C):** Significant increased surface areas occupied by ED and ID cells in 4D K treated mice compared with normal glands at day 4. **(D):** The number of salispheres formed in culture from glands of normal, 4D K, IR, and K-IR treated mice sacrificed on day 4 showed that significantly more salispheres were detected after ΔN23-KGF treatment compared with the numbers for untreated groups. **(E):** Salispheres were counted from IR and K-IR treated mice that received different radiation doses. Significantly more salispheres were recovered from glands in the K-IR group compared with the IR group. However, the irradiation dose-response curve (slope) was not altered by KGF. For all data in **(C–E)** a minimum of three mice were used. *, $p < .05$. Error bars represent SEM. Scale bar = 50 μm; inset = 20 μm. Abbreviations: 4D K, treatment with ΔN23-KGF for 4 days; 7D K, treatment with ΔN23-KGF for 7 days; IR, irradiation; IR-K, ΔN23-KGF after irradiation; K-IR, ΔN23-KGF before irradiation; K-IR-K, irradiation in the middle of ΔN23-KGF treatment; KGF, keratinocyte growth factor.

which would imply that the relative loss of salivary gland stem/progenitor cells also may be reduced after ΔN23-KGF pretreatment. In fact, our 15-Gy data (Fig. 4D) indeed suggested that the relative loss of salisphere-forming cells may be reduced after ΔN23-KGF pretreatment. To test whether this effect is indeed significant, glands of mice treated with ΔN23-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 ΔN23-KGF injection was calculated (Fig. 4E). Indeed, a dose-dependent decrease in salisphere-forming cells was observed. At all doses, the number of salispheres from irradiated glands was significantly higher after ΔN23-KGF-treatment than after irradiation alone. However, the relative radiation-induced decrease in sphere formation after an ΔN23-KGF pretreatment was equal over all doses, and the slopes of these curves were virtually the same (Fig. 4E). This result suggests that ΔN23-KGF has no effect on the intrinsic radiosensitivity of these primitive cells.

Taken together, these data show that ΔN23-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 ΔN23-KGF Treatment Expands the Number of Radiation-Surviving Stem Cells

If the protection of ΔN23-KGF pretreatment is due to an increment in the pool of progenitor/stem cells, the question

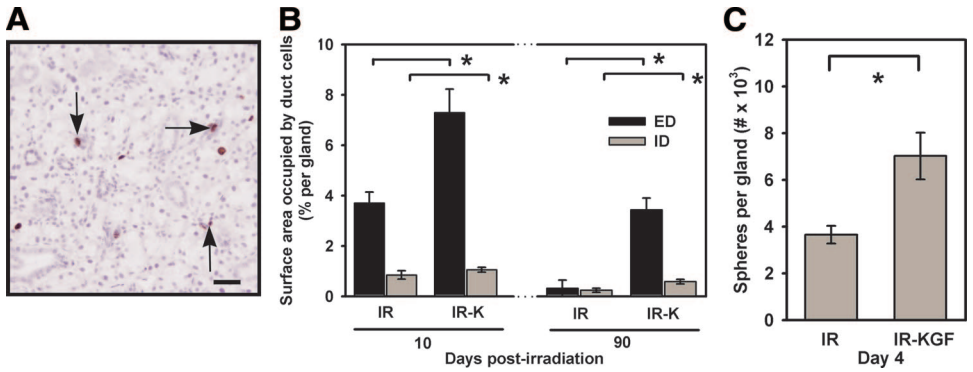

Figure 5. ΔN23-KGF stimulates surviving duct cells to proliferate after irradiation. **(A):** BrdU-retaining ID cells (arrows) were present in glands from IR-K (see Fig. 1) treated mice, 24 hours after the last ΔN23-KGF/BrdU injection. **(B):** The surface area occupied in by ED and ID cells, expressed as percentage per gland, was significantly increased in IR-K mice compared with that in IR treated mice both at 10 and 90 days after radiation. **(C):** Significantly more salispheres could be recovered from glands from IR-K mice compared with those from IR treated glands. Abbreviations: IR, irradiation; IR-K, IR-KGF, ΔN23-KGF after irradiation; KGF, keratinocyte growth factor.

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 FGFR2IIIb 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 FGFR2IIIb 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 indicate 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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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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