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Prevention of Radiation-Induced Salivary Hypofunction Following hKGF Gene Delivery to Murine Submandibular Glands

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Abstract

Purpose—Salivary glands are significantly affected when head and neck cancer patients are treated by radiation. We evaluated the effect of human keratinocyte growth factor (hKGF) gene transfer to murine salivary glands on the prevention of radiation-induced salivary hypofunction.

Experimental Design—A hybrid serotype 5 adenoviral vector encoding hKGF (AdLTR₂EF1 α -hKGF) was constructed. Female C3H mice, 8 weeks old, were irradiated by single (15 Gy) or fractionated (6 Gy for 5 days) doses to induce salivary hypofunction. AdLTR₂EF1 α -hKGF or AdControl was administered (10^8 – 10^{10} particles per gland) to both submandibular glands (SG) by retrograde ductal instillation before irradiation (IR). Salivary flow was measured following pilocarpine stimulation. Human KGF levels were measured by ELISA. SG cell proliferation was measured with bromodeoxyuridine labeling. Endothelial and progenitor or stem cells in SGs were measured by flow cytometry. The effect of SG hKGF production on squamous cell carcinoma (SCC VII) tumor growth was assessed.

Results—In 3 separate single-dose IR experiments, salivary flow rates of mice administered the AdLTR₂EF1 α -hKGF vector were not significantly different from nonirradiated control mice ($P > 0.05$). Similarly, in 3 separate fractionated IR experiments, the hKGF-expressing vector prevented salivary hypofunction dramatically. Transgenic hKGF protein was found at high levels in serum and SG extracts. AdLTR₂EF1 α -hKGF-treated mice showed increased cell proliferation and numbers of endothelial cells, compared with mice treated with AdControl. hKGF gene transfer had no effect on SCC VII tumor growth $\pm$ radiation.

Conclusions—hKGF gene transfer prevents salivary hypofunction caused by either single or fractionated radiation dosing in mice. The findings suggest a potential clinical application.

Introduction

Saliva is critical for multiple oral functions. Loss of salivary secretion, by whatever cause, leads to significant morbidity, including dysphagia, increased oral infections, and considerable discomfort. More than 60% of patients with head and neck cancer receiving

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

radiation therapy suffer from salivary hypofunction (xerostomia; refs. 1–4). As a result, there has been a substantial effort to minimize or eliminate this major side effect of radiation therapy for oral cancers.

Keratinocyte growth factor (KGF), also known as fibroblast growth factor (FGF) 7, is produced by cells of mesenchymal origin (5, 6), but is an epithelial cell-specific growth and differentiation factor acting exclusively through a subset of FGF receptors, FGFR2B (7). The human and mouse KGF receptors are 96% identical. Notably, the mouse receptor does not have an IG1 domain (8), and the KGF binding site is in the IG2 and IG3 domains (8, 9). Human KGF (hKGF) can bind functionally to the mouse KGF receptor (10, 11).

Many studies have shown that hKGF is a potentially useful agent to protect and regenerate damaged epithelial cells (12–18). Indeed, many groups have shown that recombinant hKGF can decrease the acute and chronic mucositis caused by chemoradiotherapy in animal models (12–18), and we have recently shown that hKGF gene transfer can prevent radiation-induced oral mucositis (19). Herein, we used murine submandibular glands (SG) as a target tissue and tested a hybrid adenoretroviral vector encoding hKGF, AdLTR₂EF1 α -hKGF (19, 20), for prevention of the salivary hypofunction occurring after single or fractionated radiation doses.

Materials and Methods

Construction of recombinant vectors

The vectors used (AdLTR₂EF1 α -hKGF and AdControl) were prepared exactly as described previously (19).

Cell culture

C7 cells, which are derived from 293 cells and stably express both the Ad5 preterminal protein and DNA polymerase (21), were grown in high glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen). The following supplements (Invitrogen) were included: 10% fetal bovine serum (BS), 100 U/mL penicillin G, and 100 μ g/mL streptomycin. Cells were incubated at 37°C in humidified 5% CO₂.

Experimental animals

Female C3H mice [National Cancer Institute (NCI) Animal Production Area; Frederick, MD] were used for this study. Mice were approximately 8 weeks of age at the time of experimentation. All experiments were executed under a protocol approved by the NCI Animal Care and Use Committee and were done in compliance with the Guide for the Care and Use of Laboratory Animal Resources National Research Council (1996).

In vivo viral vector delivery, blood, saliva, and tissue collection

Mice were anesthetized with ketamine (60 mg/kg) and xylazine (8 mg/kg) intramuscularly. Vectors were administered to both SGs by retrograde ductal instillation (22, 23). Groups of mice ($n = 4$ or 5 per treatment) received 1×10^{10} particles per gland of either AdControl or AdLTR₂EF1 α -hKGF on day zero (Figs. 1A and 2A). Animals that were not irradiated or irradiated alone served as control groups. For dose response experiments, groups of mice ($n = 4$ per treatment) received either 1×10^{10} particles/gland for the AdControl group or 1×10^8 , 1×10^9 , and 1×10^{10} particles per gland for AdLTR₂EF1 α -hKGF groups on day zero (Fig. 3). Blood, saliva, and SGs were collected after 9 weeks. For saliva collections, anesthetized mice were stimulated by 1 μ L/g body weight of a pilocarpine solution (0.05 mg/mL) subcutaneously. Whole saliva was collected with a 75-mm hematocrit tube (Drummond) into 1.5 mL preweighed Eppendorf tubes for 15 minutes. The tubes were then

reweighed to calculate the volume of saliva and frozen immediately thereafter. Salivary flow rates are reported as a percentage of the mean value of the nonirradiated group. Blood samples were obtained from the retro-orbital sinus following saliva collection. At the terminal time point, anesthetized animals were euthanized in a carbon monoxide chamber and SGs were removed. Soluble protein extracts of SGs and squamous cell carcinoma (SCC VII) tumors were prepared by the CelLyticM Cell Lysis Reagent (Sigma).

Animal radiation

As we previously described, the head and neck area was irradiated by placing each animal in a specially built Lucite jig so that the animal could be immobilized without anesthetics (19, 20, 24, 25). In addition, the jig was fitted with a Lucite cone surrounding the head and preventing head movement during irradiation (IR). Single-dose IR, at 15 Gy 1 day after vector administration, and fractionated IR, at 6 Gy/day for 5 days with the first fraction starting 1 day after vector administration, were delivered by a Therapax DXT300 X-ray irradiator (Precision X-ray) by using 2.0 mm Al filtration (300 kVp) at a dose rate of 1.9 Gy/minute. After IR, animals were removed from the jig, housed (4 or 5 animals per cage) in a climate- and light-controlled environment, and allowed free access to food and water.

Measurement of hKGF and FGFR2B levels

Serum, saliva, and gland extracts were assayed for hKGF with hKGF/FGF-7 ELISA kits from R&D Systems. We used this same ELISA kit, in an indirect binding assay, to determine the number of specific FGFR2B receptors that were present in crude SG membranes. Crude membranes were prepared by homogenizing SGs in 10 mmol/L NaHCO₃, centrifuging at 1,000 g for 10 minutes to remove debris, and then centrifuging the crude membranes at 16,000 g for 20 minutes. For each assay 1,000 pg (53 fmol) hKGF, crude membrane protein (0–25 µg), 1 µg/mL heparin (Sigma), 1 mg/mL BS albumin (BSA; GibcoBRL), and 25 mmol/L Hepes, pH 7.4, were present. Following incubation (37°C, 1 hour, shaking), incubation mixtures were centrifuged at 16,100 g for 20 minutes, and the resulting supernatant assayed for hKGF with the ELISA kit. Specific binding was determined by subtracting the results obtained in similar assays by using: (i) crude membranes from mouse mononuclear cells and (ii) by including FGF1 (0–1,200 pg; GenWay Biotech, Inc.) in the incubation mixtures. FGFR2 receptor binding showed specific and saturable kinetics.

QPCR assays

Genomic DNA from SGs was extracted with the Wizard Genomic DNA Purification Kit (Promega). A total of 100 ng DNA was used per quantitative PCR (QPCR) reaction. The primers E3Taq1 and E3Taq2 and probe E3Taqprobe, using sequences previously reported (19), were specific for the adenoviral E3 region and were used to measure the vector copy number. All QPCR assays were done in an ABI Prism 7700 Sequence Detector (PE Applied Biosystems) with conditions as follows: 95°C for 2 minutes, 95°C for 8 minutes, 95°C for 15 seconds, and 60°C for 1 minute for 40 cycles.

Measurements of endothelial cells and progenitor or stem cells

SGs were transduced with either AdControl or AdLTR₂EF1 α -hKGF 24 hours before IR (single dose, 15 Gy). SGs were collected at 4 and 24 hours after IR, minced with a disposable scalpel, placed in a 50 mL tube, and 15 mL of a solution containing 0.2% collagenase, 0.02% Dnase, and 0.02% hyaluronidase (Sigma) was added and tubes were placed on a shaker at 37°C for 30 minutes. A total of 5 mL of fetal BS was added to stop enzyme digestions, and samples were filtered through a 100 µm Nylon strainer (BD Falcon) to allow undigested tissue and cell clumps to be separated from dispersed single cells. After

centrifugation at 1,200 RPM for 3 minutes, the cell pellet was washed 3 times in phosphate buffered saline (PBS), resuspended and aliquoted onto a 96-well plate. Primary antibodies against VE-cadherin (BD Pharmingen) or c-kit (Santa Cruz Biotechnology) were labeled with DyLight 649 (Thermo Scientific) and then incubated with cells at a concentration of 1:100 for 1 hour at room temperature in the dark. Cells were washed 3 times for 10 minutes with PBS and gentle agitation in the dark, and finally, resuspended in 300 μ L of PBS prior to analysis by fluorescence-activated cell sorting in a fluorescence-activated cell sorting (FACS) Canto Flow Cytometer (BD Biosciences). A total of 50,000 events were captured per sample, and tissue and cell debris were gated out to exclude them from analysis. Results are displayed as a percentage of positive cells.

Bromodeoxyuridine assay

Forty-eight hours posttransduction (10^{10} particles per gland of either AdControl or AdLTR₂EF1 α -hKGF), mice were irradiated with a single dose (15 Gy). Twenty-four and 46 hours post-IR, mice were injected with Bromodeoxyuridine (BrdUrd; 25 mg/kg, Sigma) intraperitoneally. Two hours later, mice were euthanized, and SGs harvested. SGs were fixed in 10% formalin solution (Sigma) embedded in paraffin and 5 μ m sections were prepared. Each slide was incubated in xylene twice for 20 minutes each, rehydrated, and incubated for 10 minutes in 3% hydrogen peroxide. Sections were denatured in 2 N HCl for 30 minutes at room temperature and washed for 10 minutes in 0.1 mol/L sodium borate buffer, pH 8.5, following 2 washes of 10 minutes in PBS. Nonspecific antibody binding was blocked by incubation in 10% donkey serum (Jackson ImmunoResearch), 3% BSA in PBS for 1 hour at room temperature. BrdUrd was detected by incubation with an HRP-conjugated rat monoclonal anti-BrdUrd antibody (ab 74546, 1:100; Abcam) at 4°C in a humidified chamber. Sections were washed once in PBS +0.05% Tween 20 (Sigma) for 15 minutes, then 3 times for 10 minutes in PBS, stained with 3,3'-diaminobenzidine and mounted with VectaMount (Vector Laboratories). Under light microscopy, 20 fields per sample were randomly selected and all BrdUrd positive cells were counted. Results are displayed as the average of number positive cells/field $\pm$ SEM.

Immunofluorescence staining

For immunofluorescence staining, SGs and SCC VII tumors (see next) were removed, fixed in 10% formalin and embedded in paraffin. Sections of 5 μ m were de-paraffinized and rehydrated in a gradient series of ethanol then washed in PBS. Antigen retrieval was carried out with 1 mmol/L EDTA (pH 8), 0.05% Tween 20 in a microwave oven for 10 minutes. Sections were then blocked with 20% goat serum in 5% BSA for 1 hour, incubated with primary antibodies (rabbit polyclonal antimouse FGFR2; rabbit polyclonal antihuman aquaporin-5 [AQP5]; goat polyclonal antimouse aquaporin-1 [AQP1], which was used as a positive marker for endothelial cells herein; all antibodies from Santa Cruz Biotechnology) in 5% BSA in PBS for 1 hour at room temperature, and washed with PBS. Next, the slides were incubated with secondary antibodies, either Alexa Fluor488 donkey antigoat immunoglobulin (Ig) G (H+L) or Alexa Fluor546 donkey antirabbit IgG (H+L)(Invitrogen) for 1 hour, washed with PBS, and mounted with Prolong Gold antifade reagent with DAPI (4', 6 diamidino 2 phenylindole; Invitrogen).

Western blot analysis

Samples from SGs and SCC VII tumors were homogenized in 400 μ L of CelLyticTMM Cell Lysis Reagent (Sigma). Then, supernatants were mixed with NuPAGE LDS Sample Buffer (4 $\times$; Invitrogen) and loaded onto SDS-PAGE gels for Western blots, using either rabbit polyclonal anti-mouse FGFR2 antibody (Santa Cruz Biotechnology, Inc.) or rat monoclonal anti-mFGFR2 (IIIb) and (IIIc) antibodies (R&D System) for detection.

Effect of radiation and vectors on SCC VII tumor growth

To assay whether transgenic hKGF affects tumor growth with or without radiation treatment, we used the mouse SCC VII model (obtained from Dr. T. Phillips, University of California San Francisco, San Francisco, CA), propagated in C3H mice, as described previously (25). To assess this question, we did 2 types of experiments. For the first experiment, 3×10^5 viable SCC VII cells, suspended in 100 μ L PBS, were injected into the subcutaneous space of the right hind leg of 7- to 9-week-old female C3H mice and tumor size measured $\pm$ IR and $\pm$ vector. Tumor growth was followed until the diameter of tumor reached 8 to 10 mm as measured by caliper. At this time point, animals were randomly divided into 6 groups: no-IR, no-IR plus AdControl, no-IR plus AdLTR₂EF1 α -hKGF, IR, IR plus AdControl, and IR plus AdLTR₂EF1 α -hKGF. Next, either the AdControl or AdLTR₂EF1 α -hKGF vector was delivered at 10^{10} particles per gland (both glands) by retrograde ductal instillation. Animal IR was begun 24 hour post-transduction. Mice received 3 Gy to the tumor containing leg area each day for 5 days (25). According to our protocol, mice were euthanized when tumor size was approximately 15 mm. Therefore, mice from no-IR groups were euthanized on day 17, whereas the mice in the IR groups were euthanized on day 21. Tumor size was measured, and then tumors were removed for further studies. For the second experiment, examining the effect of hKGF gene transfer on tumor growth, 3×10^4 viable SCC VII cells, suspended in 100 μ L PBS, were injected into the subcutaneous space of the right hind leg of 7- to 9-week-old female C3H mice. Then, animals were randomly divided into 3 groups: no-IR, no-IR plus AdControl, and no-IR plus AdLTR₂EF1 α -hKGF. On the same day, either the AdControl or AdLTR₂EF1 α -hKGF vector was delivered at 10^{10} particles per gland (both glands) by retrograde ductal instillation. Tumor size was followed and tumors measured from days 9 to 15.

Statistical analysis

Data analyses were done by SigmaStat version 2.0 (SPSS, Inc.) and Excel (Microsoft) software. Results are presented as mean values $\pm$ SEM. One-way ANOVAs, or a Kruskal-Wallis test, followed by a Tukey test, were employed as appropriate (see figure legends).

Results

Models of salivary hypofunction

Female C3H mice were irradiated at either a single dose of 15 Gy or with 6 Gy fractions daily for 5 days, and salivary flow was measured 9 weeks post-IR. As shown in Figure 1 (single dose) and 2 (fractionated dose), salivary flow rates in the IR only group were dramatically and significantly decreased approximately 60% to 70% compared with the no-IR group ($P < 0.001$). In addition, the body weights of IR only groups were decreased presumably because the diminished saliva caused some difficulties in alimentation (Figs. 1C and 2C; $P < 0.001$). Both the single and fractionated radiation schemes used here clearly led to salivary hypofunction in the female C3H mice.

Effect of KGF gene transfer on salivary gland hypofunction

AdLTR₂EF1 α -hKGF was used to deliver the hKGF cDNA to SGs. A characteristic of this vector is its ability to mediate longer term transgene expression in salivary glands compared with conventional serotype 5 adenoviral vectors (19, 20). Indeed, we found that 9 weeks posttransduction, remaining vector in targeted glands still mediated production of transgenic hKGF protein that also was secreted (Table 1). These results were consistent with our previous studies (19, 20).

We next determined whether hKGF gene transfer could prevent salivary hypofunction after head and neck IR. Typically, these experiments included 4 study groups ($n = 4$ mice per

group): no-IR, IR only, and IR + vector (either AdControl or AdLTR₂EF1 α -hKGF). The single-dose IR (15 Gy) experiment was repeated 3 times, and the results shown in Figure 1 are combined from all 3 experiments at 9 weeks posttransduction. Mice receiving 15 Gy $\pm$ AdControl had a significant and similar reduction in their salivary flow rates (Fig. 1B, approximately 70% and 75%, respectively) compared with the no-IR group ($P < 0.001$). Furthermore, the salivary flow rates from these 2 groups were also significantly lower than those of irradiated mice treated with AdLTR₂EF1 α -hKGF ($P < 0.001$). In contrast, the salivary flow rates from the no-IR and AdLTR₂EF1 α -hKGF plus IR groups were not significantly different ($P = 0.065$). In addition, body weight measurements showed that values in the IR and IR plus AdControl groups were significantly less than those of the AdLTR₂EF1 α -hKGF plus IR group. These aggregate results suggest that the hKGF gene transfer provided some protection from salivary hypofunction after single-dose IR damage.

To more closely mimic the clinical IR paradigm, we conducted 3 fractionated IR experiments. As noted earlier, Figure 2 shows that the fractionated IR scheme used yielded generally similar reductions in salivary flow as the single-dose experiments. As found in Figure 2B, mice receiving fractionated IR $\pm$ AdControl experienced a similar and significant reduction in salivary flow rate (approximately 60%) compared with the no-IR group ($P < 0.001$). Furthermore, salivary flow rates from both of these irradiated groups were also significantly lower than those of AdLTR₂EF1 α -hKGF plus fractionated IR group (approximately 90% of nonirradiated control values; $P < 0.001$). In addition, the salivary flow rates from the no-IR and AdLTR₂EF1 α -hKGF plus IR groups were not significantly different ($P = 0.375$). Body weight results shown in Figure 2C were similar to the findings in the single-dose study (Fig. 1C).

Next, we examined the effects of vector dose on the protective effects of hKGF with both single and fractionated IR. The results shown in Figure 3 demonstrate that the hKGF gene transfer protective effect on fractionated IR-induced salivary hypofunction was vector dose-dependent, and the maximal effect was seen at the highest dose tested, 10^{10} particles per gland of AdLTR₂EF1 α -hKGF. The single-dose IR experiment gave similar results (data not shown). Transgenic hKGF expression in SG extracts and serum was also vector dose-dependent (Table 2).

Effect of hKGF gene transfer on solid tumor growth

Since KGF is an epithelial cell growth factor, it is important to determine whether hKGF gene transfer can affect the growth of a SCC, the most common head, and neck cancer. We used the SCC VII tumor model in experiments to test this possibility. By using immunofluorescence staining of tumor sections and Western blots of tumor extracts, we showed that the SCC VII tumor had the FGFR2 (Fig. 4). Next, we examined the effect of hKGF gene transfer on tumor growth without and with IR. As shown in Figure 5C, hKGF gene transfer had no effect on tumor growth with or without fractionated IR. In this study, gene therapy was initiated 24 hours prior to IR treatment for tumors of considerable size (8–10 mm diameter). To determine whether hKGF gene transfer would impact tumor growth for a smaller tumor burden, studies were conducted where 10-fold less tumor cells were injected and hKGF gene transfer was done immediately after tumor cell injection. Figure 5D shows that hKGF gene transfer had no effect on the growth of the SCC VII tumor during tumor formation. Collectively, the results shown in Figure 5 clearly show that SCC VII tumor growth was not affected by hKGF gene transfer under both experimental paradigms employed.

Possible mechanisms of hKGF action

We could not find any significant differences in the general morphology of SGs between experimental groups with hematoxylin and eosin staining (data not shown). Also, there were no differences found in the levels of immunopositive AQP5 in sections of glands from all study groups (data not shown). We also examined sections of SGs to determine whether they expressed FGFR2, the receptor necessary for KGF signaling (7, 16, 26, 27). As shown in Figure 4C, murine salivary parenchymal cells express this receptor. This result was confirmed by Western blot (Fig. 4B). Furthermore, by using the assay described in Materials and Methods, we calculated that there are approximately $1,000 \pm 52$ (SEM) fmol hKGF binding sites/mg crude SG membrane protein. In general, duct cells showed stronger signals than acinar cells. In addition, endothelial cells of blood vessels within the gland were FGFR2 positive (Fig. 4D). We then evaluated whether hKGF gene transfer could protect or enhance the proliferation of salivary epithelial cells in irradiated glands. For this experiment, the mice were irradiated with a single dose, 15 Gy. As shown in Figure 6 at 48 hours after IR, mice receiving 15 Gy alone or 15 Gy plus the AdControl vector had a significant reduction (approximately 30%–35%) in BrdUrd positive (i.e., proliferating) cells present compared with the group that was not irradiated ($P < 0.001$). Conversely, the AdLTR₂EF1 α -hKGF plus IR group showed similar levels of BrdUrd positive cells as the control nonirradiated group (Fig. 6). This suggests that the hKGF gene transfer may protect proliferating cells in murine SGs during IR.

We also tested whether hKGF gene transfer affected progenitor/stem cells in SGs by quantifying c-kit positive cells. For this experiment, mice were irradiated with a single dose of 15 Gy. The results shown in Figure 6B are somewhat similar to the BrdUrd results ($F = 4.166$, $P = 0.012$). However, a Tukey test comparing results between the AdControl and AdLTR₂EF1 α -hKGF-treated groups indicated that they were not significantly different ($P = 0.254$).

Since our previous studies showed that IR causes damage to salivary endothelial cells (28), we next examined the effects of IR with and without hKGF vector treatment on endothelial cells in SGs. For this experiment, mice were irradiated with a single dose of 15 Gy and number of cells expressing the endothelial-specific cell surface molecule, VE-cadherin, was quantified by FACS analysis. Results in Figure 6C clearly show that the AdLTR₂EF1 α -hKGF plus IR group had similar levels of VE-cadherin positive cells as the control nonirradiated group, and both were significantly elevated compared with those from IR only and IR plus AdControl mice. This result suggests that hKGF gene transfer can also protect endothelial cells in the SGs.

Discussion

Salivary hypofunction is a serious side effect in patients with head and neck cancers who receive radiotherapy that cannot be adequately prevented at present and remains a significant clinical problem (1–4). Herein, 2 radiation-induced salivary hypofunction models were used in mice to test a gene transfer strategy to prevent this condition: a 15 Gy single-dose scheme and a 5-day $\times$ 6 Gy fractionated scheme. Both models yield severe salivary hypofunction. Importantly, we show that hKGF gene transfer to SGs can effectively protect salivary epithelial cells and prevent salivary hypofunction after both single and fractionated radiation dosing, leading to near-normal salivary flow rates approximately 8 to 9 weeks after radiation.

The exact mechanism by which the transgenic hKGF provides gland protection is not clear. Salivary parenchymal cells have FGF2 receptors (Fig. 4) that are required for hKGF signaling. Thus, hKGF could act on salivary epithelial cells in an autocrine manner. In

addition, endothelial cells within the gland express these receptors (Fig. 4) and could be the targets of paracrine and/or endocrine secretion of hKGF protein from transduced salivary epithelial cells. Indeed, recently, it has been recognized that salivary microvascular endothelial cells might be early and sensitive targets of IR in both murine and porcine salivary glands (28, 29). Herein, we show that hKGF gene transfer could prevent loss of endothelial cells within irradiated SGs (Fig. 6C).

In addition to salivary parenchymal and endothelial cells being potential targets for the transgenic hKGF, it is also possible that a small subset of salivary cells, which function as a tissue-specific stem or progenitor cells, could be a target (30). Indeed, this seems reasonable given the hKGF-mediated attenuation of decreased epithelial cell proliferative activity in irradiated glands treated with the AdLTR₂EF1 α -hKGF vector (Fig. 6A), and by the results of Lombaert and colleagues (30). We also attempted to define the signal transduction pathway employed in SGs following hKGF gene transfer, but were unable to delineate any significant responses. In part, this could be a result of the long-time points used for this *in vivo* study versus the extremely short time points employed for typical signaling studies with cells cultured *in vitro*. However, whether stem/progenitor cells were the key target population for transgenic hKGF, the examination of whole gland extracts is highly unlikely to allow detection of any signals. More study is clearly needed to define the mechanism by which hKGF gene transfer can protect salivary glands from radiation damage and preserve salivary flow.

One key observation made herein and consistent with our previous findings (19) is that transgenic hKGF is secreted from transduced glands into the bloodstream. Indeed, the levels of hKGF vector particles in gland tissue correlated well with both radiation protection and serum levels of hKGF (Table 2). A significant concern of having elevated serum hKGF levels, however, is the potential for the circulating hormone to affect tumor cell development and/or treatment. Accordingly, we evaluated the effect of hKGF gene transfer on the growth and radiation sensitivity of a widely used SCC model that expresses FGFR2 receptors. As shown in Figure 5, hKGF gene transfer is without effect on SCC VII growth kinetics $\pm$ IR. While this result is encouraging, in patients with FGFR2-expressing solid tumors, it would seem prudent to minimize the systemic availability of transgenic hKGF.

The intraductal delivery of adenoviral vectors to rodent salivary glands has been shown to be quite safe in 3 detailed GLP (Good Laboratory Practice) biodistribution and toxicology studies (31–33). Furthermore, we are currently conducting a phase I clinical trial of an adenoviral vector, AdhAQP1, also administered intraductally, to correct existing IR-damage in parotid glands (ref. 34; <http://www.clinicaltrials.gov/ct/show/NCT0372320?order=>). Interestingly, we recently reported (35) the transient detection of E1-containing adenovirus [presumptive replication competent adenovirus (RCA)] in the parotid saliva of 1 patient in that trial. Importantly, the presence of RCA was detected only in saliva, never in serum, and was without any clinical consequences for the patient who apparently had a latent adenoviral infection in the targeted gland (35). The previous GLP safety studies, the RCA event, and the presence of a fibrous capsule around human salivary glands, all support the notion that intraductal adenoviral vector delivery to salivary glands can be performed quite safely.

This study clearly shows that transfer of the hKGF gene to SGs prior to both fractionated and single-dose IR substantially prevents salivary hypofunction. It is not yet clear whether salivary parenchymal, endothelial, and/or progenitor cells/stem cells are all targets of the transgenic hKGF. Importantly, the hKGF gene transfer had no effect on the growth or radiation sensitivity of a model SCC. Further study of this preventive maneuver seems

warranted, as it may be beneficial for patients receiving radiotherapy for head and neck cancers.

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Translational Relevance

Zheng and colleagues report a preclinical study by using a novel gene transfer approach to prevent a common side effect of radiotherapy for head and neck cancers—salivary hypofunction. This condition leads to significant patient discomfort and morbidity. By using a hybrid adenoretroviral vector, the authors transfer the cDNA for human keratinocyte growth factor (hKGF) into murine salivary glands 1 day prior to either single or fractionated radiation. Both radiation schemes lead to significant salivary hypofunction after 8 weeks and hKGF gene transfer dramatically prevented hypofunction in both. Gene transfer to salivary glands, via cannulation and retrograde delivery into the main excretory duct, is not difficult and mimics a common procedure used for contrast x-rays. The results suggest a potential application with head and neck cancer patients.

Figure 1.

Effect of single-dose (15Gy) IR and hKGF gene transfer on salivary flow 9 weeks posttransduction. A, experimental design. B, salivary flow rates as a percentage of values from nonirradiated control mice. C, body weight. The data shown in (B) and (C) are mean values $\pm$ SEM ($n = 12$ mice; 3 separate experiments with $n = 4$ per group). For data in (B), $F = 47.669$, $P < 0.001$. Differences between no-IR and IR and IR-AdC are significant ($P < 0.001$) by a Tukey test. Differences between IR-AdKGF and IR and IR-AdC are also significant ($P < 0.001$) by a Tukey test. For data in (C), $F = 25.512$, $P < 0.001$. By a Tukey test, the weight of animals in the no-IR group was significantly greater than that in all other groups (IR, $P < 0.001$; IR-AdC, $P < 0.001$; IR-AdKGF, $P < 0.005$). The weight of animals in the IR-AdKGF group was significantly greater than that of mice in the IR ($P < 0.001$) and IR-AdC ($P < 0.005$) groups. No-IR, nonirradiated control; IR, irradiated only; IR-AdC, irradiated + treatment with AdControl; IR-AdKGF, irradiated + treatment with AdLTR₂EF1α-hKGF.

Figure 2.

Effect of fractionated IR and hKGF gene transfer on salivary flow 9 weeks posttransduction. A, experimental design. Mice received 5 fractions of 6 Gy, as indicated. B, salivary flow rates as a percentage of values from nonirradiated control mice. C, body weight. The data shown in (B) and (C) are mean values $\pm$ SEM ($n = 12$ mice; 3 separate experiments with $n = 4$ per group). For data in (B), $F = 50.638$, $P < 0.001$. By a Tukey test, the values for no-IR and AdKGF are not different from each other, but significantly greater than those for IR and IR-AdC mice ($P < 0.001$). For data in (C), $F = 17.765$, $P < 0.001$. By a Tukey test, the weight for a no-IR group was significantly different from all other groups (IR, $P < 0.001$; IR-AdC, $P < 0.001$; IR-AdKGF, $P < 0.05$). The weights of the AdKGF-treated mice are different those of mice in the IR ($P = 0.018$) and IR-AdC ($P < 0.005$) groups. No-IR, nonirradiated control; IR, irradiated only; IR-AdC, irradiated + treatment with AdControl; IR-AdKGF, irradiated + treatment with AdLTR₂EF1 α -hKGF.

Figure 3.

Effect of different doses of AdLTR₂EF1 α -hKGF on radiation-induced salivary hypofunction 9 weeks posttransduction. Mice were administered vectors, or not, 24 hours before receiving fractionated IR (5×6 Gy). The data shown are mean values $\pm$ SEM ($n = 8$ mice; 2 separate experiments with $n = 4$ per group). $F = 17.632$, $P < 0.001$. By a Tukey test, values for the no-IR and IR-AdKGF (10^{10}) groups are not different from each other. Values for the no-IR group are significantly different from those of the IR ($P < 0.001$), IR-AdC ($P < 0.001$), and AdKGF (10^8 , $P < 0.001$; 10^9 , $P < 0.002$) groups. Values for the AdKGF group (10^{10}) are different from those of the IR ($P < 0.001$), IR-AdC ($P < 0.001$), 10^8 ($P < 0.01$), and 10^9 ($P < 0.042$) groups. No-IR, nonirradiated control; IR, irradiated only; IR-AdC, irradiated + treatment with AdControl; IR-AdKGF, irradiated + treatment with AdLTR₂EF1 α -hKGF.

Figure 4.

Evidence for the presence of FGFR2 in mouse SGs and the SCC VII tumor by immunofluorescence staining and Western blot. See Materials and Methods for details on the methods used. A, immunofluorescence staining of SCC VII. Red color indicates FGFR2 positive staining, while blue color is nuclear staining with DAPI ($\times 400$). B, Western blot showing the presence of immunoreactive FGFR2 in crude membranes from mouse SG and SSC VII tumor (SSC). With the latter samples, 2 different antibodies were used. C, immunofluorescence staining of mouse SG. Red color indicates FGFR2 staining, while blue color is nuclear staining with DAPI ($\times 400$). d = ductal cells; a = acinar cells. D, immunofluorescence staining of blood vessel in a SG. Red color indicates FGFR2-positive staining in endothelial cells (yellow arrows) and green color indicates AQP1-positive staining in endothelial cells (white arrows), while blue color is nuclear staining with DAPI ($\times 1,000$).

Figure 5.

Effect of IR and hKGF gene transfer on SCC VII tumor growth. A, general morphology of SCC VII. Sections were stained with hematoxylin and eosin stain ($\times 400$). B, a representative picture of SCC VII tumor growth (black arrow) under the skin of the right rear flank of a female C3H mouse. C, radiation induced tumor regrowth delay. Mice were irradiated (15 Gy) or not and the size of the tumor followed as described in Materials and Methods. D, effect of AdLTR₂EF1 α -hKGF on SCC VII growth. For this experiment, we used one-tenth of the cells used in (C), as described in Materials and Methods. Vector and cells were administrated at time zero, and tumor size followed. Visible tumors could be measured beginning on day 9. The data shown are mean values $\pm$ SEM ($n = 4$ mice per group). One-way ANOVAs at each day showed no significant differences in tumor size between mice in each treatment group (irradiated and nonirradiated groups compared separately). No-IR, nonirradiated control; IR, irradiated only; No-IR AdC, treated with AdControl alone; No-IR AdK, treated with AdLTR₂EF1 α -hKGF alone; IR AdC, irradiated and treated with AdControl; IR AdK, irradiated and treated with AdLTR₂EF1 α -hKGF.

Figure 6.

Effect of IR and hKGF gene transfer on salivary gland epithelial cell proliferation, number of progenitor/stem cells, and number of endothelial cells. A, cell proliferation was measured 48 hours post-IR by the incorporation of BrdUrd. The results shown are mean values $\pm$ SEM ($n = 4$ per group). Kruskal–Wallis 1-way ANOVA, $H = 78.549$, $P < 0.001$. The values for the no-IR and IR-AdKGF groups do not differ. By a Tukey test, both are significantly different from the IR ($P < 0.05$) and IR-AdC groups ($P < 0.05$). B, changes of c-kit positive cells in different study groups 24 hours post-IR. The results shown are mean values $\pm$ SEM ($n = 20$ mice; 5 separate experiments with $n = 4$ per group). $F = 4.166$, $P < 0.012$. By the Tukey test, only the no-IR group differs from the values of the IR and IR-AdC groups ($P = 0.027$ and $P = 0.042$, respectively). C, changes of VE-cadherin positive cells in different study groups 4 hours post-IR. The results shown are mean values $\pm$ SEM ($n = 20$ mice; 5 separate experiments with $n = 4$ per group). $F = 9.257$, $P < 0.001$. The values for the no-IR group are significantly greater than those in the IR group ($P < 0.009$). The values for the IR-AdKGF group are significantly greater than those for the IR ($P < 0.001$) and IR-AdC ($P < 0.007$) groups. See Materials and Methods for details. No-IR, nonirradiated control; IR,

irradiated only; IR-AdC, irradiated + treatment with AdControl; IR-AdKGF, irradiated + 10^{10} particles of AdLTR₂EF1 α -hKGF.

Table 1

Expression of hKGF in mice administered either single or fractionated IR

Radiation group	hKGF			Vector copy number/100 ng DNA ($\times 10^4$)
	Saliva (total, pg)	Serum (total, pg)	Gland extract (pg/mg protein)	
Single	0	3,222 $\pm$ 2,777	9,521 $\pm$ 1,321	8.0 $\pm$ 1.0
Fractionated	8.9 $\pm$ 6.4	975 $\pm$ 263	8,773 $\pm$ 510	7.5 $\pm$ 0.5

NOTE: Mice received either a single dose of 15 Gy, or 5 fractions of 6 Gy each, to their head and neck, as described in Materials and Methods.

Prior to radiation (-24 hours), 10^{10} particles of AdLTR2EF1 α -hKGF were administered to both SGs of each mouse ($n = 4$ mice per experimental group). Total salivary hKGF was calculated based on 100 μ L saliva per mouse. Total serum hKGF was calculated based on 2 mL serum per mouse. Data shown for hKGF levels and vector copy number are mean values $\pm$ SEM from 3 experiments. These data are from 9 weeks posttransduction. Note that the vector copy numbers shown represent approximately 0.1%–1% of the total dose administered. No hKGF was detected in samples from mice-administered AdControl.

Table 2

Summary of protein expression and vector level results in dose response experiments

	AdLTR₂EF1α-hKGF		
	10⁸ particles per gland	10⁹ particles per gland	10¹⁰ particles per gland
hKGF, gland (pg/mg protein)	1,695.1 $\pm$ 629.3	2,245.8 $\pm$ 149.2	9,316.5 $\pm$ 550.6
hKGF, serum, pg/mL	0.03 $\pm$ 0.03	14.1 $\pm$ 8.5	464 $\pm$ 69.9
Vector copy number/100 ng DNA ($\times 10^4$)	0.31 $\pm$ 0.05	1.7 $\pm$ 0.64	7.93 $\pm$ 0.64

NOTE: Data shown for hKGF levels and vector copy number are mean values $\pm$ SEM from 2 experiments with $n = 4$ mice per experiment. These data are from 9 weeks posttransduction. Note that the vector copy numbers shown represent approximately 0.1%–1% of the total dose administered.