Radiation-Induced Loss of Salivary Gland Function Is Driven by Cellular Senescence and Prevented by IL6 Modulation

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Abstract

Head and neck cancer patients treated by radiation commonly suffer from a devastating side effect known as dry-mouth syndrome, which results from the irreversible loss of salivary gland function via mechanisms that are not completely understood. In this study, we used a mouse model of radiation-induced salivary hypofunction to investigate the outcomes of DNA damage in the head and neck region. We demonstrate that the loss of salivary function was closely accompanied by cellular senescence, as evidenced by a persistent DNA damage response (γH2AX and 53BP1) and the expression of senescence-associated markers (SA-β-gal, p19ARF, and DrR2) and secretory phenotype (SASP) factors (PAI-1 and IL6). Notably, profound apoptosis or necrosis was not observed in irradiated regions. Signs of cellular senescence were also apparent in irradiated salivary glands surgically resected from human patients who underwent radiotherapy. Importantly, using IL6 knockout mice, we found that sustained expression of IL6 in the salivary gland long after initiation of radiation-induced DNA damage was required for both senescence and hypofunction.

Introduction

Radiotherapy has proven to be effective in treating a wide variety of human malignancies and is one of the most widely employed cancer therapy modalities. However, radiotherapy often generates severe side effects that can be devastating for the patient. Interestingly, the biologic mechanisms underlying a variety of radiation-induced side effects in patients are not entirely understood. For head and neck cancer, radiotherapy is currently one of the main treatment modalities. However, radiotherapy treatment achieves a 5-year survival rate of approximately 80% for early-stage and 35% for late-stage disease (1, 2). Despite recent improvements in sparing the salivary gland through the use of intensity-modulated radiation therapy (IMRT) and proton therapy, radiation treatment still leads to xerostomia in a significant proportion of patients, especially those at high risk (5–10). Current clinical management of this condition remains palliative and generally unsatisfactory, leading to difficulties in chewing, swallowing and speech, increased oral infections, dental decay, and diminished taste perception, altogether dramatically reducing the patients’ quality of life (2, 4).

Intriguingly, the salivary glands display sensitivity to radiation-induced damage far in excess of that expected for a relatively mitotically quiescent tissue (4). The fundamental cellular and molecular mechanisms underlying the loss of salivary gland function are not clearly understood (4). Evidence derived largely from animal models supports the notion that radiation-induced salivary hypofunction is a multifactorial process initiated by DNA damage to various tissue components within the gland, including the parenchymal acinar cells, endothelium, stem/progenitor cells and also parasympathetic innervation (10–16). Apoptosis, occurring within the first few days following irradiation, has also been proposed as a fundamental mechanism underlying loss of both acinar cells and glandular innervation (15, 17, 18). However, this mechanism fails to account for the characteristic time lag of weeks to months observed between radiation treatment and the onset of irreversible salivary hypofunction (13, 19).
Excess DNA damage has another potential outcome—cellular senescence (20). Senescence is a state of stable proliferative arrest that cells undergo in response to a variety of detrimental stimuli, including DNA-damaging agents and oxidative stress (20). Senescent cells also develop a complex senescence-associated secretory phenotype (SASP) that includes secreted inflammatory cytokines, such as IL6, which function to reinforce growth arrest of senescent cells (21, 22).

Our findings establish that cellular senescence, which is reinforced by IL6, is a central mechanism underlying radiation-induced salivary gland hypofunction in mice, and that IL6 blockade can reduce senescence in this tissue while strongly ameliorating the associated decrease in salivation. Surprisingly, we also demonstrate that IL6 supplementation has the same outcome, although via a different mechanism, together suggesting novel therapeutic strategies for its prevention in patients.

Materials and Methods

Animals

Female mice (C57BL/6; Harlan Laboratories Ltd.), IL6 knock-out (IL6/−/−; B6;129S2-Il6−/−; C57BL/6; The Jackson Laboratory; ref. 23) and transgenic spg130Fc (C57BL/6) mice (24) were maintained in an animal facility under SPF conditions, at 22°C and a 12-hour light–dark cycle. Mice received sterile commercial rodent chow and water ad libitum. Procedures and maintenance were performed in accordance with Institutional Animal Care and Use Committee approved animal treatment protocols (license number ORPR-A01-5011).

Mice, 7 to 8 weeks old (17–19 g), were irradiated in groups of up to 12 mice to the head and neck while immobilized in tubular Perspex jigs. The 6MV energy X-Ray radiation was delivered by a Varian linear accelerator, Clinac 2100EX model (FSD = 100 cm, field size = 7 cm × 40 cm). Radiation doses of 13 Gy (single fraction), or 28 Gy in 5 daily fractions of 5.6 Gy (5 × 5.6 Gy; fractionated dose) were empirically determined by dose–response analysis to render approximately 80% loss of salivary gland function 8 weeks after irradiation in wild-type C57BL/6 mice (data not shown). Recombinant human IL6 (PeproTech) or Hyper-IL6 (HIL6), prepared as previously described (25), were delivered in normal saline containing 10 mM NaCl. Saliva volume was determined by weight, assuming a specific gravity of 1 g/mL.

Histological and histochemical analyses

Paraffin-embedded tissue sections were stained with hematoxylin and eosin (H&E) for routine examination. The following antibodies were used for immunostaining: anti-aquaporin 5 (Almonone Labs), anti-p21 (BD Pharmingen), anti-DcR2 (Enzo Life Sciences Inc), anti-γH2AX (Millipore), anti-53BP1 (Bethyl Laboratories Inc), anti-IL6R (R&D Systems), anti-caspase-3 (Cell Signaling Technology) and anti-pSTAT3 (pTyr-705; Cell Signaling Technology). Phosphorylated STAT3 was stained using monoclonal rabbit anti-mouse pSTAT3 (Tyr 705; Cell Signaling Technology), followed by biotinylated goat anti-rabbit (Jackson ImmunoResearch Laboratories), amplified using a Tyramide Signal Amplification Kit (PerkinElmer) and developed with AEC (Dako), as previously described (25). For confocal microscopy, coverslips were mounted in Fluoro-Gel + DABCO solution (EMD) and visualized by quadraple laser-assisted confocal microscopy (FluoView FV100, Olympus), with a 60× UPLNAPo objective. γH2AX foci in salivary gland samples that were removed 48 h or more post-irradiation were quantified by manual counting in nuclei of 50–200 ductal cells per sample. In samples displaying very high levels of γH2AX (4–48 hours after irradiation), staining levels were quantified as pixels within the nuclei of the ductal cells using Image Pro Analyzer image analysis software. TUNEL analysis was performed on paraffin-embedded thin sections using the In Situ Cell Death Detection Kit (Fluorescein, Roche) according to the manufacturer’s instructions. Detection of SA-β-gal activity was performed, as described previously (26), at pH = 5.5. Frozen sections of salivary gland tissue were fixed with 0.5% glutaraldehyde in PBS for 15 minutes, washed with PBS supplemented with 1 mmol/L MgCl2, and stained for 5 to 6 hours in PBS containing 1 mmol/L MgCl2, 1 mg/mL X-Gal, and 5 mmol/L of each potassium ferrycyanide and potassium ferrocyanide and counterstained with Nuclear Fast Red (Bio-Optica).

Western blot analysis

Protein extracts prepared from fresh frozen tissue samples (~100 mg) by homogenization were subjected to Western blot analysis as described in detail in Supplementary Materials and Methods.

IL6 ELISA

Murine serum IL6 levels were determined using a mouse IL6 ELISA kit (R&D) according to the manufacturer’s instructions on serum samples stored at −20°C.

RNA

RNA was extracted from snap-frozen tissue specimens using TRIzol reagent (Invitrogen). A detailed description of methods used for qPCR can be found in Supplementary Materials and Methods.

Human salivary gland study approval

Human patient derived histological samples were obtained following approval by the Hadassah University Hospital Institutional Helsinki Committee (Request No. 0455-14-HMO) in accordance with Helsinki Declaration procedures. Submandibular salivary glands resected from human patients were embedded...
in paraffin blocks following formaldehyde fixation and stored in the patient tissue archives according to standard hospital patient care and sample storage procedures. Clinical details of the irradiated and non-irradiated patients, including medications and treatments, are summarized in Supplementary Table S1. None of the patients received chemotherapy prior to salivary gland resection.

Statistical analysis
Statistical comparisons using the Mann–Whitney test of ranks, Wilcoxon signed rank test, or χ² test as indicated, were performed with GraphPad Prism version 6.02 software (GraphPad Software), with P < 0.05 considered statistically significant.

Results
Persistent DNA damage and senescence in the salivary gland following irradiation
To determine whether ionizing irradiation induces cellular senescence in salivary gland we irradiated mice with 13 Gy directed to the head and neck, a dose shown to produce profound glandular hypofunction (4, 27). Histologic analysis of salivary glands within the first days (24, 48, and 72 hours)
after irradiation revealed little evidence of marked morphologic changes or apoptosis, and also little evidence of acute inflammatory macrophage or neutrophil infiltration even up to 4 weeks after irradiation (Fig. 1A and Supplementary Fig. S1A–S1C). TUNEL analysis confirmed the absence of apoptotic cells at 13 Gy and also at 15 Gy (Supplementary Fig. S2A). Only at 17 Gy, a dose far in excess of that used in our experimental model, was robust TUNEL staining observed. However, this damage did not represent activation of apoptosis because it was not accompanied by either a corresponding increase in caspase-3 activation (Supplementary Fig. S2B), or by morphologic changes typical of apoptotic cells (Supplementary Fig. S1A). We therefore conclude that, at radiation doses that induce salivary hypofunction in mice, apoptosis of salivary gland cells is not a substantial outcome. On the other hand, histologic analysis performed over 12 weeks after irradiation revealed a gradual increase in the appearance of large cells with notably enlarged, hyperchromatic nuclei, morphologically reminiscent of cells undergoing senescence (Fig. 1A and Supplementary Fig. S1; ref. 28). Positive aquaporin 5 immunostaining in these cells confirmed them as being of acinar origin (Supplementary Fig. S3A; ref. 29). The appearance of these morphologically distinct acini coincided with the progressive loss of normal serous acinar cells, which has been linked to a reduction in salivary gland function (4).

To further evaluate the hypothesis that cellular senescence is an inherent outcome of radiation-induced damage in the salivary gland, we examined irradiated glands for evidence of persistent DNA damage, one of the hallmarks of cellular senescence (20).

Normal cells undergo senescence in response to severe or irreparable DNA damage, especially DNA double strand breaks (DSB). One of the first proteins to respond to DSBs is ataxia telangiectasia mutated (ATM), a kinase whose substrates include histone H2AX and p53 binding protein-1 (53BP1; ref. 22). Phosphorylated H2AX (γH2AX) and 53BP1 rapidly localize to DSBs, forming characteristic foci in the nucleus. Immunostaining for γH2AX and 53BP1 in irradiated glands revealed the presence of numerous foci, indicating a robust DNA damage response (DDR; Fig. 1B). The DNA damage foci were detectable at high levels as early as 4 hours after irradiation, most notably in ductal cells, and persisted at low levels for months. These persistent DNA damage foci (PDDF) were present at approximately 3 foci per nucleus in about 84% of the ductal cells 48 hours after irradiation, and remained at roughly 2.6 foci per nucleus in approximately 50% of ductal cells after 2 months (Fig. 1B and C). Between 48 hours and 8 weeks after irradiation, most acinar cells were largely negative for γH2AX foci (data not shown). However, at 8 weeks, some of the late-appearing enlarged acinar cells were strongly positive for nuclear γH2AX foci that appeared to be 53BP1 negative (Supplementary Fig. S3B and S3C), suggesting a behavior atypical of PDDF.

To further substantiate the senescence phenotype in the irradiated salivary glands, we also performed in situ staining for senescence-associated β-galactosidase (SA-βgal), a known marker of senescent cells (30). Salivary glands collected 8 weeks after irradiation showed substantially increased SA-βgal activity located largely in ductal cells and also in some acini (Fig. 1D). In addition, real-time PCR analysis of salivary glands 2 and 8 weeks
after irradiation revealed strongly increased expression of the senescence-associated markers (31), p21, p19ARF, Dcr2, and also the SASP genes, Il6 and PAI-1, up to 21-fold (Fig. 1E). Mice irradiated with 28 Gy administered in 5 equal doses over 5 consecutive days (5 × 5.6 Gy), an experimental paradigm more closely resembling the fractionated radiotherapy used in the clinical setting (4), showed a similar upregulation of these senescence-associated markers (data not shown). Immunostaining confirmed the upregulation of p21 and Dcr2, which localized to both ductal and acinar cells (Fig. 1F).

In order to determine the relevance of cellular senescence to radiation treatment in human salivary glands, immunostaining was performed on submandibular salivary gland samples that were resected from 3 patients, 4 to 8 months following radiotherapy as part of their treatment protocol and compared with salivary gland samples from 4 nonirradiated patients. Histopathologic analysis revealed focal chronic inflammation and also mild to marked periductal fibrosis that was observed exclusively in glands resected from the irradiated patients. Immunostaining analysis for senescence and DDR proteins clearly revealed the presence of large γH2AX and 53BP1 containing cytoplasmic chromatin fragments that were observed exclusively in glands taken from the irradiated patients (Fig. 2A and B). Previous studies have shown that γH2AX containing cytoplasmic chromatin also appear following oncogene-induced senescence and result from a recently described process called nuclear blebbing (32). Immunostaining also revealed robust Dcr2 expression that was observed exclusively in the ductal cell compartment of all three of the salivary glands taken from patients who received radiotherapy, but in only 1 of 4 non-irradiated patients (P < 0.05, using a two-tailed χ² test; Fig. 2C). Similarly, p21 expression was noted in both ductal and acinar cells in salivary glands of 2 of 3 radiation treated patients, but not in the non-irradiated patients (n = 4; P < 0.05 using a two-tailed χ² test; Fig. 2C). From these findings we conclude that cellular senescence is an inherent and long-term response to irradiation of the salivary gland of humans as well as mice.

IL6 signaling enhances radiation-induced senescence in the salivary gland

Previous studies have shown IL6 upregulation to be one of the earliest detected responses to radiation-induced tissue injury (33–35). Our above findings suggested that IL6 is also linked to the later expression of senescence-associated markers in the irradiated salivary glands. We therefore further characterized the kinetics of IL6 expression following irradiation. Analysis of serum IL6 protein levels by ELISA revealed strong elevation in IL6 expression that appeared roughly 6 hours after irradiation and lasted less than 1 day (Fig. 3A). Surprisingly, a second substantial peak of IL6 protein appeared approximately 2 weeks later. The appearance of IL6 mRNA in the salivary glands preceded that of serum IL6 protein by about 3 hours on the first day, disappearing shortly thereafter, and reappearing 2 weeks later together with the second peak of IL6 protein in the serum (Fig. 3B).

Because IL6 was found to be a crucial factor of the SASP in reinforcing cellular senescence (22, 36), we next studied the effect of IL6 deficiency on radiation-induced senescence using IL6 knockout (IL6−/−) mice. We examined the PDDF levels and the expression of senescence-associated markers in submandibular glands of wild-type compared with IL6−/− mice 8 weeks following fractionated irradiation (5 × 5.6 Gy), a time point when radiation-induced hypofunction is irreversible (27). γH2AX immunostaining of irradiated glands demonstrated a significant, 2-fold (P < 0.05) reduction in PDDF in ductal cells of the
irradiated IL6−/− mice in comparison with controls (Fig. 3C and Supplementary Fig. S4). This change was accompanied by a strong, 3-fold (P < 0.001) reduction in the expression of p19 and DcR2 mRNAs (Fig. 3D). p21 mRNA levels were also moderately reduced. DcR2 immunostaining in salivary gland confirmed the reduced DcR2 expression in the irradiated IL6−/− mice (Fig. 3E). Together, these findings strongly support the notion that IL6 acts to reinforce cellular senescence in irradiated glands.

We next reasoned that if IL6 deficiency reduces senescence, then supplementation of IL6 into the submandibular gland may enhance senescence. In order to achieve increased local IL6 signaling in the salivary gland, IL6 protein was introduced via retrograde infusion through the salivary gland secretory duct (37). IL6 signaling depends on the expression of IL6R and its coreceptor, gp130, on target cells (38). However, IL6 can also signal via its soluble receptor (sIL6R) on cells that do not express IL6R, in a mechanism called IL6 trans-signaling (38). Analysis of IL6R mRNA and protein levels in non-irradiated mice by PCR and Western blot analyses showed notable IL6R expression in the glands, although lower than in liver, a positive control (Fig. 4A and B). Immunostaining confirmed IL6R expression, located mainly on ductal cells (Fig. 4C). To substantiate the IL6R activity in the salivary gland, we utilized the induction of pSTAT3 phosphorylation, one of three main IL6 signal transduction pathways (39), as a surrogate marker for IL6/IL6R signaling following infusion of IL6 into the salivary gland ducts. In addition, we also infused a recombinant IL6/sIL6R fusion protein, called Hyper-IL6 (HIL6; ref. 40). HIL6 can signal in cells deficient of membrane bound IL6R, and also benefits from a signaling half-life longer than that of IL6 alone (41). Western blot analysis showed that infusion of IL6, without irradiation, and even more so HIL6 induced substantial pSTAT3 phosphorylation in comparison with controls (Fig. 4D). Immunostaining for pSTAT3 (Tyr-705) showed that both IL6 and HIL6-induced STAT3 phosphorylation localized primarily to ductal cells (Fig. 4E). These findings demonstrate that IL6R is expressed on ductal cells, and that these cells can be efficiently stimulated by direct infusion of either IL6 or HIL6 proteins via retrograde infusion. Interestingly, we found that infusion of IL6 protein induced upregulation of IL6 mRNA expression within the salivary gland itself by ~30-fold.
(P < 0.03) after 5 hours in comparison with saline infused controls (Supplementary Fig. S5). Moreover, an additive effect was observed following irradiation of the animals, such that mice treated by IL6 infusion prior to irradiation displayed and additional upregulation of the endogenous IL6 gene (Supplementary Fig. S5).

To determine the effect of IL6 and HIL6 supplementation on radiation-induced senescence, we irradiated (5 × 5.6 Gy) mice that had been pretreated with either protein. The glands were then analyzed 8 weeks after irradiation for PDDF and expression of senescence-associated markers. Quantification of γH2AX foci in ductal cells of irradiated glands clearly demonstrated that PDDF were significantly reduced by HIL6 and to a lesser extent by IL6 infusion (Fig. 5A and Supplementary Fig. S6). Similarly, the long-term expression of senescence-associated genes, including p19, DcR2, PAI-1 and, most dramatically, p21 upregulation may be associated with alternative functions of p21 in the irradiated salivary gland, of which senescence is only one (42). These observations indicate that IL6 pretreatment reduces radiation-induced senescence in salivary glands.

**IL6 pretreatment enhances DNA damage repair to prevent senescence in irradiated salivary glands**

We next considered the apparently contradicting findings that IL6 both reinforces and also prevents radiation-induced senescence. While the critical role of IL6 within the SASP-mediated reinforcement of oncogene- and radiation-induced cellular senescence is well established (22, 36), the mechanism of IL6 in preventing senescence is not well understood. In order to investigate this, we reasoned that because the signaling from the infused IL6 protein would be expected to have a half-life of minutes (41), the effects of IL6 infusion were most likely to be observed during or shortly after to the initiation of DNA damage. We therefore hypothesized that the effects of IL6 pretreatment may include the priming of gland cells to efficiently respond to irradiation, either by preventing DNA damage or by enhancing its repair. On the other hand, in the IL6−/− mice, the deficiency of IL6 would most likely affect the long-term persistence of unrepair DNA damage.

To examine this hypothesis, we first analyzed for DNA damage levels using γH2AX immunostaining in ductal cells of...
IL6 pretreatment, but not IL6 deficiency, accelerates DNA damage repair shortly following irradiation. A, quantification of nuclear γH2AX foci in ductal cells in naïve submandibular salivary glands and glands pretreated by retrograde infusion of IL6 (30 ng) or normal saline 48 and 72 hours after irradiation (5.6 Gy). Data, means ± SEM; **, P < 0.01; *** P < 0.001; #, P < 0.05 versus all other groups using two-tailed Mann–Whitney test; naïve, n = 3; saline (48 hours) and IL6 (72 hours), n = 9; saline (72 hours) and IL6 (48 h), n = 10). B, quantification of nuclear γH2AX foci in ductal cells of submandibular salivary glands from naïve wild-type mice and wild-type and IL6 −/− mice removed 48 and 72 hours after irradiation (5.6 Gy). Data, means ± SEM; #, P < 0.05 versus all other groups using two-tailed Mann–Whitney test; naïve, n = 3; WT (48 hours) and IL6 −/− (48 hours), n = 4; WT (72 hours) and IL6 −/− (72 hours), n = 6.

Figure 6.

salivary glands pretreated with either IL6 or normal saline control shortly (4, 48, and 72 hours) following a low irradiation dose (5.6 Gy). Robust induction of γH2AX was observed 4 hours after irradiation and was equal in both IL6 and saline-pretreated salivary glands (Supplementary Fig. S7A and S7B), indicating that IL6 pretreatment did not affect the initiation of DNA damage. Between 4 and 48 hours after irradiation, the number of γH2AX foci decreased markedly and equally in both IL6 and control pretreated salivary glands. However, between 48 and 72 hours, while the levels of γH2AX foci in ductal cells of the salivary pretreated salivary glands remained nearly unchanged, in IL6 pretreated glands the γH2AX levels continued to decrease significantly (Fig. 6A and Supplementary Fig. S7A), indicating that IL6 pretreatment significantly promotes DNA damage repair. In contrast, comparison of γH2AX levels in wild-type and IL6 −/− mice at 4, 48, and 72 hours after irradiation (5.6 Gy) also showed a strong reduction in DSBs, but at identical levels in both strains (Fig. 6B and Supplementary Fig. S8A and S8B). Thus, unlike IL6 pretreatment, IL6 deficiency does not increase repair of DNA DSBs shortly (up to 3 days) after irradiation. From these findings, we conclude that although both IL6 pretreatment and IL6 deficiency ultimately lead to a reduction in persistent DDR and senescence levels, they do so through fundamentally different molecular mechanisms.

Radiation-induced loss of salivary gland function is IL6 dependent, but is also prevented by IL6 treatment

We next hypothesized that if senescence is a major underlying mechanism driving irradiation-induced loss of salivary gland function, then a reduction in senescence, either by IL6 deficiency or by IL6 pretreatment, should significantly ameliorate the salivary hypofunction seen in the irradiated mice. To this end, we assessed salivary gland function in mice after pilocarpine stimulation 8 weeks after irradiation. Functional comparison of wild-type and IL6 −/− mice 8 weeks after irradiation revealed that animals with an IL6 deficiency had significantly increased saliva flow by 2- to 3-fold in irradiated mice (Fig. 7A and B). This was in close correlation with the observed decrease in PDDF and senescence-associated markers found in these mice, and was irrespective of whether the mice were irradiated in a single dose (13 Gy) or fractionated (5 × 5.6 Gy) doses. Blockade of IL6 signaling by injection of an anti-IL6 monoclonal antibody to wild-type mice also reduced radiation-induced hypofunction to a similar extent (Fig. 7C), thus recapitulating the effect of genetic IL6 ablation. In addition, we also examined the effect of inhibition of IL6/sIL6R-mediated IL6 trans-signaling using transgenic mice expressing high levels of the specific trans-signaling inhibitor, spg130Fc—a fusion protein consisting of soluble gp130 (sgp130) moiety linked to an immunoglobulin Fc domain (24, 43). These transgenic mice were equally sensitive to radiation-induced salivary hypofunction as wild-type mice (Fig. 7D), demonstrating that the effect of IL6 on loss of function derives from the effects of radiation on IL6R expressing cells.

Local infusion of either IL6 or HIL6 to the salivary glands prior to radiation also resulted in significantly higher long-term saliva flow, by up to 3-fold, in comparison with saline pretreated controls (Fig. 7E and F). This too was irrespective of whether the mice were irradiated in single-dose (13 Gy) or fractionated (5 × 5.6 Gy) doses. Interestingly, when mice were irradiated with a single fraction (13 Gy), no advantage was found in treatment with HIL6 over that of IL6 (Fig. 7E). This suggests that IL6R is expressed on cells for which IL6 supplementation protects against hypofunction. However, when mice were irradiated in fractionated doses (5 × 5.6 Gy), the mice pretreated with HIL6 displayed significantly more saliva flow compared with those treated with IL6 (Fig. 7E). This observation was consistent with both the lower levels of PDDF in ductal cells and also the reduced levels of p21 acinar cells observed in salivary glands of the HIL6 pretreated mice (Fig. 5A and C). This may reflect the longer signaling half-life of HIL6 complex or, alternatively, the ability of HIL6 to protect cell populations that do not express IL6R (25, 41). Importantly, we noted no side effects in the salivary glands that could be attributed to the local HIL6 or IL6 treatment, including development of tumors or inflammation. Furthermore, pretreatment with HIL6 to the salivary gland did not alter the sensitivity of distally implanted murine squamous cell carcinoma cells to radiation damage (Supplementary Fig. S9). Taken together, these findings demonstrate that...
radiation-induced persistent DNA damage and cellular senescence, at least in part, drive radiation-induced loss of salivary gland function, which can be substantially ameliorated by local treatment with HIL6 or IL6.

Discussion

Our findings identify cellular senescence as an inherent physiological process associated with and driving radiation-induced loss of normal salivary gland function. Radiation-induced senescence in the salivary glands—manifest by persistent DNA damage along with the expression of senescence-associated genes and SASP—closely accompanied the ultimate loss of glandular function. As opposed to other studies (17, 18), substantial apoptosis was not observed herein.

Our results suggest a model in which radiation in high doses produces severe genotoxic stress to the salivary gland, followed by DDR signaling and SASP, including the inflammatory cytokine, IL6. Radiation of the salivary glands induced γH2AX and 53BP1-containing DNA damage foci most notably in the ductal cells that were persistent, remaining for at least 2 months. IL6 expression appears to be crucial for the persistence of both the DDR and the expression other senescence markers. This finding is in agreement with the crucial role of the SASP and the cytokine response in enforcing senescence by boosting the DDR and other pathways (44). Importantly, cells of the salivary gland express IL6R, which has been shown to be a prerequisite for the induction and maintenance of oncogene-induced senescence (36). Our finding that independent experimental perturbations that reduced senescence (both IL6 deficiency and IL6 supplementation) also ameliorated salivary gland function following irradiation strongly supports the notion that senescence is a major driving process underlying hypofunction. On the other hand, this observation raises the intriguing question of how IL6 can support two inherently contradictory processes, i.e., the enforcement of senescence and, conversely, the prevention of senescence. The answer to the

Figure 7.
IL6 and HIL6 pretreatment, and also IL6 deficiency, ameliorate radiation-induced salivary hypofunction. Salivary gland function analyzed by pilocarpine stimulation in mice 8 weeks following irradiation with single (13 Gy; A, C–E), or fractionated (5 × 5.6 Gy; B, F) irradiation doses: wild-type and IL6-deficient (IL6−/−) mice (A and B); anti-IL6 mAb (Anti-IL6)- and control mAb IgG (CNTL Ab)-treated mice (C); wild-type and sgp130Fc transgenic mice (D); and following pretreatment by infusion to the submandibular salivary glands with saline (control), IL6 (15 ng), or HIL6 (50 ng; E and F). Data, means ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001, using one-tailed Mann–Whitney test; n = 4–7.
questions of why the DDR is persistent, and what is the mechanistic role of the SASP in maintaining this persistence, is not entirely clear (45), although some studies have shown that IL1 and other SASP components induce oxidative stress, thus producing de novo DNA damage and providing a positive feedback (46). Conversely, as shown here, IL6 can also act to enhance early DNA repair and thus prevent senescence prior to its establishment. Thus, IL6 can clearly function on two independent levels. However, whether IL6 functions as an enforcer of senescence or as an enhancer of DNA repair may depend on issues such as context, timing, and expression levels. It should be noted that in the present study radiation was administered to the head and neck region of the mice rather than focused only on the salivary glands. While the regional radiation used herein is known to have short-term effects unrelated to salivary gland parenchymal damage, we cannot exclude the occurrence of any long-term extraglandular effects.

How does cellular senescence ultimately lead to salivary hypofunction? Current thinking points to radiation-induced loss of primitive mesenchymal stem-like progenitor cells as well as the loss of acinar cells, both of which may potentially contribute to repopulating the salivary gland, together with decreased parasympathetic innervation and loss of endothelial cells and microvessels as the main causes of irreversible salivary hypofunction (12, 15, 16, 27, 47–49). Previous studies indicate that progenitor populations that are capable of repopulating and restoring salivary gland function following irradiation reside in the ductal cell compartment (10, 27, 50). Our findings demonstrate that manifestations of cellular senescence following radiation appear prominently within the ductal cell compartment, but also within acini, the latter having acquired distinct giant cell morphologies reminiscent of senescent cells in tissue culture (28). Putting these aggregate observations together, it is conceivable that salivary hypofunction is likely to be the result of multiple senescence-mediated events, including the probable loss of self-renewal of the salivary gland progenitor cells. Together, this would lead to a breakdown in the regenerative capacity required for replacement of senescent and dysfunctional acini, thus culminating in the ultimate reduction of gland function. Our findings also show that local treatment with IL6 or HIL6 prior to radiation can protect the ductal cells by enhancing DNA repair and suggests a new therapeutic approach to preserve salivary glands in head and neck cancer patients treated by irradiation. An important issue in this respect is whether local IL6 or HIL6 pretreatment in the salivary gland would have radioprotective or other undesirable effects on the tumor for which radiation is intended to treat. Because the local infusion of IL6 via cannula involves relatively minute levels of administered protein, the possibility of systemic effects can be expected to be negligible. Our preliminary studies into this possibility (Supplementary Fig. S8) support this contention. Although more extensive preclinical testing is necessary for a robust conclusion, collectively, the data presented in this study provide the rationale for potentially evaluating IL6 and HIL6 in clinical trials as agents for the preservation of salivary gland function in patients with head and neck cancer being treated by radiation.

Disclosure of Potential Conflicts of Interest

Y. Marmary has ownership interest in a patent. B.J. Baum is principal consultant at Bethesda Oral Medicine and is a consultant/advisory board member for Lg3 Pharma. S. Rose-John has ownership interest in Hyper-IL6. E. Galun has ownership interest in a pending patent. J.H. Axelrod ownership interest in intellectual property. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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References


Correction: Radiation-Induced Loss of Salivary Gland Function Is Driven by Cellular Senescence and Prevented by IL6 Modulation

In this article (Cancer Res 2016;76:1170–80), which was published OnlineFirst on January 12, 2016 and appeared in the March 1, 2016 issue of Cancer Research (1), there was an error in the Supplementary Data online. In Supplementary Fig. S7A, the image for the IL6 + IR 48 h panel of the DAPI-stained (DNA) field was incorrect; the image in this position was identical to the image above it and was an image of the Saline + IR 48 h treated glands. In addition, Supplementary Fig. S1C was presented twice in the Supplementary Data file.

The Supplementary Data has been corrected and replaced online. The authors regret these errors.

Reference

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Radiation-Induced Loss of Salivary Gland Function Is Driven by Cellular Senescence and Prevented by IL6 Modulation

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