Gene Transfer-mediated Functional Restoration for Irradiated Salivary Glands

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Radiation therapy for malignant tumours in the head and neck region are inevitably associated with significant long-term injury to the salivary glands, often resulting in salivary gland hypofunction. The subsequent lack of saliva production leads to many functional and quality-of-life problems for affected patients and there is no effective method to eliminating this problem caused by radiation treatments. Although many studies have been done in animal models, the mechanism of this injury in humans is still unclear. In this review, an animal model (miniature pigs) used in irradiated research is mainly discussed. This review also presents the progress made to date on the gene transfer-mediated functional restoration of irradiated salivary glands and the possibilities provided by future interventions to prevent radiation damage to salivary glands.

Key words: *gene transfer, miniature pig, radiation damage, salivary glands, xerostomia*

Head and neck cancers are common tumours, with 40,000 new cases of oral cancer diagnosed each year in the USA[1]. Most patients with head and neck cancers are treated with radiation therapy. While irradiation (IR) is quite effective as an adjunctive therapy for the cancers, it can also damage the adjacent normal tissues. It has long been recognised that salivary glands in the radiation field can suffer irreversible complications, leading to a marked reduction in the production of saliva. Because of the decrease in salivary secretions, some essential functions of saliva are impaired that may lead to difficulties with swallowing and speech, along with the susceptibility to oral infections[2,3]. This radiation-induced hypofunction has a large impact on the quality of life for surviving oral cancer patients[4,5]. Despite the numerous studies that have been done, the mechanism of radiation damage to salivary glands is still not well understood, and the prevention and management of salivary gland dysfunction remain inadequate. This review mainly focuses on IR-induced salivary gland hypofunction in miniature pigs, and gene transfer-mediated management and prevention of the loss of the salivary gland function.

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The effect of irradiation on salivary glands

IR leads to a loss of the fluid-secreting salivary acinar cells, resulting in severe glandular hypofunction in most patients. Although several experimental models were used to study the effects of IR on the salivary glands, the mechanism underlying this injury remains enigmatic. Previous studies show that damage to the salivary glands occurs in multiple phases[6,7]. The acute phase (0-60 days) is characterised by a significant decline in the saliva flow rate to approximately 50% of control glands, loss of glandular wet weight and loss of acinar cells, and alterations in the water and protein composition of saliva. In the chronic phase (60–240 days), cell numbers and protein secretion remain unchanged compared with the acute phase, whereas saliva flow decreases to approximately 30% of control glands[8]. Typically, radiosensitive tissues are composed of primitive, undifferentiated

cells with a high mitotic rate. Salivary gland acinar cells, which are the sole site of fluid secretion in the glandular tissue, are well-differentiated cells exhibiting a low mitotic rate. However, the changes in the quantity and composition of saliva that occur shortly after radiation therapy indicate that the glandular tissue is an acutely responsive tissue[9-12].

In IR-induced salivary gland hypofunction animal models, including mouse[13-18], rat[11,19-24], rhesus monkeys[25,26], and miniature pigs (minipigs)[27,28] (Tables 1 and 2), rodents are the most extensively studied. No animal model is entirely representative of human physiology, but the larger animals could provide a more appropriately sized target and often a better predictive result for many potential therapies than rodents[29-31]. The primate is one of the best models for studying irradiation damage to salivary glands[25,32], however, this model is too expensive for most laboratories. Previous studies showed that minipig parotid glands share several anatomic and physiologic characteristics in common with human glands and provide a valuable and affordable large animal model for studying irradiation-induced salivary hypofunction[33]. A typical large animal model of IR-induced damage to parotid gland and hyposalivation could be established in the minipigs. Firstly, the minipig parotid glands were irradiated with a single dose of 20 Gy and observed for 16 weeks. By 4 weeks post-irradiation, the parotid saliva flow rates in irradiated glands decreased by 60%, and by 16 weeks post-irradiation by 80%. Additionally, at this point of post-irradiation, the weight of the irradiated parotid glands was decreased by 50%. Marked destruction and atrophy of acini were observed, whereas most of the ductal structure was well-preserved histologically[28]. Secondly, the effects of single or dual field IR with the same radiation dose on the minipig parotid glands were evaluated. Two IR groups were subjected to a single 20 Gy irradiation to one parotid gland, using the single field or dual-field modality to produce different irradiation dose-volume distributions between the two irradiation groups.

Saliva flow rates from the IR side decreased dramatically at all time points, especially in the dual field irradiation group. These findings suggest that dose-volume distribution is an important factor in evaluating the radiobiology of parotid glands[34]. Thirdly, the effect of IR on microvascular endothelial cells in the minipig parotid glands were evaluated. A single radiation dose (25 Gy) was delivered to the parotid glands of minipigs. Irradiation led to a marked increase in the frequency of apoptotic nuclei detected in the minipig parotid glands. Additionally, 24 hours post-IR a significant number of the endothelial cells of the parotid gland were both TUNEL and CD31 positive, suggesting that those endothelial cells became apoptotic after IR. Measurements of acid SMase (ASMase, L) and neutral Mg2+-dependent SMase (NSMase, M) activity in irradiated minipig parotid glands was also performed.

Local parotid gland blood flow rate was decreased rapidly at four hours post-irradiation and remained lower than that of the control throughout the 14-day observation period. Parotid microvascular density (MVD) also was decreased from four to 24 hours post-irradiation, and remained lower than that of the control thereafter.

These findings suggest that IR-induced microvascular damage might play a key role in the IR-induced damage to salivary glands[35]. Since fractionated irradiation dosing is clinically used, it is essential to understand the structural and functional sequelae of fractionated IR-induced salivary damage in this large animal model. Minipig parotid glands were irradiated with 7.5 Gy or 9 Gy for five consecutive days and the changes observed for four months. Parotid saliva flow rates steadily decreased, reaching a 65% reduction in the 7.5 Gy group, and 75% reduction reached in the 9 Gy group by 16 weeks post-irradiation. Parotid gland weights were also significantly decreased (50%) in both IR groups. All irradiated glands in both IR groups showed a reduction in acinar size and number, with light eosinophilic staining in the cytoplasm of acinar cells. An increase in adipose tissue and a dramatic increase in interstitial fibrosis, indicating atrophy and degeneration of the parenchymal cells were noted. Variable levels of inflammatory cell infiltration, predominantly mononuclear cells with scattered neutrophils, were also seen in all irradiated glands. Surviving ducts were dilated and contained cellular debris and inspissated secretions. Additionally, there was a dramatic reduction in the levels of immunoreactive AQP5 detected in the acinar and intercalated duct cell region following both IR regimens. There also were significant alterations in saliva chemistry parameters 16 weeks after fractionated IR[36].

Gene transfer-mediated functional restoration

Current management approaches for xerostomia are generally unsatisfactory. For example, the use of pilocarpine to increase salivary output has met with minimal success, as has the use of thiol-based radioprotectants[37,38]. Although amifostine appears beneficial, the management of most patients with radiation-induced salivary hypofunction remains palliative in nature, including stimulation of residual salivary gland secretory potency

Table 1 Methods for animal models for irradiation-induced damage to salivary glands and hypofunction

Animal model	Anaesthesia during IR	Radiation times	Radiation dose (Gy)	Radiation field	Radiation instrument	IR plan	Protection for the rest of the body
Mouse[13-18]	Yes or No	Single	7.5, 15	Head or salivary gland	X-ray medical linear accelerator unit (MEVATORON 74DX 40; Philips CMG 41 X)	N/A	3 mm lead
		Multiple	6 × 5				
Rat[11,19-24]	Yes	Single	15, 17.5, 21	Head or salivary gland	Co-60 γ-radiation (Alcion CGR II) or X-ray unit (Mueller MG 300, Theratron 780)	N/A	3 or 6 mm lead
		Multiple	2 × 16				
Rhesus monkeys[25,26]	Yes	Single	10	Only salivary gland	Co-60 γ-radiation or X-ray unit (Clinac 20 linear accelerator)	N/A	N/A
		Multiple	2.5 × 20				
Minipig[27,28]	Yes	Single	15, 20	Only salivary gland	Linear accelerator (SL 7520 Philips Medical Systems)	3-DTP system	Lead door (control the ray)
		Multiple	7.5 × 5, 6 × 6				

Table 2 Results of animal models for irradiation-induced damage to salivary glands and hypofunction

Animal model	Saliva sample	Saliva flow rate	Pathology	Vascular damage and local blood flow	Collect saliva tool	Gland weight
Mouse[13-18]	Whole saliva	Decrease 50%	Pronounced loss of acinar cells	Vascular damage	Polyethylene tube	N/A
Rat[11,19-24]	Salivary gland saliva or whole saliva	Decrease 50–60%	Vacuolisation of acinar cells, pyknotic nuclei, lysis of acini, lack of acinar cells, relative increase in ductal cells, fibrous tissue		Miniaturised Lashley cups or polyethylene tube	Decrease 20-40%
Rhesus monkeys[25,26]	Salivary gland	Decrease 50–90%	Inflammatory cell infiltration, dilated ducts, parenchymal cells atrophic changes, loss of acinar cells, as well as in regions of fibrosis	Thrombosis	Miniature Carlson-Crittenden cups	N/A
Minipig[27,28]	Parotid gland saliva	Decrease 60–80%	Significant fibrosis, acinar atrophy, parenchymal loss, intercalated duct proliferation, intercalated duct dilatation, striated duct dilation, infiltration of lymphocytes	Vascular damage, increased Smase, decrease of local blood flow rate	Modified Lashley cups	Decrease 50%

(e.g. chewing, taste stimuli) and wetting the oral tissues if the residual potency is negligible (e.g. saliva substitutes). Despite these efforts, many patients who survive oral cancer experience significant xerostomia[39]. For these reasons, a gene transfer-mediated corrective treatment for radiation-induced salivary hypofunction was developed in the animals.

The gene transfer-mediated corrective strategy for IR-induced salivary hypofunction, using human aquaporin-1(hAQP1) cDNA encoded within a serotype 5 adenoviral vector (AdhAQP1), was based on the understanding of salivary fluid secretion and salivary duct cell physiology[40]. It was hypothesised that if the duct cells could secrete fluid through the transfer of a gene encoding a functional, non-polarised water channel protein, then the duct cells could generate an osmotic gradient, lumen > interstitium, and the expression of a water channel in these normally water impermeable cells would in turn permit osmotically-driven transepithelial fluid flow into the lumen. To transfer the hAQP1 cDNA into duct cells, a first generation serotype 5 adenoviral (Ad5) vector was used[19]. Initial studies of the function and potential utility of this vector were performed *in vitro* with several epithelial cell lines. The transgenic hAQP1 protein was functional and resulted in the net movement of fluid from a basal to an apical direction[19].

Additionally, osmotically obliged fluid movement across the submandibular gland acinar epithelial (SMIE) cells was increased in an AdhAQP1 dose dependent manner[41]. To determine if the AdhAQP1 vector was effective in restoring saliva flow to IR-damaged salivary glands, investigators employed single radiation doses in rodents (either 17.5 Gy or 21 Gy) rather than a fractionated scheme more typical of the clinical situation. At the appropriate point in time, the animals were administered 5×10^9 pfu of the AdhAQP1 vector or a control Ad5 vector. After three days, the overall saliva flow rate from each animal was measured. The results demonstrated that delivery of the AdhAQP1 vector increased the saliva flow rate in the irradiated rats[19].

A critical step in the development of gene therapy is the demonstration of efficacy, and scaling to a large animal model. Originally for this purpose, some studies conducted experiments in rhesus macaques, but the results were equivocal, probably because of the small number of animals available[26]. Gene transfer approaches for salivary gland irradiation-induced hypofunction in minipig parotid glands were used. Firstly AdCMVluc, a recombinant type 5 adenoviral (rAd5) vector containing a luciferase reporter gene, and AdCMVlacZ, a similar rAd5 vector encoding β-galactosidase, were transferred into minipig parotid glands by intraductal cannulation[42]. Transgene expression was located in both acinar and ductal cells, and an inflammatory response to rAd5 vectors in the minipig parotid glands were similar to results seen earlier in rodent studies. Next, one minipig parotid gland was irradiated with a single dose of 20 Gy for experimental convenience. Sixteen weeks post-irradiation, the average parotid saliva flow rates in irradiated glands were decreased by 80%. All animals were treated with either the AdhAQP1 vector or a control vector (Ad5 vector encoding luciferase) the following week. The maximum total vector dose administered to each parotid gland was 109 pfu. Three days after vector delivery, a dramatic increase in parotid saliva flow rates, up to 80% of the levels of pre-irradiation control. The flow rates began to decrease at day 7 until day 14, but still higher than that of the glands with the control vector. An apparent loss of acinar cells with replacement by connective tissue was observed histologically, as seen in humans[43]. Nonetheless, these results strongly support the hypothesis that the hAQP1 gene transfer could lead to a correction in saliva flow rates in large irradiated animals.

As a result of pre-clinical efficacy studies in rats and minipigs and extensive safety studies, a phase I clinical study with AdhAQP1 is ongoing at the National Institute of Dental and Craniofacial Research at the National Institutes of Health[19,43-45]. However, the strategy of using AdhAQP1 would be relatively short lived because of the transient expression of adenoviral vector-mediated transduction of salivary glands[46]. To provide stable transfer and expression of the hAQP1 cDNA in irradiated salivary glands, a vector that induces a longer duration of expression is needed. Serotype 2 adeno-associated viral (AAV2) vectors provide stable transgene expression in several tissues, including both rodent and macaque salivary glands[47,48]. The utility of AAV2 vectors to provide extended expression of transferred genes to the parotid glands of minipigs was assessed. Transgene expression was vector dose dependent, with high levels of hEpo in saliva from transferred parotid glands detected for up to 32 weeks. What was proven was that AAV2 vectors mediate extended gene transfer to minipig parotid glands and should be useful for testing pre-clinical gene therapy strategies to correct IR-induced hypofunction of the salivary gland[49]. *In vitro* studies showed that an AAV2 vector encoding hAQP1 could increase net fluid secretion *in vitro*[50]. Then an AAV2 vector was evaluated for extended correction of IR-induced (20 Gy) parotid salivary hypofunction in minipigs. Sixteen weeks following IR, salivary flow from targeted parotid glands

decreased by 85 to 90%. AAV2hAQP1 administration at week 17 post-irradiation transduced only duct cells and resulted in a dose-dependent increase in parotid saliva flow to 35% of pre-IR levels after eight weeks. Vector-treated animals generated high anti-AAV2 neutralising antibody titers by week 4 (1:1600) and significant elevations in salivary but not serum GM-CSF levels. At the same time, the expression of the transgenic hAQP1 in transduced glands was examined. The results indicated that significant transduction of the targeted tissue occurred[51].

Gene transfer-mediated prevention of radiation damage

The most effective intervention for reduced salivary gland function is prevention, because once damage occurs, treatment of hyposalivation essentially relies upon stimulation of the residual secretory capacity of the irradiated salivary glands. Some researchers believed that gene transfer to restore the function of the damaged gland can only be an option when epithelial tissue survives the damage caused by the radiation. In the absence of any parenchymal cells, when a gland is fully replaced by fibrotic tissue, gene transfer cannot lead to an enhancement of saliva production since no system exists to produce and transport fluid into the mouth[52,53]. Regardless, preventing or minimising the damage is essential. The preclinical experiments on preventing radiation damage have made significant progress. For example, a single intravenous injection of IGF1 (insulin-like growth factor-1) prior to exposure to γ-radiation, diminishes salivary acinar cell apoptosis and completely preserves salivary gland function three and 30 days following irradiation[54]. Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) is a stable nitroxide that has been shown to be a radioprotector *in vivo* and *in vitro*. Tempol is a promising candidate for clinical applications to protect salivary glands in patients treated with radiotherapy for head and neck cancers[13,55]. Using a recombinant adenovirus serotype 5 viral vector for Tousled-like kinase 1B (TLK1B) gene transfer into rat submandibular salivary glands, after a single fraction of 16 Gy, the decline in salivary function at eight weeks was less pronounced in TLK1B-treated animals (40%) when compared with saline-treated controls (67%). Histopathological analysis showed an increase in acinar atrophy, decrease in acinar cell number, and increase in inflammatory infiltrate and fibrosis in irradiated control tissues relative to TLK1B-treated glands. These results indicate the radioprotective benefits of TLK1B and implicate its usefulness in the management of regional radiotherapy-induced xerostomia[56].

While most studies aimed at preventing IR-induced salivary hypofunction have focused on acinar cells, some studies have explored an alternative possibility; that IR primarily damages microvascular endothelial cells. The possibility that microvascular endothelial cells might be most sensitive to IR was first put forth in studies on gastrointestinal radiation damage[57]. Investigators confirmed the association between IR-induced changes in endothelial cells and the development of IR damage to salivary glands. Specifically, four hours after IR, the MVD in murine submandibular glands was significantly reduced. Furthermore, a single local administration of a modest dose (5×10^9 particles/gland) of a serotype 5 adenovirus (Ad5) vector encoding either bFGF or VEGF 48 hours prior to IR (15 Gy) prevented rapid MVD loss in submandibular glands and reduced the loss of saliva flow measured eight weeks post-IR[58]. Similar prevention studies have been designed and scheduled in minipig parotid glands.

Perspectives

After irradiation of the salivary glands, and the AdCMVAQP1 transfer-mediated clinical trial for patients with hyposalivation are carried out, the study may provide basic information about the biology of gene transfer to human salivary glands. Since adenovirus-mediated gene expression is transient, the clinical application of AdCMVAQP1 may be limited. AAV2-mediated AQP1 gene expression has a longer effect and may be an alternative way to restore salivation in patients with irradiated salivary glands. The best way to overcome this problem is to prevent or minimise therapy-related injury to the salivary glands.

Conclusions

A clear understanding of the mechanism of the irradiation-induced injury to the salivary glands in the well-established minipig large animal model, along with better and more effective prevention methods should be investigated and discovered, and, finally, used to prevent irradiation damage to salivary glands.

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