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INVITED MEDICAL REVIEW

Stem cells and the repair of radiation-induced salivary gland damage

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Hyposalivation underlying xerostomia after radiotherapy is still a major problem in the treatment of head and neck cancer. Stem cell therapy may provide a means to reduce radiation-induced hyposalivation and improve the quality of life of patients. This review discusses the current status in salivary gland stem cell research with respect to their potential to attenuate salivary gland dysfunction. Knowledge on the embryonic development, homeostasis and regeneration after atrophy of the salivary glands has provided important knowledge on the location of the salivary gland as well as on the factors that influence proliferation and differentiation. This knowledge has helped to locate, isolate and characterize cell populations that contain the salivary gland stem cell, although the exact tissue stem cell is still unidentified. The role that stem/progenitor cells play in the response to radiation and the factors that can influence stem/progenitor induced proliferation and differentiation are discussed. Finally, the mobilization and transplantation of stem cells and supportive cells and their potential to attenuate radiation-induced salivary gland damage are discussed. Based on the major advances made in the field of stem cell research, stem cell-based therapy has great potential to allow prevention or treatment of radiation-induced hyposalivation.

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Introduction

Hyposalivation is the most common condition underlying xerostomia, the subjective feeling of dry mouth. A more than 50% reduction in salivary flow is usually accompanied by xerostomia. Many people but especially those 65 years old or greater knowingly or unknowingly suffer from xerostomia. Especially, in patients using drugs, suffering from Sjögren's syndrome and those being treated for head and neck cancer with radiotherapy prevalence of hyposalivation is particularly high. Next to these a number of other conditions such as uncontrolled diabetes mellitus, sarcoidosis and renal diseases can cause hyposalivation related xerostomia (Napenas *et al*, 2009). Considering the severe impact xerostomia may have on patients quality of life, there is an unmet clinical need for an efficient treatment (Vissink *et al*, 2003a,b, 2010; Jensen *et al*, 2010a,b).

Stem cell therapy could provide an option to prevent and repair damage of tissues induced by degenerative processes due to auto-immune responses, radiation-side effects or other cytotoxic events. Basically, three stem cell types are currently being investigated for their potential use in stem cell therapy: embryonic stem cells, induced pluripotent stem cells and adult stem cells. Induced pluripotent stem cells which closely resemble embryonic stem cells may evolve to be useful in the not too far away future but first the control of cell differentiation and development into salivary glands specific lineages needs to be assessed to prevent the formation of teratomas. Therefore, in this review we will focus on the adult stem cells of the salivary glands of which in animal models it has been shown that with these cells recovery of radiation damaged salivary gland tissue can be achieved. Currently, adult stem cell therapy of the salivary glands is in the transitional stage of bringing the animal results into the clinic.

Adult stem cells of the salivary gland

Adult (somatic or tissue-derived) stem cells are generally organ restricted and only form cell lineages of the organ from which they originate (unipotent) and therefore do not form teratomas. Like any other adult stem cell population, salivary gland adult stem cells are undiffer-

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entiated but reside between differentiated cells (stem cells niche). Adult stem cells are able to self-renew and can differentiate to yield all specialized cell types. Formation, maintenance and repair of the tissue in which they reside are the primary roles of the adult stem cell. Regretfully, adult stem cells are not easy to find, but by using well-established specific stem cell characteristics it is possible to distinguish them from the other cells in the tissue.

Adults stem cells are closely related to or remnants from the embryonic development. Interestingly, during adult recovery from injury cells with an embryonic like phenotype have been associated with the regeneration process (Cotroneo *et al*, 2008, 2010; Carpenter and Cotroneo, 2010). Additionally, like during embryonic development (Cutler and Chaudhry, 1973) the interaction between mesenchymal and epithelial cells also seems important for the regeneration of the radiation-injured salivary gland tissue (Lombaert *et al*, 2006).

Embryogenesis of the salivary glands, lessons for regenerative therapies

The parotid gland and the submandibular/sublingual gland develop from different embryonic origins. The parotid gland is derived from the ectoderm, whereas the submandibular and sublingual glands are derived from endoderm. From the stem cell point of view this could make them unique and potentially not interchangeable. However, all types of salivary gland develop in a similar pattern of morphogenesis driven by cytokines, growth factors and extra cellular matrix components. Transplantation of submandibular gland stem cells in parotid glands and visa versa could shed light on the interchange ability of the stem cells. For the clinical application of stem cells this may be very important, since then after the dissection of the submandibular gland during a neck dissection procedure its stem cells could be used for therapy of the parotid gland. What is clear, however, is that both the parotid and submandibular/sublingual glands are enclosed within a well defined capsule of neural crest derived mesenchyme and that interaction with the surrounding mesenchyme is essential for the initial budding of the salivary gland (Jaskoll *et al*, 2002).

Fibroblast growth factors

Fibroblast growth factor (FGF) signalling is of major importance during development of the salivary gland (Patel *et al*, 2006). Although the initiation of the salivary gland during embryogenesis is not controlled by FGFs, secretion of members of the FGF family by the mesenchyme, for example FGF8 and FGF10, appears to control the branching of the developing salivary gland. In parallel the epithelial cells of the salivary gland express the FGF2b receptor at this stage (Jaskoll *et al*, 2005). A whole range of growth factors, cytokines, extra cellular matrix proteins and anti-apoptotic proteins are involved in the development of the salivary glands. When provided at the appropriate time, FGF7, FGF8 and tumour necrosis factor-alpha (TNFα) increase budding and branching is induced by FGF10, epidermal growth factor (EGF), transforming growth factor-β (TGF-β), TNFα, bone morphogenetic protein 7, ectodysplasin and sonic hedgehog homolog expression (see Tucker, 2007). Many of these factors have been shown to play a role in regeneration as well.

The specificity of 'salivary gland mesenchyme' is of eminent importance as demonstrated by the lack of salivary gland formation when the mesenchyme is replaced with that from other glandular tissues (Kratochwil, 1969; Kusakabe *et al*, 1985). Mesenchyme of submandibular and parotid gland however, do seem to be interchangeable (Ball, 1974) even between species (Nogawa and Mizuno, 1981), meanwhile maintaining the characteristics of the epithelial cells of origin (Tyler and Koch, 1977). Epithelium and mesenchyme are separated by the basement membrane of which components such as glycosaminogycans, collagens, fibronectins, integrins, laminin-γ1 and nidogen-1 play a crucial role in branching morphogenesis (Ekblom *et al*, 1994; Kadoya *et al*, 1997; Kadoya and Yamashina, 2005; Patel *et al*, 2006, 2007; Ho *et al*, 2008). It appears that once cytodifferentiation has started, the presence of mesenchyme is no longer required (Cutler, 1980). As a result it has been shown in adult mice that specificity of mesenchymal cells is less important since even bone marrow derived mesenchymal cells were able to stimulate regeneration (Lombaert *et al*, 2006). For future development of regenerative therapies, knowledge of the development of the salivary glands, their stem/progenitor cells and the interactions with the environment in cell fate decision is of eminent importance (Lombaert and Hoffman, 2010).

Stem cells and tissue homeostasis

In every tissue cell loss due to aging or cytotoxic insults needs to be compensated by proliferation and differentiation of the tissues progenitor and/or stem cells. In most tissue the normal homeostasis involves proliferation of progenitor cells, whereas the stem cell is relatively quiescence and becomes only active after substantial depletion of differentiated cells. Many studies have been performed to investigate the cells that are involved in the homeostasis of salivary glands. It has been shown that nearly all of the differentiated cell types of the adult salivary gland appear to retain the ability to replicate (Denny *et al*, 1993), but which cells have the potential to produce other types of cells? Moreover, which cells are able of forming all other cell types?

Most studies investigating cells that are involved in homeostasis of a tissue use the label-retaining cell (LRC) assay to define progenitor/stem cell localization. Nucleotide analogues, such as bromodeoxyuridine or tritiated thymidine, are incorporated in the DNA after a period of continuous administration, and label all dividing cells. During a subsequent chase period in which no nucleotide analogue is administered, the label will be diluted with every cell division. The less frequently dividing cells will therefore retain the label; the LRCs are considered to contain the stem cells (Potten *et al*, 2002). It has long been thought that the intercalated duct cells contain a progenitor type of cells which could differentiate into both acinar and granular convoluted

tube (GCT) duct cells, and even further downstream into striated and excretory ducts (Zajicek *et al*, 1985). The latter residence of progenitor cells, however is disputed in later studies (Man *et al*, 2001), where an extremely high labelling index was also observed for the excretory ducts suggesting a progenitor role of these cells in the formation of striated ducts. During normal homeostasis in adult male mouse submandibular glands, it was observed that from a certain age acinar cells were completely maintained through self-proliferation, subject to constraints correlated to age of the mouse, as opposed to in females where the intercalated ducts appeared to provide the stem cells until the mice retired as breeders. Hereafter the salivary gland was maintained in the same manner as in male mice (Denny *et al*, 1997). Based on these studies, it was proposed that the intercalated duct cells contained the progenitors for acinar and GCT cells and that striated duct cells were presumably replaced by more primitive excretory duct cells (Man *et al*, 2001).

Although these studies provided valuable information on mechanisms of proliferation and maintenance of the salivary glands, they may not provide information on the exact location of adult stem cells within a tissue. The short time period in which the labels usually are administered may not be sufficient to permit DNA analogue incorporation into the replicating genome of the mostly quiescent stem cells. Good examples of this are the studies of the intestine. Label-retention experiments pointed to a location four cell position luminal from the base of the intestinal crypt that might reside adult stem cells (Potten *et al*, 2009), whereas lineage labelling and stem cell self-renewing studies, based on expression of Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) suggested a location intercalated between the Paneth cells crypt base columnar cells (Barker *et al*, 2007). Next, it was shown that a single Lgr5-labelled cell was able to build a complete crypt-villus structure in vitro implying that this second location is most likely to harbour the real stem cell (Sato *et al*, 2009). Considering the rapid turnover of the intestinal tissue (days) and compared to the rather slow turnover of the salivary gland (2–4 months; Zajicek *et al*, 1985), it may not be surprising that the stem cell of the salivary gland has not been detected in LRC studies to date. The induction of damage to the salivary gland, thereby speeding the proliferation and potentially activating the stem cells, may provide more detailed information about which cells within a salivary gland can be considered the real stem cells.

The above described observations have all been made in rodents. Little is known about the stem/progenitor cells in humans. Most of what we know is derived from studies of salivary gland tissue obtained from neoplasms and assumes that the differentiation pathways manifested in these glands are similar to those found in normal tissues. Next to a mitigating role of myoepthelial differentiation, regeneration of the salivary gland duct was thought to be the preserve of a putative uncommitted stem cell population (Batsakis *et al*, 1989). In a series of publications (Ihrler *et al*, 1999, 2002, 2004) using immunohistochemistry for the detection of cytokeratin subtypes (depicting epithelial cells), α-actin (smooth muscle cells), Ki 67 (associated with proliferation) and Bcl-2 (an anti-apoptosis marker often upregulated in stem cells) it was shown that there is a baseline of proliferation in all five types of parenchymal cells of the normal human salivary gland. Proliferation in the intercalated duct and acinar cells was low, and that of the myoepithelial and oxyphilic cells lower still. Double immunohistochemical labelling of α-actin for Ki 67 or Bcl-2 combined with the cytokeratin profile indicated that the basal cells may contain the potential progenitor/stem cell for the striated and excretory ducts whereas acinar cells and intercalated duct cells undergo self-proliferation. Potentially, lineage labelling studies and in vitro cultures may provide more information on the human salivary gland stem cell.

Regeneration of atrophic salivary glands

Since salivary glands have an excellent capacity to regenerate after partial extirpation (Hanks and Chaudhry, 1971) or duct obstruction (Burford-Mason *et al*, 1993), it seems likely that they in fact do contain stem/progenitor cells. Both extirpation and duct obstruction have been used to characterize and isolate stem/progenitor cells (Scott *et al*, 1999; Takahashi *et al*, 2004, 2005).

Ligation of the excretory duct of the submandibular or parotid gland induces atrophy of the glands leading to shrinkage and apoptosis of acinar cells, luminal dilation due to degranulation and increased proportional volume of ducts, changed shape, position and proliferative activity of myoepithelial cells and obviously loss of gland weight (Carpenter and Cotroneo, 2010). Removal of cells seems to be initiated by the Fas ligand signalling pathway via activation of caspases 3 and 8 (Takahashi *et al*, 2007) and subsequent apoptosis. Inflammatory cell infiltration, denser connective tissue (Norberg *et al*, 1988) and enlarged intercellular spaces (Walker and Gobe, 1987) complete the picture. Interestingly, the remaining ductal cells seem to over express anti-apoptotic Bcl-2 (Takahashi *et al*, 2008), a marker that is also often over expressed in stem cells (Lu *et al*, 1996). In contrast, the proapoptotic Bax was over expressed in acinar cells (Takahashi *et al*, 2008). Additionally, myoepithelial cells presence appears to increase in response to ligation, in what is hypothesized to be an attempt to resist the infiltration-related pressure increase (Cotroneo *et al*, 2008).

Deligation of the main ducts induces a rapid regeneration process in which the number of acinar cells increases (Scott *et al*, 1999). In general, it seems that first residual (if any) acini proliferate followed by the formation of new acini (Takahashi *et al*, 1998, 2004, 2005), reattachment of parasympathetic nerves (Carpenter *et al*, 2009) and recovery of (nearly) normal saliva composition and gland function (Scott *et al*, 1999; Osailan *et al*, 2006; Carpenter *et al*, 2007). The new formation of acini after duct deligation and subsequent regeneration of the secretory tissue is an interesting phenomenon as it may yield information on progeni-

tor/stem cells of the salivary gland. After deligation atypical acinar-ductal branched structures appear that are normally virtual absent in ligated and normal glands (Cotroneo *et al*, 2008). Interestingly, expression of the protein submandibular gland-B (SMG-B), not normally noted in the adult salivary gland acinar cells was up-regulated and localized in acini of the deligated gland (Ball, 1974; Man *et al*, 1995; Mirels *et al*, 1998; Cotroneo *et al*, 2008, 2010). Therefore, it was suggested that during regeneration embryonic-like branching structures are the source of newly differentiated acinar cells (Cotroneo *et al*, 2010). Furthermore, it was speculated that during regeneration ductal cells in these embryonic-like branches have switched on a prenatal program and that ducts from which they originate contain pluripotent or at least undifferentiated cells. This would be in agreement with the LRC hypothesis and with other studies suggesting the presence of pluripotent cells in the intercalated ducts (Man *et al*, 1995, 2001; Kishi *et al*, 2006).

Selection and characterization of stem cells

Label retaining studies have provided important knowledge of the cells involved in maintenance and regeneration of a tissue. However, aside from rapidly dividing tissue such as the intestines, it is very difficult to pin point the exact location of the stem cell. Another approach to pin point the exact stem cell is genetical labelling of cells. Studies in intestine (Barker *et al*, 2007), hair (Jaks *et al*, 2008; Fuchs, 2009), skin (Blanpain and Fuchs, 2009) and stomach (Barker *et al*, 2010) used a multiple organ, genetic labelling approach to shown that genetical labelling of cells may result in the detection of the exact stem cell of a particular tissue. For example, using two knock-in alleles, Lgr5 or Lgr6 were exclusively expressed in cycling cells of the intestine (Barker *et al*, 2007), stomach (Barker *et al*, 2010) or in the hair follicle (Snippert *et al*, 2010). Furthermore, using an inducible Cre knock-in allele and the Rosa26-lacZ reporter strain, lineage-tracing experiments were performed in adult mice. With these powerful tools stem cells could be traced after division and differentiation, demonstrated to form all tissue lineages and shown to take part in the regeneration after wound infliction (Snippert *et al*, 2010). It is anticipated that when creating a mouse with a genetic label of a gene involved in the biology of salivary gland, it will possible to locate the real stem cell of the salivary gland.

However, even with these techniques, additional novel cell types have been identified that are able to self renew and differentiate in all lineages of the tissues. These data query the existence of one stem cell population in the whole tissue. Further evidence for the existence of several potent stem cell populations within a tissue niche is provided by the field of hematopoietic stem cells. Here multiple cells have been found to be able to repopulate the bone marrow with all specific cell types albeit in different proportions (Kent *et al*, 2009).

An alternative strategy for the isolation of tissue stem is to detect cells that express certain stem cell markers. A wide variety of such well-established markers have been characterized. This is particularly true for the bone marrow, where the selection of hematopietic stem cells using fluorescence-activated cell sorting has been proven to be a powerful tool. However, for solid tissue this approach is more complicated. First the tissue needs to be dispersed into a cell suspension using mechanical forces and enzymatic digestion. This often results in a mixture of dead and living cells which are not easy to handle. Therefore, an intermediate step of cell culturing is often necessary to loose the death cells and enrich the culture for stem/progenitor cells.

In the mammary gland this approach has resulted in the isolation of cells expressing the specific stem cell markers, CD24/CD29 (Shackleton *et al*, 2006) one such $CD24/CD29^{+}$ was capable of forming a complete mammary gland in a cleared fat pad of a mice. Since then several other tissues, such as prostate (Leong *et al*, 2008) have demonstrated presence of cells with the capacity to form large amounts of functional tissue, following the proliferation and differentiation of a single initial cell.

In salivary glands attempts at cell culturing after dispersion of the tissue have yielded several different cell types that may have stem cell properties. However as mentioned above, salivary tissue consist of a number of cells derived from different origin, such as parenchymal cells, stromal cells, blood vessel cells and neural cells. In culture, at first these cells cannot be separated from each other. Therefore, primary cultures of dispersed cells will always contain all these cell types making it difficult to pick out the specific salivary gland stem cell. The best way to detect what cell can be considered as the exact stem cell of a tissue would be the selection of cells carrying a specific marker or selecting the cells labelled with induced reporter proteins, as mentioned above. Although the genetic expression of reporter proteins in salivary glands has not been established yet, several attempts have been undertaken to select for specific stem cells of the salivary gland using stem cell markers.

Adult salivary gland stem cells and mesenchymal stem cells

Gorjup *et al* (2009) were able to isolate cells from the human submandibular glands and to grow these cells for up to 55 doubling times in adherent cultures. Such a long term culture of human cells is normally only seen for embryonic stem cells and for neonatal stem cells from the umbilical cord. Normally, specific adult tissue stem cells can barely be cultured in vitro with the exception of mesenchymal stem cells (MSC). Indeed the cells isolated by Gorjup *et al* (2009) could be induced to undergo chrondrogenic, osteogenic and adipogenic differentiation, hall marks for MSCs. Differentiation into all salivary gland lineages, however, was not shown and therefore these cells should be considered as supportive salivary gland MSC. However, differentiation into myoepithelial cells and supportive action through the secretion of growth factors (Lombaert *et al*, 2006) and immuno-modulation (Kode *et al*, 2009) during recovery and regeneration of damaged salivary

glands may offer therapeutic potential of these supportive salivary gland MSCs.

Formation of acinar and duct-like structures cells was observed from the 2D culture of human (Sabatini *et al*, 1991; Dimitriou *et al*, 2002; Hiraki *et al*, 2002), macaque (Sabatini *et al*, 1991) and rat (Horie *et al*, 1996) submandibular/parotid (duct) gland cells. More recently, investigators succeeded to grow structures expressing amylase and aquaporin 5 (AQP5) in 3D collagen/matrigel cultures (Joraku *et al*, 2005). Next, Kishi and colleagues (Kishi *et al*, 2006), where the first able to show the presence of stem/progenitor cells in vitro using a colony forming assay using cells obtained from (neonatal) rat submandibular glands. The colonies formed all cell types of the submandibular gland and expressed several specific submandibular gland markers such as AQP-5, Smooth muscle actin, N^+K^+ATPase, S-100, CK19 and c-met.

These results showed that in vitro culturing of salivary gland structure may be possible, albeit without showing which exact cell type was responsible for the formation of the structures. A certain level of division and differentiation can be shown with these models, but formation of different structures, functionality of these structures and the self-renewal of a specific cell cannot be assessed.

To this end, in vivo transplantation studies with prior selection of stem/progenitor cells have been performed. In a set of experiments, specific markers were used to select stem/progenitor cells after enrichment of the population by duct ligation. As such a population of Sca-1^+c-Kit^+, $\alpha6^+/\beta1^+$-integrin, $laminin^+$ and $CD49f^+/Thy1^+$ cells were isolated from mouse (Hisatomi *et al*, 2004), rat (Okumura *et al*, 2003; Matsumoto *et al*, 2007), swine (Matsumoto *et al*, 2007) and human (Sato *et al*, 2007) salivary glands. When transplanted through the portal vein into the partially hepatectomized livers some of these cells were able to integrate into the liver and were shown to express liver specific markers. Although these results are very interesting, these cells were not fully tested for their stem cell potential in clonogenic and functional tests.

Considering the above described studies, it is very likely that the selected cells at least contain salivary gland derived MSCs. The fate of these cells is very likely also determined by the niche in which they are grown and it is still debated whether these cells can differentiate into non-mesenchymal cells (Prockop, 2009). The adherence of these cells on the culture surface and rapid proliferation is not in agreement with what one would expect from adult tissue specific stem cells, which normally rapidly differentiate in 2D culture. However, it seems from many studies that mesenchymal stem cells improve repair of tissue by differentiation and potentially transdifferentiation, albeit in many situations the cells augment the repair and functional improvement of injured tissues without significant engraftment or differentiation. Indeed, this was suggested to occur in the salivary gland with bone marrow derived MSCs (Lombaert *et al*, 2006, 2008c), through paracrine secretions and cell-to-cell contacts.

The role of stem/progenitor cells in response of salivary glands to radiation

Because of their anatomic position, salivary glands often are (partially) located with in the radiation portal during radiotherapy for head and neck cancers. The resulting radiation-injury to salivary gland tissue may result in life-long salivary gland impairment severely reducing the posttreatment quality of life of the patients. In the response of a tissue to radiation many factors play a role (Bentzen *et al*, 2007), but ultimately, the (in)ability of stem/progenitor cells to reconstitute functional cells determines the onset and the severity of the radiation effects (Zeilstra *et al*, 2000; Coppes *et al*, 2009). Therefore, the time for a tissue to respond to radiation is determined by the tissue turnover time. Salivary glands, however, are an exception to that rule. Salivary gland is a highly differentiated and slowly dividing tissue that responds to radiotherapy very rapidly (Burlage *et al*, 2001). This effect cannot be due to stem cell sterilization, but has been ascribed to either induction of apoptosis (Grundmann *et al*, 2009) or selective radiation damage to the plasma membrane of the secretory cells, which in turn disturbs muscarinic receptor stimulated watery secretion (Coppes *et al*, 2000, 2005). Interestingly, apoptosis has been show to occur extensively and at rather low radiation doses when the whole body or the whole head of the test animals is irradiated (Stephens *et al*, 1986, 1991; Grundmann *et al*, 2009). Why apoptosis seems to play a large role in the early effects of radiation and not following more localized irradiation in these experimental models (Konings *et al*, 2005a) remains unknown and demands further investigation. Although the early postradiation effects certainly play a role in the patients feeling of well being (Jellema *et al*, 2007; Jensen *et al*, 2010a), in the long term, the survival of the tissues stem cell plays an important role. Fractionated radiation is commonly applied to spare late normal tissue effects (Joiner and van der Kogel, 2009). Indeed, using a clinically oriented fractionation scheme of 16 fractions of 2Gy/fraction in a rat model after 120 days it appeared that the parotid gland performed better than after a biological equivalent single dose of 15 Gy (α/β: 9.6 for early effects) (Coppes *et al*, 2002), indicating sparing of the tissue stem cell with consequential late recovery. Such phenomenon has also been observed in humans, where even 5 years after radiotherapy some further improvement of salivary flow rate was observed (Braam *et al*, 2005). These results predicted the presence of a relatively quiescent salivary gland stem cell responsible for late recovery that may not reside near the acini. In line with this, sparing of parts of the parotid gland using proton radiation, a radiation technique with a much steeper dose delivery within a tissue thus sparing surrounding tissues, resulted in reduced damage to the gland (Konings *et al*, 2005b). Surprisingly, it mattered which part of gland was spared. Irradiation of the cranial 50% of the gland results in a more than proportional damage than irradiation of the caudal 50%. Secondary effects and enhanced recovery, respectively, where thought to be

responsible for these two phenomena. Recently, however, it was shown that a very low dose to the cranial 50% could already completely abrogate the enhanced sparing after caudal irradiation (van Luijk *et al*, 2009). Interestingly, the most sensitive part of the salivary gland contains the largest proportion of excretory ducts which potentially harbour the salivary gland stem cells also suggesting an uneven distributed quiescent salivary gland stem cell responsible for late recovery.

Stem cell therapies

Application of agents that stimulate the division of progenitor and stem cells

Growth factors, cytokines and other drugs stimulating progenitor and stem cell proliferation understandably have been experimentally investigated for the modulation of radiotherapy complications (Joiner and van der Kogel, 2009). In addition, stimulation of proliferation and differentiation of radiation surviving stem cells have been shown to be beneficial in many organs. In the salivary gland cytokines like EGF (Ohlsson *et al*, 1997; Limesand *et al*, 2009), insulin growth factor (Thula *et al*, 2005; Limesand *et al*, 2009) and bFGF (Thula *et al*, 2005) have been suggested to inhibit apoptosis and/or enhance proliferation. Recently, in our laboratory the effects of Keratinocyte growth factor (KGF or FGF7) on radiation-induced salivary gland damage in the mouse submandibular gland were studied (Lombaert *et al*, 2008c). ΔN23-KGF treatment for 4 days prior to irradiation indeed induced salivary gland proliferation of all cell types but especially stem/progenitor cells, increasing the stem- and progenitor cell pool. Although the relative radiation sensitivity of the stem/progenitor cells was not affected, the absolute higher number of stem/progenitor cells and acinar cells that survived irradiation was higher. Postirradiation treatment with ΔN23-KGF further improved gland function, seemingly through accelerated expansion of the pool of progenitor/stem cells that survived the irradiation treatment. These results are very promising, also because, ΔN23-KGF has been shown to ameliorate radiation-induced damage in oral mucosa (Dorr *et al*, 2002), potentially attenuating radiation-induced xerostomia by reducing both hyposalivaton and mucositis. However, for future clinical use, a potential issue of concern is the possibility that KGF, like any other growth factor may stimulate tumour proliferation and may potentially interfere with anti-cancer therapies. Interestingly, postradiation KGF treatment also showed a small but significant increase stem/progenitor proliferation and salivary flow. This effect was very similar to the effects observed after pilocarpine treatment (Roesink *et al*, 1999; Coppes *et al*, 2001; Burlage *et al*, 2008). Pilocarpine, like growth factors seems to induce postirradiation proliferation (Burlage *et al,* 2009) thereby enhancing the regenerative potential of radiation surviving stem/progenitors. From the above mentioned studies it can be concluded that salivary gland that do survive the radiation-insult are able to proliferate and regenerate the tissue when stimulated properly. They also imply, however that intrinsic factors, such as wound healing induced cytokines secretion of TGF-β (known to inhibit stem cell proliferation and induce differentiation and extracellular matrix deposition by fibroblasts), are altered by radiation preventing stem cells from regenerating the tissue. Appreciation of these facts suggest new strategies for the enhancement and regeneration of radiation-damaged tissue.

Blood vessels

Blood vessels are of eminent importance for the function of the salivary glands. It has been shown that by only 4 h after irradiation the microvessel density in salivary glands decreases by ~45% (Cotrim *et al*, 2007). Whereas 90 days after irradiation blood vessel dilation (telangiectasia-like) contributes to decreased blood perfusion of the salivary gland (Ahlner and Lind, 1994). This impaired regeneration capacity is inferred from reduced endoglin expression (Lombaert *et al*, 2008b) a factor crucial for neoangiogenesis (Arthur *et al*, 2000) and vascular repair (van Laake *et al*, 2006). The early loss of microvessel density and function could be reduced by pretreatment with serotype 5 adenoviral vector-mediated transfer of basic fibroblast growth factor or vascular endothelial growth factor complementary DNAs (Cotrim *et al*, 2007), stimulating blood vessel growth. The late vascular damage could be ameliorated by postirradiation mobilization of bone marrow derived cells (Lombaert *et al*, 2008b). The subsequent homing of MSCs and endothelial progenitor cells (EPCs) not only resulted in an improvement of the parenchyma but also in a reduction of vascular damage. The latter is partly due to bone marrow derived cells (BMCs) differentiating in vascular cells but is likely to also result from direct stimulation of existing blood vessel cells by secreted factors from BMCs (Lombaert *et al*, 2006, 2008b).

Thus local secretion of growth factors stimulating blood vessel growth may reduce radiation damage to the salivary glands. Regretfully, preirradiation treatment with growth factors may not applicable for clinical use due to their pro-tumorigenic potential (Cotrim *et al*, 2007). However, postirradiation mobilization of MSCs and EPCs from the bone marrow may not be tumorigenic. Similar to what has been suggested for other diseases (Khoo *et al*, 2008) autologous EPCs derived from the bone marrow could serve to prevent radiation induced vascular damage.

Adult tissue stem cell transplantation

One of the first encouraging reports of adult stem cell transplantation raised the issue of stem cell plasticity (Orlic *et al*, 2001). Now numerous publications using different experimental models have suggested that adult stem cells are capable, under suitable circumstances, of producing a wide variety of cell types regardless of their germ layer. Although data supporting stem cell plasticity are extensive, they are also somewhat controversial; cell-cell fusion has been proposed as a more appropriate interpretation for this phenomenon. Next to this, transplanted adult stem cells may also induce repair by

stimulation of proliferation of endogenous tissue stem cells (Duffield *et al*, 2005) induced by factors secreted by these cells (Krause and Cantley, 2005). These findings, although eliciting enthusiastic responses, also indicate that the use of adult stem cells for tissue repair strategies in degenerative disease is only in its early stage of evolution.

Although adult stem cells may not be as 'powerful' or diverse as embryonic stem cells, at present they offer many advantages for the development of cellular therapeutics including lack of ethical problems, the possibility to using autologous cells, accessibility, stable phenotype and tissue type compatibility. The potential success of adult stem cell therapy has been implicated by muscle satellite cell (Collins *et al*, 2005) and cardiac stem cell transplantation (Dawn *et al*, 2005). Adult tissue-specific stem cells have now been identified in many tissues including liver (Yang *et al*, 2002), brain (Clarke *et al*, 2000; Gronthos *et al*, 2000), dental pulp (Gronthos *et al*, 2000), hair follicles and skin (Fuchs, 2009; Snippert *et al*, 2010), intestine (Barker *et al*, 2007), adipose tissue (Zuk *et al*, 2002), blood (Zhao *et al*, 2003; Yen *et al*, 2006), lung (Yen *et al*, 2006), mammary epithelium (Kordon and Smith, 1998) and salivary glands (Lombaert *et al*, 2008a).

Transplantation of healthy salivary glands stem/progenitor cells collected prior to irradiation and transplantation after treatment may reduce hyposalivation and xerostomia. In a preclinical model we have recently developed a method to isolate and culture murine submandibular gland cells as salispheres (Lombaert *et al*, 2008a) similar to mammary glands (mammospheres) (Woodward *et al*, 2005) and brains (neurospheres) (Reynolds and Weiss, 1996) both of which have been shown to contain progenitor and stem cells. The obtained salispheres were derived from putative stem cells of ductal origin and contained cells expressing stem cell markers Sca-1, c-kit and Msi-1 (Lombaert *et al*, 2008a). These spheres were able to form functional duct and acinar-like cells in vitro. Strikingly, when transplanted as single cells (75.000) into irradiated glands they were able to restore long term submandibular gland morphology and function. Further enrichment for stem cells was obtained by flow cytometric selection of c-kit$^+$ cells from salispheres. Enrichment was shown by the observation that only 300 c-Kit+ cells were necessary for an even enhanced transplantation efficacy in irradiated mice submandibular gland. Moreover, when the submandibular glands from responder mice were collected, donor derived spheres could be cultured of which c-Kit cells could be isolated. Only 100 c-Kit+ cells from the first donor clearly improved saliva secretion and morphology from secondary recipients (Lombaert *et al*, 2008a), indicative of the high potential of this subpopulation of cells. These results showed that a population of c-Kit cells derived from salispheres obtained from mice submandibular glands contain the tissue stem cells, which are able to self-renew and regenerate radiation-damaged submandibular gland tissue.

Interestingly, c-kit$^+$ cells were also detected in excretory ducts from human salivary glands as shown in mice (Lombaert *et al*, 2008a; Feng *et al*, 2009). Moreover, salispheres could be cultured from both human parotid and submandibular gland tissue. In vitro, these human based salispheres could self-renew and differentiate into ductal structures and mucin-expressing acinar-like cells in 3D matrix cultures. This indicates that also human salivary glands contain a 'putative' stem cell population comparable to that found in rodents. In the near future, these cells may have the potential to reduce radiotherapy-induced salivary gland dysfunction in patients.

Conclusions

Loss of tissue function resulting from a lack of regenerative capacity after a cytotoxic insult may not only be due to an insufficient number of viable tissue stem cells, but also due to other phenomena such as changes in environmental signals which inhibit stem cell proliferation and a compromised vascular regeneration. The compromised tissue function is most likely due to a failure to replace functional cells of the vasculature and the parenchyma. Therefore, it seems logical to transplant a combination of different cell types to induce tissue regeneration and neovascularization. Such a combined treatment has already been shown to be promising in ischemic cardiomyopathy (Memon *et al*, 2005). Moreover, posttransplantation treatment with growth factors may be necessary to enable to potential limited number of stem cells obtained by pretreatment biopsies to regenerate the tissue. Indeed, treatment with KGF has shown to increase the number of sphere forming cells in the tissue and enhance regeneration after irradiation (Lombaert *et al*, 2008c).

Stem cell research has begun to explore the unique qualities of salivary gland stem cells as well as their vast clinical potential. Although many questions remain to be answered, significant progress has been made during the last few years. In the near future, cell-based therapies may restore function of not only irradiation-damaged salivary gland but also other disorders that have resulted in damage to salivary glands, for example, Sjögren's syndrome. Hereto, the antibodies responsible for the disease should first be inhibited or the stem cells should be modified to inhibit the presentation of specific antigens.

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