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On approaches to the functional restoration of salivary glands damaged by radiation therapy for head and neck cancer, with a review of related aspects of salivary gland morphology and development

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Abstract

Radiation therapy for cancer of the head and neck can devastate the salivary glands and partially devitalize the mandible and maxilla. As a result, saliva production is drastically reduced and its quality adversely altered. Without diligent home and professional care, the teeth are subject to rapid destruction by caries, necessitating extractions with attendant high risk of necrosis of the supporting bone. Innovative techniques in delivery of radiation therapy and administration of drugs that selectively protect normal tissues can reduce significantly the radiation effects on salivary glands. Nonetheless, many patients still suffer severe oral dryness. I review here the functional morphology and development of salivary glands as these relate to approaches to preventing and restoring radiation-induced loss of salivary function. The acinar cells are responsible for most of the fluid and organic material in saliva, while the larger ducts influence the inorganic content. A central theme of this review is the extent to which the several types of epithelial cells in salivary glands may be pluripotential and the circumstances that may influence their ability to replace cells that have been lost or functionally inactivated due to the effects of radiation. The evidence suggests that the highly differentiated cells of the acini and large ducts of mature glands can replace themselves except when the respective pools of available cells are greatly diminished via apoptosis or necrosis owing to severely stressful events. Under the latter circumstances, relatively undifferentiated cells in the intercalated ducts proliferate and redifferentiate as may be required to replenish the depleted pools. It is likely that some, if not many, acinar cells may de-differentiate into intercalated duct-like cells and thus add to the pool of progenitor cells in such situations. If the stress is heavy doses of radiation, however, the result is not only the death of acinar cells, but also a marked decline in functional differentiation and proliferative capacity of all of the surviving cells, including those with progenitor capability. Restoration of gland function, therefore, seems to require increasing the secretory capacity of the surviving cells, or replacing the acinar cells and their progenitors either in the existing gland remnants or with artificial glands.

Keywords

apoptosis; atrophy; differentiation; proliferation; radiation; regeneration; salivary glands; stem cells

Saliva plays a major role in maintaining oral health. This becomes apparent when the amount and quality of saliva is significantly reduced by medications, salivary gland neoplasms,

disorders such as Sjögren's syndrome, and especially ionizing radiation (IR) therapy for tumors of the head and neck (Franke et al. 1965, Liu et al. 1990, Valdez 1991, Vissink et al. 2003a, Fox 2004, Ship and Hu 2004). Decreased saliva (symptomatically, *xerostomia*) causes difficulty in mastication and swallowing, accelerates dental caries (Fig. 1A), and exacerbates periodontal diseases. The oral mucosa is painfully dry, sticky, and more susceptible to infection and trauma. Although these problems can partially be alleviated by an intensive regimen of home and professional care, many patients are unable to maintain the diligence required to be effective.

Of particular interest here are the amount and pathways of commonly used radiation that greatly decrease the viability of the osteogenic cells and vasculature of the maxilla and mandible, which then are at high risk of developing osteoradionecrosis (Fig. 1B–D), sometimes spontaneously, but more often from dental and periodontal abscesses, extractions and deep oral ulcers (Vissink et al. 2003a). Edentulation prior to radiation, therefore, is the standard of care for patients with poor dentition due to neglect.

A number of recent reviews have provided excellent overviews of the effects of IR on oral mucosa, taste perception, maxillary and mandibular bone, teeth, periodontium and salivary glands, including those of Nagler (2002), Vissink et al. (2003a,b), Ship and Hu (2004), and Dirix et al. (2006). Except for acini, however, these have provided few details about differences in vulnerability and potential for regeneration of the several component structures of salivary glands.

I review here approaches to restoring salivary gland function after IR with emphasis on the development and functional morphology of salivary glands as these may relate to damage from IR and how the damage might be undone. Accordingly, the sequence is to provide background regarding the importance of saliva to oral and dental health, the components of salivary glands that are preferentially destroyed by IR, the anatomy and functional histology of salivary glands as these relate to their vulnerability to IR and the production of saliva, including cell lineages and other aspects of their developmental biology that bear on repair and regeneration, and experiments on the repair and regeneration of salivary glands using various methods other than IR that produce damage. With the necessary background thus established, I review the means that have been tried or are being considered to minimize the effects of IR on salivary glands and to repair the damage after it has occurred.

To provide representative illustrations of radiation damage to salivary glands, new photomicrographs were taken of paraffin sections of 4.0% formaldehyde-fixed specimens of normal (sham-irradiated) and irradiated rat submandibular glands used in a previous study (O'Connell et al. 1999). Both glands were harvested eight months after the irradiated gland was subjected to 10 Gy of IR with the brain and esophagus protected by a lead shield. Note that in this review, the units of radiation are those presented in the cited publications, although rad (radiation absorbed dose) is obsolete; one gray (Gy), the currently accepted unit, equals 100 rad. In addition, sections were cut from paraffin blocks of normal and irradiated human submandibular glands that had been fixed in 4.0% formaldehyde (commercial 10% neutral buffered formalin). These were obtained from the archives of the Pathology and Laboratory Service of the Washington, DC, Veterans Affairs Medical Center with the permission of the Human Subject Subcommittee and the Research and Development Committee. The normal human gland had been removed as an excisional biopsy of a benign salivary gland neoplasm in a 52-year old male. The irradiated gland had been removed as part of a radical neck dissection four weeks after an eight week course of therapeutic X-irradiation with concomitant chemotherapy with carboplatinum for squamous cell carcinoma of the base of the tongue in a 65-year of male. The accumulated dose to both the cancer and the submandibular gland was 70.63 Gy. The patient was unable to tolerate amifostine and after two days it was discontinued.

Sections from all specimens were stained with hematoxylin and eosin (H & E), periodic acid-Schiff and hematoxylin (PAS-H) (Mowry 1963), or alcian blue 8GX at pH 2.5 (Mowry 1963) and eosin (AB-E) or hematoxylin (AB-H). Additional sections of the human specimens were stained with mucicarmine and others were subjected to immunohistochemical (IHC) procedures with antibodies to smooth muscle actin (SMA) and α -amylase, using Envision and dual link HRP in a Dako (Carpenteria, CA) autostainer, and counterstained with hematoxylin. The chromogen for IHC was 3,3-diaminobenzidine (DAB). The anti-SMA reaction was enhanced using antigen retrieval in Reveal buffer in a pressure cooker. Antigen retrieval used with the anti-amylase antibody caused widespread nonspecific reactions. The reaction was both ample and specific without antigen retrieval. Formaldehyde and DAB were purchased from Government Scientific Solutions (Alexandria, VA). Hematoxylin, eosin and Schiff reagents were obtained as ready-to-use solutions from Surgipath Medical Industries (Richmond, IL; no lot or C.T. # listed). Alcian blue 8GX (C.I. 74240) and a ready-to-use kit (HT30) for mucicarmine (Lot 094K4359) that includes metanil yellow and iron hematoxylin counterstains, and rabbit polyclonal anti-human α -amylase were purchased from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal anti-bovine SMA (clone 1A4) was obtained from Cell Marque (Rocklin, CA).

Saliva and oral health

The health and comfort of the oropharyngeal mucosa and teeth depend on a supply of saliva that is adequate in both amount and quality. Saliva lubricates boluses of food as well as the teeth and mucosa to facilitate mastication, swallowing and speech (Baum 1987, Rudney 1989). For these functions, the volume of water is paramount, but mucins and other glycoproteins (most secretory proteins are glycoproteins) also are very important (Tabak et al. 1982). Reviews of the proteins in human saliva have been provided by Rudney (1989) and Nieuw Amerongen et al. (2004) and in rodent saliva by Ball (1993). The amounts and proportions of electrolytes including bicarbonate, calcium, chloride, fluoride, magnesium, phosphate, potassium, and sodium provide the pH and mineral reservoir that maintains homeostasis of tooth structure (Schneyer and Schneyer 1967). These inhibit chemical erosion and dental caries unless overwhelmed by dietary factors and/or poor oral hygiene. Some salivary proteins, such as mucins and statherin, facilitate this function by carrying minerals in a supersaturated state, though this also can lead to inappropriate deposition as in dental calculus. Some salivary enzymes, such as α -amylase (Kauffman et al. 1970, Keller et al. 1975), lipase (Hamosh 1979) and ribonucleases (Eichel 1964), initiate digestion and aid in clearing the oral mucosa and teeth of food debris. Other enzymes, such as salivary peroxidase, and other constituents, such as agglutinins, cystatins, defensins (Vylkova et al. 2007), histatins, lysozyme, mucins, proline-rich proteins, secretory immunoglobulins, and thiocyanate, play important roles in protection against ingested noxious substances and infection by oral microorganisms. Proline-rich proteins (PRP) are synthesized constitutively and are stored in the secretory granules of the serous acini of human parotid and submandibular glands (Bennick 1982, Sabatini et al. 1989, Carlson 1993), but their concentrations in these glands are low in the rat unless induced by dietary tannins or strong β -adrenergic stimulation such as injection of isoproterenol (Mehansho et al. 1983, Carlson 1993). Carbonic anhydrases are involved in salivary bicarbonate regulation; some are secreted by acinar cells and others dwell in the cytoplasm of striated and excretory ducts (Parkkila et al. 1990, Peagler et al. 1998, Redman et al. 2000). A number of hormones are produced and secreted by human and rodent salivary glands including epidermal and nerve growth factors (Gresik 1994). Epidermal growth factor has been shown to enhance healing of oral, esophageal and gastric ulcers (Olsen et al. 1984, Goodland and Wright 1995). Saliva also facilitates taste by serving as a solvent for timely circulation of gustatory substances around the taste buds (Matsuo 2000). In this function, the concentration of sodium chloride and other salts is important as a reference for the taste cells.

Salivary gland damage from ionizing radiation

In both human and rat salivary glands, serous acini are most labile when subjected to ionizing radiation, many undergoing degeneration and cell death after even moderate doses (Cherry and Gluckman 1959, Phillipe 1970, Sholley et al. 1974, Fajardo 1982, Abok et al. 1984). Good regeneration of acini occurs after the minor damage caused by mild (600 to 1600 rads) doses, but after heavy doses (more than 6000 rads) of IR, the serous acini of the parotid gland nearly disappear and little or no regeneration takes place. Less extensive degeneration and necrosis occur among the mucous acini of the sublingual and submandibular glands, and some regeneration may occur. The effects of mild (10 Gy) IR to a rat submandibular gland and heavy (70.63 Gy) to a human submandibular gland are illustrated in Figures 2 and 3, respectively.

The mechanism of IR damage to salivary glands has been reviewed thoughtfully by Nagler (2002), Vissink et al. (2003a) and Dirix et al. 2006). The greater lability of serous cells to radiation damage has been attributed to generation of free radicals via transition metal ions, such as copper, iron, manganese and zinc, contained in their secretory proteins. When the secretory granules harboring these proteins are discharged by administration of secretagogues prior to irradiation, the damage to and loss of acinar cells is considerably diminished in rat parotid, but not submandibular glands (Peter et al. 1995, Coppes et al. 2001). Such strong secretory stimulation can result in a significant increase in acinar cell mitosis (Schneyer 1970) and thus vulnerability to IR. However, the mitoses would have been initiated several hours after the administration of the IR, and in any event this factor probably was outweighed by the removal of the metal ions. It seems (note omission of “however”) that in serous acini, most free radicals are generated by copper and iron ions, and that most of the damage to DNA would require that these ions be in close proximity to the nucleus. A plausible explanation is that during IR, the secretory granule membranes are damaged by the IR, releasing the contents, including the free radical-generating metal ions, into the cytoplasm close to the nucleus. The dramatic drop in salivary output that occurs during the first few days after a seemingly mild single dose or the first few fractionated doses of IR apparently is not due to immediate death, but to widespread dysfunction of the acinar cells. Plausible explanations for this phenomenon include transient damage to the plasmalemma and receptor-signaling apparatuses (Coppes et al. 2001, Dirix et al. 2006). In any event, if DNA and other cellular damage is not severe enough to cause immediate death of the cell, inaccurately repaired DNA damage may be sufficient to interfere with future proliferative activity or cause delayed death. Thus, as the surviving acinar cells and their precursors in the intercalated and other ducts die during the ensuing weeks, the extent to which the acini can regenerate is severely compromised.

There may be additional reasons for radiation-induced loss of salivary gland function, such as damage to innervation, blood vessels and stroma. Chomette et al. (1981), using enzyme histochemistry and transmission electron microscopy, reported persistent damage including edema and loss of vesicles in secretory nerve endings of rat submandibular gland through 70 days after administration of single doses of 2000, 2500 or 3000 rads. This damage was interpreted as sufficient to cause loss of stimulation, thus contributing to the cycle of regeneration, engorgement with secretory granules, and death of acinar cells observed during the 70 days after IR. On the other hand, much of the secretory innervation reportedly survives radiation (Kohn et al. 1992, Forsgren et al. 1994, Aalto et al. 1995, Coppes et al. 2001), and surviving acinar cells from rat salivary glands subjected to mild (10 Gy) radiation still respond normally to secretagogues (O’Connell et al. 1999). Furthermore, although neuropeptides such as substance P and bombesin were increased transiently in rat submandibular gland ganglia at 10 days post radiation of 30 to 40 Gy IR (given in daily fractionated doses), values had returned to normal by 180 days. Changes in taste after IR also offer a clue to the importance of IR effects on nerves in salivary glands. After IR for cancer of the head and neck, the taste threshold increased dramatically at one month, but had recovered to normal baseline values by 6 months

(Sandow et al. 2006). These results indicate that the neuroepithelial (taste) cells, nerve endings, or both were destroyed or damaged by the IR. Results with experimental animals have documented damage and destruction of lingual taste buds by IR (Farbman 1972). Regeneration of taste cells via differentiation from surface epithelial cells has been shown to be dependent on appropriate innervation (Farbman 1972). From the foregoing, it seems likely that any nerve damage caused by IR for head and neck cancer would have been limited to the proximal portions of the nerve processes, allowing new nerve endings to migrate from the surviving portions of the nerves. Interestingly, taste recovered even when there was marked xerostomia, suggesting that saliva may be less important to taste perception than supposed on theoretical grounds (Matsuo 2000). There was no functional assessment of damage to the lingual glands of von Ebner, however, which serve the majority of taste buds in the troughs of the vallate papillae and foliate folds (Hand 1987). In a case pertinent to this point, Fajardo (1982) illustrated “a heavily irradiated tongue” in which “all that remained of a lingual salivary gland was a dilated and ulcerated duct forming a mucocele.”

Following moderate to high dose IR, the stroma of human parotid and submandibular glands has been described as undergoing adiposis and fibrosis, respectively (Frank et al. 1965). Some stromal fibrosis also has been observed in rat salivary glands, but only after high dose IR (Cherry and Gluckman 1959, Phillippe 1970, Sholley et al. 1974). The endothelium of blood vessels is susceptible to radiation damage and compromised blood supply has been observed after ^{131}I treatment for thyroid cancer (Mandel and Mandel 2003). Thickening of extracellular matrix components in response to high doses of IR also has been reported (Bartel-Friedrich et al. 2000). These stromal changes may restrict diffusion of nutrients, essential minerals and oxygen to parenchymal cells, and thus may adversely affect late attempts at regeneration and function by surviving acinar cells.

Because of the importance of acini as the origin of the water and most of the organic secretory products in saliva, the more dramatic effects of IR on acinar cells overshadows the considerable effects that moderate to high doses of IR have on excretory and striated ducts (Abok et al. 1984, Mandel and Mandel 2003) (Fig. 3). The diminished function of striated and other large ducts is evident in higher salivary sodium and chloride and lower bicarbonate concentrations, and consequently lower pH, in stimulated saliva (Valdez et al. 1992). In these regards, stimulated saliva from irradiated glands is more like unstimulated saliva from normal glands. The reduced flow and buffering capacity and lower pH of saliva from irradiated glands are major factors in the rapid development of dental caries in the absence of rigorous control measures. In addition to their importance in ion exchange with the luminal fluid as noted above, they may have some capacity to regenerate intercalated duct and acinar cells.

The most plausible explanation for the poor recovery of acinar cells after moderate to high doses of IR is that the lifetime proliferative capacity of the acinar cells and their progenitors is partially depleted by repeated mitoses in attempts to replace cells lost to radiation and is diminished further by DNA/chromosomal damage in still viable cells (Coppes et al. 2001, Nagler 2002). Studies in rats have shown that IR induces increased cellular proliferation among the several cell types in proportion to the extent the cells are killed. After a single dose of 15 Gy (*ca.* 1500 rad) to the parotid and submandibular glands of mature rats, proliferative activity as determined by uptake of bromodeoxyuridine (BrdU) slowed for 24 h, then resumed in the intercalated ducts after three days and in the acini, striated ducts and granular convoluted tubules after 6 to 10 days (Peter et al. 1994). By immunohistochemical localization of proliferating cell nuclear antibodies (PCNA), proliferative activity increased by factors of 12.6, 3.4 and 2.2 in the acinar, intercalated duct, and striated duct cells, respectively, in rat submandibular glands seven days after exposure to a single dose of 30 Gy (Ballagh et al. 1994). These observations indicate that the divided doses used in therapeutic IR for cancer of the head and neck in humans compounds the damage to salivary glands as each wave of

increased proliferation, when the cells have greater vulnerability compared to non-dividing cells, is subjected to another dose of radiation.

Functional morphology and development of salivary glands

In this section I review elements of the morphology and development of human and rodent salivary glands that are required to understand both the pathologic changes that occur in these glands in response to IR and the rationale and potential usefulness of approaches to restoring structure and function to glands damaged in this way.

Anatomy

The anatomic location and size of a salivary gland determines the extent to which it is in the path of therapeutic IR for a given tumor, including metastases, and thus the potential damage it may incur.

In both humans and the rodents most frequently used as models for salivary research, mice and rats, there are three major salivary glands and numerous minor glands (Young and van Lennep 1978). The paired major glands are the parotid, situated in the tissue anterior and inferior to the ears and superficial to the ramus and angle of the mandible, and the sublingual and submandibular, located inferior to the mandible and the floor of the mouth. The human minor glands are distributed in the tissues immediately subjacent to the oral mucosa in the cheeks (buccal mucosa), anterior floor of mouth, lips (labial mucosa), posterior hard and soft palate, tonsillar pillars, and posterior dorsal and anterior ventral tongue. Their distribution is similar in mice and rats except that these species have no salivary glands in the labial mucosa, hard palate and ventral tongue.

Histology

Knowledge of the morphologic features related to the recognition and function of the several types of parenchymal and stromal cells facilitates assessment of the effectiveness of methods used to minimize IR damage and to restore structure and function after IR damage has occurred.

Comprehensive presentations of the histology and ultrastructure of human and other mammalian salivary glands are available in the monograph by Young and van Lennep (1978) and a series of articles in the September, 1993 and January, 1994 issues of *Microscopy Research and Technique*.

In humans, the parotid gland is composed of serous acini, the sublingual gland has mucous acini with serous demilunes, and the submandibular gland (Fig. 3A–D) has mostly serous acini and numerous mucous acini with serous demilunes (Young and van Lennep 1978, Hand 1987). In all three glands, the secretory end pieces drain into intercalated, striated and excretory ducts, and myoepithelial cells invest the acini and intercalated ducts. Although some histologists consider the parotid acini to be seromucous rather than serous, the terminology used here is that of Young and van Lennep (1978). The terminology reflects the quality of the saliva of these glands, that of the parotid gland being watery and only slightly slippery; the sublingual gland, very viscous and slippery; and the submandibular gland, moderately viscous and slippery.

The rat parotid gland consists of serous acini with secretory granules that are electron dense and negative with mucin stains (e.g., alcian blue and mucicarmine), intercalated ducts with cuboidal cells harboring a few small, electron-dense, mucin-negative, strongly PAS-positive secretory granules in the juxta-acinar primary segments, and striated and excretory ducts (Young and van Lennep 1978). Striated ducts are prominent and although some segments may harbor a few secretory granule-like vesicles (Hand 1987), these are not sufficient in size or

number to be considered granular convoluted tubules. Otherwise, the striated and excretory ducts are similar to those of the submandibular gland. Unlike almost all other salivary glands, including human parotid gland, the myoepithelial cells of mature mouse and rat parotid glands invest only the intercalated ducts (Garrett and Parsons 1973, Redman et al. 1980, Redman 1994).

The number of acinar secretory granules is subject to circadian variation in rats, especially in the parotid gland, and this must be taken into account in experiments measuring the number of secretory granules and products per unit of tissue (reviewed by Johnson 1987). There is heavy feeding activity in the dark, which stimulates exocytosis of secretory granule contents so that the amount per unit of tissue has decreased by more than half when the lights come on the next morning. Feeding activity is slow with the lights on, as the rats mainly are asleep or at rest, so that acinar secretory granule accumulation nears its peak by early afternoon. Also by early afternoon, the synthesis of α -amylase and other acinar secretory products slows, then increases again soon after resumption of heavy feeding activity in the dark (Sreebny et al. 1971). The magnitude of the day/night difference in the number of acinar secretory granules per cell is greatly influenced by the physical consistency of the diet. The more masticatory effort the diet requires, as with a pelleted commercial diet, the stronger the secretory stimulation. With weak stimulation, such as a liquid or powdered diet, the acini initially fill with secretory granules. After a day or two of adjustment, during which many of the secretory granules undergo autophagocytosis (Hand 1987), the acini produce and store much less secretory product and the circadian variation in feeding activity is nearly abolished. Thus, at any time of the day or night, a liquid or powdered diet results in low acinar secretory granule content similar to that seen during the three hours or so before and after lights are on in a rat fed a pelleted diet. Secretion from rabbit and human salivary glands also can be strongly stimulated by taste, especially acid.

The rat submandibular gland consists of seromucous acini that appear serous in histologic sections stained with H & E, but with secretory granules varying in electron density with different fixatives, that drain into a complex duct system (Young and van Lennep 1978). A circadian variation has been demonstrated in the acinar secretory granules that is similar to, but of lesser magnitude than that of rat parotid acini (Albegger and Müller 1973). The acinar secretory granules contain true mucin and they also store glutamine/glutamic acid-rich proteins (GRP) (Moreira et al. 1989), peroxidase (Moriguchi et al. 1995, Kruse et al. 1998), and PRP, the last-named varying with dietary factors as noted above. The small amount of α -amylase in the mature rat submandibular gland has been attributed to glandular blood, with origins in the liver and pancreas (Schneyer and Schneyer 1960). The lumens and luminal cytoplasm of acini, however, reacted modestly to the anti-human amylase antibody that was used to illustrate α -amylase in human submandibular acini in Fig. 3C (unpublished observation). The acinar-intercalated duct junction is marked by the presence of juxta-acinar secretory cells that are morphologically distinct from those of the acini as well as from other granule containing cells of the intercalated ducts (Young and van Lennep 1978, Quarnstrom and Hand 1987). The walls of the intercalated duct, which reportedly constitute 20% of the parenchymal cell population, consist of a single layer of cuboidal cells (Chang 1974). At the ultrastructural level, intercalated duct cells have much less rough endoplasmic reticulum and Golgi apparatus, and fewer and smaller secretory granules than acinar cells (Young and van Lennep 1978); the secondary intercalated ducts are devoid of secretory granules. The intercalated ducts are continuous to an abrupt transition with the specialized granular convoluted tubules of rodent submandibular glands, which synthesize and secrete many bioactive proteins (Gresik 1994). Fluid from the granular convoluted tubules continues through striated and excretory ducts to the oral mucosa. The final complex solution that enters the oral cavity includes secretory products from the acini and granular convoluted tubules as well as, presumably, the products of the juxta-acinar and

intercalated duct cells. The specific composition is determined by the type, intensity and duration of secretory stimulation.

The striated and excretory ducts of all the major human and rat salivary glands, and to a lesser extent the granular convoluted tubules of the rat submandibular gland, consist mainly of columnar cells with deep basolateral invaginations and intercellular interdigitations of the plasmalemma accompanied by numerous large, elongated mitochondria (Tamarin and Sreebny 1965, Hand 1987, Tandler 1993, Tandler et al. 2006). The invaginations provide an enormously increased area for ion exchange with the interstitial fluid and the mitochondria provide the energy required to facilitate this exchange, in particular the resorption of sodium and chloride ions from the luminal fluid for their return to the circulation. The larger excretory ducts vary by gland in both rat and human salivary glands, some consisting of stratified, others of pseudostratified columnar epithelium (Tandler 1993, Tandler et al. 2006). There are several types of columnar cells in the excretory ducts, i.e., basal, dark, light and tufted cells (Shackelford and Schneyer, 1971, Sato and Miyoshi 1997). These cells seem to have different functions, e.g., in the main duct of the rat submandibular gland, the dark cells are involved in electrolyte transport (Knauf et al. 1983), while the tuft cells may have a sensory function (Sato and Miyoshi 1997).

Myoepithelium invests the acini and intercalated ducts, but not the granular convoluted tubules and striated and excretory ducts (reviewed by Redman 1994). There are, however, basal cells in the larger excretory ducts that have been shown immunohistochemically to share a number of proteins with myoepithelial cells. The long processes of the myoepithelial cells are arranged like a basket around the acini and spirally along the long axis of the intercalated ducts. Ultrastructurally, the myofilaments in these processes resemble those of smooth muscle, but differ from the latter in that their intermediate filaments are cytokeratins, not desmin. These contract rhythmically when the gland is stimulated to secrete and compress the lumen with a peristalsis-like effect that pushes the luminal contents from the acini through the intercalated ducts into the larger ducts. The terminal web, a meshwork of supporting myofilaments immediately subjacent to the luminal plasmalemma (Leblond et al. 1960), also contracts rhythmically, but more slowly, during secretion. These contractions provide additional impetus to the flow of the luminal contents not only in the acini and intercalated ducts, but also throughout the rest of the duct system.

The innervation and main blood supply enter the body of the salivary gland with the main duct (reviewed by Young and van Lennep 1978). The arterial blood supply flows upstream to the salivary flow, parallel to the branches of the duct tree, to the acini. The venous return retraces these pathways downstream. Prominent, large, thin-walled veins around the striated and excretory ducts facilitate resorption and exchange of ions among these ducts, the luminal fluid and the circulation. Parasympathetic nerves synapse in a ganglion located within the main body of the rat submandibular gland near the hilus (Ng et al. 1992); these are joined by sympathetic nerves as they arborize with the duct tree. With the major salivary glands, parasympathetic stimulation (cholinergic and muscarinic) elicits mainly the watery component of saliva, and sympathetic (α - and β -adrenergic), the organic component, mostly via exocytosis of secretory granules (reviewed by Garrett 1987). Physiologic stimulation from food mastication and taste involves both types of autonomic nerve, with modification by peptides such as substance P and vasoactive intestinal peptide (VIP). These observations are relevant to studies of the mechanisms of salivary gland atrophy with main duct ligation and IR discussed later.

In summary, the acini are responsible for almost all of the water, mucins, digestive enzymes and other organic substances in saliva. In rats, the granular convoluted tubules contribute epidermal and nerve growth factors and several proteases (Gresik 1994). One of these, kallikrein, aids the secretion processes via vasodilation of the glandular vasculature. The

myoepithelium assists the movement of fluid from acini into ducts and also may coordinate the propagation of secretory stimuli from nerves to acini (Redman 1994). The striated and other large ducts alter the ions in the salivary product by reabsorbing sodium and chloride and adding bicarbonate (Mangos et al. 1966, Schneyer and Schneyer 1967, Knauf et al. 1983). It is noteworthy that the concentrations of sodium, bicarbonate and chloride in human saliva increase with increasing flow rate (Schneyer and Schneyer 1967). This is because the resorption of sodium and chloride by the ducts cannot keep up with the rate of flow and bicarbonate is transferred into the saliva in exchange for chloride.

Development

Detailed knowledge of the changes in organization and cellular structure that a developing gland undergoes between initiation and maturity is a prerequisite to meaningful analysis of the effects of environmental and other factors on gland development. As discussed in a following section, some aspects of development are recapitulated in the regeneration of acini and granular convoluted tubules during recovery from major injury. Further, selection of agents such as hormones and growth factors for promoting the regeneration of gland structures that have been damaged or destroyed by IR can be guided by assessment of their influence on the normal development and maintenance of these structures.

Regulation of salivary gland growth and development occurs at many levels, e.g., dietary generation of secretory stimuli via specific autonomic nerves; circulating levels of the major hormones such as corticosteroids, insulin, testosterone and thyroxine; deposition, activation and destruction of components of the extracellular matrix, receptors residing in the cell membranes, and intracellular messengers; and transcription, translation and destruction of specific mRNAs. The importance of these factors has been delineated both in vivo and in vitro (Johnson 1987, Oliver et al. 1987, Redman 1987, Broverman et al. 1998, Durban 1990, Yeh et al. 1991, Lazowski et al. 1992, 1994, Durban 1990, Durban et al. 1994, Horn et al. 1996, Quissell et al. 1994, Macauley et al. 1997, LaFrenie and Yamada 1998, Ikeda et al. 2008, Inukai et al. 2008).

In the rat parotid gland, both acinar and duct cytodifferentiation occur almost entirely postnatally, with secretory granules appearing during the first day after birth and increasing progressively in number and size thereafter (Redman and Sreebny 1971). In samples taken in the early afternoon or after overnight fasting, a profound increase in acinar size and secretory granule content then occurs in conjunction with weaning, provided the pup is weaned to a pelleted diet (Redman and Sreebny 1976). Scattered cells with mucous secretory granules occur transiently in developing acini during the first week after birth (Lawson 1970, Taga and Sesso 1979, Ikeda and Aiyama 1997, 1999, Ikeda et al. 2008). The striated and excretory ducts also become functionally and structurally mature between 15 and 30 days after birth (Mangos 1978, Taga and Sesso 1979, Peagler et al. 1998, Peagler and Redman 1999, Redman et al. 2002). Myoepithelial cells differentiate around the immature acini and small ducts between birth and 15 days, but during weaning (18–25 days), they are left behind on the intercalated ducts by the more rapidly growing acinar cells (Redman et al. 1980, Tsujimura et al. 2006).

Unlike the parotid and submandibular glands, acinar differentiation in rat sublingual glands occurs mostly prenatally (Leeson and Booth 1961, Redman and Ball 1978, Taga and Sesso 1998, Wolff et al. 2002). Up to a day or so before birth, the secretory granules of many of the otherwise typical mucous acini have an eccentrically placed dense core in the midst of the flocculent mucin. The few cores still present in the newborn gland are not seen in glands of animals a few days older. The serous cells initially are arranged randomly among the mucous cells. Just before birth, they become positioned as caps or demilunes at the distal ends of the tubuloacini. At birth, the striated and excretory ducts also are well differentiated in terms of having numerous basolateral membrane infoldings and large mitochondria (Leeson and Booth

1961, Taga and Sesso 1998). However, during the four weeks after birth, these ducts undergo progressive increases in diameter (Leeson and Booth 1961) and intensity of immunohistochemically localized reactions to carbonic anhydrase (Ogawa et al. 1998, Redman et al. 2000). Myoepithelial differentiation also occurs postnatally (Redman and Ball 1979).

The development of the rat submandibular gland is more complex than that of most other salivary glands (Jacoby and Leeson 1959, Leeson and Jacoby 1959). Differentiation of the acini involves the appearance of two transient secretory cell types in the perinatal gland, continuing with the disappearance of these cells from the maturing acini, and culminating in the formation of adult seromucous cells. The transient cells were defined as Type I and Type III cells (Ball and Redman 1984) based on the ultrastructural characterization and nomenclature of their secretory granules that was developed by Cutler and Chaudhry (1974). The Type I and III cells correspond, respectively, to the terminal tubule cells and the peroxidase-secreting proacinar cells described by others (Jacoby and Leeson 1959, Strum 1971, Chang 1974, Yamashina and Barka 1974). Stimulation of secretion by β -adrenergic agonists causes degranulation of Type III cells and release of Proteins A, B1 and B2. By contrast, cholinergic stimulation causes degranulation of Type I cells and the appearance of Protein C in the secretion product (Ball and Redman 1984). Another protein, Protein D, is secreted in response to both kinds of agonists. Analysis of these proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) determined, initially, apparent MWs of 25, 26, 27, 97 and 150 kDa, for A, B1, B2, C and D, respectively.

Ball and colleagues have analyzed further some of these proteins in the developing rat submandibular gland and presented much of it in a concise synopsis (Ball et al. 2003). The B1-immunoreactive protein “family” consists of proteins that are reactive to polyclonal antibodies raised against Protein SMG-B1, a prominent substance in the neonatal submandibular gland. This family of proteins includes the major components SMG-B1, -B2 and -A, as well as several minor species (Ball and Redman 1984, Ball et al. 1988a, 1993). Molecular analysis has shown Protein SMG-A to be homologous to the parotid secretory protein (PSP) characterized by others in the mouse (Mirels and Ball 1992, Owerbach and Hjorth 1980, Shaw and Schibler 1986). Subsequently, SMG-A was found to be identical to RPSP, the product of the rat *Psp* gene, which is almost identical to the *Psp* gene described previously in the mouse parotid gland (Ball et al. 1993, Mirels et al. 1993, 1998). The second BI-IP gene of the rat, *Smgb*, is not expressed in the mouse. In the rat, the gene is expressed as a single polypeptide and the major isoforms (B1 and B2), as well as minor variants, result from differential glycosylation and/or post-translational proteolysis of the single gene product (Mirels et al. 1998).

Protein C development appears to be regulated differently than B1-IP. When the five-day submandibular gland library was screened, clones were found, none of which were full length, that hybridized to a 3 kb transcript, which is abundant in the 5-day-old gland, but is not detectable in the adult submandibular, sublingual or parotid glands. As demonstrated on Northern blots, the message disappears between 20 and 30 days post partum. Based on its size, organ specificity, abundance and developmental profile, this transcript appears to encode Protein C (Mirels et al. 1993).

Unlike the other perinatal proteins, Protein D immunoreactivity is seen in both perinatal cell types and is localized near the exoplasmic surface of the secretion granule membrane (Ball et al. 1991). This localization was striking in the Type I cells and less so in the Type III cells. In the adult, low levels of Protein D immunoreactivity are distributed diffusely in the secretion granules of the seromucous acinar cells. In the sublingual gland, membrane-associated Protein D immunoreactivity is found in the serous demilunes, and mucous acinar cells have diffuse distribution of immunoreactivity in the secretion granule contents. The different localization

of Protein D in the several cell types raises the question of the identity of these immunoreactive species.

In conjunction with the disappearance of the perinatal cell types from the maturing acini, some cells in the adjacent intercalated ducts show strong reactivity for the perinatal antigens (Ball et al. 1988a,b 1993, Moreira et al. 1990, 1991, Man et al. 1995). This indicates that, as in the rat parotid gland, there are cells with distinctive secretory proteins that translocate from the immature acini to the intercalated ducts in the developing rat submandibular gland.

The striated ducts are moderately differentiated in newborn rats and become mature in terms of increased height of the columnar cells and depth of the infoldings and interdigitations of the basolateral plasmalemma in conjunction with weaning (Jacoby and Leeson 1959, Leeson and Jacoby 1959, Cutler and Chaudhry 1975). The intensity of immunohistochemical reactions to carbonic anhydrase antibodies parallels these structural changes (Ogawa et al. 1998, Redman et al. 2000).

The granular convoluted tubules differentiate in response to sex hormones, with a much greater response to androgens resulting in progressively larger sexual dimorphism between 30 and 120 days of age in both mouse and rat (reviewed by Gresik 1994). In rats, differentiation begins at two weeks after birth in striated duct cells, often leaving a segment of striated duct interposed between granular convoluted tubule and intercalated duct segments (Cutler and Chaudhry 1975, Srinivasan and Chang 1975). As differentiation proceeds in adolescent rats, the most distal (from the oral orifice) granular convoluted tubule cells become juxtaposed to secondary intercalated duct cells, thus assuming the adult configuration.

Differentiation of the myoepithelium begins just prior to birth and is complete by age 15 days (Line and Archer 1972, Cutler 1973).

Cell lineages

A central theme in this review is which salivary gland cells have the capacity not only to make more cells of like type, e.g., acinar cells dividing to make more acinar cells, but also to serve as progenitors for other cell types, e.g., intercalated duct cells redifferentiating into acinar or striated duct cells. This is of critical importance to the choice of cells to be injected into glands to effect restoration of acini and other structures that have been destroyed by IR, or to use in creating an artificial salivary gland.

One approach to analyzing the problem of cell lineages in salivary glands has been to ascertain the proliferative activity of the various cell types in the mature gland and as they differentiate during gland development. The undifferentiated cells of the embryonic gland progressively differentiate into the highly specialized cells of the acini, myoepithelium, and striated and excretory ducts, with the agranular intercalated duct cells and basal cells of the larger ducts retaining a simpler morphology bearing some resemblance to the cells in the gland anlage (reviewed by Redman 1987). Mitotic division of acinar cells of the pancreas and salivary glands had been reported by a number of workers beginning more than half a century ago (Parker 1919, Blumenfeld 1942, Schneyer 1970). Their relationship to the development, repair and neoplasia of these organs was unclear, however, until methods such as electron microscopy, immunohistochemistry and in situ hybridization were developed.

The observation of mitoses among well-differentiated acinar cells in electron micrographs of rat parotid glands documented a fundamental difference between the epithelium of skin and mucosa and that of salivary glands; the latter do not necessarily require relatively undifferentiated progenitor cells for most of their growth and development (Redman and Sreebny 1970). Subsequently, ultrastructural observations and ³H-thymidine labeling of cells

in semithin sections showed that proliferative activity is common among well-differentiated cells of all types including myoepithelial cells, and that it is the acinar cells that have a disproportionately high rate of proliferation in the rat parotid gland at all stages of development and maturity through 42 days (Redman 1994, 1995). In older rats, there is conflicting information. Although the BrdU labeling index of intercalated ducts was higher than those of acini and striated ducts (Peter et al. 1994), the ^3H -thymidine labeling index was much higher in acinar cells than in any other cell type (Schwartz-Arad et al. 1988).

Other ultrastructural and ^3H -thymidine autoradiographic studies showed proliferation of well-differentiated acinar and other cells in rat pancreas (Sesso et al. 1973) and submandibular gland (Chang 1974, Alvares and Sesso 1975, Srinivasan and Chang 1975). Like the rat parotid gland, proliferative activity is greatest among the acinar cells at all stages of development in the rat pancreas (Sesso et al. 1973) and sublingual gland (Taga and Sesso 1998). In the rat submandibular gland, however, after the acinar cells become mature at about 30 days after birth, cell division is more frequent in the intercalated ducts than among other cell types (Chang 1974, Srinivasan and Chang 1975, Peter et al. 1994). These and subsequent studies of this gland in the rat and mouse (Denny et al. 1997) suggest that intercalated duct cells are major progenitors of the granular convoluted tubule cells, which differentiate and increase disproportionately to other cell types for many weeks after puberty.

In all three major rat salivary glands from 18 days *in utero* to 60 days after birth, the cell cycles of all cell types are of approximately equal length at all ages, with no male-female difference (Klein and Harrington 1976, 1977, Taga et al. 1994, Redman and Kruse 1999). In addition, apoptosis is rare ($\leq 0.05\%$; Redman and Kruse 1999, Tsujimura et al. 2006). In the rat submandibular gland, however, apoptosis of Types I and III cells is rather common (6% or more) as these become reduced in both size via atrophy and number between 23–30 days after birth (Hecht et al. 2000, Hayashi et al. 2000). Denny and Denny (1999), also found somewhat higher rates of apoptosis (0.15%) in the intercalated ducts than elsewhere in submandibular glands of young adult female mice. Melnick and Jaskell (2000) found several TUNEL-labeled nuclei in large ducts near the terminal buds in prenatal mouse submandibular glands. They suggested that death of these cells is a necessary part of the process of lumen formation. Redman and Kruse (1999), however, found no increase in apoptosis during prenatal and postnatal lumen formation in the ducts and terminal buds in any of the three major rat salivary glands. In addition, lumen formation in developing rat salivary glands has been shown to occur by differentiation of the elements of tripartite junctional complexes in pre-existing intercellular spaces (Shimono et al. 1981, Cutler and Mooradian 1987, Redman 1987). These observations indicate that cell death is not an integral part of lumen formation in developing salivary glands.

A comprehensive study by Man et al. (2001) provided new data on the role of intercalated ducts in the normal replacement and expansion of adult parenchymal cell populations in rat submandibular gland. Because the intercalated ducts cells are phenotypically diverse, based on their different expression of perinatal secretory proteins, these investigators examined the incorporation of a systemically injected 24 h pulse of ^3H -thymidine into cells of the different phenotypes and assessed the distribution of ^3H -thymidine labeled cells in animals killed at intervals during the ensuing month. Proliferating cells were found within all parenchymal cell compartments. They were most numerous in intercalated ducts, primarily in cells lacking immunoreactivity for the perinatal proteins SMG-B1, -C and -D. The labeling index (LI; the percent of cells that were labeled) of the intercalated duct cells reached a peak at seven days post-injection, then decreased over the next three weeks. The LI increased at the junctions of intercalated ducts with acini and with granular convoluted tubules, and within these larger parenchymal elements. These investigators concluded that the intercalated duct cells that are not reactive for perinatal proteins, i.e., harbor no secretory granules, proliferate to expand the intercalated duct compartment. The apparent migration of labeled cells suggests that many of

the intercalated duct cells adjacent to the granular convoluted tubules differentiate into new granular convoluted tubules cells, while some cells near the acini seem to differentiate into new acinar cells. Alternatively, the slower proliferation of acinar cells allows some to retain label for up to a month and their more frequent location near the acinar-ID junctions results from migration of cells from acini to ID. These data provide no evidence for differentiation of intercalated duct cells into cells of striated ducts. The small number of excretory duct profiles seen in these investigators' preparations, however, showed an extremely high LI (> 25%), suggesting that more extensive data might reveal a precursor role for the excretory duct in replacement of striated ducts cells. Because the administration of label to these rats began at the onset of adolescence, the high LI in the excretory duct profiles may also be due, at least in part, to their undergoing elongation to keep up with the growth of the neck and mandible. It also is clear that during this period, the growth of the submandibular glands occurs much more in the granular convoluted tubules and other ducts than in the acini. In addition to the apparent passage of cells from intercalated ducts to other parenchymal elements at their junctions, the occurrence of occasional clusters of B1-positive acini (BAC) among the typical B1-negative acini suggested an alternative pathway for replacement of acinar cells in which entire segments of newly expanded intercalated ducts might develop directly into a recapitulated perinatal stage of B1-reactive cells pursuant to becoming mature acinar cells. Electron microscopic visualization of specific proteins in the BAC using immunogold cytochemistry demonstrated the presence of B1-reactivity similar to that in the perinatal Type III cells during their transition into the seromucous acinar cells of the adult submandibular gland (Moreira et al. 1991). Also consistent with a precursor role in cellular replacement of acini, the BAC had a four-fold greater LI than typical adult acini. By contrast, the LI of uncommon anomalous mucous acini showing a sublingual gland phenotype was not significantly different from typical acinar cells (Man et al. 2001).

In the developing rat parotid gland, the immature acini have cells with secretory granules that either contain or are devoid of peroxidase. Between six and 14 days after birth, the cells with peroxidase-negative secretory granules appear to migrate from the immature acini into, and become the granule-containing cells of, the intercalated ducts (Redman and Field 1993). A similar migration pattern has been observed by Sivakumar et al. (1998) with prolactin-inducible protein (PIP). The translocation of these cells also has been followed using the strong reaction of their secretory granules to the periodic acid-Schiff reaction as a marker (Ikeda et al. 2008). These observations support the suggestion that the acinar cells are progenitors for the intercalated ducts in the developing rat parotid gland. A review of published illustrations reveals that a similar, but apparently heretofore overlooked, phenomenon also occurs in the developing rat submandibular gland. The Types I and III cells that do not undergo apoptosis or, in the case of some of the Type III cells, differentiate into mature (seromucous) acinar cells, migrate from the secretory end pieces ("terminal tubules") into intercalated ducts.

These findings support several conclusions regarding developing salivary glands in rats. First, in general, rates of apoptosis among the several cell types of developing and young adult rat salivary glands are too low to affect derivation of cell lineages as inferred from differences in cell proliferation, except as noted regarding a subset of intercalated duct cells in the submandibular gland. Second, the findings reduce concerns that comparisons of the rates of proliferation of the various cell types derived from the uptake of ³H-thymidine or BrdU, or by immunohistochemistry for PCNA, might be confounded by differences in cell cycle length. They do not account, however, for cells, including acinar cells, that cease dividing during the course of gland development but can resume dividing in response to strong secretory stimulation (Klein and Harrington 1977). Third, during gland development, many cells in the terminal bulbs and immature acini migrate into the developing intercalated ducts. The extent to which this happens in mature glands is not known, but it is possible that these cells and

others in G₂ in the acini and striated and excretory ducts may constitute a subset of pluripotential reserve cells.

Primary culture

Primary culture of salivary gland cells can yield important clues regarding the conditions and gene expressions involved in their differentiation. Cultures also have the potential to serve as depots for replenishing cells in injured glands. In addition, co-culture with salivary gland cells may be a necessary step for inducing stem cells to differentiate into salivary cells prior to injection into IR-damaged salivary glands.

Mature rat parotid acinar cells seem to tolerate a wide range of conditions while maintaining phenotype in primary culture, including 20% O₂ (Oliver et al. 1987, Yeh et al. 1991). In early attempts, however, mouse and rat submandibular gland cells quickly de-differentiated into duct-like phenotypes (Tapp 1967, Lamey et al. 1982, Yang et al. 1982). Therefore, Quissell and colleagues undertook studies to establish and evaluate effective culture conditions for mature acinar cells from rat submandibular gland. In their initial attempt (Quissell et al. 1986), they seeded dissociated acinar-intercalated duct complexes onto a bovine collagen gel matrix with 15% fetal bovine serum in the medium and gassed them with 20% O₂ and 4% CO₂. Almost all the acinar cells died within 48 h, leaving only the intercalated duct cells. This indicated that rat submandibular gland acinar cells do poorly under conditions of moderate hypoxia. A similar phenomenon occurs with focally reduced blood supply in human minor salivary glands (Rye et al. 1980). Serum-free primary culture conditions then were developed in which rat submandibular acinar cells proliferated and were maintained in a well differentiated state for at least two weeks (Quissell et al. 1994). The key conditions were rat tail collagen matrix with laminin, hyperoxygenation (35%), dexamethasone, retinoic acid, insulin in a narrow optimal range, and stimulation with carbamylcholine and/or norepinephrine. Although other parenchymal cell types survived in these cultures, they were rare and mitoses were observed only in acinar cells. Exocytosis of the abundant, alcian blue positive (i.e., containing mucin) acinar secretory granules was observed, indicating function in response to the secretagogue in the medium. Durban (1990) successfully induced and maintained duct differentiation, including granular convoluted tubules, in primary cultures of mouse submandibular cells by growing them in a three-dimensional collagen gel matrix and adding male sex hormone to the medium.

Cell lines

A number of rat and human cell lines have exhibited acinar differentiation. One of these, the human submandibular gland (HSG), can be induced to differentiate from intercalated duct-like to acinar phenotype by cell-substrate interaction with a gel containing laminin-1 or Matrigel (Hoffman et al. 1996) or into myoepithelial cells when grown on plastic with sodium butyrate added to the medium (Yoshida et al. 1986). Although much information has been gained from experiments with these and other cell lines (e.g., Quissell et al. 1998, Zheng et al. 1998), their use for repopulating acini and other cells in damaged salivary glands is precluded by the risk of unbridled growth that they pose.

Repair and regeneration of salivary glands after mechanical or chemical injury

Another approach to assessing cell lineages in salivary glands is to follow the cells involved in regeneration after injury. Several models have been explored. After excision or laser destruction of part of a rat submandibular gland, many cells of all types near the cut edge die. Regeneration of lobules seems to occur mainly by de-differentiation, proliferation, and budding of cells of the larger ducts, which then differentiate into acini and all types of ducts (Hanks and

Chaudhry 1971, Takahashi and Wakita 1993). Some surviving acinar and intercalated duct cells also participate. On the other hand, Tokoro et al. (2003) demonstrated the de-differentiation of acinar into duct cells in partially extirpated rat pancreas by the sharp, but not complete, decline of acinar cell markers such as α -amylase. Many of these “acinoductular cells” then redifferentiated into acinar cells during regeneration. It is noteworthy that when most of a mature exocrine gland is removed, the regeneration is incomplete; much of the original mass of the gland is not replaced. After one gland is completely removed, however, the contralateral gland is likely to show compensatory hypertrophy via hyperplasia (Alho 1961).

Ligation of the main excretory duct of rat salivary glands brings about marked atrophy of mainly the secretory cells, e.g., of acini and granular convoluted tubules. Standish and Shafer (1957) documented the serial changes in rat submandibular glands attributable to long term ligation of Wharton’s duct and the accompanying main artery. Although the nerves along the duct were not mentioned, this pioneering study set the stage for all subsequent duct ligation studies owing to its detailed description of the ligation method and establishment of the importance of ligating only the duct. Arterial ligation alone resulted in infarction of a large portion of the gland not served by vessels from the capsule and peripheral stroma. Within the infarct, the acini became necrotic and the large ducts underwent squamous metaplasia. As repair proceeded, the central zone collapsed into a small scar with a few dilated large ducts, while the acini and ducts gained collateral circulation from the capsule and survived and regenerated. After a few weeks, except for the central scar zone and the markedly diminished size, the artery-ligated glands were similar histologically to the control glands. Duct ligation alone resulted in reduction in gland size, owing mostly to atrophy of the acini into small duct-like structures, but without the large scale necrosis and duct metaplasia of the arterial ligation. This state was achieved within a week and persisted to the end of the experiment 20 weeks after ligation. Combined arterial and duct ligation resulted in the central infarct of the artery ligation and the peripheral acinar atrophy of the duct ligation. After repair of the infarct, however, there was no regeneration of acini or intralobular ducts.

In a short term study (Ahn et al. 2000), the P2Y₂ nucleotide receptor was neither expressed nor functional in the rat submandibular gland except after ligation of the main duct. When the ligation was removed, the P2Y₂ expression faded as the gland recovered. After three days, there was marked atrophy of the acini and granular convoluted tubules, but there also were scattered dead cells. The acini had recovered after one week and the granular convoluted tubules after two weeks. Mitoses were uncommon during recovery and it appeared that recovery from this short term ligation involved mostly redifferentiation of atrophic or de-differentiated acinar and granular convoluted tubule cells. With prolonged ligation, mainly acinar cells, but also some of the duct and myoepithelial cells, die and the larger ducts may undergo squamous metaplasia (Standish and Shafer 1957, Tamarin 1971, Donath et al. 1973, Walker and Gobé 1987, Burford-Mason et al. 1993, Burgess and Dardick 1998, Takahashi et al. 1998, 2001). The ligation model mimics main duct stenosis and lithiasis in humans (Harrison and Bader 1999). Tamarin (1971) suggested that the gland atrophy of prolonged duct ligation in the rat submandibular gland involved both death and de-differentiation of acini and granular convoluted tubules. Upon removal of the ligature after 30 days, regeneration proceeded regionally as lumens redeveloped in the ducts. The regeneration of gland was similar both morphologically and chronologically to the stages of development in the perinatal and adolescent rat. Thus, differentiation of the acini took two to three weeks, followed during another two to three weeks by differentiation of the granular convoluted tubules. Tamarin (1971) remarked that this was an example of “regeneration retraced ontogeny.”

The source of the regenerating acini and granular convoluted tubule was interpreted to be the de-differentiated acinar and granular convoluted tubule cells, respectively. Subsequently, both death and de-differentiation of acinar cells also have been shown to occur after main duct

ligation in the rat parotid gland. Walker and Gobé (1987) and Takahashi et al. (1998) reported that cell death destroyed almost all acini after five to seven days, with acinar restoration proceeding only after acini differentiated from the ducts. Burford-Mason et al. (1993) and Burgess and Dardick (1998), however, found that regeneration of acini occurred principally by proliferation of surviving acinar cells after removing the ligature from Stensen's duct. Takahashi et al. (2000, 2002) found that apoptosis was proportionately greater among acinar than duct cells in the ligated rat sublingual and submandibular glands, but even after 28 days there were many surviving acinar cells. They also observed both duct and acinar cells in stages of de-differentiation and redifferentiation in the sublingual gland. In addition, few myoepithelial cells in the ligated glands underwent apoptosis or atrophy; most collected around the de-differentiated, duct-like structures (Takahashi et al. 1999, 2001, 2003). During recovery, proliferative activity of the myoepithelial cells was less than among the other types of cells. Interestingly, the emerging parotid acini initially were surrounded by myoepithelial cells. As the acini became better differentiated, the myoepithelial cells translocated to the intercalated ducts. The sequence is almost identical to that occurring during normal rat parotid gland development, as previously noted, echoing Tamarin's (1971) statement of "regeneration retracing ontogeny."

Most workers have stated that they avoided inclusion of the blood vessels and nerves accompanying the duct, which would have added hypoxia and denervation to the etiology of the glandular changes observed. The differences in results or interpretation appear to be due partly to the extent to which the main duct of a salivary gland was occluded (Takahashi et al. 1998, 2001, 2004, Harrison et al. 2001). A single ligature might have allowed some escape of secretions, thus greater acinar cell survival, while the tighter occlusion of dual ligation resulted in a much greater loss of acinar cells. Osailan et al. (2006) ligated the main duct of the rat submandibular gland near the oral orifice to avoid compression of the chorda tympani nerve. Comparison of the degree of atrophy between this method and ligation at the hilum of the gland showed an additional increment of atrophy with the latter, indicating some inhibition of secretory nerve and vascular function occurs despite taking care to exclude the nerve and blood vessels from the ligature.

Administration of ethionine to rats causes destruction of mainly acinar cells of the parotid and submandibular glands, followed by regeneration within two weeks. In the parotid, regeneration involved proliferation of acinar cells almost exclusively (Leeb 1978), but no similar follow-up was done on the submandibular gland (Ulmansky et al. 1969). By contrast, administration of 2-acetylaminofluorene to young adult male rats was selectively toxic to the cells of the granular convoluted tubules; some were destroyed and the rest de-differentiated into cuboidal cells (Danz et al. 1997). Temporary plugging of these ducts caused the acinar cells to become atrophic, some into small duct-like structures. As the granular convoluted tubules became patent, the acinar cells rapidly redifferentiated. During recovery, PCNA immunohistochemistry indicated that proliferative activity of basal cells of the excretory ducts, recovering granular convoluted tubule segments, and acinar cells all were greatly elevated above controls, suggesting that all of these contributed to replenishment of the granular convoluted tubule cells.

Approaches to restoring function to salivary glands damaged by ionizing radiation

As detailed in the preceding sections, loss of acini is the principal cause of the reduced salivary function after IR. Therefore, acinar preservation during IR would be the ideal situation, but failing that, restoration of acini would be necessary. Obviously, even marginally successful methods of preservation would leave more gland structure including acini, ducts, stroma, vasculature and innervation, with which to work for restoration.

Preservation

Shield one or more salivary glands during IR—Shields can be useful if the cancer is unilateral and external to the oral cavity, as in the case of a tumor of a parotid gland or skin. With tumors of the larynx and oral mucosa, however, it is difficult to place a shield that protects the major salivary glands without also blocking one or more of the ports essential for effective radiation of the tumor and its local extensions and metastases.

The use of the three-dimensional planning technique (3-DTP) and conformational dose delivery techniques have been shown to reduce the radiation damage to the parotid and other salivary glands and thus to decrease post-IR xerostomia (Henson et al. 2001, Malouf et al. 2003). These techniques use computerized radiation dose programs guided by computed tomography to fit more precisely the radiation doses to the site(s) of the cancer and its metastases while minimizing the IR affecting the salivary glands, oral mucosa, and other structures not likely to harbor tumor cells. Some patients, however, such as those with large midline cancers and bilateral metastases, are less effectively treated using these techniques.

Stimulate the acinar cells to secrete just prior to each dose of IR—As discussed above, the production of free radicals by interactions between IR and heavier metals, such as calcium and magnesium, in secretory granules of serous acini reportedly augments the damage to these cells. In human trials, administration of pilocarpine or bethanechol prior to each dose of IR has resulted in significantly less loss of salivary flow rates, especially from the parotid gland (Vissink et al. 2003b, Fox 2004, Jham et al. 2007). In most patients whose glands receive more than 50 Gy of radiation, however, the amount and quality of saliva still is reduced enough to have seriously adverse effects on dental caries and oral mucosal comfort and health. In addition, many patients do not tolerate the side effects (e.g., profuse perspiration, cardiac arrhythmias) from pilocarpine or have medical contraindications its use.

Use agents that selectively protect salivary glands, kidneys and other organs from IR damage—The use of agents such as amifostine (WR2721; Ethyol®; Brizel et al. 2000, Vissink et al. 2003b.) in head and neck cancer patients has shown promising results with glands receiving low to moderate doses. Many patients cannot tolerate side effects such as nausea and vomiting, however, especially when IR is combined with chemotherapy. A case in point is illustrated in Fig. 3, in which nausea and vomiting were severe enough that the patient discontinued use of amifostine after only two days. Consequently, his salivary glands suffered severe damage. Although the results so far indicate an acceptably low level of protection to tumor cells, large scale studies are needed to resolve this problem with greater statistical power (Vissink et al. 2003b, Dirix et al. 2006). Administration of amifostine has decreased the radiation-induced bone growth inhibition in the femurs and tibias of rats (Tamurian et al. 1999, Damron et al. 2000) and the skulls of rabbits (La Scala et al. 2005). The radiation, however, affected mainly the immature osteogenetic cells in the cartilage or fibrous tissue and developing bone in growth plates, periosteum or sutures. The extent to which amifostine offers protection to mature bone of the maxilla and mandible in human adults has not been addressed. Nonetheless, mature bone undergoes constant remodeling, and the generation of new osteoblasts and osteoclasts from precursor cells provides a measure of parallelism to the growing bone model with regard to vulnerability to IR.

Heat shock proteins and Tempol also have shown differential protection of salivary glands from IR. Lee et al. (2006) injected an adenoviral vector carrying mouse genes for heat shock proteins 25 or 70i into the submandibular glands of young adult male rats one day before the glands received 17.5 Gy IR. Control animals were sham-irradiated, received vector only, or received an intraperitoneal injection of amifostine. The injected heat shock protein remained markedly elevated at one day and had returned to the level of controls by 40 and 90 days post-

irradiation. Measures of gland damage included histologic assessment, salivary flow and constituents, cellular aquaporin 5, and indicators of apoptosis. The heat shock proteins, like the amifostine, provided considerable, but incomplete, gland protection by all measures. Injecting salivary glands before each of the fractionated IR doses used in humans with head and neck cancer, however, risks complications such as infection and increasingly rapid destruction of the injected agents by an immune response. Multiple injections would be unnecessary if the heat shock proteins remained elevated in the glands for several weeks after one injection, but with this vector the cytoplasmic gene depots were eliminated within 10 days, even when the animals were immunosuppressed transiently with corticosteroids (Vitolo and Baum 2002). Cotrim and colleagues (2007) administered Tempol (4 hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) prior to each of five fractionated doses of IR to either the salivary glands or a tumor-bearing leg of female CH3 mice. Salivary output with pilocarpine stimulation was 60% greater in the mice receiving Tempol than in the unprotected mice, while the tumors were about equally damaged. The unique paramagnetic properties of Tempol permitted assessment of its conversion to nonprotective hydroxylamine by magnetic resonance imaging. The conversion occurred twice as rapidly in the tumors as it did in the salivary glands, providing a rationale for the observed difference in protection. Unfortunately, no histologic analysis was conducted on the extent to which Tempol protected the normal bone surrounding the tumor and the salivary gland acini and convoluted granular tubules.

Transplant the main body of a salivary gland away from the main field of radiation—After transplantation, one can either leave the gland at its new location or return it to its original site after completion of IR (Hoshino and Lin 1970, 1971, MacLeod et al. 1990). For the transplanted gland to be useful, its connection to the main duct must be maintained or re-established. In a recent study, eight human patients had one submandibular gland transplanted to the submental space contralateral to the site of the oral oropharyngeal cancer prior to chemotherapy plus IR (Al-Qahtani et al. 2006). The surgery resulted in loss of part of the blood supply, although the authors indicated that the post-IR loss of salivary flow was minor to moderate. This method has the same site problem as the use of shields, i.e., patients frequently present with such widespread local extension and regional metastases of the cancer that there are no sufficiently radiation-spared sites for transplantation. Further, only the one gland is spared and the functional loss of only one major gland substantially increases susceptibility to dental caries among the teeth where the localized salivary shortfall occurs. In addition, such major surgery could delay the start of IR as much as or more than would extractions and would not inhibit IR damage to the bones of the maxilla and mandible.

Restoration

Regeneration—After the completion of IR, one can induce the residual salivary gland cells to regenerate acini and other parenchymal elements either directly via gene transfer or indirectly by local infiltration of factors that are involved in normal salivary gland growth, development and maintenance. These methods show promise, but are in their infancy with regard to salivary gland regeneration. One interesting approach is that of Okazaki et al. (2001). These investigators induced a moderate acceleration of rat submandibular gland repair after duct ligation-unligation by infusing basic fibroblast growth factor (bFGF) into the gland via the main excretory duct.

Induce surviving cells to remedy the deficient salivary fluid and constituents via gene therapy—This very promising approach is being pursued by Baum and colleagues at the National Institute for Dental and Craniofacial Research (Adesanya et al. 1995, Baccaglini et al. 2001, Vitolo and Baum 2002, Baum et al. 2006). Beginning with rats and progressing to miniature pigs and nonhuman primates, genes have been inserted into parenchymal cells via adenoviral and nonviral vectors infused into the main duct. Because mature salivary glands

have a low rate of mitosis, vectors for incorporation of genes into the cellular DNA are not useful. Instead, they have used vectors to establish cytoplasmic gene depots to induce the cellular machinery to manufacture the desired products. Although the depots are rejected within a few days, even when the animal is immunosuppressed with corticosteroids, the results have established the usefulness of the concept. Insertion of a gene for human aquaporin 1 (important for water transport into the lumens of acinar cells) has dramatically, albeit transiently, increased salivary flow in irradiated rat and pig submandibular glands. Issues of potential toxicity and unwanted systemic distribution have been addressed successfully in vitro and in vivo. There remain the problems of inability of heavily irradiated glands to replenish cells, including those that might have long lasting gene insertions, because these continue to be lost long after the completion of IR. Gene insertion into luminal cells of the larger ducts, which survive IR better (Fig. 3), may be helpful.

Replacement with cells saved in vitro—Remove a small piece of the patient's salivary gland prior to IR, from it grow acinar and/or other cells in primary culture, and inject them into the gland after IR has been completed. The cells, unencumbered by IR, could proliferate and restore functional glandular components. Considerable groundwork has been laid for this approach. O'Dell et al. (1979, 1983, 1985, 1987) and Sharawy and O'Dell (1979, 1981) performed a number of experiments on autografts of submandibular gland cells into the tongue or submandibular gland. A portion of the donor gland was excised and diced into tiny pieces. A suspension of the pieces then was injected into the submucosa of the tongue, or in one experiment, the contralateral submandibular gland. Initially, there was much degeneration and necrosis of the injected cells, and most of the surviving cells formed dilated cystic structures lined by thin, duct-like cells. A few groups of cells, however, clearly showed acinar and striated duct differentiation. This seemed to occur mostly when they managed to make a patent connection with the oral mucosa (tongue site) or ducts (gland site). Although both adrenergic and cholinergic nerves were found among the grafted structures after several weeks, the distribution of the adrenergic nerves was limited to blood vessels. In addition, the number and size of the acinar cells were not affected by prior sympathectomy indicating that the acinar cells became or remained differentiated despite a lack of adrenergic stimulation. More recently, Sugito et al. (2004) grew rat submandibular gland cells in primary culture, labeled them with PKH 26, a fluorescent linkage marker, and injected them (together with India ink to mark the site of injection) into normal glands and glands previously rendered atrophic by main duct ligation. In the normal glands, the cells remained close to the site of injection and gradually became scarce after four weeks. In the glands recovering from the effects of duct ligation, more cells were present and some had migrated some distance away from the site of injection. Almost all stayed in the stroma, however, and few if any differentiated into acinar cells. Indeed, the lack of double labeling (IHC for PKH 26 and mucin or α -smooth muscle actin) suggested that very little of the PKH 26 was still in salivary epithelial cells. Nonetheless, these investigations demonstrated the potential for repairing damaged salivary glands by seeding them with new or banked cells. It also confirmed that the seeded cells must be able to establish connections with the lumens of residual parenchymal structures, because widespread acinar cell atrophy and death usually occur when there is no outlet for secretion, e.g., with obstructed ducts (Tamarin 1971, Donath et al. 1973, Walker and Gobé 1987, Burford-Mason et al. 1993, Burgess and Dardick 1998, Takahashi et al. 1998).

Replacement with stem cells—Selectively retrieve hematopoietic stem cells from a sample of the patient's blood and inject these into the gland, or induce them to differentiate into salivary gland cells in vitro, and inject these into the damaged gland as in the previous approach. Advantages of this approach are that there is no delay in radiation treatment while a donor gland recovers from surgery and that the stem cell-derived cells may have greater proliferative potential than gland-derived cells.

There is considerable preliminary work in this field, mostly involving other organs and tissues. In recent years, there have been reports of female recipients of bone marrow transplants from male donors having hepatocytes, cardiac muscle cells and other tissues that bear Y chromosomes (Körbling et al. 2002). This indicates that stem cells from bone marrow have the potential to differentiate into cells of non-hematopoietic lineage. A few of these stem cells circulate in the blood (Steidl et al. 2002). Although differences between marrow-bound (“stromal”) and circulating stem cells have been described, both have been shown to be capable of differentiating into tissues other than hematopoietic in humans as well as in experimental animals (Lanzkron et al. 1999, Mahmood et al. 2001, Kang et al. 2001). In animal models, it has been shown that only a few or even just one such stem cell can re-populate the marrow to near normal hematopoiesis (Krause et al. 2001). Further, methods have been developed for selectively extracting circulating stem cells from the blood. Ordinarily these cells are rare, but there are ways to mobilize them from the marrow, e.g., by administering granulocyte colony stimulating factor (G-CSF), which greatly increases the yield from peripheral blood (Krause et al. 2001). Although analysis of host livers had suggested that most of the Y chromosomes occur in fused (donor/host) cells, perhaps limiting their usefulness, this was shown conclusively not to be the case for oral mucosal epithelium (Tran et al. 2003). Up to 12% of the epithelial cells harbored the Y chromosome in five female bone marrow recipients, and further analysis of 9700 cells showed that all but two were XY only. These observations suggest the possibility of regenerating glandular structures destroyed by disease or radiation by using stem cells from the patient’s own blood, thus avoiding problems associated with immunological rejection of cells from homologous sources. This approach has been used to enhance repair of tissues, e.g., heart muscle after myocardial infarction (Wang et al. 2006, Howard et al. 2007). However, although a large number of mobilized bone marrow stem cells came to reside in mouse salivary glands damaged by IR, and the damage was partly ameliorated, none of the stem cells appeared to have transdifferentiated into salivary gland parenchymal cells (Lombaert et al. 2006).

Regardless of the source of cells, an important consideration for attempts to replace cells lost to IR is the extent to which the inserted cells can gain secretory innervation. The positive regeneration results obtained with the ligated-unligated duct model are not helpful in this regard, because in it the innervation appears to suffer little damage. There is reason for some optimism, because large nerve tracts, at least, seem to survive heavy IR (Fig. 3E). Further, some of the pieces of rat submandibular gland autografted into the tongue by O’Dell et al. (1985) developed both cholinergic and adrenergic innervation, apparently from the lingual nerve. In the developing mouse submandibular gland, there is trophic interaction between the developing parenchymal cells and their developing nerve supply (Yohro 1971, Coughlin 1975a,b). If a similar trophism does not appear spontaneously in irradiated glands, one might be stimulated by injecting nerve growth factor or other agents with the introduced cells. Perhaps better yet, one might prolong the attraction by including among the introduced salivary gland cells some that produce nerve growth factor such as granular convoluted tubule cells.

Another consideration is the extent to which alterations of the stroma by age and IR might inhibit growth and differentiation of not only the introduced parenchymal cells, but also of nerve processes, fibroblasts and blood vessels. Here, inclusion of epidermal, fibroblast and vascular growth factors with the introduced parenchymal cells might be useful, as for example, in the report by Okazaki et al. (2001).

Tissue engineering—An intriguing idea is to obtain cells from donor glands and to use these to grow new glands in vitro, or construct artificial salivary glands by lining tubular scaffolds of biodegradable or biocompatible material with the cells and transplant these into the patient’s oral mucosa. Tissue engineered autologous bladders have been used successfully used in patients needing cystoplasty (Atala et al. 2006). Candidate materials for such scaffolds have been tested in vivo (Aframian et al. 2000). The results provided a choice between a

material that was biodegradable, which might permit lining cells to develop a more nearly normal supporting structure, and one that degraded very slowly if at all, which might be necessary for maintaining the desired differentiation of the lining cells. Manipulation of culture conditions for a human submandibular gland cell line has led to acinar differentiation from the original intercalated duct phenotype (Hoffman et al. 1996, Zheng et al. 1998, Wang et al. 1999). Such cell lines are useful only for developing methodology, however, because introducing a cell line into oral tissues risks forming salivary gland neoplasms. There has been progress recently in culturing normal cells. Primary culture of human and miniature pig submandibular gland cells resulted in striated duct-like and acinar cells, respectively (Tran et al. 2005, Baum and Tran 2006, Sun et al. 2006). Like the attempts to introduce new parenchymal cells into irradiated glands, a potential problem for artificial glands is to what extent they can develop secretory innervation.

Identification of cellular phenotypes

With any approach to regeneration or replacement of salivary gland tissue, it is essential to determine the extent to which the surviving original cells and the new cells are differentiated functionally. As noted above, regeneration in the ligated-unligated duct model often involves “recapitulation of ontogeny” in which transient acinar cell types appear first and these then translocate into intercalated ducts or differentiate into mature acini. A variety of methods are available to identify the different types of parenchymal cells including both adult and immature phenotypes. Some that ought to be particularly useful in studies on preserving and restoring salivary gland function are presented below.

Routine and special stains

In formaldehyde-fixed, paraffin-embedded tissues, routine H & E staining distinguishes among serous and mucous acini, striated and excretory ducts, and stromal components, but inadequately addresses the functional differentiation of these structures. For example, in the mature rat submandibular gland, the secretory granules of the acini stain modestly blue-violet, while those of the granular convoluted tubules take up very little H & E. This can be confusing, because in specimens stained only with H & E, the serous granular convoluted tubule cells appear to be mucous and the seromucous acinar cells appear to be serous.

Special stains can be quite definitive. The secretory granules of mature seromucous acinar cells of rat submandibular gland and the secretory granules of most mucous glands can be identified clearly in routine paraffin sections using the alcian blue and mucicarmine stains (Figs. 2 and 3B,F; Mowry 1963, O’Connell et al. 1999). Most mucous acini stain intensely with PAS, while serous acini stain modestly, but there are exceptions. PAS is useful for distinguishing between the serous secretory granules of acini (lightly stained, indistinct) and intercalated ducts (more intensely stained, small, distinct) as their phenotypes diverge in the developing rat parotid gland (Ikeda et al. 2008). The secretory granules of granular convoluted tubules of rat submandibular gland stain lightly to moderately with PAS. Importantly, glutaraldehyde fixation may render many nonmucous cellular structures strongly PAS-positive, because this dialdehyde can leave many unlinked aldehyde groups available for reaction with the Schiff reagent (Quissell et al. 1986, 1994). This problem was partly alleviated by following the glutaraldehyde fixation with formaldehyde fixation. In addition, glycogen can accumulate in striated ducts, myoepithelial cells, and immature acini, and should be removed in alternate sections by diastase or amylase to avoid being confused with secretory glycoproteins (Young and van Lennep 1978).

In glutaraldehyde-fixed, epoxy resin-embedded tissues, the toluidine blue-basic fuchsin (“CPC” or Paragon) stain clearly distinguishes Type I, Type III and seromucous acinar cells in developing rat submandibular glands as well as the acini and larger duct structures of adult

glands (Ball and Redman 1984). It also distinguishes the secretory granules of intercalated ducts and acini in developing and adult rat parotid gland (Redman 1995).

Immunohistochemistry

When more exacting identification is necessary, methods for enzyme histochemistry and immunochemistry can be employed.

Mature acinar cells—GRP are unique to the seromucous acinar cells of the mature rat submandibular gland and immunohistochemistry with antibodies to GRP permits unquestionable identification of these cells (Moreiera et al. 1989, 1990). Antibodies to human blood group A mark the mucin in rat submandibular, but not sublingual, acini in a pattern similar to the 1F9 RSMG anti-mucin antibody (Moreiera et al. 1991). These proteins, along with the absence of the perinatally expressed Smgb and Psp gene products, define accurately the transition from the perinatal Type III cells to mature acinar cells (Ball et al. 1993). By contrast, salivary peroxidase is a marker of all cell types involved in the transition of the perinatal Type III cells to mature seromucous acinar cells (Strum 1971, Yamashina and Barka 1974, Moriguchi et al. 1995, Kruse et al. 1998).

Alpha-amylase and salivary peroxidase identify mature acinar cells of the human parotid and submandibular glands (Riva et al. 1978, Zimmer et al. 1984; Fig. 3C) and rat parotid gland (Hand 1987, Redman and Field 1993).

The duct system—Enzyme- and immunohistochemical procedures can be used to evaluate the full range of duct differentiation. In this regard, several, but not all, antibodies to cytokeratins 18 and 19 have been shown to react within all elements of the ductal system, but not with acini or myoepithelium (Mori 1991, Redman 1994). The granular convoluted tubules of rat submandibular glands can be recognized unambiguously by antibodies to a number of secretory proteins, e.g., esterase A, renin, and nerve growth factor (NGF) (reviewed by Gresik (1994). Striated ducts can be distinguished from acini and intercalated ducts at both early and late stages of differentiation by detection of cytochrome C oxidase, succinate dehydrogenase, and Na⁺, K⁺-ATPase by enzyme histochemistry (Peagler and Redman 1999, Redman et al. 2002), and of carbonic anhydrase (Peagler et al. 1998, Redman et al. 2000,) and atrial natriuretic peptide (Vollmar et al. 1991, Cho et al. 2000) by immunohistochemistry.

Myoepithelium—The basal cells of the large ducts and the myoepithelial cells can be distinguished by immunohistochemistry for anti-keratin 13 Ab K 8.12 (Takahashi et al. 2001) and anti-smooth muscle actin (Redman 1994), respectively. For the latter, adding PAS outlines elements of the basal lamina, helping to distinguish between myoepithelial cells within it and other anti-smooth muscle actin-positive cells, such as myofibroblasts and blood vessels, that are in the stroma (Redman and Kruse 1999).

Transmission electron microscopy

Ultrastructural evidence of functional differentiation of acini, granular convoluted tubules, and granular intercalated ducts includes characteristic size and patterns of electron density of secretory granules and granules in the process of exocytosis. The degree of striated duct differentiation can be judged by the extent of invaginations and interdigitations of the basolateral plasmalemma and the size and relative numbers of mitochondria (Tamarin and Sreebny 1965, Hand 1987, Tandler 1993, Tandler et al. 2006). Mature excretory ducts should have dark, light and tufted cells of appropriate size and distribution (Sato and Miyoshi 1997, Knauf et al. 1983). Myoepithelial cells should be situated between the basal lamina and other parenchymal cells, and their processes should display cytoplasmic bands of myofilaments with dense bodies and plasmalemmal caveolae (Redman 1994).

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Fig. 1.

Effects of therapeutic radiation for head and neck cancer on teeth, oral mucosa and bone. A) The oral mucosa is dry and irritated and dental caries have amputated many teeth at the level of the gingiva. Archival photograph courtesy of the National Institute for Dental and Craniofacial Research, National Institutes of health, Bethesda, MD. B) Intraoral view of osteoradionecrosis of the mandible. C, D) Normal and irradiated human maxillary bone. C) Normal: nuclei of osteocytes occupy most of the lacunae, and the canal has blood vessels and endosteum with no sign of inflammation. D) Irradiated: almost all lacunae are empty and the canal harbors a clump of necrotic tissue and leukocytes. H & E. Scale bars = 50 μ m.

Fig. 2.

Submandibular glands of male rats at age 10 months, taken of histologic slides that were used in a previous publication (O'Connell et al. *Radiation Res.* 151:150-158, 1999). A) Sham-irradiated gland. Acini (blue secretory granules) are tightly spaced in large bunches, granular convoluted tubules (g) are numerous and large, and the stroma is scanty except around the larger ducts. B) Gland eight months after 10 Gy irradiation. Proportionately more stroma and fewer acini and granular convoluted tubules are present, and the granular convoluted tubules are smaller, than in (A). Alcian blue and hematoxylin. Scale bar = 60 μ m.

Fig. 3.

Human submandibular glands. A–D) Normal gland from a 52-year-old male. E–H) Gland removed from a 62-year-old male four weeks after being subjected to 70.34 Gy radiation in divided doses concurrent with eight weeks of chemotherapy as part of a treatment regimen for cancer of the oropharynx. In the normal gland, serous acini contain ample secretory granules that stain darkly with hematoxylin, gray with mucicarmine, blank with anti-smooth muscle actin (SMA), and richly (brown) with anti-amylase. Secretion product in mucous acini stains magenta with mucicarmine, and myoepithelial cells (arrows) and smooth muscle in blood vessels stain brown with anti-SMA. In the irradiated gland, myoepithelial cells are plentiful, but serous acini are not identifiable as such, mucous acini are diminished in number, size, and

staining intensity with mucicarmine, the columnar cells of many of the striated and excretory ducts are reduced almost to cuboidal dimensions, and the adipose cells (large, empty-looking cells) and stroma occupy proportionately more space. Large nerves appear to be intact (E). Labels: a, serous acini; ed, excretory ducts; m, mucous acini; n, large nerves; sd, striated ducts; v, blood vessels. H & E (A, E), mucicarmine (B, F) and immunohistochemical localization (peroxidase-DAB-H₂O₂ reaction) of α -amylase (C, G) and SMA (D, H), counterstained with hematoxylin. Scale bar for A and E = 100 μ m; for B–D and F–H = 60 μ m.