SUMMARY

Radiotherapy provides an effective curative treatment for patients suffering from head and neck cancer. Due to their anatomical position at least part of the major salivary glands are included in the radiation portal. Radiation injury to the glandular tissue often leads to salivary gland dysfunction. The reduced flow and altered composition of saliva cause a wide range of clinical symptoms of which the most distressing are a sensation of a dry mouth, difficulties in swallowing and speach, nocturnal oral discomfort, and an enhanced susceptibility to oral infections and dental caries. These side effects of cancer treatment are a heavy burden to the patient during and after radiotherapy. Consequently, prevention of salivary gland injury or its reduction to a minimum is a matter of major concern. There is general agreement as to the dose-dependent extent of radiation-induced glandular damage, but the underlying mechanism is still unknown. Obviously such knowledge is a prerequisite for a rational design of treatment protocols directed to protection against radiation damage. The purpose of the experiments described in this thesis was to obtain more insight into the mechanism(s) of early radiation damage to salivary glands. To achieve this goal, *rat* studies on morphology and function were performed. Emphasis was placed on (1) the radiosensitivity of the different epithelial cell populations, (2) cell replacement following radiation-induced cell loss, and (3) the relation between the granulation level and radiosensitivity of the secretory tissue. Radiation effects were also studied *in vitro*, because in *in vivo* experiments it is difficult to quantify the radiosensitivity on the cellular level.

Chapter 1 provides an extensive review of literature related to (1) ionizing radiation with emphasis on normal tissue damage and apoptosis as a mode of cell death, (2) structure and function of the salivary glands and (3) radiation effects on salivary gland morphology and function. At the end of this chapter the scope of the thesis is described.

The study described in **Chapter 2** is conducted to obtain insight into radiation-related changes in the proliferative activity of cells in the different epithelial cell compartments (i.e. acinus, intercalated duct, granular convoluted tubule, striated duct) of the parotid and submandibular salivary glands. Following local X-irradiation with a single dose of 15 Gy, proliferative activity was measured by bromodeoxyuridine-labelling in the different epithelial cell compartments as a function of time (up to 30 days). In both glands, repopulation was observed in all cell compartments. Proliferation started in the intercalated ducts (day 3), followed by acini (day 6), granular convoluted tubules (day 6), striated ducts (day 6, parotid; day 10, submandibular). It could not be deduced if the labelled cells originated solely from progenitor cells residing in the intercalated duct or if cells of the other compartments were also stimulated. Proliferative activity was found to be higher in the intercalated duct compartment of the parotid than in that of the submandibular

gland which may be related to the suggested higher radiosensitivity and thus a greater demand for cell replenishment in the parotid gland.

Based on data from salivary gland morphology, serous cells are supposed to be more radiosensitive than mucous cells. In case of the submandibular gland data from the literature suggest a causal relationship between the presence of secretory granules and radiation death of serous cells. Therefore it was decided to focus on studies related to the role of secretory granules in radiation injury of the tissue. For this purpose, a drug had to be selected that degranulates the secretory tissues without deleterious side-effects (**Chapter 3**). Phenylephrine (α-adrenergic), isoproterenol (ß-adrenergic) and mecholine (muscarinic cholinergic) were tested. Time and degree of maximal depletion of acinar and granular convoluted tubule cells were determined morphologically. Isoproterenol was found to induce high levels of degranulation in serous acinar cells of the parotid gland (almost 100%) and mucous acinar cells of the submandibular gland (about 80%). This strong depletion could be achieved with a relatively low dosage (5 mg/kg ip) without obvious toxic side-effects. Degranulation of the serous granular convoluted tubule cells was observed after treatment with phenylephrine or mecholine. The latter drugs were not suitable for irradiation studies planned, because the magnitude of degranulation was too low (about 50%) and the dosages used caused unacceptable circulatory side-effects. Therefore, isoproterenol was chosen to investigate the possible involvement of secretory granules in radiation damage to acinar cells.

In **Chapter 4** radiation effects on morphology are described. Following local X-irradiation with a single dose of 15 Gy, cell death was observed as early as 2-4 h after irradiation. In the literature, radiosensitivity of the different cell populations has mainly been assessed in qualitative studies. To further address this issue, radiation-induced cell death was determined as a function of time by counting the number of nuclear abberations per individual epithelial cell population. This quantitative study revealed comparable degrees of radiation-induced kill in the serous and mucous secretory and intercalated duct compartments. Also some striated duct cells showed aberrant nuclei, but the variation within these tissue samples was too high to allow a reliable analysis. Subsequent to nuclear aberrations, progressive lysis occurred in secretory, but not in ductal units. Loss of tissue integrity was maximal at day 3. Similarly, radiation-induced changes in salivary gland function (**Chapter 5**) were maximal 3 days after irradiation. Flow rate and amylase secretion were decreased, while an increase in lag phase and concentrations of potassium and sodium was observed. Although tissue structure had recovered completely by day 10, function was still impaired. Isoproterenol-induced degranulation of acinar cells before irradiation did not reduce early radiation-induced cell death (**Chapter 4**). Thus, in acini secretory granules seem to play no major role in radiation damage. In the parotid gland, there was evidence for a faster recovery from radiation-induced tissue injury after isoproterenol pretreatment which may be the result of stimulation of cell proliferation by isoproterenol. This faster replacement of damaged cells by functionally intact cells may provide also an explanation for the improvement of radiation-induced changes

in flow rate and composition of parotid saliva observed at later periods (**Chapter 5**). It can, however, not contribute to the less reduction of parotid flow rate observed as early as 1 day after irradiation. Therefore, it is still possible that the presence of serous acinar secretory granules contribute to radiation-induced cell death and that degranulation leads to a certain degree of protection against radiation damage.

In **Chapter 6** a method was established to culture cells isolated from both rat parotid and submandibular salivary glands. Immunohistochemical characterization was applied to identify the origin of the cultured cells. The distribution of cell types in both isolates was comparable to their distribution in the original tissue. Exponential growth was observed from cell aggregates attached to the culture dish between days 4 and 8. Subsequently, cell numbers reached a plateau in the parotid culture, while in the submandibular culture cell numbers decreased. Only few secretory granules were observed in newly formed cells which made identification of the cultured cells with antibodies directed against (glyco)proteins impossible. Analysis of intermediate filaments demonstrated the epithelial origin of the outgrowing cells. In the areas between the epithelial outgrowth, occasionally contamination with fibroblasts was observed. Fibroblast contamination occurred more often in parotid than submandibular cultures. No fibroblastic overgrowth was observed during the culture period.

In **Chapter 7**, the established primary culture was applied to investigate the radiosensitivity of the cultured cells. In radiobiology classically two types of cell death are distinguished: reproductive cell death (it occurs during the first or subsequent attempts of the cell to divide) and interphase cell death (cells disintegrate before mitosis). In proliferating cells, like the investigated cultures of salivary gland cells, much higher radiation doses are needed for interphase death than for reproductive death. Radiation-damage was determined by three different parameters, viz. decrease in cell numbers as determined by a DNA method, trypan blue exclusion and leakage of lactate dehydrogenase (LDH). The indirect measurement of cell survival with the DNA method was performed in the dose range from 0-9 Gy (reproductive death) and a cell survival curve was constructed. The cells isolated from the parotid gland appeared to be more radiosensitive than those isolated from the submandibular gland as indicated by the slope of the exponential part of the curve. The initial slope of the survival curves was the same, but the DNA method was not sensitive enough to further quantify the capacity of the cells for sublethal damage repair. To detect radiation damage with the trypan blue and LDH assays high radiation doses were necessary leading to interphase death of these cells. With a dose of 50 Gy no differences were observed in this type of cell death by both types of cells. These observations suggest that the radiosensitivity of the plasma membrane of the cultured cells from the parotid and from the submandibular glands is comparable. Rather, differences in residual DNA damage are suggested to be the reason for the different sensitivity to radiation-induced reproductive death in both cell cultures.

In **Chapter 8** it was tested if radiation damage (e.g. decomposition products of unsaturated fatty acyl chains from phospholipids in membranes) can be detected by proton NMR spectroscopy. This technique might be worthwhile to use as it possibly can be applied noninvasively to monitor tissue damage during the course of radiotherapeutic treatment. Three days after *in vivo* X-irradiation with a single dose of 15 Gy, the major salivary glands were removed for the preparation of tissue homogenates and lipid extracts. No radiation effects were observed in proton spectra of the homogenates, but spectra obtained from lipid extracts of parotid and sublingual gland tissues showed obvious changes. Most striking was the decreased amount of methylenes next to the carbon-carbon double bond (about 50%) and the appearance of a new peak at δ 1.85 ppm. No radiation effect was observed in the lipid extract of the submandibular tissue, even not after 30 Gy. Liposome preparations from phospholipids of rat livers did not show the peak at δ 1.85 ppm after irradiation with 200 Gy as was the case in the lipid extracts of parotid and sublingual glands. This suggests that interaction with tissue components is necessary to produce this specific radiation product. The lipid may very well be a secondary product of radiation damage, e.g. as the result of cell degeneration during the 3 days after irradiation. The nature of the new product is unknown. It may possibly represent a methyl hydrogen adjacent to either an unsaturated carbon-carbon group or a carbonyl group of a ketone, or may originate from an alkyl hydrogen of an alcohol. This has to be explored further.

In **Chapter 9** the relationship between the performed studies is discussed. The extent of early cell death appeared to be comparable for both the serous and mucous acini, the serous granular convoluted tubules and the intercalated ducts. The lethally injured cells showed some morphological characteristics of apoptosis, an issue that has to be investigated in more detail. The early radiation damage was followed by repopulation in all cell compartments, starting in the intercalated duct compartment. This is in agreement with its proposed progenitor function. Pretreatment of the rats with isoproterenol before irradiation did not result in a reduction of early damage to acinar cells, indicating that secretory granules play no major role in early radiation-induced death of these cells. The observed faster recovery (morphologically as well as functionally) of the parotid glands from radiation injury may be due to a drug-induced stimulation of cell proliferation, resulting in an earlier unmasking of latent lethal damage. The proposed proliferative stimulation can, however, not contribute to the less reduction of the parotid salivary flow one day after irradiation. Therefore, it is still possible that the presence of serous secretory granules at the moment of irradiation somehow contribute to radiation-induced death of acinar cells. Moreover, data from the literature indicate that apart from isoproterenol (ß-adrenergic), pretreatment of rats with cholinergic or α-adrenergic drugs has the potential to reduce radiation-induced parotid gland dysfunction. Since these drugs act by different receptors and hence different signal transduction pathways, separate mechanisms seem to be involved in the protective action. Further study of these mechanisms may contribute to the

development of clinically applicable methods to reduce radiation damage to salivary glands.

In conclusion, the data described in this thesis contribute to the interpretation of early effects of radiation on salivary glands and provide some interesting tools for future studies on the protection of these tissues. Particularly, the following questions have to be solved:

1) Is apoptosis involved in early radiation-induced death of salivary gland cells? If apoptosis plays a role, to which extent does it affect the different epithelial cell compartments? Immunohistological studies applying antibodies specific for apoptotic cells may provide useful information on this issue.
2) Does the increased BrdU-labelling index observed in all epithelial cell compartments after irradiation imply that in all compartments cells are stimulated to proliferate due to the sudden cell loss or are solely the stemcells located in the intercalated duct responsible for the repopulation? This has to be investigated by studying the displacement of pulse-labelled cells in the different cell compartments as a function of time.
3) Does isoproterenol-induced activation of acinar cell proliferation indeed contribute to the less reduction of parotid gland function observed after irradiation? This can be tested by determining the number of proliferating cells with a BrdU-pulse-label as a function of time in isoproterenol pretreated salivary glands.
4) What is the mechanistic explanation for the observed protective effect of α-adrenergic and cholinergic agents?

Further research on the mechanism(s) of radiation damage to salivary glands and its prevention may result in adjustment of radiotherapeutic treatment protocols for patients with head and neck cancers directed to a maximal tumour cure with minimal damage of salivary gland tissues.