Imbalance between Apoptosis and Proliferation Causes Late Radiation Damage of Salivary Gland in Mouse

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Summary

Severe xerostomia is a common late radiation consequence, which occurs after irradiation of head and neck malignancies. The aim of the present study was to analyze apoptosis and proliferation and their relationship during the late post-irradiation phase. C57BL/6 mice were locally irradiated in head and neck region with a single dose of 7.5 or 15 Gy and their submandibular glands were collected at 40 and 90 days after irradiation. To identify apoptotic cells, the TUNEL method was employed and immunohistochemistry with proliferating cell nuclear antigen (PCNA) was used for detecting proliferation. Histological changes at day 40 were mild in contrast to day 90 when glands of irradiated mice showed severe atrophy, vacuolization and mononuclear infiltration. Acinar cells, granular and intercalated duct cells of mice irradiated with 7.5 and 15 Gy expressed higher apoptotic index than cells of non-irradiated, control glands at both examined time points. At 40 days, a higher proliferation index in granular and intercalated duct cells was detected only in group irradiated with 7.5 Gy. At 90 days, proliferation index for all cell types in both irradiated groups was similar to the controls. According to our results, the imbalance between apoptosis and proliferation caused by X-irradiation may be the reason for gland impairment during the late post-irradiation phase.

Key words

Irradiation • Salivary glands • Late post-irradiation phase • Apoptosis • Proliferation

Introduction

Radiotherapy plays an important role in the treatment of head and neck tumors. Unfortunately, too many patients whose salivary glands are irradiated during treatment for their cancer often results in life-long severe xerostomia ("dry mouth"). Reduction in salivary flow *per se* is not life-threatening, but deterioration of dental and oral health has significant impact on the quality of their life (Guchelaar *et al.* 1997, Taylor and Miller 1999, Taylor 2003).

Numerous studies have demonstrated that radiation-induced impairment of salivary glands could be divided into two phases: short-term crisis followed by

recovery period and late deterioration phase (Coppes *et al.* 1997, Nagler and Laufer 1998, Nagler 2001, 2002, Muhvic-Urek *et al.* 2005). In rodents, the chronic radiation damage to salivary glands is fully developed and stabilized within 60-90 days after radiation treatment (Sodicoff *et al.* 1978, Dorr 1998).

The complex relationship among cell proliferation, differentiation and apoptosis is a cardinal feature in the maintenance of normal architecture and function of submandibular gland. Enhanced apoptosis of acinar cells is hypothesized to be one of the major causes of salivary gland impairment and it can be induced with DNA damaging agents such as radiation (Baehrecke 2002, Limesand *et al.* 2003).

One can speculate that gland proliferation is increased in an attempt to overcome the irradiation damage. We therefore investigated expression of the proliferating cell nuclear antigen (PCNA) in irradiated glands. Since PCNA manifests itself mostly during S-phase of the cell cycle it is widely used as a marker of proliferation (Girod *et al.* 1998, Actis *et al.* 2002).

The purpose of this study was to analyze the relationship between apoptosis and proliferation in acinar, granular duct and intercalated duct cells during late (40 days) and prolonged (90 days) post-irradiation period.

Methods

Male C57BL/6 mice aged 8-10 weeks were randomly divided into three groups: (I) sham irradiated – control group; (II) 7.5 Gy irradiated; and (III) 15 Gy irradiated. The mice were intraperitoneally anesthetized with sodium pentobarbital (40 mg/kg b.w.), fixed in a plastic mould and locally irradiated in the head and neck regions with 6 MV X-rays from a medical linear accelerator (Mevatron MD-2, Siemens, Medical Laboratories Inc., USA) with a single dose of 7.5 Gy (n=15) or 15 Gy (n=15) (Nagler 1998, Nagler *et al.* 1998). Irradiation was performed by a standard source to surface distance (SSD) of 100 cm. Radiation field size was x=15 cm, y_1=0 cm, y_2=3 cm and a dose rate was 191 cGy/min. Prior to the irradiation beam, calibration in solid water phantom was performed using Farmer type 0.6 cc ionization chamber with PTW Unidos dosimeter. Control animals (n=15) were anesthetized, fixed and sham-irradiated. The Ethical Committee of the Medical Faculty at University of Rijeka approved all procedures using mice.

Submandibular glands were analyzed at 40 and 90 days after irradiation, i.e. at two time points that represent long and extended post-irradiation periods (Nagler 1998, Nagler *et al.* 1998). The excised submandibular glands were immediately fixed in 4 % paraformaldehyde and processed for paraffin embedding according to standard procedure. Serial sections 2 μm thick were cut and stained with hematoxylin and eosin (H&E). Nuclear aberrations were examined at 600x magnification using light microscope (Olympus, Japan). Histopathological criteria for apoptotic cells were shrunken cells with condensation of chromatin forming dense stained, sharply delineated granular masses.

To detect apoptotic cells the TUNEL method was performed using In Situ Cell Death Detection Kit-POD, (Roche Diagnostics GmbH, Germany) (Macluskey *et al.* 2000). The sections were incubated with terminal deoxynucleotidyl transferase (TdT) and fluorescein deoxyuridine triphosphate (dUTP) without proteinase K pretreatment. After phosphate buffer saline (PBS) rinsing, anti-fluorescein-peroxidase antibody was applied and the reaction was visualized by 3,3'-diaminobenzidine (DAB). Sections were counterstained with hematoxylin. Control sections were incubated with distilled water in the absence of TdT.

To examine proliferating cells, sections were incubated with anti-PCNA monoclonal antibody (DAKO, USA) (Roos *et al.* 1993), followed by staining with biotinylated rabbit anti-mouse polyclonal antibody (DAKO, USA) and the streptavidin-biotin-peroxidase complex (Boehringer, Germany). As a chromogen 3-amino-9-ethyl-carbazol substrate (AEC-Substrate-Chromogen, DAKO, USA) was used. PBS was substituted for the primary antibody as negative control. Hematoxylin was used for counterstaining.

The apoptotic and proliferation indexes were calculated for each group (Macluskey *et al.* 2000). Labeling index represented the apoptotic/proliferating cells as a percentage of the specific cell type. Five coded sections stained with PCNA and TUNEL were randomly chosen from each animal. Approximately 1000 cells from each cell population (acinar cells, granular duct cells, intercalated duct cells) were counted by two observers (MM-U, MB) at a magnification of 400x (Olympus, Japan), and the percentage of PCNA and TUNEL positive cells was calculated. Striated ducts were omitted from the study since too few of them were examined to provide useful information. The labeling index for each group was obtained by averaging the percentages of all animals, and the mean value ± standard error of mean (SEM) were

determined for the control, 7.5 and 15 Gy irradiated group. The comparison between experimental and control data was made by two-way analysis of variance, followed by Tukey´s honestly significant difference (HSD) *post hoc* test, with P<0.05 as statistically significant.
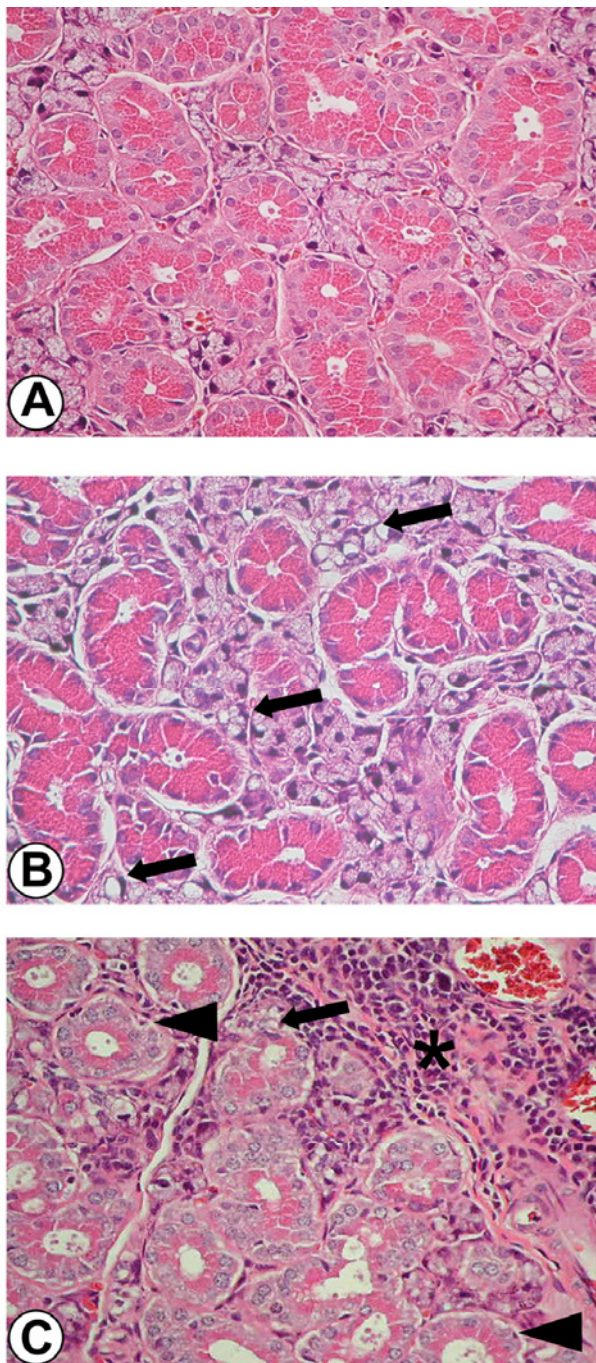
Fig. 1. Histopathological changes in submandibular gland after irradiation. Normal appearances of acinar cells in control animal (A), whereas in gland irradiated with 15 Gy at 40 days after irradiation (B) and at 90 days after irradiation (C) vacuolization (←), condensated nuclei (◄) and mononuclear infiltration (✱) is observed. H&E. Magnification x400.

Results

Survival

All animals in the control group survived the whole experimental period as well as animals irradiated with 7.5 Gy. In the group irradiated with 15 Gy, 3 mice died within 2 weeks during the acute post-irradiation phase.

Histological observation

Histological observation of control animals showed normal morphology, i.e. ducts widely separated by crowded acini (Fig. 1A) while mild histological changes were observed in glands of irradiated animals with 7.5 and 15 Gy at 40 days after irradiation. Only vacuolization of acinar cells was detected and no pathological changes in the ducts and interstitium were present (Fig. 1B). Moreover, no significant difference between two irradiation doses was observed in tissue morphology.

At 90 days, submandibular glands of irradiated animals showed acinar atrophy, reduction in the number and severe vacuolization of acinar cells. Changes were more intensive in glands irradiated with higher radiation dose. In animals treated with 7.5 Gy granular duct cells were unaffected, but in animals irradiated with 15 Gy granular duct cells were often degenerated and desquamated. In interstitium of 15 Gy irradiated glands small edema was present, but the most remarkable change concerned disseminated mononuclear infiltration observed in all irradiated glands (Fig. 1C).
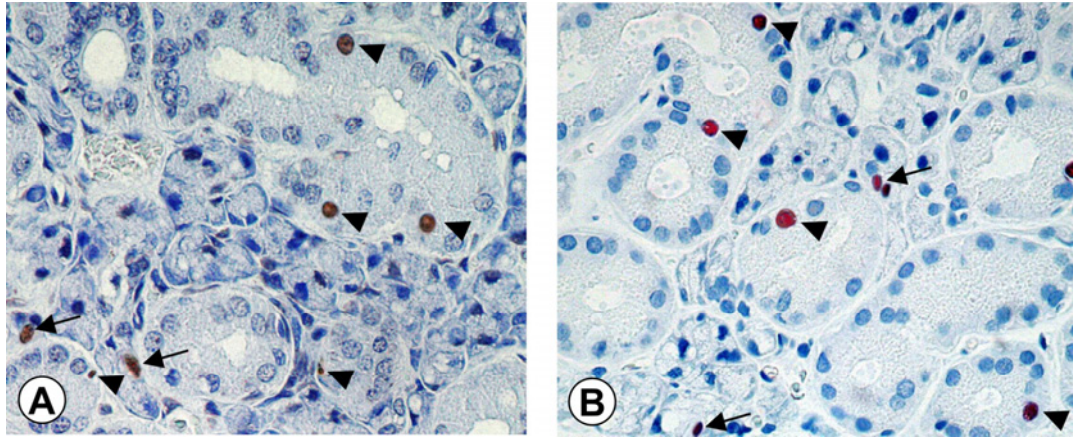
Apoptosis and proliferation

In the submandibular gland of control animals, a small number of apoptotic cells was observed in acinar cells (0.06 %), granular duct cells (0.36 %) and intercalated duct cells (0.3 %). At 40 days after irradiation, higher apoptotic activity was noted in all investigated cell compartments in both irradiated groups. Apoptotic activity in irradiated glands decreased from 40 to 90 days, but it remained above control levels. Only in the population of acinar cells irradiated with 15 Gy significantly higher apoptotic activity was observed even at day 90 (Figs 2A and 3).

Submandibular gland of control animals exhibited low proliferative activity in all gland compartments: acinar cells (2 %), granular duct cells (6.42 %) and intercalated duct cells (2.25 %). At 40 days after irradiation, labeling index for granular and

intercalated duct cells in mice irradiated with 7.5 Gy was higher than that of acinar cells. In addition, labeling index for both duct cells was significantly higher than in control mice, while mice irradiated with 15 Gy exhibited labeling index similar to control level. At 90 days after irradiation in all gland compartments (acinar cells, intercalated and granular duct cells) the proliferative activity in irradiated mice was similar to the control, sham irradiated group (Figs 2B and 4).

Fig. 2. Example of TUNEL (A) and PCNA (B) labeling in salivary gland tissue after irradiation. Apoptotic TUNEL positive cells have brown nuclei, whereas proliferative PCNA positive cells have red nuclei. Magnification x400.
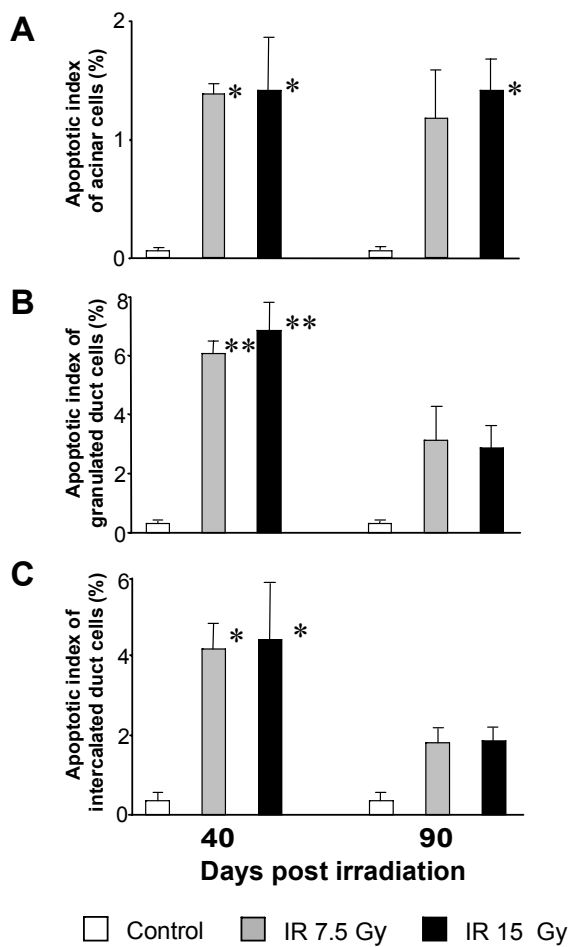
Fig. 3. Apoptotic index of individual parenchymal cell types (mean ± SEM) in submandibular gland after irradiation. (**A**) Acinar cells, (**B**) Granular duct cells, (**C**) Intercalated duct cells. * denotes statistical significance of $P<0.05$; ** denotes statistical significance for $P<0.001$.
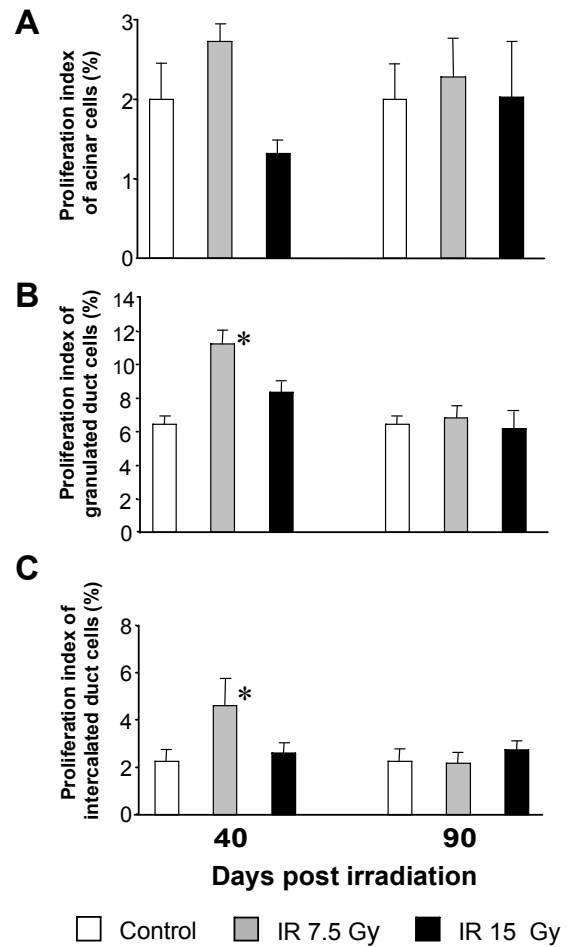
Fig. 4. Proliferation index of individual parenchymal cell types (mean ± SEM) in submandibular gland after irradiation. (A) Acinar cells, (B) Granular duct cells, (C) Intercalated duct cells. * denotes statistical significance for $P<0.05$.

Discussion

The mechanism of salivary gland pathology induced by radiation is not clearly understood and no adequate prevention and treatment is yet available. Late tissue response to radiotherapy is regarded as more important than the acute effects because of their progressive and irreversible character. Parenchymal cells, vascular endothelium and fibroblasts are considered as target cells for irradiation-induced damage (Dorr and Hendry 2001, Paris *et al.* 2001). We analyzed the apoptotic and proliferation index in parenchymal cells since their loss lead to functional impairment of salivary glands and hyposalivation (xerostomia) that are major symptoms of head and neck radiotherapy. Mouse submandibular gland is formed of parenchymal cells organized in acini and secretory ducts (intercalated, granular and striated) that converge to form the excretory ducts. Since cells of salivary glands have slow turnover rates, one could expect they would be relatively radioresistant. On the contrary, they are extremely radiosensitive. It is known that radiosensitivity is greatest in serous secretory cells followed by mucous secretory cells whereas ductal cells appeared to be relatively radioresistant.

There is convincing evidence that ionizing radiation can induce apoptosis (Langley *et al.* 1993, Bump *et al.* 1994, Roos 1999, Belka *et al.* 2004). It is believed that apoptosis plays a major role only during acute post-irradiation damage (Guchelaar *et al.* 1997), but we have observed significantly higher apoptotic index in irradiated animals during the late post-irradiation phase. Our results lead to assumption that irradiation is responsible for sublethal DNA damage that becomes manifest during late post-irradiation phase. The damaged cell is detected on the cell cycle checkpoint and instead entering mitosis it is subjected to apoptosis (Roos 1999, Nagler 2002). To our knowledge no investigation using the TUNEL method on submandibular glands of irradiated mice has been performed. Denny and Denny (1999) investigated dynamics of parenchymal cell apoptosis in normal mouse submandibular gland by the same method. These authors have found apoptotic index for acinar and other cell types similar to those we obtained for non-irradiated control mice.

The highest proliferation index was detected at the late time point (40 days after irradiation) in group irradiated with 7.5 Gy, while control and 15 Gy irradiated animals exhibit similar values. We have evidence that animals irradiated with lower radiation dose exhibit higher proliferative capacity. At the same time both ductal cells exhibited higher proliferative capacity in contrast to acinar cells whose proliferative activity was similar to the control level. Peter *et al.* (1994) investigated the early post-irradiation phase and they observed the highest proliferative activity of intercalated duct cells and suggested intercalated duct cells as stem cells.

At 40 days after irradiation mild microscopic changes (vacuolization) in the irradiated gland were noted. We have confirmed the findings of Vitolo *et al.* (2004), who observed discrete vacuolization in acinar cells in mice irradiated with 15 Gy. We have evidence that in the late deterioration phase postponed effects of irradiation emerge and aggravate gland morphology. Our observation is in accordance with the results of studies performed on rats, rabbits and primates (including humans) (Stephens *et al.* 1986, Ahlner *et al.* 1994, Price *et al.* 1995, Nagler 2001, 2002). Inflammatory infiltration detected in primates and rabbits were also found in mice (Taylor and Miller 1999). Since ionizing radiation can induce apoptosis as well as necrosis (Guchelaar *et al.* 1997), necrosis could be the reason for salivary gland´s inflammatory infiltration as we have also observed.

We also demonstrated that the proliferation index was similar as control values during extended post-irradiation period. At the same time significantly higher apoptosis was detected in acinar cells irradiated with 15 Gy. In other cell types, apoptosis was above control levels implicating that proliferation was not sufficient to sustain normal architecture of the gland.

We conclude that elevated apoptosis in the irradiated gland is major cause of imbalance between cell production and cell loss. Further research concerning the modulation of apoptotic-signaling pathway may hopefully improve the therapeutic potential and reduce late time sequelae in irradiated salivary glands.

Acknowledgements

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