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EFFECT OF IRRADIATION ON MICROVASCULAR ENDOTHELIAL CELLS OF PAROTID GLANDS IN THE MINIATURE PIG

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Abstract

Purpose—To evaluate the effect of irradiation on microvascular endothelial cells in miniature pig parotid glands.

Methods and Materials—A single 25-Gy dose of irradiation (IR) was delivered to parotid glands of 6 miniature pigs. Three other animals served as non-IR controls. Local blood flow rate in glands was measured pre- and post-IR with an ultrasonic Doppler analyzer. Samples of parotid gland tissue were taken at 4 h, 24 h, 1 week, and 2 weeks after IR for microvascular density (MVD) analysis and sphingomyelinase (SMase) assay. Histopathology and immunohistochemical staining (anti-CD31 and anti-AQP1) were used to assess morphological changes. MVD was determined by calculating the number of CD31- or AQP1-stained cells per field. A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis assay was used to detect apoptotic cells. The activity of acid and neutral Mg²⁺-dependent SMase (ASMase and NSMase, respectively) was also assayed.

Results—Local parotid gland blood flow rate decreased rapidly at 4 h post-IR and remained below control levels throughout the 14-day observation period. Parotid MVD also declined from 4 to 24 hours and remained below control levels thereafter. The activity levels of ASMase and NSMase in parotid glands increased rapidly from 4 to 24 h post-IR and then declined gradually. The frequency of detecting apoptotic nuclei in the glands followed similar kinetics.

Conclusions—Single-dose IR led to a significant reduction of MVD and local blood flow rate, indicating marked damage to microvascular endothelial cells in miniature pig parotid glands. The significant and rapid increases of ASMase and NSMase activity levels may be important in this IR-induced damage.

Keywords

Salivary glands; Radiotherapy; Microvessel density; Endothelial cells; Sphingomyelinase

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Conflict of interest: none.

INTRODUCTION

Radiotherapy is commonly used in the treatment of most head and neck cancers. A significant consequence of this ionizing radiation (IR) is the irreversible damage to salivary glands (SGs) in the irradiation field (1), which results in severe hyposalivation in most patients. Because saliva plays such a critical role in the physiology and protection of upper gastrointestinal tract tissues (2), patients experiencing reduced salivary flow suffer considerable morbidity, including mucosal infections, dental caries, and dysphagia, as well as frank discomfort (3).

While the exact damage mechanism remains enigmatic, fluid-secreting acinar cells are lost, and most studies aimed at preventing irradiation-induced salivary hypofunction have focused on acinar cells. However, SG acinar cells, which are the sole site of fluid secretion in this tissue, although highly radiosensitive, are well-differentiated cells exhibiting a low mitotic rate (4). In 2001, the general possibility that microvascular endothelial cells are an IR target in the gastrointestinal tract was suggested (5). Subsequent studies specifically evaluated the importance of SG endothelial cells to IR-induced injury in mice (6). Four h after single-dose IR, microvascular density (MVD) in SGs decreased by ~45%. However, if mice were pretreated with a serotype 5 adenoviral vector encoding either basic fibroblast growth factor or vascular endothelial growth factor 48 h before IR, the loss in MVD was significantly reduced (6).

Endothelium has long been recognized as a principal target for IR injury to lung and brain (7, 8), and ~15 years ago, reports showed that acid sphingomyelinase (ASMase), an enzyme located primarily on cell membranes, plays a critical role in mediating IR-induced endothelial cell apoptosis, both *in vitro* and *in vivo* (7, 9). Furthermore, the vulnerability of the endothelium to IR-induced apoptosis appears to be related to the high levels of ASMase expression in endothelial cells, which is approximately 20-fold higher than, for example, that in macrophages (10) and to its preferential trafficking to the plasma membrane. The aim of the present study was to evaluate the possible effect of IR on microvascular endothelial cells in the parotid glands of miniature pigs. Specifically, we measured local blood flow rate, MVD, apoptotic activity, and ASMase and NSMase activities in irradiated parotid glands of this large animal model.

METHODS AND MATERIALS

Animals and irradiation of parotid glands

These experiments were performed on nine inbred, healthy male miniature pigs, 9 months of age, weighing 30 to 40 kg, which were obtained from the Institute of Animal Science, Chinese Academy of Agricultural Sciences. Three miniature pigs were observed for 24 h, as group 1; three pigs were observed for a 2-week observation period, as group 2; and the remaining three miniature pigs served as an untreated control group. Animals were kept under conventional conditions with free access to water and food. The Animal Care and Use Committee of the Capital Medical University approved all experiments in this study.

Animals were first anesthetized with a combination of ketamine chloride (6 mg/kg) and xylazine (0.6 mg/kg) injected intramuscularly. The local surface just over the parotid glands was randomly marked at three points, using tattoos for local blood flow rate measurements. A single dose of IR (25Gy, 6mV of photon energy at 3.33Gy/min; Department of Radiation Oncology, Beijing Friendship Hospital, Capital Medical University, Beijing, China) was delivered via an electronic linear accelerator (600C; Varian, Inc.) directed at both parotid glands of 6 miniature pigs by utilizing the three-dimensional conformal radiation therapy technique (11). After IR, animals were removed to a climate- and light-controlled

environment and allowed free access to food and water. Parotid gland biopsy was performed on anesthetized group 1 animals at pre-IR and 4 h post-IR; and at pre-IR and 1 week post-IR, parotid glands were biopsied in the group 2 animals. The animals from group 1 were sacrificed at 24 h, while animals from group 2 were sacrificed at 2 weeks. Parotid gland biopsies were obtained from control group animals at pre-IR, at 1 week, and following sacrifice at 2 weeks.

Local blood flow rate measurements

Local blood flow in parotid glands was measured prior to IR and at 4 h, 24 h, 3 days, 5 days, 7 days, 10 days, and 2 weeks post-IR by an ultrasonic Doppler blood flow rate analyzer (2 MHz detector, 40-mm depth, 160% power, 4 plus; JYQTC-2000; Kingrich Science and Trade Co., P. R. China).

Determination of microvascular density

Sections of parotid glands were prepared and stained with hematoxylin and eosin as previously described (11). Immunohistochemical staining of sections used two antibodies (anti-CD31 and anti-AQP1) as markers for microvascular endothelial cells. Briefly, sections were incubated in a 1:100 dilution of either goat anti-porcine CD 31 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-porcine AQP1 (Santa Cruz Biotechnology). Next, the primary antibody was detected with an appropriate biotinylated second antibody, followed by incubation with streptavidin-conjugated horseradish peroxidase (KPL, Gaithersburg, MD) and colorimetric detection with 3,3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO). MVD was determined by calculating the number of CD31- or AQP1-stained cells per field at $\times 200$ magnification, using Image-Pro Plus version 6.0 software. An entire parotid gland section for each animal was counted with an average of 40 fields/gland.

Determination of apoptotic cells

A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis detection kit (Millipore) was used to identify apoptotic cells by following the manufacturer's instructions. Briefly, sections were covered with diluted proteinase K, incubated for 30 min at 37°, and washed with phosphate-buffered saline (PBS) four times, each for 2 min. Next, terminal deoxynucleotidyl transferase (TdT) buffer was applied for 10 min and then incubated with a TdT end-labeling cocktail for 60 min at 37°, and the reaction was stopped by immersing the cells in Tris-borate buffer for 5 min at room temperature. Thereafter, sections were washed with PBS, exposed to blocking buffer, and incubated at room temperature for 20 min. After incubation with avidin-fluorescein isothiocyanate (FITC) solution in the dark for 30 min at 37°, the sections were incubated with goat anti-porcine CD31 (Santa Cruz Biotechnology). Last, sections were washed with PBS, incubated with donkey anti-goat immunoglobulin G (Alexa Fluor 350-conjugated; Invitrogen) in the dark, and then propidium iodide (Sigma-Aldrich) was applied. Sections were viewed by fluorescent microscopy, and pictures were taken at $\times 400$ magnification. Photos of apoptotic cell nuclei (stained green by FITC) and photos of all other nuclei (stained red by propidium iodide) with CD31 (stained blue with Alexa Fluor 350; as an endothelial cell marker) were composed using Image-Pro Plus version 6.0 software. The yellow spots from FITC staining and propidium iodide staining on photos of each section were counted to quantify the presence of apoptotic cells. CD31 and TUNEL-assayed apoptotic double-stained cells were also counted to determine the percentage of apoptotic cells also labeled for CD31. Each parotid gland section was counted, with an average of 15 fields/gland.

Determination of sphingomyelinase activity

The activity levels of neutral Mg^{2+} -dependent sphingomyelinase (NSMase) and ASMase were assayed using trinitrophenylaminolauroyl (TNPAL)-sphingomyelin (Sigma-Aldrich). For assaying ASMase, acid buffer (pH 5.0) was prepared (100 mM sodium acetate, 0.1% Triton X-100, and 0.01 M ethylenediaminetetraacetic acid); while, for assaying NSMase, a neutral buffer (pH 7.4) was prepared (100 mM Tris HCl, 0.1% Triton X-100, and 0.02 M $MgCl_2$). The samples were homogenized with a Sonics Ultrasonic process (Jingtan Medical Instrument Factory, China) in cold acid and then neutral buffer, respectively. The resulting homogenates were centrifuged at $1,000 \times g$ (Anke centrifuge; Shanghai Anting Scientific Instrument Factory, China), and the supernatants were used for determination of both SMase activity and protein concentration. For each assay, 50 μ l of the supernatant was added to a tube containing 10 μ g of TNPAL-sphingomyelin in 150 μ l of the above acid or neutral buffers. Tubes were shaken at room temperature for 5 min and then incubated for 1 h in a water bath at 37°C with continuous shaking. The reaction was stopped by adding 750 μ l of a mixture of isopropanol–heptane–5% H_2SO_4 (40:10:1, vol/vol/vol). Next, 400 μ l of heptane was added and the tube was shaken and then centrifuged ($1,000 \times g$) to separate the two phases. The upper, heptane phase contained TNPAL-ceramide, which was formed from TNPAL-sphingomyelin by the SMase present in the sample. The absorbance of the heptane phase was read at 330 nm using a Spectra-Max Plus (Molecular Devices, Sunnyvale, CA). We generated a standard curve of SMase activity, using human placental SMase (Sigma-Aldrich). SMase activity was expressed in nanomoles of ceramide per hour per milligram of protein. Protein content was measured with a bicinchoninic acid protein assay kit (Sigma-Aldrich) according to the manufacturer's instructions. Bovine serum albumin (fatty acid-free; Sigma-Aldrich) was used as a standard for protein assays.

Statistical analyses

SPSS version 13.0 software was used for statistical analyses. Descriptive summaries (means $\pm$ standard deviation [SD]) of the data were determined first. One-way and two-way analyses of variance were used to analyze data, with a p value of <0.01 considered significant.

RESULTS

Local blood flow rate in parotid glands after irradiation

A maximum decline of parotid blood flow rate was observed at 4 h after IR (Fig. 1A). At that time, the local blood flow rate had decreased by $>40\%$ compared to that seen initially with non-IR control animals; the average ($\pm$ SD) parotid blood flow rate measured in the control group was 6.68 (± 1.27) cm/sec, while for the irradiated group, this value was 3.87 (± 0.90) cm/sec ($p < 0.01$). At 24 h, the local blood flow rate in the irradiated group partially recovered to 4.41 (± 0.67) cm/sec, and on day 3, it was 5.74 (± 0.61) cm/sec ($\sim 85\%$ of normal controls). Thereafter, parotid blood flow rate was fairly stable and $\sim 20\%$ lower than control values, *e.g.*, on day 14 it was 4.89 (± 1.21) cm/sec (Fig. 1).

Histological and MVD changes in parotid glands

At 4 h after IR, there was some eosinophilic fiber-like structure in the basement membrane region around acini (Fig. 1B), which partially abated at 24 h (Fig. 1C). By 2 weeks after IR (Fig. 1D), increased fibrosis was evident, and some acinar regions were atrophic. The basement membrane around surviving acinar cells appeared thickened.

At 4 h after IR, the parotid MVD decreased by $\sim 20\%$ (when measured by CD31 immunostaining) or was essentially unchanged (when measured by AQP1 immunostaining). However, at 24 h after IR, both CD31 and AQP1 immunolabeling showed similar and

marked MVD losses; with CD31 staining, the MVD was ~72% of values of a control group that did not receive any IR, while with AQP1 staining, it was reduced to ~79% (Fig. 2). The average ($\pm$ SD) MVD in non-IR control parotid glands labeled by CD31 was 62.98 ($\pm$ 14.40) optical density unit (ODU), while 24 h after IR, the MVD was 45.07 ($\pm$ 8.57) ODU ($p < 0.01$). Similarly, the MVD in the control parotid glands labeled by AQP1 was 64.54 ($\pm$ 16.05) ODU, and for the irradiated group at 24 h, the MVD was 50.70 ($\pm$ 8.55) ODU ($p < 0.01$). At 2 weeks after IR, the MVD remained lower than pre-IR levels and was decreased ~40% (by CD31) or ~33% (by AQP1).

Apoptosis in parotid glands after irradiation

Irradiation led to a marked increase in the frequency of apoptotic nuclei detected in miniature pig parotid glands (Fig. 3A–K). Since the acinar regions became atrophic and glandular fibrosis was increased after IR, the cellular density (presence of nuclei) differed between parotid gland sections at pre- and post-IR. To account for this difference, we determined the percentage of apoptotic nuclei for the total number of nuclei present in gland sections. By 4 h after IR, this percentage increased to 2.55% ($\pm$ 0.67%), about 5-fold greater than that of non-IR controls (0.48% [$\pm$ 0.44%]), while at 24 h post-IR, this value increased further to 3.30% ($\pm$ 1.24%). Two weeks after IR, the percentage of apoptotic nuclei in the IR group had declined but was still substantially higher (1.93% [$\pm$ 0.62%]) than that of the non-IR group (0.17% [$\pm$ 0.18%]). Additionally, a significant amount (2.43% [$\pm$ 0.69%]) at 24 h) of the cells in the post-IR parotid gland sections were both TUNEL- and CD31-positive, *i.e.*, 73.6%, suggesting that many endothelial cells became apoptotic after IR (Fig. 3).

ASMase and NSMase activity

From 4 to 24 h after IR, SMase activity in parotid glands increased rapidly and then declined gradually after 1 to 2 weeks (Fig. 3L and M). By 4 h post-IR, ASMase activity increased to 220.70 ($\pm$ 29.05), a level >4-fold that of non-IR controls (51.97 [$\pm$ 13.99]), and at 2 weeks after IR, the level of ASMase activity (128.90 [$\pm$ 4.99]) was still higher (261%) than non-IR values (49.33 [$\pm$ 10.11]). Similarly, NSMase activity in parotid glands was also increased after IR, albeit not to the extent seen with ASMase. At 4 h post-IR, NSMase activity had increased to 64.62 ($\pm$ 8.60), nearly 2-fold higher than that of control levels (35.53 [$\pm$ 9.14]). Thereafter, as with ASMase activity, NSMase activity declined and returned to normal levels by 2 weeks after IR.

DISCUSSION

The primary purpose of this study was to evaluate the hypothesis that microvascular endothelial cells are targets of IR damage in parotid glands of a large animal model, the miniature pig. This is an extremely useful animal model for studies of IR-induced salivary hypofunction and, importantly, is ~1,000-fold larger than that in mice (11, 12). Previous studies in a mouse model of IR damage to SGs showed that within 4 h after exposure to 15 Gy, an ~45% loss in MVD was observed in mouse submandibular glands, as measured with two separate endothelial cell membrane markers, CD31 and AQP1 (6). In the present study, we generally used the same experimental strategy with miniature pig parotid glands, and severe loss in MVD was observed at 24 h after irradiation, ~25% reduction from nonirradiated controls. Furthermore, local blood flow rates in parotid glands showed an ~40% decline at 4 h after IR, with significant reductions still apparent from 24 h post-IR until the end of the study (2 weeks). These data provide strong evidence for both a structural and functional alteration of parotid microcirculation occurring shortly (4–24 h) after single-dose IR.

Previous studies have suggested that SMase activity, leading to endothelial cell apoptosis, can contribute to IR-induced damage in the intestine, brain, and lungs of wild-type mice (5, 7, 8, 12). SMase hydrolysis of sphingomyelin leads to ceramide generation, which then acts as a second messenger in a ubiquitous, evolutionarily conserved signaling systems (13–15). There are several isoforms of SMase, distinguished by the pH optima for their activity, *i.e.*, acid (ASMase), neutral (NSMase), or alkaline SMase. The pH optima appear to regulate the on–off rate of substrate rather than the catalytic activity of the enzyme (16). Both ASMase and NSMase can be rapidly activated by diverse stress stimuli, such as UV light and IR, leading to increased ceramide levels within minutes to h (10, 16). Ceramide is a signal for stress-induced apoptosis, and there is evidence from many cell systems demonstrating that an increase in ceramide levels precedes other biochemical and morphologic manifestations of apoptosis (17–19).

Interestingly, several studies demonstrated that ASMase-deficient T and B lymphocytes, rodent embryonic fibroblasts, oocytes, and endothelial cells failed to release ceramide on IR and, indeed, were resistant to γ -IR-induced cell death (20). Additionally, ASMase-deficient mice are resistant to γ -IR and do not generate ceramide in endothelial cells in lung, small intestine, or brain, as observed in normal mice (7). Also, ASMase appears to regulate microvascular function (21). While the notion of IR-induced endothelial cell apoptosis is still quite controversial (*e.g.*, see ref. 22), previous studies showed endothelial apoptosis developed as an early, dose-dependent event after single-dose IR exposure, peaking at 4 to 10 h after IR. Moreover, histopathological observations showed that damage to the endothelium was confined to the microvasculature, while larger vessels appeared resistant to this effect. The maximal apoptotic effect was observed in the intestinal mucosa at 15 Gy, in the lungs at 25 Gy, and in the central nervous system at 40 Gy (5, 7, 8).

Since SMase appears to play a critical role in mediating IR-induced endothelial apoptosis both *in vitro* and *in vivo*, we studied the activity levels of ASMase and NSMase in parotid glands before and after IR. Both SMases were markedly increased immediately after IR, with ASMase activity increasing by >4-fold at 4 h and >6-fold at 24 h. Additionally, we found a marked and temporally associated increase in the percentage of apoptotic cells in parotid glands, which also were CD31-positive. These results are consistent with previous studies in mice.

Compared with small animal models, such as mice and rats, large animal models are especially valuable for studying many features of human pathologies, as well as for developing preclinical therapies. The oral and maxillofacial regions of miniature pigs, including the salivary glands, are similar to that of humans in anatomy, development, physiology, pathophysiology, and disease occurrence (11, 12, 23, 24). Previously, we have shown the value of miniature pigs as an IR-induced salivary hypofunction animal model, as well as a preclinical model for gene therapy of this condition (11, 25). In the present study, we used a single IR dose of 25 Gy, which is roughly comparable to a total clinical IR dose of 65–70 Gy (given as 2-Gy fractions three times/week). The results herein support both the need for further study of the possible role of damaged SG microvasculature in IR-induced salivary hypofunction and the use of this large animal model for such studies, particularly when using fractionated IR doses more analogous to the clinical situation. Importantly, these studies also suggest the possibility that pharmacological inhibitors of SMase activity may be useful in preserving blood flow in irradiated SGs and, thus, in limiting the IR-induced salivary hypofunction that occurs in patients with head and neck cancers, a hypothesis that is directly testable.

CONCLUSIONS

In summary, we have shown that single-dose IR rapidly leads to changes in microvascular endothelial cells and blood flow in miniature pig parotid glands. Furthermore, we showed that these changes are associated with increased parotid SMase activity and apoptosis of parotid gland endothelial cells.

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Fig. 1.

Local blood flow rate in irradiated miniature pig parotid glands (A) and histological appearance of irradiated miniature pig parotid glands following irradiation (B–E). Miniature pigs received a single dose of 25 Gy to their parotid glands, and glandular blood flow was measured, as described in Materials and Methods. The results shown are the averages ($\pm$ SDs) for three control animals and six (up to 24 h) or three (remaining time points) irradiated animals. The differences observed in blood flow in irradiated versus control animals was significant ($p < 0.01$; $F_{8/117} = 9.93$) (A). Panels B–E show parotid gland sections stained with hematoxylin and eosin, viewed at $\times 200$ magnification. (B) 4 h; (C) 24 h; (D) 2 weeks post-IR. (E) Nonirradiated. At 4 h there is some eosinophilic fiber-like

structure in the basement membrane region around acinar cells (B, arrowheads). At 24 h (C), some increase in connective tissue between glandular lobes is seen (arrows). At 2 weeks (D) there is a marked increase in the interlobular connective tissue, and some acinar cells are becoming atrophic (*)).

Fig. 2.

Assessment of microvascular density in irradiated miniature pig parotid glands.

Determination of microvascular density (MVD) was based on immunodetection of CD31+ cells (A and C) and AQP1+ cells (B and D) and was performed and quantified as described in Materials and Methods. Data shown in panels C and D are averages ($\pm$ SD) of the total number of microvessels (y axis) measured for three control animals and six (up to 24 h) or three (remaining time points) irradiated animals. Overall differences in the MVD of irradiated and nonirradiated parotid glands were significant ($p < 0.01$; $F = 44.67$ for CD31 and 58.75 for AQP1). At 24 h post-irradiation both CD31 and AQP1 labeled MVD showed a marked reduction ($p < 0.01$).

Fig. 3.

Apoptotic cell assays (A-K) and sphingomyelinase (SMase) activity measurements (L and M) in irradiated miniature pig parotid glands. Sections of non-IR parotid glands (G-I) and 4 h (A-C) or 24 h (D-F) after IR are shown. Nuclei of apoptotic cells were labeled by FITC (green, A, D, G), while all other nuclei were labeled by propidium iodide (red, B, E, H). Images composed of overlaid photos (C, F, I) were also used to eliminate false-positive nuclei. The percentage of apoptotic nuclei (yellow, thin arrows, C, F, I) compared to the total number of nuclei observed in each section (J) was determined. Also, the percentage of CD31-positive (blue, thick arrow, B-I) cells with apoptotic nuclei was assayed, and the results showed significant differences between the non-IR and IR groups, both in total

apoptotic nuclei ($p < 0.01$, $F = 14.27$) and in apoptotic CD31-positive cells ($p < 0.01$; $F = 14.26$). As shown in panel K, a significant level (2.43% [$\pm 0.69\%$] at 24 h) of the cells with apoptotic nuclei in the post-IR parotid gland sections were CD31-positive (73.6%). Activity measurements of acid SMase (ASMase, L) and neutral Mg^{2+} -dependent SMase (NSMase, M) activity in irradiated miniature pig parotid glands was also performed. SMase activities were determined as described in Materials and Methods and are expressed as nanomoles of ceramide formed/hour/mg protein. The data shown are the average ($\pm$ SD) ASMase (L) and NSMase (M) activity levels measured for three control animals and six (up to 24 h) or three (remaining time points) irradiated animals. Overall differences in ASMase and NSMase activities between irradiated and nonirradiated parotid glands were significant ($p < 0.01$; $F = 85.00$ for ASMase and 9.26 for NSMase).