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Peroxynitrite formation in radiation-induced salivary gland dysfunction in mice

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ABSTRACT

Xerostomia frequently arises in patients with head and neck malignancies that are treated by radiation. However, the mechanisms responsible for the destruction of the salivary gland remain unknown. We previously established a xerostomia model of mice and identified the pathway through which nitric oxide (NO) affects the pathogenesis of radiation-induced salivary gland dysfunction. Although the toxicity of NO alone is modest, NO with superoxide anion ($O_2^{\cdot-}$) rapidly forms peroxynitrite ($ONOO^-$), a more powerful toxic oxidant. In this study, we used the experimental model to examine: 1) when NO and $O_2^{\cdot-}$ production is maximum in the salivary gland after irradiation; 2) whether peroxynitrite, as assessed by nitrotyrosine production, is responsible for salivary gland dysfunction; and 3) the effect of the iNOS selective inhibitor, aminoguanidine (AG), on nitrotyrosine formation. The increases in production of NO and $O_2^{\cdot-}$ in the salivary gland peaked on day 7 after irradiation. Nitrotyrosine detected immunohistochemically was significantly reduced by AG in the salivary gland. On the basis of these results, we concluded that NO together with $O_2^{\cdot-}$ forms the more reactive $ONOO^-$, which might be an important pathogenic factor in radiation-induced salivary gland dysfunction.

Radiotherapy is an established therapy for head and neck malignancies, but the treatment field might include normal several tissues and organs. As a result, xerostomia due to salivary gland dysfunction is a clinically important side effect that is often irreversible (23, 25). The actions of radiation include a direct effect of the radiation itself and the indirect DNA injury caused by the $\cdot OH$ radical, which induces cell death and salivary gland atrophy (3, 17). However, the actual pathogenesis of radiation-induced xerostomia is obscure, and an effective treatment has not been established.

We previously established a xerostomia model us-

ing mice and identified the inflammatory pathway through which NO affects the pathogenesis of radiation-induced salivary gland dysfunction (27). NO is a radical molecule and has been identified as a vascular endothelium-derived relaxing factor (7, 9, 20). NO is generated by three types of nitric oxide synthase (NOS): neural NOS, endothelial NOS and inducible NOS (20). The expression of inducible NOS (iNOS) is induced by inflammatory cytokines (1, 19) and that large amounts of NO are continuously released from activated macrophages through the NADPH-dependent oxidant deamination of L-arginine (9).

The toxicity of NO is due both to NO itself and to NO-derived reactive oxidants (4). The modest toxicity of NO is increased by rapidly binding with NO together with $O_2^{\cdot-}$ to form $ONOO^-$, which is a more powerful, toxic oxidant (25). However, most of these reactive oxidants can not be directly detected *in vivo*. Thus, nitrotyrosine is used as a biomark-

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er of ONOO⁻ (4), and tyrosine nitration might alter protein structure and cellular function (4, 31). In fact, nitrotyrosine has been detected in various human pathological conditions, as well as in animal and cellular models of disease (5, 14, 30, 31).

This present study examines the following in the irradiated salivary gland: 1) when NO and O₂⁻ production is maximal; 2) whether ONOO⁻, assessed as nitrotyrosine, is responsible for the dysfunction; and 3) the effect of an iNOS selective inhibitor (aminoguanidine, AG) on nitrotyrosine formation.

MATERIALS AND METHODS

Animals. ICR female (purchased from CLEA JAPAN, INC.), 5–6-week-old mice (35–38 g body weight) were used in all experiments. After a week of adaptation, the mice were used in this study. All experimental protocols complied with the guidelines for animal experiments at the Tokyo Dental College.

Reagents. All reagents used in these experiments were of the highest purity grade commercially available and dissolved in water purified by ultrafiltration using Milli-Q[®]. Chelex[™]-100 resin (sodium form) was purchased from Bio-Rad Co. (Hercules, CA, USA). We purchased EDTA-2Na from Dou-jin Chemical (Kumamoto, Japan), and AG from Sigma Chemical Company (St. Louis, MO, USA).

Irradiation. The mice were anesthetized with 60 mg/kg of pentobarbital sodium injected intraperitoneally. Both submandibular glands on both sides were irradiated with a dose of 15 Gy in a single acute exposure using a MEVATORON 74DX 40[®] X-ray unit (27).

Experimental group. The mice were divided into the following groups: 1) control (C), 2) irradiated (R), and 3) irradiation plus aminoguanidine (R+AG) groups. The control group was not irradiated. In the R + AG group, mice were treated with AG 2 h before irradiation, and then received a daily subcutaneous injection of AG at a dose of 50 mg/kg, as well as 2 g/L in the drinking water. The animals in the AG group received AG on the same schedule as the R + AG group.

Detection of NO₂⁻/NO₃⁻ in the submandibular glands. NO₂⁻/NO₃⁻, the stable end products of NO, were assayed using the Griess reaction (16). On days 0, 1, 2, 3, 5, 7, and 14 after irradiation, NO₂⁻/NO₃⁻ levels were assayed for each group. After the tissues were

perfused with phosphate buffered saline (PBS, pH 7.4), they were isolated and homogenized on ice in PBS containing 5 mM EDTA. Subsequently, they were centrifuged (15,000 rpm, 4°C, 30 min), filtered through 0.45 and 0.22 μm syringe filters, and ultrafiltered in a 10,000 M.W cut filter (15,000 rpm, 4°C, 40 min). The concentration was detected using a Nitrite/Nitrate colorimetric assay kit (Bio Dynamics Laboratory Inc., Tokyo, Japan).

Measurement of O₂⁻ generation in the submandibular glands. On days 0, 1, 2, 3, 5, 7, and 14, O₂⁻ levels were assayed. After perfusion with PBS (pH 7.4) the isolated tissues were briefly minced and incubated in Hank's balanced salt solution with 0.1% trypsin at 37°C for 20 min, then filtered through a 100-μm pore diameter filter into RPMI 1640 medium containing 10% fetal calf serum. O₂⁻ production in the isolated cells from the submandibular gland was examined with the chemiluminescence reaction. As a probe, 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo(1,2-*α*)pyrazin-3-one (MCLA) (Tokyo Kasei, Japan) (16) stimulated by 50 ng of phorbol 12-myristate 13-acetate (18) was used. To terminate O₂⁻ production, 50 U of superoxide dismutase were added. The amount of O₂⁻ production was expressed as the number of counts per second per cell × total cell count (30).

Immunohistochemistry. Nitrotyrosine, a biomarker of ONOO⁻, was stained on day 7 after irradiation because the nitrotyrosine concentration was maximal at this time. The submandibular glands were isolated, fixed with periodate-lysine-paraformaldehyde solution and then frozen. Cryostat sections were stained using the avidin-biotin peroxidase complex method. Endogenous peroxidase in the sections was blocked with 0.03% hydrogen peroxide in methanol for 30 min, followed by three PBS washes (for 5 min each). Nonspecific antibody binding was blocked with 3% normal horse serum. The sections were incubated for 1 h with a mouse monoclonal antibody against nitrotyrosine (Sigma Chemical Company) diluted 1 : 100 in PBS containing 0.1% sodium azide. After washing in PBS the sections were incubated for 60 min with biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, California) diluted 1 : 100 in PBS : BSA, and followed by the avidin-peroxidase complex (Vectastain, Vector Laboratories). Peroxidase activity was visualized using glucose oxidase diaminobenzidine with nickel enhancement. Stained sections were dehydrated, cleared, and mounted in Pertex Mounting Medi-

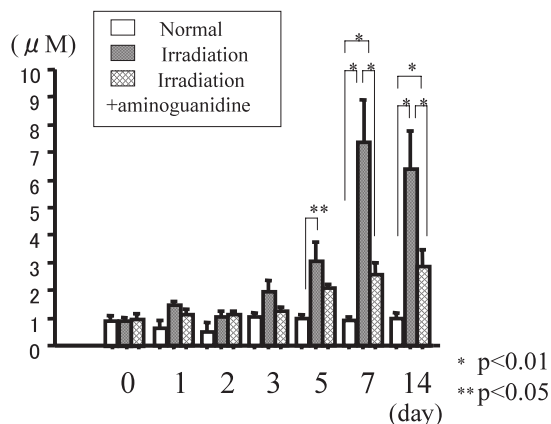


Fig. 1 Measurement of concentration of $\text{NO}_2^-/\text{NO}_3^-$ in the submandibular gland as measured using the Griess assay method. Values are means \pm SD; $n = 5$ mice/group.

um (CellPath, Hemel Hempstead, United Kingdom). Normal sections were stained in the same manner without irradiation. Additional controls were incubated without the primary antibody for nitrotyrosine.

RESULTS

Detection of $\text{NO}_2^-/\text{NO}_3^-$ in the submandibular glands

The production of $\text{NO}_2^-/\text{NO}_3^-$ obviously increased in the R group (Fig. 1). The concentration began to increase on the day 5 and reached a maximum on day 7. In contrast, the concentration did not change in group C. The generation of $\text{NO}_2^-/\text{NO}_3^-$ was significantly inhibited by AG in the R+AG group. Figure 1 shows the concentration increase in the R group on days 5 (3.03 ± 1.64), 7 (7.37 ± 3.68), and 14 (6.41 ± 3.12), compared with the control group (day 5, 0.97 ± 0.35 ; day 7, 0.91 ± 0.35 ; day 14, 0.94 ± 0.59). Compared with the R group, the concentration in the R + AG group was inhibited on days 7 (2.60 ± 0.82) and 14 (2.86 ± 1.41).

Measurement of O_2^- production in the submandibular glands

The production of O_2^- obviously increased in the R group (Fig. 2). The O_2^- concentration began to increase on day 5 (13.82 ± 2.00) and reached a maximum on day 7 (17.41 ± 3.37), which continued to day 14 (15.90 ± 3.95). In contrast, the O_2^- concentration did not change in the C group (day 5, 8.17 ± 1.814 ; day 7, 6.52 ± 1.33 ; day 14, 6.44 ± 2.77).

Immunohistochemistry. Nitrotyrosine staining was negative in the C group (Fig. 3A, B and C), but obviously positive throughout the submandibular gland

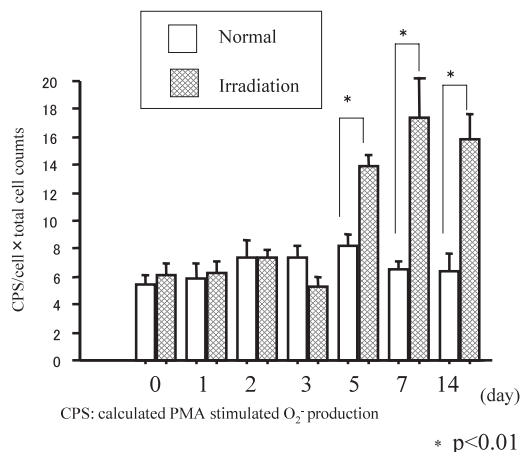


Fig. 2 Phorbol 12-myristate 13-acetate (PMA)-stimulated O_2^- production by inflammatory cells in the salivary gland. O_2^- production was measured using the chemiluminescence method with an O_2^- -specific chemiluminescent probe 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo(1,2- α)pyrazin-3-one. Each value was calculated as PMA-stimulated O_2^- production/cell \times total cell number. CPS, counts/s. Values are means \pm SD; $n = 5$ mice/group.

in the R group (Fig. 3D, E and F). Intensely positive nitrotyrosine regions tended to coincide with salivary gland atrophic sites, located in the acinar and duct areas. In the R + AG group, the staining was not as marked as in the R group, and was restricted to some atrophic sites in the salivary gland (Fig. 3G, H and I).

DISCUSSION

The pathway of radiation damage has been elucidated. Radiation not only induces double-strand breaks in DNA (3, 29) but also produces $\bullet\text{OH}$ radicals from H_2O . In another mechanism, inflammatory cytokines induce iNOS and amount of NO are released. Radiation also induces cell cycle arrest in the salivary gland (6). The present study found an increased concentration of $\text{NO}_2^-/\text{NO}_3^-$ in the submandibular glands on days 5, 7, and 14 after irradiation. The iNOS inhibitor, aminoguanidine, attenuated, but did not completely block formation of nitrotyrosine, which suggest the possibility that NO is derived from other NO sources, such as endothelium nitric oxide synthase (eNOS). Takeda *et al.* (27) showed that NO production in the same xerostomia model could be inhibited with N^G -monomethyl-L-arginine acetate (L-NMMA), a nonspecific NOS inhibitor. In this study, we showed that the iNOS-specific inhibitor, aminoguanidine, attenuated NO production. These results suggest that iNOS is the major source

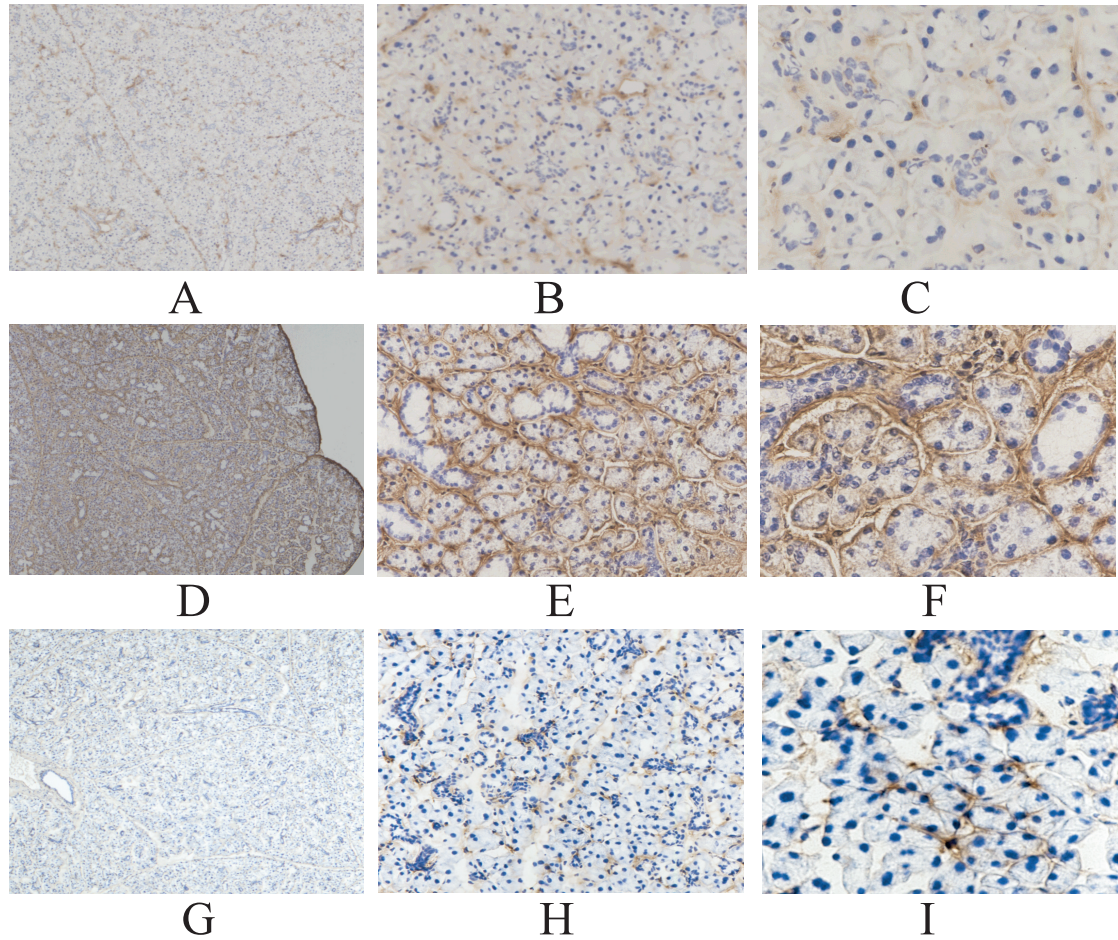


Fig. 3 Immunostaining with antinitrotyrosine antibody in the submandibular gland in C group (A, B, and C), R group (D, E and F), and R + group (G, H and I). Nitrotyrosine formation is shown as a brown color. A, D, and G original magnification $\times 50$; B, E, and H original magnification $\times 200$; C, F, and I original magnification $\times 400$.

of NO production in the salivary gland after irradiation, and is responsible for the irradiation-induced injury. Although the role of eNOS in radiation-induced salivary gland injury remains unknown, it might be related to epithelial cell regeneration, since a marked increase in eNOS occurs in the denuded aorta and during the repair stage of inflammation (21, 24).

The nitrotyrosine-positive cells in the R group were identified as being duct cells and acinar cells, which express iNOS protein (13), suggesting that these cells may be a major source of NO production. $O_2^{\cdot-}$ is another mediator of injury and has been shown to play an important role in ischemia-reperfusion injury, as well as in hypoxic and hyperoxic injury (12, 28). In the R group, $O_2^{\cdot-}$ generation was greater than in the C group. NO reacts rapidly with $O_2^{\cdot-}$ ($k = 6.7 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$), and forms $ONOO^-$, which is a more reactive oxidant (8, 22) at a rate

that is over 3-fold faster than the scavenging of superoxide dismutase (SOD) $O_2^{\cdot-}$ ($k = 2.0 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$) (8). Furthermore, $ONOO^-$ nitrates inactivates manganese SOD, furthering the progression of injury (9, 14). The nitration of structural proteins can produce major pathological consequences. Studies using antibodies to nitrotyrosine have demonstrated nitration in human atherosclerosis, myocardial ischemia, septic and distressed lungs, inflammatory bowel disease, and amyotrophic lateral sclerosis (4), indicating that proteins nitration is associated with pathogenesis.

In conclusion, $ONOO^-$ might play an important role in radiation-induced salivary gland dysfunction. The control of $ONOO^-$ production might offer a therapeutic strategy for dealing with the clinically severe side effect of head and neck radiation therapy.

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