acclimatization. All experiments were performed in accordance with the animal experiment guidelines of Tokyo Dental College.

Irradiation

Each mouse was anesthetized by intraperitoneal injection of 60 mg/kg sodium pentobarbital. The animals in the irradiated groups were placed in defined positions (Fig. 1). A single acute exposure of 10 MV X rays (MEVATRON 74 DX40; Toshiba Medical Systems, Tokyo) at a dose rate of 3 Gy/min at a distance of 1000 mm. The position of each salivary gland was confirmed by computed tomographic (CT) scanning under the same conditions as used for the irradiation. The effective radiation dose to the salivary gland was set using the percentage depth dose and was over 95% of the maximum dose delivered.

Collection of Saliva

Saliva flow in each group ($n = 5$) was measured 1, 2, 3 and 4 weeks after irradiation. All mice were weighed and then anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg). Ten minutes after the pentobarbital injection, pilocarpine (0.1 mg/kg) was injected into the tail vein of the mouse to stimulate salivation. Total saliva was collected from the floor of the mouth with a capillary micropipette 5 min after the pilocarpine injection (22). The mice were fasted during the 6 h prior to the experiment but were given access to water.

Analysis of NO in the Salivary Glands In Vivo

Seven-week-old female ICR mice were studied 1 week after irradiation. The unirradiated control group consisted of animals bred and raised under conditions that were the same as in the irradiated groups. Two groups were irradiated, one with 10 Gy and the other with 15 Gy. All groups ($n = 5$) were anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg), and after they were killed the submandibular gland was excised using aseptic surgical techniques and cut into small fragments with a scalpel. These fragments were cultured in 48-well plates in 0.5 ml DMEM (Gibco BRL, Gaithersburg, MD) containing 10% FBS (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (23), and 0.5 mM L-NMMA (Research Biochemicals International, Natick, MA) was added to some of the cultures to inhibit NOS activity. All of the cultures were incubated at 37°C in 95% air/5% CO_2 for 24 h. After incubation for the times indicated, supernatants were collected, centrifuged for 10 min at 5000 rpm, and frozen at −20°C. NO was measured using a chemiluminescence assay (24) with a Model 270 B NO analyzer (Taiyo Toyo Sanso, Osaka).

In Vitro Salivary Gland Epithelial Cell Culture

Cells of the human salivary gland cell line HSG-AZA3 (kindly provided by Prof. M. Sato of Tokushima University School of Dentistry) were cultured in 0.5 ml DMEM (Gibco) containing 10% FCS (Gibco), 1000 U/ml penicillin, and 2 mM L-glutamine (25–28). Cells were detached with trypsin/EDTA in calcium and magnesium-free phosphate-buffered saline, and approximately 3×10^4 cells/ml were cultured in 24-well plates. The culture medium was supplemented with 10 ng/ml Il1b, 2.86×10^3 U/ml Tnfa, and 0.1 ng/ml Ifng, and incubation was performed for 24 h in 95% air/5% CO_2 at 37°C. After incubation for the times indicated, supernatants were collected, centrifuged for 10 min at 5000 rpm, and frozen at −20°C. NO was measured with a Model 270 B chemiluminescence system (Taiyo Toyo Sanso).

Effect of the NOS Inhibitor

Seven-week-old female ICR mice were used 1 week after 15 Gy irradiation. Each mouse ($n = 5$) was anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg), and 10 min later the same mice were injected intravenously with different doses of L-NMMA (3, 30, 300 mg/kg) (29) and then immediately injected intraperitoneally with pilocarpine (3 mg/kg). The mice were divided into four experimental groups: an L-

NMMA 300-mg/kg group, an L-NMMA 30-mg/kg group, an L-NMMA 3-mg/kg group, and a pilocarpine 3-mg/kg alone group. Saliva was collected from the floor of the mouth with a capillary micropipette after 5, 10, 15, 20 and 30 min.

Statistics

The experiments were repeated two or three times. Values given are the means of measurements. Student's t test was used to test the significant differences between groups.

RESULTS

Establishment of the Murine Xerostomia Model

There were no significant differences in water or food consumption between the mice exposed to 10 Gy or 15 Gy radiation and the control mice over the 4-week period after irradiation, nor were there any marked differences in body weight among the three groups.

Effect of Radiation on Salivation

The volume of saliva secreted 1 week after irradiation in the 15-Gy group was 0. 27 ± 0.08 μl/g and was significantly lower than in the control group (Fig. 2). In contrast, there was no significant difference between the control group and the 10-Gy group at 1 week. Two weeks after irradiation, the volumes of saliva secreted in both the 10-Gy and the 15-Gy groups were significantly lower than that in the control group ($P < 0.01$). The saliva volume in the 10-Gy group tended to recover somewhat at week 3 ($P < 0.05$), and there was no longer any significant difference from the control group at week 4. The saliva volume in the 15-Gy group remained low at weeks 3 and 4 ($P < 0.01$), and at week 4 it was significantly lower than in the control group.

Quantification of NO

The NO level was determined in irradiated (10 Gy, 15 Gy) and unirradiated salivary glands. The results are shown in Fig. 3. The NO level was 0.02 ± 0.005 pmol/mg in the 10-Gy group and 0.013 ± 0.007 pmol/mg in the control group; these values were not significantly different. The corresponding value (0.036 ± 0.004 pmol/mg) in the 15-Gy group was significantly higher than those in either the 10-Gy or the control group ($P < 0.05$). There was not a marked decrease in the NO level in the 10-Gy or control groups when L-NMMA was added, but the NO level decreased significantly to 0.023 ± 0.002 pmol/mg in the 15-Gy group ($P < 0.05$). There were no significant differences among the three groups in NO levels in the presence of L-NMMA.

We also investigated whether production of NO by the salivary glands can be regulated by inflammatory cytokines present in irradiated tissues. The value for cells with added inflammatory cytokines (1.055 ± 0.009 pmol/μl) was significantly higher than the control value (0.934 ± 0.013 pmol/μl, $P < 0.05$).

Mouse
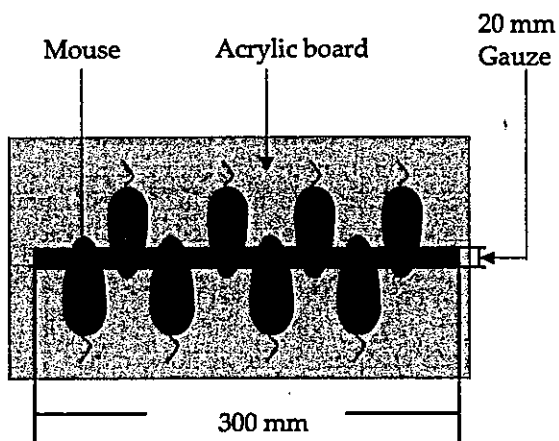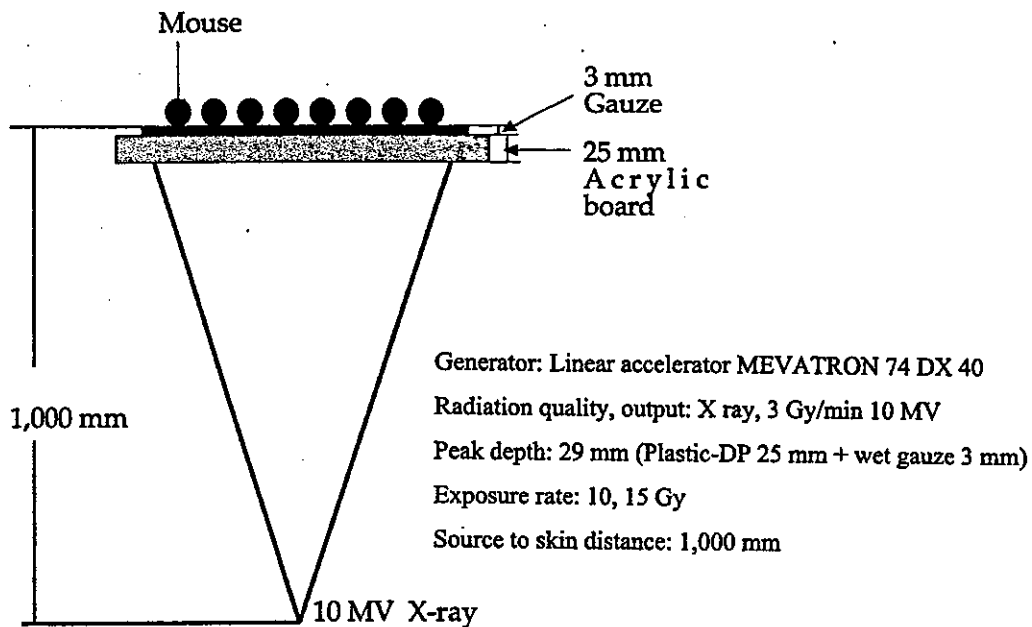
3 mm
Gauze

25 mm
Acrylic
board

1,000 mm

Generator: Linear accelerator MEVATRON 74 DX 40

Radiation quality, output: X ray, 3 Gy/min 10 MV

Peak depth: 29 mm (Plastic-DP 25 mm + wet gauze 3 mm)

Exposure rate: 10, 15 Gy

Source to skin distance: 1,000 mm

10 MV X-ray

Mouse   Acrylic board

20 mm
Gauze

300 mm

ISODOSES

ISODOSES

FIG. 1. Method of irradiation. Mice were placed in the decubitus position so that there was no clearance between the skin and the wet gauze. The field extended from the foreleg to 20 mm below the lower lip and was 300 mm wide. CT scanning of the mice confirmed that the position of each salivary gland was within the 95% isodose curve (yellow).
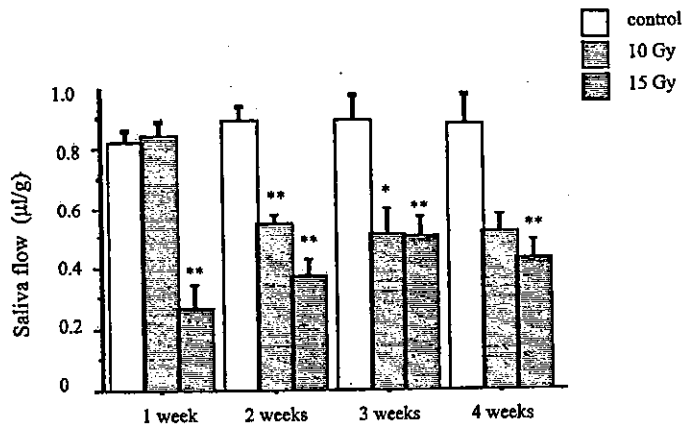
FIG. 2. Saliva flow is expressed as the total output of saliva during the first 5 min after pilocarpine stimulation normalized to total body weight on the day of saliva collection. Five mice in each group were analyzed. Error bars indicate the standard errors of the mean. The asterisks indicate significant decreases in comparison to the control for each week. $*P < 0.05$, $**P < 0.01$, compared to the respective control.
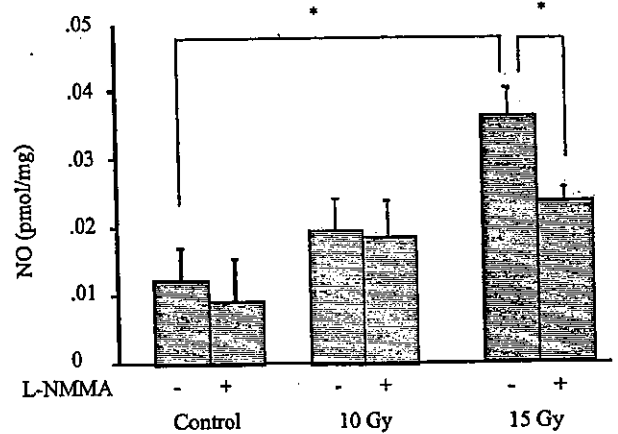
FIG. 3. The amounts of NO in the culture medium of the irradiated (10, 15 Gy) and control salivary glands were compared, and the amount of NO in each group in which L-NMMA was added to the culture medium was also determined. Five mice in each group were examined. Error bars indicate the standard errors of the mean. Asterisks indicate significant decreases in the 15-Gy group ($P < 0.05$).

Effects of NOS Inhibitor on Secretory Function of Salivary Glands

The saliva volume in all groups peaked 15 min after stimulation with pilocarpine, and there were no significant differences in saliva volume among the three groups at 30 min or later (Fig. 4). More specifically, saliva volumes were 0.41 ± 0.06 μl/g, 0.38 ± 0.11 μl/g, and 0.2 ± 0.1 μl/g 10 min after administration of an NOS inhibitor at doses of 3, 30 and 300 mg, respectively. These values were significantly higher than the control value, 0. 063 ± 0.1 μl/g ($P < 0.01$, $P < 0.05$). At 15 min, saliva volumes were still higher in the 30-mg ($P < 0.05$) and 300-mg ($P < 0.01$) groups (0.65 ± 0.06 μl/g and 1.09 ± 0.08 μl/g, respectively) than in the control group (0.37 ± 0.06 μl/g). The volume in the 3-mg group, however, was similar to that in the control group at 15 min and thereafter. At 20 min and later, the volume of saliva secreted remained higher in the 300-mg group (0.68 ± 0.09 μl/g, $P < 0.05$) than in the control group (0.34 ± 0.06 μl/g), but the value in the 30-mg group was similar to the control value. There were no clear differences in the volume of saliva secreted among the three groups (L-NMMA 3, 30, 300 mg/kg) at 30 min.

DISCUSSION

In clinical radiation therapy, the radiation field is made as small as possible to avoid injuries to normal tissues. This is one of the factors that make it difficult to use clinical data to understand in detail how radiation-induced xerostomia develops. Recently, although radiation-induced salivary gland dysfunction has been studied, the decision regarding the size of the radiation field has varied (*30–32*). Nagler *et al.* studied changes in body weight after irradiation and reported a significant decrease in the body weight of the animals after 15 Gy irradiation compared to unirradiated controls (*33*). However, there were no significant dif-

ferences in the body weights of irradiated and unirradiated mice in our study. In our study, the field of irradiation was determined precisely before irradiation by accurately identifying the anatomical position of the salivary glands by CT scan. This presumably minimized the systemic effects of irradiation, thereby preventing weight loss in the irradiated mice compared to the unirradiated animals. We therefore believe that our animal model of salivary gland dysfunction is useful for precise analyses of the direct effects of radiation on the salivary glands.

Circulatory disturbances that result from radiation-induced changes in blood flow, rather than direct injury of the tissue by radiation, have been noted to be important as a mechanism of radiation-induced salivary gland dysfunction (*34*). Body fluids have also been reported to become
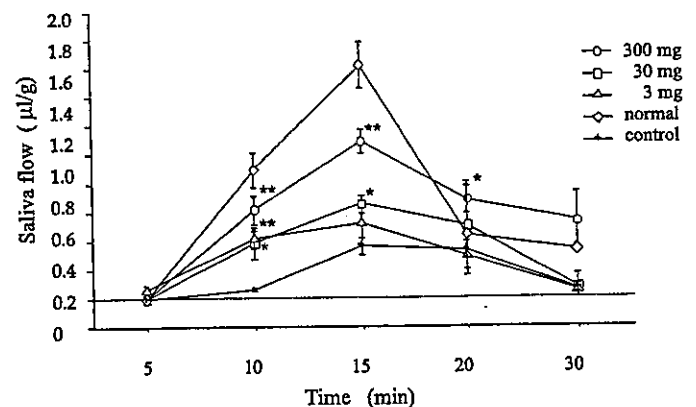

FIG. 4. Saliva flow is expressed as total saliva output after pilocarpine stimulation normalized to total body weight on the day of saliva collection. Mice were used 1 week after 15 Gy irradiation. Five mice in each group were analyzed. Error bars indicate the standard error of the mean. All groups also showed a peak increase in the rate of secretion after stimulation for 15 min. $*P < 0.05$, $**P < 0.01$, compared to the respective control.

harmful when irradiated, and excessive *in vivo* and *in vitro* expression of the inflammatory cytokines Ifng and Il1b at the site of γ irradiation is apparently involved in the pathological condition (6, 35). Although salivary gland dysfunction presumably occurs through these indirect mechanisms, the details of their effects on the salivary glands remain unclear. Our observations showed that radiation promoted production of NO and decreased saliva secretion, and these findings together with our inference that NO is produced by iNOS induced by inflammatory cytokines suggest that NO produced in response to radiation-induced expression of cytokines is involved in the production of abnormalities in the secretory mechanisms.

NO is a diffusible free radical that plays many roles as a signaling and an effector molecule in diverse biological systems, including transmission of neuronal messages and vasodilation (12). In the nervous system, NO appears to have both neurotoxic and neuroprotective effects, and it may have a role in the pathogenesis of stroke and other neurodegenerative diseases, as well as in demyelinating conditions (e.g. multiple sclerosis, experimental allergic encephalopathy, X-adrenoleukodystrophy) associated with infiltrating macrophages and the production of proinflammatory cytokines (36). NO and peroxynitrite (reaction product of NO and O_2^-) are potentially toxic to neurons and oligodendrocytes; these molecules may mediate toxicity through the formation of iron–NO complexes of iron-containing enzyme systems (37), nitration of proteins, and nitrosylation of nucleic acids and DNA strand breaks (38). Although monocytes/macrophages are the primary source of iNOS in inflammation, other cytokines induce a similar response in arthritides (24). NO generated by macrophages, microglia and astrocytes has been implicated in the damage to myelin-producing oligodendrocytes in demyelinating disorders like multiple sclerosis and in neuronal death during neuronal degenerative conditions, including brain trauma (39). Our findings demonstrate that radiation may accelerate production of NO by salivary gland epithelial cells, thereby inducing dysfunction of the glands. They also suggest that inhibition of radiation-induced production of NO ameliorates salivary dysfunction, indicating a possible effect of NO inhibitors in the treatment of salivary dysfunction. Our study suggests that NOS inhibitors represent a possible avenue of research in the development of therapeutic agents directed against nitric oxide-mediated salivary gland disorders, particularly in xerostomia.

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