Effects of Amifostine Administration Prior to Irradiation to the Submandibular Gland in Mice: Autoradiographic Study using $^3$H-leucine

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Summary: We histochemically investigated the radioprotection of organelle in acinus on the submandibular gland using amifostine. Mice were divided into those without amifostine administration or irradiation (group A), with amifostine administration and without irradiation (group B), without amifostine and with 5 Gy of irradiation (group C), and with amifostine and 5 Gy of irradiation (group D). In groups B and D were given 100 mg/kg of amifostine at 30 minutes prior to irradiation to the area equivalent to the submandibular gland. HE staining revealed that group D showed atrophy of the acinar cells, though the change was milder than that seen in group C. In AZAN-Mallory staining, fibrogenesis were found more often in group C as compared to the other groups. In Light Microscopic Autoradiography findings, the ratio of reduced silver particles in the acinar cells was lower in group C than in the other groups at 30 minutes after RI administration. Microstructure findings showed the findings were very similar to those of group A. In Electron microscopic Autoradiography, the ratio among the secretory granules was lower in group C at 120 minutes after RI administration. Our results indicate that amifostine can alleviate xerostomia caused by late effect from radiation.

Introduction

The incidence of head and neck cancer is increasing, with surgery and radiotherapy performed for cases in the early stages. For unresectable cancer, both radiotherapy and chemotherapy are generally applied as optional therapies. Of those, radiotherapy is an excellent option, as it has the advantage of retaining functional status of the patients. During implementation, in order to avoid radiation damage to normal tissue as much as possible, the shape of the exposure field and method of irradiation utilized are determined on the basis of information provided by a CT simulator, while the dose distribution is obtained by computed treatment planning. However, normal tissue such as the salivary gland can not be excluded from the exposure field in many cases, depending on the irradiation procedure$^1$.

Long-term result of radiotherapy induces fibrosis of the acinar cells and a reduction in the salivary secretion, resulting in xerostomia$^2$, dysphagia$^3$, dental caries occurrence$^4$, agenesia$^5$, oral candidosis, malnutrition, and late effect such as osteoradionecrosis$^6$, which adversely impacts patients' quality of life. In an attempt to prevent those complications, it is necessary to protect the healthy salivary glands. One potential preventive measure undergoing clinical trials$^7$ and animal experiments$^8$ mainly in the United States is administration of a radioprotective agent prior to radiation to prevent acute and late effect of radiation therapy.

In vivo studies of cell protection from radical oxygen generated by ionizing radiation have been conducted over the years, and Patt et al. $^9$ and Bacq et al. $^{10}$ reported on the cell protective actions of cysteine and cysteamine against radiation more than 50 years ago. However, cysteine and cysteamine are not applied in a clinical setting, as they are very toxic. Thereafter, the US Army Walter Reed Institute (Washington, DC) synthesized a number of compounds with radioprotective activities, among which amifostine (WR-2721, Ethyol) was shown to be low in toxicity and have a high cell protective action toward the...
salivary glands at a relatively low concentration. In addition, amifostine was found to be organ specific, and considered to protect normal tissue from acute and late effect by radiation in patients with head and neck cancer. The Food and Drug Administration (FDA) of the United States authorized it as a radioprotective agent in 1999.

A previous report found that amifostine is toxic to human cultured cells at a high concentration, even though it has radioprotective activities, since it is a derivative of cysteamine and an aminothiol. However, most studies of the radioprotective activities of amifostine using rodents examined pathological changes and the metabolism of salivary amylase, and sodium and potassium metabolism, while there is no known report that used autoradiography, which enables simultaneous observation of cell morphology and amino-acid metabolism. In the present study, we studied acinar cells from the submandibular glands of mice and present histopathological findings, as well as amino acid uptake and excretion findings, and the reduced in silver particles in the organelle, which are involved in amino acid secretion.

Materials and Methods

Experimental animals

The male BALB/c mice of eight weeks of age, each weighing about 20 g (Tokyo Jikken-dobutsu-sha, Japan) were used. They were reared in cages at 25°C and 50% humidity, and given Lab. Animal Diet (MF, Oriental Kobo, Japan) and tap water ad libitum. Prior to the experiments, 20 mice were divided into four groups (A–D, Table 1) and kept in preliminary rearing cages for one week. The experiments were performed in line with the guidelines for experimental animals of the Rules for the Care and Use of Laboratory Animals, Nippon Dental University.

Amifostine administration

Amifostine (WR-2721, Ethyl, MedImmune Oncology Inc., USA) was diluted with 20 mM of physiological saline and 0.4 mL of the solution was injected intraperitoneally at a rate of 100 mg/kg to the mice in groups B and D, which was performed 30 minutes prior to irradiation for group D. The same amount of physiological saline only was injected intraperitoneally into the mice in groups A and C.

Table1 Classification of an experimental animal

<table>
<thead>
<tr>
<th>irradiation</th>
<th>amifostine administration</th>
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<td>0 Gy</td>
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<td>5 Gy</td>
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X-ray irradiation

For X-ray irradiation, a Hitachi X-ray generator (MBR-1520R-3: Hitachi, Tokyo, Japan) was used. The device was set at 2.0 Gy/min for the mean amount of irradiation to the submandibular gland, with the X-ray tube voltage set at 150 kV, the X-ray tube current at 20 mA. Following intraperitoneal anesthesia with pentobarbital sodium (Nembutal®, Dainippon Pharmaceutical Co, Japan), the all mice were fixed in the dorsal position on an acrylic plate. The exposure field was determined precisely to irradiate the submandibular portion. A 5 Gy of X-ray irradiation was administered. Mice in groups A and B were only given anesthesia with Nembutal®.

Radioisotope administration

The mice in each group were given only tap water from 12 hours prior to radioisotope (RI) administration. Then, 370 kBq/g of $^3$H-lucine ([4,5-$^3$H]2.22-4.44TBq/mol, Moravek Biochemicals Inc., USA) was administered intraperitoneally. The submandibular glands were extracted under general anesthesia at 30 and 120 minutes after administration.

Preparation of histological specimens

All mice were euthanized under deep anesthesia 30 day after irradiation. The submandibular gland was immediately removed, and fixed in neutral buffered formalin for 24 hours and embedded in paraffin using an ordinary method. Sections of Four-μm-thick were obtained, then HE and AZAN-Mallory stains for observation under a light microscope (DMRE, Leica, Germany).

Preparation for light microscopic autoradiography (LMARG)

The center of the secretory portion was shredded into a portion about 1 mm$^3$ in size, then it was fixed with 2.5% glutaraldehyde for 2 hours, followed by 1.0% osmium acid for 1 hour, following the routine procedure, and dehydrated with an ascending ethanol series and embedded in Epon-Araldite mixed resin. Sections about 1.0 μm thick were sliced, then emulsifiable concentrate for LMARG (NTB2, Eastman Kodak, USA) was applied to make a single layer of silver particles. After 10 days of exposure, development and fixation were performed, after which the sections were statistically processed with the differential interference mode using a confocal laser scanning microscope (TCS SP, Leica, Germany). Fifteen different points in each bilateral submandibular gland extracted from the mice were photographed, and used for measuring the area and counting the grains to calculate the reduced silver particles per 1 mm$^2$ of acinar cells.

Preparation for electron microscopic autoradiography (EMARG)

Ultra-thin sections about 90 μm thick were sliced from identical embedded samples used for LMARG.
The sections were mounted on a slide glass on which a celloidin membrane had formed. After uranium acetate staining and Reynold’s lead staining in ice, carbon shadowing of about 10 nm in thickness was applied for preventing chemography. As the diameter of the emulsion for EMARG (Ilford L-4, Ilford, U.K.) is about 140 nm, the emulsion was diluted so as to enable a single-layer application. After confirming the interference color, the emulsion was applied using a dipping procedure. After 50 days of exposure, phenydon development and fixation with 30% sodium thiosulfate were performed. After rinsing with distilled water, the celloidin membrane was peeled off from the slide glass and floated on the water surface. After mounting on a 200-mesh grid, the sections were examined under a transmission electron microscope (JEM-200EX-II, Nihon Denshi, Japan). Twenty points were randomly selected for each group for observation. The ratio of localization in the organelles was obtained on the basis of the number of silver particles in the acinar cells and reduced of silver particles localized in the granular endoplasmic reticulum, which is deeply involved in protein synthesis in the secretory granules, Golgi apparatus, and secretory granules. Cytoplasmic organelles, which are involved in amino-acid active transport, were examined on the basis of the distribution at 30 and 120 minutes by EMARG.

Statistical analysis

For statistical analysis, we used statistical software (JSTAT ver. 8.1 for Windows). The number of silver particles per 1 mm$^2$ and reduced silver particles localized on the cytoplasmic organelles examined by EMARG were determined by counting the grains to calculate the ratio. The difference in the ratios between groups A and B, C and D, A and C, B and D, and A and D at the identical time after administration were studied using Welch’s t-test. A risk rate of 1% was regarded as significant.

Results

Histological study

HE staining findings for each group are shown in Fig. 1. In group A, a few conical cells aggregated and formed...
the acinus in the terminal portion. The nucleus was mainly closer to the base of the cell, and demonstrated an oval or circular shape. In comparison to the cells from the group A specimens, those from group B showed atrophy in some parts. The morphology of the acinar cells was very similar to that of those in group A, whereas the acinar cells in group C showed marked atrophic changes and an irregular morphology, while they also had flat nuclei and varied in size. The cells in group D showed atrophic changes of the acinus, as seen in group C, though the changes were milder, closer to groups A and B. The findings obtained by AZAN-Mallory staining are shown in Fig. 2. The stainability of groups A and B was very similar. In group C, fibrogenesis were slightly more abundant than in the other groups, while that distribution in group D was very similar to that in groups A and B.

Change in number of argentum grain ratio in acinar cells as shown by LMARG

Rodents have a granular ampullary part from which serous saliva is excreted to the capillaries that is not present in the human salivary structure. In the present study, only the acinus was observed. The LMARG findings for each group at 30 and 120 minutes after RI administration are shown in Figs. 3 and 4. Moreover, the numbers of silver particles per 1 mm² of acinar cells are shown in Fig. 5. There was no difference in reduced silver particles in the acinar cells at 30 minutes after RI administration between groups A [(4.8±0.2)x10⁴/mm²] and B [(5.0±0.2)x10⁴/mm²]. In comparison to group D [(5.0±0.3)x10⁴/mm²], the number was significantly smaller in group C [(0.9±0.1)x10⁴/mm²], while that in group C [(0.9±0.1)x10⁴/mm²] was significantly smaller than in group A [(4.8±0.2)x10⁴/mm²]. At 120 minutes after RI administration, the number of silver particles was [(4.8±0.2)x10⁴/mm²] for group A, [(4.1±0.2)x10⁴/mm²] for group B, [(4.7±0.3)x10⁴/mm²] for group C, and [(4.1±0.2)x10⁴/mm²] for group D, thus the groups were nearly equal.

EMARG

The findings at 30 and 120 minutes after ¹H-leucine administration are shown in Figs. 6 and 7. In group A, the endoplasmic reticulum and Golgi apparatus were developed, while the pyknosis, a precursor of electron-lucent secretory granules, was present sporadically. The secretory granules varied in size and had a nearly uniform

Fig. 2. AZAN-Mallory staining in each group. Fibrous changes were scarcely observed in groups A, B, and D, while they were observed sporadically in group C. Scale bar: 20 μm.
Fig. 3. LMARG findings at 30 minutes after RI administration in each group. Group C had a smaller reduction of silver particles than the other groups. Scale bar: 10 μm.

Fig. 4. LMARG findings at 120 minutes after RI administration in each group. The reduction in silver particles was nearly equal among all of the groups. Scale bar: 10 μm.
content. In group B, as in group A, the granular endoplasmic reticulum and Golgi apparatus were abundantly present, while vacuolar degeneration was observed in some parts. In comparison to group A, the secretory granules varied in size slightly more, though they had a nearly equivalent electron density. In group C, the contrast of the acinar cells as a whole was strong. The granular endoplasmic reticulum and Golgi apparatus showed disaggregation and great morphological changes, while the secretory granules had a twisted and fused configuration in comparison to group A. In the nucleus, the distinction between metachromatic and orthochromatic could not be made in some instances. In group D, swelling of the granular endoplasmic reticulum and Golgi apparatus was partially observed. However, the findings were similar to those of group A in most parts.

Fig. 5. Numbers of silver particles at 30 (A) and 120 (B) minutes after RI administration in LMARG. At 30 minutes after RI, group D showed a number similar to groups A and B, while the number was lower in group C as compared to the others.

Fig. 6. EMRG findings at 30 minutes after RI administration in each group. Group D showed fewer morphological changes than the others, while group C demonstrated marked morphological changes. In that group, the cells as a whole were deeply stained and the reduction in silver particles was scant. Scale bar: 1 μm.
The ratio of reduced silver particles localized in the granular endoplasmic reticulum at 30 minutes after RI administration was (43.2±2.3%) for group A, (48.8±1.6%) for group B, (43.7±6.4%) for group C, and (44.3±2.0%) for group D. At 120 minutes those were (14.6±1.7%), (22.0±1.8%), (16.8±6.7%) and (18.4±1.8%), respectively, demonstrating no significant difference. Furthermore, the ratio of reduced silver particles localized in the Golgi apparatus at 30 minutes after RI administration was (27.1±1.4%) for group A, (21.8±1.8%) for group B, (24.3±3.4%) for group C, and (26.5±2.0%) for group D, thus no significant differences were seen among the groups. Those ratios at 120 minutes after RI administration were (31.4±1.9%), (26.9±2.3%), (49.8±3.5%), and (39.0±2.2%), respectively, demonstrating a significantly higher ratio for group C as compared to group A (Fig. 8). In addition, the ratio for reduced silver particles localized among the secretory granules at 30 minutes after RI administration was (23.1±2.0%) for group A, (22.0±2.2%) for group B, (20.1±3.6%) for group C, and (16.9±1.5%) for group D, demonstrating the same levels among the groups. The ratios at 120 minutes after RI administration were (47.0±2.3%), (43.7±3.6%), (22.2±2.1%), and (33.8±2.1%), respectively, showing that the ratio for group C was significantly lower than that for group A (Fig. 9), as well as compared to group D. The ratio of reduced silver particles in other cells was around 10%.

Discussion

Amifostine is metabolized by alkaline phosphatase, which binds to the cell membrane. As the levels of alkaline phosphatase in tumor cells are lower than those in normal cells, it is considered that the phosphatase portion selectively protects only normal cells\(^{16}\).

During radiotherapy for progressive malignant tumors in the oropharyngeal region, radiation damage in the parotid\(^{17}\) and submandibular glands\(^{1}\) occurs in 30–90% of reported cases. In experimental laboratory animals, Yachi et al.\(^{18}\) reported the efficacy of amifostine on reduced radiation damage. In the present study, we studied the morphological and functional effects of amifostine on reducing radiation damage to acinar cells in

Fig. 7. EMARG findings at 120 minutes after RI administration in each group. In group C, a reduction in silver particles in the Golgi apparatus occurred more frequently as compared with the other groups. Scale bar: 1 μm.
the submandibular gland. Amifostine is rapidly decomposed by alkaline phosphatase in blood plasma when administered intravenously, with a half-life within 10 minutes. Although it has a short half-life, its metabolite WR-1065 remains longer in normal tissue and is retained in the salivary gland, kidney, and liver tissues for up to 30 minutes after administration, and in normal cells like those in bone marrow for up to 24 hours. In the present study, we used intraperitoneal injections of amifostine at 100 mg/kg in mice, which is equivalent to a dose of 20 mg/kg in humans, 30 minutes prior to X-ray irradiation at 5 Gy for an absorbed dose of 150 kV at 20 mA to the area equivalent to the submandibular gland. Furthermore, since amifostine has a greater protective effect on delayed damage time than on acute damage, the observation period was set for 30 days after irradiation, as previously reported by Coppes et al. Histological studies with HE staining revealed that morphological changes of the nucleus occurred more frequently in group B than in group A, demonstrating the effects of amifostine on morphology. Yuhas et al. studied the cytotoxicity of amifostine in mice and reported a 30-day survival dose of 784 mg/kg of body weight. For the present study, we considered that the effects of amifostine on the salivary gland were demonstrated as histological abnormalities. In group C, attrition and nuclear concentration were more conspicuous as compared to group D, suggesting that amifostine administration before irradiation can alleviate tissue damage. With AZAN-Malloy staining, extensive fibrosis was observed in group C as compared to group D, which was similar to the report of Sagowski et al. In the mechanism of late effect, it is thought that a number of interstitial cells, such as vascular endothelial cells, fibroblasts, and macrophages, accumulate excessive extracellular matrix and collagen at the early stage of irradiation, resulting in an imbalanced tissue structure. Giannopoulou et al. reported that amifostine administration before irradiation protected the vessels. Thus, it is considered that amifostine administration performed in the present study likely reduced fibrosis in the submandibular gland and protected the acinar cells.

In our autoradiographic study, intracellular uptake was examined at 30 minutes after RI, when a sufficient amount of leucine had entered the cells, and at 120 minutes, when synthesized protein was excreted. LMARG findings were compared between group B, in which only amifostine was administered without irradiation, and group A, without amifostine administration or irradiation, and found to be similar. Furthermore, in group D,
amino acid uptake and excretion were nearly the same as in groups A and B. On the basis of these results, it is suggested that amifostine is effective to prevent functional damage caused by irradiation. In the group of mice irradiated without amifostine administration, leucine uptake and protein synthesis were abnormal, causing a delay in uptake and excretion at 30 and 120 minutes after irradiation. In group D, in which amifostine was administered with aminothiol radix, it was speculated that the effects of radiation on the cell membrane would be alleviated by the antagonistic activities between free radicals and repairing action inside and outside of the acinar cells generated by irradiation. On the basis of our findings, we concluded that amifostine exerts nearly no effect on amino-acid excretion, while it has a slight effect on morphological changes.

In our morphological observations using electron microscopy, group C showed seemingly irreversible marked changes as compared to group A. Meanwhile, group D demonstrated a morphology more similar to group A than group C. Thus, effects of amifostine on the microstructural changes in the submandibular gland induced by X-ray irradiation were shown in the present study. Trocha et al. reported that amifostine administration before γ-ray irradiation protected the functions of nucleotides and lysosomes in rats. In the present study, amifostine alleviated the effects of radiation on the biofunctions of cells, such as organelles, which are deeply involved in the secretory process, in regard to nucleic acid, endocytosis, and autophagy. In group B, vacuolar degeneration was observed in some areas, suggesting the possible effect of amifostine itself. In an examination of the morphological effects of amifostine on the parotid glands of rats, Pratt et al. reported that they could not specify whether vacuolization of the Golgi apparatus due to secretion of enzymes and granules was caused by WR-2721 or by ordinary secretary activity. Thus, it cannot be concluded whether it reflects the effects of amifostine itself. Moreover, in the present group D, the morphology of the nucleus was retained. Accordingly, we considered that an administration of amifostine with fewer adverse side effects might protect the function by binding with DNA, in addition to a covering action against damage to the acinar cells by radiation.

As for the change in number of reduced silver particles in acinar cells over time revealed by EMARG, the ratio of reduced silver particles present in the granular endoplasmic reticulum at 30 and 120 minutes after RI administration was nearly the same among the groups. In contrast, the ratio of reduced silver particles localized in the Golgi apparatus was high at 120 minutes after RI administration in group C, while that localized in the secretory granules was nearly equal among the groups at 30 minutes after RI administration. However, at 120 minutes after RI administration, the ratio in the secretory granules was the same in groups B and A, and significantly lower in group C. Since the ratio of reduced silver particles localized in the Golgi apparatus was high at 120 minutes after RI administration, it is suggested that irradiation to the submandibular gland delays the time from amino-acid uptake to substance synthesis, and that amifostine administration before irradiation has a cell protective action against dysfunction and morphological changes of acinar cells in the submandibular gland.

Clinical trials are being conducted to examine the side effects of amifostine and its action toward alleviating radiation damage, and are gathering data in regard to dosage, regimen, concentration, and individual immunity. Thus, amifostine is not yet used in routine clinical practice. However, a number of clinical reports and basic studies have clarified that amifostine is effective for patients undergoing radiotherapy. In cases of radiation therapy for the head and neck, it is necessary to take into account the number and period of amifostine administration, as well as determine the exposure field and method. Furthermore, application of heavy particle radiotherapy with fewer side effects might be necessary. Thus, it is important to examine radiotherapy methods for alleviating xerostomia, not only from the use of radioprotective agents, but also from a comprehensive viewpoint.

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