Effect of Preliminary Amifostine Administration in Irradiation of Parotid Glands

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Abstract: To investigate the effect of amifostine, we administered amifostine, a radioprotective agent, 30 minutes before exposing the maxillary region of mice, including the parotid gland, to 5 Gy or 10 Gy X-ray irradiation. The survival rate was recorded, and changes in the parotid gland morphology were investigated by examining the hematoxylin and eosin (HE)-stained specimens and light microscope autoradiography (LMARG) images obtained 30 days after irradiation. A survival rate of 100% was not observed in any group administered 200 mg/kg amifostine with or without irradiation. Among the groups irradiated with 10 Gy X-rays, the survival rate was higher and the survival period was longer in the 100 mg/kg amifostine group than in the no amifostine group. The histological findings in the group that received 5 Gy irradiation without amifostine were as follows: auxetic growth of acinar cells, nuclei of all sizes, cells in the mitotic phase, and cells undergoing apoptosis. Further, the treated groups were compared with the no amifostine and no irradiation group (untreated control). LMRG imaging revealed that the number of reduced silver grains per mm² of acinar cells after 30 min of ³H-leucine administration was higher than that after 120 min in mice treated with 100 mg/kg amifostine with or without 5 Gy irradiation. This observation was similar to that in the untreated control.

This finding suggests that although amifostine administration reduces the adverse effects of irradiation on the parotid gland, higher doses of amifostine may be fatal.

Key words: parotid gland, X-ray irradiation, amifostine

Introduction

Radiotherapy is often the treatment of choice for malignant tumors in the head and neck region because it ensures the maintenance of function and morphology. However, when a salivary gland is included in the radiation field, there is an increased risk of abnormalities in the oral cavity function, such as xerostomia, eating disorders, mastication/deglutination disorders, increased rate of infection, and frequent occurrence of dental caries1,2. Therefore, a decrease of salivary gland disorders is warranted in cases of radiotherapy to this region.

Amifostine3, which was screened in the 1980s, shows lower toxicity than the previously used aminothiol substances and is quickly absorbed by healthy tissues. Furthermore, gradual absorption of amifostine by poorly vascularized tumors limits its protective effect on tumors. In the USA, am-
Amifostine has been widely used in radiotherapy to the head and neck region. However, some reports have indicated its side effects such as nausea, emesis, and induction of hypotension or hypocalcemia. Thus, its optimal dose and administration period remain to be established. Further, several animal studies have measured only the quantity and constituents of the saliva secreted from irradiated salivary glands. Considering all these aspects, in this study, we histochemically investigated the effect of different doses of amifostine on the parotid gland and the relationship between amifostine administration and radiation damage.

Materials and Methods

1. Experimental Animals and Breeding Conditions

Eight-week-old Balb/c male mice weighing approximately 30 g (Tokyo Laboratory Animals Science Co. Ltd., Japan) were used as experimental animals. For the adaptation of the animals to the experimental set-up, the mice were maintained for one week at room temperature (25°C) under a 12-h light-dark cycle. Subsequently, the mice were randomly divided into 9 groups from A to K according to the doses of amifostine (0, 100, and 200 mg/kg) and irradiation (0, 5, and 10 Gy, Table 1) administered. Each group included 4 mice. Throughout the experimental period, the mice were allowed unrestricted access to standard food and water; however, at 12 h prior to amifostine administration, the intake of only tap water was allowed. The animals were handled and treated in accordance with the guidelines for experimental animals issued by the Nippon Dental University School of Life Dentistry.

2. Administration of Amifostine

Amifostine (WR2721, Ethyol; Medimmune Oncology, Inc., Gaithersburg, MA, USA) was diluted using physiological saline solution. Intraabdominal injection of amifostine was administered 30 min prior to X-ray irradiation at a dose of 100 mg/kg into the mice of groups D, E, and F, and at a dose of 200 mg/kg into the mice of groups G, H, and K. Physiological saline solution (dose, body weight (g) × 0.02 ml) was injected intraabdominally into the mice of groups A, B, and C.

3. X-ray Irradiation

A Hitachi X-ray generator (MBR-1520R-3; Hitachi Medical Corporation, Japan) was used for X-ray irradiation, which was applied to the experimental mice under general anesthesia with an intraabdominal injection of 10 ml/kg pentobarbital sodium (Somnopentil®; Schering-Plough Animal Health Corporation, USA). The maxillary region, including the parotid gland, was irradiated; the mice in groups B, E, and H received 5 Gy irradiation, while those in groups C, F, and K received 10 Gy irradiation under the following conditions: tube voltage, 20 mA; tube current, 20 mA; filter, 1.0-mm Al; and dosage rate, 2.1 Gy/min.

4. Survival Rate and Period

The survival rate and period of the mice in each group were determined at 30 days after the irradiation according to the procedure described by Pamujula et al.

5. Preparation of Histological Specimens

Specimens of the parotid glands in groups A, B, D, and E were obtained 30 days after X-ray irradiation. These specimens were prepared for histological analysis as follows: After the mice were sacrificed by overdose of pentobarbital sodium, their parotid glands were enucleated and fixed with 10% formalin neutral buffer solution for 24 h. The fixed parotid glands were then dehydrated using an ethanol series and embedded in paraffin. The paraffin-embedded parotid glands were sliced to obtain approximately 4-μm-thick sections; these sections were then stained with hematoxy-

Table 1 Classification of the experimental groups

<table>
<thead>
<tr>
<th>Amifostine</th>
<th>X-ray (Gy)</th>
<th>0</th>
<th>5</th>
<th>10</th>
</tr>
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<tbody>
<tr>
<td>0 mg/kg</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>D</td>
<td>E</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>200 mg/kg</td>
<td>G</td>
<td>H</td>
<td>K</td>
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6. Light Microscope Autoradiography (LMARG)

After 30 days of irradiation, the mice in groups A, B, D, and E were administered an intraabdominal injection of 370 kBq/g of $^3$H-leucine (L-leucine-4,5-$^3$H, 2.22–3.33 TBq (60–90 Ci)/mmol; Moravek Biochemicals, Inc., USA) (hereafter referred to as RI administration). The mice in each of the abovementioned groups were sacrificed under deep anesthesia at 30 and 120 min after RI administration, because in LMARG, the reduced silver grains in the acinar cells of the parotid gland show a maximum value at 30 min after $^3$H-leucine administration and thereafter decreases with time. The parotid glands were enucleated and their central portion was cut into fine particles of approximately 1 mm$^3$ in volume. These pieces were fixed with 2.5% glutaric aldehyde for two hours and 1.0% osmic acid for one hour. The embedded specimens in Epon-Araldite resin were thinly sliced to approximately 1-$\mu$m-thick sections. The obtained sections were finally dipped in an emulsion for LMARG (NTB; Eastman Kodak Company, USA) so that silver grains formed a single layer on each section. After exposing the sections for one week, they were processed. To obtain images of the specimens, the fixed sections were photographed under a confocal laser-scanning microscope (NTB SP; Leica Microsystems GmbH, Germany) in the differential interference mode. The number of reduced silver grains on the image was counted and then converted to a value indicating the number of grains per mm$^2$ of acinar cells. The values obtained at 30 and 120 min after RI administration were compared between group A and groups B, D, and E. Further, in order to examine the effect of amifostine on the irradiated parotid glands, the values were compared between groups B and E. In addition, the value obtained at 30 min after RI administration was compared with that obtained at 120 min for each group. The deviation from the average value was calculated using Welch’s t test.
administration were higher than those obtained at 120 min. A similar tendency was observed in group E. In contrast, in group B, the value obtained at 120 min after RI administration was significantly higher than that obtained at 30 min.

**Discussion**

Under the action of alkaline phosphatase, amifostine acts as a phosphoric acid-type prodrug and binds to the SH group to transform to a new type of prodrug (WR-1065) containing an active SH group\(^{12}\). This new prodrug has a protective effect and low toxicity. Since healthy tissues have sufficient blood supply, they benefit the most from such drugs because of the rapid absorption of these drugs by the blood. Conversely, the protective effect of such drugs on tumors is considered to be less because tumors have poor vasculature and

<table>
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<tr>
<th>Group</th>
<th>Survival rate (%)</th>
<th>Average survival period (days ± SE)</th>
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</thead>
<tbody>
<tr>
<td>A,B,D,E</td>
<td>100</td>
<td>&gt; 30</td>
</tr>
<tr>
<td>G</td>
<td>75</td>
<td>28.8 ± 2.5</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>8.3 ± 3.0</td>
</tr>
<tr>
<td>C</td>
<td>25</td>
<td>16.0 ± 3.9</td>
</tr>
<tr>
<td>F</td>
<td>50</td>
<td>26.5 ± 2.8</td>
</tr>
<tr>
<td>K</td>
<td>25</td>
<td>11.0 ± 5.5</td>
</tr>
</tbody>
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**Fig. 1** Changes in the survival rate in each group

**Fig. 2** a: HE-stained image of group A, b: HE-stained image of group B, c: HE-stained image of group D, d: HE-stained image of group E (Bar: 10 μm)
therefore inadequate drug absorption. Furthermore, salivary glands, etc. have been reported to absorb high concentrations of drugs\textsuperscript{13}. In the USA, amifostine has been used clinically to protect healthy salivary glands during radiotherapy to the head and neck region\textsuperscript{4,5}. However, side effects of amifostine, such as nausea, emesis, and tendency of hypotension and hypocalcemia, have been reported\textsuperscript{6,7}. Therefore, the optimal dose and administration period of amifostine remain to be determined for its clinical application. Inadequate data on amifostine has hindered its effective use

**Fig. 3** LMARG images of each group at 30 min and 120 min after RI administration (Bar: 10 μm)

a: group A, 30 min, b: group A, 120 min, c: group B, 30 min, d: group B, 120 min, e: group D, 30 min, f: group D, 120 min, g: group E, 30 min, h: group E, 120 min

**Table 3** Number of reduced silver grains per mm\textsuperscript{2} of acinar cells

<table>
<thead>
<tr>
<th>Group</th>
<th>A</th>
<th>B</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time after RI administration (min.)</td>
<td>30</td>
<td>120</td>
<td>30</td>
<td>120</td>
</tr>
<tr>
<td>Average value</td>
<td>12.5</td>
<td>11.1</td>
<td>5.1</td>
<td>11.8</td>
</tr>
<tr>
<td>SD</td>
<td>1.3</td>
<td>1.3</td>
<td>1.0</td>
<td>2.1</td>
</tr>
</tbody>
</table>
1. Methods

There exist numerous reports on various irradiation conditions, including the type, intensity, and region of irradiation, applied in mice experiments. Some of the irradiation conditions studied are as follows: 7.5–12.5 Gy X-ray irradiation locally\(^{14}\), 15 Gy X-ray irradiation to the whole body\(^{15}\), and \(^{60}\)Co \(\gamma\)-ray irradiation at a dose of 6 Gy locally\(^{16}\). In this study, the doses of X-ray irradiation were set at 5 Gy and 10 Gy on the basis of the survival results obtained in a preparatory experiment.

Previous reports indicated that amifostine has been administered at doses of 400 mg/kg\(^{17}\), 200 mg/kg\(^{14}\), and 100 mg/kg\(^{18}\) to the Rodentia family (mainly the mouse and rat). Damron et al.\(^{19}\) studied the effect of amifostine in the dose range of 0–250 mg/kg. They found that amifostine exhibited its radioprotective effect at doses above 100 mg/kg and that its toxicity increased at doses above 200 mg/kg. On the basis of these findings, amifostine doses of 100 mg/kg and 200 mg/kg were selected for the current experiment. The most effective timing of amifostine administration has been reported to be 15 min\(^{20}\), 15–30 min\(^{17}\), 20–30 min\(^{10}\), 25 min\(^{21}\), and 60 min\(^{10}\) prior to radiation therapy. Since the effective administration timing ranged between 15 and 60 min before irradiation in all these studies, we administered amifostine at 30 min before irradiation. The observation time selected for this experiment was 30 days after X-ray irradiation. Coppes et al.\(^{22}\) irradiated the neck region of mice with 15 Gy X-rays, and observed the consequent changes in the mice over a 240-day period that was divided into 4 subperiods of 60 days each; decreases in the number of acinar cells and amylase secretion were then observed over a period of 10–60 days. In the report by Pamujula et al.\(^{10}\) wherein the effects of whole-body irradiation with 9 Gy \(^{137}\)Cs \(\gamma\)-rays was observed over a 30-day period, the effect of amifostine was found to be greater a few days after administration than immediately after administration. A survival rate of 50% at 30 days after exposure of an animal group (LD50/30 (lethal damage dose 50%, 30 days)) has been used as one of the criteria for investigating the optimal dose of medication, reaction to medication, and influence of irradiation. Hence, in the present study, 30 days after irradiation was selected as the observation period.

2. Survival Period and Rate

The relationship between amifostine administration and the effects of whole-body irradiation were investigated by analyzing the survival rate and period. A 100% survival rate at 30 days after irradiation was observed in groups A, B, D, and E. The dose of 100 mg/kg amifostine is considered as an appropriate dose for reducing radiation injuries because among the groups irradiated with 10 Gy X-rays, the group administered 100 mg/kg amifostine (group F) exhibited a better survival rate and period than the group administered no amifostine (group C). In contrast, the groups administered 200 mg/kg amifostine had worse survival rates and periods. Although the underlying mechanism is not known, it is considered that amifostine and radiation have contrasting but synergistic effects.
on a living body. Since a 100% survival rate could not be achieved in group G, we consider that in mice, the intraabdominal injection dose of amifostine should be less than 200 mg/kg. Furthermore, Damron et al.\textsuperscript{19} and Yuhas et al.\textsuperscript{25} reported that doses greater than 200 mg/kg amifostine were fatal; this result was validated in the present study.

3. Morphology of Acinar Cells

Since slight morphological changes were observed in group D, the influence of amifostine on morphology is confirmed. Among the groups irradiated with 5 Gy X-rays, morphological changes were greater in the group administered no amifostine (group B) than in the group administered 100 mg/kg amifostine (group E). This confirmed that the administration of 100 mg/kg amifostine prior to irradiation was effective in reducing the radiation-induced morphological changes. Cameron\textsuperscript{24} reported that the survival period of acinar cells in the mouse submandibular gland was 185 days, while that of exocrine cells of the mouse pancreas was 520 days; therefore, the cells were considered to require a long turnover time. In the present study, the cells in the mitotic phase and apoptosis-like cells were not observed in the tissue images of group D\textsuperscript{25}. However, these cell types were observed in many tissue images of group B. This is considered to reflect the phenomenon wherein irradiation induces apoptosis\textsuperscript{26} and the number of acinar cells decreases at 30 days after irradiation\textsuperscript{17}.

4. Leucine Quantity in Acinar Cells

Onodera \textit{et al.}\textsuperscript{11} sequentially observed the leucine absorption ability of the acinar cells of the mouse parotid gland by using LMARG. The amount of leucine in the acinar cells of the parotid gland was highest at 30 min after RI administration (\textsuperscript{3}H-leucine was injected intraabdominally into the mice). Thereafter, leucine was mainly excreted from the cells in the form of secretion granules, and the amount secreted decreased with time. This study clarified that amino acid absorption and excretion were slower in the irradiation groups than in the non-irradiation groups; this is because irradiation causes abnormalities in the transport system of the cell membrane and intracellular metabolic system of the acinar cells of the parotid glands. This study also clarified that amifostine relieves the irradiation-induced functional disorder of the acinar cells since amifostine inhibited the action of free water radicals that absorb radiation energy and molecules that compose the cells. Appropriate administration of amifostine not only reduces the harmful effects of irradiation but also alleviates acute heart toxicity (heart failure symptoms such as cardiac muscle softening, palpitation, and breathlessness) induced by Adriamycin, which is an anticancer agent\textsuperscript{28}. As expected, fundamental data from various fields are required to establish the optimum conditions for amifostine administration. Thus, amifostine has a potential role in future cancer therapy.

References

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