Effects of Local and Whole Body Irradiation on Appearance of Osteoclasts During Wound Healing of Tooth Extraction Sockets in Rats

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We examined effects of local and whole body irradiation before tooth extraction on appearance and differentiation of osteoclasts in the alveolar bone of rat maxillary first molars. Wistar rats weighting 100 g were divided into three groups: non-irradiation group, local irradiation group, and whole body irradiation group. In the local irradiation group, a field made with lead blocks was placed over the maxillary left first molar tooth. In the whole body irradiation group, the animals were irradiated in cages. Both groups were irradiated at 8 Gy. The number of osteoclasts around the interradicular alveolar bone showed chronological changes common to non-irradiated and irradiated animals. Several osteoclasts appeared one day after tooth extraction, and the maximal peak was observed 3 days after extraction. Local irradiation had no difference from non-irradiated controls. In animals receiving whole body irradiation, tooth extraction one day after irradiation caused smaller number of osteoclasts than that 7 day after irradiation during the experimental period. Whole body-irradiated rats had small osteoclasts with only a few nuclei and narrow resorption lacunae, indicating deficiency of radioresistant osteoclast precursor cells. Injection of intact bone marrow cells to whole body-irradiated animals immediately after tooth extraction recovered to some content the number of osteoclasts. These findings suggest that bone resorption in the wound healing of alveolar socket requires radioresistant, postmitotic osteoclast precursor cells from hematopoietic organs, but not from local sources around the alveolar socket, at the initial phase of wound healing.

INTRODUCTION

Active bone resorption is a characteristic feature of the early period of the healing process following tooth extraction. During wound healing of tooth extraction sockets in rats, interradicular alveolar bone is resorbed by osteoclasts 2 to 3 days after extraction, newly formed bone in the socket increases gradually, and the extraction socket is filled with newly formed bone tissue 7 days after extraction.1,2) Active bone resorption appears to be indispensable for new bone formation in an extraction socket. However, histometric analysis of healing sockets has shown that the percentage of bone formation areas does not differ between animals irradiated immediately and 7 days after tooth extraction, whereas the total alveolar bone volume is lowest and the percentage of bone resorption areas is highest in animals irradiated immediately after tooth extraction.3) This indicates that the maximal interval (7 days) of irradiation-timing results in the recovery of alveolar extraction sockets through rapid wound healing, as described above. By contrast, the delays of wound healing and the increased bone resorption in animals irradiated immediately may result from chronic damage to the microvasculature responsible for supply of osteoclast progenitor cells, rather than acute injury of rapidly dividing cells such as fibroblasts and osteoblastic stem cells. Thus, supply of osteoclast progenitor cells to the extraction socket and differentiation into multinucleated osteoclasts seem to be important for the prompt wound healing of extraction sockets, even under the influence of irradiation.

Osteoclasts are large, multinucleated cells that are capable of resorbing calcified bone matrix, and participate in remodeling of bone tissues through matrix resorption by coupling
to osteoblasts. Early studies demonstrated that bone resorption in osteopetrotic mice is restored by parabiotic cross-circulation with normal littermates and transplants of bone marrow and spleen cells.\(^6\)\(^7\)\(^8\) Similarly, parabiotic exchange between lethally irradiated rats and normal, non-irradiated animals labeled with radioactive compound as a contrast medium has revealed that osteoclasts are derived from the non-irradiated animal.\(^9\) More direct evidence has been obtained in recent studies showing that multinuclear cells are derived from hematopoietic stem cells of monocyte/macrophage lineage and are recruited from hematopoietic tissues such as bone marrow, spleen, and circulating blood to bone tissue.\(^7\)\(^8\)\(^9\)\(^10\) Interestingly, Güngör et al.\(^11\) have reported that multinucleated osteoclasts are more resistant than the progenitor cells in bone marrow and spleen to sublethal and lethal irradiation. This implies that irradiation may have a great influence on wound healing through irreparable injury to osteoclast progenitor cells, because active bone resorption plays a crucial role in the early period of the wound healing process. However, the influence of irradiation on the differentiation and maturation of osteoclast progenitor cells and the appearance of multinucleated osteoclasts in the healing socket remains to be fully elucidated.

To examine chronological changes in the appearance of osteoclasts in the alveolar extraction socket under the influence of local or whole body irradiation prior to tooth extraction, we counted the number of osteoclasts and observed the histopathological features characteristic of the irradiated osteoclasts in the interradicular alveolar bone of healing extraction sockets. Furthermore, recovery of osteoclast appearance in healing alveolar sockets of rats that had received whole body radiation before extraction was examined at different times after irradiation and transplantation of bone marrow cells from intact animals.

![Diagram](attachment:diagram.png)

**Fig. 1.** Diagrammatic representation of the experimental design. All rats in local and whole body irradiation were exposed with 8 Gy. (a) Experiment 1. NIR: non-irradiated control, LIR-1: local irradiation one day before tooth extraction, LIR-7: local irradiation 7 days before extraction, WBI-1: whole body irradiation one day before extraction, and WBI-7: whole body irradiation 7 days before extraction. (b) Experiment 2. WBI-4: whole body irradiation 4 days before extraction. (c) Experiment 3. NBR: no injection of bone marrow cells into rats receiving whole body irradiation one day before tooth extraction. IBR: injection of bone marrow cells immediately after tooth extraction in NBR. ▼: tooth extraction, ▲: irradiation, ◇: injection of bone marrow cells, and ↓: animal sacrifice.

MATERIALS AND METHODS

Animals and irradiation

Animals were maintained and the experiments were conducted, according to the Principles of Laboratory Animal Care established by the NIH. The experimentation protocol used in this study was previously approved by the Animal Ethics and Research Committee, and was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Health Sciences University of Hokkaido. Male Wistar rats weighting 100 g (N = 108) were divided into three groups: non-irradiation group (N = 21), local irradiation group (N = 30), and whole body irradiation group (N = 57). In the local irradiation group, a field (10 × 10 mm) made with lead blocks was placed over the maxillary left first molar tooth to ensure as constant an exposure dose as possible. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutor, Abbott Laboratories, Abbott Park, IL, USA) and fixed in a lateral position so that the left teeth were facing upwards. In the whole body irradiation group, the animals were irradiated in cages. Both groups were irradiated at 8 Gy (1 fraction, dosage rate 0.65 mGy/min, FSD 80 cm) using a 60Co-ray unit (Toshiba, Japan). The exposure doses at 8 Gy were measured by DD-system (R-TECH, Nagano, Japan) before these experiments. This was based on a general occurrence of bone marrow death in rats having received a whole body irradiation dose of 8 Gy. In the non-irradiation control group, the rats were subjected to only anesthesia, in the same

Fig. 2. Histopathological features of the alveolar socket after tooth extraction. (a) Healing socket one day after extraction in a non-irradiated rat. S: dental socket, I: interradicular alveolar bone, B: buccal side, O: oral cavity. H-E, ×13 (b) Higher-magnification view of interradicular alveolar bone 3 days after tooth extraction in a non-irradiated rat. Multinucleated osteoclasts (arrows) are evident on the alveolar bone. B: bone. TRAP, ×200.

Fig. 3. Chronological changes in white blood cell counts in peripheral blood. NIR: non-irradiated control, LIR: local irradiation (8 Gy), and WBI: whole body irradiation (8 Gy).

way as the rats in the local and whole body irradiation groups.

Details of the experimental designs are shown in Fig. 1. The aim of experiment 1 was to examine the influence of time after local or whole body irradiation on the appearance of osteoclasts in the healing socket (Fig. 1a). Tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts were counted every day up to day 5 after tooth extraction. In experiment 2, we explored the number of osteoclasts in the alveolar extraction socket, in order to examine the influences of whole body irradiation prior to tooth extraction (Fig. 1b).

The number of TRAP-positive osteoclasts was measured in the healing alveolar sockets. In experiment 3, to clarify the derivation of osteoclast precursors that would appear in the extraction sockets, bone marrow from intact rats was transplanted into whole body-irradiated rats (Fig. 1c). Femurs of intact rats were cut off at both extremities with scissors, and a bone marrow cell suspension was prepared by rinsing the femoral cavity with minimum essential medium. The cell suspension was made up to $1 \times 10^7$ cells/mL, after which the bone marrow cells were injected into the abdominal cavity of whole body-irradiated rats.

Fig. 4. Histopathology of femoral bone marrow of mice subjected to whole body irradiation. (a) Non-irradiated control. (b) One day after local irradiation. (c) One day after whole body irradiation. (d) Five days after whole body irradiation. (e) Eight days after whole body irradiation. Note regeneration of hematopoietic cells. (f) Fourteen days after whole body irradiation. Regeneration of hematopoietic cells is marked. H-E, $\times 100$.

Fig. 5. Numbers of osteoclasts appearing on the interradicular alveolar bone after tooth extraction in experiment 1. Note the maximal peak 3 days after tooth extraction in non-irradiated controls (NIR), locally irradiated (LIR) and whole body-irradiated rats (WBI).

Tissue preparation

The maxillary left upper molar was extracted before or after irradiation. On the designated day, three rats in each group were killed by intraperitoneal injection of an overdose of anesthetic. The animals were immediately fixed by perfusion with 4% paraformaldehyde (PFA) buffered with 0.1 M sodium cacodylate (pH 7.4), and then the removed maxillae were re-fixed in freshly prepared PFA for 24 h at 4°C. The specimens were decalcified in 5% ethylenediaminetetraacetic acid (EDTA), and then embedded in paraffin. The sections cut in a buccal-lingual direction were cut at the level of the distal socket of the first molar. For light microscopic observation, these sections were stained with haematoxylin and eosin (H-E). TRAP activity in differentiated osteoclasts was also detected using a reaction mixture consisting of 0.1 M acetic buffer (pH 5.0), naphthol AS-BI phosphate (Sigma, St Louis, MO, USA), fast red violet LB salt (Sigma) and 50 mM sodium tartrate. All TRAP-positive multinucleated osteoclasts facing a resorption bone lacuna were counted in an area of interradicular alveolar bone using each of five tissue preparations (Fig. 2).

Observation of circulating blood cells and bone marrow

To examine the influences of local or whole body irradiation on circulating blood cells, blood was obtained from the tail vein of each rat, and white blood cells were counted once a day for 14 days after irradiation with a hemocytometer. Furthermore, to evaluate the influences of irradiation on the bone marrow, the femurs were dissected from another three animals of each group at the time of tooth extraction. The specimens were fixed as described above, and the sections were stained with H-E.

Statistical analysis

The average numbers of osteoclasts were analyzed statistically by ANOVA, and then the significance was tested by Student’s t test (P = 0.05). SPSS was the statistical software employed.

RESULTS

Influences of irradiation on white blood cells in circulating blood and femoral bone marrow

The numbers of white blood cells in peripheral blood are shown in Fig. 3. There was no significant difference in the counts of white blood cells between the control and local irradiation groups. In the whole body-irradiated rats, the number was markedly decreased immediately after irradiation. The low value continued up to 6 days, and thereafter

Fig. 6. Histopathological features of interradicular alveolar bone in healing sockets 3 days after tooth extraction. (a) Interradicular alveolar bone in non-irradiated rats. Large osteoclasts have several nuclei and actively erode the surface of the interradicular bone. (b) Interradicular alveolar bone in rats receiving local irradiation one day before extraction. There is no histopathological difference between non-irradiated controls and local irradiation groups. (c) Characteristics of osteoclasts in rats receiving whole body irradiation 7 days before tooth extraction. Note small osteoclasts with only a few nuclei. (d) Histopathological features of interradicular alveolar bone after injection of bone marrow cells into whole body-irradiated rats. Note large osteoclasts with several nuclei comparable to those in non-irradiated controls (see Fig. 6a). H-E, × 200.

the number of white blood cells increased gradually toward the end of the study period.

There was no histopathological difference in the femoral bone marrow between the non-irradiation (Fig. 4a) and local irradiation groups (Fig. 4b). Numerous myelocytes and erythroblasts were evident. In the whole body-irradiated animals, bone marrow cells were markedly decreased one day after irradiation (Fig. 4c). In particular, hardly any erythroblasts and myelocytes were observed, whereas vacuolization was frequently evident. Few bone marrow cells were observed 5 days after irradiation, but numerous adipose cells were present (Fig. 4d). Eight days after irradiation, hyperemia became milder, and megakaryocytes and aggregations of hematopoietic cells were seen sporadically in the femoral bone marrow (Fig. 4e). Hematopoietic cells regenerated and increased around myelocytes 14 days after irradiation (Fig. 4f).

Experiment 1

Chronological changes in the numbers of osteoclasts showed features common to both the non-irradiated and irradiated groups (Fig. 5). Immediately after tooth extraction, a small number of osteoclasts appeared around the interradicular alveolar bone, reaching a maximum at 3 days after extraction in each of the experimental groups. The local irradiation group showed no difference from the controls during the experimental period, and the number of osteoclasts declined 4 and 5 days after extraction. In animals that received whole body irradiation, on the other hand, the number of osteoclasts was lower in the rats subjected to tooth extraction one day after irradiation than in those subjected to tooth extraction 7 days after irradiation. In addition, the numbers were scarcely decreased 4 and 5 days after extraction.

Figure 6 shows histopathological features of osteoclasts in the interradicular alveolar bone 3 days after extraction. A number of multinucleate cells were observed around the alveolar bone in the non-irradiated control (Fig. 6a) and in rats subjected to local radiation one day before extraction (Fig. 6b). The multinucleated osteoclasts were large with several nuclei, and the resorbing bone lacunae were deep and large, in accordance with the size of the osteoclasts. By contrast, small osteoclasts with only a few nuclei and narrow bone lacunae were seen in alveolar bone of rats that had been subjected to whole body irradiation (Fig. 6c).

Experiment 2

Teeth were extracted at different intervals after whole body irradiation. The number of osteoclasts in animals subjected to tooth extraction one day after whole-body irradiation was lower than in rats subjected to tooth extraction 4 days and 7 days after irradiation (Fig. 7). The number of osteoclasts increased with the period after irradiation.

Experiment 3

Figure 8 shows the number of osteoclasts 3 days after

Fig. 7. Numbers of osteoclasts 3 days after tooth extraction in experiment 2. The number of osteoclasts increases depending on the interval from irradiation to tooth extraction.

Fig. 8. Osteoclasts in rats subjected to injection of bone marrow cells in experiment 3. The number of osteoclasts in non-irradiated rats (NIR), rats subjected to whole body irradiation without injection of bone marrow cells (NBR), and rats subjected to whole body irradiation immediately after tooth extraction (IBR). Note recovery of the number of osteoclasts to some degree after injection of bone marrow cells immediately after tooth extraction.

tooth extraction when bone marrow cells from intact femurs were injected into rats immediately after tooth extraction one day after whole body irradiation (see Fig. 1c). The injection of bone marrow cells increased the number of osteoclasts counted in the healing socket. However, the number did not recover to a level comparable with that in the non-irradiated controls. In histopathological observation,
osteoclasts with several nuclei comparable to those in non-irradiated groups were often found at this stage of the wound healing (Fig. 6d).

DISCUSSION

During normal healing process of tooth extraction sockets in rats, interradicular alveolar bone is resorbed by osteoclasts 2 to 3 days after extraction, newly formed bone in the socket increases gradually, and the socket becomes filled with newly formed bone tissue 7 days after the extraction.\textsuperscript{1,2} However, the sequence of events leading to the appearance of osteoclasts in the healing alveolar socket is an important yet poorly understood phenomenon. In this study, irrespective of the local or whole body irradiation and post-irradiation intervals, a small number of osteoclasts were observed in the injured alveolar socket one day after tooth extraction and showed reactivity at least for TRAP, a marker of bone resorption. These cells may be preexisting multinucleated osteoclasts that are more radioresistant than their progenitor cells and have a life span of more than 1 week, possibly 9 to 10 days.\textsuperscript{11,13} In addition, the peak of osteoclast appearance occurred 3 days after tooth extraction, and there was no difference in the number of osteoclasts between non-irradiated and locally irradiated rats during the experimental period. This may indicate that local irradiation has no influence on wound healing and the differentiation of osteoclasts in the alveolar socket. However, the radiosensitivity of osteoclasts appears to differ depending on the stage of differentiation. Osteoclast development from proliferating progenitor cells is selectively inhibited by irradiation, whereas fusion of preexisting postmitotic precursors into multinucleated osteoclasts is radioresistant.\textsuperscript{14-16} These findings suggest that, at least locally, there may not be a pool of radiosensitive osteoclast progenitor cells around the healing socket.

Whole body irradiation, on the other hand, resulted in a decrease of osteoclasts when performed up to 4 days after extraction, compared with the situation in control and locally irradiated animals. However, the chronological changes in osteoclast appearance showed a maximal peak 3 days after tooth extraction, followed by a plateau at roughly the same level. The number of osteoclasts increased slowly up to 3 days in animals subjected to whole body radiation one day before tooth extraction. Osteoclasts are formed by fusion of postmitotic precursor cells.\textsuperscript{14,15} In addition, the inhibition of osteoclast formation by irradiation is due principally to inhibition of osteoclast progenitor proliferation, and not fusion of postmitotic precursor cells into multinucleated osteoclasts.\textsuperscript{16} Therefore, the slight increase of osteoclasts in animals subjected to irradiation one day before extraction may have resulted from multinucleation of preexisting postmitotic precursor cells. By contrast, the maximal interval (7 days) of whole body irradiation may permit the osteoclast progenitor cells to differentiate into multinucleated osteoclasts through recovery of hematopoietic potential to some extent, in addition to fusion of preexisting postmitotic precursor cells into multinucleated osteoclasts. This is supported by the histopathological features of the femoral bone marrow in animals that had been subjected to whole body irradiation. Indeed, the number of osteoclasts increased depending on the period between whole body radiation and tooth extraction (Fig. 1b and 7). Furthermore, injection of bone marrow cells evoked an increase in the number of multinucleated osteoclasts in the healing socket (Fig. 1c and 8). Osteoclasts are derived from hematopoietic stem cells of monocyte/macrophage lineage and are recruited from hematopoietic organs such as the bone marrow, spleen, and circulating blood to bone tissue.\textsuperscript{17-19} In addition, multinucleated osteoclasts are formed by fusion of postmitotic mononuclear precursor cells derived from circulating progenitor cells.\textsuperscript{14,18} Therefore, the present results suggest that bone resorption during the initial phase of alveolar socket healing may require principally the re-supply of circulating osteoclast progenitor cells rather than their precursor cells around the alveolar socket.

Of interest was the appearance of small osteoclasts with only a few nuclei in the healing sockets of animals that had received whole body irradiation. Since, in general, active mitotic cells are often very sensitive to ionizing irradiation, the radiation-induced formation of small osteoclasts is likely due to radiation interference with proliferating osteoclast progenitor cells or hematopoietic stem cells.\textsuperscript{14,19} In this study, although the number of osteoclasts was markedly decreased in animals subjected to tooth extraction one day after whole body irradiation (Fig. 1b and 7), osteoclasts recovered to some degree when bone marrow cells were injected immediately after tooth extraction (Fig. 1b and 8). Similar data have been reported by Gürpür et al.,\textsuperscript{11} who described that osteoclasts were decreased in the sternum of wholly irradiated rats, whereas this decrease was ameliorated by an injection of bone marrow cells. Therefore, radiation injury may induce a deficiency of osteoclast progenitor cells in the hematopoietic organs.

The decreased number of small osteoclasts continued for up to 5 days after tooth extraction in whole body-irradiated animals (Fig. 5), indicating the slow process of bone resorption by newly formed osteoclasts. The delay of wound healing may have been due to failure in re-supply of osteoclast precursor cells from hematopoietic organs via the circulation. Therefore, the present results suggest that, in addition to activation of preexisting mature osteoclasts, initial replenishment of osteoclast progenitor cells and the subsequent formation of osteoclasts are essential for rapid wound healing of alveolar extraction sockets. However, the present results do not rule out the involvement of inflammatory signals from the immune system and osteoblast-derived regulatory signals in the differentiation and maturation of multinucleated osteoclasts.\textsuperscript{20,21}

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