Effect of radiation on the expression of osteoclast marker genes in RAW264.7 cells

BING YANG[1], HUI ZHOU[2], XIAO-DONG ZHANG[1], ZHENG LIU[1], FEI-YUE FAN[1] and YUAN-MING SUN[1]

[1]Tianjin Key Laboratory of Molecular Nuclear Medicine, Institute of Radiation Medicine, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin 300192; [2]School of Pharmacy, Tianjin Medical University, Tianjin 300070, P.R. China

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Abstract. Cancer radiation therapy can cause skeletal complications, such as osteopenia and osteoporosis. To understand the mechanism responsible for the skeletal complications, the expression profiles of osteoclast marker genes in RAW264.7 cells were observed. Osteoclast formation was established by RAW264.7 cells that were treated with the receptor activator of nuclear factor (NF)-κB ligand (RANKL) and detected using immunochemistry and morphological observations. Quantitative real-time polymerase chain reaction was used to assess the expression of a panel of osteoclast markers, including the receptor activator of NF-κB (RANK), tartrate-resistant acid phosphatase (TRAP), integrin β3 and the calcitonin receptor (CTR). RANKL-induced osteoclasts were TRAP-positive and multinucleated, and displayed a distinct morphology. RANKL-induced osteoclast precursor cells had increased TRAP and RANK expression and decreased CTR expression compared to the control cells not treated with RANKL. RAW264.7 cells irradiated with 2-Gy γ-rays had upregulated integrin β3 and RANK expression and downregulated CTR expression compared to the control RAW264.7 cells. The effect of radiation on RANKL-induced osteoclast differentiation enhanced the expression of CTR and inhibited the expression of RANK and TRAP. Therefore, radiation damage from 2-Gy γ-rays can promote the activities of osteoclast precursor cells, but not those of osteoclasts.

Introduction

Previous studies have shown that radiation therapy can cause unavoidable post-radiation impairments, such as osteopenia and osteoporosis. Skeletal complications of radiation therapy have been described in breast, brain and pelvic cancer as well as in leukemia (1-4). Osteoclast precursors have the potential to differentiate into osteoclasts and are hypersensitive to radiation. We hypothesized that irradiated osteoclast precursors are associated with bone loss caused by radiation. To date, little information is available on the effects of irradiated osteoclast precursors on osteoclast dysfunction.

RAW264.7 cells are mouse monocyte/macrophage cells; they are regarded as osteoclast precursors (5) and differentiate into tartrate-resistant acid phosphatase (TRAP)-positive multinuclear osteoclasts following treatment with the nuclear factor (NF)-κB ligand (RANKL) (6-8).

To investigate the role of irradiated osteoclast precursors in the formation of abnormal osteoclasts, RAW264.7 cells were irradiated and differentiated into osteoclasts *in vitro*. In the present study, quantitative real-time polymerase chain reaction (QRT-PCR) was used to assess the expression of a panel of osteoclast marker genes.

Correspondence to: Dr Yuan-Ming Sun, Tianjin Key Laboratory of Molecular Nuclear Medicine, Institute of Radiation Medicine, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin 300192, P.R. China
E-mail: sym0702-dc@hotmail.com

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Materials and methods

Cell culture. RAW264.7 cells were obtained from the Cell Bank of the Institute of Basic Medicine at the Chinese Academy of Medical Science (Beijing, China) and maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and 100 μg/ml of penicillin/streptomycin, in a humidified atmosphere of 5% CO_2 at 37˚C, until reaching 80% confluency. The medium was changed every 3 days.

RAW264.7 cells were divided into 4 groups: Group A, normal RAW264.7 cells used as the control group; group B, RAW264.7 cells cultivated in the presence of 50 ng/ml of RANKL (PeproTech) for osteoclast formation (9); group C, RAW264.7 cells exposed to 2-Gy γ-rays to irradiate the osteoclast precursor cells; and group D, RAW264.7 cells treated with both 2-Gy γ-rays and 50 ng/ml of RANKL to induce osteoclast formation with radiation damage. All groups were maintained for 7 days. As a radiation source, ^{137}Cs was used.

Assessment of TRAP-positive cells. The TRAP kit was purchased from the Science and Technology Company of the Institute of Hematology at the Chinese Academy of Medical Sciences (Tianjin, China). After the cells were fixed with para-

Table I. Sequences of primers for quantitative real-time polymerase chain reaction.

Target	Primers (5'-3')
TRAP	Forward: AGGACGTGTTCTCTGACCG Reverse: CGCAAACGGTAGTAAGGG
CTR	Forward: TAGGAGGTGGAGGATAGC Reverse: TGACTTGGTGTTGAGGAC
Integrin β3	Forward: CCTTCGGATTGGCTTTGG Reverse: TCATTGAAGCGGGACACC
RANK	Forward: GTCTGCAGCTCTTCCATG Reverse: TCCCTTCCTGTAGTAAACG
β-actin	Forward: GGGTGTGATGGTGGGAATG Reverse: CTCATTGTAGAAGGTGTGGTGC

TRAP, tartrate-resistant acid phosphatase; CTR, calcitonin receptor; RANK, receptor activator of nuclear factor-κB.

formaldehyde and incubated with the TRAP solution at 37˚C for 1 h, the cells were washed in distilled water and counterstained with hematoxylin. Multinucleated TRAP-positive cells were observed using an inverted phase contrast microscope, and their images were captured.

RNA extraction, reverse transcription and QRT-PCR analysis. Total RNA was extracted using the TRIzol reagent (Invitrogen). First-strand cDNA was synthesized using the reverse transcription kit (Takara) with total RNA (4 μg). QRT-PCR analyses for TRAP, calcitonin receptor (CTR), integrin β3 and receptor activator of NF-κB (RANK) were performed using the ABI Prism 7000 sequence detection system (Applied Biosystems) with Platinum® SYBR-Green qPCR SuperMix-UDG (Invitrogen). The reaction conditions were: 50˚C for 2 min and 95˚C for 10 min, followed by 40 cycles of 95˚C for 30 sec and 60˚C for 45 sec. The levels of β-actin mRNA were used as the internal control, and the gene-specific mRNA expression was normalized against β-actin expression. The sequences of the primers used in these analyses are listed in Table I.

Statistical analysis. Data are presented as the means ± SD. The data were compared using two-tailed unpaired Student's t-test (SPSS 13.0 for Windows). $P<0.05$ was indicative of a statistically significant difference.

Results

Morphological features of osteoclasts derived from RAW264.7 cells. After RAW264.7 cells were plated in flasks and treated with RANKL for 7 days, they were stained with TRAP and counterstained with hematoxylin. The adherent osteoclasts displayed a ruffled membrane (Fig. 1A), pseudopodia and a multinuclei phenotype (Fig. 1B).

Expression of TRAP, CTR, integrin β3 and RANK mRNA. To examine the effect of RANKL on the initial proliferation of osteoclast precursor cells, RAW264.7 cells were grown

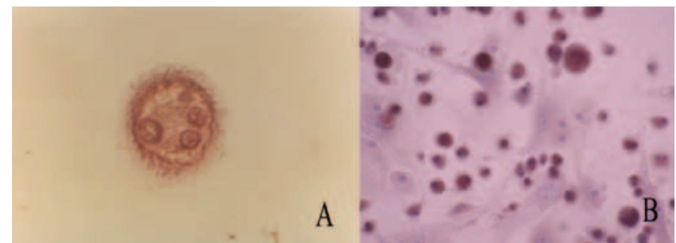

Figure 1. Morphological features of osteoclasts that were derived from RAW264.7 cells. (A) A large mature osteoclast was stained for TRAP and displayed a ruffled membrane. (B) Osteoclast was counterstained with hematoxylin.

in the presence of 50 ng/ml of RANKL for up to 7 days. RANKL-induced osteoclast precursor cells had increased TRAP and RANK expression and decreased CTR expression compared to the control cells not treated with RANKL (Fig. 2).

To investigate the effect of radiation on osteoclast precursor cells, RAW264.7 cells were exposed to 2-Gy γ-rays and maintained without RANKL for up to 7 days. These osteoclast precursor cells had upregulated integrin β3 and RANK expression and downregulated CTR expression compared to the control cells (Fig. 2).

To evaluate the effect of radiation on RANKL-induced osteoclast differentiation, we compared an expression panel of osteoclast marker genes in RAW264.7 cells that were treated with RANKL alone or in combination with radiation. The irradiation of RANKL-induced osteoclasts led to an increased expression of CTR and a decreased expression of RANK and TRAP compared to the non-irradiated RANKL-induced osteoclasts (Fig. 2).

Discussion

Bone metabolism is a dynamic and continuous remodeling process that is normally maintained in a tightly coupled balance between resorption of old or injured bone and the formation of new bone. This coordinated regulation of bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts) is regulated by a complex network of cytokines, cell surface receptors and various signaling pathways (10). Osteoclasts are derived from hematopoietic progenitor cells of a monocyte/macrophage lineage and multinucleated cells that degrade the mineralized bone matrix (11). Recent studies have identified activated osteoclasts as a pathological feature of osteopenia and osteoporosis (12). Osteoclast precursors synthesize DNA and proliferate. Therefore, osteoclast precursors are more vulnerable to radiation injury than osteoclasts. Little attention has been given to evaluating the effects of radiation on osteoclast precursor function.

TRAP, a metallophosphatase that is highly expressed in osteoclasts, is secreted into the resorption lacuna and is associated with the resorbing matrix (13-16). TRAP expression is dramatically upregulated during osteoclast differentiation. Hence, TRAP activity is commonly used as a histochemical marker of osteoclasts (17). When activated by proteolytic processing, TRAP exhibits protein phosphatase activity toward several bone matrix proteins, including osteopontin (15,16). In accordance with previous reports (18), our results show that RANKL-induced RAW264.7 cells had increased TRAP

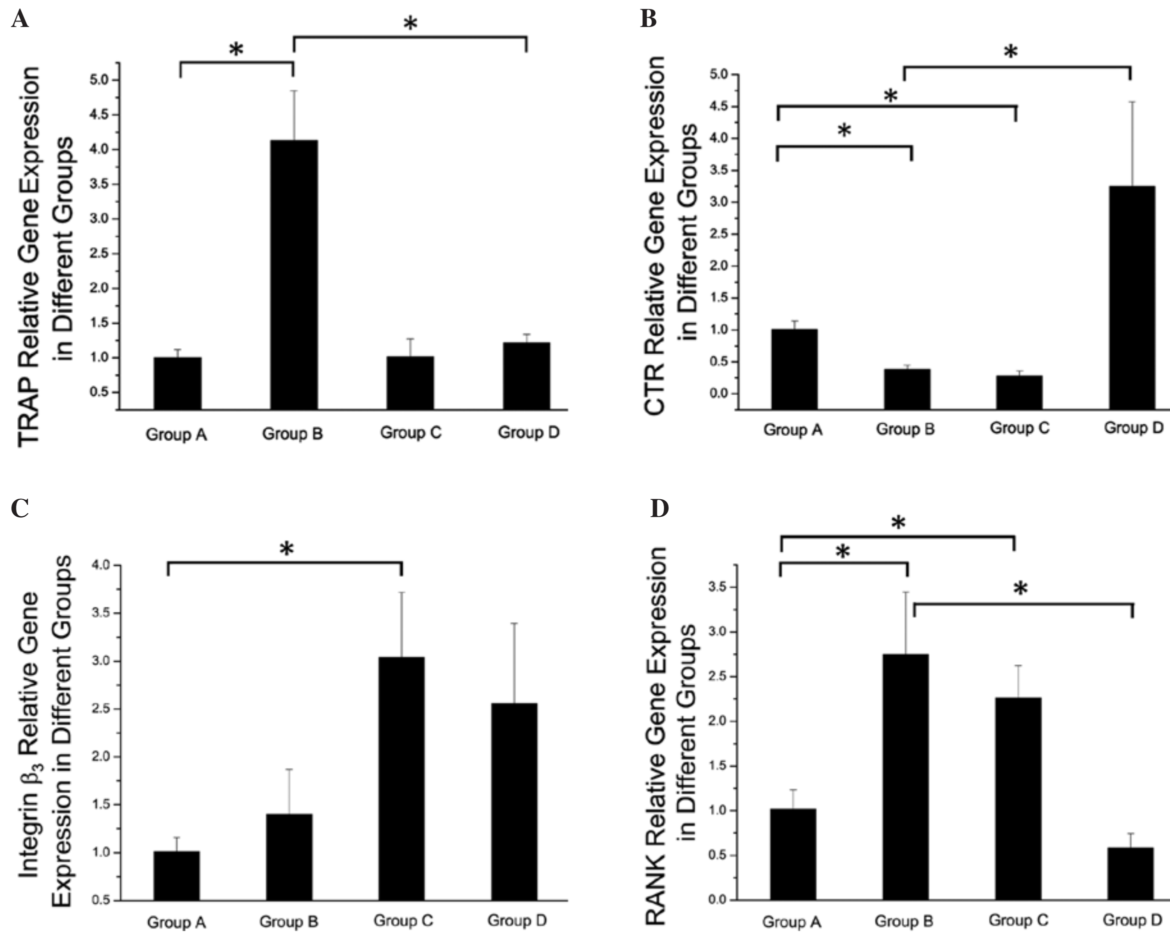

Figure 2. Relative expression of TRAP, CTR, integrin β3 and RANK (*$P<0.05$). The expression profiles of (A) TRAP, (B) CTR, (C) integrin β3 and (D) RANK were determined using the $2^{-\Delta\Delta Ct}$ method.

expression (group B). We demonstrated that the expression of TRAP was not significantly different between groups A and C, suggesting that radiation may have little impact on TRAP expression in osteoclast precursors. We initially hypothesized that TRAP expression in irradiated mature osteoclasts may be higher compared to non-irradiated counterparts according to skeletal complications in patients receiving radiotherapy. Notably, group D, which comprised RANKL-induced RAW264.7 cells that were irradiated with 2-Gy ^{137}Cs γ-rays, showed lower levels of TRAP than group B, in which the cells were cultivated in the presence of 50 ng/ml of RANKL for osteoclast formation. These results indicate that radiation may have a negative effect on TRAP expression, although the underlying mechanism remains unknown.

As part of bone remodeling, osteoclasts bind to the bone matrix, form an actin ring-mediated sealing zone, secrete enzymes and acid to degrade the bone and then migrate to a new site. Each of these functions is regulated in part by integrins that are located on the membrane surface of the osteoclast and interact with neighboring cells and the extracellular matrix (19). The predominant integrin in osteoclasts is αvβ3. Antibody inhibition of αvβ3 inhibits osteoclast attachment to the bone matrix and osteoclast-mediated bone resorption (20). The β3 subunit, which is a component of αIIβ3 and αvβ3 integrins, plays an important role during early fracture healing (21). Our findings indicate that irradiated RAW264.7 cells expressed significantly higher levels of integrin β3 than the control group. However, the expression levels of integrin β3 between groups B and D showed no significant differences. These results suggest that irradiation promotes the activity of osteoclast precursor cells, but not that of osteoclasts.

In the bone, CTR is a specific marker of osteoclasts (22-25), particularly osteoclast differentiation (26), and osteoclasts are normally associated with osteolysis. The binding of calcitonin to its receptor is known to dampen osteoclast activation (27). We found that cells in the RANKL-induced group and the irradiated group expressed lower levels of CTR than those in the control group, suggesting that mature osteoclasts express lower levels of CTR than osteoclast precursors. However, cells in the group receiving RANKL in combination with radiation treatment expressed higher levels of CTR compared to those in the control group. Our results suggest that radiation exposure may increase the activity of osteoclast precursors, but may damage the resorption ability of osteoclasts.

RANK expression on hematopoietic precursor cells is required in the murine model for osteoclast differentiation and activation, the resorption of bone and the regulation of calcium homeostasis by calcitropic hormones (28,29). Our results showed that cells in the RANKL-induced group (group B) and the irradiated group (group C) expressed higher levels of RANK. The RANK mRNA expression did not significantly differ between groups C and D. The irradiation

of RANKL-induced osteoclasts (group D) led to a decreased RANK expression compared to the cells in group B. These results suggest that radiation exposure may promote RANK expression in osteoclast precursors, but not in osteoclasts.

In conclusion, our experiments revealed that RAW264.7 cells differentiated into functional osteoclasts in the presence of RANKL. Radiation damage may promote the activities of osteoclast precursors, but it decreases those of osteoclasts. We inferred that radiation impairs the function of osteoclasts and stimulates the differentiation of osteoclast precursors. Therefore, irradiated osteoclast precursors may play a significant role in bone damage and may mediate skeletal complications in patients receiving radiotherapy.

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