EXPERIMENTAL BIOLOGY

Different Effects of Carbon Ion and γ-Irradiation on Expression of Receptor Activator of NF-kB Ligand in MC3T3-E1 Osteoblast Cells

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We investigated the effects of carbon ion and γ-irradiation on osteoblastic MC3T3-E1 cells by comparing mRNA expression levels for RANKL and osteoprotegerin by RT-PCR. MC3T3-E1 cells were irradiated with 2, 4, or 6 Gy of carbon ions or γ-rays, and total RNA was harvested 1, 2, 3, 5, or 7 days after irradiation. The RANKL mRNA/OPG mRNA ratio in carbon ion-irradiated MC3T3-E1 cells was lower, while in γ-irradiated MC3T3-E1 cells this ratio was higher than in non-irradiated cells. To evaluate osteoclastogenesis of MC3T3-E1 cells, carbon ion- or γ-irradiated cells were co-cultured with non-irradiated cells from murine bone marrow. Staining for tartrate-resistant acid phosphatase (TRAP) in co-cultures showed that carbon ion irradiation suppressed osteoclastogenesis. This result is consistent with the lower RANKL/OPG mRNA ratio for carbon ion-irradiated cells. These results suggest that carbon ion irradiation acts primarily on osteoblastic cells, leading to a decrease in the RANKL/OPG mRNA ratio. This effect, in turn, leads to a decrease in osteoclastogenesis and osteoclast activity, which results in an increase in bone volume.

Key Words: *carbon ion; jsteoblast; jsteoclastogenesis; RANKL; OPG*

Osteoclasts are bone-specific, multinucleated, giant cells with the capacity to resorb mineralized tissues. They are derived from hematopoietic progenitors of the monocyte-macrophage lineage. Osteoclast formation from hematopoietic precursors requires factors that promote their differentiation and maturation. These factors are produced by stromal/osteoblastic cells that originate from mesenchymal progenitors residing in the bone marrow [10,11]. RANKL (receptor activator of NF-κB ligand) is essential for and, together with macrophage colony-stimulating factor (MCSF), sufficient for osteoclast differentiation. RANKL acts by binding to its receptor (RANK) on the surface of hematopoietic precursors, thus stimulating their differentiation into mature osteoclasts [1,5,14]. The action of RANKL is prevented by osteoprotegerin (OPG), a soluble decoy receptor that

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competes with RANK for binding to RANKL [9]. The ratio between RANKL and OPG mRNA expression levels in osteoblastic cells is postulated to be a key factor in osteoclast regulation [6].

We have previously shown that focal irradiation with carbon ions induces bone responses that are qualitatively and quantitatively different from those induced by an equivalent dose of γ-irradiation. In our study, carbon ion irradiation resulted in a dose-dependent increase in bone volume, whereas γ-irradiation resulted in a loss of bone volume [7]. Osteoclasts in the carbon ion-irradiated group were smaller than those in the γ-irradiated group, suggesting that carbon ion irradiation severely inhibits osteoclast maturation. A possible explanation for this effect is that carbon ion irradiation inhibits the production of osteoblastic cells or matrix-derived osteoclast-stimulating factors, resulting in a reduction in both the size of osteoclasts and their bone-absorption capacity [8].

In the present study, we examined this hypothesis by assessing the effects of carbon ion and γ-irradiation on the expression of osteoclast differentiation factors RANKL and OPG, as measured by RT-PCR. We also investigated the effects of irradiation on osteoclastogenesis in co-cultures with non-irradiated murine bone marrow cells.

MATERIALS AND METHODS

MC3T3-E1 cells derived from calvaria of newborn mice were obtained from the Riken Cell Bank (Tsukuba, Japan). MC3T3-E1 cells were cultured in α-MEM (GIBCO, Grand Island, NY) supplemented with 10% charcoal-stripped fetal bovine serum (FBS; Bioscience, Lenexa, KS), 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO) at 37°C in an atmosphere of 5% CO_2 and 95% air. The medium was changed every 2 days.

MC3T3-E1 cells were exposed to 2, 4, or 6 Gy of carbon ion or γ-irradiation. Carbon ion irradiation was carried out using a 290-MeV/μ, 6-cm, spread-out Bragg peak (SOBP) carbon-ion beam at the Heavy Ion Medical Accelerator in Chiba (HIMAC), at the National Institute of Radiological Sciences, in Japan. The estimated linear energy transfer (LET) averaged 40 keV/μ for the proximal SOBP, and the dose rate was 3 Gy/min. The beam intensity was measured using dose monitors installed in the beam path. Binary filters made of 0.5- to 128-mm-thick polymethylmethacrylate plates were used to adjust the LET. γ-Irradiation was performed at a dose of 0.52 Gy/min using a remotely controlled medical cobalt-60 source (Isoton-21; Shimadzu, Kyoto, Japan).

Total cellular RNA was harvested from irradiated and control MC3T3-E1 cells after 1, 2, 3, 5, or 7 days. TRIzol reagent (Invitrogen, Carlsbad, CA) was used to isolate RNA according to the manufacturer's protocol. Total RNA was used as the template for cDNA synthesis in reverse transcription reactions with oligo-$(dT)_{20}$ primers and RevertraAce enzyme (TOYOBO, Osaka, Japan).

Aliquots (1-μl) of the resulting cDNA were PCR-amplified in 25-μl reactions with Taq Plus DNA polymerase (TOYOBO) in a thermal cycler (MJ Research, Waltham, MA) using 35 cycles of 94°C for 1 min, 60°C for 30 sec, and 72°C for 1 min.

Commercially synthesized primers for RANKL, OPG, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Sigma-Genosys, Sapporo, Japan) were used for separate PCR reactions. RANKL primer sequences (shown (5'-3') were CCTGAGGCC CAGCCATTT (forward) and CTTGGCCCAGCC TCGAT (reverse). OPG primer sequences were TCCTGGCACCTACCTAAAACAGCA (forward) and CTACACTCTCGGCATTCACTTTGG (reverse). GAPDH primer sequences were CACCATGG AGAAGGCCGGGG (forward) and GACGGACA CATTGGGGGTAG (reverse).

The RT-PCR products (10 μl) were mixed with 2 μl of bromophenol blue loading buffer and electrophoretically separated in 1.5% agarose gels (L 03; TaKaRa, Shiga, Japan) in Tris-boric acid-EDTA buffer (Tefco, Tokyo, Japan). The gels were stained with ethidium bromide, and UV images were captured using an ATTO Bioinstruments imaging system (Tokyo, Japan). The amount of RNA on the gels was measured using a densitometry imaging system. The relative amounts of RANKL and OPG mRNAs were normalized to the amount of GAPDH mRNA in the same sample. The ratio of RANKL mRNA to OPG mRNA was used as a measure of osteoclastogenesis. The data shown are representative of three independent experiments.

Immediately after irradiation, MC3T3-E1 cells (5×10^4 cells/well) were plated in 24-well plates (Falcon Labware, Oxnard, CA) in 0.5 ml of (α-MEM supplemented with 10% FBS. Responder bone marrow cells were obtained from 4-week-old C57Bl/6 mice by flushing the femoral shafts using a sterile 26-gauge needle. These cells were added (10^5 cells/well) to the irradiated MC3T3-E1 cells 18 h after irradiation. The co-cultures were supplemented with 10% 1,25-dihydroxycholecalciferol (1,25-dihydroxy Vitamin D) and 100 nM dexamethasone (Sigma, St. Louis, MO). The medium was changed every 2 days. After 6 days, the cells were fixed in 10% formaldehyde in phosphate-buffered saline (PBS), treated with ethanol-acetone (1:1), and stained for

tartrate-resistant acid phosphatase (TRAP) [12]. TRAP-positive cells were considered to be osteoclasts. The number of TRAP-positive cells was expressed as the mean±standard deviation (SD) of triplicate cultures in 10-mm diameter wells.

The use of animals in this study was approved by the Animal Care and Use Committee of Hiroshima University. All protocols were in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Three independent RNA analysis experiments were conducted, each with two replicates. TRAP assay data are from five separate co-culture experiments and represent the means of at least two replicates in each experiment. Statistical comparisons were carried out using analysis of variance (ANOVA).

RESULTS

In cells exposed to 2 or 4 Gy of carbon ion irradiation, RANKL expression was observed shortly after irradiation, whereas in cells exposed to 6 Gy carbon ion irradiation, RANKL mRNA expression was barely detectable (Fig. 1, Table 1). Two days after exposure to 2 or 4 Gy of γ-irradiation, the amount of RANKL mRNA in the irradiated cells decreased, as compared to non-irradiated cells; however, 3 days after exposure, the amount of RANKL mRNA increased to a level surpassing that in non-irradiated cells. More RANKL mRNA was present in cells exposed to 6 Gy of γ-irradiation than in non-irradiated cells, from day 1 after irradiation, onward. As a rule, expression of RANKL mRNA was lower in carbon ion-irradiated cells than in non-irradiated cells, whereas γ-irradiated cells had increased RANKL mRNA levels. Although expression of RANKL mRNA in cells irradiated with 2 or 4 Gy of carbon ions remained the same, or increased shortly after exposure, as compared to non-irradiated cells, it was lower than in non-irradiated cells from day 2 onward.

Carbon ion and γ-irradiation had opposite effects on OPG mRNA expression levels, but no significant correlations between OPG mRNA expression levels and radiation dose, or time after irradiation, were observed for either group (Fig. 1, Table 2). Compared to that of non-irradiated cells, the amount of OPG mRNA tended to increase as the dose of carbon ion irradiation increased or as the dose of γ-irradiation decreased. The two types of irradiation had significantly different effects on the RANKL/OPG mRNA ratio ($p<0.05$), mostly due to changes in RANKL expression (Table 3). The RANKL/OPG mRNA ratio was generally significantly lower in cells irradiated with carbon ions

TABLE 1. RANKL mRNA Expression Levels over the Time Course of the Experiment

Group (irradiation dose)	Time, days				
	1	2	3	5	7
Non-irradiated	0.058948±0.011790	0.045037±0.011259	0.012903±0.003871	0.009721±0.003889	0.005752±0.002588
Carbon ion irradiation					
2 Gy	0.082932±0.009123	0.033512±0.005027	0±0	0±0	0.005717±0.000572
4 Gy	0.058824±0.007647	0.031250±0.003125	0±0	0±0	0±0
6 Gy	0.003233±0.000517	0.003426±0.000651	0±0	0±0	0±0
γ-Irradiation					
2 Gy	0.041101±0.016440	0.029229±0.010230	0.014275±0.003569	0.018921±0.003784	0.024518±0.005639
4 Gy	0.052383±0.018334	0.049674±0.022353	0.032571±0.004886	0.116526±0.027384	0.140252±0.021038
6 Gy	0.157035±0.036118	0.145985±0.036496	0.108932±0.025054	0.040208±0.006031	0.066489±0.012301

Note. Results for the carbon ion- and γ-ray-irradiated groups differ significantly ($p<0.01$) at equivalent time points after equivalent doses of irradiation, starting at 3 days after irradiation. Data shown are representative of three independent experiments.

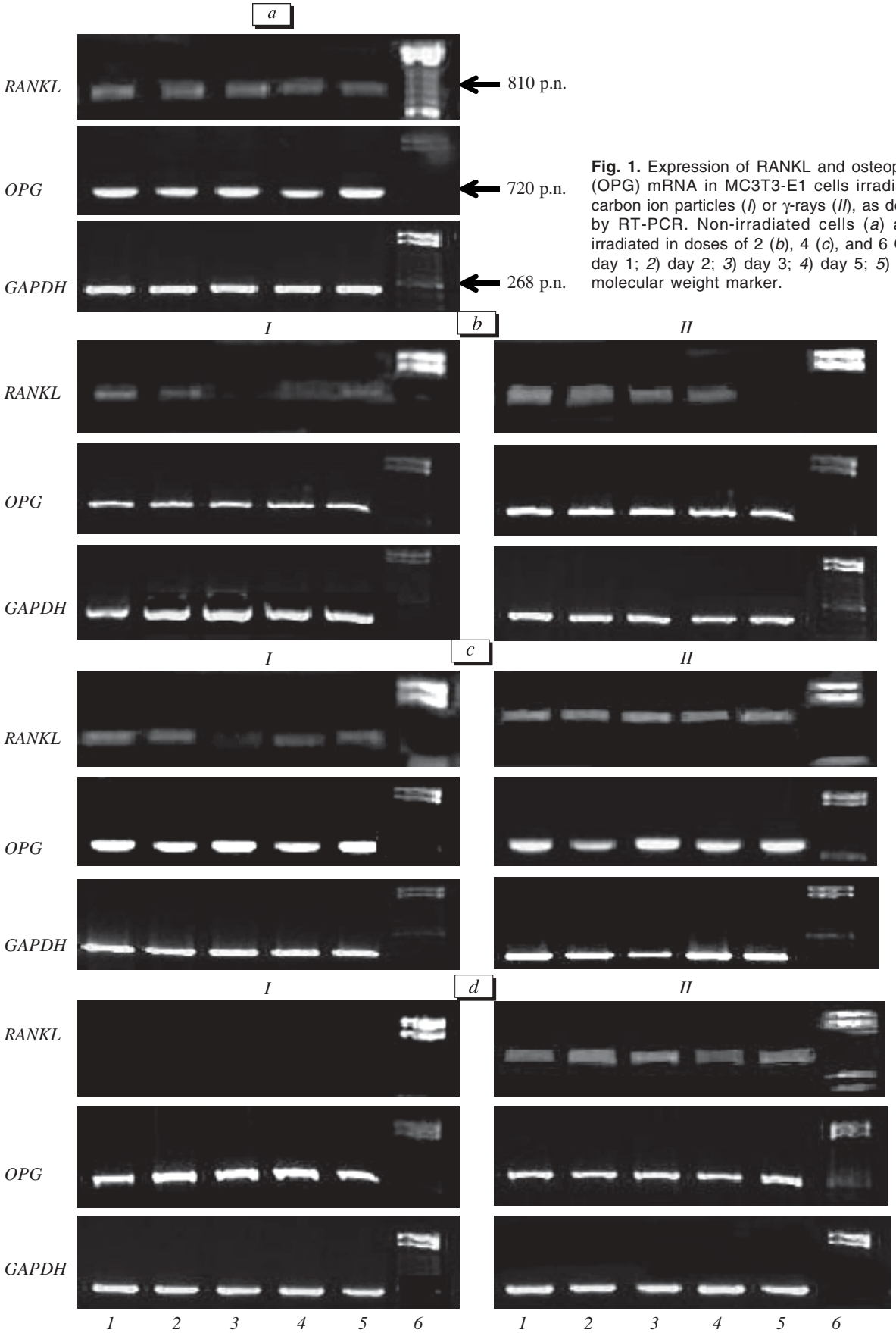

Fig. 1. Expression of RANKL and osteoprotegerin (OPG) mRNA in MC3T3-E1 cells irradiated with carbon ion particles (*I*) or γ-rays (*II*), as determined by RT-PCR. Non-irradiated cells (*a*) and cells irradiated in doses of 2 (*b*), 4 (*c*), and 6 Gy (*d*). *1*) day 1; *2*) day 2; *3*) day 3; *4*) day 5; *5*) day 7; *6*) molecular weight marker.

TABLE 2. OPG mRNA Expression Levels over the Time Course of the Experiment

Group (irradiation dose)	Time, days				
	1	2	3	5	7
Non-irradiated	1.251460±0.250292	0.923695±0.083133	0.910±0.091	0.875891±0.105107	0.972965±0.097297
Carbon ion irradiation					
2 Gy	0.629972±0.100796	0.6800±0.1156	0.6600±0.1386	0.64±0.16	0.6500±0.0975
4 Gy	0.62900±0.08177	0.730±0.146	0.7200±0.1656	0.7400±0.1406	0.700±0.119
6 Gy	0.628839±0.188652	1.064748±0.266187	1.004003±0.240961	0.981626±0.176693	0.768190±0.107547
γ-Irradiation					
2 Gy	1.077451±0.150843	0.855864±0.102704	0.920±0.092	0.7500±0.0675	0.687480±0.082498
4 Gy	1.022001±0.102200	0.8100±0.0891	0.8800±0.0528	0.700±0.056	0.650±0.052
6 Gy	0.800±0.112	0.662409±0.066241	0.791576±0.071242	0.680±0.068	0.629987±0.044099

Note. Results for carbon ion- and γ-ray-irradiated groups do not differ significantly at equivalent time points after equivalent doses of irradiation, but expression levels tended to increase with the dose of carbon ion irradiation, whereas they decreased with the dose of γ-irradiation, as compared to non irradiated cells. Data shown are representative of three independent experiments.

TABLE 3. Ratio of RANKL mRNA to OPG mRNA over the Time Course of the Experiment

Group (irradiation dose)		Time, days				
		1	2	3	5	7
Non-irradiated		0.047104±0.023000	0.048758±0.026000	0.014179±0.005100	0.011099±0.010000	0.005912±0.004000
2 Gy	carbon ion irradiation	0.172966±0.041000	0.049282±0.032000	0±0	0±0	0.036283±0.022000
	γ-irradiation	0.003868±0.002000	0.012807±0.002000	0.017844±0.001800	0.025229±0.016000	0.035663±0.018000
4 Gy	carbon ion irradiation	0.093519±0.029000	0.054074±0.017000	0±0	0±0	0±0
	γ-irradiation	0.025628±0.023000	0.061326±0.025000	0.037013±0.005000	0.19421±0.03400	0.215773±0.039000
6 Gy	carbon ion irradiation	0.005141±0.003000	0.003218±0.002000	0±0	0±0	0±0
	γ-irradiation	0.196294±0.042000	0.220386±0.052000	0.137615±0.070000	0.05913±0.01400	0.105541±0.028000

Note. Results for the carbon ion- and γ-ray-irradiated groups differ significantly ($p<0.01$) at equivalent time points after equivalent doses of irradiation, except for the results obtained on the second day after 4 Gy of irradiation. Data shown are representative of three independent experiments.

than in cells irradiated with γ-rays; this effect was not evident on days 1 and 2 after 2-Gy exposure, and on day 1 after 4-Gy exposure exceptionally, and was caused by a transient increase in RANKL mRNA of carbon ion irradiated cell. The RANKL/OPG mRNA ratios tend to be suppressed by carbon ion irradiation.

Co-cultured MC3T3-E1 and bone marrow cells were fixed one week after irradiation, and the number of TRAP-positive cells was determined for each radiation exposure level. Incubation of bone marrow cells with γ-irradiated MC3T3-E1 cells resulted in the formation of considerable numbers of TRAP-positive osteoclasts. In contrast, carbon ion irradiation suppressed osteoclastogenesis in a dose-dependent manner (Fig. 2). Fewer TRAP-positive osteoclasts were generated in the carbon ion-irradiated group than in the non-irradiated group, whereas more osteoclasts were generally observed in the γ-irradiated group than in the non-irradiated group. At the same dose of radiation, the numbers of osteoclasts in the two irradiated groups differed significantly ($p<0.05$).

Extremely large osteoclast-like cells were formed from bone marrow cells co-cultured with γ-irradiated MC3T3-E1 cells (Fig. 3, *c*). These cells were larger than osteoclast-like cells formed in co-cultures with non-irradiated MC3T3-E1 cells and exhibited strong TRAP activity. In contrast, only small TRAP-positive cells were found in co-cultures of carbon ion-irradiated MC3T3-E1 cells (Fig. 3, *b*).

Conventional irradiation commonly causes a loss of bone density via osteoclast-mediated bone resorption; however, the mechanism of osteoclast activation is still not clear. In our previous *in vivo* study we demonstrated that carbon ion irradiation in a dose of 15-30 Gy causes a significant increase in bone volume and in the thickness of the trabeculae in experimental animals, whereas γ-irradiation at the same dose results in significantly decreased bone volume [7]. Exposure to 15 or 22.5 Gy of carbon ions or γ-rays also induced a lot of clearly small osteoclasts in the tibiae of experimental animals immediately after irradiation; the appearance of numerous immature osteoclasts appeared to compensate for the irradiation-induced decline in bone absorption capability. In the animals irradiated with carbon ions, the number of osteoclasts subsequently decreased sharply. These previous results resemble those of the present study, in which the RANKL/OPG ratio in carbon ion-irradiated cells increased transiently, immediately after irradiation, and then acute decreased. The mean osteoclast size was smaller in rats irradiated with carbon ions than in rats irradiated with γ-rays [8].

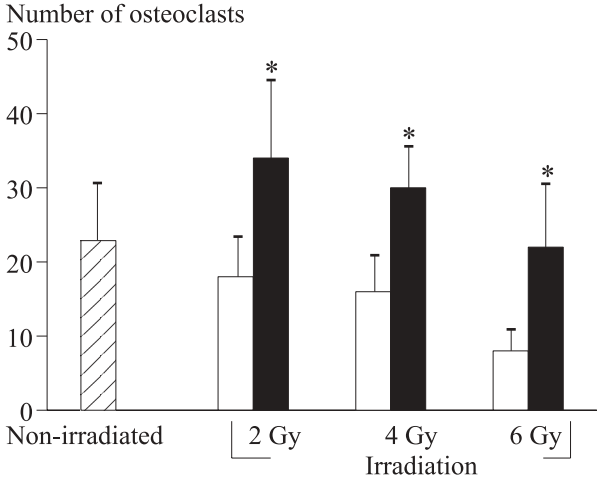

Fig. 2. Numbers of osteoclasts generated from bone marrow cells co-cultured for one week with carbon ion-irradiated, γ-irradiated, or non-irradiated MC3T3-E1 cells. $p<0.05$ compared to cells exposed to carbon ion irradiation in the equivalent dose.

The stimulation of osteoclast formation by osteoblastic MC3T3-E1 cells involves several extracellular regulators of osteoclastogenesis, including RANKL [4,15]. Therefore, in the present study, we examined the effects of carbon ions and g-rays on the production of osteoclast differentiation factors. We found that an increased ratio of osteoblast RANKL mRNA to OPG mRNA was correlated with osteoclast maturation and bone resorption. This finding is consistent with previous reports that the RANKL/OPG mRNA ratio in the microenvironment regulates osteoclast formation [2,3].

When mouse bone marrow cells were cultured with 10^{-10} M 1,25-dihydroxycholecalciferol, TRAP-positive multinucleated cells formed abundantly [13]. To our knowledge, this study is the first to use a co-culture system consisting of bone marrow cells and carbon ion- or γ-ray-irradiated MC3T3-E1 cells. This co-culture technique was used to induce bone marrow precursors to differentiate into osteoclast-like cells. γ-Irradiation resulted in increased formation of TRAP-positive osteoclasts, whereas carbon ion irradiation suppressed the formation of TRAP-positive cells. The production of osteoclast differentiation factors was suppressed by the incubation of bone marrow stromal cells with carbon ion-irradiated MC3T3-E1 cells.

We postulate that the different effects of carbon ions and γ-rays on the RANKL/OPG mRNA ratio are behind these results. Our data suggest that the microenvironment of osteoclastic precursors, with regard to osteoblasts, is an important factor in the local regulation of bone resorption after bone irradiation, and that carbon ion irradiation increases bone volume by reducing RANKL expression and

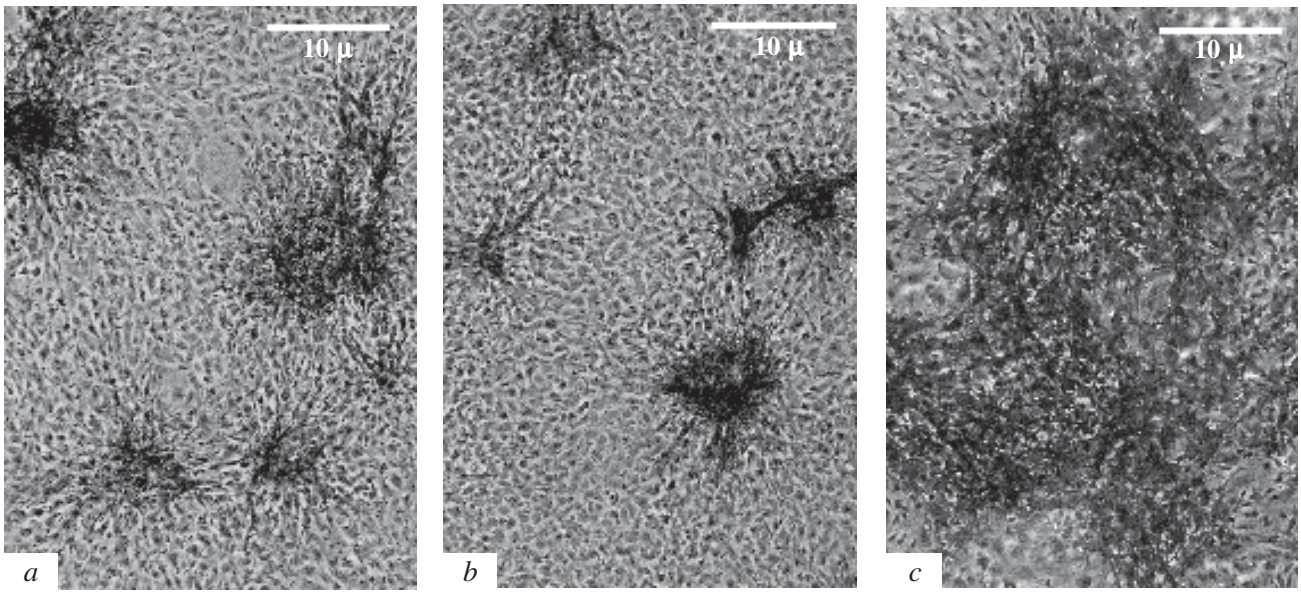

Fig. 3. TRAP-staining of osteoclast-like cells on day 6 after initiation of co-culture of bone marrow cells with irradiated or non-irradiated MC3T3-E1 cells. *a***)** osteoclast-like cells in co-culture with non-irradiated MC3T3-E1 cells. *b***)** osteoclast-like cells in co-culture with MC3T3-E1 cells exposed to 6 Gy of carbon ion irradiation. *c***)** osteoclast-like cells in co-culture with MC3T3-E1 cells exposed to 6 Gy of γ-irradiation.

subsequently inhibiting osteoclastogenesis and bone resorption. Our study focused on the dynamic interaction between the RANKL/OPG mRNA ratio and osteoclasts. As the first demonstration of carbon ion or γ-irradiation-induced expression of RANKL with concomitant stimulation of osteoclastogenesis by osteoblasts, this study substantially advances our understanding of the molecular mechanism by which carbon ions or γ-rays affects osteoclastogenesis. Further research to identify other radiation-affected osteoclast differentiation factors is needed. Future studies might address the range of cytokines generated in this system.

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