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Early Increase in Osteoclast Number in Mice after Whole-Body Irradiation with 2 Gy X Rays

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Abstract

Bone loss is a consequence of exposure to high-dose radio-therapy. While damage to bone vasculature and reduced proliferation of bone-forming osteoblasts has been implicated in this process, the effect of radiation on the number and activity of bone-resorbing osteoclasts has not been characterized. In this study, we exposed mice to a whole-body dose of 2 Gy of X rays to quantify the early effects of radiation on osteoclasts and bone structural properties. Female C57BL/6 mice (13 weeks old) were divided into two groups: irradiated and nonirradiated controls. Animals were killed humanely 3 days after radiation exposure. Analysis of serum chemistry revealed a 14% increase in the concentration of tartrate resistant acid phosphatase (TRAP)-5b, a marker of osteoclast activity, in irradiated mice ($P < 0.05$). Osteoclast number (+44%; $P < 0.05$) and osteoclast surface (+213%; $P < 0.001$) were elevated in TRAP-stained histological sections of tibial metaphyses. No significant change was observed in osteoblast surface or osteocalcin concentration or in trabecular microarchitecture (i.e. bone volume fraction) as measured through microcomputed tomography ($P > 0.05$). This study provides definitive, quantitative evidence of an early, radiation-induced increase in osteoclast activity and number. Osteoclastic bone resorption may represent a contributor to bone atrophy observed after therapeutic irradiation.

INTRODUCTION

Bone atrophy and increased risk of bone fracture are consequences of exposure to radiation for cancer treatment (1,2). Osteopenia and osteoporosis have been characterized as pathological conditions after therapeutic irradiation (3,4). There is an increased incidence of spontaneous hip fractures demonstrated by patients receiving radiation to treat pelvic cancers, with the fractures generally occurring 1 to 5 years after therapy (1,5-7). As long-term survivorship increases with improved diagnosis and treatment, the morbidity and mortality associated with osteoporosis and hip fractures within this population is becoming a significant concern.

This loss of bone mass after radiotherapy has been hypothesized to occur as a result of damage to bone-forming osteoblasts and the bone vasculature itself (1-4,8). While previous studies typically observed atrophy as a late effect, loss of volumetric bone mineral content has been reported in cervical cancer patients 5 weeks after treatment and has been described as a low-turnover type of osteoporosis (4). An inhibition of osteoblasts and osteoblast progenitors after

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radiation exposure has been further described both *in vitro* (9-12) and *in vivo* (13-15), and we have reported a long-term reduction of bone mass in irradiated mice (16). Despite evidence that bone loss can occur soon after irradiation, a putative increase in osteoclast activity has received little attention as a potential contributor to radiation-induced osteoporosis.

The effect of radiation on the number and activity of osteoclasts is varied in published reports, from observed decreases in osteoclast numbers (13,17,18), to stable numbers (19,20), to a qualitative description of an increase in the osteoclast population (21). A better understanding of the effects of radiation on osteoclasts is needed to reduce or prevent the subsequent bone atrophy and fracture risk.

In the present study we examined the effects of acute, whole-body irradiation of mice with 2 Gy of X rays on bone histology, trabecular microarchitecture, and markers of bone resorption. Since loss of bone mineral density has been observed several weeks after exposure to radiation, we wanted to determine whether osteoclast activation very early after exposure might contribute to this relatively acute loss of bone. Thus we examined the response after 3 days. This particular time was chosen to provide the framework for future, longer-term time-course studies characterizing bone cell number and function after irradiation. We sought to determine whether an increase in osteoclast number and activity is an early response to radiation exposure.

MATERIALS AND METHODS

Thirty-two C57BL/6 mice (13 weeks old; Charles River Breeding Laboratories, Wilmington, MA) either were irradiated or served as nonirradiated controls ($n = 16$ per group). Subsets of these mice were used for one of three assays: histological analysis, microcomputed tomography (microCT), and serum chemistry analysis. All animals were allowed a 1-week acclimation period prior to the start of the study, with food and water provided *ad libitum*. The Institutional Animal Care and Use Committee at Clemson University approved of all procedures. All animals were killed humanely 3 days after irradiation to collect data on body mass, tibial length and morphology.

While under anesthesia (1.5% isoflurane), mice were irradiated in the prone position with a single field of 140 kVp X rays to a single-fraction mid-plane dose of 2 Gy. Irradiation was performed at a nominal dose rate of 1.37 Gy/min with an exposure time of 1.46 min. A 150 kV industrial portable X-ray unit was used (Philips Medical Systems, Bothell, WA). Anesthetized control mice were placed inside the inactive X-ray unit for a similar amount of time as the treated animals for sham irradiation.

To examine the postirradiation activity of osteoclasts, a set of 12 mice ($n = 6$ /group) were used for histological analyses. Three days after whole-body irradiation, tibiae were isolated, cleaned of soft tissue, fixed in formalin for 48 h, and stored in 70% ethanol. The right tibia was decalcified in a weak formic acid solution (Immunocal™, Decal Chemical Corporation, Tallman, NY). Radiographs assessed the earliest time of complete decalcification. Then tibiae were embedded in a glycol methacrylate resin (Immunobed™, Polysciences, Warrington, PA) and cut into sagittal sections with a thickness of 1.5 μm . The presence of osteoclasts was determined by tartrate-resistant acid phosphatase (TRAP) staining of the slides using a commercial kit (Sigma, St. Louis, MO) and then counterstaining with hematoxylin (Sigma).

Histomorphometric evaluation was performed from captured micro-graphs (400 $\times$) throughout the metaphysis, starting approximately 0.25 mm distal from the growth plate (to exclude the primary spongiosa) and extending a further 0.5 mm. Bone histomorphometric parameters for the proximal metaphysis of the tibia were measured as described in the report of the American Society of Bone and Mineral Research (ASBMR) Histomorphometry Nomenclature Committee (22). Surface measurements were quantified relative to total bone surface (BS). These

measurements included osteoblast surface (Ob.S/BS; %), osteoclast surface (Oc.S/BS; %), eroded surface with the inclusion of osteoclast surface (surface covered by Howship's lacunae plus osteoclasts, [ES(Oc+)/BS], %), and eroded surface with the exclusion of osteoclast surface (surface covered by Howship's lacunae, [ES(Oc-)/BS], %). The number of osteoclasts (N.Oc) within the region of interest along trabeculae of the secondary spongiosa was also determined (N.Oc/BS, mm⁻¹).

Twenty mice ($n = 10$ /group) were treated identically for examination of trabecular microarchitecture and serum chemistry markers of bone resorption. The right tibiae were scanned with microCT (Scanco Medical AG, Bassersdorf, Switzerland), with isotropic voxels of 9 μ m/side. A 3-mm section of bone immediately distal to the proximal growth plate was scanned. Evaluation of the scanned region began immediately adjacent to the primary spongiosa and extended 1 mm. Trabecular bone parameters including bone volume fraction (BV/TV), connectivity density (Conn.D), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) were quantified.

To confirm osteoclast activation with a systemic analysis technique, serum was collected from these mice after they were killed by cardiac puncture and exsanguination. Sufficient blood for further serum analysis was collected from eight of the ten mice in each group. ELISAs were used to determine the blood serum concentration of tartrate-resistant acid phosphatase (TRAP)-5b, a marker of osteoclast activity that is associated with bone resorption (ImmunoDiagnostic Systems Inc., Fountain Hills, AZ), and osteocalcin, a marker of osteoblast activity that is associated with bone formation (Biomedical Technologies Inc., Stoughton, MA).

Serum and other histomorphometric analyses were tested for statistical significance using SigmaStat Version 3.5 (Systat Software Inc., Richmond, CA). A Student's t test was performed to reveal significant differences between the control and irradiated groups. A P value of less than 0.05 was considered a significant difference. All data are presented as means $\pm$ SEM.

RESULTS

TRAP-stained sections of bone were used to identify osteoclasts within tibial metaphyses (Fig. 1A, B) and to quantify surface measurements (Table 1, Fig. 1C) from mice 3 days after irradiation with 2 Gy. Large, multinucleated cells were evident along the trabeculae in both irradiated and control mice (Fig. 1A, B). Flattened, TRAP-positive cells with few nuclei or a single nucleus were also present along irradiated trabeculae (Fig 1B). No significant differences were observed in ES(Oc-)/BS (eroded surface excluding osteoclast surface), BS or Ob.S/BS (Table 1; $P > 0.05$). A 79% increase in ES(Oc+)/BS (eroded surface including osteoclast surface) and a 213% increase in Oc.S/BS was observed within the irradiated tibiae compared to nonirradiated controls (Table 1, Fig. 1C; $P < 0.001$). In addition, there was a 44% increase in N.Oc/BS in irradiated mice (Table 1, Fig. 1D; $P < 0.05$).

Relative to nonirradiated controls, there was no significant difference in trabecular architectural parameters as determined by microCT (Table 1, $P > 0.05$).

At the time of killing there was no change in the body mass of irradiated (18.9 ± 0.2 g compared to 19.2 ± 0.2 g) or nonirradiated mice (18.8 ± 0.3 g compared to 18.8 ± 0.3 g) ($P > 0.05$). The concentration of TRAP-5b in serum harvested from mice 3 days after irradiation was 14% greater than in the nonirradiated controls (Table 1; $P < 0.05$). There was 13% decline in the concentration of serum osteocalcin, although this difference was not statistically significant (Table 1, $P > 0.05$).

DISCUSSION

We have demonstrated that an acute, low dose of ionizing radiation (2 Gy) has the capacity to significantly increase *in vivo* osteoclast number and activity early after exposure. Three days after irradiation we observed increases in circulating TRAP-5b, an indicator of bone resorption produced by osteoclasts (Table 1). There was also an increase in the number of osteoclasts and the extent of resorption surfaces lining trabeculae (Table 1, Fig. 1C, D). The general morphology (i.e., size and number of nuclei) of osteoclasts from control and irradiated individuals was largely similar. However, more flattened TRAP⁺ cells, with few nuclei or a single nucleus, were present after irradiation. TRAP⁺ mononuclear cells play a role in bone resorption (23), and osteoclasts with fewer nuclei are consistent with other observations of osteoclasts after irradiation (13). Despite evidence of increased osteoclast activity, no differences were observed for BV/TV or other microarchitectural indices as determined by microCT analysis (Table 1). Similarly, BS (two-dimensional value determined from histological samples) was unchanged between groups. Because the number of active osteoclasts along the trabeculae of the metaphyses were substantially elevated 3 days after exposure, it is likely that appreciable bone loss (at least within the region examined) would occur on subsequent days.

This study represents robust and quantitative evidence of a significant increase in osteoclast activity and number soon after irradiation. Previous studies noting the presence and activity of osteoclasts within irradiated bone and cell culture examined the response at a range of times, doses, radiation types, energies and ages/strains of mice, with varied results. In embryonic mice irradiated on days 14–16 of gestation, osteoclast numbers decrease in a dose-dependent manner (18). Osteoclasts cultured from 17-day-old embryos seem more resistant; however, no increase was observed after irradiation. The majority of studies of older animals commenting on osteoclast numbers after radiation exposure documented either no change in number or a marked decrease, depending on the dose and location (13,19,20). However, these studies were limited, in that examinations were performed at times greater than 1 week. Other studies that have discussed an increase in osteoclast number have done so in the context of qualitative, non-histomorphometric data obtained from the tibiae of weanling rats, in combination with reduced body mass, vascularity and decreased osteoblast activity (21).

The reduction in bone mineral density and deterioration of trabecular microarchitecture quantified weeks to months after high-dose radiation exposure in humans is substantial (4). However, these effects have been attributed primarily to reduced bone formation and sclerotic occlusion of the vasculature (3). We have previously described long-term (i.e. 4-month) loss of trabecular bone in mice exposed to 2 Gy of radiation (16). Ionizing radiation inhibits osteoblast proliferation, promotes cell cycle arrest, increases sensitivity to agents that induce apoptosis, and reduces collagen production (9,11,12). Arrested *in vivo* bone formation can persist for months after radiation exposure (14). Although a progressive decline in osteoblast number occurs in the first month after irradiation (13,15), the results of the present study demonstrated no change in osteoblast surface or serum osteocalcin, a marker of bone formation, after 3 days (Table 1). An early/acute increase in osteoclast activity after irradiation could contribute to the reduction in bone volume and density in concert with the presumed eventual reduction in osteoblast function. Bone loss has been documented within the secondary spongiosa of the proximal tibia in weanling rats starting 5 days after exposure of the knee joint to single doses of 15–30 Gy of γ rays; however, these effects were quantified from very small (50 μm^2) regions of the metaphysis using histology (24). This may support an early, osteoclast-mediated loss of bone early after irradiation. More advanced three-dimensional imaging techniques (i.e. microCT) throughout a greater area of the metaphysis are necessary to identify how early changes in bone cell populations can influence trabecular volume and, perhaps more importantly, microarchitecture.

In summary, osteoclast number and activity in mice are increased early after exposure to 2 Gy X rays. How activated osteoclasts may contribute to bone atrophy and fractures over time requires additional study. Additionally, no mechanistic information is currently available to explain how radiation exposure may increase osteoclast number and resorption. The mice in the present study were exposed to whole-body radiation, such that multiple organs capable of influencing bone homeostasis were irradiated. Future studies should appropriately model therapeutic radiation exposure and better isolate the response of bone and bone cells by employing partial-body or targeted exposure. The early elevation of osteoclast number and activity reported in the present study could provide a viable pharmacological target for the prevention of osteoporosis or fractures after radiotherapy.

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REFERENCES

1. Baxter NN, Habermann EB, Tepper JE, Durham SB, Virnig BA. Risk of pelvic fractures in older women following pelvic irradiation. *J. Am. Med. Assoc* 2005;294:2587–2593.
2. Ergun H, Howland WJ. Postradiation atrophy of mature bone. *CRC Crit. Rev. Diagn. Imaging* 1980;12:225–243. [PubMed: 6985580]
3. Hopewell JW. Radiation-therapy effects on bone density. *Med. Pediatr. Oncol* 2003;41:208–211. [PubMed: 12868120]
4. Nishiyama K, Inaba F, Higashirara T, Kitatani K, Kozuka T. Radiation osteoporosis—an assessment using single energy quantitative computed tomography. *Eur. Radiol* 1992;2:322–325.
5. Bliss P, Parsons CA, Blake PR. Incidence and possible aetiological factors in the development of pelvic insufficiency fractures following radical radiotherapy. *Br. J. Radiol* 1996;69:548–554. [PubMed: 8757658]
6. Grigsby PW, Roberts HL, Perez CA. Femoral neck fracture following groin irradiation. *Int. J. Radiat. Oncol. Biol. Phys* 1995;32:63–67. [PubMed: 7721641]
7. Ogino I, Okamoto N, Ono Y, Kitamura T, Nakayama H. Pelvic insufficiency fractures in postmenopausal woman with advanced cervical cancer treated by radiotherapy. *Radiother. Oncol* 2003;68:61–67. [PubMed: 12885453]
8. Huh SJ, Kim B, Kang MK, Lee JE, Lim do H, Park W, Shin SS, Ahn YC. Pelvic insufficiency fracture after pelvic irradiation in uterine cervix cancer. *Gynecol. Oncol* 2002;86:264–268. [PubMed: 12217746]
9. Sakurai T, Sawada Y, Yoshimoto M, Kawai M, Miyakoshi J. Radiation-induced reduction of osteoblast differentiation in C2C12 cells. *J. Radiat. Res. (Tokyo)* 2007;48:515–521. [PubMed: 17928745]
10. Gevorgyan A, La Scala GC, Sukhu B, Leung IT, Ashrafpour H, Yeung I, Neligan PC, Pang CY, Forrest CR. An *in vitro* model of radiation-induced craniofacial bone growth inhibition. *J. Craniofac. Surg* 2007;18:1044–1050. [PubMed: 17912079]
11. Gal TJ, Munoz-Antonia T, Muro-Cacho CA, Klotch DW. Radiation effects on osteoblasts *in vitro*: a potential role in osteoradionecrosis. *Arch. Otolaryngol. Head Neck Surg* 2000;126:1124–1128. [PubMed: 10979127]
12. Szymczyk KH, Shapiro IM, Adams CS. Ionizing radiation sensitizes bone cells to apoptosis. *Bone* 2004;34:148–156. [PubMed: 14751572]
13. Sawajiri M, Mizoe J, Tanimoto K. Changes in osteoclasts after irradiation with carbon ion particles. *Radiat. Environ. Biophys* 2003;42:219–223. [PubMed: 13680258]
14. Sugimoto M, Takahashi S, Toguchida J, Kotoura Y, Shibamoto Y, Yamamuro T. Changes in bone after high-dose irradiation. Bio-mechanics and histomorphology. *J. Bone Joint Surg. Br* 1991;73:492–497. [PubMed: 1670456]

15. Ma J, Shi M, Li J, Chen B, Wang H, Li B, Hu J, Cao Y, Fang B, Zhao RC. Senescence-unrelated impediment of osteogenesis from Flk1+ bone marrow mesenchymal stem cells induced by total body irradiation and its contribution to long-term bone and hematopoietic injury. *Haematologica* 2007;92:889–896. [PubMed: 17606438]
16. Hamilton SA, Pecaut MJ, Gridley DS, Travis ND, Bandstra ER, Willey JS, Nelson GA, Bateman TA. A murine model for bone loss from therapeutic and space-relevant sources of radiation. *J. Appl. Physiol* 2006;101:789–793. [PubMed: 16741258]
17. Anderson ND, Colyer RA, Riley LH Jr. Skeletal changes during prolonged external irradiation: alterations in marrow, growth plate and osteoclast populations. *Johns Hopkins Med. J* 1979;145:73–83. [PubMed: 470292]
18. Scheven BA, Burger EH, Kawilarang-de Haas EW, Wassenaar AM, Nijweide PJ. Effects of ionizing irradiation on formation and resorbing activity of osteoclasts *in vitro*. *Lab. Invest* 1985;53:72–79. [PubMed: 4010232]
19. Goblirsch M, Lynch C, Mathews W, Manivel JC, Mantyh PW, Clohisy DR. Radiation treatment decreases bone cancer pain through direct effect on tumor cells. *Radiat. Res* 2005;164:400–408. [PubMed: 16187742]
20. Vit JP, Ohara PT, Tien DA, Fike JR, Eikmeier L, Beitz A, Wilcox GL, Jasmin L. The analgesic effect of low dose focal irradiation in a mouse model of bone cancer is associated with spinal changes in neuro-mediators of nociception. *Pain* 2006;120:188–201. [PubMed: 16360279]
21. Furstman LL. Effect of radiation on bone. *J. Dent. Res* 1972;51:596–604. [PubMed: 4501299]
22. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J. Bone Miner. Res* 1987;2:595–610. [PubMed: 3455637]
23. Domon T, Wakita M. Electron microscopic and histochemical studies of the mononuclear osteoclast of the mouse. *Am. J. Anat* 1991;192:35–44. [PubMed: 1750380]
24. Sawajiri M, Mizoe J. Changes in bone volume after irradiation with carbon ions. *Radiat. Environ. Biophys* 2003;42:101–106. [PubMed: 12768291]

FIG. 1.

Representative images after tartrate-resistant acid phosphatase (TRAP) staining within the proximal metaphysis from (panel A) nonirradiated control mice and (panel B) mice irradiated with 2 Gy 3 days previously. Original magnification 400 $\times$. Sections were stained to indicate the presence of red-colored TRAP⁺ osteoclasts along the trabecular surfaces (indicated by arrows). For the histomorphometric variables determined from these sections, values were quantified within the secondary spongiosa, extending 0.5 mm distal from the primary spongiosa. Panel C: Osteoclast surface as a percentage of total bone surface (Oc.S/BS; %), eroded surface with the exclusion of osteoclast surface [ES(Oc-)/BS]; and eroded surface with the inclusion of osteoclast surface [ES(Oc+)/BS]. Panel D: The number of osteoclasts (N.Oc/BS) as a percentage of total bone surface. Error bars represent SEM. ^a $P < 0.001$ and ^b $P < 0.05$, after *t* test.

TABLE 1
Histological Parameters, Trabecular Microarchitectural Parameters, and Bone Metabolism Markers Collected from Mice Three Days after Whole-Body Irradiation with 2 Gy of X Rays

	Nonirradiated	Irradiated (2 Gy)	Percentage change
Histological parameters (<i>n</i> = 6/group)			
BS (mm)	6.26 ± 0.57	5.69 ± 0.33	−9%
ES(Oc+)/BS (%)	19.0 ± 2.3	34.1 ± 1.9 ^a	+79% ^a
ES(Oc−)/BS (%)	14.2 ± 2.0	19.6 ± 2.2	+38%
Oc.S/BS (%)	4.8 ± 0.9	15.0 ± 1.5 ^a	+213% ^a
Ob.S/BS (%)	14.6 ± 2.2	14.2 ± 1.9	−3%
N.Oc/BS (mm ^{−1})	4.5 ± 0.4	6.5 ± 0.5 ^b	+44% ^b
Trabecular microarchitecture (<i>n</i> = 10/group)			
BV/TV (%)	6.76 ± 0.47	7.45 ± 0.10	+10%
Conn.D (N/mm ³)	27 ± 5	35 ± 4	+30%
Tb.Th (μm)	43.7 ± 0.5	43.2 ± 0.5	−1%
Tb.N (N/mm ³)	3.37 ± 0.12	3.56 ± 0.08	+6%
Tb.Sp (μm)	297 ± 12	280 ± 7	−6%
Serum bone metabolism markers (<i>n</i> = 8/group)			
TRAP-5b (U/liter)	9.84 ± 0.24	11.17 ± 0.55 ^b	+14% ^b
Osteocalcin (ng/ml)	732 ± 55	638 ± 58	−13%

Notes. BV/TV, bone volume fraction, connectivity density (Conn.D); trabecular thickness (Tb.Th.); trabecular number (Tb.N); trabecular separation (Tb.Sp.); bone surface (BS); eroded surface with the inclusion of osteoclast surface [ES(Oc+)/BS]; eroded surface with the exclusion of osteoclast surface [ES(Oc−)/BS]; osteoclast surface (Oc.S); osteoblast surface (Ob.S); number of osteoclasts (N.Oc/BS). All values are mean ± SE.

^a $P < 0.001$

^b $P < 0.05$ after *t* test.