Inhibitory effects of autologous γ-irradiated cell conditioned medium on osteoblasts *in vitro*

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Received March 29, 2014; Accepted January 15, 2015

DOI: 10.3892/mmr.2015.3354

Abstract. Skeletal complications from radiation therapy have been reported in patients with breast, brain and pelvic cancer, and types of blood cancer. However, it remains to be elucidated whether localized radiotherapy may result in systemic adverse effects on the unirradiated skeleton through an abscopal mechanism. The present study investigated the abscopal effect of radiation on osteoblasts mediated by autologous γ-irradiated cell conditioned medium. Osteoblasts obtained from calvarial bones were incubated with irradiated cell conditioned medium (ICCM) and changes in cell viability, alkaline phosphatase (ALP) activity, mineralization ability, cell apoptosis and the gene expression levels of ALP, osteocalcin (BGP), osteoprotegerin (OPG), receptor activator of nuclear factor-κB ligand (RANKL) and caspase 3 were observed. Notably, ICCM regulated osteoblast function, inhibiting viability and differentiation, resulting in apoptosis or cell death. ICCM at 10 or 20%, from osteoblasts irradiated with 10 Gy γ-rays, significantly inhibited the proliferation of osteoblastic cells ($P<0.001$). In addition, an increase in apoptosis was noted in the osteoblasts incubated with ICCM at 40% with increasing doses of radiation, accompanied by an upregulation in the mRNA expression of caspase 3. In addition, ICCM at 20% inhibited the ALP activity in the 5 and 10 Gy groups and osteoblast mineralization, particularly at 10 Gy ICCM. Additionally, the mRNA expression levels of ALP, BGP, OPG and RANKL of the cells treated with ICCM at 20% were downregulated significantly compared with those treated with medium from unirradiated cells. The present study provided novel evidence to elucidate radiation-therapy-associated side effects on the skeleton.

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Key words: abscopal effect, radiotherapy, osteoblast, irradiation

Introduction

Radiation therapy is an important approach for cancer treatment worldwide. It was reported by The American Cancer Society that ~4 million new cases of cancer were recorded in the USA in 2006, and ~50% of these patients received radiation therapy as treatment (1). Radiation therapy has been important in the-improving survival rates of cancer patients. Currently 62% of adults and >75% of patients with pediatric cancer survive over five years in the United States (2). However, early and late toxicity limits the deliverable intensity of radiotherapy and may affect a patient's long-term quality of life. The present study investigated the effects on bone loss, which is an area of clinical concern. The improved survival rates of cancer patients receiving radiotherapy increases the importance of understanding the causal mechanisms and possible effects of radiation-induced bone loss (2).

Radiotherapy may induce bone loss, which can result in pathological fractures. In a previous study of >6,000 females aged >65 years, treated for cervical, rectal and anal cancer, it was reported that radiation therapy significantly increased the risk of pelvic fracture by a relative risk of 1.66, 1.65 and 3.16, respectively (3). Abe *et al* (4) reported that pelvic insufficiency fractures were identified in 27 of 80 (34%) Asian patients with uterine cancer. Blomlie *et al* (5) reported prospectively that 16 of 18 (89%) advanced cervical carcinoma patients exhibited findings compatible with radiation-induced insufficiency fractures through magnetic resonance imaging. Therefore, radiation compromises bone health and can have a severe impact on the functional capabilities of patients. The rate and severity of radiation-induced bone mass loss is greater compared with bone damage due to postmenopausal hormone changes or due to treatment with glucocorticoids (6,7).

However, there remains no consensus on the mechanism underlying bone damage from radiation exposure, based on the anatomical location of the bone relative to the radiation exposure. While Baxter *et al* (3) found that the incidence of arm or spine fractures, outside the treatment volume, was similar in irradiated and non-irradiated groups, Zuppinger and Minder (8) demonstrated a non-targeted effect of radiation on bone metabolism. In another study, the unirradiated tibiae of animals that had received 800 rad to another hind extremity exhibited significant differences in tibial lengths compared with the bones of control animals, indicating significant

abscopal growth retardation (9). Jia *et al* (10) also provided evidence that post-radiotherapy fractures in the unirradiated skeleton may result from the abscopal impact of local irradiation. Decreases in serum levels of a bone formation marker, the rate of *in vitro* osteoblast differentiation and bone mineral density following administering male C57BL/6 mice with a single dose of 15 Gy x-rays to the abdomen (10). In addition, clinical investigations involving local radiotherapy have revealed that a femoral fracture rate of 1,716 breast cancer patients 1 year after treatment to the chest wall is >20 times higher compared with the overall annual hip fracture rate (11).

The direct effect of γ-irradiation on osteoblastic cells was examined in our previous study and the osteoblasts were significantly affected (12). However, the mechanism of the abscopal effect remains to be elucidated. The aim of the present study was to investigate the cellular mechanisms underlying the abscopal degradation of the unexposed skeleton, which remains essentially undefined. The elucidation of these mechanisms may have important clinical implications as these mechanisms may define improved preventive or curative solutions for bone loss. Thus, the radiation-induced bystander effect is an attractive target for investigation as alterations in osteoblast function may be, in part, associated with abscopal effects of bone loss. Using a medium transfer procedure, the present study aimed to investigate the complex interplay between radiotherapy and bone loss.

Materials and methods

Radiation exposure. For irradiation, osteoblasts ($5x10^5$) were plated in 25 cm^2 flasks, cultured for one day at 37˚C in an atmosphere containing 5% CO_2 and then exposed to γ-rays generated by a ^{137}Cs source (Gammacell 40 Exactor; Nurdion International Inc., Kanata, ON, Canada) at a dose rate of 0.76 Gy/min. The irradiation doses were selected as 0, 1, 2, 5 and 10 Gy, which are biologically equivalent doses of clinical relevance.

Chemicals. Minimum essential medium (MEM) and fetal bovine serum (FBS) were purchased from Gibco-BRL (Gaithersburg, MD, USA). Penicillin-G was purchased from Shanghai Asia Pioneer Pharmaceutical Co., Ltd. (Shanghai, China). Streptomycin was purchased from North China Pharmaceutical Group Corporation (Shijiazhuang, China). Oligonucleotide primer sets were synthesized by Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). An Alexa Fluor 488 Annexin V/dead cell apoptosis kit was purchased from Invitrogen Life Technologies, (Carlsbad, CA, USA).

Animals. Sprague-Dawley (SD) male rats at 8 weeks old were obtained from the Department of Laboratory Animal Science, Fudan Univeristy (Shanghai, China) weighing 180-200 g. The rats were fed *ad libitum* and were housed in specific pathogen free animal facilities under controlled conditions (temperature 21±1˚C; relative humidity 50±10% and 12:12 h light-dark cycle).

Cell isolation and primary culture of osteoblasts. The procedures used in the present study were reviewed and approved by the Committee for Ethical Use of Experimental Animals at Fudan University. Osteoblasts were prepared from the calvarias of 1 or 2 day old SD rats using a sequential enzymatic digestion method, as described previously (12). Briefly, the calvarias were incubated at 37˚C for 10 min with 0.1% collagenase and 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) in calcium- and magnesium-free phosphate-buffered saline (PBS). After 10 min the supernatant was discarded, more enzyme solution was added and the incubation continued at 37˚C for a further 10 min. This process was repeated four times. The cells obtained during the last four digestions were pooled in MEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin). The cells were then centrifuged at 250 x g for 5 min and the pellets were suspended in MEM containing 10% FBS. The cells ($1x10^5$cells/ml) were grown in MEM supplemented with 10% FBS, 100 U/ml penicillin-G and 100 μg/ml streptomycin in flasks. The cells were maintained in an incubator at 37˚C in an atmosphere of 5% CO_2. Subculture was routinely performed using a 1:1 solution of 0.25% trypsin and 0.02% EDTA (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) at 37˚C. The medium was replaced every 2 days. All assessments were performed at the first subculture of osteoblasts. The osteoblastic cells were observed and images were captured using an inverted microscope (DMI3000B; Leica Microsystems GmbH, Wetzlar, Germany). The phenotype was identified via alkaline phosphatase (ALP) staining using a nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) staining kit (Beyotime Institute of Biotechnology, Haimen, China).

Collection of irradiated cell conditioned medium (ICCM). Culture flasks (25 cm^2, 40 ml flasks; Corning, Inc., Acton, MA, USA) containing ~$5x10^5$ cells were seeded and irradiated at room temperature using a ^{137}Cs instrument delivering ~0.76 Gy/min during the period of the experiment. Control flasks were sham-irradiated and the medium collected was considered the control medium. The cells were returned to the 37˚C incubator immediately following irradiation. The medium was removed from the donor flasks 24 h following irradiation and the cells were washed twice with PBS. The osteoblasts were then cultured in 5.0 ml serum-free MEM for an additional 24 h at 37˚C in an atmosphere containing 5% CO_2. The serum-free culture medium was collected and filtered through a 0.22 μm filter to sterilize the solutions and to ensure that no cells remained in the transferred medium. The medium was then divided into 1 ml aliquots. No intact cells were present, as detected by the examination of the aliquots of the medium under a microscope (DMI3000B; Leica Microsystems GmbH). The medium was then divided into aliquots in 1 ml volumes, stored at -20˚C and thawed only once when required for subsequent experiments. The donor medium generated was referred to as ICCM.

Cellular viability of osteoblasts. Osteoblasts were incubated in 96 well plates at $4x10^3$ cells/well and cultured in medium for 24 h at 37˚C in an atmosphere containing 5% CO_2. The cells were treated, as described in Table I. The extent of cellular viability in response to different doses of the conditioned medium was determined using an MTT assay (Amresco LLC, Solon, OH, USA), as described previously (13). The culture

Table I. Experimental groups of osteoblasts treated with different irradiation doses and concentrations of ICCM.

Irradiation dose (Gy)	Concentration (%ICCM+%MEM)		
	10% ICCM group	20% ICCM group	40% ICCM group
0	10+90	20+80	40+60
1	10+90	20+80	40+60
2	10+90	20+80	40+60
5	10+90	20+80	40+60
10	10+90	20+80	40+60

ICCM was collected without serum to ensure that serum serotonin is not involved in the production of bystander signals by irradiated cells (43). The serum-free ICCM was added to the complete culture medium. ICCM, irradiated cell conditioned medium; MEM, minimum essential medium.

medium was removed and 110 μl fresh culture medium (without serum) containing 10 μl MTT solution (5 mg/ml in PBS) was added to each well. The cells were then incubated for 4 h at 37˚C and the insoluble formazan crystals were dissolved in 100 μl 10% sodium dodecyl sulfate (Sigma-Aldrich). After 2 h incubation at 37˚C, the optical density was measured at 570 nm using a Multiskan FC reader (Thermo Scientific, Rockford, IL, USA). The results are expressed as the mean ± standard deviation of 8 wells for each group.

Flow-cytometric analysis. Apoptosis was measured by analyzing the membrane redistribution of phosphatidyl serine (PS), based on the binding properties of annexin V to PS and the DNA-interacting capabilities of propidium iodide (PI). The percentage of apoptotic cells was determined by flow cytometry, using an Annexin V fluorescein isothiocyanate (FITC) and PI staining kit (Invitrogen Life Technologies). Briefly, the osteoblasts ($7.5x10^4$ cells/well) were cultured in 6-well culture plates in a humidified 5% CO_2 incubator at 37˚C for 24 h. Following the addition of 20% or 40% conditioned medium or control medium, the cells were cultured for a further 48 h. Following incubation, the cells were harvested by centrifugation at 1,000 x g for 5 min. Subsequently, the cell suspension was obtained and annexin V-FITC and PI staining was performed, according to the manufacturer's instructions.

The cells were resuspended in 100 μl 1X binding buffer supplemented with 1 μl annexin V-FITC and 5 μl PI and maintained at room temperature in the dark for 15 min. Following the incubation period, 400 μl 1X binding buffer was added and the stained cells were maintained on ice and assessed using a FACSCanto flow cytometer (Beckman Coulter, Miami, FL, USA) for flow cytometric analysis.

ALP activity of osteoblasts. Osteoblasts ($4x10^3$ cells/well) were plated into 96-well plates and the ALP activity and protein content were examined, as described previously (14). The cells were lysed in 0.05% Triton X-100 for 1 h at 4˚C and subsequently by sonication (FS-600; U&STAR Ultrasonic Technology Co., Ltd., Hangzhou, China). The total cellular ALP activity was measured in 2-amino-2-methyl-1-propanol buffer, with p-nitro phenyl phosphate (Fluka, Milwaukee, WI, USA) as a substrate, at 37˚C. A total of 0.5 mmol/l NaOH was added to terminate the reactions. Absorbance of the reaction was measured at 405 nm using a Multiskan FC reader. The total protein content in the lysates was measured using a bicinchoninic acid assay (Beyotime Institute of Biotechnology). Subsequently, ALP activity was adjusted to the cell protein content and expressed as U/mg protein.

Mineralized nodule formation in osteoblasts. Mineralization of the nodules in the cultures was assessed by Alizarin red S (ARS; Sinopharm Chemical Reagent Co., Ltd.) staining. A total of six wells were analyzed for each group to determine the area of the mineralized matrix nodules. The osteoblasts ($5x10^4$ cells/well) were plated into 48-well plates and cultured in MEM (1.0 ml/well) containing 15% FBS for 24 h at 37˚C in an atmosphere containing 5% CO_2. Subsequently, the matrix was replaced by MEM culture medium mixed with 20% ICCM of 0, 2, 5 and 10 Gy. The medium was replaced every 2 days. After 7 days, the medium was aspirated and the cells were treated with mineralization medium [MEM supplemented with 15% FBS, 50 μg/ml ascorbic acid and 10 mmol/l β-glycerophosphate (Sigma-Aldrich)] for 21 days. Subsequently, cells were rinsed with PBS and the mineralization nodules were visualized by fixing the cells in 0.25% glutaraldehyde and staining with ARS for 10 min at room temperature. Mineralization was measured by visual counting under an optical microscope (magnification, x100). All six wells from each group were visualized, and the sizes of the nodules were analyzed for each group. Simple PCI (Compix Inc., Cranberry Township, PA, USA) imaging software was used to analyze the area of the mineralization nodes.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Osteoblasts ($5.0x10^4$ cells/well) were plated into 6-well plates and cultured in medium for 24 h at 37˚C in an atmosphere containing 5% CO_2. The cells were cultured with 20% ICCM from osteoblasts irradiated with 0, 2 or 10 Gy γ-rays. The total RNA was isolated using TRIzol reagent (Tiangen Biotech, Beijing, China) and the first-strand cDNA was generated using Transcript First-Strand cDNA Synthesis Supermix (Tiangen Biotech), according to the manufacturer's instructions. The primer sequences used are shown in Table II. The RT-qPCR was performed using SYBR Green supermix (Takara Bio Inc. Ohtsu, Japan) using a Light Cycler 2.0 (Roche

Table II. Primer sequences.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
β-actin	agccatgtacgtagccatcc	ctctcagctgtggtggtgaa	228
ALP	ctgagcgcacgcgagcaac	ggcgtggttcacccgagtgg	116
BGP	gaacagacaagtcccacac	gagctcacacacctccctg	270
OPG	tgggaatgaagatcctccag	gaggaaggaaagggcctatg	109
RANKL	agccgagactacggcaagta	gcgctcgaaagtacaggaac	208
Caspase 3	gccctggcacacgggacttg	gcacagacgccctgatgggg	106

ALP, alkaline phosphatase; BGP, osteocalcin; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; BP, base pairs.

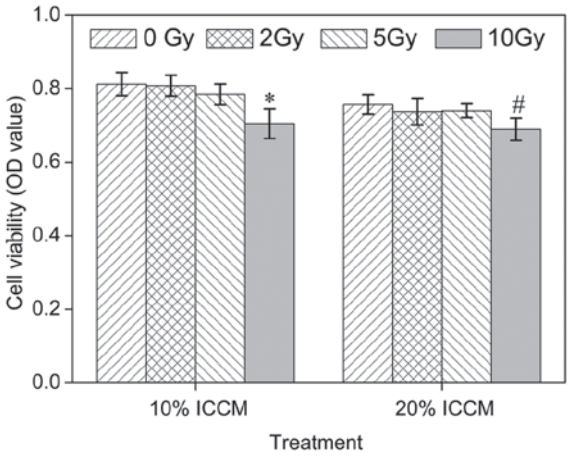

Figure 1. Effect of ICCM on the viability of osteoblasts determined using MTT assays. Data are expressed as the mean ± standard deviation of at least three independent experiments. *P<0.05, compared with 10% group control, #P<0.05, compared with 20% group control. ICCM, irradiated cell conditioned medium; OD, optical density.

Diagnostics, Mannheim, Germany). The relative mRNA expression of the target gene was quantified by measuring the threshold cycle (Ct) and normalized against the mRNA expression of β-actin. Each sample was assessed in triplicate. The fold change was determined using the $2^{-\Delta\Delta CT}$ method (15).

Statistical analysis. Statistical analysis was performed using SPSS 16 (SPSS, Inc, Chicago, IL, USA). All data are expressed as the mean ± standard deviation. A one-way analysis of variance, followed by Dunnett's T3 or Dunnett's test were used to analyze the data. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of ICCM on the viability of osteoblasts. An MTT assay was performed to identify the cell survival in cells cultured with ICCM (10%), or ICCM (20%) for 5 days. As shown in Fig. 1, no significant differences were identified between the responses to 0, 2 and 5 Gy ICCM. However, the viability of cells was significantly decreased in the 10 Gy ICCM group compared with the control cultures in the 10 and 20% groups. The MTT absorbance of the control cells was 0.809±0.031 and 0.757±0.026 in the 10 and 20% groups, respectively, but only 0.705±0.040 and 0.690±0.030 in the 10 Gy ICCM-treated cells in the 10 and 20% groups, respectively.

ICCM-induced cell apoptosis in osteoblasts. The present study subsequently investigated whether ICCM induces cell death through an apoptotic mechanism. The number apoptotic and/or necrotic cells were assessed by measuring the binding of annexin V-FITC to PS expressed at the membrane (early apoptosis) and by staining the nuclei with impermeable fluorescence PI to indicate lost plasma membrane integrity (late apoptotic and/or necrotic cells). These fluorescence-stained cells were analyzed by flow cytometry. A significant increase of apoptosis in the osteoblastic cells was found in the 40% ICCM-treated cultures (P<0.05; Fig. 2A). These data suggested that ICCM induced apoptosis in the osteoblasts.

In addition, it was observed that the expression of caspase 3 was upregulated in the conditioned medium treated cells (Fig. 2B). The ICCM (40%) from osteoblasts irradiated with 5 and 10 Gy upregulated the mRNA expression of caspase 3 compared with the 0 Gy ICCM group and were elevated in a dose-independent manner.

Effects of ICCM on the ALP activity of osteoblasts. Following 5 days of incubation, the production of ALP was evaluated using an NBT-BCIP assay. Compared with the vehicle-treated group, no significant differences were identified in the enzyme production when the osteoblasts were incubated in the presence of ICCM irradiated by 2 Gy γ-ray in the 10 and 20% group. In the groups treated with 5 and 10 Gy, the ICCM attenuated the activity of ALP (Fig. 3).

Effects of ICCM on the mineralization capacity of osteoblasts. The mineralized nodules were stained with ARS (Fig. 4A-4D) and the areas were analyzed using Simple PCI imaging software. As shown in Fig. 4E, 20% ICCM inhibited the formation of the mineralized matrix (P<0.05) and had a significant effect at all doses compared with the control with inhibitory rates of 34, 41 and 49%, respectively. Quantization of the ARS deposition areas revealed that ICCM suppressed the mineralization of osteoblasts at all doses.

ICCM-induced mRNA expression changes in osteoblasts. To better understand the effect of ICCM in osteoblastogenesis, its effect on the expression of several osteoblast markers was examined using RT-qPCR analysis. The results are presented as

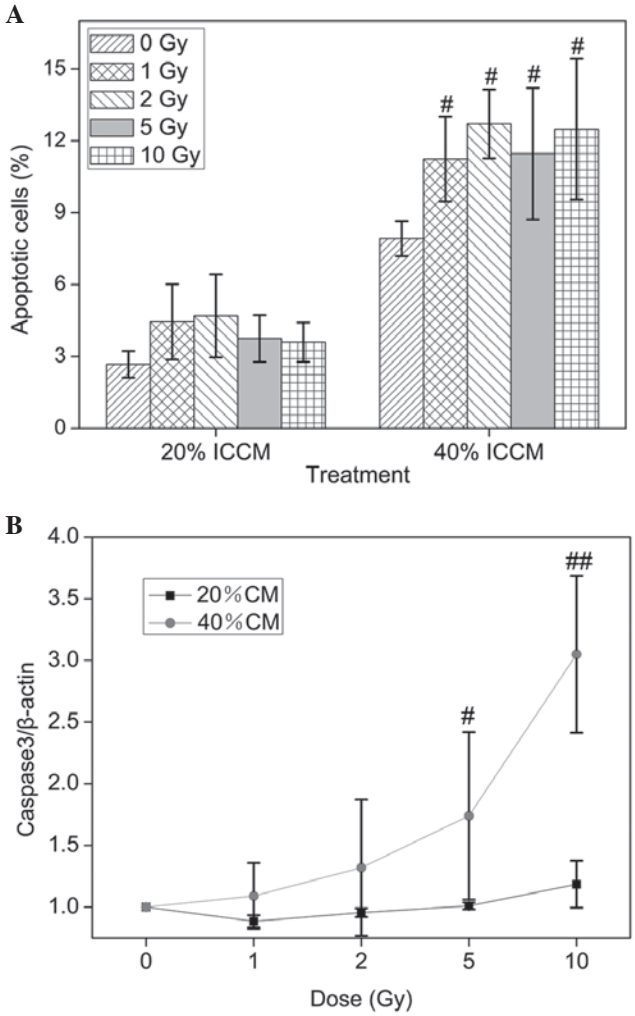

Figure 2. ICCM-induced apoptosis in osteoblastic cells. (A) Percentage of osteoblast cells undergoing apoptosis following treatment with various doses of ICCM for 48 h. The percentage of annexin V/PI cells wasobtained from three independent experiments. Data are expressed as the mean ± standard deviation. #P<0.05, compared with control. (B) Reverse transcription quantitative polymerase chain reaction detection of the expression levels of caspase 3 in osteoblast cells cultured with ICCM at 20 and 40%. #P<0.05 and ##P<0.01 compared with the control. ICCM, irradiated cell conditioned medium; PI, propidium iodide.

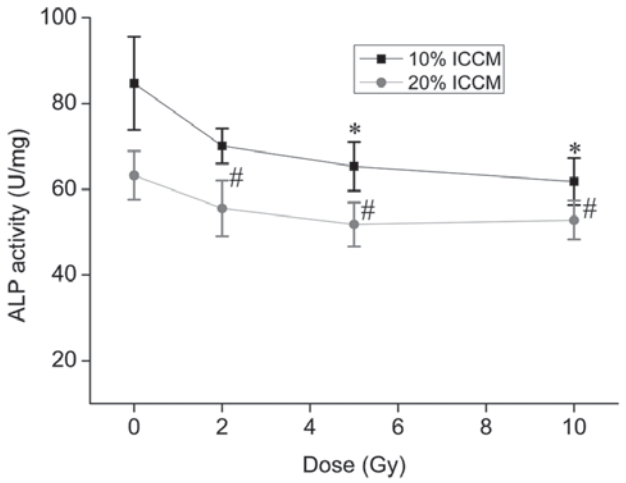

Figure 3. Effects of ICCM on the alkaline phosphatase activity of osteoblastic cells. Values are expressed as the mean ± standard deviation. Data are representative of results from three separate experiments. *P<0.05, #P<0.05, compared with each control. ICCM, irradiated cell conditioned medium.

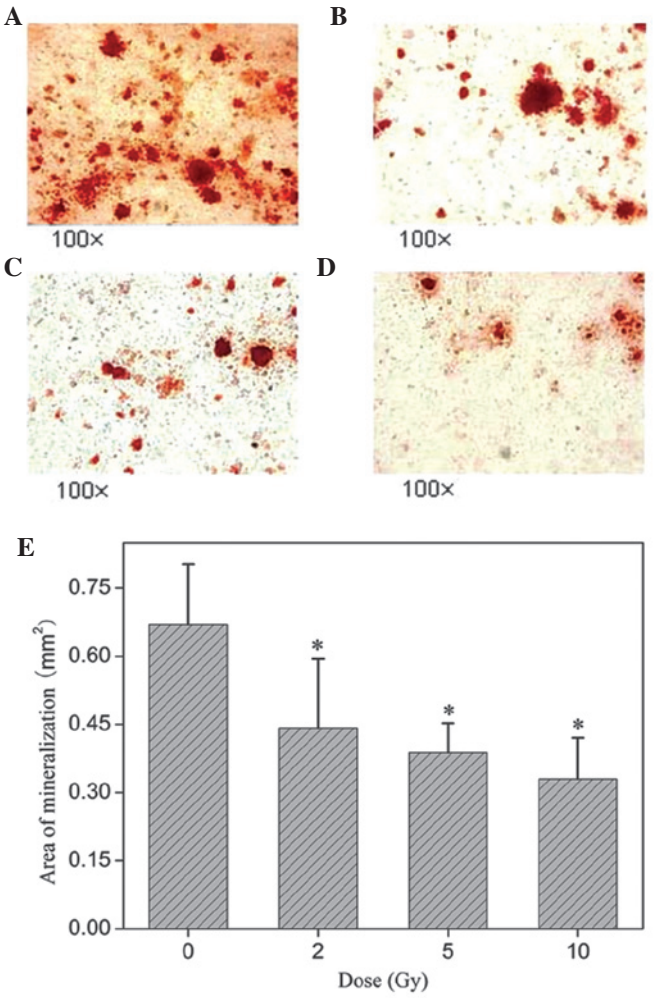

Figure 4. Effect of 20% ICCM on the formation of mineralized matrix nodules as a function of dose to the osteoblasts. (A-D) Images of mineralized nodules stained by ARS (magnification, x100). (A) Cells treated with 0 Gy ICCM. (B) Cells treated with 2 Gy ICCM. (C) Cells treated with 5 Gy ICCM. (D) Cells treated with 10 Gy ICCM. (E) Mineralization inhibitory rate was determined by quantification of ARS staining using Simple PCI software. Data are expressed as the mean ± standard deviation. *P<0.05, compared with the control group. ICCM, irradiated cell conditioned medium; ARS, Alizarin red S.

histograms in Fig. 5. The osteoblasts cultured with 1 or 10 Gy ICCM exhibited different gene expression patterns compared with the controls. ALP is an early osteoblastic marker and a single dose of x-rays (4 Gy) can induce significant changes in osteoblast differentiation 6 days after radiation (16). As shown in Fig. 5A, incubation of the cells with ICCM inhibited the mRNA expression of ALP. In addition, ICCM induced a more marked downregulation of the mRNA expression of osteocalcin (BGP; Fig. 5B). Furthermore, the mRNA expression levels of osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL) were decreased markedly following ICCM administration. However, no significant difference was identified between the responses to 1 or 10 Gy ICCM.

Discussion

Radiotherapy-associated bone complications have been recognized in patients since the early application of ionizing

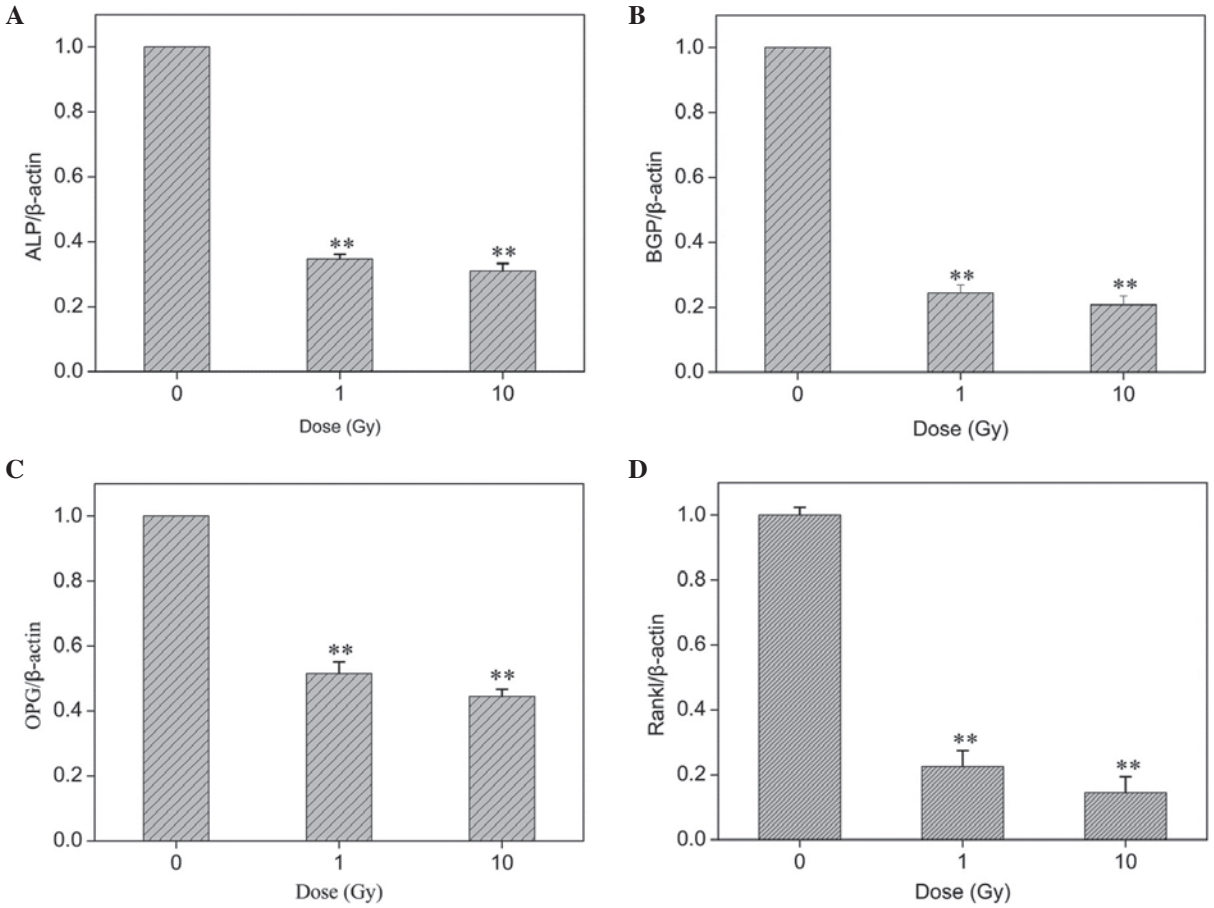

Figure 5. Effects of 20% ICCM on osteoblastogenic markers. Osteoblast cells were cultured in the presence of ICCM for 5 days. Reverse transcription quantitative polymerase chain reaction was used to analyze the mRNA expression levels of (A) ALP, (B) BGP, (C) OPG and (D) RANKL, normalized against β-actin. Data are expressed as the mean ± standard deviation. **P<0.01, vs. the 0 Gy ICCM group. ALP and RANKL groups were analyzed by Dunnett's test; OPG and BGP groups were analyzed by Dunnett's T3 test. ALP, alkaline phosphatase; BGP, osteocalcin; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; ICCM, irradiated cell conditioned medium.

radiation almost a century ago. It has continued to be one of the treatment-associated side-effects, which considerably compromises the quality of life of the patient (17). Non-malignant bone complications, frequently in the form of bone loss and often only diagnosed following the occurrence of fractures, are a common complication post-irradiation. The etiology of non-malignant bone complications in cancer patients treated with radiotherapy remains to be elucidated and appears to be multifactorial. Cumulative evidence suggests abscopal effects are involved in the development of injuries in normal tissues, including the skeleton, following local radiotherapy (11,17). Jia *et al* (18) and Travis (19) observed potent effects on bone density and other parameters even when single organs, including the gut, are irradiated. These abscopal effects further highlight the importance of developing a better understanding of the complete mechanism by which direct and indirect radiation exposure affects bone quality.

Bone damage occurs following exposure to low linear energy transfer ionizing radiation (γ and x-rays) and is hypothesized to be a result of physiological changes that occur to vasculature and bone cells, including bone-forming osteoblasts and resorbing osteoclasts (20). To date, whether ICCM affects bone metabolism by affecting the proliferation, differentiation, and mineralization function of primary osteoblasts *in vitro* has not been reported. Osteoblasts are specialized, terminally differentiated products of mesencyhmal stem cells. They synthesize very dense, cross-linked collagen, and various additional specialized proteins in smaller quantities, including osteocalcin and osteopontin, which comprise the organic matrix of bone (21). Osteoblasts cultured from newborn mouse skulls retain more somatic cell functional characteristics compared with cell lines and simulate biological changes *in vivo* (21). The present study investigated the γ-ray-induced ICCM-bystander-type effects on osteoblasts at the cellular level at clinically therapeutic doses. It has been established that irradiated cells release cytotoxic factor(s) into the surrounding medium, which causes the death of unirradiated cells (22,23). The present study demonstrated that 10 Gy ICCM exposure significantly inhibited osteoblast proliferation. ALP activity, which is an early characteristic marker of osteogenic differentiation, was also significantly inhibited in cells treated with 5 and 10 Gy ICCM. Mineralization is a necessary condition of osteoblasts to form bone calcification (14), and the present study observed that 2, 5 and 10 Gy ICCM exposure suppressed the mineralization function of osteoblasts. Radiation is a well-known inducer of apoptosis in numerous cell lines and there is evidence that apoptotic death is a prominent feature of cultures demonstrating bystander effects (22,24). This is

supported by data presented in the present study. Analysis of the cells revealed that ICCM increased the percentages of osteoblasts processing apoptosis in a dose-independent manner. In addition, upregulation in the mRNA expression of caspase 3was observed. Caspase 3 is a frequently activated protease in mammalian cell apoptosis, essential for certain characteristic changes in cell morphology and biochemical events associated with the execution and completion of apoptosis (25). In the present study, RT-qPCR was performed on the samples to investigate the effect of ICCM on the expression of a series of osteoblast markers. As expected, ICCM also significantly suppressed the expression levels of ALP, BGP, OPG and RANKL to various degrees. These data, although preliminary, are indicative that ICCM has potential deleterious effects on osteoblasts.

In terms of the implications for radiotherapy, there is a body of data that remains to be fully understood concerning the abscopal effects of radiation, including the response of an organ or tissue that was not in the field of treatment to experimental irradiation or radiotherapy (9,26-31). Radiation-induced bystander effects may provide an explanation for certain abscopal effects. Previous studies using a range of epithelial cells, including normal and tumor cells, indicated a wide variation in the production of the signal and the expression of the effect (24,32-36), therefore, a 'holistic' approach to understanding the process. may be required For example, there are multiple inducers, which have multiple possible consequences in different cell systems, and the precise consequence of a specific trigger may depend on numerous factors. Lyng *et al* (37) hypothesized that irradiated cells release toxic factors other than reactive oxygen species (ROS) into the medium; ROS appear to be involved in the signaling pathway for specific end points, however, the existence of other signaling mechanisms is indicated, particularly for cell death. It has been demonstrated that specific long-lived signaling factors may cause apoptosis in unirradiated cells exposed to ICCM by triggering calcium fluxes and the mitogen-activated protein kinase signaling pathway (38). Increases in transforming growth factor β1 and interleukin-8 have been demonstrated previously in bystander cell supernatants (39-41). Mothersill *et al* (42) suggested that a signal transduction mechanism may control cell death or survival via the bystander effect rather than by release of a directly cytotoxic factor. While Mothersill *et al* demonstrated that serum has the ability to produce bystander factors and that serotonin in serum is important in the mechanism of signal production, this possibility is excluded in the present study, in which the ICCM is serum-free (43). These conflicting experimental results imply that there are complex mechanisms underlying these effects, which remain to be elucidated. Whether radiation-induced abscopal damage to the skeleton is associated with the systemic effects of radiation injury, where damage to a critical organ may have consequences for another organ, and its precise mechanism are not fully understood and require further investigation.

The morbidity of bone irradiation and its attendant complications are significant. Subsequent investigations aim to identify irradiation-induced changes in the expression of cytokines, which are involved in inhibitory effects, and to include co-culture and an *in vivo* model. In conclusion, the present study has improved current understanding of the skeletal complications associated with localized radiotherapy and, if preventing or minimizing post-radiation-induced bone loss is a valid investigative goal, further understanding of in-field and out of-field damage is required. A potentially important outcome may be the identification of treatments, which protect the bone from irradiation and/or facilitate bone repair.

Acknowledgements

The present study was supported by the Shanghai Municipal Health Bureau (grant nos. 2009003 and 12GWZX0401).

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