

Indirect effects of X-irradiation on proliferation and osteogenic potential of bone marrow mesenchymal stem cells in a local irradiated rat model

RUILIAN SUN, GUOYING ZHU, JIANPING WANG, LING TONG and JIANGLONG ZHAI

Department of Radiation Protection, Institute of Radiation Medicine, Fudan University, Shanghai 200032, P.R. China

Received October 15, 2016; Accepted April 3, 2017

DOI: 10.3892/mmr.2017.6464

Abstract. Cancer survivors after radiotherapy may suffer a variety of bone-related adverse side effects, including radioactive osteoporosis and fractures. Localized irradiation is a common treatment modality for malignancies. Recently, a series of reactions and injuries called indirect effects (remote changes in bone when other parts of the body are irradiated) have been reported on the indirect irradiated area of bone tissue after radiotherapy. To address this issue, we developed a rat localized irradiation model. Rats were irradiated with a single dose of X-rays to the left hind limbs, and bone marrow mesenchymal stem cells (BMMSCs) were isolated from bone marrow of the left (direct irradiated) and right (indirect irradiated) hind limbs 3, 7 and 14 days after irradiation, and assayed for the proliferation ability and osteogenic potential by alkaline phosphatase (ALP) activity, mineralization assay, RT-PCR and western blot analysis. The results showed that there were significant morphology changes in the BMMSCs from direct and indirect irradiated bone tissue with bigger cell bodies and increased granules. The proliferation of BMMSCs decreased both in the direct irradiated and non-irradiated bone tissue. The ALP expression and activities of BMMSCs from direct irradiated bone was consistently defected following a transient enhancement, the mRNA levels of RUNX2 and OCN, the protein expression of RUNX2, and the mineralization ability also showed the same trend. Simultaneously, in indirect irradiated group, the osteogenic potential indicators of BMMSCs decreased in the early stage of post-irradiation and were still impaired 14 days after irradiation. Our data demonstrate that localized irradiation may have both direct and indirect adverse effects on BMMSCs' proliferation and osteogenic potential into osteoblast, which may be the mechanism of

radiation-induced abscopal impairment to the skeleton in the cancer radiotherapy-induced bone loss.

Introduction

As the incidence of cancer continues to rise progressively, radiotherapy plays a vital role in the treatment of tumor. However, survivors after radiotherapy were plagued by a series of adverse side effects (1), including various degrees of radioactive bone injuries (2), such as radioactive osteoporosis, radioactive osteomyelitis, radioactive fractures, osteoradionecrosis or radioactive bone development disorders, which have been confirmed by animal experiments and population epidemiological studies. It has been reported that pelvic irradiation substantially increases the risk of pelvic fractures in women (3,4), and rib fracture is frequently seen on CT after stereotactic body radiotherapy for lung cancer (5,6). Additionally, even if patients show no obvious signs of symptoms, they often face a higher risk of bone mineral density (BMD) decline (7). Meanwhile, in a mouse model, it has been proved that irradiation could induce acute cancellous bone loss in the tibiae and lumbar vertebrae (8). Therefore, it is very important to uncover the mechanism underlying the radioactive bone injury and develop appropriate preventive and treatment measures.

Recently, evidence suggested a new hidden danger of radioactive bone injury resulting from radiotherapy, which is in addition to the already known dangers of direct irradiated bone injury after radiotherapy, bone loss or injury known as indirect effect (9-11) may arise at indirect irradiated bone tissue (12). Radiation induced indirect effects on bone refer to the responses detected in unirradiated bone when neighboring or remote bone tissue is irradiated. It has been reported that postmenopausal women with uterine cervical cancer treated with concurrent chemoradiation had a lower BMD and were confronted with a higher risk of osteoporosis (13). Moreover, it has been reported that bone formation activity and total-body BMD of rats decreased after abdominal irradiation (11). In conclusion, it has been proved that irradiation could cause systemic adverse effects on the non-irradiated skeleton, however, the indirect effects and the mechanism of bone injury after radiotherapy has not been well-studied.

It is common knowledge that the interaction between osteoblasts and osteoclasts keeps the bone remodeling balance, which

Correspondence to: Mrs. Guoying Zhu, Department of Radiation Protection, Institute of Radiation Medicine, Fudan University, 2094 Xie-Tu Road, Shanghai 200032, P.R. China
E-mail: zhugy@shmu.edu.cn

Key words: local irradiation, bone mesenchymal stem cells, osteogenesis, indirect effect, rat model

plays a key role in maintaining bone health (14). The osteoblasts are differentiated from BMMSCs, which is a type of heterogeneous and radiosensitive cell, with the ability to differentiate into osteoblasts, neural cells, chondrocytes, adipocytes and myoblasts (15,16). It has been confirmed that BMMSCs' proliferation and its osteogenic differentiation abilities were inhibited after irradiation (17,18), and the possibility of radiation-induced bone injury by damaging bone marrow microenvironment for stem cells (19) has also been put forward. However, there were sufficient uncertainties about the indirect effects of radioactive bone injuries. Thus, further investigations are needed to characterize the potential hazard of radiation-induced bone injury and their associations to the dysfunction of BMMSCs. In this regard, we established a local irradiated rat model to evaluate the effects of irradiation on the proliferation and osteogenic potential of BMMSCs obtained from direct and indirect irradiated bones of rats, in order to develop a more thorough understanding of the complete mechanism of indirect radioactive bone injuries.

Materials and methods

Animals. Male Sprague-Dawley rats aged 6 weeks were obtained from the Department of Experimental Animals of the Fudan University (Shanghai, China). The rats were housed at 20–26°C with 16-h light and 8-h dark cycle, and provided *ad libitum* food and water. All the animal handling and experimental procedures were approved by the Committee for Ethical Use of Experimental Animals at Fudan University (Shanghai, China), and the ethical approval registration number for animal study was 20150559A183.

Ionizing radiation procedure. The rats were locally irradiated with 2 Gy by an Animal X-ray Irradiator (X-RAD320, PXi, USA) at a rate of 185.5 cGy/min after being anesthetized. The irradiation site was restricted in the region between distal femur and proximal tibia, which was sensitive to irradiation (20). In addition, the irradiation area is limited with the collimators, only the left hind limbs of rats were exposed to irradiation directly, and the remaining parts of the rats (including opposite right hind limbs) were covered by a shielding device. The Source-to-Surface-Distance of irradiation is 60 cm, and the irradiation field is 2.54 cm². Control groups of rats were anesthetized and placed in the irradiator but no exposure to irradiation.

Isolation and culture of BMMSCs. The BMMSCs were obtained from three male Sprague-Dawley rats in each group at 3, 7 and 14 days after irradiation. The rats were sacrificed by cervical dislocation and the left and right femur and tibia of the local irradiated rats and control rats were dissected, and then bone marrow mesenchymal stem cells (BMMSCs) were obtained by density gradient centrifugation. The BMMSCs isolated at 3, 7 and 14 days post-irradiation were named D3, D7 and D14, respectively. BMMSCs isolated from direct irradiated bones (left hind limbs of local irradiated rats) and indirect irradiated bones (right hind limbs of local irradiated rats) were named direct irradiated group and indirect irradiated group. BMMSCs obtained from the non-irradiated rats were named control group. The cells were cultured in culture media

(DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco/Invitrogen, Grand Island, NY, USA), 100 U/ml penicillin and streptomycin (North China Pharmaceutical Co., Ltd., Shijiazhuang, China) at 37°C in 5% humidified CO₂. As soon as the cells reached 80% confluence, they were detached using 0.25% trypsin-EDTA and replated into cell culture flasks.

Proliferation assay. The proliferation ability of BMMSCs was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The cells isolated from direct, indirect irradiated bones as well as the control group at 3, 7 and 14 days post-irradiation were seeded in 96-well plates with 3,000 cells per well. When the cells were cultured for 2, 3, 5, 7, 9 and 11 days, the MTT assay were performed as described below. The culture medium was removed and 100 μ l of fresh culture medium containing 10 μ l of MTT (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to each well (21,22). The cells were then incubated at 37°C for 4 h. The color was extracted with 100 μ l 10% sodium dodecyl sulfate (China Pharmaceutical Shanghai Chemical Reagent Co., Ltd., Shanghai, China) at 37°C for 2 h. OD values, which were directly related to the viable cell numbers, were determined at room temperature using a microplate reader (Epoch 2 Microplate Spectrophotometer; BioTek, Milan, Italy) and cell growth curves were plotted.

Osteogenic induction of BMMSCs. When the third passage of BMMSCs isolated from direct, indirect irradiated bones at 3, 7 and 14 days post-irradiation were cultured for 48 h, we changed the medium into osteogenic inductive medium. Specifically, the medium was prepared by supplementing DMEM with 15% FBS, 50 mg/l ascorbic acid and 10⁻⁸ M dexamethasone (both from Sigma-Aldrich), 10 mM β -glycerol phosphate (China Pharmaceutical Shanghai Chemical Reagent Co., Ltd.) (17,23) and 100 U/ml penicillin and streptomycin (North China Pharmaceutical Co., Ltd.). The medium was changed every 3 days.

Alkaline phosphatase activity and staining. The BMMSCs isolated from direct, indirect irradiated bones as well as the control group at 3, 7 and 14 days post-irradiation were seeded in 96-well plates with 3,000 cells per well for ALP activity assay. On the 7th day after osteogenic induction, ALP activity assay was performed as described below. After being rinsed twice with phosphate-buffered saline (PBS), the BMMSCs were lysed with 0.1% Triton X-100 (Sigma-Aldrich) at 4°C for 2 h. Then the cells were lysed three times by ultrasonication (VCX130PB Serial; Sonics & Materials, Inc., Newtown, CT, USA) for 10 sec at 20 kHz on ice. The cell supernatant was collected. Next, 2-amino-2-methyl-1-propanol buffer containing p-nitrophenyl phosphate (Fluka, Co., Milwaukee, WI, USA) were subsequently added to the supernatant and the mixture was incubated for 30 min at 37°C. The reaction was stopped by adding 0.1 M NaOH. Finally, the ALP activity was determined at the wavelength of 405 nm. Protein content was measured using a BCA assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. All results were normalized in relation to protein content (24). The ALP staining assay was also

performed on the 7th day after osteogenic induction, and the BMMSCs isolated from direct, indirect irradiated bones and unirradiated bones at 3, 7 and 14 days post-irradiation were seeded in 48-well plate with 6,000 cells per well. The staining procedure was washed the cells twice with PBS and fixed them in 2.5% glutaraldehyde solution for 5 min, then, a BCIP/NBT Alkaline Phosphatase Color Development kit (Beyotime Institute of Biotechnology) was performed according to the manufacturer's instructions.

Mineralization assay. The BMMSCs from direct and indirect irradiated groups as well as the control group in D3, D7 and D14 were cultured in 48-well plates (5×10^4 cells/well) with osteogenic inductive medium for 3 weeks. Subsequently, the differentiated cells were stained with Alizarin Red for osteogenic mineralized nodules on the 21th day after osteogenic induction. Cells were first washed with PBS and fixed in cold 95% (v/v) ethanol for an hour, then the fixed BMMSCs were incubated with staining solution (Alizarin Red-Tris-HCl, pH 8.3) at room temperature for 10 min. The areas of positively stained mineral nodules were measured and analyzed (25) with an optical microscope and Simple PCI 5.2.1. imaging software.

RNA isolation, cDNA synthesis and gene expression. The BMMSCs isolated from direct, indirect irradiated bones as well as the control group at 3, 7 and 14 days post-irradiation were cultured in 6-well plates (5×10^5 cells/well) with osteogenic inductive medium for a week. Then the total RNA was harvested from cells using RNeasy Pure Cell/Bacteria kit (Qiagen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocols. The RNA was then used to synthesize complementary DNA (cDNA) by using a Quantscript RT kit (Qiagen Biotech Co., Ltd.). According to the manufacturer's instructions, RT was performed in a 20- μ l reaction mixture. Then cDNA was analyzed on Real Time PCR Amplifier (Light Cycler 2.0; Roche Diagnostics GmbH, Mannheim, Germany) with SYBR Premix Ex Taq Mix (Takara Bio Inc., Otsu, Japan) for the expression of RUNX2 (also called Cbfa1, initially expressed in osteoprogenitor cells) and osteocalcin (OCN, also called Bglap, a late period marker in matrix maturation). The PCR primers were designed by Primer 5. The gene sequences were searched in MEDLINE. Rat RUNX2 forward, 5'-TGCCACCTCTGACTTCTGC-3' and reverse, 3'-GATGAAATGCCTGGGAAGT-5'; Rat OCN forward, 5'-GAACAGACAAGTCCCACAC-3' and reverse, 3'-GAGCTCACACACCTCCCTG-5'; Rat β -actin forward, 5'-CACCCGCGAGTACAACCTTC-3' and reverse, 3'-CCCA TACCACCATCACACC-5'. All detections were in triplicate for each sample and data were normalized to β -actin levels ($\Delta\Delta Cq$). The qPCR performed 40 cycles at 95°C for 5 sec, and then at 60°C for 20 sec.

Western blot analysis. Western blot analysis was used to detect protein expression levels of RUNX2 and ALP. The BMMSCs isolated from direct, indirect irradiated bones as well as the control group at 3, 7 and 14 days post-irradiation were cultured in osteogenic inductive medium for a week. Then total cytoplasmic protein was isolated with RIPA lysis buffer and supplemented with phenylmethylsulfonylfluoride (PMSF)

(both from Beyotime Institute of Biotechnology). Next, the samples were centrifuged at 20,000 \times g for 10 min at 4°C. The protein contents were determined using a BCA protein assay kit (Beyotime) according to manufacturer's instructions. Equal quantities of total proteins were separated by 10% (w/v) Tris glycine SDS/PAGE (Beyotime) and transferred to PVDF membranes (Millipore). The membranes were then blocked with 5% (w/v) non-fat milk in Tris-buffered saline with Tween-20 for an hour at room temperature. We then incubated the membranes with an optimal concentration of the primary antibodies: anti-RUNX2 (1:1000, cat. no. 8486S; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-ALP (1:1000, cat. no. sc-98652; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-Tubulin (Beyotime) overnight at 4°C. Immunoreactive bands were detected with anti-rabbit (Cell Signaling Technology, Inc.) or goat anti-mouse (Beyotime) fluorescein-conjugated secondary antibody and visualized with chemiluminescent substrate (ECL Plus; Beyotime) by Gel Imaging system (Omega Lum C; Aplegen, San Francisco, CA, USA). The protein expression levels were quantified by the optical density ratio of the target and loading control proteins using Image-Pro Plus 6.0 software.

Statistical analysis. Data are expressed as the mean \pm standard deviation (SD) for separate experiments. Statistical analysis was done using a one-way ANOVA by SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA). Values of $P < 0.05$ were considered to be statistically significant.

Results

Cell morphology. The BMMSCs isolated from the rats 3, 7 and 14 days after local irradiation were named D3, D7 and D14, respectively. Under a light microscope, no obvious morphological change was observed 3 days post-irradiation in the direct and indirect irradiated groups. However, there were significant morphological changes in the cell shape in the direct and indirect irradiated groups at 14 days post-irradiation compared to the control group. In detail, the control group was presented with long spindle-shaped adherent growth, with equal cytoplasm and oval nuclei (Fig. 1A), while the BMMSCs of direct and indirect irradiated group were irregularly shaped, such as short fusiform or polygons, with bigger cell bodies and increased granules at 14 days post-irradiation (Fig. 1B and C).

Cell proliferation. The proliferation ability of BMMSCs showed different trends at 3, 7 and 14 days post-irradiation. At 3 days post-irradiation, the proliferation ability of direct and indirect irradiated group decreased slightly, with the decrease rate reaching 20.9 and 32.0%, respectively (after 7 days culture). However, at 7 days post-irradiation, the proliferation of BMMSCs in the direct irradiated group was damaged significantly by 35.9%, and the viability of BMMSCs in the indirect irradiated group was still impaired (Fig. 2B). At 14 days post-irradiation, the proliferation of BMMSCs obtained from direct and indirect irradiated bones were both markedly inhibited by 40.4 and 39.9%, respectively (Fig. 2C).

Osteogenic potential of BMMSCs. ALP is a marker for early osteoblast differentiation, and its activity was analyzed to

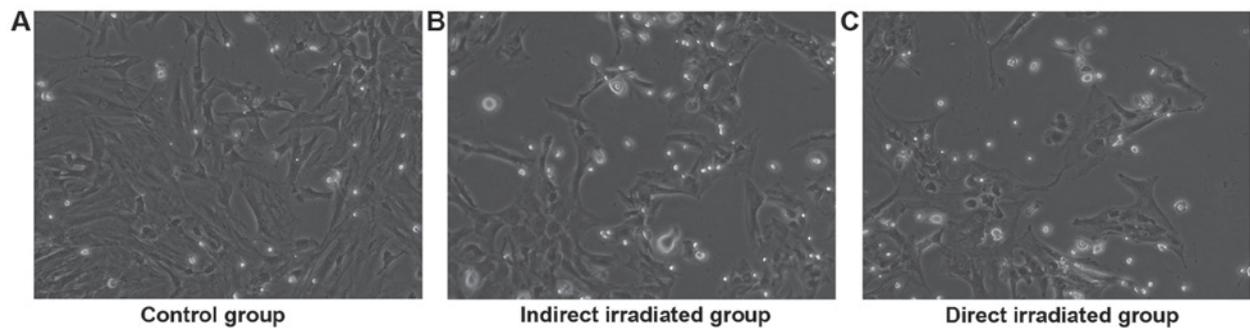


Figure 1. Morphological changes of BMMSCs in different groups at 14 days post-irradiation. (A) Control group, BMMSCs showed long spindle-shape, more likely to confluent and cells tend to grow in same direction. (B) Indirect irradiated group, cell shaped irregular shapes and cell number was decreased. (C) Direct irradiated group, cell morphology was changed significantly with polygon shapes and bigger cell bodies. Microphotographs were taken at x100 magnification.

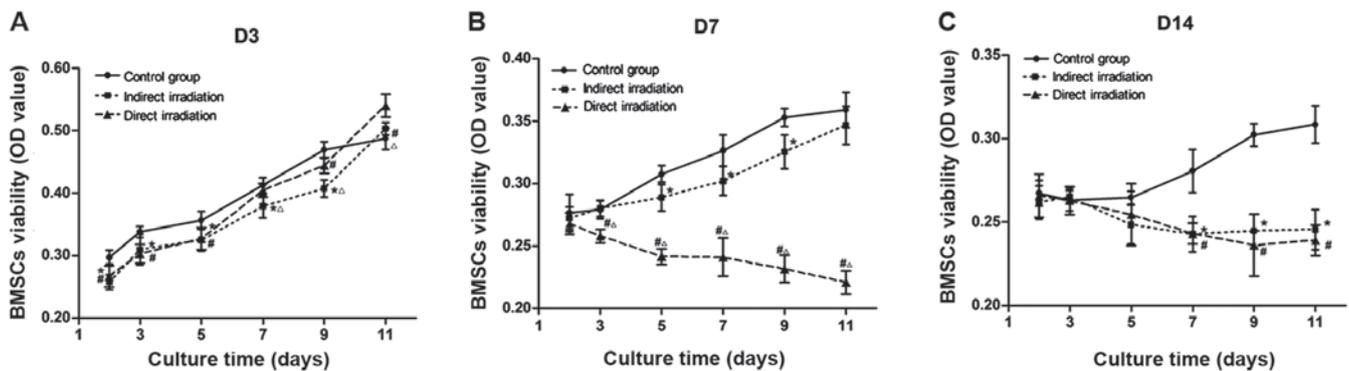


Figure 2. Proliferation ability of BMMSCs obtained from direct and indirect irradiated bones was damaged. The data are expressed as mean \pm SD. * $P < 0.05$, indirect irradiated group with control group. # $P < 0.05$, direct irradiated group with control group. $\Delta P < 0.05$, direct irradiated group with indirect irradiated group ($n = 8$). (A) In D3, cell proliferation ability of indirect and direct irradiated group was slightly declined. (B) In D7, the proliferation of BMMSCs was sharply dropped in direct irradiated group, while slightly declined in indirect irradiated group. (C) In D14, cell proliferation ability of indirect irradiated group as well as direct irradiated group was both sharply decreased.

reflect the early osteogenic potential of BMMSCs. The results of ALP activity normalized by protein content (IU/mg protein) were shown as Fig. 3A, and the ALP staining was shown as Fig. 3C. In direct irradiated groups, a transient increased ALP activity was observed at 3 days post-irradiation, then a sharp drop of ALP activity was observed at 7 days post-irradiation, the decrease rate reaching 67.2% compared with the control group, and only a little recovery was observed at 14 days post-irradiation compared to 7 days post-irradiation. Interestingly, in indirect irradiated groups, ALP activity was found to increase both at 3 and 7 days post-irradiation, with the increase rate reaching 29.9 and 31.3%, respectively ($P < 0.05$), and a decline of ALP activity was observed only at 14 days post-irradiation, (decrease rate reaching 16.0%, $P < 0.05$). The results of western blot analysis were shown as Fig. 3B, and the results were well consistent with ALP activity and staining.

Mineralization ability. Alizarin Red staining was applied to reflect the capacity of mineralization of osteoblast *in vitro* (Fig. 4A), and the area of bone mineralization nodules quantified by Simple PCI 5.2.1. imaging software were showed as Fig. 4B. Typical nodular structure of mineralized tissue with clear cell accumulation around the nodules can be observed in the control group. In the direct irradiated group, a slight increase of staining for mineralized nodules were observed

3 days post-irradiation (the increase rate reaching 19.6%), while almost no mineralization structure was observed 7 days post-irradiation (the area of bone mineralization nodules decreased by 91.9%), and only a little recovery was observed at 14 days post-irradiation (the decrease rate reaching 57.9%). Additionally, in the indirect irradiated group, there has been a modest decrease of the calcium-richer mineralized matrix at 3, 7 and 14 days post-irradiation, with the decrease rate reaching 19.7, 27.9 and 36.1%, respectively.

Differential expression of lineage-specific genes and proteins.

By using the real-time PCR, we found the expressions of osteogenesis-related genes RUNX2 (Runt-related transcription factor 2) and OCN (osteocalcin) altered in BMMSCs after irradiation (Fig. 5A). In the direct irradiated group, the mRNA expression levels of RUNX2 and OCN were clearly higher than that of the control group at 3 days post-irradiation, while a sharp decrease was observed at 7 days post-irradiation, and no obvious recovery was found at 14 days post-irradiation. In other words, in the direct irradiated group there has been a more powerful osteogenic potential at 3 days post-irradiation, but the enhanced potential decreased at 7 and 14 days post-irradiation. In the indirect irradiated group, the expressions of RUNX2 and OCN dropped in varying degrees at 3, 7 and 14 days post-irradiation. Thus, the osteogenic potential

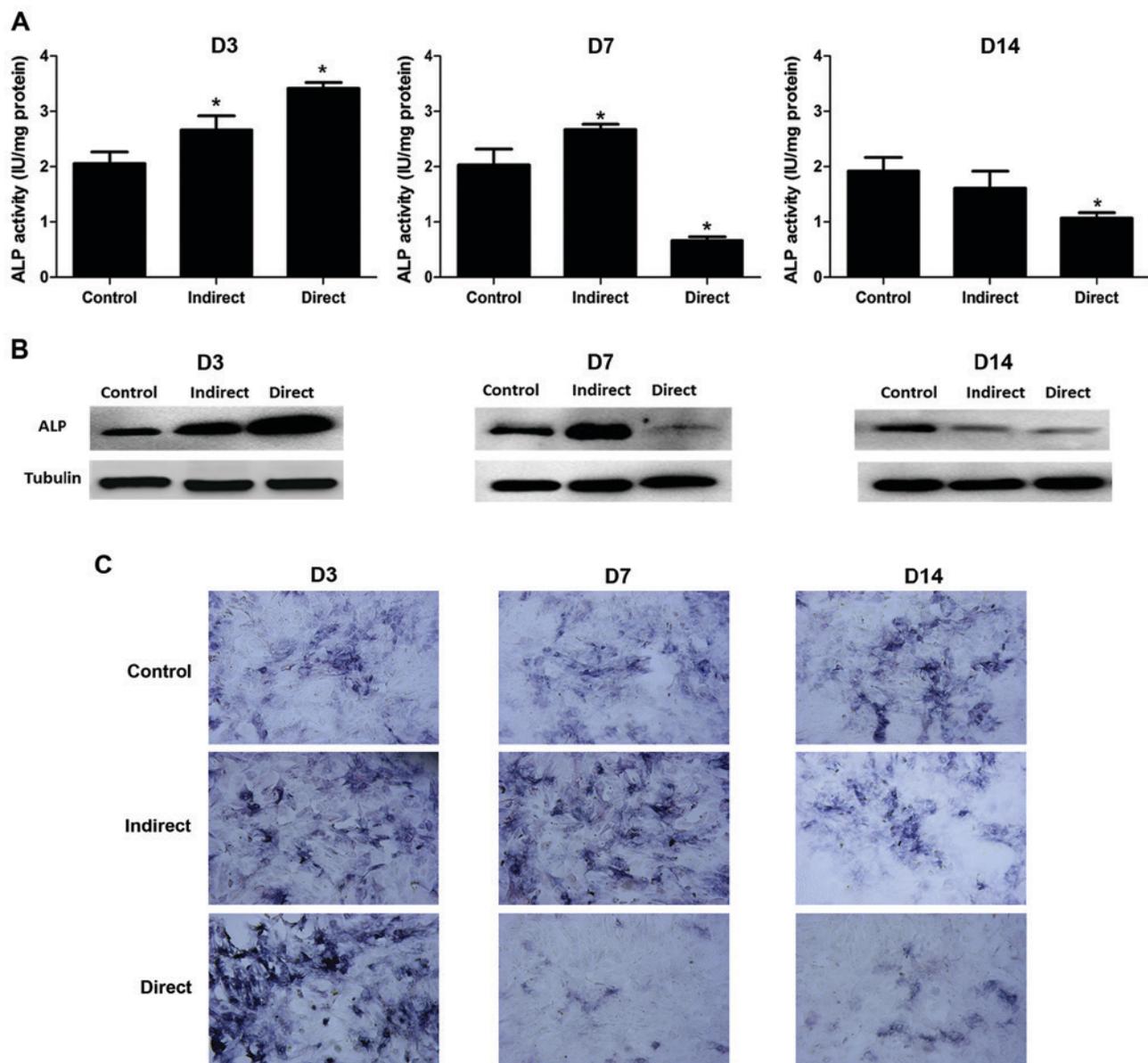


Figure 3. Irradiation can decrease ALP activity and ALP expression of BMSCs from direct and indirect irradiated bone in the long term. (A) ALP activity of different groups in D3, D7 and D14. The data were expressed as mean \pm SD. * $P < 0.05$, (n=8). (B) Protein expression of ALP of differentiated BMSCs in different groups. (C) The ALP staining of control, direct and indirect irradiated group in D3, D7 and D14. The differentiated BMSCs positive for alkaline phosphatase were stained purple. Microphotographs were taken at x100 magnification.

of BMSCs obtained from indirect irradiated bones was impaired at early stages of irradiation. The results of western blot analysis were well consistent with the gene expression levels (Fig. 5B). The protein expression of RUNX2 at D3, D7, D14 groups were shown as Fig. 5B with the control groups showed the basic level. The optical density of the protein bands of RUNX2 (related to tubulin) was quantified, and the results were shown as Fig. 5C.

Discussion

Clinical studies (26,27) and murine model experiments (28) have proved that irradiation can cause bone loss and other bone complications, which considered to be a type of late radiation damage (3,4,29). In preclinical models, sublethal irradiation may lead to impaired bone formation and increased

osteoclast activity, thereby contributing to a rapid collapse in bone quantity and quality (30-33). Currently, clinical treatment strategy usually adopted irradiation with fractionation dose of 2 Gy every time (34,35) rather than disposable high dose treatment. According to our pre-experiment, too high dose of irradiation would result in BMSCs' death. Therefore, we built a local irradiated rat model with 2 Gy irradiation dose to investigate the effect of irradiation on BMSCs. It is worth mentioning that we specially selected male SD rats to avoid estrogen affecting the results of the experiment (36,37). And in the future, combine irradiation with the ovariectomized rat model to investigate the effects of irradiation on BMSCs will be a promising research. In our local irradiated rat model, we found that ionizing radiation can lead to radioactive bone injury and indirect effects by affecting BMSCs' proliferation and osteogenic differentiation ability.

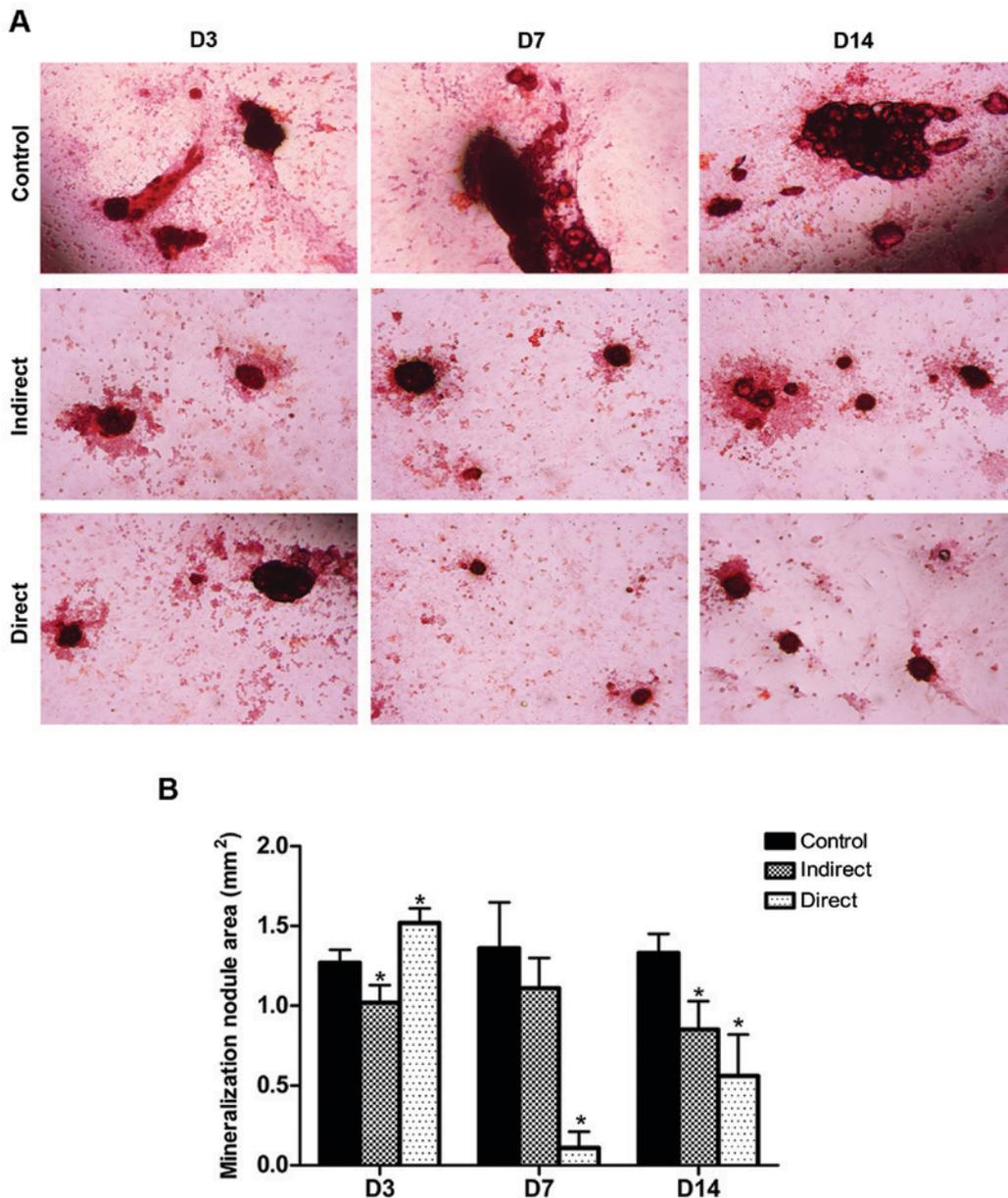


Figure 4. Irradiation affected mineralization ability of differentiated BMMSCs from direct and indirect irradiated bone in D3, D7 and D14. (A) Calcium accumulation and mineralized nodules positive for alizarin red were stained red or reddish black. Microphotographs were taken at x100 magnification. (B) Quantification of mineralization nodule area. Data were expressed as the mean \pm standard deviation (n=4). *P<0.05, compared with the control group.

In present study, as the results of MTT assay shown, the proliferation of BMMSCs in the direct irradiated group had no significant change at 3 days post-irradiation, whereas there was a sharp decline at 7 days post-irradiation, and no obvious recovery was observed at 14 days post-irradiation. Although the proliferation of BMMSCs in the indirect irradiated group has no much difference with that of the control group at 3 days post-irradiation, it declined at 7 days post-irradiation, and the downward trend was more obvious at 14 days post-irradiation. Therefore, we infer that the machinery of self-renewal of BMMSCs may have been injured after irradiation, which deserves further investigation. At the same time, the decreased proliferation of BMMSCs may associate with the damage of the microenvironment of BMMSCs. As an important component of the microenvironment of BMMSCs, vasculatures were also seriously damaged after irradiation, which may

be a potential mechanism lead to subsequent impairment of bone formation (19). Simultaneously, the impairment of angiogenesis is also an important factor lead to the poor nutrition and decreased number of BMMSCs (38,39). On the other hand, we thought irradiation may cause the shrink of the BM mesenchymal stem/progenitor cells pool (40). In addition, it has been proved that the levels of free radicals were dramatically increased after irradiation, which may have great relationships with the survival and self-renewal of BMMSCs. Consistent with our study, some research found that irradiation can reduce the number of osteoblasts from the irradiated area at 1 and 4 weeks after irradiation (19).

It is now well established that the osteodifferentiation of BMMSCs is marked by sequential stages of cellular proliferation and bone extracellular matrix maturation, ALP activity peaks at the end of the proliferation stage and before matrix

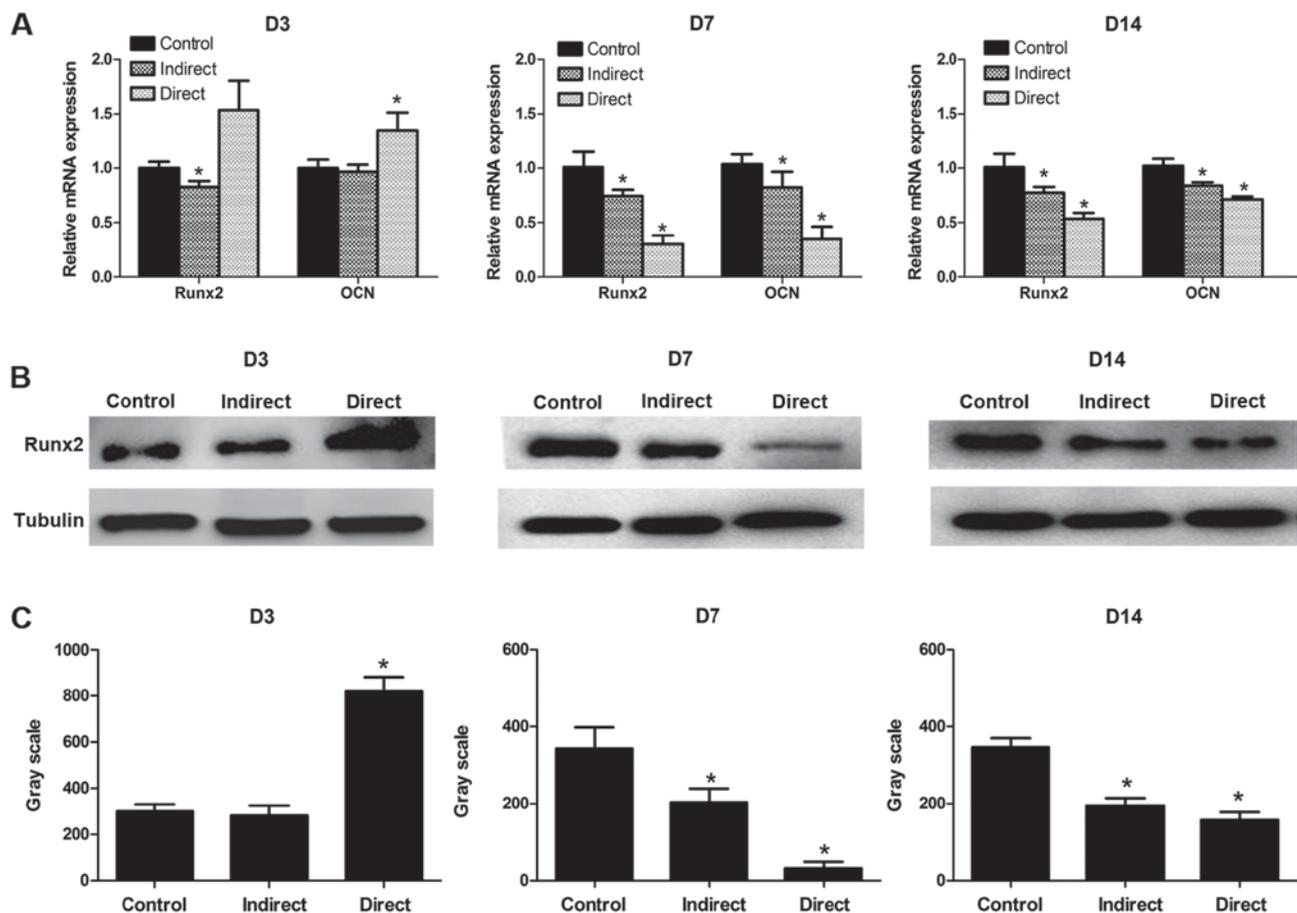


Figure 5. In the long term, irradiation can decrease osteogenic potential of BMMSCs from direct and indirect irradiated bones. (A) Osteogenesis-related genes expression levels in D3, D7 and D14. Data were shown of three independent experiments. (B) Protein expression of RUNX2 in different groups. (C) The grey scale of RUNX2 (relative to tubulin) in different groups. Data were expressed as the mean \pm standard deviation (n=3). *P<0.05, compared with the control group.

maturation (41). Since active osteoblast has high expression of ALP (42), ALP is regarded as a transient early marker of osteodifferentiation. In present study, ALP activity was analyzed to indicate the early osteogenic differentiation potential of BMMSCs. As shown by our results, an enhanced ALP activity in the direct irradiated group was observed at 3 days post-irradiation, while declines were found at 7 and 14 days post-irradiation. This indicates that, as a kind of stress response after irradiation, an increase in early differentiation ability was seen in BMMSCs from the direct irradiated group, but the early differentiation potential decreased as time elapsed. Surprisingly, an increase in ALP activity was found in the indirect irradiated group at both 3 and 7 days post-irradiation, and it finally dropped at 14 days post-irradiation. We assumed that BMMSCs in the indirect irradiated group made corresponding compensation with the early osteogenic differentiation ability of BMMSCs in the direct irradiated group decreasing over time. However, the early osteogenic differentiation ability of indirect irradiated group declined in the long-term.

RUNX2, a differentiation regulator in the osteoblast lineage, is necessary for osteoblast differentiation. Its expression is maintained postnatally in fully differentiated osteoblasts and is crucial for regulating the rate of bone matrix deposition (43,44). OCN is a marker only expressed by fully differentiated osteoblasts before the mature phase (42). As the results of RT-PCR and Western-blot in direct irradiated group

showed at 3 days post-irradiation, the expression levels of RUNX2 and OCN were consistently defected following a transient enhanced expression. The mineral deposition showed an identical trend. In accord with our findings, it has been found that irradiation could alter BMMSCs' osteogenic potential in a murine TBI (total body irradiation) model (40). The down-regulated RUNX2 potentially affects the EZH2 expression in mature osteoblasts, which may be one of the most prominent epigenetic enzymes during the osteogenic differentiation of BMMSCs. A recent study proved that the expression of EZH2 increased after irradiation (20). We assumed that the decreased expression of RUNX2 may influence the osteogenic potential and bone formation through LncRNA-ANCR/EZH2/RUNX2 feedback loop (45). In particular, our study also emphasized the osteogenic differentiation process of BMMSCs was accelerated in the early phase (3 days post-irradiation) and delayed thereafter.

But in the indirect irradiated group, our results showed that the mRNA expression of RUNX2 and OCN declined at 3 days post-irradiation, and no significant recovery was found at 7 and 14 days post-irradiation. The results of western blot analysis and mineralization assay in indirect irradiated group were well consist with the results mentioned above. It may relate to some cytokines secreted from the irradiated area or that irradiation may cause some systemic response. We inferred that irradiation altered the osteoblast differentiation

program of the BMMSCs in the distal limbs without direct irradiation, further causing indirect effects of radioactive bone injuries. Overall, it was proved that the differentiation potential of BMMSCs obtained from direct and indirect irradiated bone tissues were both impaired after irradiation. Therefore, it could be inferred that radioactive bone injury may be associated with the dysfunction of BMMSCs. This highlights the importance of preventing BMMSCs' injuries from radiotherapy to accelerate cancer patients' recovery from radioactive bone injuries.

By comparing results of the direct and indirect irradiated groups, we found that although the function of BMMSCs in the indirect irradiated group was impaired (11), it was less severe than the direct irradiated group. Meanwhile, the results of changes in ALP activity, mineral deposition, along with the mRNA and protein expressions suggested that irradiation may alter the osteoblast differentiation program of the BMMSCs in the distal limbs without direct irradiation, further causing indirect effects of radioactive bone injuries. Other studies also revealed that irradiation can affect BMMSCs indirectly in the proximal femur without direct irradiation (11,19). However, further research on how irradiation intervenes the differentiation signal pathway of BMMSCs in indirect irradiated area are still need in order to determine the underlying mechanisms of govern BMMSCs' proliferation and differentiation, providing us more effective approaches to making progress in stem cell-related therapies. In this study, the results give us a clue that the complications involving radioactive bone injuries after radiotherapy may be associated with the dysfunction of BMMSCs. Our results also highlight the importance of enhancing applications of BMMSCs in cell therapy and regenerative medicine (15,16,46,47).

The life qualities of patients after radiotherapy were deeply affected by radioactive bone injuries. It has been reported that osteoporosis may be relevant to stem-cell dysfunction (48), and it has also been proved that irradiation may influence bone formation by interfering with BMMSCs' proliferation and osteogenic potential (17,19). On the other hand, some studies suggested that the mobilization of stem cells in the circulation can occur in response to irradiation (49-51). Interestingly, hematopoietic stem cells in the circulation can differentiate into osteoclast precursors, gathered onto bone remodeling surfaces and differentiated into osteoclasts. It is still unclear if the changes in the hematopoietic stem cells after irradiation related to the numbers and activities of osteoclasts. And the relationship between hematopoietic stem cells and bone resorption need further investigation.

In conclusion, the indirect effects of radioactive bone injury at the BMMSCs' level were explored in a local irradiated rat model. Our results suggest that radioactive bone injury may relate to the BMMSCs' dysfunction, and irradiation has indirect effects on the proliferation and osteogenic ability of BMMSCs, which may be an important mechanism leading to subsequent impairment of bone formation after radiotherapy.

Acknowledgements

The present study was sponsored by the Shanghai Natural Science Fund (grant no. 14ZR1401600) and Shanghai

Municipal Commission of Health (grant nos. 2013ZYJB0801 and 20154Y0202).

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