Inhibition of Adipogenesis Is Involved in the Protective Effects of 1,25-Dihydroxy Vitamin D3 on the Radiation-Injured Bone Marrow Microenvironment in Mice

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Summary To explore the protective effects of 1,25-dihydroxy vitamin D3 (1,25-(OH)2D3) on the bone marrow microenvironment in mice after irradiation and the underlying molecular mechanisms, a total of 150 7-wk-old male BALB/c mice were randomly divided into a normal group, an irradiation (IR) group and an irradiation/H11001 1,25-(OH)2D3 (IR/H11001 VD3) group. The mice in the IR/H11001 VD3 group were treated with 6.0 Gy 60Co rays, and 1,25-(OH)2D3 (dissolved in DMSO, 2.5 g/kg) was administered once per day from 2 d before to 8 d after irradiation. Mice in the IR group were treated with the same dose of rays and an equal volume of DMSO. Subsequently, the body weights and the numbers of peripheral white blood cells (WBCs) were measured. Histological analysis of femur bone marrow was conducted to determine the proportion of adipose area as well. Finally, the expression of peroxisome proliferator-activated receptor-gamma (PPARγ) in bone marrow was detected by immunohistochemistry. After irradiation, the percentage of adipose area in the bone marrow was significantly increased, and the WBC number and body weight were markedly reduced. Compared with irradiation alone, the co-administration of 1,25-(OH)2D3 with irradiation markedly attenuated radiation-induced adipogenesis in bone marrow, resulted in fewer bone marrow stromal cells expressing PPARγ and enhanced the recovery of body weight and WBCs. These results indicate that 1,25-(OH)2D3 could accelerate the recovery of body weight and WBCs in irradiated mice and protect the bone marrow by inhibiting radiation-induced adipogenesis via the down-regulation of PPARγ expression.

Key Words 1,25-dihydroxy vitamin D3, protect, bone marrow niche, adipogenesis, radiation

As medical technology has developed, radiation has become widely utilized in medical diagnosis and treatment. Medical radiographic images have increasingly become an important basis for clinical disease diagnosis, and radiotherapy has become an important technique for cancer treatment. However, myelosuppression induced by radiation greatly affects patients’ quality of life by causing dramatic declines in body weight and peripheral white blood cells (WBCs) and decreasing immunity. The essence of myelosuppression relates to injured hematopoietic stem cells and their microenvironment. Research on protecting hematopoietic stem cells from radiation is in an early stage; however, no particularly effective method for achieving this objective has been identified. Recently, researchers have focused on ameliorating irradiation-induced damage to the bone marrow microenvironment. It is well known that adipogenesis increases as bone marrow hematopoietic function declines. A recent study (1) showed that the adipose tissue of the bone marrow, a negative regulator of the hematopoietic microenvironment, directly inhibits hematopoiesis in the bone marrow by suppressing the proliferation of primitive hematopoietic cells. Thus, hematopoietic recovery may be improved by enhancing the hematopoietic microenvironment, such as by inhibiting adipogenesis in the bone marrow after irradiation.

The hematopoietic microenvironment is composed of cells, extracellular matrix, and other factors. Cells in this microenvironment include endothelial cells, osteoblasts, mesenchymal stem cells (MSCs), and adipocytes. Both osteoblasts and adipocytes are differentiated from MSCs. Osteoblasts, endothelial cells and MSCs are important positive regulators of hematopoiesis, whereas adipo-
cytes negatively regulate hematopoiesis. Adipogenesis includes two key phases: the conversion of MSCs to preadipocytes and differentiation of preadipocytes to mature adipocytes (2). During the process of adipogenesis, but not during osteogenic differentiation, peroxisome proliferator-activated receptor-gamma (PPARγ), a member of the nuclear receptor superfamily (3), is activated. Ishida et al. demonstrated that 1,25-dihydroxy vitamin D3 (1,25-(OH)2D3) could inhibit preadipocyte differentiation and triglyceride aggregation (4). Hida et al. further demonstrated that 1,25-(OH)2D3 could inhibit the thiazolidinedione-induced differentiation of 3T3-L1 preadipocytes by down-regulating PPARγ2 expression (5). Duque et al. found that 1,25-(OH)2D3 could inhibit bone marrow steatosis by down-regulating PPARγ protein expression in aged mice (6). However, it is unclear whether 1,25-(OH)2D3 can protect the bone marrow microenvironment in mice after radiotherapy by inhibiting preadipocyte differentiation via the down-regulation of PPARγ expression. Moreover, Vitamin D3, as an important nutrient, has many physiological functions. The present study was designed to evaluate the effects of 1,25-(OH)2D3 on bone marrow adipogenesis and hematopoietic recovery after irradiation with a sub-lethal dose (6.0 Gy) of 60Co (6).

MATERIALS AND METHODS

Mice. All animal experiments were performed according to the guidelines of the Animal Ethics Committee of the State Scientific and Technological Commission for Animal Experiments, and approval of the protocol was granted by the ethics committee of Jinan University (approval number: 20111008001). A total of 150 7-wk-old male SPF BALB/c mice with a mass of 20±2 g were obtained from the Laboratory Animal Center of Guangzhou Province (animal certification number: SCXK (Guang Dong) 2008-0002). All mice were equally and randomly divided into a normal group, an irradiation (IR) group and an irradiation + 1,25-(OH)2D3 (IR+VD3) group.

Experimental materials. 1,25-(OH)2D3 in pill form was obtained from Roche (Basel, Switzerland), with each pill containing 0.25 μg of 1,25-(OH)2D3. A rabbit anti-mouse PPARγ monoclonal antibody and a goat anti-rabbit IgM antibody were purchased from Abcam (Cambridge, UK). DAB developer and 10% goat serum for blocking were obtained from Boster (Wuhan, China). Hematoxylin was purchased from Gibco (Thermo Fisher Scientific, Waltham, MA).

Marrow fat conversion by irradiation with 60Co γ. The mice in the IR and IR+VD3 groups were exposed to a sub-lethal dose (6.0 Gy) of 60Co γ with a 1-n source-skin distance at a rate of 1.25 Gy/min. From 2 d before to 8 d after irradiation, the mice in the IR+VD3 group were treated with 2.5 μg/kg·d of 1,25-(OH)2D3 (diluted in DMSO) based on our pre-experiment via daily intra-gastric administration. The mice in the IR group were injected with the same volume of DMSO instead of 1,25-(OH)2D3. The normal group received no treatment.

Measurement of body weight and counting of peripheral blood leukocytes. Before irradiation (day 0) and on days 4, 8, 16 and 24 after irradiation, 10 mice at a time were randomly selected from each of three groups, and their body weights were measured. Then, 40 μL of blood was obtained from each mouse via retro-orbital bleeding and transferred into anticoagulant tubes containing EDTA. The number of WBCs was measured by a blood analyzer after thorough mixing.

Preparation of pathological bone marrow sections and hematoxylin–eosin (HE) staining. After the blood was obtained from the retro-orbital sinus, 10 selected mice from each group were euthanized using a cervical dislocation method. The bilateral thighbones of the mice were removed, and the muscle and muscular fasciae attached to the surface of the bone were cleared. Then, the thighbones were decalcified and fixed in hydrochloric acid formaldehyde solution (4% volume fraction). The bone marrow hematopoietic tissue, which had previously been treated with regular dehydration, paraflin embedding, sectioning and HE staining, was imaged and analyzed by microscopy.

Calculation of the fat vacuole area in the marrow cavity. Marrow sections were placed under a 100× high-power microscope (Leica, Wetzlar, Germany), and 20 random fields of the marrow cavity were observed in each group. Areas of fat vacuoles in the marrow cavity were calculated using the PAS-8000 pathology image analysis system (Leica).

Immunohistochemistry. After dewaxing and hydration, the marrow sections were washed with PBS and retrieved with citric acid buffer. Then, the sections were blocked for 20 min with 10% goat serum and incubated in 100 μL of diluted monoclonal antibody overnight at 4°C, followed by incubation in labelled secondary antibody for 45 min after washing with PBS. Finally, the sections were sealed with neutral resin after DAB coloration and counterstaining with hematoxylin.

Statistical analysis. Study data were presented as means±standard deviation (±s) and differences between groups were analyzed by one-way ANOVA following by the Student-Newman-Keuls post hoc test using OriginPro 8 (OriginLab, Northampton, MA). Differences were considered significant at a value of p<0.05.

RESULTS

Observation of the body weight of mice after irradiation

No mice died within the 24-d observation period after irradiation. The mice in the normal group were healthy and exhibited good dietetic condition, flexible reactions, smooth skin and increasing body weight. The mice treated with radiation alone were generally in poor condition and exhibited a lower level of activity, dry skin, less food intake and obvious weight decreases during the observation period. Compared to the IR group, the mice in the IR+VD3 group were in better condition and exhibited a lower level of activity, dry skin, less food intake and obvious weight decreases during the observation period. Compared to the IR group, the mice in the IR+VD3 group had...
clearly decreased compared to the normal group, but the difference between the two groups was not significantly different (Fig. 1). On day 16 after irradiation, the mice in the IR+VD3 group had a higher body weight than those in the IR group, and this difference was significant (*p<0.05, Fig. 1). The body weight of mice in the IR group slowly increased, while the body weight of mice in IR+VD3 group rapidly recovered. A larger gap between the body weight of the two groups was observed on day 24 after irradiation, as shown in Fig. 1. Thus, we suggest that 1,25-(OH)2D3 can prevent the loss of body weight induced by irradiation and accelerate body weight recovery.

Effect of 1,25-(OH)2D3 on the quantity of WBCs in mice after irradiation

On day 4 after irradiation, the mice in the IR+VD3 and IR groups both exhibited dramatic and significant (*p<0.05) decreases in the number of WBCs compared to the normal group (Fig. 2), which indicated that WBCs were reduced and bone marrow hematopoietic function was inhibited after irradiation with 6.0 Gy 60Co γ. During the following observation period, the number of WBCs of mice in the IR+VD3 group and the IR group increased, and the difference between the two groups was significant (*p<0.05, Fig. 2) on days 8, 16 and 24 after irradiation, which indicated that mice in the IR+VD3 group had more WBCs than mice in the irradiation group. The differences were significant compared to the normal group on days 4 and 8 after irradiation (**p<0.01, Fig. 2), which indicated that the numbers of WBCs of mice in the IR+VD3 group and the IR group were still much lower than in the normal group. On day 24 after irradiation, the number of WBCs in the IR+VD3 group presented little difference compared to the normal group (Fig. 2). Nevertheless, the number of WBCs in the IR group had not returned to the normal level, and compared to the normal group and the IR+VD3 group, the difference was significant (*p<0.05, Fig. 2). Restoration of WBCs indirectly indicates the gradual restoration of the hematopoietic function of the bone marrow. Overall,
these data indicated that radiation with 6.0 Gy 60Coγ inhibits WBCs that are derived from bone marrow and that 1,25-(OH)2D3 can accelerate the restoration of the peripheral hemogram in radiation-damaged mice. 

**Effects of 1,25-(OH)2D3 on pathological changes in the bone marrow of irradiated mice**

During the observation period, the fat vacuole area in the normal group was less than 3%. On the 4th day after irradiation, the number of adipose cells in both the IR group and the IR+VD3 group began to increase (Fig. 3C, 3D and Fig. 4) and then increased markedly on the 8th day after irradiation (Fig. 3E, 3F and Fig. 4). As shown in Fig. 4, compared to the normal group, the area of adipocytes in both the IR+VD3 group and the IR group markedly increased during the following observation period (**p<0.01), especially in the IR group. This indicated that the bone marrow hematopoietic function of the mice was seriously damaged, and the number of available hematopoietic cells was substantially reduced concomitant with an increase in the number of adipose cells after radiation with 6.0 Gy 60Coγ. However, compared to the IR group, the adipose area in the IR+VD3 group exhibited a significantly smaller increase after treatment with 1,25-(OH)2D3 on days 16 and 24 after irradiation (Fig. 4). Thus, our study suggests that 1,25-(OH)2D3 can reduce the formation of adipose cells in the bone marrow after irradiation to protect the hematopoietic tissue from damage.

**DISCUSSION**

Normal hematopoiesis is maintained by the interregulation and interaction of hematopoietic cells and the bone marrow microenvironment. The bone marrow cavity of newborn mammals is occupied by red bone marrow containing hematopoietic cells. However, due to aging or other stimuli, the number of adipocytes in the marrow cavities increases (7, 8); the bone marrow then is called yellow bone marrow. The red-to-yellow conversion is reversible (9). Bone marrow adipogenesis is the cardinal pathological change in the bone marrow niche after irradiation, and the function of the marrow is severely restrained rather than lost. Under suitable conditions, yellow bone marrow can revert to red bone marrow, and the restrained function of bone marrow also can return to normal (10). In this study, a bone marrow damage model was constructed by treating mice with 60Coγ radiation. After irradiation, the mice had fewer peripheral leukocytes and stronger bone marrow pimelosis, indicating that 6.0 Gy 60Coγ radiation could successfully generate the marrow fat conversion model used in our study.

Some recent studies have found that the number of adipocytes in the bone marrow cavity is negatively correlated with hematopoietic activity. In vitro, adipocytes inhibit the differentiation of polymorphonuclear granulocytes by blocking the expression of neuropilin-1 in megakaryocytes (11). Adipocytes can directly sup-
press the proliferation of primitive hematopoietic cells in vivo, thereby inhibiting bone marrow hematopoiesis (1). These studies suggest that adipocytes are a major negative regulator of hematopoiesis in the bone marrow microenvironment, and thus, inhibition of bone marrow adipogenesis could promote hematopoietic reconstitution.

As we know, WBCs mainly include neutrophils and lymphocytes, most of which have a short life (2–5 d). Moreover sub-lethal irradiation inhibited the function of bone marrow and led to less production of WBCs. Additionally, irradiation damaged the tissues and organs of the body and resulted in bleeding in such areas as the lungs, intestinal mucosa and subcutaneously. So production of WBCs in irradiated mice could not counteract the loss of WBCs. As shown in Fig. 2, on days 0 and 4, the quantity of WBCs decreased. Having declined to a certain level the quantity of WBCs began to increase during the following observation (days 8, 16 and 24), which means the hematopoietic function of bone marrow was being restored. Notably, mice in the IR+VD3 group had a higher recovery rate of WBCs than mice in the IR group. Beyond that, radiation-induced loss of body weight was alleviated, and the general condition of the mice improved. All of these results indicated the protective effects of 1,25-(OH)2D3 in radiation-injured mice.

1,25-(OH)2D3 is an activated form of vitamin D3. In addition to regulating calcium and phosphorus homeostasis, this steroid hormone also has a wide range of in vivo biological activities, such as anti-tumor effects and immunoregulation (12, 13). In this study, fat vacuoles in the marrow cavity were detected by HE staining. During the process of specimen preparation, lipid in the adipocytes dissolved in dimethylbenzene, and finally there would be some rounded or suborbicular defect regions in sections, reflecting the shape of adipocytes. It is easy to distinguish the lipid defect regions from other irregular defect regions in the bone marrow. Our results suggested that intragastric intervention with 1,25-(OH)2D3 could indeed reduce the formation of adipose cells in the bone marrow to improve the hematopoietic microenvironment of irradiated mice.

In adipogenesis, 1,25-(OH)2D3, as a high-affinity ligand of the vitamin D3 receptor (VDR), activates or inhibits vitamin D3-inducing genes in a VDR-dependent manner to inhibit the expression and decrease the transactivation activity of PPARγ (14, 15). Adipocytes have the unique morphological features seen after routine histologic section and HE staining: circular vacuoles and nucleus squeezed at the edge of cells. These features are more intuitive than other markers. Therefore, in this study, the expression of PPARγ, a member of the nuclear receptor superfamily, in bone marrow of mice was detected by immunohistochemical staining. As shown in Fig. 5, PPARγ not only existed at the rim of adipocytes, but also presented in the non-adipocyte area. Overall, brown color in the normal group was the least; the most was seen in the IR group, followed by the IR+VD3 group. Consistent with reported results in vitro (5) and in vivo (6), our results confirmed that 1,25-(OH)2D3 inhibited bone marrow pimelosis in bone marrow via down-regulation of expression of the PPARγ protein.

Our study investigated the inhibitory effects of 1,25-(OH)2D3 on adipocytes in bone marrow after irradiation via down-regulation of the expression of PPARγ. More animal trials will be needed to verify that 1,25-(OH)2D3 decreases the transactivation activity of PPARγ in vivo. Our results suggest the novel approach of restoring the hematopoietic cells alone for hematopoietic reconstitution after radiotherapy and chemotherapy, as well as for the treatment of aplastic anemia and other bone marrow adipogenic diseases.

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Author contributions
Zhao Zhou, Xiao-Yu Chen and Ge-Xiu Liu conceived and designed the study. Zhao Zhou, Xiao-Yu Chen, Ai-Zhen Zhu, Cheng-Cheng Liu and Jin-Can Zhu performed the experiments. Xiao-Yu Chen, Ai-Zhen Zhu, Cheng-Cheng Liu and Jin-Can Zhu acquired the data. Zhao Zhou, Xiao-Yu Chen and Ge-Xiu Liu performed statistical analysis. Zhao Zhou and Xiao-Yu Chen wrote the paper. Ai-Zhen Zhu, Cheng-Cheng Liu, Jin-Can Zhu and Ge-Xiu Liu reviewed and edited the manuscript. All authors read and approved the manuscript.

Zhao Zhou and Xiao-Yu Chen contributed equally to this work and are co-first authors.

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