Osteoporosis increases chondrocyte proliferation without a change in apoptosis during fracture healing in an ovariectomized rat model

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Abstract. Osteoporotic fractures commonly occur in the elderly. Current studies regarding cell proliferation and apoptosis during osteoporotic fracture healing are limited. In this study, we established an osteoporotic fracture healing model. Bone loss and callus formation were monitored with DXA, cell proliferation was examined using immunohistochemistry with BrdU monoclonal antibody and apoptotic cells were detected using the TUNEL method. Both cell proliferation and apoptosis occurred during the entire period of the study. BrdU immunostaining showed a decreasing tendency in the process of fracture healing. On days 20 and 30 post-fracture, the percentage of BrdU-positive cells in ovariectomized rats was significantly higher compared to sham-operated rats. TUNEL-positive chondrocytes reached a peak on day 20 post-fracture. There was no significant difference between the two groups. Our results indicate that osteoporosis markedly delays the fracture healing process, mostly due to increased chondrocyte proliferation without a change in chondrocyte apoptosis.

Introduction

Osteoporosis is defined as a systemic skeletal disease characterized by low bone mass and micro-architectural deterioration of bone tissue, with a concomitant increase in bone fragility and fracture risk (1). There are increasingly more studies focusing on the pathophysiology, epidemiology, diagnosis and monitoring of the disease, on its metabolic and cellular basis and on the effects of novel therapeutic concepts. However, only a few have placed emphasis on the study of fracture healing, a complex cascade of cellular events. Skeletal conditions affect fracture treatment and healing initially but the effects of osteoporosis on this complex process remain unclear, with studies presenting contradictory results (2-6).

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Cell proliferation and apoptosis play an important role in the process of fracture healing. Previous studies have found that cell proliferation and apoptosis co-exist during the entire period of fracture healing (7). The chondrocytes of fracture callus change their functions and morphology and are ultimately removed by the process of apoptosis (8). There is currently a lack of data regarding cell proliferation and apoptosis during osteoporotic fracture healing. In order to investigate this issue, we established an osteoporotic fracture healing model in ovariectomized (ovx) rats and investigated the impact of osteoporosis on cell proliferation, apoptosis and on associated factors, during the fracture healing process.

Materials and methods

Animals and the osteoporotic fracture model. Sixty female Sprague-Dawley rats, with an average weight of 200±50 g, were used in this experiment. All animals, approved by Sichuan University, were 4 months old at the beginning of the study. Thirty animals underwent ovariectomy (ovx, n=30) and thirty sham operation (sx, n=30). Three months following the ovariectomy, osteoporosis was confirmed in the ovx rats by BMD measurement. Subsequently, an open right femoral midshaft fracture was created and stabilized by intramedullary pins in rats from both the ovx and sx groups (n=30 in each group) in accordance with previous reports (2). Animals were injected with 30 mg/kg of BrdU (Sigma-Aldrich Corp., St. Louis, Mo, USA) 6 h before sacrifice on days 10, 20 and 30 post-fracture. During the experiment, no infection was observed and all animals were maintained in cages with free access to food and water.

BMD measurement of the fracture site. The bone mineral density (BMD) (g/cm^2) was performed using a dual energy X-ray absorptiometer (DXA) (Lunar Radiation Corp., Madison, WI, USA). Three months following the ovariectomy, 7 rats were randomly selected from both the ovx and sx group and the total BMD was measured. The fractured rats were sacrificed on day 10 (n=7 both ovx and sx), 20 (n=7 both ovx and sx), and 30 (n=7 both ovx and sx). Post-fracture and fracture calluses were harvested, and BMD measurement was performed using DXA with small animal mode. An area 7 mm in height and 8 mm in width of the scanning region was centered on each fracture site.

Histological and immunostaining examination. Following the DAX scan, all femurs (n=7 both in ovx and sx) were dissected clean from the surrounding soft tissue and fixed with 4% paraformaldehyde and 0.1 mol/l phosphate-buffered saline at pH of 7.0 at 4˚C for 24 h. The tissues were decalcified at 4˚C in 10% EDTA (Sigma-Aldrich Corp.) at a pH of 7.0, then bisected sagittally in the median plane and embedded in paraffin. Midsagittal sections (6 μm) were made and stained with haematoxylin and eosin (Sigma-Aldrich Corp.).

For immunohistochemistry, the sections were deparaffinized in xylene and immersed in graded ethanol and distilled water. Immunohistochemistry was carried out using the avidin-biotin peroxidase complex (ABC) method according to the manufacturer's instructions. The concentrations of the primary antibody in this experiment were monoclonal antibody against BrdU (1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The primary antibody was omitted as a negative control for the immunostaining method.

Detection of cell apoptosis. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) was performed to detect apoptotic cells according to the manufacturer's instructions (Promega, Madison, WI, USA). In brief, sections were deparaffinized by immersion in xylene and through a graded series of ethanols. After washing in Tris-buffered solution (TBS), the sections were incubated with proteinase K (20 μg/ml) for 20 min. The DNA fragments of apoptotic cells were end-labelled by biotinylated nucleotide mix through the action of the terminal deoxynucleotidyl transferase. The labelled apoptotic cells were identified via a streptavidin-alkaline phosphatase complex and NBT. Negative controls without the transferase were included in each assay.

Cell analysis. All coded sections of the specimens were analyzed in a blinded manner. The stained cells in the calluses were examined under a light microscope (Olympus) at a x400 magnification. The $BrdU^+$ and $TUNEL^+$ cells were calculated independently. Five areas per section were counted and five slides from each animal were examined. The percentage of positive chondrocytes in the total number of chondrocytes in each area was calculated and used for further statistical analysis. All data were expressed as the mean ± SE.

Statistical analysis. Data for the individual time points are presented as the mean and the standard error of the mean. Comparisons between ovx rats and sx rats were made at each time point using the Student's t-test (SPSS, V11).

Results

Bone mineral density measured by DAX. Three months after the ovariectomy, the BMD of ovx rats was significantly decreased compared to that of the sx rats ($p<0.05$) (Fig. 1A). During the fracture healing process, the BMD of the fracture callus in both groups increased gradually. However, the BMD of the ovx rats was significantly lower than the BMD of the sx rats on days 20 and 30 post-fracture ($p<0.05$) (Fig. 1B).

Detection of cell proliferation by BrdU immunostaining. Cell proliferation was evident 10 days post-fracture in the tissue around the fracture gap both in the ovx (Fig. 2A-I) and sx rats (Fig. 2A-II). Most of the BrdU-positive cells were undifferentiated mesenchymal cells found adjacent to the fracture site. There was no significant difference between the ovx and sx rats. With the development of fracture callus, the decline of chondrocyte proliferation in the sx rats from day 10 to 20 was significant, since at that point the typical appearance of hypertrophic chondrocytes was clearly visible within the zone of cartilage matrix (Fig. 2A-IV); In the ovx rats, however, a large portion of proliferating chondrocytes were found in the cartilage islands which extended to span the fracture gap (Fig. 2A-III). On day 30, the majority of the BrdU-positive chondrocytes were noted in the remaining cartilage in the sx rats (Fig. 2A-VI) while in the ovx rats, there were still many more proliferating chondrocytes in the fracture callus (Fig. 2A-V). On days 20 and 30 post-fracture, the percentage of BrdU positive cells in the ovx rats was significantly higher than that in the sx rats. A summary of the statistical analysis of cell proliferation is provided in Fig. 2C.

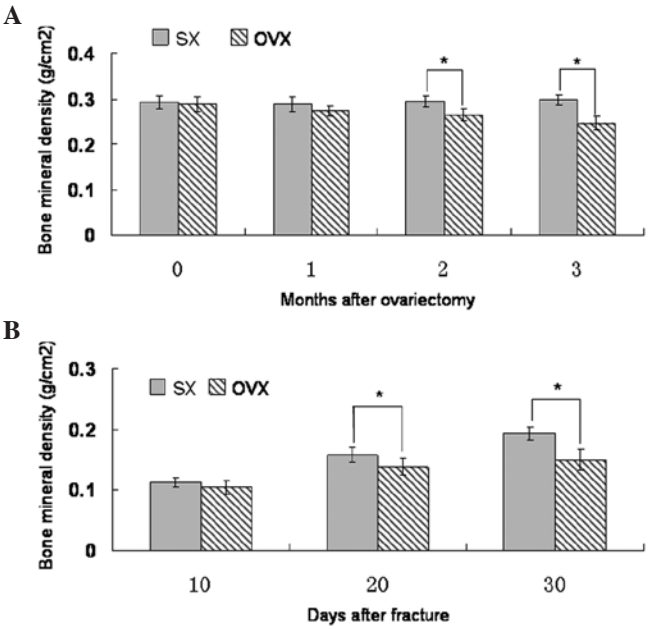

Figure 1. Bone mineral density of the total bone and fracture callus. (A) BMD of total bone after ovariectomy; (B) BMD of fracture callus; ($^*p<0.05$; ovx vs. sx).

Detection of cell apoptosis by TUNEL. TUNEL-positive cells were labelled dark blue (BICP/NBT substrate was used). TUNEL-labelled cells were observed in each period throughout the fracture healing process. During this process, some positive TUNEL-labelled cells were observed in the fibrous callus and the newly formed soft callus near the fracture gap; there was no difference in spatial distribution between the sx and ovx rats. On day 10 following the fracture in the sx rats, some TUNEL-postive chondrocytes were observed in the soft callus (Fig. 2B-II), followed by an increase in TUNEL labelling in chondrocytes in the callus on day 20 post-fracture (Fig. 2B-IV). The number of TUNEL-labelled chondrocytes in the fracture callus peaked on day 20 post-fracture (Fig. 2B-IV) and then declined on day 30, when the mature or hypertrophic chondro-

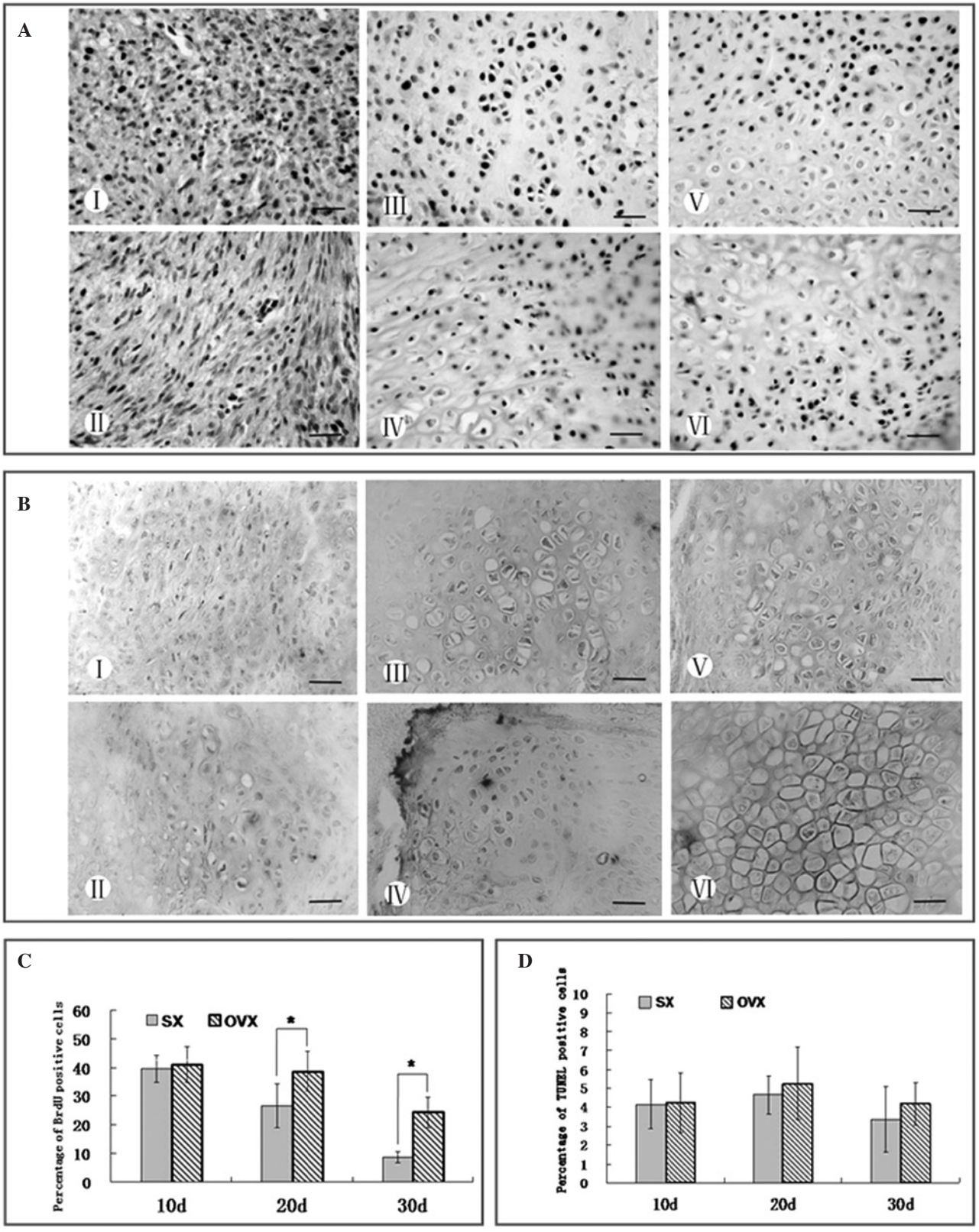

Figure 2. Detection of cell proliferation and apoptosis in the fracture callus of the ovx and sx rats. (A) BrdU immunostaining of the fracture callus; (B) TUNEL staining of the fracture callus. Bar, 50 μm. (C) Summary of cell proliferation during the fracture healing in ovx and sx rats ($^{*}p<0.05$, ovx vs. sx). (D) Summary of cell apoptosis during the fracture healing in ovx and sx rats.

cytes were labelled by TUNEL (Fig. 2B-VI). Compared with the sx rats, the number of TUNEL-labelled chondrocytes in the ovx rats was greater than those in the sx rats on days 10 (Fig. 2B-I), 20 (Fig. 2B-III) and 30 (Fig. 2B-V) post-fracture, but the difference was not significant. A summary of the statistical analysis of cell apoptosis is provided in Fig. 2D.

Discussion

Osteoporosis is a major problem in elderly people and often results in fracture. In recent years, there have been several studies on fracture healing in osteoporosis in animals and most of them have focused on the microstructure and mechanical properties of fracture callus. In the present study, we examined the impact of osteoporosis on the proliferation and apoptosis of chondrocytes of the fracture callus in an ovariectomized rat model. Compared to the control group, the osteoporosis group showed that there was a significant increase in chondrocyte proliferation and a delay in the maturation of the fracture callus. The BMD of the fracture callus was also reduced significantly, but there was no marked difference in the apoptosis of chondrocytes between the two groups. Taken together, these findings indicate that osteoporosis affects chondrocyte proliferation and the remodelling of soft calluses into bony calluses, but it had no influence on chondrocyte apoptosis during the early fracture healing process.

In the present study, we examined chondrocyte proliferation using immunohistochemistry with the BrdU monoclonal antibody, which has been used and reported on previously (9,10). Cell proliferation appears to be a major cellular response during the early period of fracture healing (7,9). In sx rats, the cell proliferation revealed by BrdU labelling first appeared in the fibroblasts and chondrocytes in fracture edges on day 10 post-fracture, and then declined on day 20 and 30 post-fracture. With the development of fracture healing, the chondrocytes underwent differentiation from proliferating to mature and hypertrophic chondrocytes and then the terminal differentiating chondrocytes were removed from the fracture callus. The decline in chondrocytes contributed to the fact that the chondroid callus was replaced with immature osseous tissue. In the ovx rats, however, there was an increase in chondrocyte proliferation and it was noted that the infantile cartilage tissue remained for a longer time in the fractue callus, especially on days 20 and 30 post-fracture. These changes, together with alterations in histological features and BMD of the fracture callus, may contribute to the prolonged endochondral calcification and the reduction in the capacity of endochondral ossification, resulting in a delay in fracture healing. Estrogen deficiency is one of the most important factors in the pathogenesis of osteoporosis. Osteoporotic changes due to estrogen deficiency have been associated with an imbalance between bone formation and bone resorption (11). We established the osteoporotic fracture model by ovariectomy. Thus, the level of estrogen may have had an influence on chondrocyte proliferation and differentiation. As reported by previous studies (12-14), ovariectomy significantly stimulates chondrocyte proliferation and matrix synthesis in the growth plate cartilage, probably through IGF-I (13). This may be one of the reasons contributing to chondrocyte proliferation and inhibition of the removal of hypertrophic chondrocytes.

Accordingly, apoptosis is an important regulatory mechanism of skeletal development and repair. Investigations into the removal of cells from the chondro-osseous junction in growth plate tissue have shown that terminal hypertrophic chondrocytes are removed from the epiphysis via apoptosis. Apoptosis of the last hypertrophic chondrocytes at the transition from cartilage to bone plays a key role in the regulation and coordination of endochondral bone formation (15-17). Our study found that chondrocyte apoptosis occurred during the entire period of fracture healing; this finding is consistent with previous findings (18). Chondrocyte apoptosis increased during the process of fracture healing, reaching a peak on day 20 post-fracture, at which point the proliferation of chondrocytes was in decline, Numerous typical hypertrophic chondrocytes were observed in cartilage tissue, suggesting that apoptosis enhanced the maturation of cartilage tissue and endochondral ossification (19). In the present study, no significant difference in chondrocyte apoptosis was noted between the sx and ovx rats. However, due to the increase in chondrocyte proliferation in the osteoporosis group, there were more mature or hypertrophic chondrocytes existing in the fracture callsus than in the control group. This may have resulted directly in the delay of endochondral ossification.

In conclusion, the present study provides evidence that osteoporosis influences the fracture healing process significantly and that the delay in fracture healing is due mostly to increased chondrocyte proliferation without chondrocyte apoptosis. The findings of our study may implicate a novel viewpoint regarding the features of fracture healing of osteoporosis bone and may provide new ideas into therapies for osteoporotic fracture.

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