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Anti-osteopontin monoclonal antibody prevents ovariectomy-induced osteoporosis in mice by promotion of osteoclast apoptosis

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ABSTRACT

Osteopontin (OPN) is abundant in mineralized tissues and has long been implicated in bone remodeling. However, the therapeutic effect of targeting OPN in bone loss diseases and the underlying molecular mechanism remain largely unknown. Here, we reported that anti-OPN mAb (23C3) could protect against ovariectomy-induced osteoporosis in mice, demonstrated by microcomputed tomography analysis and histopathology evaluation. In vitro assay showed that 23C3 mAb reduced osteoclasts (OCs)-mediated bone resorption through promotion of mature OC apoptosis. Thus, the study has important implications for understanding the role of OPN in OC bone resorption and survival, and OPN antagonists may have therapeutic potential for osteoporosis and other osteopenic diseases.

1. Introduction

Most prevalent metabolic bone diseases, such as osteoporosis, are due to an imbalance in bone remodeling with excessive bone resorption compared with bone formation. Bone resorption has been a target for modern pharmaceutical therapy, such as bisphosphonates and calcitonin. The rate of bone resorption is determined by both the number and activity of osteoclasts (OCs) [1–3]. The number of OCs is dependent upon relative rates of cell differentiation and death. OCs have limited lifespan in vivo and in vitro. Current understanding of the molecular mechanism by which these survival factors prevent OC death is limited, although some studies have reported that the survival of OCs is regulated by certain cytokines and hormones, including estrogen, TGF-β, M-CSF, IL-1, and RANKL [4–11].

Osteopontin (OPN; "bone-bridging" protein), is one of the more abundant non-collagenous proteins in bone matrix that is produced by osteoblasts and OCs [12,13]. Evidence has suggested that OPN is a potent stimulator of the osteoclastogenesis and resorptive activity of mature OCs [14]. Although OPN does not appear to be required for normal development of bones [15], the absence of OPN makes the animal less sensitive to ovariectomy- and unloading-induced bone loss [16–18]. OPN may facilitate OC attachment to the mineralized extracellular matrix and controlling cytoskeleton through integrin αvβ3 [19,20]. However, whether OPN can promote OC survival remains still unclear. Soluble OPN inhibits growth factors and cytokine depletion- induced apoptosis in adherent endothelial cells, which involves enhanced Bcl-xL expression [21]. OPN contributes to anti-apoptotic signaling of melanocytes in dermal collagen through interaction with integrin αvβ3 [22]. OPN-deficient cardiac fibroblasts undergo increased cell death in response to hydrogen peroxide through a caspase-3-independent pathway [23]. OPN null mice show a significant delay in 7,12-dimethylbenzanthracene-induced papilloma development as a result of enhanced apoptotic effects [24]. Hur and colleagues reported that OPN promoted the survival of activated T cells by inhibiting transcription factor forkhead box O3a [25]. These results suggest that OPN exerts its survival activity by way of a cell type-specific mechanism.

To date, no available information regarding the therapeutic effect of targeting OPN in bone loss diseases. Recently, a murine anti-human OPN monoclonal antibody (23C3 mAb), which cross-reacted with murine OPN, was prepared in our lab, and could reduce the severity of established arthritis in mice through promotion of activated T cell apoptosis [26]. In the present study, we investigated whether application of the mAb to OPN could protect against ovariectomy-induced bone loss in mice through induction of OC apoptosis.

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2. Materials and methods

2.1. Mice and reagents

Eight-week-old C57BL/6 mice were purchased from the Second Military Medical University, Shanghai, People's Republic of China. All animals used in this study were housed under pathogen-free conditions and maintained in accordance with guidelines of the Committee on Animals of the Second Military Medical University, Shanghai, China. All animal study protocols (A20090611) were approved by the Animal Care Committee of the Second Military Medical University.

Anti- human OPN monoclonal antibody (23C3), cross-reacting with mouse OPN, was produced as described previously [26]. Purified mouse IgG were purchased from Sigma and used as a control antibody. Recombinant mouse soluble RANKL and M-CSF were purchased from PeproTech EC (London, United Kingdom).

2.2. Generation of mouse OCs

OCs were prepared from bone marrow (BM) derived- cells using a standard method [30]. In brief, BM derived- cells were cultured with 100 ng/ml M-CSF for 3 days to obtain OC precursor cells (OCps) of the monocyte/macrophage lineage. OC precursors were cultured in the presence of 30 ng/ml of M-CSF and 50 ng/ml of RANKL for 6 days. Culture medium was changed every two days. Multinucleated OCs were identified by TRAP activity assay. TRAP-positive cells with three or more nuclei were counted for quantification and represented per well. Rhodamine- phalloidin for F-actin staining was also carried out as described previously [27].

2.3. Bone resorption assay

Single cell suspensions of OCs were seeded onto dentine slices (ALPCO Diagnostic, Windham, NH), incubated at 37 °C, 5% CO_2 in the presence of M-CSF and RANKL. Following 2 days of culture, the slices were rinsed with PBS, then left overnight in 1 M ammonium hydroxide, and stained with 1% toluidine blue in 0.5% sodium tetraborate solution. The number of resorptive areas or "pits" per low power field on each bone slice was counted using reflective light microscopy. The area (mm^2) of each pit was evaluated by measuring width x length using QCapture Pro (Version 5.1) by an investigator who was blinded to the experimental groups.

2.4. OC survival assay

Survival assay was conducted according to the previous report [28]. Briefly, mature OCs were cultured in the presence of anti-OPN mAb for various time and harvested by adding trypsin-EDTA. 1×10^6 cells were suspended in binding buffer containing annexin V-PE (BD Pharmingen) incubated for 20 min at room temperature in the dark. The cells were then analyzed by flow cytometry. Propidium iodide positive cells were excluded from analysis. In situ apoptotic cells were detected using the TUNEL assay (ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit; Chemicon, Millipore) according to the manufacturer's instructions. Cells with positive

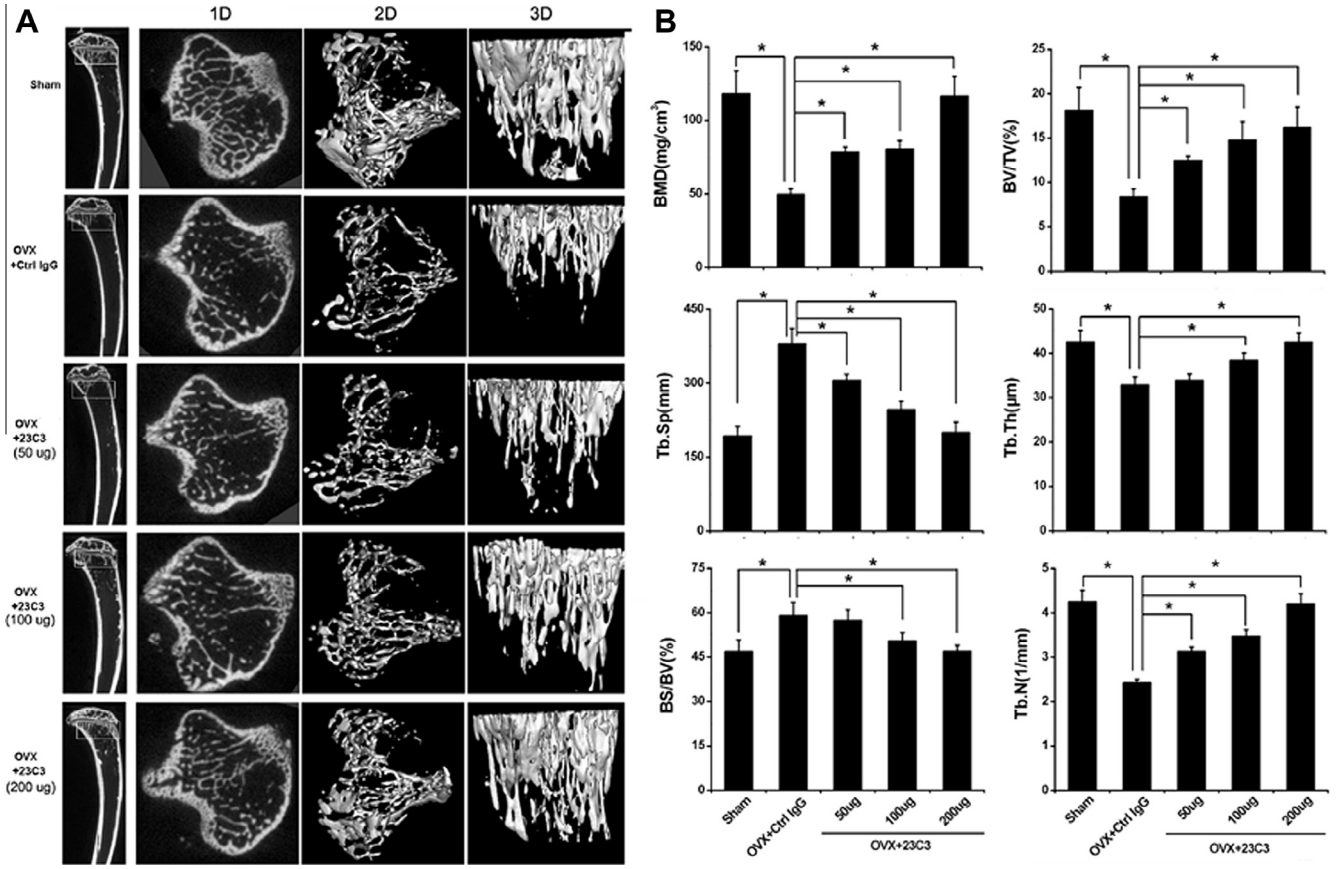

Fig. 1. Therapeutic effect of anti-OPN mAb on ovariectomy-induced bone loss by μCT analysis of the tibiae of mice. (A) Representative micro-computed tomography (μCT) of tibiae bone. (B) 3D trabecular structural parameters in tibiae: bone volume fraction (BV/TV), trabecular number (Tb.N), bone surface/bone volume (BS/BV), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), and bone mineral density (BMD). Data represent mean ± SD. $^*P < 0.05$. Data represent mean ± SD. $^*P < 0.05$.

ApopTag labeling were counted and expressed as a percentage of total cell number.

2.5. Analysis of disease models

Models of osteoporosis induced by OVX-induced model of bone loss were described previously [16]. In these models, more than 10 female C57BL/6 mice were examined in each group. Treatment with anti-OPN mAb (23C3) or control IgG commenced 0 day after OVX by i.p. administration. We continued the treatment at 2-day intervals for 14 days and terminated the experiment on day 31. Histopathology and μCT imaging were carried out for analysis as described previously [27].

2.6. Statistical analysis

Data are expressed as mean ± SD from at least 3 independent experiments. Statistical analyses were performed using the 2-tailed Student's *t*-test to analyze differences among groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Therapeutic effect of anti-OPN mAb on ovariectomy-induced bone loss

We investigated the effect of anti-OPN mAb on the bone loss in ovariectomized (OVX) mice, a classical osteoporosis model. Micro-computed tomography (μCT) revealed that a large reduction in bone volume after ovariectomy in control-Ig treated- mice, whereas OVX mice treated with the mAb 23C3 displayed the marked inhibition in bone loss in a dose dependent manner (Fig. 1A). Quantification evaluation of 3D trabecular structural parameters in tibiae, including bone volume fraction (BV/TV), trabecular number (Tb.N), bone surface/bone volume (BS/BV), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), and bone mineral density (BMD), also supported the potential effect of anti-OPN mAb (Fig. 1B). Histopathology of the proximal tibia showed that the mAb 23C3 treatment significantly improved the loss of cortical and trabecular mass (Fig. 2A), and reduced the number of TRAP$^+$ cells, compared with control Ig-injected OVX animals (Fig. 2B). Furthermore, we found that number of TUNEL$^+$ cells in bone tissue was significantly increased in OVX mice after the mAb 23C3 treatment (Fig. 2C). Taken together, these data suggested that anti-OPN mAb also could prevent ovariectomy-induced osteoporosis in vivo.

3.2. Anti-OPN mAb reduces mature OC mediated- bone resorption in vitro

We further investigated whether anti-OPN mAb could affect the capacity of mature OCs to resorb mineralized matrix by pit formation on dentine slices. As shown in Fig. 3, increasing concentration of mAb 23C3 significantly decreased the number and area of OC mediated-bone pits, compared with control-Ig treatment (Fig. 3A). However, the formation of an actin ring, a cytoskeletal structure that is essential for OC functions such as bone resorption, showed no significant difference between anti-OPN and control Ig treated-OCs (Fig. 3B). Thus, the data indicated that the inhibition effect of anti-OPN mAb on bone resorption by OCs was mediated by cytoskeleton integrity-independent mechanism.

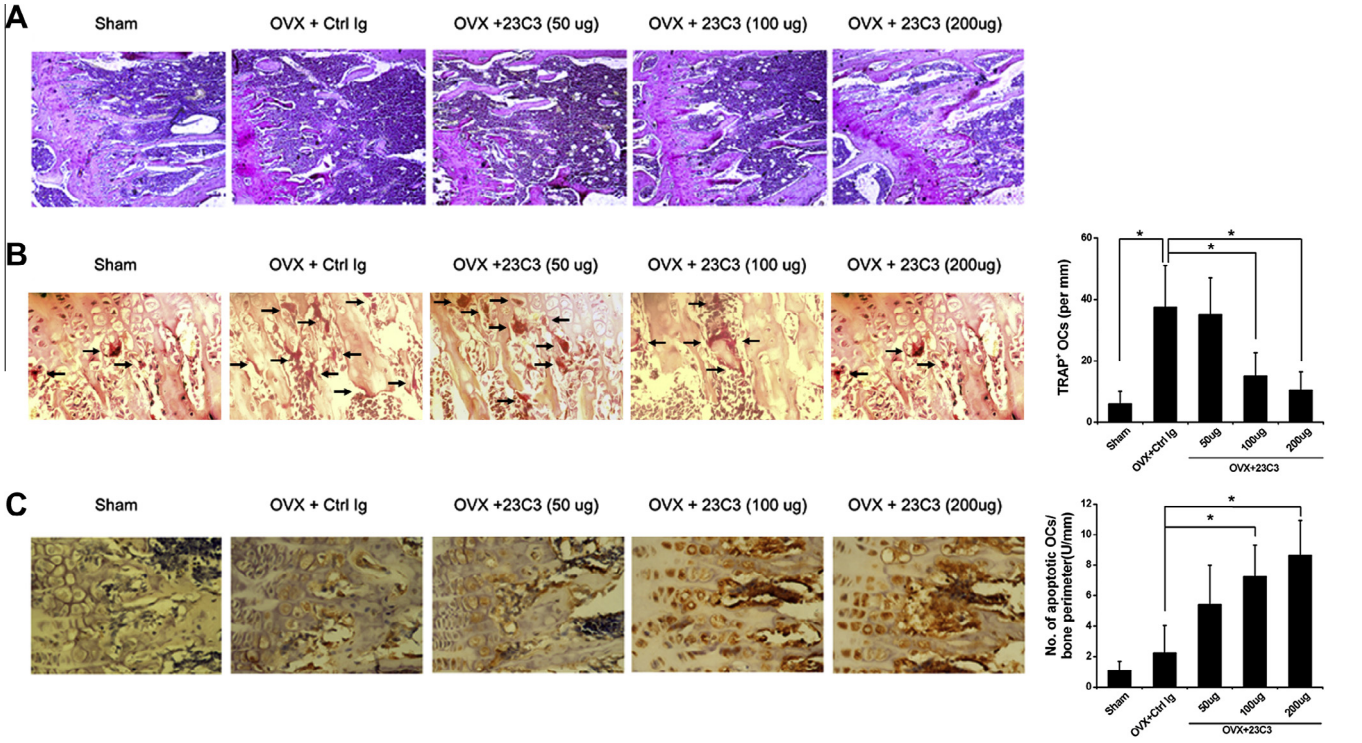

Fig. 2. Therapeutic effect of the anti-OPN mAb on ovariectomy-induced bone loss by histological analysis of the tibiae of mice. (A) Histological analysis of the tibiae from ovariectomized mice by hematoxylin (original magnification, ×100). (B) Histological analysis of the tibiae from ovariectomized mice by TRAP staining. Left panel, representative photographs of OCs for TRAP staining (original magnification, ×320). Right panel, quantification for the percentage of TRAP$^+$ OCs. Data are presented as means ± SD. $^*P < 0.05$. (C) Apoptosis detection of OCs in bone tissue of ovariectomized mice by TUNEL staining. Left panel, photographs are representative of several independent experiments (original magnification, ×320); Right panel, quantification for the percentage of apoptotic OCs. Data are presented as means ± SD. $^*P < 0.05$.

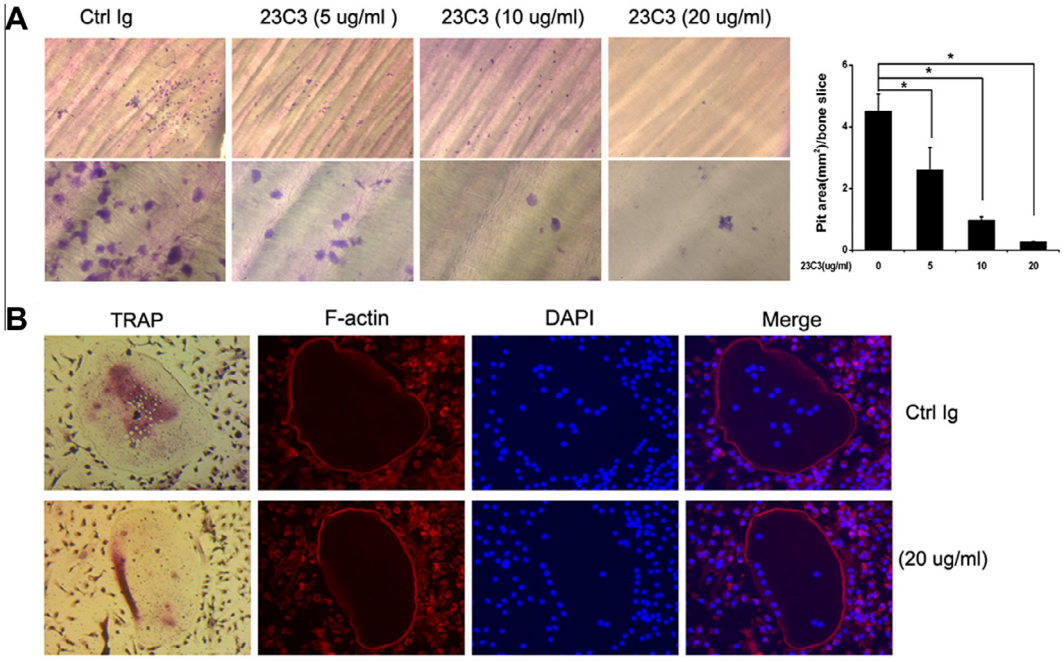

Fig. 3. Effect of anti-OPN mAb on OC bone resorption. (A) Pit formation on dentine slices by OCs in the presence of 23C3 mAb. Left panel, typical image of pits (original magnification, upper, ×40; below, ×200). Right panel, quantification for pits area of resorbed regions. Data represent mean ± SD. $^{*}P < 0.05$. (B) F-actin organization after 23C3 treatment. Representative photomicrograph of mature OCs by F-actin staining is shown (original magnification, ×200).

3.3. Anti-OPN mAb promotes OC apoptosis in vitro

In vivo, we observed the OC apoptosis in anti-OPN mAb treated- osteoporosis mice. Thus, we hypothesis that the inhibition effect of anti-OPN mAb on bone resorption by OCs might be exerted through promotion of OC apoptosis. Expectedly, in the absence of M-CSF, The cultured OC apoptosis was significantly increased in the presence of anti-OPN mAb 23C3, compared with that of control-Ig treated- OCs (Fig. 4A). Flow cytometry analysis by Annexin V staining showed that 23C3 mAb reduced the OC survival in a dose-dependent manner (Fig. 4B and C). TUNEL in situ staining for OCs supported similar results (Fig. 4D). Thus, these data demonstrate that OPN is important for matured OCs survival, and anti-OPN can induce the apoptosis of OCs.

4. Discussion

Many cytokines and factors such as TNF-α, IL-6 and IL-1, are known to induce OC differentiation and function [29,30]. Recently, OPN is known as early T cell activation gene-1 (Eta-1), which has provided a link between the immunity and bone development. Yamamoto et al. demonstrated that treatment of the mice with anti-OPN mAb protected cartilage destruction in a murine model of RA [31]. However, some important parameters of bone loss in vivo were not determined in this study. Moreover, we could not exclude that bone erosion in the collagen-induced arthritis model was result from inflammation cytokines, since anti-OPN reduced the level of TNF-α, IL-6 and IL-1 [26,31]. Thus, in the present study, we rigorously evaluate the therapeutic effect of anti-OPN mAb in OVX-induced bone loss in mice, a classical osteoporosis model, using μCT for quantification evaluation of 3D trabecular structural parameters in tibiae, TRAP and TUNEL staining for OCs in vivo. Collectively, these data showed that OPN might be an appropriate target for the treatment of bone loss diseases involving OCs, including withdrawal of estrogen-induced osteoporosis. Importantly, we first report that neutralization of OPN could promote the apoptosis of mature OC, which lead to reduction of bone resorption by OC, and the improvement of bone loss in mice.

OPN is known to play multiple roles in OC development and function, such as bone resorption. Previously it has been reported that the absence of OPN makes the animal less sensitive to ovariectomy- and unloading-induced bone loss [16–18]. Moreover, we have not entirely excluded the possibility that the mAb 23C3 has other therapeutic functions, such as capacities to inhibit migration and attachment of OCs and osteoclastogenesis that could contribute to the improvement of bone loss. Thus, we investigated whether anti-OPN mAb could affect the capacity of mature OCs to resorb mineralized matrix by pit formation on dentine slices and the formation of an F-actin ring. Our data indicated that the inhibition effect of anti-OPN mAb on bone resorption by OCs was mediated by cytoskeleton integrity-independent mechanism.

Compared with the large body of knowledge about OC formation, bone resorption, migration and adhesion, relatively little is known about the role of OPN in OC survival. Previous studies demonstrated that OPN could protect many type cells from apoptosis [21–25]. Recently, 23C3 mAb could reduce the severity of established arthritis in mice through promotion of activated T cell apoptosis [26]. Here, we observed the OC apoptosis in 23C3 mAb treated- osteoporosis mice in vivo and first report the anti-apoptotic activity of OPN in OCs. These observations clearly indicate that OPN plays a critical role in the OC apoptosis of events that cause OVX-induced bone loss.

In summary, this study is first investigation to define the role of OPN in mature OC survival. The findings may have implications regarding the role of OPN in the bone disorders. Application of 23C3 mAb could protect against ovariectomy-induced bone loss in mice at least partially via promoting OC apoptosis. Thus, targeting OPN may be a promising treatment for various bone resorptive disorders.

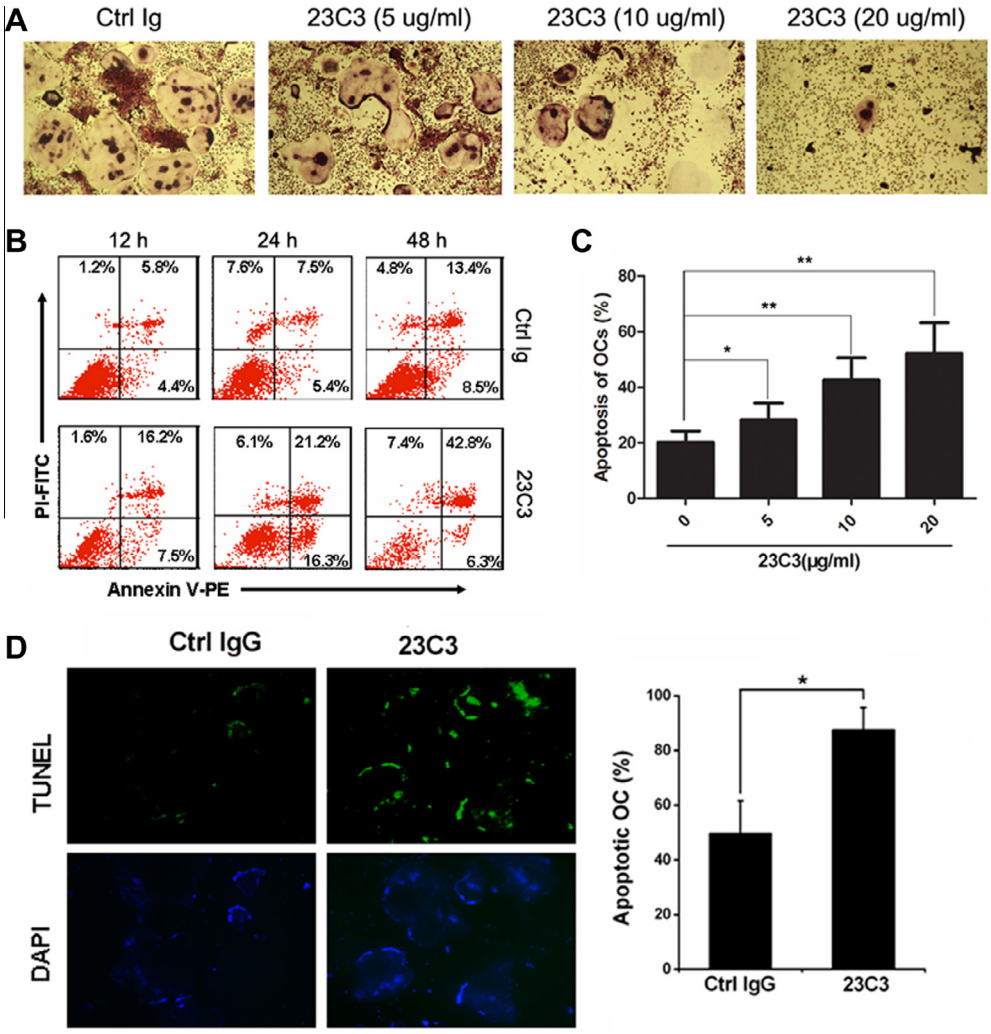

Fig. 4. Anti-OPN mAb promotes apoptosis of mature OCs. (A) Photomicrographs showed apoptotic morphologic changes in OCs by TRAP staining (magnification, ×200) with various concentrations of 23C3 treatment for 48 h. (B) OCs were cultured for various times in the presence of 23C3 (20 μg/ml) treatment. Apoptotic cells were detected by fluorescence flow cytometry with Annexin V staining. The experiments were repeated 3 times. (C) Apoptosis of OCs was determined after various concentrations of 23C3 treatment. (D) Apoptosis of OCs was analyzed by in situ TUNEL staining. Left panel, representative photographs of apoptotic OCs for TUNEL staining (original magnification, ×100); Right panel, quantification for the percentage of apoptotic OCs. Data are presented as means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

Acknowledgments

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