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Fluid Shear Stress Increases Osteocyte and Inhibits Osteoclasts via Downregulating Receptor-Activator of Nuclear Factor κB (RANK)/Osteoprotegerin Expression in Myeloma Microenvironment

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ACDEFG 1 Xiaotao Wang
BDF 1 Yuchan He
BCD 1 Shen Tian
BF 2 Fangxiao Zhu
BE 1 Bo Huang
BF 1 Junyan Zhang
BF 1 Zhong Chen
BF 1 Hangfei Wang

1 Department of Hematology, The Second Affiliated Hospital of Guilin Medical University, Guilin, Guangxi, P.R. China
2 Department of Rheumatic Immunology, The Affiliated Hospital of Guilin Medical University, Guilin, Guangxi, P.R. China

Corresponding Author: Xiaotao Wang, e-mail: wxttjl@126.com
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Background: The aim of this study was to determine the effects of myeloma cells exposed to fluid shear stress on osteocytes and osteoclasts, and clarify the potential underlying mechanisms.

Material/methods: A flow and a non-flow model were established using a flow fluid chamber. The myeloma cell line U266 and murine osteocytic MLO-Y4 cells were cultured *in vitro*. The osteocytes and osteoclasts were examined under a microscope. Osteoclasts were stained for tartrate-resistant acid phosphatase (TRAP) activity. RANKL and osteoprotegerin (OPG) gene expression were detected using reverse transcription-quantitative polymerase chain reaction.

Results: Compared with the controls, Y4 cells cultured with U266 culture supernatant showed altered morphology, fewer osteocytes, increased RANKL gene expression, a higher RANKL/OPG gene ratio, and a greater number of TRAP-positive osteoclasts ($P<0.05$ for all). Compared to the no-flow model, the flow model showed a higher number of Y4 cells, increased OPG gene expression, decreased RANKL gene expression, a lower RANKL/OPG gene ratio, and fewer TRAP-positive osteoclasts ($P<0.05$ for all).

Conclusions: Our study revealed that fluid shear stress ameliorated the inhibitory effects of myeloma cells on osteocyte growth and inhibited osteoclast proliferation by means of decreasing RANKL/OPG gene expression. This may have clinical implications in patients with multiple myeloma in that mechanical loading with low-intensity vibration or mild exercise may prevent the progression of myeloma bone disease.

MeSH Keywords: Multiple Myeloma • Osteoclasts • Osteocytes • RANK Ligand • Shear Strength

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Background

Multiple myeloma is a malignancy characterized by the clonal proliferation of plasma cells in the bone marrow. Myeloma bone disease is a common and serious complication of multiple myeloma characterized by progressive bone destruction and multiple osteolytic bone lesions. Myeloma bone disease involves a breakdown of the metabolic balance in the bone, which leads to increased bone resorption and impaired bone formation. Myeloma bone disease is the main complaint and major cause of disability in multiple myeloma patients. Myeloma bone disease seriously affects the quality of life of multiple myeloma patients and increases the risk of death by 40%. Myeloma bone disease may render patients bedridden for prolonged periods and accelerate bone loss [1]. Furthermore, myeloma bone disease tends to be refractory to treatment.

The metabolic changes in myeloma bone disease are mediated by bone marrow stromal cells and myeloma cells, partially via classical signaling pathways such as receptor-activator of nuclear factor κB (RANK) and its ligand RANKL. These cells increase osteoclast activity and decrease osteoblast activity via RANK signaling, leading to increased osteolysis and hindering new bone formation [2]. Osteoclast and osteoblast activities are regulated by osteocytes, which can sense and integrate mechanochemical signals in the skeleton and coordinate the downstream activities of osteoblasts and osteoclasts. Thus, osteocytes regulate the balance between bone formation and resorption [3]. Although it is known that osteocytes contribute to a microenvironment that favors multiple myeloma and myeloma bone disease progression, their precise role in metastatic multiple myeloma and their effect on the interaction between osteoclasts and myeloma cells remain to be elucidated.

Recently developed therapeutics have decreased bone-related events in myeloma bone disease patients and increased progression-free survival; however, multiple myeloma remains an incurable cancer. In multiple myeloma, the disease process itself as well as the strategies used to treat myeloma can cause considerable bone damage. Increased mechanical loading can promote bone formation by upregulating osteoblasts and inhibiting osteoclasts. Unfortunately, as most multiple myeloma patients are at a high risk for developing fractures, even moderate exercise may not be suitable for these patients [4]. Low-intensity vibration simulates the effects of muscle contraction, and was able to preserve bone quality in a murine model of ovarian cancer [5]. In the absence of mechanical loading, low-intensity vibration can reduce osteoclast activity, decrease bone resorption, and enhance cytoskeletal protein formation [6]. The effects of mechanical loading and low-intensity vibration on bone remodeling, particularly bone resorption and osteoclast differentiation, and the underlying mechanisms are still not fully understood in multiple myeloma patients.

Therefore, in this study, we determined the effects that myeloma cells subjected to fluid shear stress have on osteocytes and osteoclasts. We also explored the potential mechanisms underlying these effects. We used a flow-fluid chamber to simulate fluid shear stress, a mechanical signal involved in the response of bone to mechanical load. We first characterized the response of osteocytes to culture with myeloma-conditioned media and to mechanical loading. We also evaluated the gene expression of RANKL and OPG to assess changes in downstream remodeling. We hope that our results may provide a foundation for the use of mechanical loading as a new physical therapeutic approach in myeloma bone disease treatment.

Material and Methods

Materials

The clonal murine osteocytic cell line (MLO-Y4, BD Laboratories) were used as osteoblastic cells, human multiple myeloma cells (U266, University of Toronto, Canada) and mouse macrophage strain RAW264.7 cells were procured from Bioengineering Laboratory of University of Toronto, Canada. The other materials included α-minimum essential medium (α-MEM) cell culture medium (Sigma Corporation, USA). The flow fluid chamber (device of mechanical engineering of University of Toronto Research Laboratory, Canada) and culture dishes and flasks (BD Biosciences) were used in experiment.

Methods

Cell culture and reagents

Multiple myeloma U266 cells were suspension-cultured in α-MEM medium containing 10% fetal bovine serum (FBS), 5% calf serum (CS), 100 U/mL penicillin, and 100 g/mL streptomycin in an incubator. MLO-Y4 cells were cultured on collagen-coated plates in α-MEM containing 10% FBS and 5% CS, 100 U/mL penicillin, and 100 g/mL of streptomycin. These 2 kinds of cell cultures were followed by incubation at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in a CO_2 incubator. After cells covered the bottom of culture flask (usually after 2 to 3 days), the cell concentration was adjusted into 5×10^5/mL, followed by digestion with 0.25% trypsin and passage under aseptic conditions.

RAW264.7 cells, a murine monocyte/macrophage cell line, were cultured in Dulbecco-modified Eagle medium (DMEM) supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, 1% CS, 100 U/mL penicillin (Lukang Pharma Co., Ltd., Jining, China), and 100 mg/mL streptomycin (Lukang Pharma) at 37°C in a humidified atmosphere with 5% CO_2. Cell digestion and passaging were performed when 80–90% of the flask

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sidewalls were covered by the cells. The culture medium was removed, and the cells were washed thrice with phosphate-buffered saline (PBS) and predigested for 1 minute with 1 mL of 0.25% trypsin (Amresco Inc., Solon, OH, USA). The flask was tapped to dislodge the cells, which were resuspended in alpha-modified minimum essential medium (α-MEM) supplemented with 10% FBS, as described previously. Every 3 days, the culture media were changed, and the cells were passaged.

Mechanical loading

Flow fluid chamber is a mechanical stimulus that osteocytes experience *in vivo* with habitual loading, 2-hour exposure to the pressure at 1 Pa, the current at 1 Hz and the flow rate at 2 cm/sec was selected as the mechanical stimulus. Under these conditions was defined as flow mode. For the non-flow mode, the samples were not exposed to flow. The cells and their media were collected from each group for ensuring experiments. The cells were morphologically observed and counted under a microscope. Each experiment was repeated 5 times.

Study groups

U266 cells, which are a human myeloma cell line, and Y4 cells, which are osteocyte-like cells, were separately cultured with their corresponding culture media for 48 hours and then centrifuged. The supernatants thus obtained were collected for the culture of Y4 cells. In the experimental group, U266 supernatant was used, while in the control group, Y4 supernatant was used for the culture. Samples from both groups were loaded onto a flow fluid chamber. The samples from each group were further subdivided into a flow-mode subgroup and a non-flow subgroup.

Detection of RANKL and OPG gene expression

Under RNase-free conditions, mRNA was isolated using the TRIzol extraction method. The total RNA was quantified using spectrophotometry. Next, 1 μg total RNA was reverse transcribed in a volume of 20 μL by using the OneShine™ First Strand cDNA Synthesis Kit (BioRad, CA, USA) to obtained first-strand cDNA. Finally, 50 ng of the cDNA thus obtained was subjected to reverse transcription quantitative polymerase chain reaction (RT-qPCR) in a final volume of 20 μL. Gene expression analysis was conducted using TaqMan Gene Expression Assay and TaqMan Universal PCR Master Mix (Applied Biosystems, CA, USA; final volume, 20 μL) for the following genes: RANKL (Hs00243522_m1), osteoprotegerin (OPG; Hs00900358_m1), and GAPDH (Hs02758991_m1). All assays were performed in triplicate on a 7500 Fast Real-Time PCR system (Applied Biosystems). Gene expression was calculated using the ΔΔCt method, and each sample was normalized according to the mean GAPDH expression.

Osteoclast formation and differentiation inhibition assay

To determine the inhibitory effect of fluid shear stress on osteoclast formation, the fifth generation of cultured RAW264.7 cells was digested with trypsin, and plated in a 96-well culture plate (density, 2×10^4 cells/well). After overnight incubation, 100 μL of α-MEM containing MLO-Y4 culture supernatant or U266 culture supernatant, 25 ng/mL M-CSF (Peprotech Inc., Rocky Hill, NJ, USA) and 50 ng/mL soluble RANKL (Oriental Yeast, Tokyo, Japan) was replaced in each well, and the cells were cultured for another 6 days in the flow fluid chamber presence or absence of mechanical loading for inducing differentiation into osteoclasts. The medium containing the relevant reagents was changed every 2 days during this period.

Identification of osteoclasts

RAW264.7 cells were treated as previously described, after being cultured for 6 days, the cells were washed 2 times with 1× PBS and fixed with 4% paraformaldehyde for 10 minutes. The cells were then stained for tartrate-resistant acid phosphatase (TRAP) by using an acid phosphatase kit (Sigma-Aldrich, Saint Louis, MO, USA) according to the manufacturer's instructions. Under a light microscope, we classified the TRAP-positive cells in each well as follows: mononuclear cells, multinuclear cells (2–10 nuclei), and giant cells (≥10 nuclei). Each experiment was performed in duplicate.

Statistical analysis

The experimental data were analyzed using Graphpad Prism 5 (Graphpad Software, San Diego, CA, USA). All experiments were conducted separately at least 3 times, and all data are presented as the mean ± standard deviation. Statistically significant differences were assessed by analysis of variance (followed by Bonferroni multiple comparisons test) or Student's *t*-test with *P*-values <0.05 considered significant.

Results

Effects of myeloma and fluid shear stress on the morphology and number of MLO-Y4 cells

Microscopically, the Y4 cells incubated with Y4 culture supernatant (control group) showed a normal, long-spindle shape, while the Y4 cells incubated with the U266 culture supernatant (experimental group) showed greatly changed morphology, transforming from their typical long spindle shapes into various forms, ranging from short shuttle, triangular, thorn-like, and other irregular shapes. They also showed cell-membrane thickening, nuclear enlargement, and cytoplasm darkening (Figure 1). In addition, the number of adherent cells was

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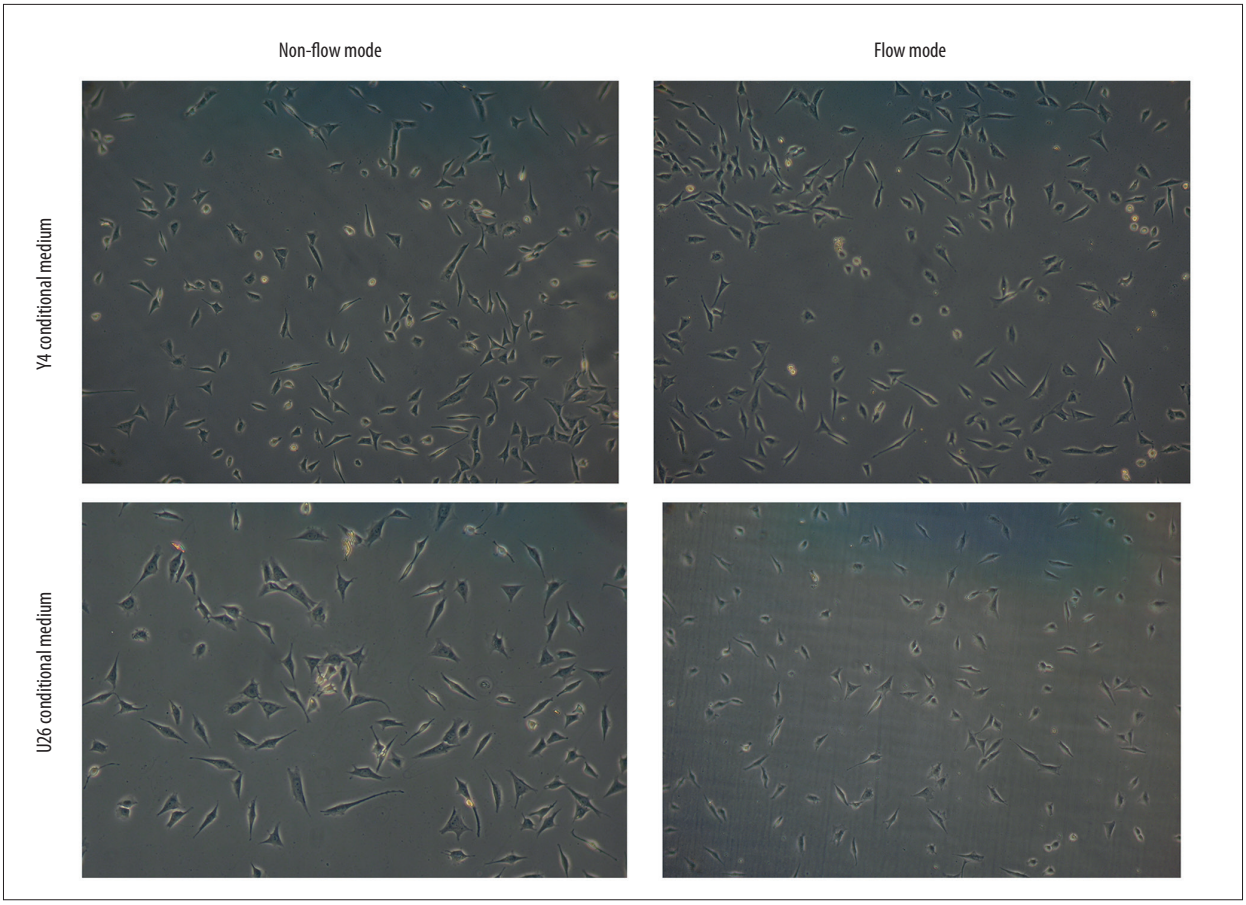

Figure 1. Y4 and U266 culture medium and flow fluid regulated the morphology of MLO-Y4 cells. The morphology under microscope (100×).

reduced. When Y4 cells (regardless of whether they were incubated with U266 or Y4 culture medium) were subjected to fluid shear stress (flow-mode group), their morphology changed, and they showed an increased number of spindle cells as compared with the non-flow group. However, this increase in cell number was not significant compared to the non-flow subgroup ($P>0.05$, Figure 2A). When Y4 cells were incubated with U266 culture supernatant, regardless of whether they were subjected to fluid shear stress, they significantly decreased in number as compared with their counterparts incubated with Y4 culture supernatant ($P<0.05$, Figure 2A). These findings suggest that myeloma cells inhibited osteocyte proliferation and differentiation. Fluid shear stress could alleviate the inhibitory effects of myeloma cells on osteoclast differentiation. Thus, fluid shear stress might promote the differentiation of Y4 cells.

Effects of myeloma and fluid shear stress on RANKL/OPG expression in MLO-Y4 cells

To determine the mechanisms underlying the effects of fluid shear stress MLO-Y4 osteocytes and osteoclasts, we evaluated the expression of RANKL and OPG, which are key genes regulating downstream bone remodeling. RT-qPCR showed that RANKL expression (Figure 3A) and the RANKL/OPG ratio (Figure 3C) were significantly higher in cells cultured with U266 supernatant than in cells cultured with Y4 supernatant in both the flow-mode and non-flow subgroups ($P<0.05$). In both the Y4 (control) and U266 (experimental) groups, the RANKL expression and RANKL/OPG ratio in MLO-Y4 osteocytes were significantly lower in the flow-mode subgroup than in the non-flow subgroup ($P<0.05$; Figure 3B). OPG expression was significantly lower in the U266 group than in the Y4 group, regardless of the presence of fluid shear stress ($P<0.05$, Figure 3B). Furthermore, in both the U266 and Y4 groups, OPG expression was significantly higher in the flow-mode group than in the non-flow group ($P<0.05$, Figure 3B).

Effects of myeloma and fluid shear stress on the morphology and number of osteoclasts

To determine the role of fluid shear stress in osteoclastogenesis, we treated cultured RAW264.7 cells with RANKL to induce osteoclast differentiation. In both the U266 and Y4 groups, osteocytes facilitated osteoclast differentiation in the presence

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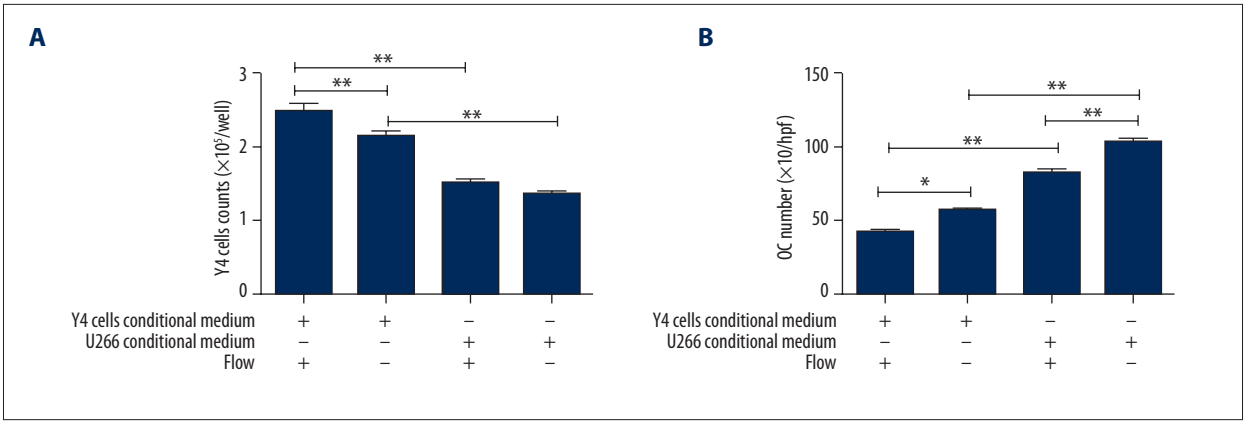

Figure 2. (**A**) Y4 and U266 culture medium and flow fluid regulated the number of MLO-Y4 cells (* *P*<0.05, ** *P*<0.01). (**B**) The number of osteoclast and multinucleated osteoclast-like cells after differentiation of RAW264.7 cells in different group (* *P*<0.05, ** *P*<0.01).

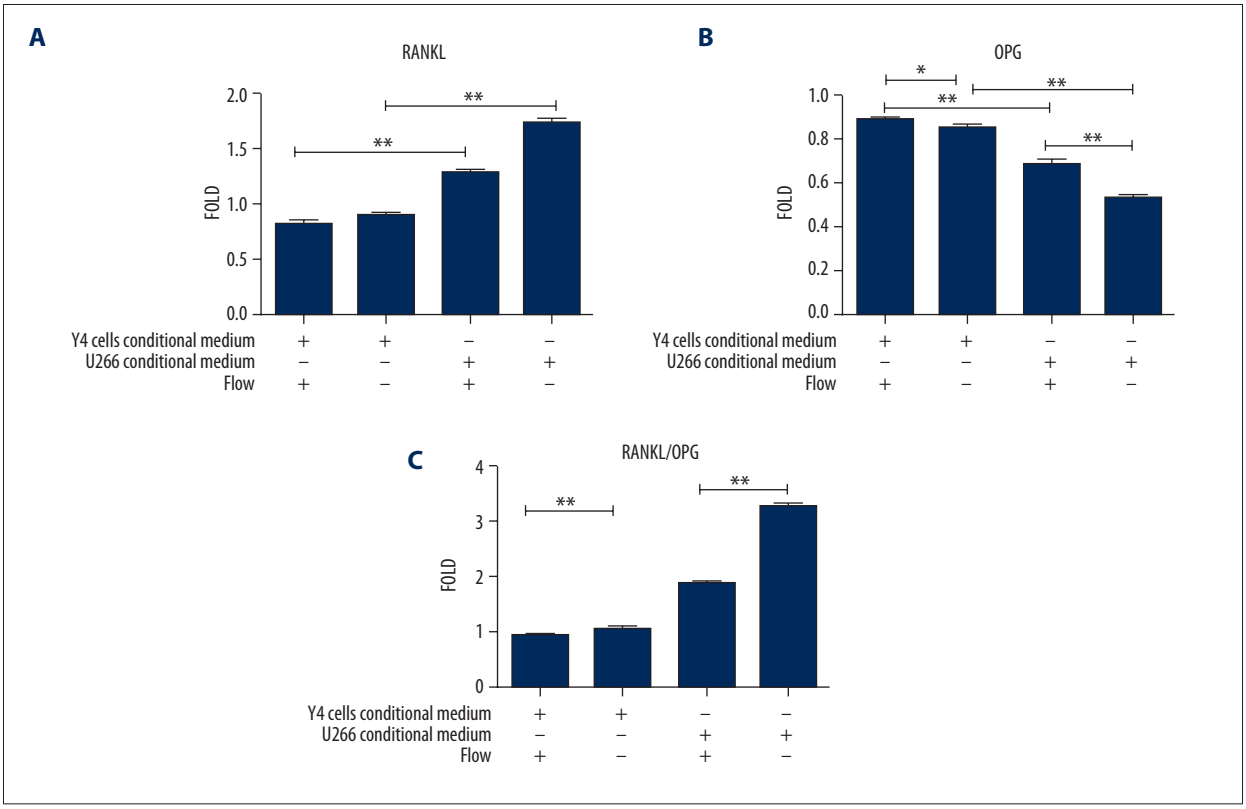

Figure 3. U266 culture medium and flow fluid regulated osteoclastogenesis by regulating RANKL (**A**), OPG (**B**) and the ratio of RANKL/OPG (**C**) in MLO-Y4 osteocytes. (* *P*<0.05, ** *P*<0.01).

of RANKL. TRAP staining revealed that the number of giant and multinuclear cells was significantly greater in the U266 group than in the Y4 group (Figure 4). Additionally, in both the U266 and Y4 groups, fluid shear stress inhibited osteoclast differentiation, as indicated by a decrease in the number of giant and multinuclear cells as compared with the non-flow subgroups (Figure 2B). Additionally, regardless of the presence of fluid shear stress, the number of osteoclasts was significantly greater in the U266 group than in the Y4 group (Figure 2B). These findings suggested that myeloma cells promoted the osteocyte proliferation and differentiation into osteoclasts, and that fluid shear stress could alleviate these effects of myeloma cells.

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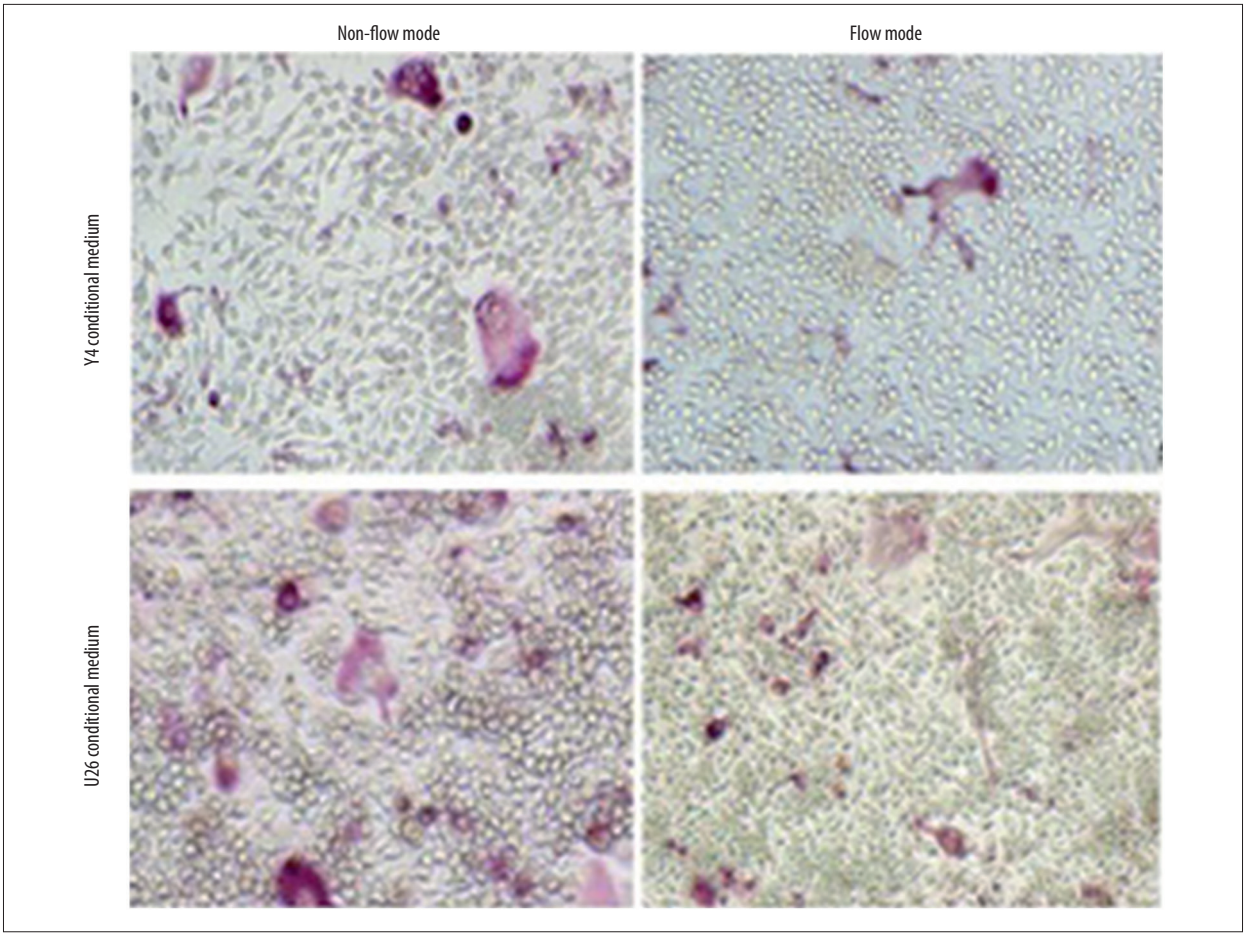

Figure 4. The osteoclast and multinucleated osteoclast-like cells under microscope with TRAP staining after differentiation of RAW264.7 cells in different group.

Discussion

In this study, we demonstrated that compared with Y4 culture supernatant, U266 culture supernatant significantly inhibited MLO-Y4 cell proliferation, altered MLO-Y4 cell morphology, and accelerated osteoclast proliferation in the presence of exogenous RANKL. These effects were ameliorated by exposure to fluid shear stress. These findings suggest that soluble signaling factors from myeloma cells modulate osteocyte mechanosensing and induce osteocyte apoptosis. The apoptotic osteocytes might attract more osteoclast precursors, stimulating osteoclast recruitment, and initiating and/or sustaining bone resorption [7]. These findings are consistent with those of previous studies on myeloma-induced osteoblast inhibition and osteoclast activation [8]. In normal bone, osteoblasts and osteoclasts maintain a dynamic balance between bone resorption and formation, via interactions among the OPG/RANKL/RANK system [9], Wnt signaling pathway, and ubiquitin proteasome pathway [10]. In myeloma bone disease patients, however, this balance is upset due to the interaction of myeloma cells and the bone marrow microenvironment, leading to increased activity and number of osteoclasts and decreased activity and number of osteoblasts, resulting in excessive bone resorption and eventually osteolytic bone destruction. We previously found that mip-1a and sclerostin gene expression are enhanced in culture media containing primary human myeloma cells [11]. Sclerostin inhibits osteoblastic bone formation, and mip-1a is an osteoclast-activating factor. Furthermore, many studies report that U266 cell culture media contain other autocrine factors such as interleukin (IL)-6 and IL-1, which are generated by malignant plasma cells [12]. IL-6 is associated with myeloma-cell proliferation, bone resorption, and osteoclastogenesis. IL-1 is considered to be an osteoclast-activating factor, and can induce large amounts of IL-6 [13]. Malignant plasma cells stimulate osteoclasts via IL-1 production and autocrine or paracrine IL-6 secretion, leading to osteolytic disease [14]. The aforementioned findings indicate that myeloma cells induce osteolysis by promoting osteoclastogenesis and inhibiting osteoblastogenesis through the action of various autocrine factors.

The perturbed bone metabolism in multiple myeloma increases fracture risk and alters the immune system. Exercise regulates

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the growth and development of bone tissues and helps maintain bone mass. Numerous *in vivo* studies have revealed that mechanical unloading leads to osteocyte apoptosis, which can be reduced through exogenous mechanical stimulation [15,16]. Leisure-time physical activity has been associated with a lower risk of myeloma, and low-intensity shaking delayed tumor progression and helped conserve bone mass in multiple myeloma mice [17,18]. However, the underlying mechanisms are not yet understood. Furthermore, although exercise is an effective non-pharmaceutical treatment for the bone-weakening effects of both multiple myeloma and osteoporosis, strenuous activity can induce pathological or compression fractures in multiple myeloma patients, and is best avoided. In this study, we showed that fluid shear stress stimulated osteoblast differentiation, as indicated by the increase in the number of MLO-Y4 cells and dendrite formation in the flow-mode subgroup as compared to the non-flow subgroup. This indicates that fluid shear stress attenuated the stimulatory effect of the myeloma culture medium on osteocyte-mediated osteoclastogenesis. Our data indicate that fluid shear stress might be unfavorable to osteocyte apoptosis and thereby help maintain the number of bone cells and facilitate recovery from myeloma bone disease. Osteocytes subjected to fluid shear stresses ranging between 0.5 and 2 Pa *in vitro* release important messengers, including intracellular calcium, nitric oxide, prostaglandin E2, and adenosine triphosphate [19]. These small molecules may transfer mechanical loading-induced signals via connexins, resulting in connexin upregulation, increased osteogenesis, and increased osteocyte differentiation [20]. Another possible mechanism via which fluid shear stress inhibits osteocyte apoptosis is that fluid flow may accelerate oxygen and nutrient supply to the osteocytes, increasing cell viability [21].

When RAW 264.7 cells were exposed to fluid flow for 2 hours in a co-culture system with U266 cell culture medium and exogenous RANKL for 3 days, the number of TRAP-positive multinuclear-cells significantly decreased as compared to cells not exposed to fluid flow. This demonstrated that fluid shear stress might suppress osteoclast activity and promote osteoclast apoptosis, which is consistent with a previous report [20,22]. The aforementioned findings indicate that fluid shear stress may inhibit bone resorption and promote recovery from myeloma bone disease.

Osteoclastic bone resorption is dependent on RANKL expression. OPG, a decoy receptor for RANKL, competitively inhibits RANKL binding to RANK, which not only blocks the formation of new osteoclasts but also shortens the survival of osteoclasts already present in the bone tissue. Thus, the balance between RANKL and OPG expression determines the volume of bone resorption. In this study, we found that fluid shear stress decreased RANKL expression and increased OPG expression in MLO-Y4 cells, significantly decreasing the RANKL/OPG ratio. Changes in the RANKL/OPG ratio have been shown to directly affect osteoclast formation in *in vitro* experiments [23]. Thus, fluid shear stress is a potent regulator of osteocyte differentiation, as it suppresses osteoclastogenesis via OPG upregulation and RANKL downregulation. The results of our *in vitro* experiment suggest that low-intensity vibration or mild exercise (analogous to fluid shear stress) may help multiple myeloma patients recover from myeloma bone disease. Further studies are warranted to understand the specific mechanisms underlying these findings and evaluate their clinical value.

Conclusions

Our study revealed that fluid shear stress ameliorated the inhibitory effects of myeloma cells on osteocyte growth and inhibited osteoclast proliferation by means of RANKL and OPG signaling. This may have clinical implications in patients with multiple myeloma in that mechanical loading with low-intensity vibration or mild exercise may prevent the progression of myeloma bone disease.

Conflict of interest

None.

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