FTY-720P Suppresses Osteoclast Formation by Regulating Expression of Interleukin-6 (IL-6), Interleukin-4 (IL-4), and Matrix Metalloproteinase 2 (MMP-2)

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Background:
Osteoclast formation is closely related to the immune system. FTY720, a new immunosuppressive agent, has some functions in immune regulation. Its main active ingredients become FTY-720P in vivo by phosphorylation modification. The objective of this study was to determine the effects of FTY-720 with various concentrations on osteoclasts in vitro.

Material/Methods:
RAW264.7 cells and bone marrow-derived mononuclear phagocytes (BMMs) were treated with RANKL to obtain osteoclasts in vitro. To investigate the role of FTY-720 in osteoclast formation, trap enzyme staining was performed and the number of osteoclasts was counted. Bone slices were stained with methylene blue, we counted the number of lacunae after bone slices were placed into dishes together with osteoclasts, and we observed the effect and function of FTY-720 in osteoclasts induced by RAW264.7 cells and BMMs. Then, we used a protein array kit to explore the effects of FTY-720P on osteoclasts.

Results:
The results of enzyme trap staining and F-actin staining experiments show that, with the increasing concentration of FTY-720P, the number of osteoclast induced by RAW264.7 cells and BMMs gradually decreased (P<0.05), especially when the FTY-720P concentration reached 1000 ng/ml, and the number of osteoclasts formed was the lowest (P<0.05). With bone lacuna toluidine blue staining, the results also show that, with the increasing concentration of FTY-720P, the number of bone lacuna gradually decreased (P<0.05), and the number of lacunae is lowest when the concentration reached 800 ng/ml. Finally, protein array results showed that IL-4, IL-6, IL-12, MMP-2, VEGF-C, GFR, basic FGF, MIP-2, and insulin proteins were regulated after FTY-720P treatment.

Conclusions:
FTY-720P can suppress osteoclast formation and function, and FTY-720P induces a series of cytokine changes.

MeSH Keywords:
Bone Diseases • Interleukin-4 • Interleukin-6 • Matrix Metalloproteinase 2 • Osteoclasts

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Background

The skeletal system constantly repairs itself in response to resisting pressure. In this process, bone repair depends mainly on the continuous removal of necrotic bone tissue and producing new bone tissue. Osteoclasts are known as “scavengers” in bone tissue, which appear in the later stage of embryo development. They reconstruct and update primary cancellous bone and epiphyseal cartilage of the long bones. After birth, it updates the primary cancellous bone for bone density and fracture repairs [1–3]. In osteoclast function-deficiency mice (c-fos gene knock in mice or c-Src gene knock in mice) with osteoporosis, osteoclasts mainly appear in high-density bone and extended bone-presenting resorption performance [4,5]. In addition, osteoclasts play a key role in bone defects and osteoporosis. Controlling osteoclasts has become a key treatment. Osteoclasts activity in vivo can be affected by estrogen and other hormones [6–10]. In addition, osteoclasts are associated with the human immune system. The role of osteoclasts in orthopedics and the innovation of treatment has important significance [11,12].

Osteoclasts and macrophages are similar in morphology and function. Their functions can be explained by phagocytosis theory [13]. Researchers used macrophages to induce osteoclasts, which supports this hypothesis. Udagawa et al. [14] successfully induced mononuclear cells derived from bone marrow cells, proving that osteoclasts are most likely derived from monocytes. Osteoclasts and the immune system are closely related, which can be used to inhibit the role of osteoclasts. Kusano et al. showed that IL-1 and IL-6 can effectively promote the synthesis and secretion of matrix metalloproteinases, which can enhance the function of osteoclasts [115]. Ota et al. also reported that TGF can induce CXCL16 and LIF and increase the osteogenic function of osteoblasts [16].

FTY720 is a component extracted from Cordyceps sinensis [17–19], and has been certified by the United States FDA as a new type of immunosuppressive agent. Its main active ingredient is FTY-720P, created in vivo by phosphorylation modification. Its mechanism is to inhibit the S1P receptor pathway [20–23], which affects the differentiation of T lymphocytes and also affects bone formation. Masaru reported that FTY720 competes with S1P to compete with its S1P receptor to reduce bone loss in the bone surface [24]. These studies show that FTY-720 can inhibit immune response and affect the differentiation and function of osteoclasts, but the effect of FTY-720 on osteoclasts remains unclear.

In this study, BMMS and RAW264.7 cells were induced into osteoclasts with RANKL in vitro. We observed the bone lacuna, analyzed the effect of FTY-720P on osteoclasts, and explored osteoclast phagocytic function. Protein array was used to explore the effects, and it is useful in learning how FTY-720P improves and affects osteoclasts.

Material and Methods

Cell culture

The RAW264.7 mouse monocyte cell line was obtained commercially from ATCC (USA). The cells were maintained in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin/streptomycin solution at 37°C in 5% CO². Cells were plated at a density of 3×10⁴ cells/well in 6-well plates and grown in DMEM containing 50 ng/ml RANKL (R&D, MN, USA). At about 7 days they differentiated into osteoclasts, and then the medium was changed every 2 days.

Bone marrow cells were collected from the femurs and tibias of 4-week-old male SD mice. They were suspended in DMEM supplemented with FBS (10%) and antibiotics (1%) and cultured in flasks with 10 ng/ml M-CSF (Sigma, USA). After 24 h, non-adherent cells were removed and suspended in DMEM medium with FBS (10%), antibiotics (1%), and M-CSF (10 ng/ml). Next, RANKL (30 ng/ml) was added to the medium and cultured for 7 days; bone marrow cells differentiated into osteoclasts during this period. All experiments were conducted in accordance with a protocol approved by the Southern Medical University Animal Care Committee.

TRAP staining

The induced RAW246.7 and BMCs were washed with PBS 3 times, then 4% paraformaldehyde (PFA) was added and fixed for 15 min. The cells were washed 3 times again and fixed with 4% PFA. We added TRAP enzyme reagent into dishes in accordance with the instructions. The cells were incubated at 37°C for about 1 h, and then we removed the supernatant, washed the cells with 37°C deionized water 3 times, and co-stained them with hematoxylin. The results were observed under an inverted phase-contrast microscope (OLYMPUS, CK40-F200, Japan). Cells were counted and analyzed using Image-Pro Plus 5.0 software.

F-actin staining

Cells were washed with PBS 3 times. Acetone solution was used to fix the cells for 10 minutes, then they were washed 3 times again using PBS 4 minutes each time. We used 0.1% Triton X-100 solution to treat cells for 25 min, then they were washed twice with PBS for 10 min each time. After blocking with 10% goat serum at 37°C, cells were incubated with primary antibody (Phalloidin labeling with Rhodamine, Sigma, USA) at 4°C overnight, and then washed again 3 times for 5 min.
DAPI (4’,6-diamidino-2-phenylindole) was used to stain cell nucleus, observed under fluorescence microscopy.

**Osteoclasts were treated with FTY-720P**

We induced 5×10⁵ RAW264.7 cells per well into the osteoclasts. To investigate which concentration of FTY-720P (Cayman USA) is best, the concentrations of FTY-720P were divided into groups with 0, 500, 600, 700, 800, 900, 1000, 1500, 2000, and 2500 ng/ml. We also added the corresponding concentration into BMMs. All of the above culture systems were changed at 2 days in order to maintain the appropriate concentration of FTY-720. After incubating for 4 days, cells were observed under a microscope. These breaking cells were identified in accordance with the method of bone osteoclasts identification. After 10 days in culture, TRAP enzyme staining and F-actin staining were performed.

**Osteoclast phagocytosis test**

In the various concentrations of FTY-720P, some of the induced BMMs and RAW264.7 turned into osteoclasts. Cells morphology was observed and the bones were devoured. After 10 days in culture, the bone fragments were removed and the bone slices were placed for 10 min at 4°C in the 2.5% glutaraldehyde solution, then treated with 0.25 mol/L ammonium hydroxide and washed with distilled water 3 times. Slides were immersed into 50%, 70%, 80%, and 95% ethanol for about 1 min for dehydration. Staining was performed with 1% toluidine blue stain for 4 min at room temperature, washed with distilled water 3 times for 1 min, and then we observed osteoclast resorption pits under a microscope.

**Protein arrays**

We added 700 ng/ml FTY-720P and DMSO to the experimental groups and control group, respectively. Cells were cultured for 10 days, then proteins were extracted with a kit (Cat: AAR-BLM-1, Cytokines arrays kit, RayBiotech, Guangzhou, China). A protease inhibitor cocktail was added into cell lysis buffer and mixed thoroughly. Proteolytic extraction was carried out about 30 min after mixing well and cell lysate. The supernatant was obtained by centrifuge and kept at ~80°C for protein microarray analysis. Protein arrays were performed in accordance with kit instructions. The results were analyzed with protein array kit software.

**Statistical significance**

The results are presented as mean ±S.D. All statistical analyses were performed by SPSS 13.0 (San Rafael, CA,USA) using ANOVA or the t test. Significance was set at p<0.05.

**Results**

**FTY-720P suppressed osteoclast formation induced by RAW264.7**

RAW264.7 cells were induced into osteoclasts with RANKL. In this study, various concentrations of FTY-720P were used to treat the induced osteoclasts from RAW264.7. The results show that they have no effects on osteoclasts formation (P>0.05) when FTY-720P concentration are 500 ng/ml and 600 ng/ml. However, at the concentration of 700 ng/ml, the positive rate of osteoclasts begins decreasing. In the FTY-720P group, we found that 800 and 900 ng/ml also could suppress osteoclast formation (P<0.05), but 1000, 1500, 2000, and 2500 ng/ml in the FTY-720P group had no differences in inhibiting osteoclasts formation among each other (P>0.05, Figure 1A). Figure 1B also demonstrates similar results in TRAP staining, indicating the 700 ng/ml group had dramatically suppressed osteoclast formation (yellow staining).

**FTY-720P also suppresses osteoclast formation derived from BMCs**

Bone marrow cells were extracted from mouse bone marrow. Under a light microscope, the round and polygonal cells can be observed. The few cells with a single nucleus were BMMs. These cells was treated with M-CSF and RANKL for 24 h, then the cells showed spindle portion changes, gradually becoming necrotic and floating on the culture medium surface, and these were discarded. Continuing incubating the culture, after 4 days, a small number of larger cells appeared, which were multi-nuclear cells with extending tentacles. At about 7 days, there were many visible multi-nuclear giant cells growing more pseudopods. By F-actin staining, the results show that these giant multi-nucleated cells were osteoclasts. After treating them using various concentrations of FTY-720P, the data show that the FTY-720P concentration of 700 ng/ml had a significant reduction in the number of osteoclasts formed (Figure 2).

**The phagocytic function of osteoclasts is suppressed by FTY-720P**

The above results demonstrated that FTY-720P could suppress the number of osteoclasts formed. To investigate the phagocytic function of osteoclasts when BMMs were induced into osteoclasts, we co-cultured it with bone fragments, and after 7 days, toluidine blue staining was performed. The results suggest that osteoclast phagocytic function was decreased in the FTY-720P group compared to the control group, while in the FTY-720P group the bone fragments remained better preserved than in the control group (Figure 3A). Under the microscope, the group of 500 ng/ml FTY-720P began suppressing osteoclast formation, and less zona pellucida was formed. Osteoclast phagocytosis was lower in the 500 ng/ml concentration of 700 ng/ml, the positive rate of osteoclasts better preserved than in the control group (Figure 3A). Under the microscope, the group of 500 ng/ml FTY-720P began suppressing osteoclast formation, and less zona pellucida was formed. Osteoclast phagocytosis was lower in the 500 ng/ml concentration of 700 ng/ml, the positive rate of osteoclasts better preserved than in the control group (Figure 3A). Under the microscope, the group of 500 ng/ml FTY-720P began suppressing osteoclast formation, and less zona pellucida was formed. Osteoclast phagocytosis was lower in the 500 ng/ml concentration of 700 ng/ml, the positive rate of osteoclasts better preserved than in the control group (Figure 3A). Under the microscope, the group of 500 ng/ml FTY-720P began suppressing osteoclast formation, and less zona pellucida was formed. Osteoclast phagocytosis was lower in the 500 ng/ml concentration of 700 ng/ml, the positive rate of osteoclasts better preserved than in the control group (Figure 3A). Under the microscope, the group of 500 ng/ml FTY-720P began suppressing osteoclast formation, and less zona pellucida was formed. Osteoclast phagocytosis was lower in the 500 ng/ml concentration of 700 ng/ml, the positive rate of osteoclasts better preserved than in the control group (Figure 3A).
FTY-720P group compared to the control group, and the 800 and 900 ng/ml FTY-720P group had a reduced number of damage dots in bone fragments. The number of lacunae per bone chip or fragment was counted. The results suggest that the control group without FTY-720P, and the 500 and 600 ng/ml groups had more lacunae than in the 700 ng/ml FTY-720P group (P<0.05). The 700 ng/ml group had more osteoclasts than in the 800 and 900 ng/ml FTY-720P groups (P<0.05). The lowest osteoclast formation was in the 1000, 1500, 2000, and 2500 ng/ml groups and there are no differences between each other (P>0.05). (B) Representative images of cells treated with different concentrations of FTY-720P. Osteoclasts were observed under a light microscope. The osteoclasts were stained with TRAP staining. The number of osteoclasts formed was decreased with the gradually increased FTY-720P concentration.

Cytokine alteration in osteoclasts with FTY-720P treatment

To investigate which cytokines had been involved after FTY-720P treatment, protein array was carried out using protein array kits. Image Quant LAS4000 Scanner software was used to analyze the results. The fold change values of greater than 1.2 and less than 0.8 were regarded as showing a difference between the control group and FTY-720P treatment groups (Figure 4A, 4B). The results suggest that interleukins, transforming growth factor family of proteins, Fas protein, IL-6, IL-4, and MMP2 were altered.

Figure 1. FTY-720P suppressed the osteoclasts formation being induced by RAW264.7 (A) RAW264.7 cells were treated with FTY-720P, which induced RAW264.7 into osteoclasts. The rate of osteoclasts formation with different concentrations of FTY-720P was assessed. When the concentration was 500 or 600 ng/ml, it induced more osteoclasts formation than 700 ng/ml FTY-720P (P<0.05). The 700 ng/ml group had more osteoclasts than in the 800 and 900 ng/ml FTY-720P groups (P<0.05). The lowest osteoclast formation was in the 1000, 1500, 2000, and 2500 ng/ml groups and there are no differences between each other (P>0.05). (B) Representative images of cells treated with different concentrations of FTY-720P. Osteoclasts were observed under a light microscope. The osteoclasts were stained with TRAP staining. The number of osteoclasts formed was decreased with the gradually increased FTY-720P concentration.
Toll-like receptor, insulin-like protein and other proteins, and chemokine expression are obviously different in the control group and the experimental group, mainly IL-4, IL-6, IL-12/IL-23p40, MMP-2, VEGF-C, GFR alpha-1, basic FGF, insulin, and MIP-2 expression, all of which were higher in the experimental groups (Figure 4C).

Discussion

In recent years, it was discovered that osteoclasts can restore human bone gradually, and another researcher found that osteoclasts and the immune system are inextricably linked [12]. Therefore, osteoclasts have an important role in the treatment of orthopedic disorders. Osteoclasts are considered to be difficult to culture in vitro, but there are no criteria for deciding on culture conditions. Currently, there are 3 main theories about osteoclasts: (1) osteogenic theory; (2) blood origin theory, and (3) the monocyte-macrophage system theory. Technological developments have led to acceptance of the monocyte-macrophage system theory. In our previous study, we found that tumor necrosis factor superfamily, OPG, and RANKL are the 3 factors that can influence the NF-kB pathway, which is consistent with a previous report [25]. Osteoclast formation was induced by BMMs and RAW264.7 cells with M-CSF and RANKL. The method is simple and easy to carry out in experiments; therefore, it has been more widely accepted.

FTY-720P is an ingredient extracted from Cordyceps sinensis, which affects the S1P receptor and interferes with dendritic cells, NK cells, NKT cells, and macrophages, as well as regulating immune function. Using RAW264.7 cells, we induced osteoclasts. Various concentrations of FTY-720P can hinder bone cell formation, and we found that 700 ng/ml is an appropriate concentration for controlling osteoclast formation in our study. For 1000 ng/ml, there are also lower positive rates of osteoclast formation, indicating that the various concentrations of FTY-720P can effectively inhibit osteoclasts formation. The results of TRAP and

Figure 2. FTY-720 suppressed BMMs being induced into osteoclasts. BMMs were induced into osteoclasts, then it was stained with F-action antibody in different FTY-720P concentrations. The number of osteoclasts was dramatically decreased (magnification: ×100), especially when FTY-720P was 1000 ng/ml.
F-actin staining demonstrate that when the FTY-720P concentration reaches 700 ng/ml, the number of osteoclasts induced from BMMs significantly reduced their ability to swallow bone pieces. FTY-720P decreased the number of osteoclasts derived from RAW264.7 cells, which is consistent with that of BMMs. Osteoclast formation and function are affected by FTY-720P. In our study, many proteins were being regulated. Osteoclast formation and function is involved with the following proteins: tumor necrosis factor superfamily (TNFs), transforming growth factor family (TGFs), colony stimulating factors (CSFs), and interleukin 4. Interleukins such as IL-1, -3, -6, -8, and -10 play an important role in osteoclast differentiation and maturation of osteoclast function [26]. Experimental results show that IL-1 and IL-6 have combined effects, promoting osteoclasts to synthesize and secrete metalloproteinases, which enhances the function of osteoclasts. IL-3 also can effectively improve the osteoclasts and enhance their functions [15,27]. IL-4 can directly inhibit osteoclast precursors to differentiate into mature osteoclasts, but also can inhibit the JNK/STAT signaling pathway and the RANKL expression of inducible transcription factors to reduce the differentiation and function of osteoclasts [28,29]. IL-6 and PGE2I act as RANK-RANKL-OPG pathways to regulate osteoclast maturation and function [30].

In this study, in the FTY-720P group, IL-4 expression was increased but IL-1 and IL-3 expression was reduced, showing that FTY-720P treatment reduced the positive rate of osteoclast formation and osteoclast function.

The transforming growth factor family also plays an important role in osteoclast formation, suggesting that TGF-β affects osteoclast maturation, inhibiting osteoclast maturation but not promoting maturation of osteoclasts. Moreover, Ota et al. [31] found that TGF-β can induce osteoclasts producing CXCL16 and LIF, which are 2 biologically active molecules, thereby increasing osteoblastic function.

The members of the tumor necrosis factor superfamily (TNFs) are the most important factors influencing osteoclast maturation [16]. TNFs such as RANKL and RANK appear to be osteoclast maturation proteins [25,32]. After FTY-720 treatment, TNF did not increase, suggesting that FTY-720P does not affect TNF-α.

Colony-stimulating factor (CSFs) can promote osteoclast precursor cells to differentiate into mature osteoclasts [40] in the presence of RANKL. Yao et al. [33] showed that M-CSF can induce the synthesis of C-Fos. Yamada et al. [34] suggested that M-CSF can effectively influence the synthesis and secretion of OPG, thus
affecting the maturation of osteoclasts. The study used FTY-720P to treat cells, but GM-CSF expression did not increase, suggesting that FTY-720P is not affected by colony-stimulating factor.

In our study, MMP-2, VEGF-C, GFR alpha-1, basic FGF, insulin, and MIP-2 were increased in the treatment group. VEGF-C is angiogenic, and the closely related lymphatic protein FTY-720P can stimulate cells to produce VEGF-C to promote the formation of lymphatic vessels; basic FGF has an important role as a protein in blood vessels [35]. These studies indicate that FTY-720P plays an important role in angiogenesis. The literature also suggests that using FTY-720P to treat joint allogeneic bone skull defects would increase angiogenesis. In addition, FTY-720P can affect MMP-2, GFR α-1, insulin, and MIP-2, suggesting that it may affect the central nervous system and immune function, but the specifics need further study.

**Conclusions**

The experiments detailed above show that FTY-720P can suppress osteoclast formation and function, and FTY-720P induces a series of cytokine changes. The mechanism by which this occurs needs further study.
The conflict of interests

All authors declared have no conflict of interests.

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