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Original Article

Effect of r-Mt-Cpn10 on human osteoblast cells

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Abstract: Objective: To observe the effect of recombinant mycobacterium tuberculosis heat shock protein 10 (r-Mt-Cpn10) on human osteoblast proliferation, cell cycle, alkaline phosphatase, calcium nodules and the expression of Receptor Activator of Nuclear Factor KB Ligand (RANKL) and Osteoprotegerin (OPG). Methods: Osteoblasts were cultured in the medium with different concentration of r-Mt-Cpn10. No drug was added to the medium in the control group. The effect of r-Mt-Cpn10 on osteoblast proliferation was detected by MTT. The 3rd generation of osteoblasts was taken and detected the effect on the activity of osteoblasts secreted alkaline phosphatase on 1, 3, 5, 7 and 9 d of cell culture. The effects of different concentrations of r-Mt-Cpn10 on the expression of RANKL and OPG were detected. Results: The r-Mt-Cpn10 blocked osteoblasts in the G2/M phase and G1 to S phase. Compared with the control group, the r-Mt-Cpn10 with different concentrations inhibited the proliferation and alkaline phosphatase activity of osteoblast ($P<0.05$), the number of calcium nodules formation was significantly reduced. The r-Mt-Cpn10 increased the expression of RANKL in a dose-dependent manner and reduced the expression of OPG ($P<0.01$). Conclusion: The inhibition of r-Mt-Cpn10 on the osteoblast proliferation and alkaline phosphatase activity was achieved by osteoblasts arrest in G2/M phase and G1 to S phase, it can also regulate the expression of RANKL and OPG which affecting local bone metabolic balance.

Keywords: Human osteoblasts, proliferation, RANKL, OPG, alkaline phosphatase activity

Introduction

Bone and joint were the most susceptible tissues except for lung in tuberculosis, which accounts for about 3-5% of incidence of tuberculosis [1] and more than 35-50% of the incidence of extra-pulmonary tuberculosis. The incidence of spinal tuberculosis is the highest in bone-joint tuberculosis, which accounted for more than 1/3. One result of the pathological changes is bone resorption (destruction). Pott's disease can cause vertebral structural damage, spinal cord compression and neurological dysfunction. The number and function of osteoclast play a critical role in inflammation-induced bone loss and joint destruction. The increase formation and active situation of osteoclast is an important pathological mechanism of bone mass loss and bone destruction, but the specific causes and mechanisms were still unclear. Under normal condition, osteoblast play an important role in the differentiation and activation of osteoclast by RANKL and Osteoprotegerin (OPG). The ratio of OPG and RANKL [2, 3] determines the differentiation and maturation of osteoclast. In pathological status, many factors in vitro and in vivo can impact the secretion of RANKL and OPG to change the osteoclast formation and function within the bone microenvironment. OPG/RANKL imbalance can cause the formation and active situation of osteoclast, which result in bone loss and bone destruction.

Among many factors that can cause bone destruction of bone tuberculosis, heat shock protein CPN10 secreted by mycobacterium tuberculosis is one of the main factors which cause bone dissolution and absorption. Mycobacterium tuberculosis heat shock protein 10 is a chaperonins which secreted into the surrounding environment by mycobacterium tuberculosis. It can induce osteoclast cells proliferation and inhibit osteoblast's proliferation, which

Table 1. The sequences of primers used in this study

Gene	Length	Sequence (5'-3')
RANKL	279 bp	F: GGAGTTGGCCGCAGACAAGA
		R: TAGAAGAACAGGGCGACGCT
OPG	200 bp	F: CCTCTGTGAAAACAGCGTGC
		R: AGGTGTCTTGGTCGCCATTT
GAPDH	202 bp	F: TGTTGCCATCAATGACCCCTT
		R: CTCCACGACGTACTCAGCG

result in the imbalance between osteoclasts and osteoblasts. This has been confirmed by bone resorption experiments of cultured calvaria of mice in vitro: After r-Mt-Cpn10 acting on calvaria of mice for 48 hours, there are significant release of calcium and formation of osteoclasts. Experimental results showed that was caused by loop spanning residues 65-70, but the molecular mechanisms which cause the osteoclasts is still not clear. There is a hypothesis that [6] CPN10 need to chelate with divalent metal cation to maintain the stability of loose collar. Chelating with divalent metal cation calcium ion in the bone will lead to bone decalcification and osteolysis absorption. There is another hypothesis that [7] osteoclast differentiation factor (RANKL) and receptors on the surface of osteoclasts were located at the TSIKIPSS sequence, which is very similar to mycobacterium tuberculosis sequence SGLVIPDT CPN10. This makes CPN10 directly combined with RANK receptor on the osteoclast surface, substitute for the role of RANKL to induce a series of changes in osteoclast precursors and form osteoclasts. Currently when we use antituberculosis drugs to kill TB bacilli and treat bone tuberculosis, we should also consider how to prevent and reduce the degree of bone absorption and destruction, how to protect bony anatomy structure and prevent neurological damage which caused by spinal cord compression after the bone resorption.

So we supposed in this study that the r-Mt-Cpn10 blocked osteoblasts in the G2/M phase and G1 to S phase, inhibited the proliferation and activity and osteogenic capacity of osteoblast. By influencing the secretion and expression of RANKL and OPG, it can result in OPG/RANKL imbalance then increase the local osteoclast formation and active situation, which result in bone destruction.

Materials and methods

The effect of r-Mt cpn10 on osteoblast cell cycle

The experiment was carried out when were in the logarithmic growth phase. Cells were cultured with 1×106 cells/mL per 2 mL in 6-well plates. Changed the medium into DMEM culture medium which contain 0.1 mg/L, 1 mg/L, 10 mg/L of r-Mt cpn10. The culture was terminated after 24 h and 48 h of the dosing. Cell fixation: 800 rpm centrifuged for 5 min, collected cells precipitation, then abandoned supernatant. Washed twice with pre-cooling PBS, added pre-cooling 75% ethanol, fixed at 4°C for 4 h. Cell staying: 1500 rpm centrifuged for 5 min, discarded the supernatant, washed with 3 mL PBS, added 400 uL ethidium bromide (PI, 50 ug/mL), 100 ul RNase A (100 ug/mL), and incubated at 4°C for 30 min (avoid light). They were analyzed by flow cytometry and ModFit software.

The effect of r-Mt cpn10 on osteoblasts proliferation (MTT assay)

The passage 3 osteoblasts were cultured in the DMEM medium which containing 10% Fetal Bovine Serum and inoculated into three 96-well plates. Cellular concentration was 6×10^6/ml; 100 ul per well cultured for 24 hours and observed under microscope. After adhering to the wall, the cells were divided into four groups by 36 wells (n=9). Cells in the control group were cultured in 100 uL DMEM medium in 9 wells, cells in three experimental groups were cultured in DMEM medium 100 uL which containing 0.1 mg/L, 1 mg/L, 10 mg/L r-Mt cpn10. The three 96-well plates were cultured for 24, 48, 72 h. Methyl thiazolyl tetrazolium (20 ul, 5 g/L) were added into each well 4 h before the end of culture. After 4 h of culturing, the thiazolyl tetrazolium was extracted. 150 ul DMSO were added into each well, swayed for 1 min until the blue crystals were dissolved. The absorbance value (A value) of each holes were determined by enzyme mark instrument (wavelength 492 nm).

The effect of r-Mt cpn10 on ALP activity of osteoblasts

The passage 3 osteoblasts were cultured in the DMEM medium which containing 10% Fetal

Table 2. The effect of r-Mt cpn10 on osteoblast cell proliferation

Group	24 hours	48 hours	72 hours
Control	0.667 ± 0.041	0.728 ± 0.021	0.812 ± 0.059
0.1 mg/L	0.660 ± 0.034	0.544 ± 0.021	0.498 ± 0.021
1 mg/L	0.657 ± 0.034	0.491 ± 0.010	0.352 ± 0.081[a]
10 mg/L	0.513 ± 0.019	0.398 ± 0.053	0.253 ± 0.012[a]

[a]$P<0.05$, compared with control group at the same time point.

Bovine Serum and inoculated into five 96-well plates. Cell concentration was 6×10^6/ml; the 24 wells were divided into four groups (n=6). The passage 3 osteoblasts were divided into four groups according to r-Mt cpn10 concentration, the 0.1 mg/L group, 1 mg/L group, 10 mg/L group and the control group. The medium was discarded after cells were cultured for 1, 3, 5, 7, 9 d. The medicine was added, collected and lysed the cells, then added cell lysis buffer into 96-well plates, operated according to the instructions of ALP determination kits and determined ALP by enzyme mark instrument (wavelength 490 nm).The activity of ALP was calculated according to formula. ALP (King Armstrong unit/100 ml) = Absorbance of the sample tube × phenol content of standard tube (0.005 mg) ×100 ml/Absorbance of standard tube/0.05 ml.

The effect of r-Mt cpn10 on the expression of OPG/RANKL in osteoblasts

Drug treatment: use r-Mt cpn10 for the preparation of 10 ug/ml mother liquor, then diluted to experiment concentration. The passage 3 osteoblasts were cultured in the DMEM medium which containing 10% Fetal Bovine Serum and inoculated into four 6-well plates. Cell concentration was 1×10^8/L; 2 mL per well cultured for 24 hours and observed cell adherence under microscope. Cells were cultured for 72 hours in the DMEM medium which containing 10% Fetal Bovine Serum with or without 0.1 mg/L, 1 mg/L, 10 mg/L r-Mt cpn10. Intracellular total RNA was extracted from the medium of 6-well plates after cultured for 3 days, rinsed three times in phosphate buffer solution, and added 1 mL methyl thiazolyl tetrazolium (20 ul, 5 g/L) into each well according to the manual. The RNA content were detected by nucleic acid protein analyzer, and the ratio of A260/A280 of extracted RNA content were detected by nucleic acid protein analyzer to determine its purity. Extracted total RNA were analyzed by 1% agarose gel electrophoresis, the integrity of RNA were detected.

The effect of r-Mt cpn10 on the osteogenic activities of osteoblasts

The passage 3 osteoblasts were cultured and inoculated by $2\times10^6/cm^2$ into 6-well plates. The experimental groups were treated with 10 mg/L r-Mt cpn10, the control group was not treated with r-Mt cpn10, changed medium every two days. After cultured for two weeks, the calcium nodules of osteoblast were stained by Alizarin red, medium was abandoned from petri dish. Washed twice with PBS buffer, fixed by 95% ethanol for 10 min, washed three times by double distilled water, stained by 0.1% alizarin red-Tris-HCl (pH8.3) at 37°C for 30 mins. The number and size of calcium nodules in the experimental group and control group were observed. Primer design of Rt-PCR: The primers of RANKL, OPG and internal reference were designed according to Genebank, synthetized by Shanghai Biological Engineering Company (**Table 1**). cDNA was obtained by reverse transcription according to the Rt-PCR kit and used for amplifying. The reaction conditions were as follows: 95°C predenaturation for 2 mins, anneal at 60°C for 60 s, extended at 72°C for 1 min; after 40 cycles extended at 72°C for 10 mins again.

Cells in each group were detected for 3 times. The changes of fluorescence intensity in each reaction tube were collected by PCR instrument and the curve of kinetics was drew. The average Ct value of the samples until fluorescence intensity increased to threshold was obtained. The ΔCt value of the GAPDH comparison between the housekeeping genes and awaiting genes was automatically calculated by analysis software, then the ΔΔCt value of the comparison between experimental group and control group was obtained. The comparison between the relative levels of RANKL and OPG of the awaiting genes were calculated according to this formula: relative levels of gene=1/2ΔΔC.

Western-blotting detection of the RANKL and OPG

The passage 3 osteoblasts were cultured in the DMEM medium which containing 10% Fetal Bovine Serum and inoculated into four 6-well

Table 3. r-Mt cpn10 effects on osteoblast activity of alkaline phosphatase ($\bar{x}$ ± s, n=6, Absorbance)

Time	control group	0.1 mg/L	1 mg/L	10 mg/L
1 day	7.170 ± 4.756	5.050 ± 1.796	3.161 ± 1.730[a]	2.535 ± 1.283[a]
3 days	1.228 ± 0.219	1.202 ± 0.115	1.082 ± 0.070	1.322 ± 0.035
5 days	5.377 ± 4.767	4.058 ± 2.745	2.430 ± 0.533[a]	2.044 ± 0.927[a]
7 days	1.414 ± 0.206	1.518 ± 0.183	1.678 ± 0.142	1.526 ± 0.424
9 days	1.319 ± 0.467	1.430 ± 0.207	1.371 ± 0.207	0.944 ± 0.040

[a]*P*<0.05 *vs*. Control group at the same time point.

plates. Cellular concentration was 1×10^8/L and 2 mL per well. They were cultured for 24 hours and observed under microscope. After adhering to the wall, cells were cultured for 72 hours in the DMEM medium which containing 10% Fetal Bovine Serum with or without 0.1 mg/L, 1 mg/L, 10 mg/L r-Mt cpn10. The total proteins were extracted and their concentration was determined by BCA method. They were denatured by boiling for 5 min, analyzed by gel electrophoresis and transferred to membrane. Added rabbit anti-human RANKL, OPG polyclonal first antibody to each well, then incubated overnight at 4°C refrigerator. Secondary antibody was added at room temperature and shocked at uniform speed for 1 h in the oscillated instrument, DAB coloration for 1 min. GAPDH was used as control.

Statistical analysis

All data was analyzed and statistics processed with SPSS 17.0 software. Data were expressed as mean ± SD values. The differences between the experimental group and control group were tested by single factor analysis of variance. The cell proliferation experiment and ALP activity were determined by repeated measures analysis of variance. P<0.05 had statistically significant.

Results

The effect of r-Mt cpn10 on osteoblast cell cycle

There were significant differences (P<0.05) between the proportion of cells in G1 phase between the control group and the experimental group on the second day. The control group has a relatively small proportion in the G1 phase, while has a higher proportion in S phase. Cell proliferation enter the S phase in order, and r-Mt cpn10 made more cells stay in G1 phase, so the proportion of S phase is relatively small, cells were blocked from G1 to S phase; There was a significant difference between the proportion of cells in G2 phase of the experimental group from the first day to the second day (P<0.05); There were more cells detained at G2/M phase, which showed cells were blocked from the G2/M phase to the G1 phase. r-Mt-Cpn10 can significantly inhibited osteoblast proliferation ,which was achieved by the arrest of osteoblasts in G2/M phase and G1 to S phase (**Table 2**).

The effect of r-Mt cpn10 on osteoblast cell proliferation

The effect of r-Mt cpn10 on osteoblast cell proliferation which tested by MTT showed that the absorbance of osteoblasts in the control group is gradually increased with time. Compared the experimental group with the control group, the osteoblasts absorbance in the 0.1 mg/L, 1 mg/L, 10 mg/L r-Mt cpn10 group gradually decreased with time, the osteoblast proliferation showed time-dependent manner. The proliferation of the 10 mg/L group at 72 hours was most significant (P<0.05), which was also significant in the 1 mg/L group at 72 hours (P<0.05). The 0.1 mg/L group also have the proliferation effect, but there is no statistically significant at each time point (P>0.05) (**Table 2**).

ALP quantitative determination

The results of the effect of r-Mt cpn10 on the ALP activity of osteoblast showed that ALP activity gradually decreased with time. Compared with other time points, the ALP activity on the first day and the fifth day were much higher (P<0.01). Compared with the control group, the ALP activity of the 1 mg/L group and the 10 mg/L r-Mt cpn10 group decreased significantly on the first day and the fifth day. There was no significant difference between the control group and the experimental group at other time points (P>0.05) (**Table 3**).

The effect of r-Mt cpn10 on human osteoblast calcified nodule formation

There were calcified nodules in both groups after treated by 0, 10 mg/L r-Mt cpn10 human

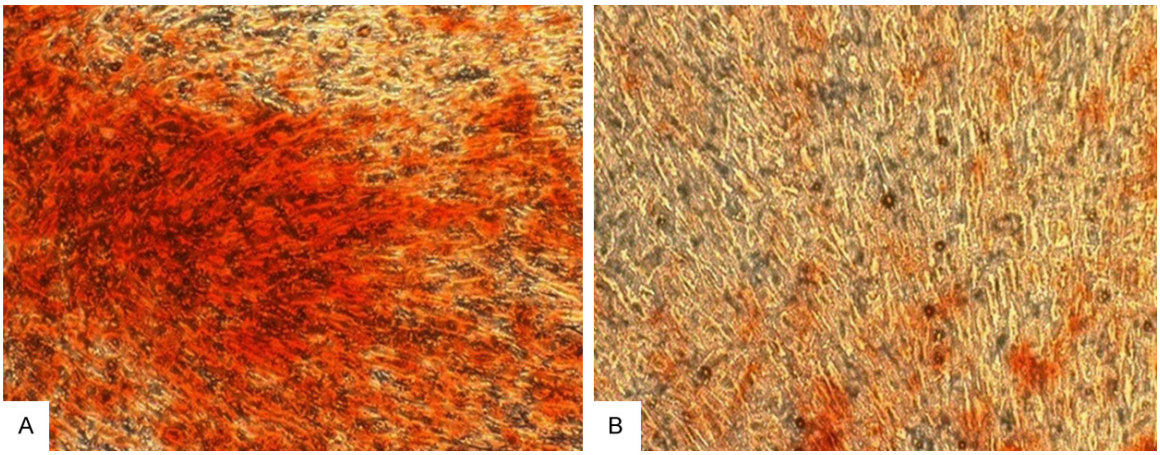

Figure 1. The effect of r-Mt cpn10 on human osteoblast calcified nodule formation. A: the control group; B: 10 mg/L r-Mt cpn10 group.

osteoblasts for 14 days. However, compared with the control group, the number of calcified nodules in the 10 mg/L r-Mt cpn10 group decreased significantly and the color is lighter (**Figure 1**).

r-Mt cpn10 effect on the expression of OPG, RANKL in osteoblast

After 72 hours intervention of r-Mt cpn10 on human osteoblasts, the RANKL mRNA expression increased in a concentration dependent manner. It had the greatest effect of the 10 mg/L group, ($P<0.05$). After 72 hours intervention, the r-Mt cpn10 decrease OPG mRNA expression in a concentration dependent manner. The effect is most significant of the 10 mg/L group ($P<0.05$). The OPG/RANKL value can dynamically reflect two opposite trends which were osteoblasts or osteoclasts (**Table 4**). The results of Western blotting showed the same effects.

Discussion

The inhibition effect of r-Mt cpn10 on the osteoblasts proliferation and ALP secretion

The result of this study showed that compared the experimental group with the control group, the osteoblasts absorbance in the 0.1 mg/L, 1 mg/L, 10 mg/L r-Mt cpn10 group gradually decreased with time, the osteoblast proliferation showed time-dependent manner. The proliferation of the 10 mg/L group at 72 hours was most significant ($P<0.05$), which was also significant in the 1 mg/L group at 72 hours ($P<0.05$), the inhibition rate reached 50%. This experiment used human osteoblast cell line MG63, the inhibition rate reached 60%. The osteoblast activity was measured by the activity of ALP. Because ALP is a necessary enzyme for bone formation and is an early indicator to identify and evaluate osteoblast differentiation degree. It can decompose organic phosphoric acid and release inorganic phosphorus and also increase the local concentration of inorganic phosphate to help the mineralization process. The result of ALP activity showed that different concentrations of r-Mtcpn10 inhibited the secretion of ALP, the ALP activity of the 1 mg/L group and the 10 mg/L r-Mt cpn10 group decreased significantly on the first day and the fifth day ($P<0.05$), which inhibit approximately 30-40% ALP secretion. The result showed that r-Mtcpn10 can inhibit the osteogenic capability and the differentiation of human osteoblast.

The experimental results of the effect of r-Mt cpn10 on osteoblast cell cycle showed that most of the cells in the r-Mt cpn10 group were in the G1 phase, which means cells were blocked from G1 to S phase. The cells in the experimental group were arrest at the G2 phase, which means cells were blocked from G2/M to G1 phase. By the view of cell cycle, r-Mt-Cpn10 can significantly inhibited osteoblast proliferation, which was achieved by the arrest of osteoblasts in G2/M phase and G1 to S phase. After treated by 0, 10 mg/L r-Mt cpn10 human osteoblasts for 14 days, there were calcified nodules in both groups. The inhibition of r-Mtcpn10 on human osteoblast proliferation,

Table 4. r-Mt cpn10 effects on the expression of OPG, RANKL in osteoblast

Group	RANKL	OPG	OPG/RANKI
control	0.1529 ± 0.0183	1.029 ± 0.1821	6.689 ± 0.5612
0.1 mg/L	0.2562 ± 0.0216[a]	0.5437 ± 0.0317[a]	2.166 ± 0.2909[a]
1 mg/L	0.4743 ± 0.0605[a]	0.1201 ± 0.0045[a]	0.2590 ± 0.0232[a]
10 mg/L	0.6670 ± 0.1107[a]	0.1027 ± 0.0086[a]	0.1560 ± 0.0233[a]

[a]$P<0.05$ *vs*. Control group.

cycle and ALP inhibition were all reflected in the osteogenic capability.

The effect of r-Mt cpn10 on the expression of human osteoblasts OPG, RANKL

The tuberculosis bacilli and their products of bone tuberculosis can cause the accumulation of inflammatory cells. The secretion of inflammatory cytokines such as LI-1, IL-6 and TNF-α and so on can direct or indirect activate RANK-RANKL-OPG axial [8-16] system, which brook the normal RANKL and OPG ratio secreted and maintained by osteoblasts [17-25], and OPG/RANKL imbalance can cause the formation and active situation of osteoclast, which result in bone loss and bone destruction. The heat shock protein 10 secreted by mycobacterium tuberculosis [26-29] can maintain the proteins structure of itself, previous studies also confirmed that it can cause osteoclast formation and osteoblast cell proliferation. The mechanism is still unclear, related studies focused on its molecular conformation. In this study, we observed the effect of r-Mt cpn10 on the expression of OPG and RANKL in human osteoblast so as to illustrate the reason of mycobacterium tuberculosis heat shock protein 10 breaking the bone metabolism balance under pathological conditions.

The effects of r-Mt cpn10 on the expression of RANKL and OPG were detected by RT-PCR and Western-blotting, the result showed that r-Mt cpn10 can increase the expression of RANKL and decrease the expression of OPG in a concentration dependent manner, which result in OPG/RANKL imbalance. Osteoblasts secreted excessive RANKL in the paracrine manner and bond with the RANK receptor of osteoclast precursors. RANK intracellular portion bond with the tumor necrosis factor receptor-associated protein TRAF6 in the cells [11], induced nuclear factor κB pathway (NF-κB) activated and transported into the cell nucleus [12]. NF-κB can increase the expression of c-Fos gene. The c-Fos can combine with NFATc1 and start OC specific gene transcription, so that the osteoclast precursor cells can differentiate into mature OC. OPG as RANKL antagonist can combine with RANK-RANKL complex and block osteoclast formation. But its expression was decreased, which means the factors prevent osteoclast formation were weaken, thus increased the osteoclasts production and cause bone destruction and bone loss. The results of the effect of r-Mt cpn10 on the mRNA and protein expression RANKL and OPG suggested that we can apply the anti-osteoporosis medicine such as bisphosphonates to assist the treatment of bone tuberculosis and improve OPG/RANKL ratio. It can be used as an idea and supplementary treatment and the further cell drug intervention experiments are necessary.

In summary, we considered that the r-Mt-Cpn10 blocked osteoblasts in the G2/M phase and G1 to S phase, inhibited the proliferation and maturation of osteoblast, it also inhibited the activity of ALP and significantly reduced osteogenic capability. r-Mt-Cpn10 can significantly inhibit the expression of OPG and increase the expression RANKL, it can also reduce OPG/RANKL proportion and promote osteoclast differentiation and maturation. This is probably one of the reasons that mycobacterium tuberculosis cause bone destruction and bone loss. In addition to the protein that secreted by mycobacterium tuberculosis, the factors which can cause bone destruction in bone tuberculosis were a number of inflammatory cytokines, such as LI-1, IL-6, TNF-α and other inflammatory cytokines [30-35]. Most of them play a role by RANKL-RANK-OPG axis, or indirectly by RANKL-RANK-OPG axis. This prompted us that when we use anti-TB drugs, apply drugs which improve the OPG/RANKL ratio can maximize the protection of the spine and other bones, and reduce the incidence of complications.

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Disclosure of conflict of interest

None.

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