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The Osteoclast Development in Patients with Rheumatoid Arthritis and the Influence of the Bisphosphonate on Its Development

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= 국문초록 =

류마티스관절염 환자에서 파골세포의 분화 및 비스포스포네이트의 영향

울산대학교 의과대학 강릉아산병원 내과학교실, 인하대학교 의과대학 내과학교실*

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목적: 첫 번째, 류마티스관절염과 골다공증 환자들의 말초혈액단핵구에서 파골세포의 분화 정도를 분석하였다. 두 번째, 류마티스관절염 환자와 나이가 비슷한 골다공증 환자, 그리고 젊은 정상인을 대상으로 비스포스포네이트의 단기간 처리가 파골 세포 생성 및 관련된 사이토카인의 발현에 미치는 영향을 비교하고자 하였다.

방법: 미국류마티스협회의 기준에 의해 진단된 류마티스관절염 12명과 그들과 나이가 비슷한 일차성 저골밀도 환자 12명을 대상으로 하였다. 이들에서 사이토카인 발현과 파골세포의 분화의 정도를 관찰하였다. 두 번째 실험에서는 혈청양성 저골밀도 류마티스관절염 환자와 나이가 비슷한 일차성 골다공증, 젊은 정상성인, 각각 6명씩에서 말초혈액단핵구를 얻었다. 이들 세포에서 사이토카인의 발현 정도는 real time RT-PCR로 확인하였고, tartrate-resistant acid phosphatase (TRAP) 양성 거대세포의 형성과 칼슘판의 흡수구멍형성을 측정하여 파골 세포의 발현과 그 기능을 확인하였다. 각각의 실험은 24시간 비스포스포네이트 처리 전후로 시행하여 그 효과를 관찰하였다.

결과: 첫 번째 실험의 경우 기본적인 사이토카인 mRNA의 발현과 TRAP 양성 거대세포의 형성과 흡수구멍의 형성 정도는 류마티스관절염 환자와 일차성 골다공증 환자 사이에 차이가 없었다.

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두 번째 실험은 비스포스포네이트 단기처리 후 macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor-κB ligand (RANKL), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interferon-γ (IFN-γ) 등의 mRNA 발현은 증가하였고 receptor activator of nuclear factor-κB (RANK), cathepsin K, TRAP 등의 발현은 세 그룹 모두에서 감소하였다. 그 중 IL-6와 IFN-γ의 증가가 가장 저명하였다. IL-1 mRNA 의 발현은 변함이 없었다. 그들의 비스포스포네이트에 의한 mRNA 변화는 증가와 감소 모두 IL-6를 제외하면 류마티스관절염 환자에서 전반적으로 그 변화 폭이 적었다. 세 그룹 모두에서 TRAP 양성 거대세포의 발현과 흡수구멍의 생성은 현저히 저해되었다.

결론: 비스포스포네이트는 말초혈액단핵구에서 염증성사이토카인의 발현을 증가시켰다. 사이토카인의 변화 중 가장 두드러진 것은 INF-γ의 증가였다. 이 결과는 IFN-γ가 T세포를 통한 파골세포 생성저해의 매개체일 수 있다는 것을 시사한다. 이들 사이토카인의 반응은 다른 그룹에 비해 류마티스 관절염 환자에서 의미 있게 감소하였다. 비스포스포네이트의 단기 처리는 파골세포의 발달을 전구세포 단계에서 저해하여 후속되는 배양에서 파골세포의 발현을 억제하였다. 그러나 이 반응도 다른 그룹에 비해 류마티스관절염 환자에서 의미 있게 감소하였다. 그러므로 류마티스 관절염 환자에서 발생하는 골다공증과 골미란의 감소에 비스포스포네이트가 도움을 주며, 특히 골다공증이 동반된 경우를 치료할 때는 그 용량이나 투여방법을 파제트병이나 전이성종양의 경우처럼 더욱 강력하여야 할 필요가 있다.

Key Words: Rheumatoid arthritis, Osteoclast, Bisphosphonate

INTRODUCTION

Rheumatoid arthritis (RA) is characterized by the systemic inflammation with the joint and bone destruction. The bone loss can be localized around the joint but also be generalized. Patients with RA are also known to have lower bone mineral density (BMD) and are at risk of pathological fracture (1-5). The low BMD was also found in RA patients not taking the glucocorticoid and can be aggravated by the use of the glucocorticoid. In patients with RA the best independent predictors of bone mass at the femoral neck were glucocorticoid use, osteocalcin levels, age, height, the presence of rheumatoid factor, and the Health Assessment Questionnaire score, and they explained 61.6% of the variance in femoral neck BMD (6). Femoral neck osteoporosis was found in 31% and increased fall risk in 68% of women with RA.

A role of increased osteoclastic bone resorption for causing the generalized osteoporosis in RA was suggested by studies which have shown that there are increased biochemical markers of bone resorption in RA patients with low skeletal BMD (7). Several cause of the generalized bone loss in RA were suggested, the changes in vitamin D and mineral metabolism and certain cytokines [e.g. IL-1, TNF-α, and IL-6] have been implicated in the pathogenesis of systemic bone loss in RA (8,9).

Osteoclasts are specialized multinucleated giant cells absorbing the bone. Recent studies emphasized the immunologic stimulation of the osteoclast in the pathogenesis of the bone loss especially in the inflammatory bone destructive disease. Activated T cells were demonstrated to provide the RANKL for the osteoclast differentiation in rat adjuvant arthritis resulting in bone loss and the joint destruction (10). The RANK/RANKL pathway has been shown to be essential for osteoclast differentiation in inflammatory arthritis. In addition, *in vitro* and *in vivo* studies have demonstrated that many cytokines and growth factors elaborated by inflamed synovial tissues may contribute to osteoclast differentiation and activation (11). Bone- resorbing osteoclasts have been identified as important effector cells in inflammation-induced bone loss in human rheumatoid arthritis, juvenile RA, and psoriatic arthritis (12,13). Inflammatory macrophages isolated from the RA synovium are also capable of differentiating into osteoclasts

in the presence of M-CSF and RANKL (14). Hirayama et al (15) showed that osteoclast formed from RA patients exhibited increased resorptive activity but there was no difference in the relative proportion of circulating osteoclast precursors or the osteoclast formation between RA patients and normal controls. There are ample evidence that the inflammatory cytokines are involved in the production and functional activation of the osteoclast.

In the treatment of the rheumatoid arthritis, studies show that the current anti-rheumatic therapy is not satisfactory enough (16,17). The bisphosphonates are the current anti-resorptive treatment, which are most frequently used together with the anti-rheumatic agents to treat the RA. The bisphosphonate has been studied in animal and human to treat the arthritis resulting in the favorable response. Previous studies (18,19) suggest that the bisphosphonates are still promising candidates to be added to the current anti-rheumatic treatment to maintain joint integrity and prevent the bone loss in patients with rheumatoid arthritis. Considering the progressive bone loss in patients with RA receiving both kinds of the medication, the patients with RA may respond to the bisphosphonate differently from the patients with primary osteoporosis. The bisphosphonates can inactivate the osteoclast. Their influence on the immune system has also been suggested.

In this study we tried to compare the osteoclastic differentiation from the peripheral blood mononuclear cells (PBMC) of the RA patients and the normal controls. And we wanted to define the effect of short term bisphosphonate pulse on the osteoclastogenesis and related cytokine expression and compare the effects between the PBMCs from patients with RA and controls.

MATERIALS AND METHODS

1. Blood sampling and isolation of the PBMC

At first part of this study, as a preliminary study, we compared the baseline cytokine mRNA expression in the PBMC in 12 definite active seropositive RA patients by the 1987 American College of Rheumatology (ACR) classification criteria and 12 age-matched normal controls are recruited. The normal control individuals did not have the risk of bone loss except for the osteoarthritis of the knee.

At the second part of the study, we selected 6 patients with seropositive RA with low BMD (age, 62.3±5.92, range 55～69 years, 5 female and 1 male, all patients were taking prednisone 2.5～7.5 mg/day), 6 age matched patients with low BMD without secondary cause of the osteoporosis (age, 62.8±11.62; range 48～80 years; 5 females & 1 male), and 6 healthy young normal volunteer (age, 31.7±7.06; range 26～45 years; 4 female & 2 males). The definition of the low BMD was the T-score of the lumbar spine, total hip, or femoral neck is −2.5 or less according to the ISCD guideline.

After an informed consent peripheral blood of 15 mL were obtained into the heparin containing syringe. The blood was separated and processed within a few hours after sampling. After centrifugation, the blood cells were diluted 1 : 1 with RPMI 1640 wash media (2% FCS), the dilute was layered over the Histopaque (HISTOPAQUE-1077, Sigma, 1077-1, St. Louis, MO, USA) and centrifuged at 693 g for 30 min at 25°C. The PBMC layer at the inter-phase between the plasma and Histopaque was collected and washed twice in RPMI 1640 and the cell pellet was resuspended in RPMI1640/FCS. The number of PBMCs in the cell suspension was counted in a haemocytometer.

2. RNA extraction and reverse transcription

Cell pellets of 1×10^6 cells were used to extract the RNA. Cell pellet was lyses by addition of 1 mL Trizol reagent (Invitrogen, CA, USA), and kept in −70°C. The total RNA was prepared according to the manufacturer's instructions (RNAgent, Promega, Z5110). The cDNA was synthesized using a reverse transcription system (Promega A3500, USA).

3. Culture of mononuclear cells

The 2×10^6 cells were cultured in the 24 well culture plate and 5×10^5 cells were cultured in the 96 well plate in RPMI 1640 (Hyclone, Logan, Utah, USA) [supplemented with 2 mM glutamine and 100 IU/mL of benzyl penicillin], streptomycin (10 g/mL) (Gibco 15240-062, USA), 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, SH30070.03, USA), and human m-CSF (Sigma M 6518, St. Louis, MO, USA) in a humidified atmosphere of 5% CO_2 at 37°C. The half of the medium was replaced every 4～5 days.

4. Pre-incubation (pulse) of the PBMCs with alendronate

Cells were incubated with or without alendronate (100 M) for 24 hours in separate culture tube and washed before further processing for culture or the RNA extraction to see the short-term pulse effect of the bisphosphonate. Again, 1×10^6 cells were harvested for the RNA extraction.

5. Expression of the osteoclastogenesis related cytokine mRNA from PBMC's

The primer and TaqMan probes for the real time RT-PCR was made commercially (TIB-Molbiol, Berlin, Germany). Real-time RT-PCR was performed on iCycler iQ Real-Time PCR Detection System (Bio-RAD, Hercules, CA) by iQ Supermix (Bio-RAD). The amplification reactions were performed in 25 μL final volume containing 12.5 μL of iQ Supermix, 1 μL of 5 pmolar of each primer, 2 μL of 5 pmolar TaqMan probe, 1 μL of cDNA, and 7.5 μL of distilled water. Thermal cycling conditions were initial 10 minutes at 95°C and 50 cycles of 30 seconds at 95°C and 1 minute at 60°C.

6. Cytochemical assessment of osteoclast formation (TRAP stain)

After 3 weeks of culture, cells were washed and fixed with citrate/acetone solution for 30 seconds. The cells were stained for TRAP using Acid Phosphatase kit (Sigma 386-A, St. Louis, MO, USA) and according to the manufacturer's instruction. The numbers of the osteoclasts were expressed as the total counts per each 96 well plate. The TRAP positive multinucleated giant cells appeared in the culture of the PBMC after adding only the 10 ng/mL of M-CSF without adding RANKL.

7. Functional evidence of osteoclast differentiation

PBMCs (2×10^6 cells/well) were added on the 24-well tissue culture plate coated with calcium-phosphate nano crystals (OAAS plate, Oscotec, South Korea) prepared as described previously. After 4 weeks of the culture. The OAAS plates are then washed with 5% sodium hypochlorite (Sigma, USA) to remove cell debris and plates were stained with Alizarin Red S.

8. Statistical analysis

The mean of numbers were analyzed by the T-test at the first part of this study comparing the data from the 12 patients with RA and 12 age matched controls. The expression of the cytokines measured by real time RT-PCR were converted to the $2^{-\Delta\Delta Ct}$ value and the relative gene expression using the GAPDH as a internal standard were analyzed by the non-parametric *Mann-Whitney* and *Kruskal-Wallis* test. Statistical analysis of the measurements will be performed using the SPSS (Ver. 12.0). p values <0.05 were considered significant. Each experiment was carried out in duplicate.

RESULTS

We established the osteoclast development from the PBMC of the patients with RA and low BMD, and normal control persons only in the presence of the M-CSF.

The cytochemical analysis by TRAP staining, and functional analysis of the osteoclast by the calcium phosphate coated plate (OAAS) confirmed the generation of the osteoclasts. The expression of the osteo-

clastogenesis related cytokine mRNA, generation of TRAP＋ giant cells, and lacunar resorption were not different between the PBMC's of the patients with RA and normal individuals.

The expression of the mRNA of the human IL-1, IL-10, TNF-α, RANK, RANKL, M-CSF, INF-γ, TRAP, cathepsin K, and osteoprotegerin (OPG) were evaluated semi quantitatively on the agar-rose gel before the each real time RT-PCR. RT-PCR showed that the mRNA of the RANK, RANKL, M-CSF, IL-10, IL-1, TNF-α, TRAP, and cathepsin K is clearly expressed in human PBMC (Fig. 1). The expression of osteoclastogenesis related cytokine mRNA was not different between the PBMC's of 12 patients with RA with low BMD and 12 ages matched normal individuals. As shown in figure 3, the amount of the cytokine expression varied too much between individuals to compare each other in each group.

TRAP positive giant cells with more than 3 nuclei were regarded as osteoclast (Fig. 2). The number of TRAP (＋) giant cells formed after 3 weeks of PBMC culture were 23.9±9.41 in patients with rheumatoid arthritis and 18.2±7.73 in control group. They were not different between the groups (p=0.117) (Fig. 3). Resorption pits were examined under magnifying glass and the percentage surface area of lacunar resorption on each dentine slice was determined as manufacturer's guide (Fig. 4). The grade of the lacunar resorp-

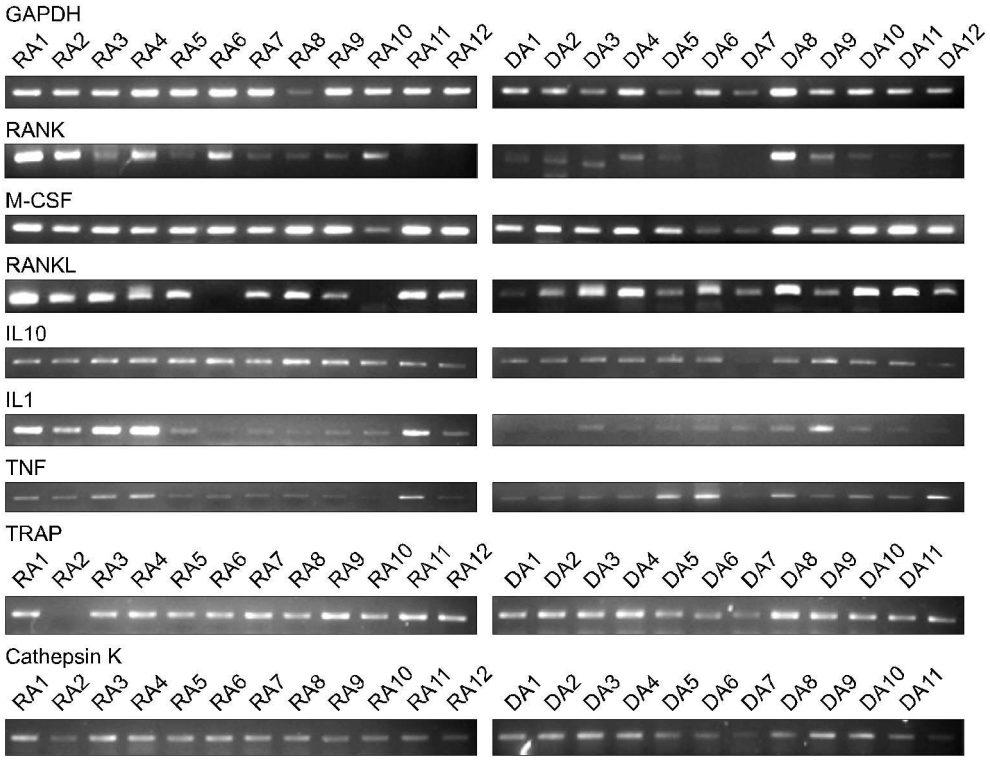

Fig. 1. RT-PCR for the osteoclastogenesis related cytokine mRNA from PBMCs of 12 patients with RA with low BMD and 12 age-matched normal individuals. The expression of osteoclastogenesis related cytokine mRNA was not different between the PBMCs of 12 patients with RA with low BMD and 12 age-matched normal individuals. The amount of the cytokine expression varied too much between individuals to compare each other in each group. The PCR condition was initial 10 minutes at 95°C, 35 cycles at 95°C for 30 seconds and 56°C for 60 seconds, and last 10 minutes at 72°C.

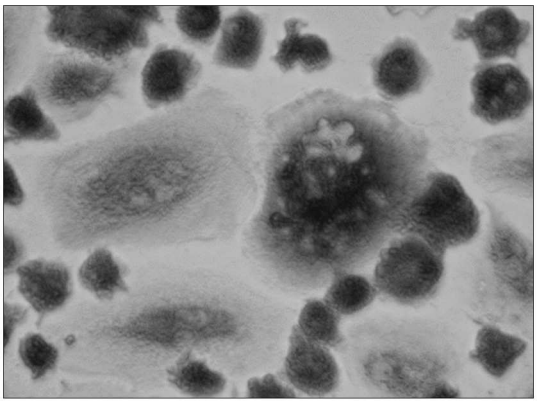

Fig. 2. After culture the osteoclast from a patient with the RA (×200, light microscope, Counter stain with the Hematoxylin). TRAP positive giant cells with more than 3 nuclei were regarded as osteoclast. TRAP positive giant cells were generated from the human PBMCs after 3 weeks of culture (×400).

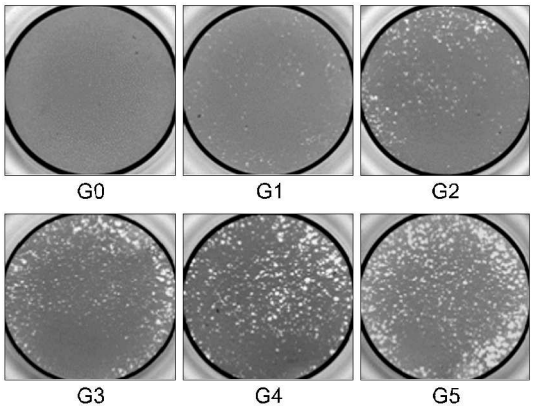

Fig. 4. Resorption pits were examined under magnifying glass and the percentage surface area of lacunar resorption on each dentine slice was determined as manufacturer's guide. Grade 0: 0%, Grade 1: 20%, Grade 2: 40%, Grade 3: 60%, Grade 4: 80%, Grade 5: 100% (×10).

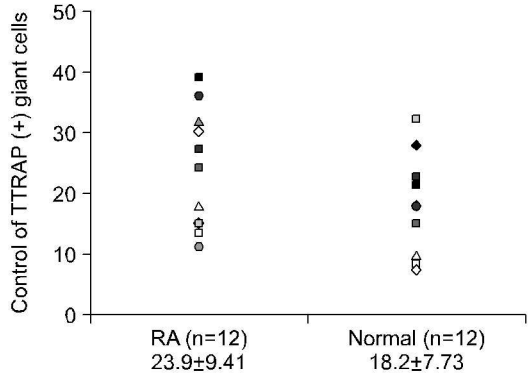

Fig. 3. The number of the TRAP＋ giant cell formation from PBMCs of 12 patients with RA with low BMD and 12 age-matched control individuals. They were not different between the groups (p= 0.117). Cell counts are per each well in 96-well culture plate expressed as mean±SD of triplicate culture.

tion pit formation after 4 weeks of the culture over the calcium phosphate coated plate 2.6±1.24 in patients with RA and 2.9±1.57 in control group which was not different significantly each other (p=0.685).

In preliminary study, we concluded that the cytokine expression, TRAP (＋) giant osteoclast generation, or the lacunar pit formation experiment is not sensitive or stable enough to find their difference between the individuals or groups of individuals with different disease states.

1. Quantitative evaluation of the cytokines expression after alendronate pulse

The RT-PCR shows that the most prominent change after incubation of the PBMCs in alendronate for 18 hours are decreased expression of the RANK and increased expression of the IFN-γ (Fig. 5). The real time RT-PCR showed that the mRNA expression of the M-CSF, RANKL, TNF-α, IL-6, and IFN-γ increased. Among them the increase of IFN-γ was most prominent. But those of the RANK, TRAP, and cathepsin K decreased after alendronate pulse of the PBMCs. The IL-1 expression did not change significantly (Table 1).

The TRAP (＋) giant cell count (cytochemical assessment of osteoclast formation) was inhibited marked after alendronate pulse in the PBMCs of the patients with idiopathic osteoporosis and normal young persons.

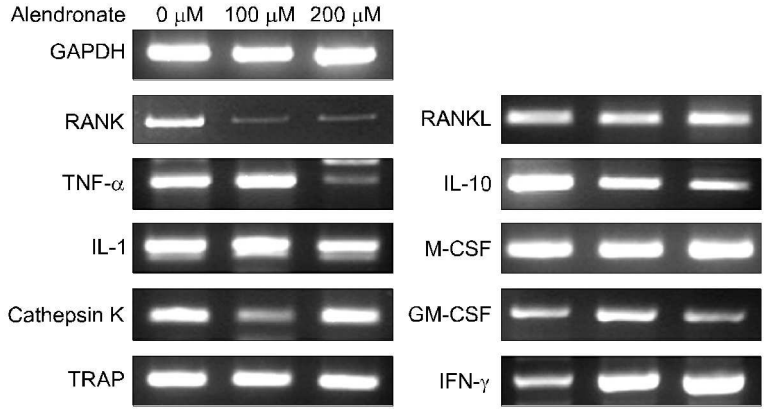

Fig. 5. The effect of the 18 hours alendronate pulse on the subsequent expression of the osteoclastogenesis related cytokines in a normal control. The increased IFN-γ expression and decreased RANK and IL-10 expression is noted.

Table 1. Response of osteoclastogenesis related gene expression in PBMCs after 24 hours of alendronate (100 microM) incubation. The real time RT-PCR showed that the mRNA expression of the M-CSF, RANKL, TNF-α, IL-6, and IFN-γ increased. Among them the increase of IFN-γ was most prominent. But those of the RANK, TRAP, and cathepsin K decreased after alendronate pulse of the PBMCs. The IL-1 expression did not changed significantly. The Results of Real Time RT-PCR by "$2^{-\Delta\Delta CT}$ Method"

Target group	M-CSF	RANKL	RANK	TNF-α	IL-1	IL-6	IFN-γ	Cathepsin K	TRAP
RA (n=6)	1.73*	1.91	0.65	1.94	1.03	43.61	5.36†,***	1.45	1.09‡
Young normal (n=6)	6.48*	2.75	0.13**	3.27	1.29	31.23	56.23***	0.54***	0.49‡,**
Age matched low BMD (n=6)	2.95	2.21	0.82**	3.81	2.01	16.86	33.41†	2.39***	1.13**

The difference between groups are *p=0.041, **p=0.02, ***p=0.015, †p=0.026, ‡p=0.009, and between the groups marked with same symbols.

The decrease is blunted in those of RA patients. The number of the giant cells tends to be more in the culture of the PBMC from the RA patients before alendronate pulse, but it was not significant statistically. Adding $1,25(OH)_2D_3$ ($10^{-7}\sim10^{-9}$ M) to the culture media did not affected the osteoclast development significantly.

The number of TRAP (+) giant cells formed after 3 weeks of PBMC culture were 23.9±9.41 in patients with rheumatoid arthritis and 18.2±7.73 in control group. They were not different between the groups (p=0.117). The decrease of the TRAP (+) giant cells after alendronate pulse was almost complete (Fig. 6). The developments of the TRAP (+) giant cells were inhibited markedly after alendronate pulse, but less markedly in RA ($p<0.001$)(Table 2). The lacunar absorption pit formation (cytochemical assessment of osteoclast formation) was inhibited completely after alendronate pulse of the PBMCs but no difference between the groups (Fig. 7)(Table 3).

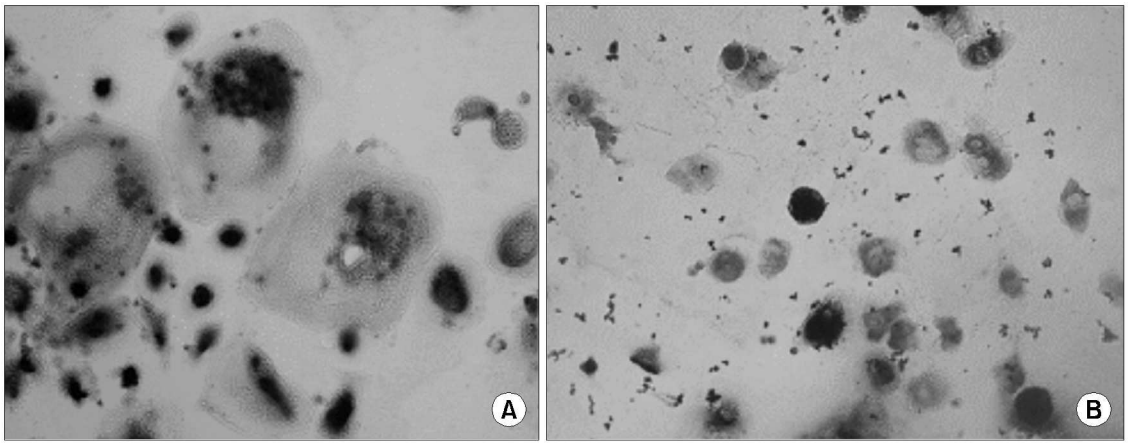

Fig. 6. (A) The culture of the whole PBMCs for 3 weeks showed many TRAP (+) giant cells (×400). (B) The pre-incubation of the whole PBMCs with the alendronate (100 M) for 24 hours before plating on the culture plate inhibited the development of the TRAP (+) giant cells almost completely (×400).

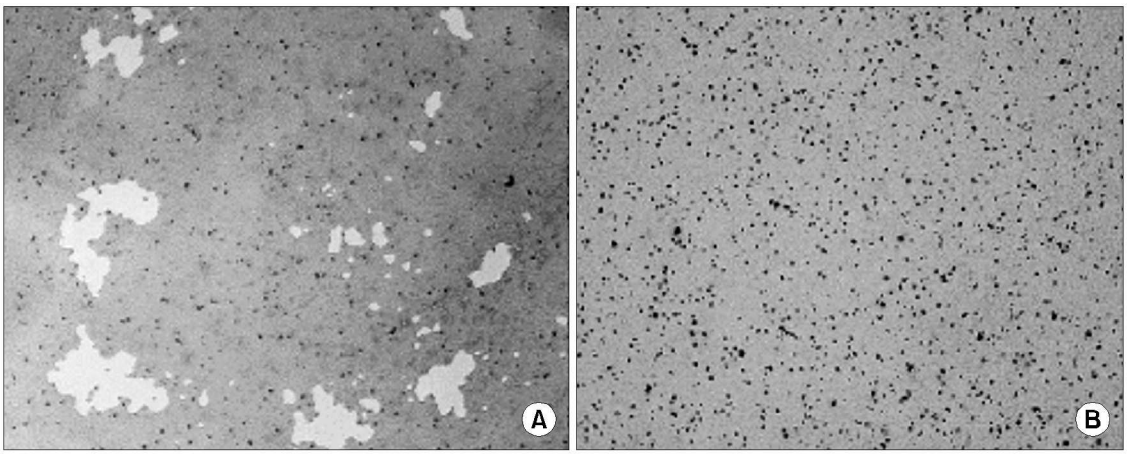

Fig. 7. (A) The culture of the whole PBMCs on the OAAS™ plate for 4 weeks showed the many resorption pits, Grade 2 (×100). (B) The pre-incubation of the whole PBMCs with the alendronate (100 M) for 24 hours before plating on the OAAS™ plate inhibited the development of the resorption pits almost completely (×100).

We think that the lacunar pit formation experiment is less sensitive than other experiments like TRAP (+) giant cell experiment.

DISCUSSION

The goal of the therapy in RA is to reduce the pain, inflammation, articular damage, functional disturbance, and systemic complication. Until now, most of the anti-inflammatory therapies including the disease modifying anti-rheumatic drugs (DMARDs) are targeted mainly on the pain and inflammation, but their long term effect on the bone and joint reservation is uncertain or at most minimal.

Previous studies we discussed above shows that the bone destructing pathologic changes are mainly due to the enhanced osteoclasts development or activation in the RA patient. In animal study, Redlich et al (18)

Table 2. The count of the TRAP positive cells in 96 well culture plates before and after alendronate pulse. They were not different between the groups (p= 0.117). The decrease of the TRAP (+) giant cells after alendronate pulse was almost complete. The developments of the TRAP (+) giant cells were inhibited markedly after alendronate pulse, but less markedly in RA

Alendronate dose	RA (n=6)	Age matched control (n=6)	Young normal (n=6)
0 μM	23.9 $\pm$9.41	22.8 $\pm$6.31	18.2 $\pm$7.73
100 μM	7.1 $\pm$4.12	0.5 $\pm$0.84	0
200 μM	1.8 $\pm$0.75	0	0

Table 3. Change of the grade of lacunar resorption after the Alendronate injection. The lacunar absorption pit formation was inhibited completely after alendronate pulse of the PBMCs

Group	Baseline	Alendronate pulse 100 μM
RA	2.5 $\pm$1.24	0
Age matched	2.9 $\pm$1.57	0

suggested that the TNF-α dependent bone erosion is mediated by osteoclasts and that the absence of osteoclasts alters TNF-mediated arthritis from a destructive to a nondestructive arthritis. The control of the osteoclastic activity in RA by the suppression of the inflammatory process has not been satisfactory. Although rare, clinically relevant progression of joint damage does occur in patients with RA in prolonged remission. And they suggested the need for markers that predict progression during periods of low disease activity and for drugs that prevent damage that is independent of disease activity (16). In another study with RA, the combination therapy with infliximab and methotrexate, marked clinical responses were associated with a global reduction in the synovial infiltrate and expression of cytokines, notably IL-18 and TNF-α, but low grade disease activity persisted.

The bisphosphonate is known to act on the osteoclast directly after it is adsorbed on the bone. When it is taken up by the osteoclast during the remodeling it inactivates or causes it to go under apoptosis (21). TNF-α potently enhances RANKL mediated osteoclast activity. Interactions between TNF-α and IL-1 are together also in osteoclastic activity directly from the progenitors independently of RANKL (22). And TNF-related activation induced cytokine/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis (23). In human TNF- transgenic mice, repeated administration of bisphosphonate at the onset of arthritis almost completely blocked the bone erosion and dramatically increased systemic bone mass, without improving the synovial inflammation. But calcitonin had no effect on synovial inflammation, bone erosion, cartilage damage, or systemic bone mass. Anti-TNF entirely blocked synovial inflammation, bone erosion, synovial osteoclast formation, and cartilage damage but had only minor effects on systemic bone mass (24).

In RA, the uptake of the bisphosphonate is not confined only to the skeleton, but it is also retained in joints, which could have implications for dose regimens. The osteoclast can come from the mononuclear cells of the PBMC's. And there is much evidence that the bisphosphonate can act on the PBMCs directly. Immunologic response induced by the bisphosphonate was described by the a few reports. Up to 60% of patients receiving their first infusion of the pamidronate experience an acute-phase reaction. Pamidronate infusion in patients with malignancy decreased lymphocyte and leukocyte count, and increased the body temperature, plasma level of IL-6, TNF-α, and CRP. These changes were not found in the patients treated with clodronate or ibandronate (25), But the pamidronate did not changed IL-1 level (26). Pechestorfer (26) described substantial difference in the hematologic response to initial treatments with different bisphosphonate. He found pamidronate decreased the counts of circulating lymphocytes, natural killer cells, T cells, and

CD4＋ and CD8＋ T cell subsets. But ibandronate treatment did not affect median body temperature, and increased the total lymphocyte count, B cells, T cells, and CD4＋ and CD8＋ T cell subsets. And clodronate treatment did neither induced changes in body temperature nor affect the number of circulating T cells and NK cells (26). Papadaki et al (27) reported that 10 mg alendronate daily per os in 16 women with chronic idiopathic neutropenia (CIN)-associated osteoporosis and 9 with CIN- associated osteopenia decreased the elevated levels of serum TNF-α and IL-1β by day 30 and thereafter. Alendronate-induced changes in the levels of both cytokines correlated inversely with the changes in neutrophil counts and BMD measurements. The effect of the alendronate may lie in its property to inhibit the production of TNF-α and IL-1β by cells of the monocyte/macrophage system, in which osteoclasts are, included (27). Takayanagi et al (28), showed that T cell production of IFN-γ strongly suppresses osteoclastogenesis by interfering with the RANKL-RANK signaling pathway. IFN-γ induces rapid degradation of the RANK adapter protein, tumor necrosis factor receptor-associated factor 6 (TRAF6), which results in strong inhibition of the RANKL-induced activation of the transcription factor NF-κB and c-Jun-N-terminal kinase (28). Udagawa (29) summarized the osteoimmunologic mechanism of osteoclast formation by activated T cells in rheumatoid arthritis. Activated T cells present in the synovial tissues produce membrane-associated RANKL, some of which is cleaved enzymatically from the plasma membrane, resulting in soluble RANKL (*sRANKL*). Activated T cells also produce IL-17, which induces RANKL via prostaglandin (PG) E2 synthesis in osteoblasts. The IL-6, together with soluble IL-6 receptors (*sIL-6R*), IL-1, and TNF-α derived from macrophages, induces RANKL in osteoblasts (29).

Among the cytokines we showed in this study, the increased pro-inflammatory cytokine expression in response to the alendronate, the M-CSF, RANK, TNF-α, and IL-6 are known to be involved in the osteoclast development, but the INF-γ is known to ablates the RANKL induced osteoclastic differentiation of cells by inhibiting cathepsin K promoter activity and ablating its induction by RANKL (30). We found the cathepsin K expression in PBMCs. There is strong evidence that cathepsin K is highly and quite selectively expressed in osteoclasts (31). Furthermore, our *in vitro* experiment showed some the potential direct bisphosphonate influence on cytokine production by the PBMC. Therefore, the bisphosphonate which inhibits the osteoclastic bone resorption has some potential to inhibit bone destruction in inflammatory arthritis such as RA.

We think that the current therapy strategy is focused too much on subsiding the inflammation and the pain which resulted in very little effort for bone and joint protection until now. Although, there is no statistical significance of increased osteoclastogenesis from the PBMC of the patients with RA than that of the OA patients. We hypothesize that the osteoclast, which is supra-physiologically activated by the inflammatory signal, is the most dominant contributor for the destruction of the bone in rheumatoid arthritis. We suggest that the bisphosphonate would provide the better bone and joint protection in patients with RA by the direct inhibition of the osteoclastic activity when combined with conventional anti-inflammatory therapy. And the patients with RA do not respond to the bisphosphonate therapy as well as the normal control or the age matched patients with osteoporosis. Other agents or the different dosing is warranted.

CONCLUSION

In preliminary study, we concluded that the cytokine expression, TRAP (＋) giant osteoclast generation, or the lacunar resorption pit formation experiment is not sensitive or stable enough to find their difference between the individuals or groups of patients with different disease states.

Alendronate, by combined effect on these cytokines, might have beneficial effect in patients with the RA

which is not only inflammatory but also bone destroying disease. We found the evidence that bisphosphonates act directly on the PBMC's before they arrive to the bone surface to make the osteoclasts and might have beneficial effect on the bone protection.

In the future, it would be desirable to find more detailed and direct evidence about the effect of the bisphosphonate by separating the PBMC's into the T cells, B cells, or monocytes to differentiate the anti-resorptive effect from the pro-inflammatory effect.

We hypothesize that the current therapy regimen for the treatment of RA is not satisfactory whether it is against the inflammation or the bone destruction. We might need different dose schedule or the different agents. The anti-osteoclastic and anti-rheumatic agents should be combined for the better results.

In conclusion, we hope to define the mechanism of the osteoclast differentiation causing the bone and joint destruction in the RA patients, and therefore design the more specifically osteoclast targeted treatment for the RA. We also need to define the effect of the bisphosphonate on the disease process of the RA as a combined agent to the standard anti-rheumatic therapy. We may need different dose schedule or the different agents to overcome blunted ant-osteoclastic response to the bisphosphonate in PBMC for RA patients.

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