JRD
Journal of Rheumatic Diseases Vol. 23, No. 6, December, 2016
https://doi.org/10.4078/jrd.2016.23.6.356

Original Article

Tumor Necrosis Factor Blockade Stimulates Circulating Osteoblastic Lineage Cells Activity while Reducing Circulating Osteoclasts

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Objective. This study examines the effects of tumor necrosis factor (TNF) blockade on markers of bone metabolism in peripheral blood from active rheumatoid arthritis (RA) patients. **Methods.** Eighteen patients (16 women, 2 men) aged 50 years (range 37-63 years), with persistently active RA (mean disease duration 7 years) were studied. Most took methotrexate (mean dose 12.5 mg) and all except one received corticosteroid (mean dose 5.7 mg). Four were treated with etanercept, eight received adalimumab and six received infliximab. Before and six months after taking TNF blockers, blood was sampled to obtain peripheral blood mononuclear cells (PBMCs), and serum bone turnover markers and acute phase reactants were measured. PBMCs were seeded and cultured to produce osteoblastic lineage cells and osteoclasts. **Results.** The formation of calcified nodules by osteoblastic lineage cells from PBMC increased from 205.7 ± 196.3 μmol/well at the baseline to 752.5 ± 671.9 μmol/well after TNF blockade ($p < 0.024$). The serum levels of bone formation markers, including bone specific alkaline phosphatase and osteocalcin also increased. The number of circulating osteoclasts and area of bone resorption pits made by osteoclasts were reduced after TNF blockade. **Conclusion.** The activity of circulating osteoblastic lineage cells increased after TNF blockade, whereas peripheral osteoclastogenesis tended to be suppressed. This is the first study of cultured human peripheral osteoblastic lineage cells in RA patients. Given that peripheral bone formation is difficult to study using radiologic methods, culture of these cells may provide a new modality for studying bone metabolism in RA. **(J Rheum Dis 2016;23:356-362)**

Key Words. Rheumatoid arthritis, Osteoblast, Osteoclast, Biologic therapy, Bone

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammatory synovitis, progressive joint destruction and systemic osteoporosis [1]. Inflammatory cytokines such as tumor necrosis factor (TNF)-α are responsible for synovial inflammation and irreversible joint destruction [2]. TNF blockers used in combination with methotrexate have revolutionized the treatment of RA, and the effective control of disease activity by TNF blockade has been proven in a number of clinical trials [3,4]. TNF-α classically plays a major role in the regulation of bone homeostasis by promoting osteoclastogenesis and inhibiting osteoblast function [1]. Because TNF-α stimulates the differentiation of osteoclast precursor cells into osteoclasts, which are responsible for bone loss, bone resorption is a central topic for understanding the role of bone metabolism in RA. However, many studies have reported on the gain in peripheral bone at the sites of bony erosions in RA patients after TNF blocker treatment [5-7], and the focus is now shifting to the process of bone formation.

Osteoblasts are derived from mesenchymal progenitor cells and are responsible for the synthesis and mineralization of bone. Previous studies have demonstrated the ability of TNF-α to inhibit multiple osteoblast functions

Received : August 24, 2016, **Revised :** October 14, 2016, **Accepted :** November 1, 2016

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pISSN: 2093-940X, eISSN: 2233-4718

in vitro as well as to repair fractures in vivo [1]. The effect of TNF blockade on bone metabolism, especially the effect on the activity of circulating osteoblastic lineage cells, has not been reported in RA patients. The primary aim of this study was to determine whether TNF blockade affects bone metabolism markers in peripheral blood obtained from RA patients after 6 months of treatment. The secondary aim was to study the relationship between disease activity and markers of systemic inflammation and bone metabolism.

MATERIALS AND METHODS

Patients

Eighteen patients (16 women, 2 men) aged 50 years (range, 37～63 years) from the outpatient clinic of the department of rheumatology in tertiary hospital were included in this study. All patients fulfilled the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for RA. The mean duration of disease was 7 years (range, 1～20 years) and all patients had persistently active RA, despite combination treatment of anti-rheumatic drugs. The demographics of patients were shown in Table 1. All patients except one took methotrexate throughout the study (mean dose 12.5 mg/week). No patients received bisphosphonate therapy and nine patients took calcium and Vitamin D supplements. All patients except one received corticosteroid at a mean daily dose of 5.7 mg. Four patients were treated with etanercept, eight received adalimumab and six received infliximab. All TNF blockers were used according to the dosages approved for the treatment of RA. Clinical disease activity was determined using the Disease Activity Score based on 28 joints (DAS28) for RA patients at baseline and after 6 months treatment of TNF blocker [8]. Blood was drawn from each individual before and after 6 months of TNF blocker treatment and was processed for routine laboratory tests including erythrocyte sedimentation rate, C-reactive protein and interleukin (IL)-6 concentrations (R&D Systems, Minneapolis, MN, USA) as markers of systemic inflammation. Serum was stored at −70°C and used in a blinded collective analysis at the end of the study. This study was approved by Inha University Hospital Institutional Review Board (IRB 14-016) and a written informed consent was obtained from all patients.

Table 1. Demographics of patients enrolled in the study

Variable	Patient (n=18)
TNF blockers used (n)	
Etanercept	4
Adalimumab	8
Infliximab	6
Age (yr)	49.7±8.3
Women (%)	88.9
Disease duration (yr)	7.0±6.6
Dose of prednisolone (mg/d)	5.7±2.7
Duration of prednisolone use (yr)	5.6±6.4
Methotrexate prescribed (%)	94.4
Dose of methotrexate (mg/wk)	12.5±4.46
Calcium and Vitamin D prescribed (%)	50.5
Bisphosphonates prescribed (%)	0

Values are number only or mean±standard deviation. TNF: tumor necrosis factor.

Cell preparation and culture

Cell culture was performed on blood samples obtained from each study subject. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density gradient centrifugation. For culture of osteoblastic lineage cells PBMCs were suspended in alpha minimal essential medium (alpha-MEM; Welgene, Seoul, Korea) containing 1% penicillin/streptomycin (Gibco, Carlsbad, CA, USA) and 15% fetal bovine serum, and plated at a density of 2×10^6 cell per well in 24-well plates. When cell multilayering was observed on days 7 to 10 after plating, media were changed to differentiation medium (alpha-MEM, 15% foetal bovine serum, 10^{-8} M dexamethasone, 1.8 mM KH_2PO_4, 2 mM glutamine, 0.1 mM L-ascorbic acid, 100 U/mL penicillin-streptomycin) and the cells were cultured for an additional 3 weeks at 37°C in 5% CO_2. Cells were then fixed and stained with Alizarin red S (Merck Millipore, Darmstadt, Germany) for detection of calcified nodules. Samples were made in duplicate and the optical density (OD) of Alizarin red S staining was measured quantitatively using an osteogenesis assay kit ECM 815 (Merck Millipore).

For culture of osteoclast precursor cells, isolated PBMCs were suspended in alpha-MEM containing 1% penicillin-streptomycin and 10% fetal bovine serum, and plated at a density of 1×10^6 cells per well in 96-well plate. Cells were incubated in culture medium for 1 week which was subsequently changed to differentiation medium supplemented with human receptor activator of nuclear factor kappa B (RANK) ligand and macrophage colony-stimulating factor (both 50 ng/mL; Peptro Tech EC

Ltd., London, UK). Cells were then fixed and stained histochemically for tartrate-resistant acid phosphatase (TRAP) using an acid phosphatase kit (386-A; Sigma-Aldrich, St. Louis, MO, USA). TRAP positive giant cells with more than 3 nuclei were regarded as osteoclasts. The total number of osteoclasts in three wells were counted and the average number of cells per well was calculated. For resorption assay, PBMCs were plated on bovine bone slices and incubated for 1 week in culture medium and for another 2 weeks in differentiation medium. Cells were removed, and the pits in the bone slices were stained with 0.5% toluidine blue. All bone slices were placed on glass slides for microscopic analysis. The slides were reviewed by two researchers who were blinded to their identity, and the resorption areas on the bone slices were quantified morphometrically, using our newly devised computer image analysis program. Normal bone structures such as Haversian canals and canaliculi tended to be stained lighter than osteoclast-mediated bone resorption pits. Thus, we used gray scale to determine a threshold to ensure that we would detect only the areas with stronger staining than that found in normal bone structure. The threshold could be adjusted according to the intensity of bone staining for each sample. The program detected the areas with stronger staining which were bone resorption pits and summed the whole area of resorption pits for each bone slice. The percentage of pit resorption area per bone slice were calculated and compared from before to 6 months after TNF blocker treatment.

Measurement of markers of bone metabolism

Enzyme-linked immunosorbent assay (ELISAs) were used to measure the serum levels of the markers of bone resorption C-terminal telopeptide (CTX; Immunodiagnostic Systems Ltd., Boldon, UK) and cathepsin K (Biomedica, Vienna, Austria). Serum levels of osteocalcin and bone-specific alkaline phosphatase (BSALP), markers of bone formation, were measured by ELISA (both from Quidel, San Diego, CA, USA).

Statistical methods

The results are expressed as mean±standard deviation. Data were compared using paired t-tests. p-values less than 0.05 were considered to be significant.

RESULTS

Disease activity decreased markedly in patients treated with a TNF blocker (Table 2). However, the acute phase reactants levels did not decrease significantly after treatment, and there was only a slight reduction in serum IL-6 level. Blockade of TNF-α resulted in increased formation of calcified nodules by PBMC (Figure 1). The OD of calcified nodules increased from 205.7±196.3 μmol/well at the baseline to 752.5±671.9 μmol/well after TNF-blocker treatment (p=0.024). The bone resorption pit area decreased from 36.0%±22.7% at the baseline to 18%±17% after treatment (p=0.005). The number of multinucleated osteoclasts from cultured PBMCs also decreased (565±295 μmol/well at baseline, 304±121 μmol/well,

Table 2. Changes in disease activities and biochemical markers of bone turnover after TNF blocker treatment

Variable	At baseline	At 6 months	p-value
Tender joint counts	16±7	8.0±5.7	<0.001
Swollen joint counts	16.0±7.3	8.1±5.6	0.001
Pain scale	75.0±14.2	40.0±23.8	<0.001
ESR (mm/hr)	34.0±19.6	28.0±28.1	0.148
CRP (mg/dL)	1.64±1.44	0.86±1.68	0.124
DAS28-ESR	6.62±0.93	4.75±1.57	<0.001
DAS28-CRP	6.2±0.8	4.24±1.31	<0.001
IL-6 (pg/mL)	23.82±26.40	17.06±32.50	0.54
BSALP (U/L)	22.17±6.99	25.82±8.89	0.007
Osteocalcin (ng/mL)	5.81±1.94	8.60±3.23	<0.001
CTX (ng/mL)	0.52±0.27	0.64±0.29	0.118
Cathepsin K (pmol/L)	4.99±1.97	4.17±2.08	0.123

Values are presented as mean±standard deviation. DAS28 is Disease Activity Score based on 28 joints for rheumatoid arthritis patients [8]. TNF: tumor necrosis factor, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, IL: interleukin, BSALP: bone specific alkaline phosphatase, CTX: c-terminal telopeptide.

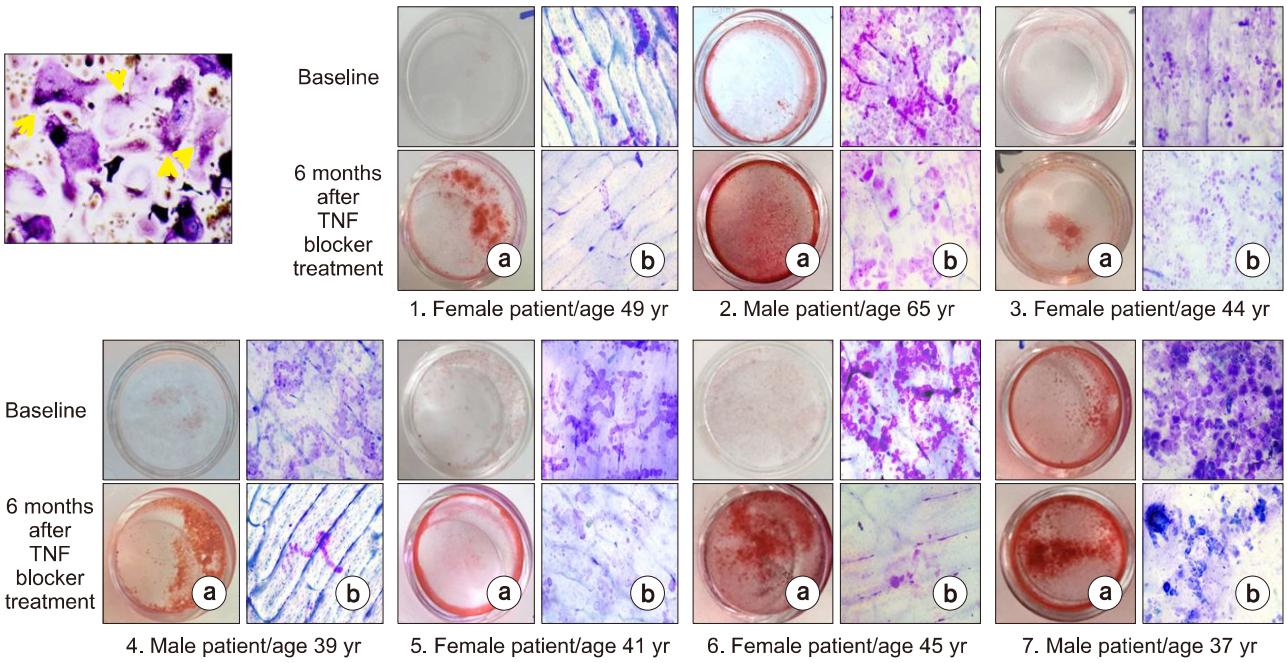

Figure 1. Calcified nodule formation (a) and bone resorption pits (b) before and after tumor necrosis factor (TNF) blocker treatment. The multinucleated osteoclasts which are responsible for bone resorption is shown in left upper corner. Patients 1～3 were treated with adalimumab, patients 4～5 with etanercept and patients 6～7 with infliximab. TRAP staining, 200×.

Table 3. Changes in disease activities and biochemical markers of bone turnover in DAS28 responders after TNF blocker treatment

Variable	At baseline	At 6 months	p-value
ESR (mm/hr)	29.0±17.7	17.0±14.3	0.002
CRP (mg/dL)	1.60±1.42	0.20±0.47	0.001
DAS28-ESR	6.48±0.94	4.09±0.96	<0.001
DAS28-CRP	6.19±0.81	3.68±0.68	<0.001
IL-6 (pg/mL)	28.07±28.32	10.52±29.72	0.149
BSALP (U/L)	22.48±7.62	26.07±9.83	0.023
Osteocalcin (ng/mL)	5.87±1.61	8.59±2.86	<0.001
CTX (ng/mL)	0.48±0.19	0.66±0.31	0.018
Cathepsin K (pmol/L)	4.66±1.80	3.69±2.26	0.1

Values are presented as mean±standard deviation. DAS28 is Disease Activity Score based on 28 joints for rheumatoid arthritis patients [8]. TNF: tumor necrosis factor, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, IL: interleukin, BSALP: bone specific alkaline phosphatase, CTX: c-terminal telopeptide.

p=0.007). The serum levels of the bone formation markers BSALP and osteocalcin increased significantly after 6 months of treatment, whereas the levels of the bone resorption markers serum CTX and cathepsin K did not change (Table 2).

According to European League Against Rheumatism (EULAR) response criteria [8], patients were classified into two groups, responder group (n=14) and non-responder group (n=4). In DAS28 responder group, formation of calcified nodules increased from 227.3±203.8 μmol/well to 917.4±674.7 μmol/well after TNF treatment (p=0.024). The number of osteoclasts decreased from 589.0±269.4 μmol/well to 285.0±111.9 μmol/well (p=0.005) and bone resorption pits by osteoclasts were also reduced from 37.0%±22.3% to 13.0%±12.3% after the treatment (p<0.001). The RA disease activity, systemic inflammation and bone turnover markers were shown in Table 3. In non-responder group, there was no change in all parameters including formation of calcified nodules, number of osteoclasts, bone resorption pits and bone turnover markers (Table 4).

Table 4. Changes in optical densities of osteoblasts, number of osteoclasts and area of bone resorption pits in addition to disease activities and biochemical markers of bone turnover in DAS28 non-responders after TNF blocker treatment

Variable	At baseline	At 6 months	p-value
Optical density of calcified nodule (μmol/well)	133.90±184.77	202.8±266.84	0.784
Bone resorption pit by osteoclasts (%)	29.0±28.4	45.0±3.4	0.392
Number of osteoclasts (/well)	547.0±419.1	437.0±124.8	0.655
ESR (mm/hr)	53.0±15.8	67.0±30.9	0.465
CRP (mg/dL)	1.80±1.72	3.10±2.56	0.465
DAS28-ESR	7.10±0.82	7.00±1.03	0.715
DAS28-CRP	6.3±0.9	6.18±1.09	1.0
IL-6 (pg/mL)	8.97±9.90	39.97±35.72	0.465
BSALP (U/L)	20.70±3.16	24.70±1.53	0.162
Osteocalcin (ng/mL)	5.52±3.62	8.66±5.51	0.102
CTX (ng/mL)	0.72±0.52	0.58±0.14	1.0
Cathepsin K (pmol/L)	5.92±2.42	5.47±0.43	0.715

Values are presented as mean±standard deviation. DAS28 is Disease Activity Score based on 28 joints for rheumatoid arthritis patients [8]. TNF: tumor necrosis factor, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, IL: interleukin, BSALP: bone specific alkaline phosphatase, CTX: c-terminal telopeptide.

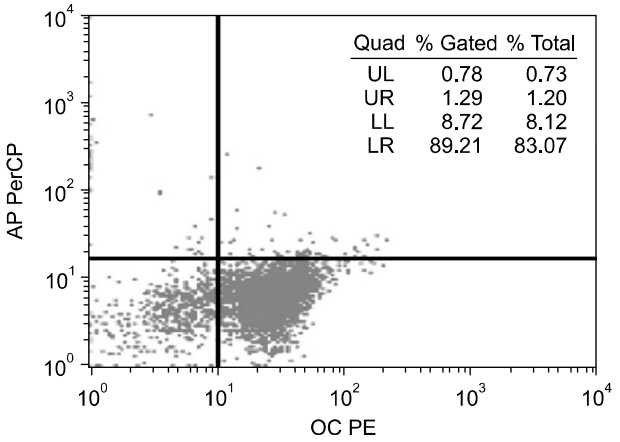

Figure 2. Flow cytometry analysis of cells from peripheral blood to sort for both osteocalcin, bone specific alkaline phosphatase (BSALP)-positive cells. Cells positive for osteocalcin (OC) were stained with phycoerythrin (PE) and cells positive for BSALP (AP) were conjugated with peridinin chlorophyll protein (PerCP). Cells positive for both osteocalcin and BSALP were turned out to be less than 1%. UL: upper left, UR: upper right, LL: lower left, LR: lower right, Quad: quadrant.

DISCUSSION

The first finding of this study is that the formation of circulating osteoblastic lineage cells increased and peripheral osteoclastogenesis tended to be suppressed after 6 months of TNF-blocker treatment. Previous studies have reported either improvement in bone mineral density [9,10] or peripheral bone gains [5-7], but ours is the first study to demonstrate bone metabolism in ex vivo using cells obtained from peripheral blood in RA patients after TNF-blocker treatment.

Progenitor stem cells in the bone marrow comprise two populations: non-adherent, circulatory hematopoietic stem cells and adherent, non-circulatory osteogenic stromal stem cells [11]. Osteoblastic cells in the bone marrow have traditionally been identified by their adherence to standard tissue culture plastic and by the subsequent formation of mineralized nodules [12,13]. Long et al. [14,15] identified a population of cells in the bone marrow that have osteogenic potential and are non-adherent and positive for osteocalcin, BSALP, or both. Eghbali-Fatourechi et al. [16] extended these studies to isolate a cell population from peripheral blood that was not plastic adherent. They showed that the percentages of cells positive for osteocalcin and BSALP were higher during the adolescent growth spurt than in adulthood and that this difference may be associated with stimulation of bone formation. They also sorted osteocalcin-positive cells from peripheral blood and detected the formation of mineralized nodules by osteocalcin-positive cells in vitro [16]. Using the parabiotic animal model, Kumagai et al. [17] showed that circulating osteogenic connective tissue progenitors are mobilized to fracture sites where they contribute to osteogenesis in the early stage of fracture healing. From these studies, we postulated that circulating osteoblastic lineage cells are present in the peripheral blood and would be responsible for bone repair. Flow cytometry was used to sort for both osteocalcin, BSALP-positive cells in our study (Figure 2). It turned out that the

percentage of sorted cells was too small (0.73%) and huge volume of blood was required for cell culture after sorting by flow cytometry. Because of this technical difficulty, we decided to culture unsorted PBMCs in osteoblast culture media. After 6 months of TNF blocker treatment, both OD of calcified nodules ex vivo and serum bone formation markers such as osteocalcin and BSALP were increased. This result shows clearly that inhibition of TNF-α stimulates the activity of osteoblastic lineage cells from peripheral blood.

In our study, the number of osteoclasts and their bone-resorption capacity were reduced after TNF-blocker treatment, although the serum bone resorption markers did not change significantly. TNF-α exerts its effects on RA-associated bone resorption through two mechanisms: (1) an indirect effect on osteoclastogenesis by increasing cell accumulation in synovial tissue and production of inflammatory cytokines and (2) direct involvement in the differentiation of osteoclast precursor cells into osteoclasts and activation of osteoclasts [18]. In our study, PBMCs were used as a defined source of osteoclast precursor cells; thus, a direct influence of TNF-α was seen, by a decrease in the number and function of circulating osteoclasts after TNF blockade. RA patients in our study showed marked improvement in disease activity but smaller reduction in the levels of acute phase reactants and systemic inflammatory marker IL-6 after TNF blocker treatment. Our data suggest that the 6-month treatment with TNF blockers inhibited the development of osteoclasts from PBMC but may have been of insufficient duration to show any effects on systemic bone resorption. In DAS28 responder group, the number of osteoclasts and the area of bone resorption pits both decreased whereas serum level of CTX increased. There is no explanation for increased serum level of CTX at this time point and we believe further investigation is warranted. The disequilibrium between osteoblast and osteoclast activities is a main factor related to bone loss in RA. Although the effect of TNF blockers on osteoclastogenesis has been reported [18,19], no reports have described the effects of TNF blockers on osteoblastic lineage cells in RA patients. Refilling of peripheral erosions in RA, as shown radiologically as a decrease in erosion depth, is incomplete even after 1 year of treatment [6]. Thus, studies of the repair of bone erosion are limited in RA despite the use of effective drug therapy. Dalle Carbonare et al. [20] found a greater number of circulating mesenchymal stem cells in osteoporosis patients compared with normal donors and proposed the study of circulating mesenchymal stem cells as a useful tool for studying the bone remodeling process. Our data also showed increased formation of calcified nodules from peripheral osteoblastic lineage cells which suggested improved function of peripheral osteoblasts after TNF blocker treatment. Our method is non-invasive and may provide a new modality for the study of bone metabolism in RA.

CONCLUSION

This is the first study to show the effects of TNF-blockers on circulating osteoblastic lineage cells and peripheral osteoclastogenesis by their treatment in RA patients. Our method is non-invasive and may provide a new modality for the study of bone metabolism in RA.

ACKNOWLEDGMENTS

The authors are grateful to Sang Min Lee, PhD for valuable contribution in developing the computer program to assess bone resorption pits.

CONFLICT OF INTEREST

Author Won Park received fee for consultation from Celltrion, Co. Any other authors have no conflicts of interest regarding this study.

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