Receptor activator of nuclear factor kappa B ligand-mediated osteoclastogenesis is elevated in ankylosing spondylitis

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Abstract

Objective

Ankylosing spondylitis (AS) is an inflammatory arthritis involving the axial skeleton. Decreased bone mineral density has also been reported in AS patients. This study sought to determine whether osteoclastogenesis and osteoclast activity are increased in AS.

Methods

Twenty patients with AS were evaluated using the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and other clinical parameters. Mononuclear cells were separated out from peripheral blood samples taken from AS patients and normal healthy controls and cultured with monocyte colony stimulating factor and receptor activator of the nuclear factor kappa B ligand (RANKL). Multi-nucleated, tartrate-resistant acid phosphatase stain-positive osteoclasts were counted after 9 days, and the areas of calcium absorption on calcium-coated plates were determined.

Results

Osteoclastogenesis was significantly greater in AS patients than in normal controls (number of osteoclasts/1×10^6 mononuclear cells, median, 518.0 vs. 362.5, $p=0.036$). No differences were observed between AS patients and controls in terms of calcium absorption areas or the serum concentrations of tumor necrosis factor-α and RANKL. Osteoclastogenesis was greater in AS patients with sacroiliac joint ankylosis than in those without. Osteoclastogenesis and the calcium absorption area were not found to be correlated with BASDAI nor with other clinical parameters including age, erythrocyte sedimentation rate, and C-reactive protein levels.

Conclusion

Osteoclastogenesis is elevated in AS patients, especially in those with sacroiliac joint ankylosis. Increased osteoclastogenesis may be related to osteopenia in AS patients.

Key words

Ankylosing spondylitis, osteoclastogenesis, RANKL, TNF-α.

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Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory disease that primarily affects the axial skeleton (sacroiliac joints and spine). The principal musculoskeletal lesions associated with AS are enthesitis and synovitis, with sacroiliitis also involving the adjacent bone (1). New bone formation and progressive ankylosis of the lesions are well known manifestations of AS, but local bony erosions, juxta-insertional osteopenia and systemic osteoporosis are also frequently detected. Furthermore, the associated risk of vertebral fracture in AS patients increases the likelihood of disability.

The pathogenesis of osteopenia in AS is not clearly understood. Spinal stiffness and immobilization have been suggested as causes, but many studies have documented systemic osteopenia in mild and early AS without immobility or stiffness (2-4). In addition, strong correlations between systemic osteopenia and AS disease activity have been reported recently. For example, Donnelly *et al*. found femoral neck bone mineral density (BMD) to be significantly correlated with clinical disease severity (Schober index) and disease duration in AS (5). Marhoffer *et al*. presented evidence of impaired cartilage/bone turnover in active AS patients based on significant positive correlations between the levels of urinary excretion of pyridinoline cross-links and acute phase reactant parameters such as the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels, but no significant correlation between ESR or CRP and markers of bone formation (6). Meirelles *et al*. found significant differences between patients with active and inactive AS, as measured by the mean Ward's triangle BMD (7). Moreover, it has also been suggested that pamidronate, which inhibits osteoclasts and is indicated for the treatment of systemic osteoporosis, may decrease disease activity in AS patients with systemic osteopenia (8).

Osteoclasts are multi-nucleated giant cells, which differentiate from their hematopoietic precursors by the activation of RANK (receptor activator of nuclear factor kappa B) via the binding of the RANK ligand (RANKL). Osteoclasts are characterized by the presence of tartrate-resistant acid phosphatase (TRAP), calcitonin receptor, and vitronectin receptor. They are the cells principally responsible for systemic osteopenia and the bony erosions of inflammatory arthritides, such as rheumatoid arthritis (RA) (9) and psoriatic arthritis (PsA) (10). Furthermore, Ritchlin *et al*. showed that osteoclastogenesis is markedly elevated in the peripheral blood of PsA patients (10). AS and PsA have the same genetic risk factor (human leukocyte antigen (HLA)-B27), produce similar skeletal changes (enthesitis, sacroiliitis, and systemic osteopenia), and have similar clinical responses to tumor necrosis factor (TNF)-α blocking therapy. Here we investigated osteoclastogenesis and its correlations with disease activity and radiographic changes in AS.

Patients and methods

Patients at a rheumatology clinic in Seoul National University Hospital who had been diagnosed with AS based on the modified New York criteria (11) were enrolled from July 2004 to November 2005. Demographic and clinical data including age, initial symptoms, and ESR and CRP levels were collected from their medical records. Radiographic changes in the sacroiliac joint were reviewed and graded according to the modified New York criteria, and radiographic changes in the lumbar spine were reviewed using the Bath Ankylosing Spondylitis Radiology Index (BASRI). All patients were interviewed to determine their Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) using 10 cm visual analogue scales. After obtaining their informed consent, heparinized blood and serum were obtained from 20 patients, and from 20 age- (difference ≤3 years) and sex-matched healthy controls.

To quantify osteoclastogenesis, peripheral blood mononuclear cells (PBMCs) were collected from heparinized blood by centrifuging samples 3 times on a Ficoll gradient (500 g, 15 min). After each centrifugation, mononuclear cell layers were aspirated and re-suspended in α MEM media containing 10% fetal

bovine serum to a final concentration of 1×10^6 PBMC/mL. PBMCs suspended in media (1 mL) were then aliquoted to 24-well culture plates or to calcium-coated culture plates. Monocyte colony stimulating factor (M-CSF) 50 ng/mL and RANKL 100 ng/mL were added to each well, and the PBMCs were cultured in a 5% CO_2 incubator at 37ºC. The culture media containing M-CSF and RANKL were changed every 3 days, and after 9 days the osteoclasts – identified as the TRAP-stained (+) and multi-nucleated (≥3 nuclei) cells – were counted. Areas of calcium absorption from the plate surfaces were also measured.

Using commercial kits, the concentrations of serum TNF-α (ultrasensitive TNF-α [hTNF-αUS] ELISA Kit, Biosource, Camarillo, California) and RANKL (Total RANKL, Soluble (human) ELISA kit, Apotech, Epalinges, Switzerland) were determined.

The Mann-Whitney U-test was used to identify differences between AS patients and healthy controls, and Spearman's rank test was used to identify the differences between continuous variables such as the osteoclast count, areas of calcium absorption, BASDAI, and serum RANKL and TNF-α concentrations. Statistical analyses were conducted using SPSS 12.0 throughout.

Table I. Clinical characteristics of ankylosing spondylitis (AS) patients.

Clinical characteristics	AS patients (n=20)
Gender (male: female)	17: 3
Age (year, mean ± SD)	33.2 ± 8.0
Onset age (year)	23.4 ± 8.3
Height (cm)	169.6 ± 5.0
Body weight (kg)	65.5 ± 10.2
BASDAI (mm)	20.9 ± 11.0
ESR (mm/hr)	30.1 ± 24.4
CRP (mg/dL)	1.44 ± 1.04
HLA-B27 positivity	100%
Current medication	
NSAID	11 (55%)
DMARD*	2 (10%)
TNF-α blockade	0 (0%)

BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; ESR: erythrocyte sedimentation rate (Westergren); CPR: C-reactive protein; HLA-B27: human leukocyte antigen-B27; NSAID: non-steroidal anti-inflammatory drug; DMARD: disease-modifying anti-rheumatic drug; TNF-α: tumor necrosis factor-alpha.
*Sulfasalazine, methotrexate.

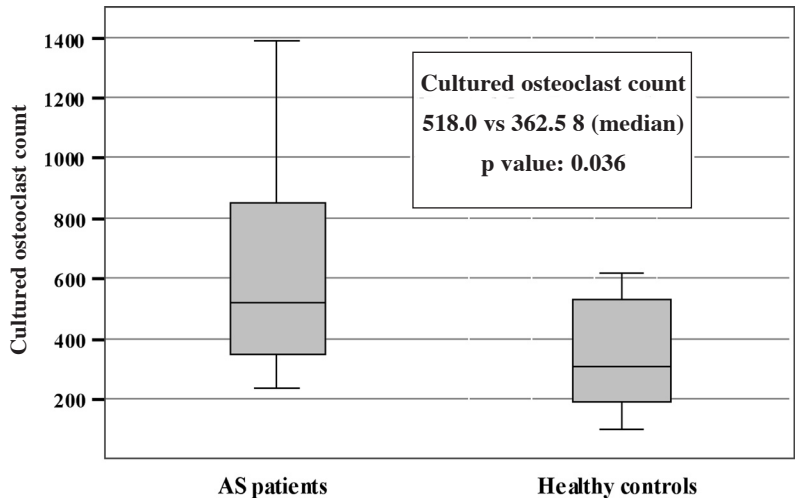

Fig. 1. Number of osteoclasts cultured from peripheral blood mononuclear cell ($1x10^6$ cell) in ankylosing spondylitis (AS) patients and healthy controls with monocyte-colony stimulating factor (M-CSF) and receptor activator nuclear factor kappa B ligand (RANKL). Box covers the interquartile range with the median indicated by the line within the box. Whiskers extend either to the minimum and maximum values. *P*-value by Mann-Whitney U-test.

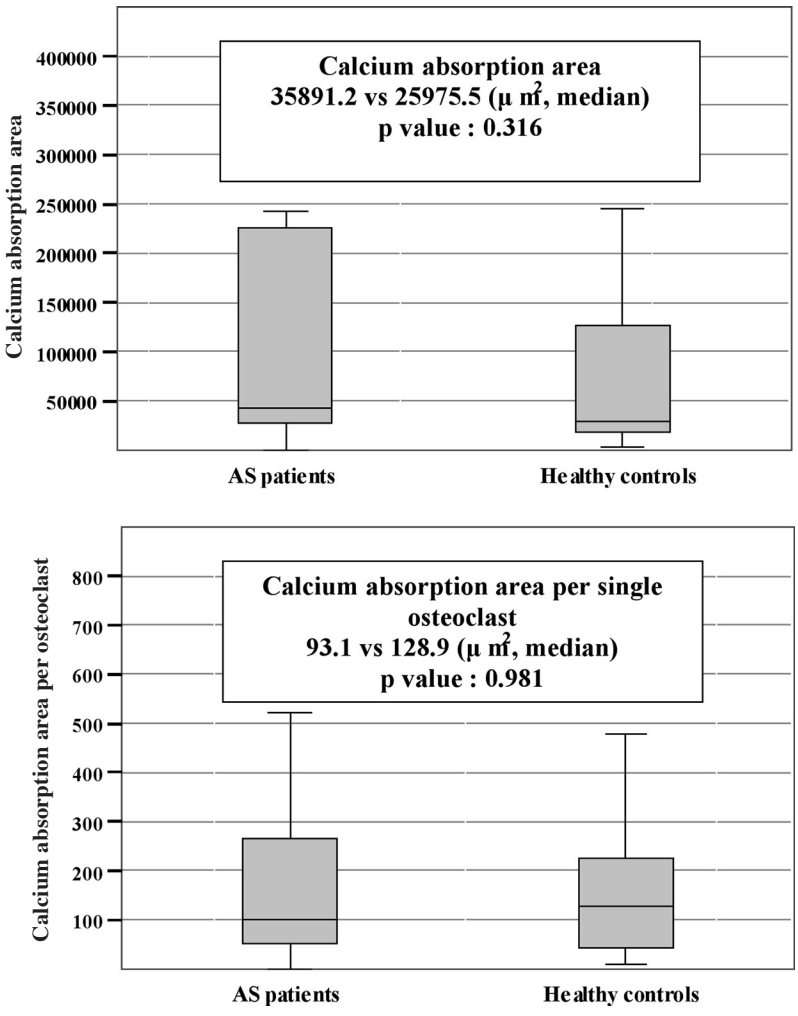

Fig. 2. Calcium absorption area (μm^2) after osteoclast culture, calcium absorption area per single osteoclast (total area divided by number of cultured osteoclasts) in ankylosing spondylitis (AS) patients and healthy controls. Box covers the interquartile range with the median indicated by the line within the box. Whiskers extend either to the minimum and maximum values. *P*-values by Mann-Whitney U-test.

Results

Twenty AS patients and 20 healthy normal controls matched for age and sex were enrolled in this study. In the AS group the M:F ratio was 17:3 and the mean age was 33.2 years; the clinical characteristics of the patients are shown in Table I.

Well-differentiated osteoclasts with multiple nuclei (3 or more) and TRAP (+) cytoplasmic staining were detected in culture plates after 9 days. No differences in morphology between the cultured osteoclasts of AS patients and healthy controls were observed, but the numbers of cultured osteoclasts were greater for AS patients than for healthy controls (Fig. 1). The pattern and amount of calcium absorption from calcium-coated culture plates did not differ significantly between AS patients and healthy controls, and adjustment for the number of osteoclasts did not affect this finding (Fig. 2). No significant differences were observed between patients and controls in serum RANKL or TNF-α concentrations (Fig. 3).

In AS patients the cultured osteoclast count and calcium absorption areas were not correlated with clinical characteristics such as age, BMI, BASDAI, serum ESR, CRP, serum RANKL and TNFα, radiographic grade for the lumbar spine and sacroiliac joints, or BMD of the lumbar spine and femoral neck (Table II). The osteoclast count and calcium absorption areas also did not differ significantly with gender or medication (non-steroidal anti-inflammatory or disease-modifying anti-rheumatic drugs) (data not shown).

AS patients with sacroiliac joint ankylosis (radiographic grade 4) were found to have a higher number of differentiated osteoclasts than patients without ankylosis (radiographic grades 2 or 3) (Fig. 4). However, the areas of calcium absorption were not significantly different between AS patients with or without sacroiliac joint ankylosis (Fig. 4). No differences in osteoclast numbers or calcium absorption were observed between AS patients with and without definite lumbar spine radiographic changes (erosion, squaring, sclerosis, or syndesmophytes, BASRI-spine 1 versus 2 and 3, Fig. 5).

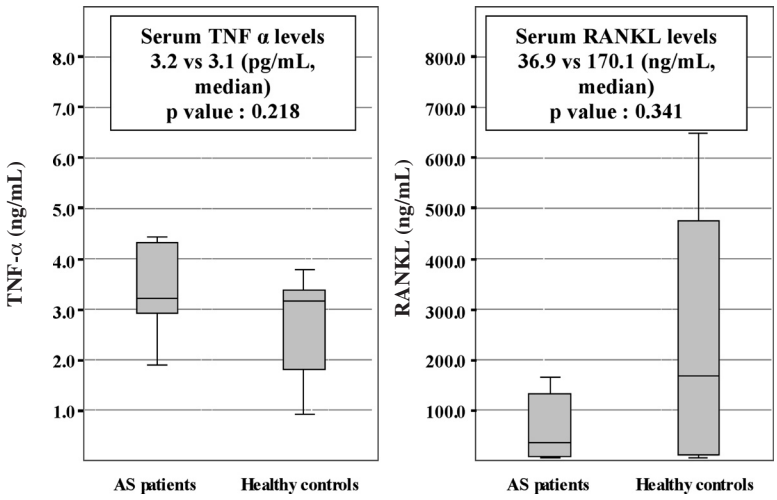

Fig. 3. Serum tumor necrosis factor (TNF) α and receptor activator nuclear factor kappa B ligand (RANKL) concentrations in ankylosing spondylitis (AS) patients and healthy controls. Box covers the interquartile range with the median indicated by the line within the box, whiskers extend either to the minimum and maximum values. *P*-value by Mann-Whitney U-test.

Table II. Osteoclastogenesis, area of calcium absorption (divided by the number of osteoclasts), and clinical and laboratory data on the ankylosing spondylitis patients.

Data	Osteoclastogenesis		Calcium absorption	
	Coefficient	*p*-value	Coefficient	*p*-value
Age	0.035	0.890	0.297	0.302
Body mass index	0.211	0.417	0.308	0.306
BASDAI	-0.141	0.576	0.121	0.681
Radiographic grade				
sacroiliac joint	0.382	0.130	-0.194	0.506
lumbar spine	0.477	0.138	0.027	0.946
Westergren ESR	-0.008	0.974	-0.143	0.626
C-reactive protein	-0.038	0.888	0.294	0.354
Serum RANKL	0.266	0.404	-0.476	0.233
Serum TNF-α	0.294	0.354	0.452	0.260

Coefficients and *p*-values were determined by Spearman's rank test.
BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; ESR: erythrocyte sedimentation rate; TNF: tumor necrosis factor.

Discussion

In the present study, RANKL-induced osteoclastogenesis from PBMCs in culture was greater in AS patients, and particularly in AS patients with sacroiliac joint ankylosis. Sacroiliac joint damage may be a marker of severity in AS, and also causes immobility. Furthermore, both disease severity and immobility can influence osteoclast precursor cell differentiation. However, no definite differences were observed between AS patients and healthy controls in the amount of calcium absorption per osteoclast, or serum RANKL and TNF-α concentrations. In addition, osteoclastogenesis in AS patients was not found to be correlated with disease activity, serum RANKL, serum TNF-α, bone mineral density, or lumbar spine radiographic changes.

The finding that the numbers of osteoclasts from AS patients cultured with M-CSF and RANKL were higher than those of controls means that levels of osteoclast precursor cells were greater in the peripheral blood of AS patients. In RA, periarticular bone destruction and systemic osteopenia have formed the subject of several studies. Takayanagi *et al*. reported that RANKL expressed on synovial fibroblasts is involved in rheumatoid bone destruction because it induces osteoclastogenesis (12). In

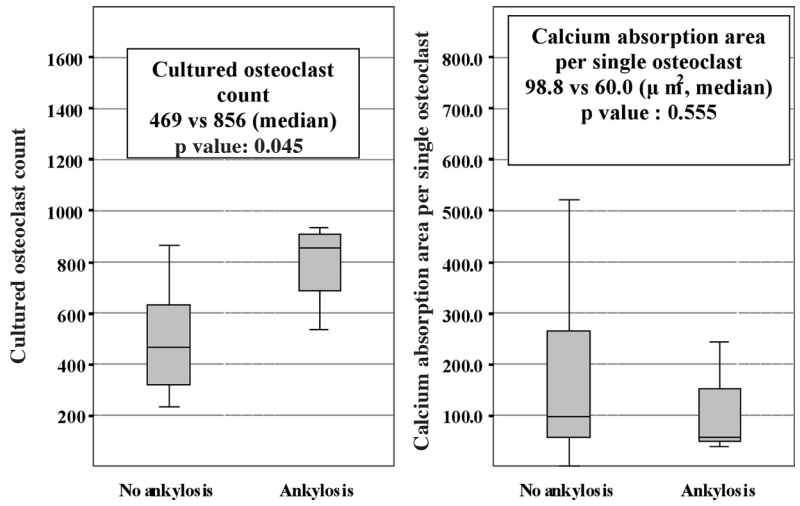

Fig. 4. Osteoclastogenesis (number of cultured osteoclasts) and calcium absorption area by osteoclast in ankylosing spondylitis patients with or without sacroiliac joint ankylosis. Box covers the interquartile range with the median indicated by the line within the box. Whiskers extend either to the minimum and maximum values. *P*-value by Mann-Whitney U-test.

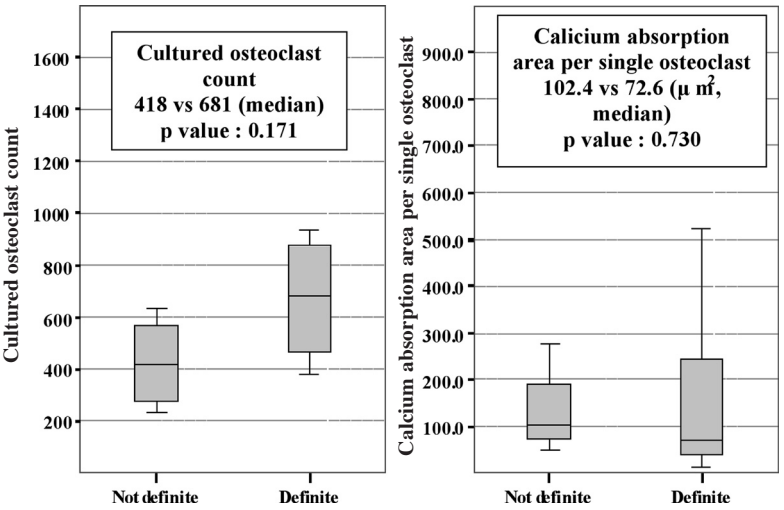

Fig. 5. Osteoclastogenesis (number of cultured osteoclasts) and calcium absorption area by osteoclast in ankylosing spondylitis patients with or without definite lumbar spine radiographic changes (erosion, squaring, sclerosis, or syndesmophytes, Bath Ankylosing Spondylitis Radiology Index-spine 1 *vs*. 2 and 3). Box covers the interquartile range with the median indicated by the line within the box. Whiskers extend either to the minimum and maximum values. *P*-value by Mann-Whitney U-test.

addition, excess production of RANKL by activated T lymphocytes increased soluble RANKL levels in the synovial fluid, and was suggested to contribute to osteoclastic bone resorption in RA patients. Ziolkowska *et al*. found that serum levels of RANKL are higher in RA patients than in healthy individuals, and that anti-TNF-α treatment normalizes these levels (13). Hirayama *et al*. reported that osteoclastogenesis from PBMCs cultured in the presence of RANKL is not increased in RA patients, but that the bone absorption activity of cultured osteoclasts is higher in RA patients than in healthy controls (9). They concluded that joint destruction and systemic osteopenia in RA patients are not associated with increased osteoclastogenesis, but rather with increased osteoclast activity. However, this differs from our findings, suggesting that osteoclast differentiation and activation are not the same in RA and AS.

Psoriatic arthritis is an inflammatory arthritis and a type of spondyloarthropathy. Ritchlin *et al*. reported that osteoclastogenesis from PBMCs in culture (mean 168±39.9 *vs*. 3.7±1.1 osteoclasts per 10^6 PBMCs; $p<0.006$) and calcium absorption (eroded bone wafer area, mean 0.49%±0.31% *vs*. 0.08%±0.12%; $p<0.009$) by cultured osteoclasts is elevated in PsA patients compared to healthy controls (10). Osteoclast precursor cells arise from TNFα-activated PBMCs. In the present study osteoclastogenesis from PBMCs cultured with RANKL and M-CSF was significantly greater in AS patients. However, the difference observed between AS patients and healthy controls was not greater than that found by Ritchlin *et al*. in PsA. In addition, in our study the areas of calcium absorption were not significantly greater in AS patients than in healthy controls. These differences between AS and PsA could explain the higher levels of severe periarticular bone destruction seen in PsA patients. We hypothesize that AS involves the same pathophysiologic processes (increased osteoclast differentiation) as PsA, but that these processes are more aggressive in PsA than AS.

The small patient number is a limitation of this investigation. However, the numbers in previous studies of osteoclastogenesis in RA (10 patients) and PsA (24 patients) were similar to that of our study of AS (20 patients).

In conclusion, it was found that levels of peripheral blood osteoclast precursor cells are elevated in ankylosing spondylitis, but that osteoclast activity is not. Furthermore, increased osteoclastogenesis was found to be associated with radiographic ankylosis of the sacroiliac joint, but not with disease activity or parameters of systemic inflammation.

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