The effect of anti-TNF treatment on osteoblastogenesis in ankylosing spondylitis: the number of circulating osteoblast-lineage cells in peripheral blood decreased after infliximab therapy in patients with ankylosing spondylitis

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Abstract

Objective

The full effect of anti-TNF therapy on new bone formation is still in debate in spondylitis fields. We sought to obtain circulating osteoblast-lineage cells in peripheral blood from ankylosing spondylitis (AS) patients and healthy control subjects, and to evaluate the effect of before and after anti TNF-α therapy on osteoblastogenesis in patients with AS.

Methods

Sixteen male patients with AS slated for infliximab therapy and 19 controls were recruited. We cultured osteoblast-lineage cells from peripheral blood and measured the optical density of their aAizarin red S staining. We also measured serum P1NP (procollagen type 1 N-terminal propeptide) as an early osteoblast differentiation marker, osteocalcin as a late osteoblast differentiation marker, and inflammatory markers.

Results

There were significantly more circulating osteoblast-lineage cells in patients than in controls. The number of circulating osteoblast-lineage cells and optical density of Alizarin red S staining decreased 14 weeks after infliximab therapy (p=0.028); serum level of P1NP decreased, but that of osteocalcin increased (p=0.002 and 0.007, respectively).

Conclusion

Our data reveals that first, the circulating osteoblast-lineage cells are recoverable and increased in AS patients, and also that they decrease after infliximab therapy; second, infliximab therapy resolves early inflammation, but allows mature osteoblast differentiation in late inflammation. The culture of osteoblast-lineage cells in peripheral blood may be a candidate for a new modality with which to study spondylitis and other autoimmune diseases.

Key words

ankylosing spondylitis, osteoblasts, inflammation, infliximab

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Introduction

It has been reported in a number of publications that treatment with tumour necrosis factor (TNF)-α inhibition does not prevent syndesmophyte formation in patients with ankylosing spondylitis (AS) (1-3). However, more recently, a protective effect of TNF-α blockers on new bone formation has been reported (4). In another study with 8 years of follow-up, there was evidence for less radiologic bony progression in patients on anti-TNF-α therapy (5). It was postulated that early vertebral inflammation resolves after anti-TNF-α therapy, whereas resolution of late inflammation allows reparation and bony progression (6-7). The full effect of anti-TNF-α therapy on the natural history of AS is thus still not resolved.

A major impediment to investigation of the biology of the axial skeleton in AS is the relative inaccessibility of tissue from the spine or sacroiliac joints. As a result, investigators have for the most part relied on indirect modalities, including magnetic resonance imaging (MRI) and animal models.

Bone marrow contains both osteoblast and osteoclast precursors that can differentiate into mature osteoblasts and osteoclasts, respectively. It is well established that abundant osteoclast precursors are found in the peripheral circulation as well as in the bone marrow. However, less is known as to whether there is a comparable population of circulating osteoblast-lineage cells. In 2005, Eghbali-Fatourechi *et al*. showed the existence of circulating osteoblast-lineage cells expressing gene markers of the osteoblast phenotype, which formed mineralised nodules, a hallmark of osteoblastic differentiation (8). They were able to culture these osteoblast-lineage cells from peripheral blood, and implantation of the osteoblast-lineage cells in immunosuppressed mice resulted in new bone formation. We sought to obtain these circulating osteoblast-lineage cells in peripheral blood from AS patients and healthy control subjects, and to use them to evaluate the relationship between inflammation and osteoblast-lineage cell activity in AS before and after anti-TNF therapy with infliximab.

Patients and methods

Patients

Sixteen male patients with AS were enrolled. They met 1984 modified New York criteria and were candidates for infliximab therapy because they were unresponsive to non-steroidal anti-inflammatory drugs (NSAIDs). Nineteen sex- and age-matched control participants were also recruited. Patients with AS received 5 mg/kg infliximab infusion at weeks 0, 2, 6, and 14. The study was approved by our local ethics committee (IRB no. 13-029), and patients signed written informed consent forms according to the principles of the Declaration of Helsinki before participating.

Primary culture of osteoblast-lineage cells

Whole blood samples were overlaid on Ficoll-Paque density gradients. Mononuclear cells were extracted and suspended in osteoblast growth medium, composed of 15% fetal bovine serum and alpha-minimal essential medium (Welgene, Seoul, Republic of Korea), and layered at a density of 2 x 10^6 cells in 2ml of medium per well in 24-well plates. After multilayering of cells had been observed (about 7 to 10 d), cells were transferred to osteoblast differentiation medium (15% fetal 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 (Gibco, Carlsbad, CA, USA)) and cultured for 3 weeks at 37°C in 5% CO_2. Cells were then fixed and stained with Alizarin red S stain dye (Millipore, Darmstadt, Germany) to mark any calcified nodules. The optical density (OD) of Alizarin red S staining was measured for quantitative analysis using an osteogenesis assay kit ECM 815 (Merck Millipore, Darmstadt, Germany).

Clinical outcomes and laboratory methods

Fasting serum samples were obtained at baseline and after 14 weeks of infliximab therapy in patients with AS. Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) levels, and the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) (9) were measured. Osteoblast differentia-

tion is stimulated through the expression and response to several transcription factors and families of growth factors (10). We used the P1NP (total procollagen type 1 N-terminal propeptide) as a marker of osteoblast proliferation (early osteoblast differentiation marker), and osteocalcin as a marker of mineralisation (late osteoblast differentiation marker) (11).

Fasting serum samples used for the measurement of the levels of P1NP, C-terminal telopeptide of type I collagen (CTX-1) as a bone resorption marker, osteocalcin, were frozen rapidly in liquid nitrogen and stored at -80°C until analysed. Serum levels of P1NP, CTX-1, and osteocalcin, were measured using commercially available enzyme-linked immunosorbent assay kits; P1NP, Assay Designs (Ann Arbor, MI, USA); CTX-1, Immunodiagnostic Systems Ltd. (Boldon, UK); and osteocalcin, Quidel (Hanover, Germany).

Statistics

Results are presented as mean ± SD. Statistical analysis was conducted using the IBM SPSS statistics for Windows v. 19.0 (IBM Corp., Armonk, NY, USA). Comparisons in the levels of biomarkers in patients and controls were analysed using a Mann-Whitney *U*-test. Changes in the levels of biomarkers between baseline and 14 weeks after infliximab therapy in patients were analysed using a Wilcoxon rank sum test. Correlations between the biomarkers were evaluated using a Spearman correlation test. Differences were considered significant at $p<0.05$.

Results

Characteristics of the AS patients and controls

Sixteen patients were enrolled from March 2011 to November 2013. The mean ± SD value of age of patients was 39.3±12.9 years; that of controls was 38.3±11.4. Patients and controls did not differ significantly with respect to age. The mean ± SD value of years after diagnosis of AS was 9.5±10.2 years. Of the 16 patients, 9 had only axial involvement and 7 had both axial and peripheral involvement. Three patients had uveitis. The mean ± SD value of modified Schober test was 3.0±1.4 cm, chest expansion test was 3.0±1.5 cm. Fifteen patients took NSAIDs and 8 were smokers. All of the patients took sulfasalazine. All patients were human leukocyte antigen (HLA) B27 positive. Among the 19 controls, 9 were smokers. The mean ± SD value of Bath Ankylosing Spondylitis Disease Activity Index in patients was 9.1±0.9.

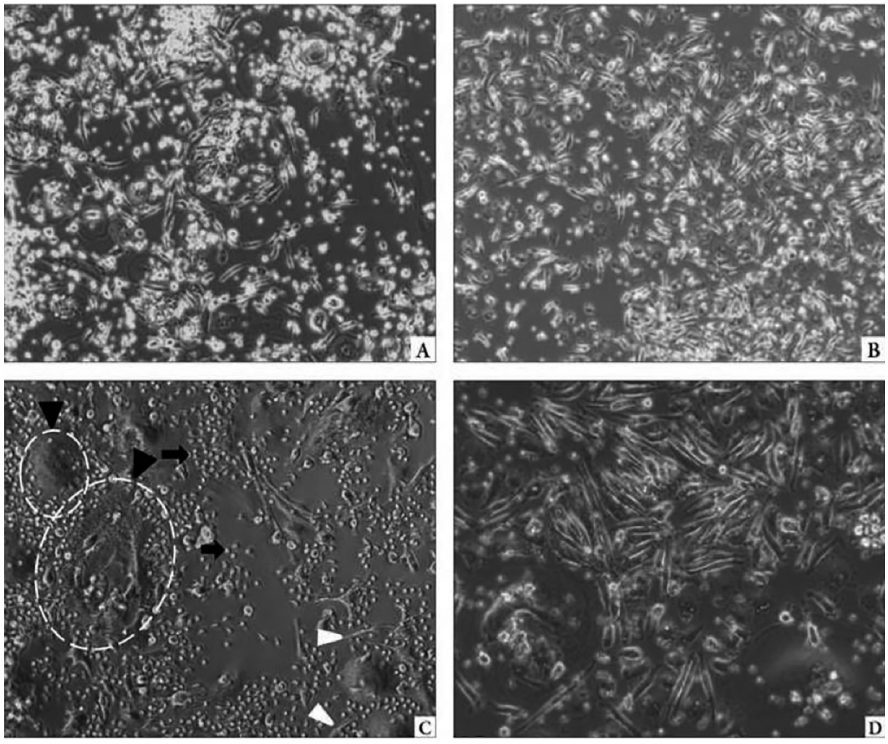


Fig. 1. Morphology of osteoblast-lineage cells derived from peripheral blood mononuclear cells of human blood. The morphology of cells cultured with osteoblast differentiation medium for 10 d (**A**, **C**), and 21 d (**B**, **D**). (**C**) Black arrow indicates that round cells look like monocytes; black triangle, multinucleated cells resemble macrophages; white triangle, elongated cells morphologically appearing fibroblasts (**A**, **B**: Phase contrast: magnification ×100, **C**, **D**: Phase contrast: magnification × 200).

Circulating osteoblast-lineage cells

We obtained circulating osteoblast-lineage cells for culture from 8 patients and 19 controls. In patients, circulating osteoblast-lineage cells for culture were obtained before and after infliximab therapy. After 7 to 10 d of culture, starting with peripheral blood mononuclear cells (PBMCs) two morphologically different subgroups were distinguishable: round monocyte-like cells or multinucleated cells resembled macrophage like cells; and elongated fibroblast-like cells (Fig. 1). Cell numbers in the cultures of circulating osteoblast-lineage cells from AS patients were far more abundant than those from controls (Fig. 2). The numbers of culturable circulating osteoblast-lineage cells decreased 14 weeks after infliximab therapy in every patient (Fig. 2). There is no statistical difference in number of circulating osteoblast-lineage cells between smoker and non-smoker group in patients with AS.

Bone biochemical markers in patients with AS and controls at baseline

Mean serum P1NP (marker of early osteoblast differentiation) and Alizarin red S OD levels in the cultures were significantly higher in patients with AS than in the controls ($p<0.008$, and $p<0.0001$ respectively; Table I). Mean serum osteocalcin and CTX levels were not statistically different in patients with AS and in controls; smoker and non-smoker groups.

Bone biochemical markers following 14 weeks of infliximab therapy in patients with AS

Serum osteocalcin (a late osteoblast differentiation marker) levels increased significantly after infliximab therapy

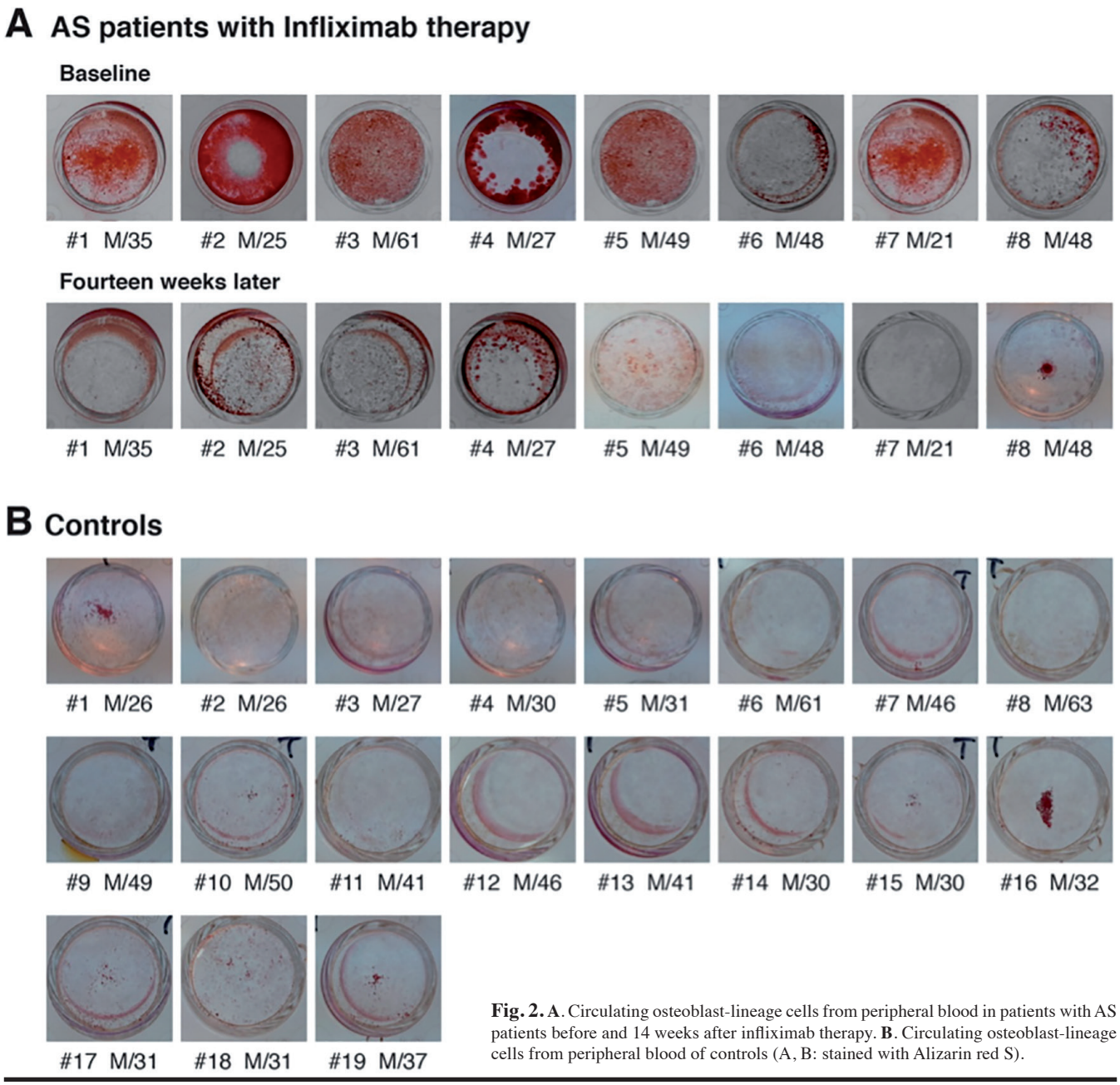


Fig. 2. A. Circulating osteoblast-lineage cells from peripheral blood in patients with AS patients before and 14 weeks after infliximab therapy. **B**. Circulating osteoblast-lineage cells from peripheral blood of controls (A, B: stained with Alizarin red S).

(p=0.0007), while serum P1NP (an early osteoblast differentiation marker) levels and Alizarin red S OD decreased (p=0.002, 0.028 respectively, Table I). Serum CTX-1 levels did not change after 14 weeks of infliximab therapy. The change of mean serum osteocalcin, PINP, CTX-1 levels were not different between smoker and non-smoker group.

Discussion

To our knowledge, this is the first study to clearly show that the circulating osteoblast-lineage cells are recoverable and increased in AS patients, and also that they decrease after infliximab therapy. Circulating osteoblast-lineage cells from the peripheral blood of patients may be a candidate for a new modality in studying spondylitis and other autoimmune disease. PBMCs are easier to obtain from patients with AS than bone marrow, so investigators can make many attempts with PBMCs to obtain results, that are unable from imaging or animal studies.

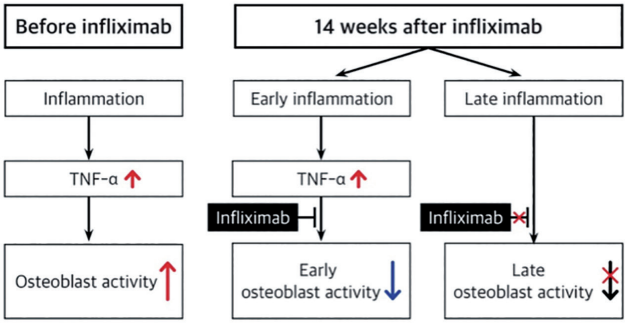
Circulating osteoblast-lineage cells were decreased after infliximab therapy in patients with AS. Serum P1NP levels and Alizarin red S OD were both higher in AS patients compared with controls, whereas both were significantly decreased following 14 weeks of infliximab therapy.

Among bone formation biomarkers, P1NP is a collagen synthesis by-product that is specific for osteoblasts. Type I collagen is produced in the early phase of bone formation. At this stage, the amino and carboxy propeptides of type I procollagen (P1NP and carboxy-terminal propeptide of type 1 procollagen) are discharged into the circulation (11). Osteocalcin is also a marker of bone formation. It is a bone matrix protein made by osteoblasts that is released at the mineralisation phase of bone formation. Osteocalcin is absorbed into

Table I. Comparison of bone biochemical markers at baseline and 14 weeks of infliximab therapy in patients with ankylosing spondylitis and controls.

	Controls (n=19)	AS: Before infliximab therapy (n=16)	AS: 14 weeks after baseline infliximab therapy (n=16)	p^*	p^{**}
P1NP, pg/mL	2914 (1574)	5303 (3255)	2824 (2144)	0.008	0.002
Osteocalcin, ng/mL	12 (4)	9 (3)	11.6 (4.5)	0.09	0.007
CTX-1, ng/mL	0.4 (0.2)	0.5 (0.3)	0.5 (0.3)	0.34	0.4
Alizarin red S OD in the cultures	29 (15)	1196 (791)	322 (290)	<0.0001	0.028

*p between Controls and 'AS: before infliximab therapy'. Mann-Whitney U test. **p between 'AS: before infliximab therapy' and 'AS: 14 weeks after baseline infliximab therapy. Wilcoxon rank sum test. Values for demographic characteristics are mean (SD) unless otherwise indicated. CTX-1: C-terminal telopeptide of type I collagen; P1NP: procollagen type 1 N-terminal propeptide; OD: optical density.



Circulating OB lineage cells	Alizarin S ↑	Alizarin S ↓	
Early OB differentiation	P1NP ↑	P1NP ↓	
Late OB differentiation	Osteocalcin		Osteocalcin ↑

Fig. 3. Effect of infliximab therapy on acute (baseline) *versus* mature (14weeks after) inflammatory lesions in patients with ankylosing spondylitis. TNF: tumour necrosis factor; OB: osteoblast; P1NP: procollagen type 1 N-terminal propeptide.

the bone matrix, and a small portion is present in the circulation both as intact molecules and as fragments.

If P1NP and osteocalcin were considered as markers of bone formation, it would be difficult to explain why P1NP was decreased, whereas osteocalcin was increased in AS patients after infliximab therapy. However, if P1NP is viewed as a marker of osteoblast proliferation (*i.e.* an early osteoblast differentiation marker) and osteocalcin as a marker for mineralisation (*i.e.* a late osteoblast differentiation marker), this result is consistent with data from MRI studies examining the relationship between inflammation and subsequent osteophyte formation in patients with AS (6). Using short tau inversion recovery (STIR) sequences, the investigators identified two different types of vertebral corner inflammatory lesions (CIL), an acute inflammatory (type A) CIL and an advanced (type B) CIL. The acute inflammatory type A lesion shows increased signal intensity on vertebral corner, while the advanced type B lesion has receded increase intensity on vertebral corner margin and increased signal intensity on rest of vertebral corner. New syndesmophytes formed more commonly from type B CIL measured with type A CIL or no CIL. They insisted that their results supports the hypothesis that new bone formation is more likely in advanced inflammatory lesions.

In untreated AS, the generally accepted sequence of events in pathogenesis is, inflammation, followed by osteoclast activation and bone erosion, followed by ossification (9). It is well established that TNF-α stimulates osteoclast differentiation. In contrast to major theory, van Duivenvoddrde *et al*. reported the relationship between inflammation, bone destruction, and osteoproliferation in the animal model of spondylitis (12). In mild inflammation, the inflammation began in the connective tissue at the intersection between the vertebra and the annulus fibrosis. There were no histologic evidences of bone destruction or bony proliferation. In moderately inflamed samples, they displayed the pannus-like tissue, enclosed many osteoclast-like cells with bone erosion in the vertebra outside the cartilage end plate. They also showed inflammatory signs in the bone marrow, together with new foci of hypertrophic chondrocytes and new bone formation, but outside the joint.

For the effects of TNF-α on osteoblasts, negative effects of TNF-α on osteoblast differentiation are well-known in several studies (13, 14). However, on the contrast, other human mesenchymal stem cell studies have shown that TNF-α can also stimulate osteogenic differentiation. Human experimental models reported an analogous osteogenic activity for TNF-α. In these models, osteogenic differentiation was enhanced via induction of bone morphogenetic protein-2, Osterix, Runx2, and osteocalcin (15,16).

On the evidence of our results, the study of van Duivenvoddrde *et al*. (12), and several other results, our hypothesis is as follows: before infliximab therapy in AS, inflammation is severe, then TNF-α would be increased, causing increased osteoblast activity (Fig. 3). This was supported by our results that Alizarin red S OD, representing circulating osteoblast-lineage cells, and serum level of P1NP as a marker of early osteoblast differentiation were higher than those of controls before infliximab therapy. In early inflammation, inflammation would be decreased and osteoblast differentiation would be also decreased after infliximab therapy. According to the study of Maksymowych *et al*. (6), the acute lesion resolved completely with TNF-α blocker therapy. Our study showed the similar results that Alizarin red S OD (marker of circulating OB

lineage cells) was decreased, and mean serum P1NP (marker of early osteoblast differentiation) was also decreased after infliximab therapy. However, in late inflammation, infliximab therapy could not alter osteoblast differentiation. The serum level of osteocalcin as a marker of late osteoblast differentiation was increased, not decreased, after infliximab therapy. To our knowledge, these results represent the first experimental evidence from patients supporting the sequence of events and effect of of anti-TNF therapy hypothesised from MRI studies (6) and histopathologic study (12).

How to explain the paradoxical effect of TNF-α in inhibiting or activating osteoblastogenesis? Osta *et al*. insisted that it depends on the differentiation stage of the responding cells (17). At the early stage of mesenchymal stem cells differentiation, TNF-α attaches its receptors and prefers osteogenic differentiation through activation of several signaling pathways, especially nuclear factor-kappa B (NF-kB) (15). However, TNF-α inhibits osteoblastogenesis by inducing DKK-1 expression, which inhibits Wnt pathway (18). Little is known about this topic, so details of the mechanism need clarification.

The strengths of this work are as follows: first, this is a human osteoblast-lineage cell study. Therefore, we could show direct effects on inflammation or osteoblast-lineage cell differentiation of infliximab therapy. Second, this osteoblast-lineage cell culture method with peripheral blood may be a candidate for a new modality in the study of spondylitis and other autoimmune diseases. Third, this model helps to explain the relationship between inflammation and osteoblast-lineage cell activity in patients with AS. However, a potential weakness of this study is the use of bulk cultures of the osteoblast-lineage cells together with other elements of peripheral blood. We attempted to culture PBMCs stained with anti-osteocalcin and anti-bone specific alkaline phosphatase antibody sorted by flow cytometry[8]. However, yields were very low (about 0.6%), and it was difficult to grow them to form multilayered colonies. Because of this technical difficulty, we decided to culture all PBMCs together. In the study of Eghbali-Fatourechi *et al*. (8), they compared the formation of mineralised nodules *in vitro* between the osteocalcin (+) and unsorted cells. The osteocalcin (+) cells formed more mineralised nodule, unsorted cells also could form mineralised nodule. Freshly sorted osteocalcin (+) cells, as compared unsorted cells were enriched for expression of the bone-related genes, but unsorted cells also could express the bone-related genes. They implanted osteocalcin (+), unsorted cells subcutaneously into immunocompromised mice. The osteocalcin (+) cells could form larger mineralised bone *in vivo*, but unsorted cells also could form mineralised bone *in vivo*.

Grcevic *et al*. (19) reported that the expression of osteoblast differentiation gene Runx2 was was decreased in patients with RA and increased in patients with AS in peripheral blood. This results are in accordance with our results that circulating osteoblast-lineage cells were increased in patients with AS than that of controls.

One more result is stated by Lim *et al*. (20). They reported that circulating osteoblast lineage cells in patients with rheumatoid arthritis (RA) were increased after TNF blockade. In RA, inflammation suppresses bone formation. The pro-inflammatory cytokine TNF-α plays a key role in actively suppressing bone formation. So, circulating osteoblast lineage cells in RA patients could be increased after after TNF blockade.

Moreover, Sieper *et al*. (9). reviewed that the inflammation in the axial skeleton of patients with AS was initially dominated by mononuclear cell infiltrates, with macrophages and T cells being the dominating cell types. As mononuclear cells could play a major role in the pathogenesis of AS, the whole cell culture of PBMC could show the somewhat similar results with those of anti-osteocalcin (+) anti-bone specific alkaline phosphatase (+) cells. Further work is needed to resolve this problem.

In summary, our study reveals first, TNF-α stimulates osteoblastogenesis before infliximab therapy in patients with AS; second, infliximab therapy resolves early inflammation, but allows mature osteoblast differentiation in late inflammation. The culture of osteoblast-lineage cells in peripheral blood may be a candidate for a new modality in the study of spondylitis and other autoimmune diseases.

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