Analysis of Mitogen-Activated Protein Kinases in Bone and Cartilage of Patients with Rheumatoid Arthritis Treated with Abatacept

Katsuaki Kanbe, Koei Oh, Junji Chiba, Yasuo Inoue, Masashi Taguchi and Akiko Yabuki
Department of Orthopaedic Surgery, Tokyo Women’s Medical University, Tokyo, Japan.

ABSTRACT: The aim of this study was to analyze the histological changes related to mitogen-activated protein (MAP) kinases in bone and cartilage treated with abatacept for rheumatoid arthritis (RA). A total of 20 patients of bone and cartilage were assessed: 10 abatacept with methotrexate (MTX)-treated RA patients were compared with 10 MTX-treated RA patients (control). The histology of bone and cartilage was observed by staining with hematoxylin and eosin and analyzed immunohistochemically for the expression of tumor necrosis factor-α, interleukin-6, CD4 (T cell), CD68 (macrophage), receptor activator of nuclear kappa-B ligand, osteoprotegerin, osteopontin, CD29 (β-1 integrin), phospho-p38 MAPK (Tyr180/Tyr182), phospho-p44/42 MAPK (extracellular signal-regulated kinase, ERK1/ERK2), and phosphor-c-Jun N-terminal kinase. The expressions of CD29 known as mechanoreceptor and ERK known as mechanotransduction signal protein in MAP kinases in the bone and cartilage of patients treated with abatacept were significantly different from those of control. These findings suggest that increases in CD29 and ERK in MAP kinases may change the metabolism of bone and cartilage in RA patients treated with abatacept.

KEYWORDS: abatacept, bone, cartilage, histology, MAPK, rheumatoid arthritis

Introduction
Abatacept has been reported to significantly inhibit the progression of structural damage to joints. Abatacept caused an ≈50% reduction in change from baseline in the Genant-modified Sharp scores compared with that of placebo at one year. In another study looking at early rheumatoid arthritis (RA), changes from baseline in the total Sharp score and erosion score were significantly lower in the abatacept group than in the control group. These changes were concomitant with mean changes in joint space narrowing scores, which were minimal and comparable between the two groups. However, there is no evidence in histological and experimental analyses regarding how abatacept changes bone and cartilage.

Abatacept is constructed genetically by fusing the external domain of human CTLA-4 and the Fc domain of human immunoglobulin G1, producing CTLA-4-Ig. Abatacept has proven efficacy in controlling the disease activity of RA. The mode of function of abatacept is the inhibition of the signal transduction of mechanical stress. Abatacept has been reported to significantly inhibit the cytokine generation, the metalloproteinase production, and the signal transduction of mechanical stress.

However, there are no studies to investigate the immunohistological findings of bone and cartilage regarding MAP kinases that differ for abatacept therapy versus MTX therapy in RA treatment. The signal transduction pathways in bone and cartilage could be elicited by abatacept treatment, if changing histological patterns of MAP kinases can be identified. Based on the hypothesis that abatacept treatment involves the induction of specific expression patterns in MAP kinases, we performed histological evaluation of 11 molecules, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, CD4, CD29, CD68, antihuman receptor activator of nuclear kappa-B ligand (RANKL), osteoprotegerin (OPG), osteopontin (OPN), as well as ERK, JNK, and p38 MAPK in the bone and cartilage of RA patients treated with...
abatacept. This preliminary report describes the findings related to histological differences in bone and cartilage during abatacept treatment.

**Methods**

The study protocol was approved by the Ethics Committee of Tokyo Women’s Medical University (No. 3433). All patients provided written informed consent to be included in this study. The research was conducted in accordance with the principles of the Declaration of Helsinki. A total of 10 patients (1 male and 9 females) underwent total knee arthroplasty (TKA) after the treatment of abatacept for an average of 12 months (10–18 months) in histological analyses of the bone and cartilage. The mean age of the patients in the abatacept group was 66.1 years (54–79 years), and the mean duration was 10 years (5–21 years). The average disease activity score in 28 joints (DAS28) of the abatacept group was 3.1 ± 0.6. Table 1 shows patients’ background at the histological examination of this study. RA Patients were categorized according to Steinbrocker criteria, 4 four patients were categorized as stage III and six as stage IV. Abatacept was applied for intravenous infusion at 500 mg for subjects with a body weight (BW) of <60 kg and 750 mg for those with BW of 60–100 kg on days 1, 14, and 28, and every month. Bone and cartilage samples were extracted at an almost similar site on the medial femoral condyle at TKA. None of the patients took disease-modifying antirheumatic drugs, had histories of hormone therapy, such as estrogen, and had treatment with bisphosphonates or parathyroid hormone.

Ten RA patients (2 males and 8 females) who were not treated with abatacept or other biologics were used as control samples. The mean age of control patients was 64.6 years (55–78 years). The average DAS28 of this control group was 3.2 ± 0.5. These subjects received 8.8 mg/week of MTX (8–16 mg) as well as 2.7 mg/day of prednisolone (2.5–10 mg) at the time of TKA. Patients were categorized according to Steinbrocker criteria, 8 three patients in the control were categorized as stage III and seven patients with RA were categorized as stage IV (Table 1). The patients in this study were diagnosed according to the American College of Rheumatology criteria. 9

For immunohistological analysis, 5-μm-thick serial paraffin sections of the bone and cartilage were stained with hematoxylin and eosin (HE). For immunostaining, the tissue sections were blocked for 10 minutes with phosphate-buffered saline plus 20% rabbit serum and then incubated overnight in a refrigerator at 4 °C with the following antibodies usually used in our laboratory. The experimental primary antibodies were described earlier, 6 such as anti-TNF-α mouse monoclonal antibody (1:1,000 dilution; Biogenesis), anti-human IL-6 rabbit polyclonal antibody (1:500, Rockland), anti-human CD4, anti-human CD68 mouse monoclonal antibody (1:1,000; DAKO), anti-human OPN mouse monoclonal antibody (1:250; Novoceastra), anti-human OPG, rabbit polyclonal antibody (1:200; Santa Cruz Biotechnology), anti-human RANKL (FL-317 rabbit polyclonal antibody (1:200; Santa Cruz Biotechnology), anti-human CD29 (beta-1 integrin) mouse monoclonal antibody (1:350; Novoceastra), anti-human phospho-p38 MAPK (Tyr180/Tyr182) mouse monoclonal antibody (1:500; Cell Signaling), anti-human phospho-p44/42 MAPK (Tyr202/Tyr204, ERK1/ERK2) mouse monoclonal antibody (1:500; Cell Signaling), and anti-human phospho-JNK (1:500; Santa Cruz Biotechnology). After treatment with secondary antibody, we compared the expression patterns of TNF-α, IL-6, CD4, CD68, RANKL, OPG, OPN, CD29, JNK, ERK, and p38 MAPK in the abatacept group with the control group according to the methods described earlier. 10

The immunohistologically stained samples were evaluated by the mean percentage of positive staining cells in three different areas in the same sample with high-power fields, at a magnification of 200× (Olympus, PM-C35DX). Statistical analyses were performed using the Mann–Whitney U-test between the two groups using the IBM SPSS Statistics 15 software program (International Business Machines Corp.), with a significant difference of P < 0.05.

**Results**

Cell proliferation was not significantly different in HE staining of the bone and cartilage with abatacept treatment compared to the subjects in the MTX control group (Figs. 1A–1D). The percentages of positive staining cells for the expression of TNF-α, IL-6, CD4, CD68, RANKL, OPG, and OPN in the bone were not significantly different between control and abatacept [mean (SD): 1.3(1.2), 12.5(4.8), 2.8(2.1), 2.1(1.5), 1.4(0.8), 1.8(1.1), and 1.1(0.5) vs. 1.5(2.3), 10.3(3.5), 3.5(1.6), 6.5(2.4), 1.3(0.5), 1.5(1.6), and 1.5(0.4)]. The percentages of cells stained positive for TNF-α, CD4, CD68, RANKL, OPG,
and OPN in cartilage were also not significantly different between control and abatacept except for IL-6 [mean (SD): 2.1(1.8), 1.5(2.3), 4.1(3.5), 1.8(1.7), 2.6(2.0), and 2.1(3.5) vs. 2.5(2.2), 2.8(2.7), 5.5(5.4), 1.2(1.1), 3.8(2.8), and 3.4(3.2); 3.5(2.4) vs. 25.1(7.5) for IL-6]. However, for the immunostaining of MAP kinases, there were significant differences in the expression of CD29 and ERK between control and abatacept with regard to bone and cartilage [mean (SD): 1.3(1.2) and 3.4(1.8) vs. 22(8.3) and 32.3(6.5), P = 0.026 and P = 0.014 in bone marrow; 2.8(2.6) and 5.2(4.7) vs. 34(12.1) and 43.1(15.8), P = 0.016 and P < 0.001 in cartilage] (Tables 2 and 3; Figs. 2A, B, E, and F and 3A, B, E, and F). Therefore, the expressions of CD29 known as mechanoreceptor and ERK known as mechanotransduction signal protein in MAP kinases in the bone and cartilage of patients treated with abatacept were significantly different from control. The patterns of JNK and p38 MAPK were expressed to almost no extent in bone and cartilage in both groups [mean (SD): 2.8(1.1) and 1.1(0.9) vs. 3.5(1.6) and 1.5(1.4) in bone marrow; 4.9(3.1) and 3.2(2.5) vs. 6.5(5.3) and 4.7(4.1) in cartilage] (Tables 2 and 3; Figs. 2C, D, G, and H and 3C, D, G, and H).

Discussion
Histopathological analyses have demonstrated a significant reduction in inflammation, bone and cartilage destruction, and pannus formation with abatacept in a rat model of collagen-induced arthritis. However, it is reported that in the analysis of synovium treated with abatacept, there is no clinical evidence regarding the relationship between abatacept administration and destruction with bone and cartilage. With respect to the function of abatacept, CTLA-4 (CD152) is a surface protein on T cells that negatively regulates the co-stimulation process between APCs and T cells. Co-stimulation has been reported to be the second essential signal for T-cell activation apart from antigen presentation through the T-cell receptor. The lack of co-stimulation does not allow T-cell activation but promotes the inverse reaction: T-cell anergy. CTLA-4 competes for the binding of CD28 on T cells with the co-stimulatory molecules CD80 and CD86 on APCs. Owing to more than tenfold higher affinity for CD80 and CD86, CTLA-4 disrupts the co-stimulation signal for T-cell activation. Thus, the administration of CTLA-4 affects various T cell-dependent models of autoimmune diseases in animals, such as experimental collagen-induced arthritis. CTLA-4 not only inhibits the signs and symptoms of human RA but also is utilized as a therapy for RA refractory to other disease-modifying drugs. However, clinical trials have shown that it also inhibits the progression of bone destruction. However, how the signal by CTLA-4 and CD80/86 translates to inhibit the binding of CD28 on T cells to inhibit the destruction of bone and cartilage is not known. We found that the ERK of MAP kinase was upregulated compared with control during the abatacept treatment. ERK is a gene associated with mechanical stress through the mechanical stress receptor CD29. CD29 was also upregulated in the present study, suggesting that abatacept may be associated with an increase in CD29 expression. This action may stimulate ERK to act upon a mechanical stress signal into the cells of the bone.
marrow and cartilage. The activation of a mechanotransduction cascade via ERK could stimulate bone formation or remodeling of the erosion observed in the destructive change of RA.

We found less expression of RANKL in both groups, suggesting that RANKL in bone and cartilage stimulates macrophages to initiate osteoclast formation. CTLA-4 has been reported to directly inhibit osteoclast formation in human peripheral blood mononuclear cells in vitro. Based on such laboratory data and the results of the present study, bone formation via mechanotransduction signals by MAPK, such as ERK, may be one of the key roles in the mechanism for abatacept treatment in patients with RA.

**Figure 2.** Immunohistochemical comparison of the expression of MAPK in bone (magnification, 200×; black arrow shows strong positive). (A) and (B): CD29 (β-1 integrin); (C) and (D): JNK; (E) and (F): ERK; (G) and (H): P38 MAPK. (A), (C), (E), and (G): control group (MTX); (B), (D), (F), and (H): abatacept group.
The present study had two main limitations: (i) small number of samples and (ii) only immunohistochemical examination was carried out without noting the mRNA level of MAP kinases. More experiments should be carried out to ascertain if ERK stimulation facilitates the remodeling or formation of bone to increase osteoblast function. However, the mechanism of the inhibition of progressive destruction of bones and joints by abatacept remains unclear. Future investigation of ERK in MAP kinases for other biological agents associated with the inhibition of bone destruction will be useful for RA treatment.

In summary, the results of our study enhance the understanding that increases in CD29 and ERK in MAP kinases...
may change the metabolism of bone and cartilage in RA patients treated with abatacept.

**Author Contributions**

Conceived and designed the experiments: KK. Analyzed the data: KK. Wrote the first draft of the manuscript: KK. Contributed to the writing of the manuscript: KK. Agree with the manuscript results and conclusions: KK, KO, JC, YI, MT, AY. Jointly developed the structure and arguments for the paper: KK. Made critical revisions and approved final version: KK. All authors reviewed and approved of the final manuscript.

**REFERENCES**

1. Kremer JM, Genont HK, Moroland LW, et al. Effects of abatacept in patients with methotrexate-resistant active rheumatoid arthritis: a randomized trial. *Ann Intern Med.* 2006;144:865–76.

2. Westhovens R, Robles M, Ximenes AC, et al. Clinical efficacy and safety of abatacept in methotrexate-naive patients with early rheumatoid arthritis and poor prognostic factors. *Ann Rheum Dis.* 2009;68:1870–7.

3. Kremer JM, Westhovens R, Leon M, et al. Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. *N Engl J Med.* 2003;349:1907–15.

4. Moreland L, Bate G, Kirkpatrick P. Abatacept. *Nat Rev Drug Discov.* 2006;5:185–6.

5. Kumar S, Boehm J, Lee JC, p38 MAP kinases: key signaling molecules as therapeutic targets for inflammatory diseases. *Nat Rev Drug Discov.* 2003;2:717–26.

6. Kanbe K, Chen Q, Nakamura A, Hobo K. Inhibition of MAP kinase in synovium by treatment with tocilizumab in rheumatoid arthritis. *Clin Rheumatol.* 2011;30:1407–13.

7. Van der Heijde DM, Van’t Hof MA, Van Riel PL, et al. Judging disease activity in clinical practice in rheumatoid arthritis: first step in the development of a disease activity score. *Ann Rheum Dis.* 1990;49:916–20.

8. Steinbrocker O, Traeger CH, Battman RC. Therapeutic criteria in rheumatoid arthritis. *JAMA.* 1949;140:659–62.

9. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* 1988;31:315–24.

10. Farahat MN, Yanni G, Poston R, Panayi GS. Cytokine expression in synovial membranes of patients with rheumatoid arthritis and osteoarthritis. *Ann Rheum Dis.* 1993;52:870–5.

11. Kliwinski C, Kukral D, Postelnek J, et al. Prophylactic administration of abatacept prevents disease and bone destruction in a rat model of collagen-induced arthritis. *J Autoimmun.* 2005;25:165–71.

12. Kanbe K, Chiba J, Nakamura A. Immunohistological analysis of synovium treated with abatacept in rheumatoid arthritis. *Rheumatol Int.* 2013;33:1883–7.

13. Brunet JF, Denizot F, Luciani MF, et al. A new member of the immunoglobulin superfamily-CTLA-4. *Nature.* 1987;328:267–70.

14. Schwartz JC, Zhang X, Nathenson SG, Almo SC. Structural mechanisms of costimulation. *Nat Immunol.* 2002;3:427–34.

15. Knoerzer DB, Karr RW, Schwartz BD, Mengle-Gaw LJ. Collagen-induced arthritis in the B rat. Prevention of disease by treatment with CTLA-4-Ig. *J Clin Invest.* 1995;96:987–93.

16. Axmann R, Herman S, Zais M, et al. CTLA-4 directly inhibits osteoclast formation. *Ann Rheum Dis.* 2008;67:1603–9.