Requirement of Methotrexate in Combination with Anti-Tumor Necrosis Factor-α Therapy for Adequate Suppression of Osteoclastogenesis in Rheumatoid Arthritis

HIROAKI MATSUNO, KAORU YOSHIDA, AKIRA OCHIAI, and MASAHIRO OKAMOTO

ABSTRACT. ***Objective.*** To determine that concomitant use of methotrexate (MTX) is required to achieve adequate suppression of bone destruction in treating rheumatoid arthritis (RA) with tumor necrosis factor-α (TNF-α)-inhibiting biologic therapy. We quantitatively compared the suppressive effects of treatment with a combination of infliximab and MTX and treatment with each of these 2 agents alone on bone destruction in SCID-HuRAg-pit mice.

Methods. Tissue derived from human RA pannus was implanted with a slice of dentin subcutaneously in the backs of SCID mice (SCID-HuRAg-pit model). Infliximab was administered daily to SCID-HuRAg-pit mice using an osmotic pump for 2 weeks with or without oral administration of MTX. Histological changes in tissue and the pits formed on the dentin slice were examined 8 weeks after transplant. Serum concentrations of TNF-α and interleukin 6 (IL-6) were also measured.

Results. Treatment with a combination of infliximab and MTX suppressed pit formation significantly, while treatment with neither infliximab alone nor MTX alone had a significant effect on pit formation. Synovial inflammation and serum TNF-α and IL-6 levels were suppressed by infliximab with or without MTX.

Conclusion. This is the first evidence in an animal model of arthritis that concomitant use of MTX is required to achieve adequate suppression of bone destruction when treating RA with a TNF-α-inhibiting biologic. Our findings suggest that infliximab suppresses bone destruction through a mechanism of action different from that mediating its antiinflammatory effects in the treatment of RA. (First Release Nov 15 2007; J Rheumatol 2007;34:2326–33)

Key Indexing Terms:
RHEUMATOID ARTHRITIS
TUMOR NECROSIS FACTOR
OSTEOCLASTOGENESIS
SCID MOUSE

Rheumatoid arthritis (RA) is an autoimmune disease characterized by proliferation of inflammatory synovial cells accompanied by infiltration of inflammatory cells and bone destruction[1,2]. In RA, several inflammatory cytokines [tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1), and IL-6] produced in synovial tissue cause imbalance of bone homeostasis resulting in cartilage destruction and osteoporosis[3]. These inflammatory cytokines induce the production of matrix metalloproteinase (MMP), accumulation of T cells, and metabolism of cartilage, and also differentiation of monocytes into osteoclasts[4,5]. Osteoclasts, which play a principal role in bone resorption of RA-affected areas, are multinucleated cells formed by fusion of mononucleated precursor cells[6]. Osteoclast precursors, which express the receptor activator of nuclear factor-κB (RANK)[7-9], recognize the RANK ligand (RANKL)[10] on osteoblasts or stromal cells, and these cell to cell interactions induce differentiation of osteoclast precursors into osteoclasts in the presence of macrophage colony-stimulating factor (M-CSF)[11,12]. The inflammatory cytokines produced in synovial tissue are thought to play important roles in each step of differentiation of osteoclast precursors into osteoclasts[3].

Among these inflammatory cytokines, TNF-α has been reported to play a central role in increase in disease activity of RA as reflected in laboratory test data (e.g., C-reactive protein levels and erythrocyte sedimentation rates) and synovitis, and to play an important role in the progression of bone destruction in patients with RA through its involvement in the differentiation of osteoclast precursor cells into osteoclasts[3,13,14]. As TNF-α-targeting drugs for the treatment of RA, 3 biolog-

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ics (infliximab, etanercept, and adalimumab) have been commercialized and prescribed extensively[15-19]. The excellent efficacy of these agents is widely recognized. Regarding the efficacy of these 3 biologics in improving laboratory test data and suppressing the activity of RA, clinical trial data and postmarketing clinical data have described suppressive effects several weeks after initiation of treatment. However, their efficacy in suppressing bone destruction has not been adequately documented, for several reasons, including (1) the long period of time (at least 2 years) required for observation of suppressive effect on bone destruction; and (2) lack of an experimental model to demonstrate suppressive effects through TNF-α neutralization.

In our laboratory, a SCID-HuRAg (severe combined immunodeficiency) animal model reproducing synovitis in RA-affected joints has been developed[20-22]. With this model, we have evaluated the suppressive effects of infliximab on synovitis and bone erosion[23]. These studies revealed that infliximab improved inflammatory changes in synovial tissue (derived from patients with RA and including some bone tissue as well) implanted subcutaneously into SCID mice, and reduced numbers of infiltrating cells by stimulating apoptosis. However, contrary to our expectations, no significant effect of infliximab on bone erosion was observed in this model. On the other hand, the recently reported ASPIRE study[24], a 54-week clinical study of infliximab in patients with early RA (within 3 years of disease onset), demonstrated that the combination of infliximab with MTX suppressed synovial inflammation and bone destruction more strongly than MTX alone. To verify the effectiveness of the combination therapy, it is essential to develop a system permitting more quantitative evaluation of bone destruction, since the current SCID-HuRAg model allows only qualitative evaluation of bone destruction. We recently modified the SCID-HuRAg model and developed a SCID-HuRAg-pit model, an animal model allowing quantitative evaluation of bone erosion[25]. We have used this new model for evaluation of drugs for the treatment of RA. The present study was undertaken to compare the effects on synovitis and bone erosion among treatment with a combination of infliximab with MTX and treatment with either one of these 2 agents alone, using this SCID-HuRAg-pit model, to obtain data confirming that infliximab must be used in combination with MTX to achieve sufficient suppression of the bone destruction associated with RA.

MATERIALS AND METHODS

Animals. Male CB17/Icr SCID mice, aged 4–5 weeks, were purchased from Charles River Japan (Yokohama, Japan). The mice were acclimated for at least 1 week in our university facilities and were used at the age of 6 to 7 weeks. Mice were housed in specific pathogen-free facilities, with water and food provided freely. A total of 18 SCID mice were used for this study. All SCID mice were sacrificed for pathological analysis prior to 15 weeks of age. Bosma, *et al*[26] showed that the chance of development of leaky SCID is small (< 5%) under 9 months of age. All animal experiments were performed under the ethical code for experimental animals of the center for experimental animals at Toin University of Yokohama.

Preparation of SCID-HuRAg-pit mice. Synovial tissues obtained during synovectomy or total joint replacement surgery in 4 RA patients [Disease Activity Score (DAS28) < 3.2] were used for implantation. Informed consent was obtained from all 4 patients. A slice of dentin (Kureha Special Laboratory, Fukushima, Japan) was covered with RA synovium and grafted subcutaneously on the backs of SCID mice that had been anesthetized by inhalation of ethyl ether as described[25]. The weight of the synovium for transplantation was adjusted to 0.5 g (± 0.05 g). Each animal had an osmotic pump surgically inserted (Alza, Palo Alto, CA, USA) on its back. All surgical procedures were performed under sterile conditions.

Protocol for infliximab treatment. SCID-HuRAg-pit mice were randomly divided into 4 groups. The treatment protocol for anti-TNF antibody and MTX with SCID-HuRAg-pit mice was performed as described[23]. Initial treatments were started 4 weeks after implantation, when it was observed that the implanted tissue was accepted by the SCID-HuRAg mouse[20-23]. Infliximab (cA2; Centocor, Malvern, PA, USA) or human IgG (BioPur, Bubendorf, Switzerland) was administered daily using osmotic pumps (0.6 μg/h, for 2 wks, total 0.2 mg). MTX (0.3 mg/kg/day) or vehicle alone, 0.5% carboxymethylcellulose (CMC) as a control, was administered orally for 2 weeks. Group 1 was treated with human IgG and CMC. Group 2 was treated with human IgG and MTX. Group 3 was treated with infliximab and CMC. Group 4 was treated with infliximab plus MTX.

Measurement of resorption area on dentin slice. Mice were euthanized 4 weeks after the initial treatment, and the implanted tissues and dentin slices were removed. The dentin slices were fixed with 4% paraformaldehyde, and resorption pits were stained with acid hematoxylin (Sigma, St. Louis, MO, USA). Percentage of acid hematoxylin-stained area in 0.1 mm areas of a grid was analyzed by ImageJ 1.37. Ten 0.1 mm areas were analyzed to determine the percentage of the resorption area on the dentin slice from each individual mouse.

Histological assessment. After the implanted tissue was removed, it was fixed in 4% paraformaldehyde. Each tissue sample was cut into 6 μm sections, and stained with hematoxylin and eosin (H&E) at room temperature. The degree of synovial inflammation was assessed as described in our own previous study, Koizumi's method[27], and other reports, including Rooney's method[28]. Briefly, the characteristic histopathological features of RA synovitis were summarized as 7 items (synovial cells, palisading, giant cells, lymphocytes, plasma cells, granulation tissue, fibrin) using Koizumi's method. Then, in order to evaluate the histological severity of these 7 items, scores from 1 to 3 points were assigned for each item and total scores were calculated. In Rooney's method, each of 6 histological features (synovial hyperplasia, fibrosis, blood vessels, perivascularis, lymphoid follicles, diffuse infiltrates of lymphocytes) was scored separately on a scale of 0–10.

Measurement of serum cytokines. Mouse blood was obtained by cardiac puncture on the day of euthanasia (8 weeks after implantation). Cytokines (TNF-α, IL-6) were measured using a sandwich ELISA according to the manufacturer's instructions (Gibco BRL, Grand Island, NY, USA).

Statistical analysis. Results are expressed as means ± SEM (standard error of mean). Statistical analysis was carried out with Dunnett's t-test or Tukey test using Exsas v.7.1.6. P values less than 0.05 were considered significant.

RESULTS

Characteristics of implanted tissue following treatment with the combination of infliximab with MTX and treatment with MTX or infliximab alone. Figure 1 compares the wet weight of tissue before implantation and 8 weeks after implantation in each group. In the control group treated with human IgG and drug-free vehicle, the wet weight of tissue remained unchanged 8 weeks after implantation, consistent with a previous report[25]. There were no significant intergroup differences in wet weight of tissue 8 weeks after implantation. The external appearance of the tissue also exhibited no change

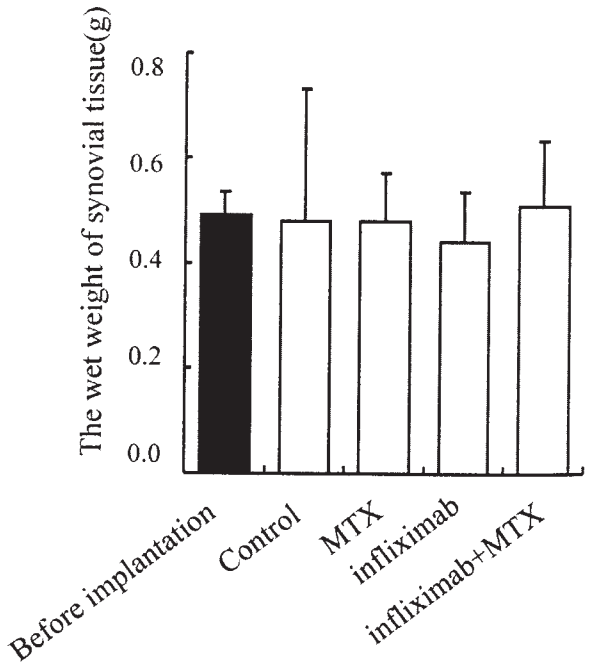

Figure 1. No reduction observed in wet weight of grafted synovial tissue in SCID-HuRAg-pit mice. There were no significant differences among the treatment groups.

(data not shown). In our previous study, we confirmed successful implantation of human rheumatoid synovium and the retention of the features of RA at 8 weeks after implantation by pathological observation[22]. In this study, the results of pathologic examination of the control group were consistent with the result in our previous study (Figures 2A and 3). In the group treated with infliximab alone and that treated with combination infliximab and MTX, on the other hand, replacement of implanted synovial tissue by fat tissue or fibrous tissue was marked (Figures 2C, 2D, and 3). No histological difference was noted between the infliximab-treated group and the infliximab + MTX-treated group (Figures 2C, 2D, and 3).

Serum TNF-α and IL-6 levels. As shown in Figure 4, we observed significant elevation of serum levels of human TNF-α (34.5 ± 18.4 pg/ml) and IL-6 (725 ± 1100 pg/ml) in the control group. This finding suggests that these cytokines were produced in the implanted synovial tissue, since serum levels of human TNF-α and IL-6 were below the limit of detection in SCID mice without implantation. In the infliximab-treated group (7.5 ± 9.0 pg/ml) and the infliximab + MTX-treated group (3.0 ± 0.6 pg/ml), serum TNF-α level was significantly lower than that in the control group (Figure 4A). In terms of serum IL-6 level, there was no statistically significant intergroup difference, but the level was evidently lower in the infliximab-treated group (75 ± 50 pg/ml) and the infliximab +

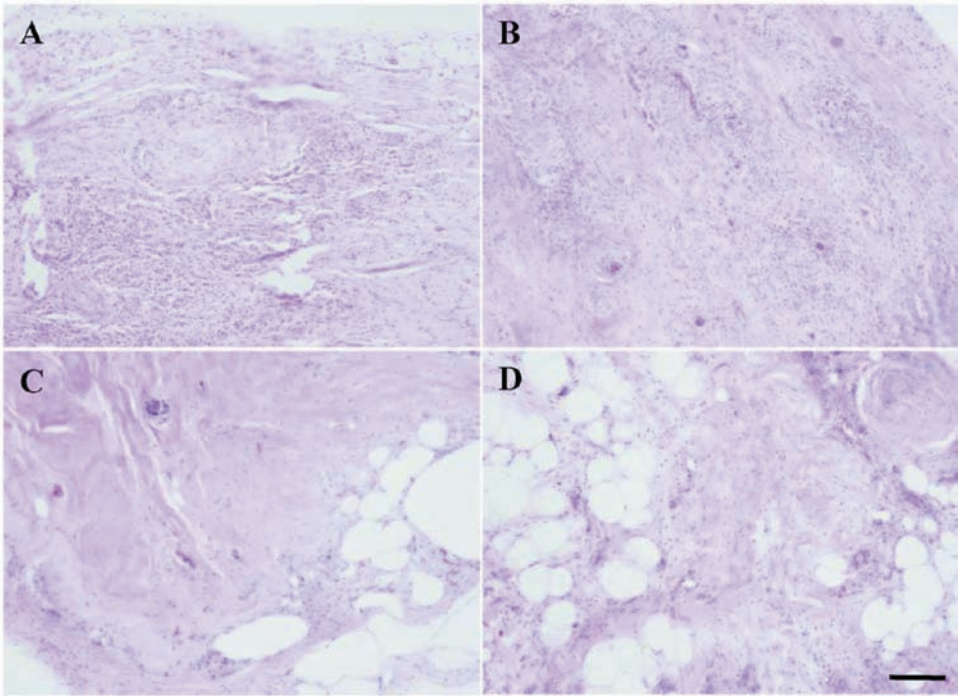

Figure 2. Light-microscopic features in each treatment group. Between the control group (A) and MTX-treated group (B), no clear differences in pathological features of implanted tissue were observed after treatment. In contrast, in the infliximab-treated group (C) and infliximab + MTX-treated group (D), numbers of inflammatory cells were markedly decreased, and inflammatory regions had been replaced by fibrous and adipose tissue. Scale bar = 200 μm.

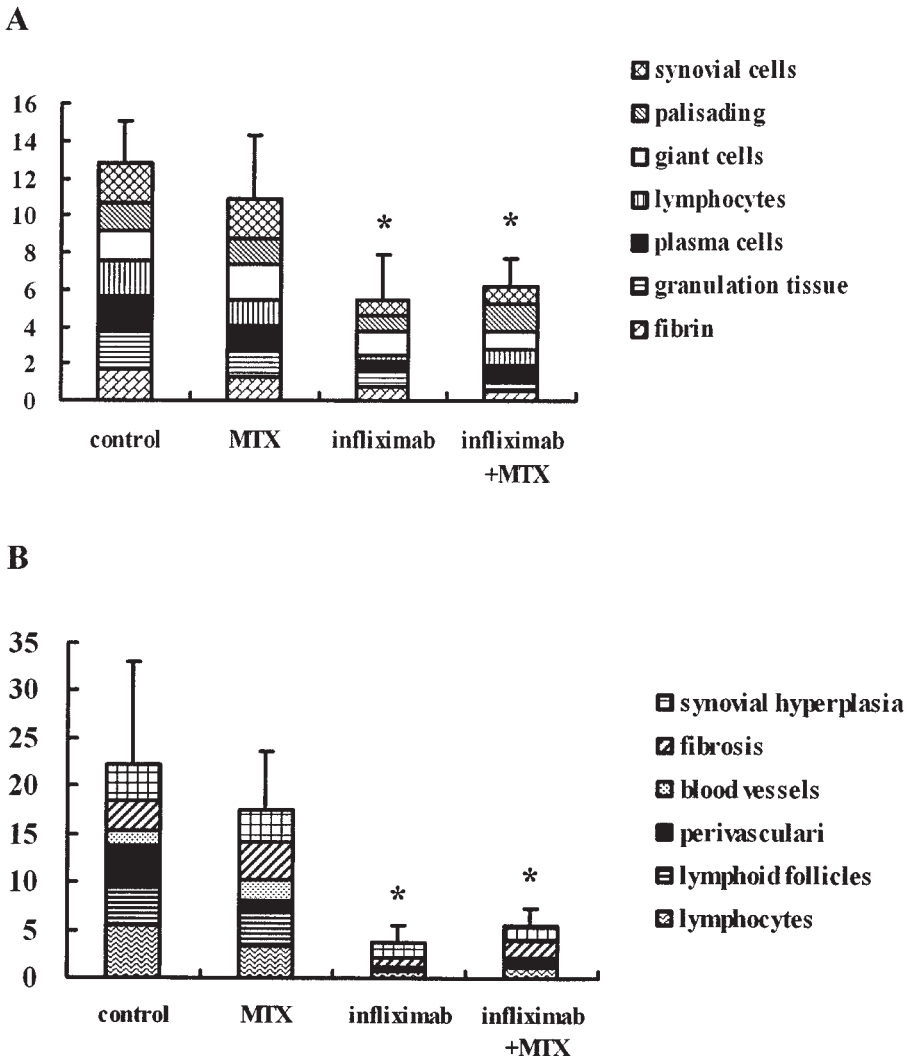

Figure 3. Histological findings were divided into individual findings, each assessed using an arbitrary score on a scale of 0–10 (A) or 0–3 (B), where the maximum value (10 or 3) indicates histological findings for the control group and 0 indicates no such findings were detectable. Four of 5 SCID-HuRAg-pit mice were used for each treatment group.

MTX-treated group (100 ± 75 pg/ml) than in the control group (Figure 4B). These results were consistent with a report that showed TNF-α played an essential part in IL-6 production in RA synovium[23].

Effects of treatment with a combination of infliximab and MTX on osteoclastogenesis in SCID-HuRAg-pit mice. Histological evaluation (Figures 2 and 3) revealed the change of implanted tissue into noninflammatory tissue in the infliximab-treated group and the infliximab + MTX group. Further, production of inflammatory cytokines in implanted tissue was suppressed by treatment with infliximab even when infliximab was used alone, without MTX. These findings suggest that combination with MTX is not required for infliximab to suppress inflammation of synovial tissue. However, when effects on osteoclastogenesis were evaluated, based on resorption area on the dentin slices (implanted together with synovial tissue), only the infliximab + MTX-treated group exhibited a significant reduction in resorption area compared to the control group (Figure 5). Figure 6 shows micrographs of the dentin slices stained with acid hematoxylin following removal 8 weeks after implantation from each group of mice. The dentin slices from the infliximab + MTX-treated group exhibited markedly less pit formation.

DISCUSSION

In our study using a SCID-HuRAg-pit model, inflammatory changes (synovitis, production of inflammatory cytokines, etc.) were suppressed both by treatment with infliximab alone and by treatment with a combination of infliximab with MTX, while osteoclastogenesis was suppressed only by treatment with the combination of the 2 agents. Our study thus yields the first results in an animal model confirming that infliximab

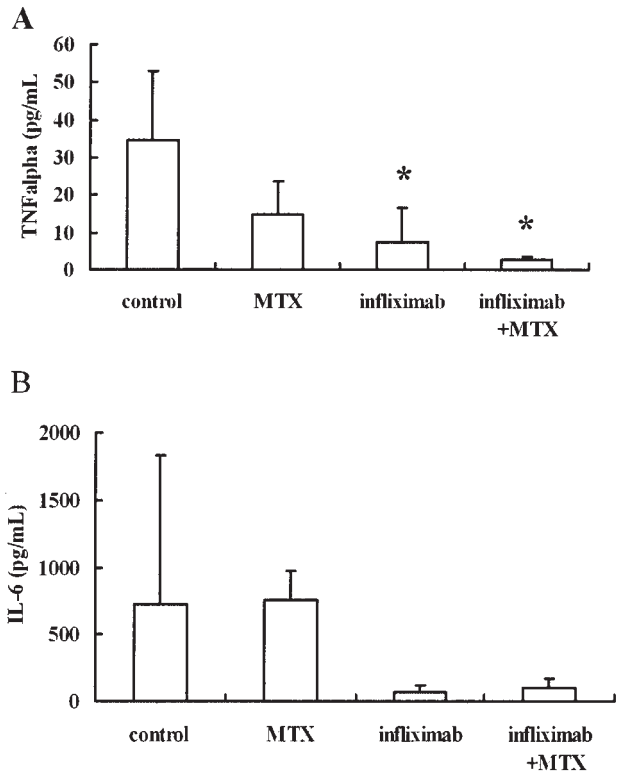

Figure 4. Levels of human TNF-α and IL-6 in sera were decreased in the infliximab-treated group. Serum levels of TNF-α (A) were significantly decreased in the infliximab-treated group and infliximab + MTX-treated group compared with control. *Significant difference ($p < 0.05$) compared with control.

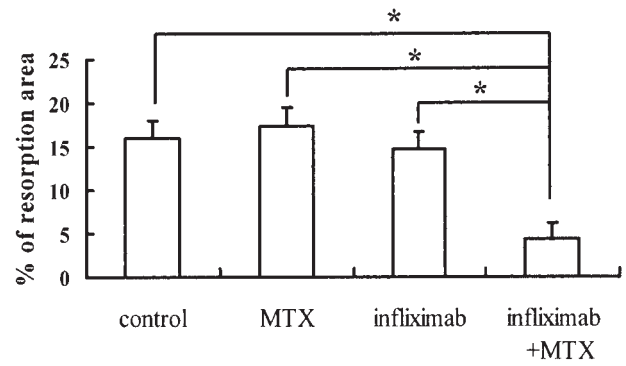

Figure 5. Suppressive effect of treatment with the combination of infliximab + MTX on bone resorption in SCID-HuRAg-pit mice. The resorption area on the dentin slices was significantly reduced by treatment with the combination of infliximab with MTX compared to control. Significant difference was observed between the MTX-treated group and infliximab + MTX-treated group. *Significant difference ($p < 0.05$) between 2 groups.

needs to be used in combination with MTX to sufficiently suppress the bone destruction associated with clinical RA. This finding confirms the importance of treatment with a combination of a TNF-inhibiting biologic and MTX as a means of treatment for RA, with the primary goal of improving patient quality of life.

There were differences between the results using an animal model and clinical results. In evaluation of this model, treatment with infliximab alone did not suppress osteoclastogenesis. In clinical studies, i.e., the TEMPO study[29], involving 2-year evaluation of etanercept, and the PREMIER study[19], involving 2-year clinical evaluation of adalimumab, significant suppression of bone destruction was noted following treatment with a TNF-α-inhibiting biologic alone or MTX alone, although the efficacy achieved was clearly less marked than that with combined use of a TNF-α-inhibiting biologic and MTX. This discrepancy might be explainable as follows. TNF-α appears to exert its efficacy against RA-associated bone destruction through 2 effects: (1) an indirect effect on osteoclastogenesis through enhancement of cell accumulation in synovial tissue[5], production of inflammatory cytokines, etc.[30-32]; and (2) direct involvement in the differentiation of osteoclast precursor cells into osteoclasts and activation of osteoclasts[3,33-35]. Of the steps in osteoclastogenesis noted in RA-affected joints, that of cell accumulation in synovial tissue is skipped in the SCID-HuRAg-pit model used in our study. Treatment with TNF-α-inhibiting biologics alone suppressed cell infiltration of synovial tissue, resulting in indirect suppression of osteoclastogenesis in clinical RA. Since osteoclasts, which play a central role in the bone destruction associated with RA, can also differentiate from mononucleated cells invading synovial tissue, the effect of suppression of cell invasion on osteogenesis could be important.

In this study, osteoclastogenesis was not affected by treatment with infliximab alone or with MTX alone, but was markedly suppressed by treatment with the combination of infliximab and MTX. This finding suggests that, so far as differentiation and activation of osteoclasts in RA-affected synovial tissue is concerned, the target suppressed by TNF-α and that suppressed by MTX both play important roles, although there are multiple routes of differentiation and activation, which require both of these targets and which are coordinated with each other in a complex way[34,36-38]. Lee, *et al* reported that when RA-affected synovial cells and mononucleated cells from peripheral blood were incubated in the presence of both 1,25-dihydroxyvitamin D3 and M-CSF *in vitro*, osteogenesis was suppressed by MTX, salazosulfapyridine (SSZ), IL-4, and infliximab. According to that report, these DMARD and IL-4 suppressed osteoclastogenesis in a dose-dependent manner when used alone. Further, in a study of the involvement of these drugs in the expression of RANK, RANKL, and osteoprotegerin mRNA and proteins, infliximab suppressed the expression of RANK and RANKL, while MTX and SSZ each suppressed expression of RANKL alone. It thus appeared that the target in suppression of osteoclastogenesis differs between TNF inhibitors and MTX or SSZ. We are unable to explain why our results differ from those of the *in vitro* experiment noted above, based only on findings currently available. However, peripheral blood mononucleated cells (PBMC) were used as a definite source of osteoclast precursor cells in the *in vitro* experiment conducted by Lee, *et al*, whereas the

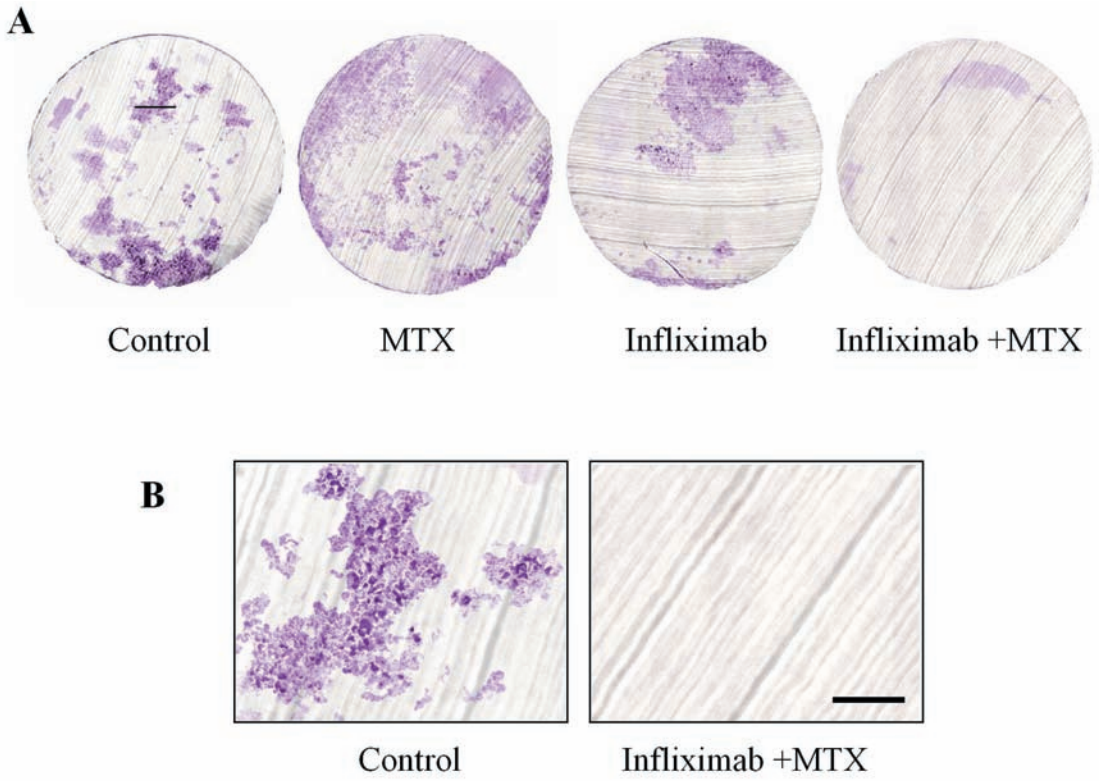

Figure 6. Reduction in number of resorption pits formed on dentin slices in SCID-HuRAg-pit mice of the infliximab + MTX-treated group (A). Implanted dentin slices from mice in the control group and the infliximab + MTX-treated group (B). The number of resorption pits was reduced by treatment with a combination of infliximab and MTX, compared with the control group. Scale bar = 500 μm.

SCID-HuRAg-pit model we used includes multiple types of cells (in addition to PBMC) able to serve as osteoclast precursor cells, since whole synovial tissue derived from RA patients was implanted into the mice. It thus appears that the discrepancy in findings between the 2 studies does not contradict the hypothesis that there are at least 2 routes involved in osteoclastogenesis in RA-affected synovial tissue, which are strongly affected by TNF-α inhibition and MTX. Further, the similarities between the pathological features of implanted tissue and clinical findings regarding drug efficacy suggest that the SCID-HuRAg-pit model reflects the osteoclastogenesis that occurs clinically more faithfully than the *in vitro* experimental system.

In conclusion, the inhibitory effects of monotherapy with a TNF inhibitor on osteoclastogenesis observed in clinical study could not be detected in the SCID-HuRAg-pit model. One possible reason for this result is that the process of permeation of cells from the bloodstream, which may be a target of monotherapy with a TNF inhibitor, was not adequately reconstructed in this model. Another is that the infiltrating "naive" blood monocytes, which may be a therapeutic target of monotherapy with a TNF inhibitor, did not exist in sufficient numbers in the implanted synovial tissues.

Our study revealed that the use of TNF-inhibiting biologics in combination with MTX plays an important role not only in suppression of the onset of human antichimeric antibodies (HACA) but also in suppression of bone destruction. Several studies have demonstrated possible mechanisms of action of MTX in RA, including inhibition of biosynthesis of purines or pyrimidines[39] and pooling of adenosine through inhibition of its metabolism[40,41]. However, the target of MTX in suppressing osteoclastogenesis remains unclear. Among the TNF-inhibiting biologics available, infliximab requires concomitant use of MTX to suppress the onset of HACA. Etanercept and adalimumab are also expected to suppress bone destruction powerfully when used in combination with SSZ, since SSZ has a mechanism of action similar to that of MTX[42,43] and is known to suppress bone destruction even when used alone[44,45]. This alternative combination therapy is thus promising as a means of suppressing bone destruction in cases of RA in which MTX cannot be used due to side effects or does not yield adequate responses. Further, some investigators have reported that potent effects in suppressing bone destruction were observed in patients with RA treated with a combination of MTX and drugs other than TNF-inhibiting biologics (e.g., bucillamine, a DMARD with a mechanism of action different from that of MTX)[46]. The SCID-HuRAg-pit mouse used in our study is a useful model for evaluation of the efficacy of these various combinations of drugs in suppressing the bone destruction associated with RA.

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