Co., Ltd. (Shiga, Japan), and all other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Cell culture

RSFs were prepared from synovial tissue as described previously (12). RA tissue specimens were obtained from patients undergoing total knee replacement who fulfilled the revised criteria (13) for the classification of RA. The protocol for this study was approved by the Toho University Ethics Committee (approval number: 19021), and all patients gave written consent to the use of their tissue for the research. Synovial tissue was digested for 2 hours with 0.25% (weight/volume) bacterial collagenase (ImmunoBiological Laboratories, Gunma, Japan) and then was suspended in RPMI 1640 medium with 10% (v/v) FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The cells were incubated at 37°C under 5% CO_2 for several days, after which nonadherent cells were removed. Fibroblast-like adherent cells from the third or fourth passages were used as RSFs at a concentration of 2.5×10^6 cells/75 cm^2 flask.

Reverse transcription – polymerase chain reaction (RT-PCR)

Cells were seeded in culture medium containing 10% (v/v) FBS, and total RNA was extracted with an RNeasy mini kit (Qiagen GmbH., Hilden, Germany), in accordance with the manufacturer's instructions. Reverse transcription was performed with a SuperScript first-strand synthesis system for RT-PCR (Invitrogen Corp.) was employed according to the manufacturer's instructions, using 2 μg of total RNA from the cells as a template. Equal amounts of each reverse-transcribed product were amplified by PCR with HotStar Taq polymerase (Qiagen GmbH). The primer sequences and number of cycles were 5'-TTCAGGTGCGCTGTAAGAGGCT (sense) and 5'-AGGCTCCAAAAGAAGAGGACCACA (antisense) with 38 cycles for Ob-Rb (920 bp), 5'-TCCCATATCTGAGCCCAAAG and 5'-CATCAGGGGCTTCCAAAGTA with 32 cycles for Ob-Re (565 bp), and 5'-CCTCGCCTTTGCCGATCC and 5'-GGATCTTCATGAGGTAGTCAGTC with 28 cycles for β-actin (626 bp). After initial denaturation for 15 minutes at 95°C, PCR involved amplification for a variable number of cycles of 30 seconds at 95°C (β-actin and Ob-Re) or 94°C (Ob-Rb), 30 seconds at 56°C (β-actin) or 59°C (Ob-Re) or 55°C (Ob-Rb), and 45 seconds (β-actin and Ob-Re) or 30 seconds (Ob-Rb) at 72°C, followed by elongation for 5 minutes at 72°C. The amplified complementary DNA (cDNA) fragments were resolved by electrophoresis on 2% (w/v) agarose gel, and were detected under ultraviolet light using an LAS-3000 (Fujifilm Corp. Tokyo, Japan) after the gel was stained with ethidium bromide.

Real-time PCR

To semi-quantitatively evaluate the expression of messenger RNA (mRNA) for IL-6, IL-1β, and TNF-α, real-time PCR was performed using real-time TaqMan technology with a Sequence Detection System model 7000 according to the manufacturer's recommendations (Applied Biosystems, Foster City, CA, USA). Cells were cultured under various conditions in medium containing 1% (v/v) FBS, after which extraction of total RNA and synthesis of cDNA were performed as described above. Specific probes for IL-6, IL-1β, and TNF-α were obtained from TaqMan Gene Expression Assay (Applied Biosystems), with the ID numbers of the products being Hs99999032_m1 for IL-6, Hs99999029_m1 for IL-1β and Hs00174128_m1 for TNF-α. The threshold cycle was calculated from a standard curve and expression of the target mRNA was normalised for the expression of β-actin mRNA.

Western blot analysis

Cells were cultured under various conditions at a density of $5\times10^4/cm^2$ in medium containing 1% (v/v) FBS. Subsequently, the cells were lysed in mammalian protein extraction reagent containing Halt™ phosphatase inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA). The protein content of the lysates was determined with bicinchoninic acid protein assay reagent (Pierce Biotechnology), using bovine serum albumin as the standard. Then cell lysates were adjusted to 10 μg of protein and were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel (10–15% [w/v]) electrophoresis. Next, the proteins were electroblotted onto Immobilon-P poly (vinylidene difluoride) membranes with a semidry blotter (Atto Corp., Tokyo, Japan). After the membranes had been blocked in 10 mM Tris-buffered saline (TBS) containing 0.1% (v/v) Tween 20 (TBST) and 5% (w/v) skim milk, the primary antibody (anti-human STAT3 antibody or anti-human phospho-STAT3 antibody) was added at a dilution of 1:1000 in TBST, and incubation was done for 18 hours at 4°C. After the membranes had been washed with TBST, the secondary antibody (HRP-conjugated goat anti-rabbit antibody) was added at a dilution of 1:10,000 in TBST and incubation was performed for 1 hour. After further washing with TBST, protein bands were detected with an enhanced ECL Western blotting detection reagent (GE Healthcare UK Ltd.) using LAS-3000 (Fujifilm Corp.).

Measurement of cytokines in the culture medium

Cells were seeded in 24-well plastic plates (1×10^5/well) and cultured for 24 hours under various conditions in medium containing 1% (v/v) FBS under an atmosphere of 5% CO_2. Then the concentrations of IL-6, IL-1β, and TNF-α in the medium were measured with an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's recommendations (Invitrogen Corp.). Experiments using RSFs were done in triplicate wells, and the concentrations of IL-6, IL-1β, and TNF-α was measured in triplicate.

RNA interference assay with Ob-Rb

An RNA interference assay was performed to assess the effect on RSFs of down-regulating Ob-Rb expression. Small interfering RNA (siRNA) for Ob-Rb (Stealth™ RNAi) and negative control siRNA were purchased from Invitrogen Corp. For gene knockdown

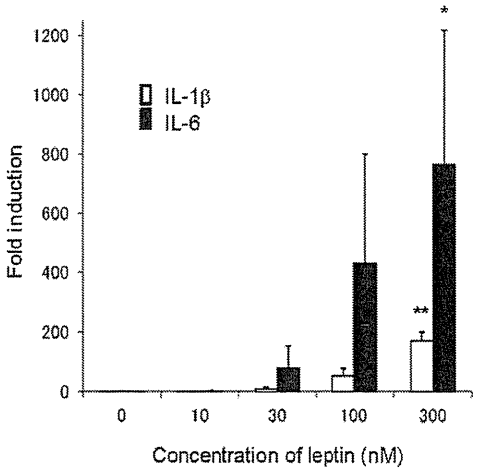

Fig. 1. Effect of leptin on mRNA expressions of inflammatory cytokines by RSFs. Rheumatoid synovial fibroblasts (RSFs) were incubated with leptin for 6 hours and the expression of interleukin (IL)-1β and IL-6 mRNA by RSFs was assessed by real-time polymerase chain reaction. The results showed that leptin significantly increased the expression of IL-1β and IL-6 mRNA by RSFs in a concentration-dependent manner. Expression of the target mRNA was normalised for the expression of β-actin mRNA, and fold induction was determined relative to expression by cells incubated without leptin. Bars show the mean and SEM (n=3). *$p<0.05$; **$p<0.01$ *vs.* no treatment. Significance was evaluated by one-way analysis of variance with Dunnett's *post hoc* test.

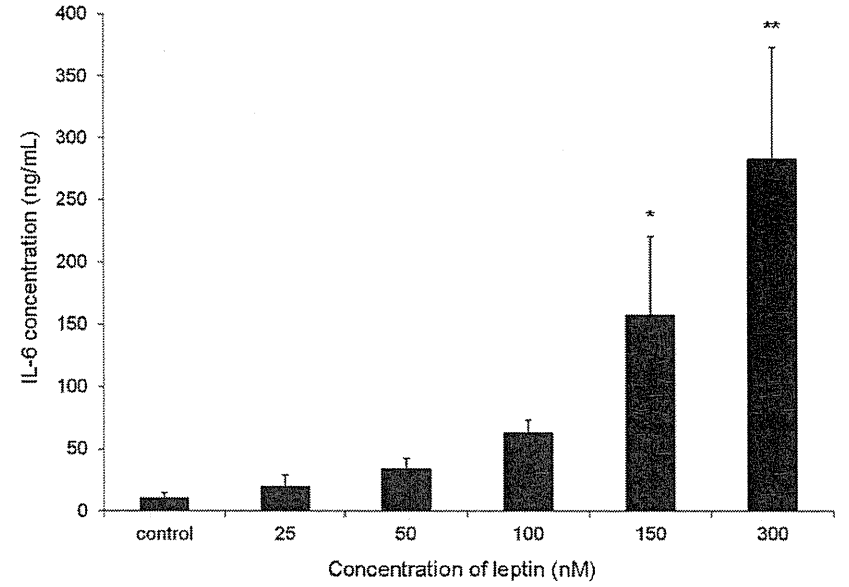

Fig. 2. Effect of leptin on the production of IL-6 by RSFs. Rheumatoid synovial fibroblasts (RSFs) were incubated with leptin at the indicated concentrations for 24 hours and the interleukin (IL)-6 concentration in the culture medium was measured by enzyme-linked immunosorbent assay. Leptin significantly increased IL-6 production by RSFs in a concentration-dependent manner. Bars show the mean and SEM (n=3). *$p<0.05$; **$p<0.01$ *vs.* no treatment. Significance was evaluated by one-way analysis of variance with Dunnett's *post hoc* test.

experiments, RSFs were plated in 10 cm plastic dishes (3×10^5/dish) in RPMI 1640 medium with 10% (v/v) FBS and cultured for 18 hours. Then the medium was changed to serum-free RPMI 1640 medium, and the cells were transfected with siRNA (10 pmol/ml) for Ob-Rb or with control siRNA (10 pmol/ml) using Lipofectamine™ RNAiMAX (Invitrogen Corp.) according to the manufacturer's recommendations. After 72 hours, the cells were replated into 35-mm plastic dishes for PCR or into 96-well plastic plates for measurement of IL-6 by ELISA.

Statistical analysis

Data are expressed as the mean ± SEM. Statistical analysis was performed with Prism ver. 5.0 software (Graphpad Software, San Diego, CA, USA). To compare two groups, Student's *t*-test was employed. Groups (≥3) were compared by using one-way analysis of variance (ANOVA). One-way ANOVA with Bonferroni's *post hoc* test was used to determine differences among all the groups. One-way ANOVA with Dunnett's *post hoc* test was used for comparison to control (no treatment). In all analyses, $p<0.05$ was considered significant.

Results

Effect of leptin on production of inflammatory cytokines by RSFs

To determine whether leptin increased the production of IL-1β, IL-6, and TNF-α by RSFs, real-time PCR was performed. The results showed that leptin significantly increased the expression of IL-1β and IL-6 mRNA by RSFs in a concentration-dependent manner (Fig. 1). In contrast, expression of TNF-α mRNA was not increased by leptin (data not shown). To confirm the production of IL-1β and IL-6 proteins, we measured the concentrations of these cytokines in the culture medium of RSFs incubated with leptin (Fig. 2). We found that leptin significantly increased IL-6 production by RSFs in a concentration-dependent manner. In contrast, concentrations of IL-1β and TNF-α were not detectable ever after stimulation with leptin.

Effect of siRNA for leptin receptor on IL-6 production by RSFs

mRNAs for both leptin receptors (Ob-Rb and Ob-Re) were expressed by cells from 3 patients with RA (data not shown). RSFs were transfected with siRNA targeting Ob-Rb (the leptin receptor) or with negative control siRNA, and then expression of Ob-Rb and Ob-Re mRNA was detected by RT-PCR. This showed that Ob-Rb mRNA expression by RSFs was decreased after exposure to the siRNA for Ob-Rb (Fig. 3A). When cells were seeded in 96-well plates and incubated with leptin for 18 hours, IL-6 production by RSFs transfected with the siRNA targeting Ob-Rb was significantly lower than that by RSFs transfected with negative control siRNA (Fig. 3B).

Effect of leptin on STAT3 phosphorylation in RSFs

We then examined more details of the signal transduction involved in these effects of leptin. To determine whether leptin induced STAT3 phosphorylation in RSFs, Western blotting was performed. This revealed that leptin increased STAT3 phosphorylation in a concentration-dependent manner (Fig. 4A). To investigate whether phosphorylation of STAT3 was related to the induction of IL-6 production by leptin, RSFs were

incubated with an anti-IL-6 antibody (Fig. 4B). Phosphorylation of STAT3 in response to leptin was not inhibited by addition of the anti-IL-6 antibody, but STAT3 phosphorylation in response to IL-6 was inhibited by the antibody.

Effects of signalling pathway inhibitors on leptin-induced IL-6 production by RSFs

We examined the effects of inhibitors of major signalling pathways on leptin-induced IL-6 upregulation in RSFs. As a result, leptin-induced IL-6 production was significantly inhibited by addition of AG490, a JAK2 inhibitor (Fig. 5), but not by LY294002, a PI3K inhibitor (Fig. 6A) or PD98059, a MAPK inhibitor for ERK (Fig. 6B). These findings suggested that leptin induces IL-6 production in RSFs *via* the JAK2/STAT3 pathway.

Discussion

In the present study, we demonstrated that leptin induced the expression of IL-6 mRNA and protein in RSFs *via* the JAK2/STAT3 pathway. This finding is supported by data obtained in leptin-deficient *ob/ob* mice by Busso *et al.* (14), who reported that leptin-deficient mice were partly protected against antigen-induced arthritis, showing less synovial tissue proliferation and a weaker humoral response to the injected antigen. Moreover, Sugioka *et al.* (15) reported that acquired leptin resistance by high-fat feeding reduces inflammation from collagen antibody-induced arthritis in mice.

Harigai *et al.* (16) reported that TNF-α induced IL-6 production by synovial fibroblasts in a dose dependent manner. On the other hand, the present study showed that leptin stimulated IL-6 production. Gonzalez-Gay *et al.* (17) reported that leptin concentration was not changed by administration of anti-TNF-α-blocker infliximab. Therefore, TNF-α-induced IL-6 production might not be mediated by leptin.

IL-6 is a pleiotropic cytokine that is overexpressed in the synovial tissue of RA patients, who have elevated concentrations of IL-6 in both serum and synovial fluid (18). IL-6 influences the function of neutrophils, T cells, B cells, monocytes, and osteoclasts. It is a major inducer of the hepatic acute phase response, which is also a key feature of RA that is correlated with disease activity and joint destruction. Thus, IL-6 is thought to play a pivotal role in RA. Tocilizumab is a humanised anti-IL-6 receptor monoclonal antibody that has shown efficacy for treating RA in clinical trials (19). The average levels of IL-6 in serum and synovial fluid of

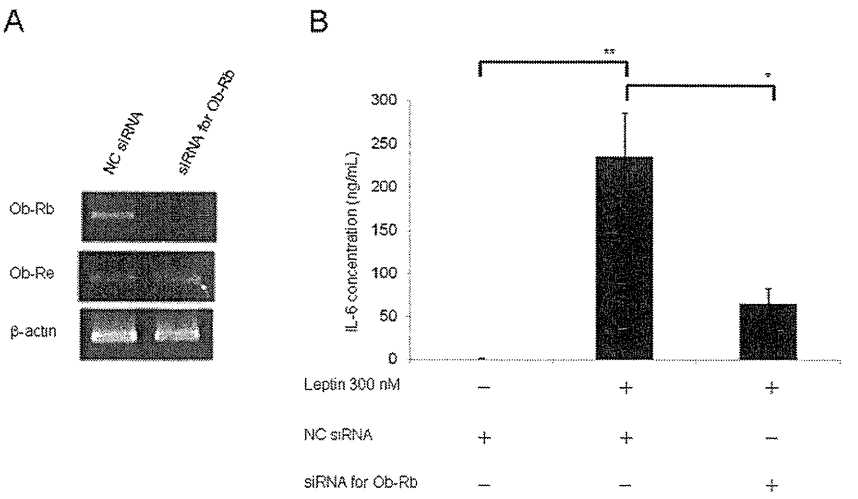

Fig. 3. Effect of siRNA targeting the leptin receptor on IL-6 production by RSFs. (**A**) Rheumatoid synovial fibroblasts (RSFs) were transfected with small interfering RNA (siRNA) for Ob-Rb or negative control (NC) siRNA, and Ob-Rb and Ob-Re mRNA levels were analysed by reverse transcription – polymerase chain reaction. Representative results obtained with fibroblasts from 3 patients are shown. Ob-Rb mRNA expression by RSFs was decreased after exposure to the siRNA for Ob-Rb when compared with exposure for NC siRNA. (**B**) After transfection with siRNA, RSFs were treated with 300 nM leptin or phosphate-buffered saline for 18 hours, and the interleukin (IL)-6 concentration in the culture medium were measured by enzyme-linked immunosorbent assay. IL-6 production by RSFs transfected with the siRNA targeting Ob-Rb was significantly lower than that by RSFs transfected with NC siRNA. Bars show the mean and SEM (n=3). $*p<0.05$; $**p<0.01$. Significance was evaluated by one-way analysis of variance with Bonferroni's *post hoc* test.

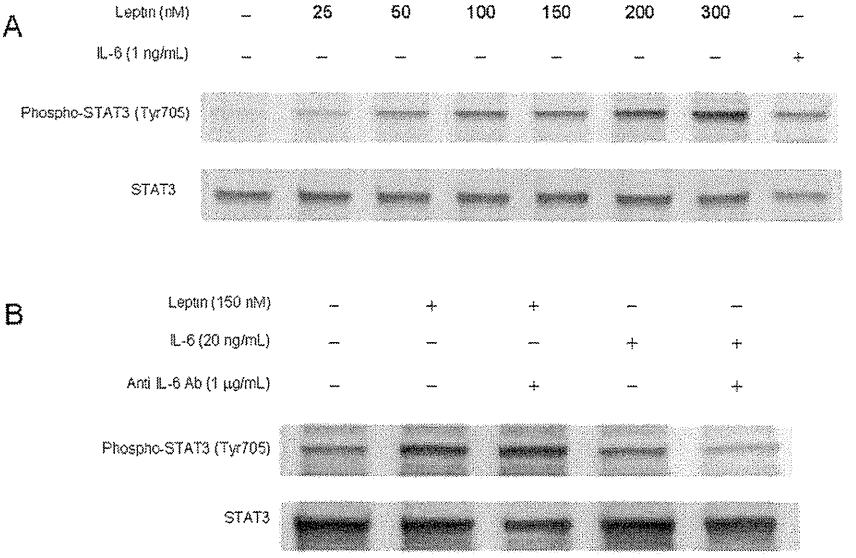

Fig. 4. Effect of leptin on phosphorylation of STAT3 in RSFs. (**A**, **B**) Rheumatoid synovial fibroblasts (RSFs) were incubated with leptin, interleukin (IL)-6, or anti-IL-6 antibody (at the indicated concentrations), and then Western blot analysis was performed. Leptin increased signal transducer and activator of transcription (STAT)3 phosphorylation in a concentration-dependent manner (**A**). Phosphorylation of STAT3 in response to leptin was not inhibited by addition of the anti-IL-6 antibody, but STAT3 phosphorylation in response to IL-6 was inhibited by the antibody (**B**). Representative results obtained with fibroblasts from 3 patients are shown.

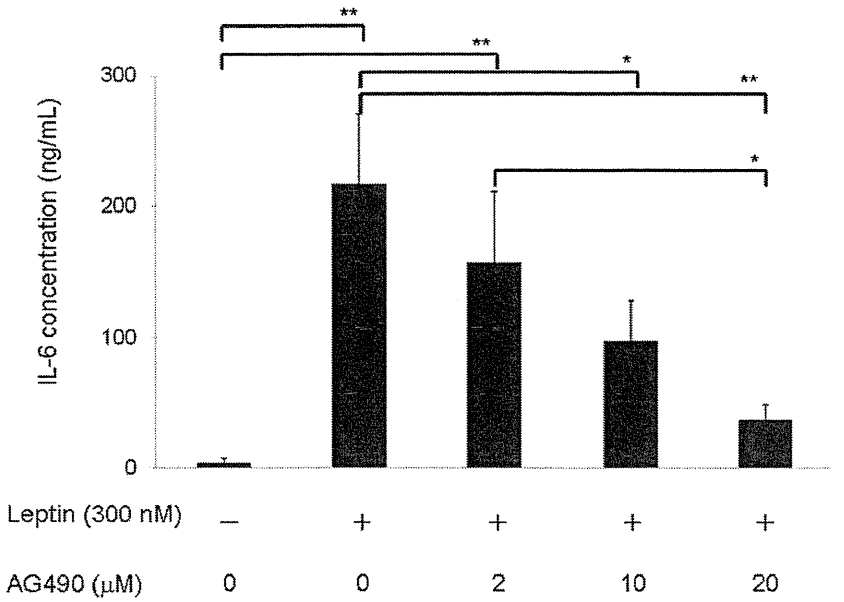

Fig. 5. Effect of a JAK2 inhibitor on IL-6 production by RSFs. Interleukin (IL)-6 level in culture medium of rheumatoid synovial fibroblasts incubated for 18 hours with/without leptin and AG490. The IL-6 concentration in the culture medium was measured by enzyme-linked immunosorbent assay. Leptin-induced IL-6 production was significantly inhibited by addition of AG490, a janus kinase 2 inhibitor. Bars show the mean and SEM (n=3). $^{*}p<0.05$; $^{**}p<0.01$. Significance was evaluated by one-way analysis of variance with Bonferroni's *post hoc* test.

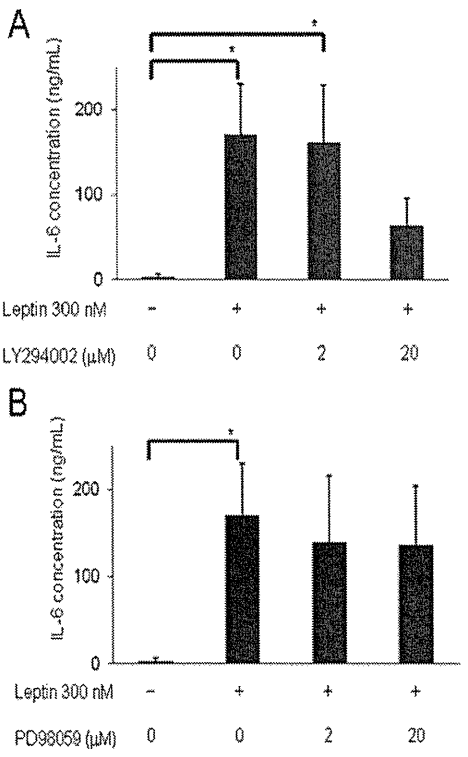

Fig. 6. Effect of signalling pathway inhibitors on IL-6 production by RSFs. (**A, B**) Interleukin (IL)-6 level in culture medium of rheumatoid synovial fibroblasts incubated for 18 hours with/without leptin, LY294002, and PD98059. The IL-6 concentration in the culture medium was measured by enzyme-linked immunosorbent assay. Leptin-induced IL-6 production was not inhibited by LY294002, a phosphatidylinositol 3-kinase inhibitor (**A**) or PD98059, a mitogen-activated protein kinase inhibitor for extracellular signal-regulated kinase (**B**). Bars show the mean and SEM (n=3). $^{*}p<0.05$; $^{**}p<0.01$. Significance was evaluated by one-way analysis of variance with Bonferroni's *post hoc* test.

RA patients were 14 pg/mL and 4 ng/mL, respectively (20). In our study, 20–283 ng/mL of IL-6 were produced by 25–300 nM of leptin (Fig. 2). Thus, our present results suggest a contribution of leptin to the pathogenesis of RA *via* its influence on IL-6.

Although IL-1β mRNA in RSFs was increased by leptin, IL-1β protein was not detectable in culture medium of RSFs. In general, synovial fibroblasts are not the principal sources of inflammasome-mediated IL-1β production in the synovium (21). This might be one of the reasons of the discrepancy between changes in mRNA and protein of IL-1β in our study.

Six isoforms of the leptin receptor have been identified (22). The Ob-Re isoform is a soluble receptor that lacks the transmembrane and cytoplasmic domains, while Ob-Rb is a long form that has an intracellular signalling domain and is thought to be involved in intracellular signalling. In the present study, we found that both Ob-Rb and Ob-Re mRNAs were expressed by RSFs. In addition, the response of IL-6 to leptin was reduced when RSFs were transfected with siRNA targeting Ob-Rb. Therefore, the induction of IL-6 production by leptin was mediated by Ob-Rb.

It is known that JAK/STAT pathway is activated by leptin in several kinds of human cells, that is hepatocellular carcinoma (23), peripheral blood mononuclear cells (24), colorectal adenoma (25). However, these report have not shown upregulation of IL-6 by leptin. In addition, this is the first report that leptin stimulates IL-6 production *via* JAK2/STAT3 in RSFs. Although leptin has been shown to stimulate IL-6 production in human osteoarthritic cartilage (26), this was mediated by the nuclear factor κB and MAPK pathway rather than the JAK2/STAT3 pathway. Since Migita *et al.* (27) reported that IL-6 induced acute-phase serum amyloid A genes *via* JAK2/STAT3 activation in RSFs, we determined whether STAT3 phosphorylation was affected by the leptin-induced upregulation of IL-6. Phosphorylation of STAT3 in response to leptin was not inhibited by the anti-IL-6 antibody, suggesting that STAT3 phosphorylation might be due to a direct effect of leptin on RSFs.

A previous study demonstrated that leptin activated two signalling pathways (PI3K and MAPK) in RSFs and human peripheral blood mononuclear cells (28, 29). Therefore, we investigated the effect of LY294002 (a PI3K inhibitor) and PD98059 (a MAPK inhibitor for ERK) on RSFs incubated with leptin. As a result, leptin-induced

IL-6 production was not mediated by signalling of PI3K and/or MAPK.

The serum leptin level in RA patients was reported to be in the 1–30 nM range (10), so the concentration of leptin used in this study was higher, but it might be possible that leptin stimulates a vicious cycle of inflammation by a paracrine effect in the articular cavity (30). Further studies will be necessary to confirm the mechanism by which leptin influences RA.

Acknowledgements

We wish to thank Prof. Chiaki Nishimura for advice regarding the statistical analysis, Dr Rika Yarita for support conducting *in vitro* experiments, and Ms Sonoko Sakurai for secretarial assistance.

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Mod Rheumatol (2013) 23:1045–1052
DOI 10.1007/s10165-012-0817-4

ORIGINAL ARTICLE

Prevention of joint destruction by tacrolimus in patients with early rheumatoid arthritis: a post hoc analysis of a double-blind, randomized, placebo-controlled study

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Received: 28 September 2012 / Accepted: 11 December 2012 / Published online: 28 December 2012

Abstract
Objectives A multicenter, randomized, double-blind, placebo-controlled study of the oral calcineurin inhibitor tacrolimus was performed in patients with early rheumatoid arthritis who had responded poorly to disease-modifying antirheumatic drugs (DMARDs), and factors related to suppression of joint destruction were investigated.
Methods The change in the total Sharp score (ΔTSS) was assessed by univariate analysis in patients with X-ray films to identify the main determinant of a ΔTSS of <0.5 in week 52. Patients with this factor were then investigated further.
Results Univariate analysis showed that a baseline C-reactive protein (CRP) level of <1.5 mg/dL was the major determinant of ΔTSS <0.5 at week 52 in the tacrolimus group. Detailed analysis of patients with a baseline CRP of <1.5 mg/dL revealed no significant differences in background factors between the two groups. In week 52, ΔTSS was significantly smaller in the tacrolimus group than in the placebo group (2.67 ± 5.40 vs. 8.05 ± 10.32, respectively, $p = 0.017$). Both groups had a similar incidence of adverse reactions.
Conclusions Adding tacrolimus to DMARDs significantly suppressed disease activity and joint destruction in patients with early rheumatoid arthritis, a disease duration ≤3 years, a CRP <1.5 mg/dL, and a poor response to oral DMARDs.

Keywords DMARD · Rheumatoid arthritis · Tacrolimus

Introduction

Tacrolimus is a macrolide antibiotic that was first identified as a metabolic product of the actinomycete *Streptomyces tsukubaensis*. It is a calcineurin inhibitor that shows strong immunosuppressive activity by selectively blocking T-cell activation [1, 2]. Tacrolimus was initially used clinically in Japan in organ transplantation, after which its efficacy for myasthenia gravis, rheumatoid arthritis (RA), lupus nephritis, and ulcerative colitis was also demonstrated.

In Japan, oral tacrolimus was approved for the treatment of RA in April 2005 (it is indicated for patients in whom conventional therapy is inadequate), after its efficacy and safety had been confirmed in clinical studies of RA patients who showed a poor response to disease-modifying antirheumatic drugs (DMARDs) [3, 4]. Recently, tacrolimus has often been used concomitantly with DMARDs, including methotrexate (MTX), and the improvement of symptoms through the use of this concomitant therapy has been reported [5, 6]. However, its effect on joint destruction is yet to be clarified.

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Previously, we performed a double-blind placebo-controlled study [7] to investigate the efficacy and safety of tacrolimus, as well as its capacity to prevent joint destruction, in 123 patients with early RA <3 years in duration, who had a diagnosis of RA based on the 1987 criteria of the American College of Rheumatology (ACR). According to the ACR criteria [9], the ACR20 improvement rate was significantly higher ($p = 0.005$) in the tacrolimus group than the placebo group. Also, according to the European League Against Rheumatism (EULAR) criteria [10, 11], a significantly higher ($p < 0.001$) percentage of the tacrolimus group showed a moderate or good response compared with the placebo group. Furthermore, the percentage of patients with a final disease activity score in 28 joints (DAS28) [12] of <2.6 was significantly higher ($p = 0.005$) in the tacrolimus group than in the placebo group. There was no significant difference between the two groups with regard to the incidence of adverse events or discontinuation due to adverse events.

However, evaluation of joint destruction in week 52 by the modified Sharp method [13] revealed the following results for the tacrolimus group and the placebo group, respectively: the total Sharp score (TSS) was 6.16 ± 10.84 (mean ± SD) versus 7.73 ± 12.23 and the bone erosion score was 2.50 ± 4.56 versus 4.27 ± 7.53 ($p = 0.090$). There were no significant differences in the change in TSS (ΔTSS) between the two groups, but the bone erosion score was lower in the tacrolimus group, suggesting that tacrolimus has the potential to reduce the progression of bone erosion. In the present study, therefore, we performed subanalyses to determine factors related to the prevention of joint destruction by tacrolimus therapy, and we found that tacrolimus suppressed disease activity and joint destruction in patients with early rheumatoid arthritis and lower levels of serum CRP (<1.5 mg/dL).

Materials and methods

Patients and study protocol

The enrollment criteria for this study were as follows: (1) males or females aged 20–65 years with a diagnosis of RA according to the ACR criteria [8]; (2) duration of disease ≥6 to ≤3 years; (3) at least 6 tender joints out of 68 joints surveyed; (4) at least 3 swollen joints out of 66 joints surveyed; (5) a C-reactive protein (CRP) level ≥1.0 mg/dL or erythrocyte sedimentation rate (ESR) ≥30 mm/h; (6) radiographic bone erosion at more than one site in the hands or lower limbs; and (7) current treatment with MTX (6–8 mg/week), salazosulfapyridine (1 g/day), or bucillamine (100–300 mg/day), and a compliance rate of ≥75 % during the minimum administration period (8 weeks before baseline, 8 weeks, or 12 weeks).

The exclusion criteria were (1) previous treatment with tacrolimus; (2) class 4 of Steinbrocker's functional classification; (3) treatment with biological products (infliximab or etanercept) or leflunomide within 12 weeks before the study for suppression of joint destruction; (4) steroid therapy at >7.5 mg/day (as prednisolone equivalent) within 4 weeks before the study, (5) administration of >2 tablets/suppositories of nonsteroidal anti-inflammatory drugs (NSAIDs) daily; and (6) diseases such as renal dysfunction, pancreatitis/impaired glucose tolerance, hyperkalemia, advanced hepatic dysfunction, cardiac disease (ischemic heart disease, arrhythmia requiring treatment, cardiac failure, etc.), severe respiratory disease, severe infection, drug hypersensitivity, or malignancy.

Subjects who fitted the above criteria, and who gave written informed consent, were randomized to a tacrolimus (3 mg/day) or a placebo group. Study drugs were administered once a day after the evening meal for a period of 52 weeks. The dosages of concomitant MTX, salazosulfapyridine, bucillamine, and NSAIDs were not changed, while dose reduction was allowed for steroids, but an increase above the baseline dose was not permitted. Initiation of new antirheumatic drugs or steroids was also not permitted.

At enrollment, in week 28, and in week 52 (or at discontinuation), plain X-ray films of both hands and both lower limbs were taken. Two blinded evaluators employed the modified Sharp method to determine the bone erosion score (ES) and the joint space narrowing (JSN) score from the X-ray films, and the sum of the ES and JSN scores was calculated as the TSS [9, 10]. The change in TSS from baseline (ΔTSS) was used to assess the progression of joint destruction. All participating institutions received the approval of their governing institutional board or equivalent, and the trial was implemented in accordance with the ethical principles of the Declaration of Helsinki and good clinical practice (GCP) guidelines, as well as relevant laws or regulations promulgated by the Institutional Review Boards for clinical trials. This study is registered at ClinicalTrials.gov (NCT00319917).

Statistical analysis

Factors with an influence on the suppression of joint destruction by tacrolimus were extracted by univariate analysis, employing gender (male, female), age (<49, ≥49 years), disease duration (<1.3, ≥1.3 years), stage (stages I/II, stages III/IV), functional class (class 1, classes 2–4), CRP (<1.5, ≥1.5 mg/dL), ESR (<41.5, ≥41.5 mm/h), DAS28-CRP (≤5.1, >5.1), DAS28-ESR (≤5.1, >5.1), TSS (<11.0, ≥11.0), ES (<5.0, ≥5.0), JSN score (<3.5, ≥3.5), yearly progression (<9.2, ≥9.2), rheumatoid factor

(<63.5, ≥63.5 IU/mL), matrix metalloproteinase-3 (MMP-3) (<187.5, ≥187.5 ng/mL), concomitant MTX therapy (yes, no), and the dose of MTX (<8, ≥8 mg/week) at the start of tacrolimus administration. CRP = 1.5, which was the median value of the population, was used in the analysis to keep the number of cases in the two groups uniform. Age, disease duration, CRP, ESR, TSS, ES, JSN, yearly progression, rheumatoid factor, MMP-3, and baseline dose of MTX were analyzed after being dichotomized at the median value (<median, ≥median).

For each factor extracted by univariate analysis, the effect on ΔTSS was compared between the tacrolimus group and the placebo group, and the factors that showed a significant difference between the two groups were selected. Next, the patients in whom a significant difference in these factors was observed were selected and used to perform a comparison between the tacrolimus and placebo groups with respect to each patient background factor, use of DMARDs, dose of DMARDs, changes in the Sharp score in week 52, improvement according to the EULAR criteria, and adverse events.

Changes in Sharp scores were examined by an analysis of variance in relation to the baseline score and the use of MTX as a covariate. The improvement rate according to the EULAR criteria was examined by logistic regression analysis. Background factors and adverse events were compared between the tacrolimus group and the placebo group by Fisher's exact test, the *t* test, or the Wilcoxon rank-sum test. Statistical significance was accepted at $p < 0.05$ (two-sided). Results are reported as the mean ± SD.

Missing radiographic data were compensated for using the linear extrapolation method, while other missing values were compensated for using the last-observation-carried-forward method.

Adverse events were classified by system organ class and preferred terms were taken from the ICH Medical Dictionary for Regulatory Activities (MedDRA Ver.11.1).

Results

Among the 123 randomized patients (61 in the tacrolimus group and 62 in the placebo group) registered in this study, 116 patients (58 in each group) had TSS data (Fig. 1). There were no differences between the background factors of the 116 patients and those of the 123 patients (data not shown). Also, there were no differences in background factors between both groups (tacrolimus and placebo) within this set of 116 patients, and the results were similar to the profile observed for all 123 patients.

Factors that influenced the achievement of ΔTSS <0.5 in week 52 were investigated in the tacrolimus group ($n = 58$) by univariate analysis, and this revealed significant influences of CRP, ESR, and DAS28-CRP (Table 1). When stratified analysis was carried out using these factors, a significant difference in ΔTSS in week 52 between the tacrolimus and placebo groups was (only) observed in the subgroup of patients with a baseline CRP of <1.5 mg/dL.

Among the 116 patients for whom the TSS was calculated, 29 patients from the tacrolimus group and 31 patients

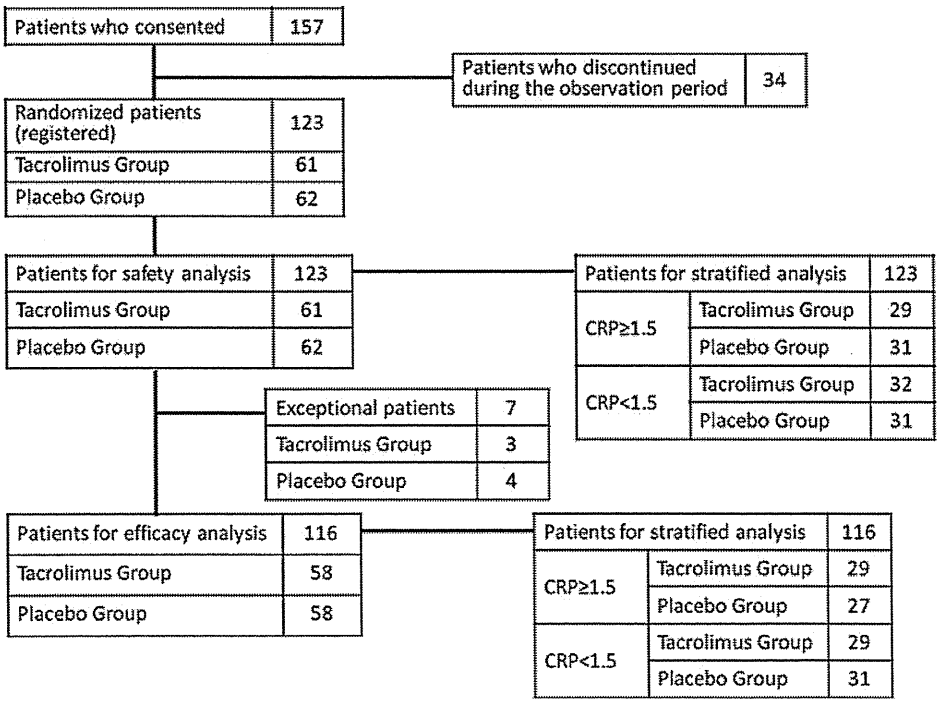

Fig. 1 Patient disposition. One hundred twenty-three RA patients were registered in this study. A total of 123 patients were randomized to either the tacrolimus group (61 patients) or the placebo group (62 patients) for safety analysis. The patients were then stratified according to CRP (<1.5, ≥1.5). Also, a total of 115 patients were randomized to either the tacrolimus group (58 patients) or the placebo group (58 patients) for efficacy analysis. Again, these patients were stratified according to CRP (<1.5, ≥1.5)

Table 1 Univariate analysis of the influence of background factors on the achievement of ΔTSS <0.5 in the tacrolimus group ($n = 58$)

		p value
Gender	Male (0/6) vs. female (14/52)	0.972
Age	<49 (8/29) vs. ≥49 (6/29)	0.541
Disease duration (years)	<1.3 (10/29) vs. ≥1.3 (4/29)	0.073
Stage classification	I, II (10/42) vs. III, IV (4/16)	0.925
Functional classification	1 (3/12) vs. 2–4 (11/46)	0.938
CRP (mg/mL)	<1.5 (11/29) vs. ≥1.5 (3/29)	0.021
ESR (mm/h)	<41.5 (11/29) vs. ≥41.5 (3/29)	0.021
DAS28-CRP	≤5.1 (13/32) vs. >5.1 (1/26)	0.009
DAS28-ESR	≤5.1 (6/15) vs. >5.1 (8/43)	0.103
Total score (modified Sharp method)	<11.0 (8/29) vs. ≥11.0 (6/29)	0.541
Bone erosion score (modified Sharp method)	<5.0 (6/27) vs. ≥5.0 (8/31)	0.751
Joint space narrowing score (modified Sharp method)	<3.5 (9/29) vs. ≥3.5 (5/29)	0.225
Yearly progression	<9.2 (7/29) vs. ≥9.2 (7/29)	1.000
Rheumatoid factor (IU/mL)	<63.5 (8/29) vs. ≥63.5 (6/29)	0.541
MMP-3 (ng/mL)	<187.5 (8/29) vs. ≥187.5 (6/29)	0.541
Concomitant MTX at the start of administration	with (8/39) vs. without (6/19)	0.358
MTX dose at the start of administration (mg/week)	<8 (9/33) vs. ≥8 (5/25)	0.523

CRP C-reactive protein, *ESR* erythrocyte sedimentation rate, *DAS28* Disease Activity Score 28, *MMP-3* matrix metalloproteinase-3, *MTX* methotrexate

from the placebo group had a baseline CRP of <1.5 mg/dL. Among all 123 patients, 32 patients in the tacrolimus group and 31 in the placebo group were subjected to safety analysis. With regard to background factors and concomitant medications, no significant differences were observed between both groups (Table 2).

Analysis of the clinical response of patients with a CRP level of <1.5 mg/dL at baseline was performed. In week 52, the tacrolimus group ($n = 32$) included 18 patients with a good response (56.3 %), 7 patients with a moderate response (21.9 %), and 7 patients with no response (21.9 %). Accordingly, 78.1 % of patients showed a moderate or good response. In the placebo group ($n = 31$), there were 10 patients with a good response (32.3 %), 6 patients with a moderate response (19.4 %), and 15 patients with no response (48.4 %), so 51.6 % of the patients had a moderate response or better. Again, there was a significant difference between the two groups ($p = 0.030$) (Fig. 2).

Evaluation of joint destruction showed the following yearly progression of TSS by week 52 in the tacrolimus group and the placebo group, respectively: ΔTSS was 2.67 ± 5.40 versus 8.05 ± 10.32 ($p = 0.017$), the change in the ES was 1.16 ± 3.10 versus 4.12 ± 6.28 ($p = 0.034$), and the change in the JSN score was 1.52 ± 3.19 versus 3.94 ± 5.14 ($p = 0.050$). ΔTSS and the change in ES were significantly smaller in the tacrolimus group than in the placebo group, and the change in the JSN score was also smaller in the tacrolimus group (Fig. 3a).

When the cumulative probability of ΔTSS up to week 52 was plotted, the cumulative probability of ΔTSS ≤0 was 37.9 % (11/29 patients) in the tacrolimus group and 16.1 % (5/31 patients) in the placebo group (Fig. 3b). This was approximately 1.57 times higher than the cumulative probability of ΔTSS ≤0 of 24.1 % for the tacrolimus group in the overall study (and 1.15 times higher for the placebo group), but this difference between groups was not significant ($p = 0.056$).

Regarding safety, the occurrence of adverse events among patients with a CRP of <1.5 mg/dL was noted in 81.3 % of those from the tacrolimus group (26/32 patients, 102 events) versus 90.3 % of such patients from the placebo group (28/31 patients, 85 events) (Table 3). Assessment according to system organ class showed no significant differences in event incidence between the two groups, and the event incidence revealed by this analysis was similar to that obtained in the overall study. Severe adverse events were observed in one patient from the tacrolimus group (3.1 %) versus seven patients from the placebo group (22.6 %), while discontinuation of administration due to adverse events occurred in two patients (6.3 %) and five patients (16.1 %), respectively.

Discussion

Since joint destruction progresses from the early stage of RA and eventually causes irreversible functional impairment, appropriate diagnosis and early treatment are needed. The 2012 ACR Recommendations [14] state that treatment with DMARDs should be initiated before joint destruction is evident. Moreover, to minimize the progression of joint destruction in patients with a disease duration of six-months or longer, administration of DMARDs alone or concomitantly is recommended, with re-evaluation every

Table 2 Comparison of the patients included in the safety analysis with CRP <1.5 mg/dL

		Tacrolimus group ($n = 32$)	Placebo group ($n = 31$)	p value
Female sex	Patients (%)	28 (96.6)	28 (90.3)	0.613[a]
Age (years)	Mean ± SD	48.6 ± 9.8	51.5 ± 11.6	0.291[b]
Height (cm)	Mean ± SD	156.9 ± 6.1	157.2 ± 8.1	0.866[b]
Weight (kg)	Mean ± SD	52.7 ± 6.0	53.0 ± 10.2	0.881[b]
Disease duration (years)	Mean ± SD	1.5 ± 0.7	1.6 ± 0.7	0.590[b]
Stage classification (stage)				
I (early stage)	Patients (%)	0	1 (3.2)	0.632[c]
II (middle stage)	Patients (%)	21 (72.4)	23 (74.2)	
III (advanced stage)	Patients (%)	8 (27.6)	5 (16.1)	
IV (terminal stage)	Patients (%)	0	2 (6.5)	
Functional classification (class)				
1	Patients (%)	7 (24.1)	7 (22.6)	0.888[c]
2	Patients (%)	22 (75.9)	24 (77.4)	
3	Patients (%)	0	0	
4	Patients (%)	0	0	
Number of painful joints	Mean ± SD	12.1 ± 7.8	11.5 ± 5.2	0.746[b]
Number of swollen joints	Mean ± SD	10.0 ± 5.1	8.5 ± 5.2	0.283[b]
Physical function evaluation by patients	Mean ± SD	0.4 ± 0.4	0.5 ± 0.3	0.214[b]
CRP (mg/dL)	Mean ± SD	0.5 ± 0.4	0.7 ± 0.4	0.257[b]
ESR (mm/h)	Mean ± SD	36.1 ± 18.1	42.4 ± 20.3	0.214[b]
Rheumatoid factor (IU/mL)	Mean ± SD	98.3 ± 126.5	115.9 ± 118.8	0.581[b]
DAS28-CRP				
≤3.2	Patients (%)	2 (6.9)	1 (3.2)	–
>3.2, ≤5.1	Patients (%)	21 (72.4)	25 (80.6)	
>5.1	Patients (%)	6 (20.7)	5 (16.1)	
	Mean ± SD	4.4 ± 0.8	4.3 ± 0.7	0.927[b]
DAS28-ESR				
≤3.2	Patients (%)	0	0	–
>3.2, ≤5.1	Patients (%)	11 (37.9)	11 (35.5)	
>5.1	Patients (%)	18 (62.1)	20 (64.5)	
	Mean ± SD	5.3 ± 0.8	5.2 ± 0.8	0.934[b]
Total score (modified Sharp method)	Mean ± SD (min–max)	15.9 ± 17.0 (2.0–75.0)	16.7 ± 17.1 (0.0–65.5)	0.858[b]
Bone erosion score (modified Sharp method)	Mean ± SD (Min–Max)	9.0 ± 8.3 (2.0–33.0)	7.7 ± 8.2 (0.0–40.5)	0.531[b]
Joint space narrowing score (modified Sharp method)	Mean ± SD (min–max)	6.9 ± 10.6 (0.0–42.0)	9.0 ± 12.1 (0.0–41.0)	0.471[b]
Yearly progression	Mean ± SD (min–max)	10.4 ± 8.7 (1.3–32.1)	10.7 ± 10.9 (0.0–46.0)	0.891[b]
Concomitant agents				
Methotrexate				
Dose (mg/week)	Patients (%)	16 (55.2)	18 (58.1)	1.000[a]
	Mean ± SD	7.0 ± 1.0	7.3 ± 1.0	0.339[b]
Salazosulfapyridine				
Dose (g/day)	Patients (%)	10 (34.5)	6 (19.4)	0.247[a]
	Mean ± SD	1.0 ± 0.0	1.0 ± 0.0	–
Bucillamine				
Dose(mg/day)	Patients (%)	3 (10.3)	7 (22.6)	0.302[a]
	Mean ± SD	166.7 ± 57.7	142.9 ± 53.5	0.545[b]

Table 2 continued

		Tacrolimus group ($n = 32$)	Placebo group ($n = 31$)	p value
Steroids				
Dose (mg/day)	Patients (%)	15 (51.7)	11 (35.5)	0.297[a]
	Mean ± SD	5.0 ± 2.2	4.8 ± 1.7	0.802[b]

CRP C-reactive protein, *ESR* erythrocyte sedimentation rate, *DAS28* Disease Activity Score 28

[a] Fisher's exact test

[b] t test

[c] Wilcoxon rank sum test

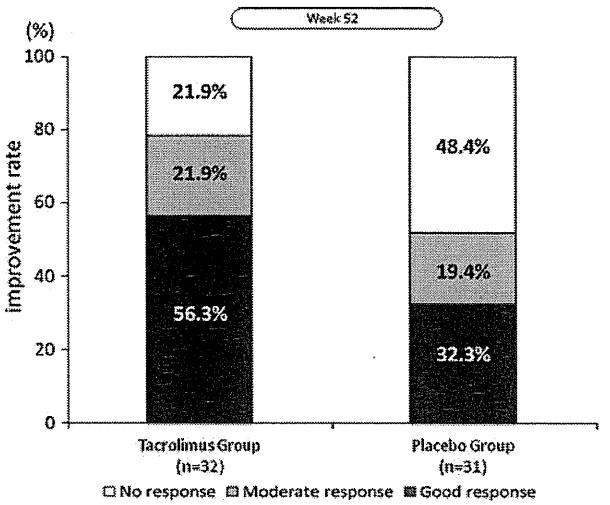

Fig. 2 Improvement rates [according to the EULAR (DAS28-CRP) criteria] in the tacrolimus group and the placebo group. *$p < 0.05$ by the Wald test for estimated parameter values

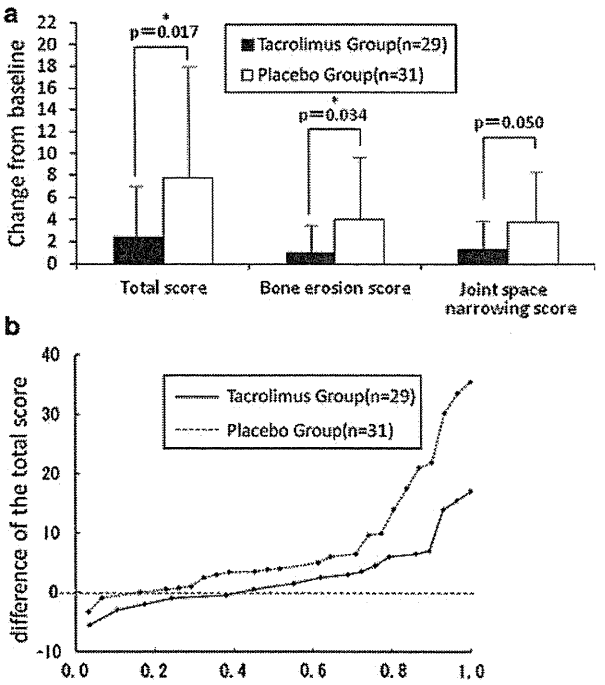

Fig. 3 **a** Evaluation of joint destruction. Joint destruction was evaluated by monitoring ΔTSS, the change in the ES, and the change in the JSN score by week 52 in the tacrolimus group and the placebo group, respectively. *TSS* total Sharp score, *ES* erosion score, *JSN* joint space narrowing; *$p < 0.05$ by analysis of covariance versus before administration in patients with or without MTX. **b** Cumulative probability of ΔTSS ≤0 up to week 52 in the tacrolimus group and the placebo group

three months until remission or low disease activity is achieved. However, there is insufficient evidence regarding the prevention of joint destruction by DMARDs apart from MTX. Tacrolimus is approved for the treatment of RA in Japan and shows good efficacy, suggesting that it could be useful for controlling joint destruction.

We previously investigated patients with a disease duration of RA of less than three years who showed an inadequate response to DMARDs. A double-blinded, placebo-controlled study of tacrolimus treatment was carried out for 12 months, with suppression of joint destruction as the primary outcome measure. Although baseline TSS showed no significant differences between the tacrolimus group and the placebo group, ΔTSS ≤0 was achieved in 24.1 % of the tacrolimus group versus 14.0 % of the placebo group [7]. Accordingly, the present subgroup analysis was performed, and CRP <1.5 mg/dL was identified as a factor that influenced the suppression of joint destruction by tacrolimus therapy according to univariate analysis. There were significantly smaller changes in TSS and ES in the patients with CRP <1.5 mg/dL from the tacrolimus group compared with those from the placebo group, as well as a smaller change of the JSN score, indicating that tacrolimus suppressed the progression of joint destruction in patients with early RA who had low disease activity and comparatively low CRP levels.

There have already been several reports about the prevention of joint destruction by biological agents [15–20],

Table 3 Adverse events (patients targeted for safety analysis and patients with CRP < 1.5 mg/dL)

	Tacrolimus group ($n = 32$)	Placebo group ($n = 31$)	Test[a]
Adverse event rate	81.3 % (26 patients, 102 events)	90.3 % (28 patients, 85 events)	$p = 0.474$
Severe adverse event rate	3.1 % (1 patient)	22.6 % (7 patients)	$p = 0.026$
Discontinuation rate due to adverse events	6.3 % (2 patients)	16.1 % (5 patients)	$p = 0.257$
Infections and infestations	40.6 % (13 patients, 25 events)	38.7 % (12 patients, 24 events)	$p = 1.000$
Benign, malignant and unspecified neoplasms (incl. cysts and polyps)	3.1 % (1 patient, 1 event)	3.2 % (1 patient, 1 event)	$p = 1.000$
Blood and lymphatic system disorders	6.3 % (2 patients, 2 events)	3.2 % (1 patient, 1 event)	$p = 1.000$
Psychiatric disorders		3.2 % (1 patient, 1 event)	$p = 0.492$
Nervous system disorders	9.4 % (3 patients, 4 events)	9.7 % (3 patients, 6 events)	$p = 1.000$
Eye disorders	9.4 % (3 patients, 3 events)	3.2 % (1 patient, 1 event)	$p = 0.613$
Vascular disorders	3.1 % (1 patient, 1 event)	6.5 % (2 patients, 2 events)	$p = 0.613$
Respiratory, thoracic, and mediastinal disorders	15.6 % (5 patients, 7 events)	16.1 % (5 patients, 6 events)	$p = 1.000$
Gastrointestinal disorders	31.3 % (10 patients, 15 events)	19.4 % (6 patients, 11 events)	$p = 0.387$
Skin and subcutaneous tissue disorders	21.9 % (7 patients, 7 events)	22.6 % (7 patients, 7 events)	$p = 1.000$
Musculoskeletal and connective tissue disorders	9.4 % (3 patients, 4 events)	9.7 % (3 patients, 3 events)	$p = 1.000$
Reproductive system and breast disorders	3.1 % (1 patient, 2 events)		$p = 1.000$
Congenital, familial and genetic disorders	3.1 % (1 patient, 1 event)		$p = 1.000$
General disorders and administration site conditions	6.3 % (2 patients, 3 events)	6.5 % (2 patients, 2 events)	$p = 1.000$
Investigations	40.6 % (13 patients, 26 events)	35.5 % (11 patients, 17 events)	$p = 0.797$
Injury, poisoning and procedural complications	3.1 % (1 patient, 1 event)	9.7 % (3 patients, 3 events)	$p = 0.355$

[a] Fisher's exact test

and the 2012 ACR Recommendations [14] suggest the use of biological agents combined with MTX for patients with early RA whose disease activity is high. However, it was reported that patients with early RA show no difference in their response to biological agents plus MTX versus DMARDs with regard to improvement of symptoms and suppression of bone erosion [21]. Thus, biological agents prevent further joint damage in patients with early RA who have higher disease activity and significant joint destruction, while the present study suggested that tacrolimus can suppress joint destruction in patients with early RA and CRP <1.5 mg/dL.

Tacrolimus has been reported to suppress the production of inflammatory cytokines, such as tumor necrosis factor-α, interleukin-1, and interleukin-6 [2, 22, 23], and it also delays the maturation of osteoclasts by inhibiting calcineurin and prevents the activation of T cells. In fact, animal studies have revealed the dose-dependent suppression of collagen-induced arthritis in rats by tacrolimus [24, 25], as well as the concentration-dependent induction of chondrocyte differentiation of progenitor cells in mouse [26]. In addition to an indirect action via the suppression of inflammatory cells, tacrolimus inhibits the maturation of osteoclasts by reducing the activation of NFATc1, a key regulator of osteoclast differentiation. Thus, tissue repair due to the promotion of bone/cartilage differentiation through direct action on osteoclasts helps tacrolimus to lessen joint destruction, and such a mechanism seems to support the results of the present subgroup analysis.

Our analysis revealed that joint destruction was prevented by adding treatment with tacrolimus at 3 mg daily in patients with early RA and CRP < 1.5 mg/dL who showed resistance to DMARDs. These results suggest that the combination of DMARDs and tacrolimus safely achieves clinical improvement in patients with early RA and CRP <1.5 mg/dL by preventing the progression of joint destruction. However, further studies will be required to confirm the suppression of joint destruction by tacrolimus in other patient populations.

Acknowledgments The authors thank Astellas Pharma Inc., which sponsored the study and provided editorial support. Statistical analysis was also performed by Astellas Pharma Inc. under the supervision of the authors.

Conflict of interest Y. Tanaka, S. Kawai, T. Takeuchi, K. Yamamoto, and N. Miyasaka have received consulting fees from Astellas Pharma Inc.

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Japan College of Rheumatology
MODERN RHEUMATOLOGY

http://informahealthcare.com/mor
ISSN 1439-7595 (print), 1439-7609 (online)

Mod Rheumatol, 2014; 24(1): 8–16

DOI 10.3109/14397595.2013.854074

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ORIGINAL ARTICLE

Post-marketing surveillance of the safety and effectiveness of tacrolimus in 3,267 Japanese patients with rheumatoid arthritis

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Received: 19 December 2012 / Accepted: 8 April 2013

Abstract

Objectives A post-marketing surveillance (PMS) program was implemented to assess the safety and effectiveness of tacrolimus (TAC) in Japanese rheumatoid arthritis (RA) patients and to identify risk factors related to adverse drug reactions (ADRs).

Methods Patients were registered centrally and monitored for all adverse events (AEs) for 24 weeks. Effectiveness was evaluated using the Disease Activity Score 28-CRP (DAS28-CRP).

Results Data from 3,172 patients (mean age 62.2 years) were evaluated in the safety analysis. Of the safety population, 78.5 %were female and 25.9 % were in Steinbrocker's functional class 3 or 4. TAC was prescribed as monotherapy in 52.5 % and the most common concomitant disease modifying antirheumatic drug (DMARD) was methotrexate, used in 28.9 % of the patients. The incidence of AEs, serious AEs (SAEs), ADRs and serious ADRs were 41.2, 6.4, 36.0, and 4.9 %, respectively. The most frequent serious ADR category was infections and infestations. Age ≥65 years, concurrent renal dysfunction, and concurrent diabetes mellitus were identified as significant risk factors for ADR. Based on EULAR response criteria, 65.4 % of the patients showed moderate or good response.

Conclusions The results demonstrate that TAC is well tolerated by Japanese patients with active RA, including those receiving concomitant methotrexate, in the real world.

Keywords Effectiveness · Post-marketing surveillance · Rheumatoid arthritis · Safety · Tacrolimus

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Published online: 14 May 2013

Introduction

Rheumatoid arthritis (RA) is characterized by persistent synovitis and structural damage of joints in part through the abnormal activation of immunocompetent cells, including T cells. It has been reported that pathogenesis of RA remains elusive in terms of active T cell- or macrophage-induced cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1, and IL-6, and molecules that cause interaction between antigen presenting cells and T cells [1–4].

Tacrolimus (TAC), a macrolide lactone discovered in 1984, mainly exerts its immunosuppressive effects through

inhibition of T cell activation and production of inflammatory cytokines, such as TNF, IL-1β, and IL-6, all involved in the pathogenesis of RA [5–7]. TAC has been used for the prevention of rejection in organ transplantation and graft-versus-host disease after bone marrow transplantation, as well as for the treatment of myasthenia gravis, lupus nephritis, and ulcerative colitis. In addition, it was approved for RA patients with inappropriate response to conventional treatments in Japan in April 2005, and subsequently approved for RA in Canada, Korea, and Hong Kong.

The efficacy of TAC against RA has been demonstrated in several clinical trials [8–12]. Although the information obtained from the clinical trial settings is straightforward and robust, it has several limitations. For example, in post-marketing settings, TAC is used in patients with various comorbidities or in patients concomitantly taking a variety of drugs, including corticosteroids, DMARDs, and even biological agents. In this regards, the safety and effectiveness of TAC in clinical practice settings remains to be investigated. To address these issues, we implemented a nationwide post-marketing surveillance (PMS) program on safety and effectiveness of TAC in RA patients with central registration and a six-month tracking period in each patient.

Materials and methods

This study was conducted in accordance with a protocol approved by the Ministry of Health, Labor and Welfare (MHLW). A Prograf post-marketing surveillance committee consisting of rheumatologists was convened, which evaluated the obtained interim results in collaboration with Astellas Pharma Inc.

During the study period from April 2005 to March 2009, cases were collected from 406 institutions in Japan. Patients were registered centrally at an independent patient registration center over a 2-year period. The planned study sample size of 3,000 patients was calculated to provide a 95 % confidence level of detecting any adverse event (AE) that occurs at in least 1 of 1,000 exposed individuals.

A written agreement was obtained from participating institutions. The study was also in accordance with the standards for Good Post-Marketing Study Practice (GPSP) provided by the MHLW in Japan.

The MHLW instructed the investigators to perform the PMS study according to GPSP, which is the authorized standard for PMS studies of approved drugs in clinical practice; therefore, no formal ethics committee approval was necessary. The PMS study in Japan is allowed to be conducted without informed consents.

This study was conducted in clinical practice settings in Japan. RA patients who had shown inappropriate response to conventional treatments for RA and who started treatment with TAC for the first time during the registration period (April 2005 to March 2007) were enrolled. Each enrolled patient was followed up for up to 24 weeks. Information regarding background of the patients, status of the TAC treatment, and use of concomitant drugs were collected.

In accordance with the approved dosage and method of administration, adult patients received 3 mg of TAC once daily after dinner. For elderly patients, TAC was started at 1.5 mg once daily after dinner and could be increased to 3 mg if signs and symptoms were not well controlled.

For 24 weeks after the start of treatment with TAC, all AEs and laboratory values were prospectively monitored. Terminology of the Medical Dictionary for Regulatory Activities/Japanese edition (MedDRA/J) version 11.1 was used for summarizing and reporting AEs. AEs were recorded with the physician's assessment of causality, and seriousness according to the International Conference on Harmonization standards.

Of 3,347 patients enrolled, case report forms from 3,267 patients were collected who had at least one follow-up visit after the first dose of TAC. Categorized by clinical department, 1,396 subjects (42.7 %) were from rheumatology departments, 871 (26.7 %) were from internal medicine departments, and 778 (23.8 %) were from orthopedic surgery departments. In accordance with the warnings section of the tacrolimus package insert, which states that "tacrolimus should be administered only by physicians familiar with the treatment of rheumatoid arthritis", the survey was conducted by clinical departments staffed by physicians familiar with the treatment of rheumatoid arthritis. Ninety-five patients were excluded because of unknown status of AEs ($n = 33$), no follow-up visit after the first dose of TAC ($n = 26$), outside enrollment period ($n = 8$), no administration of TAC ($n = 7$), overlapping patients among institute ($n = 7$), no enrollment ($n = 3$), use of TAC before the survey ($n = 2$), and others ($n = 9$). As a result, 3,172 patients were included in the safety population.

Seventeen patients were excluded because of off-label use of, or unknown response to TAC out of 3,172 patients in the safety population. As a result, 3,155 were included in the effectiveness population.

Of 3,155 patients in the effectiveness population, disease activity scores (DAS28) were reported in only 680 patients due to the observational study. Thus, it should be noted that the results of effectiveness [European League Against Rheumatism (EULAR) response rate, DAS28 scores] obtained in this study are difficult to generalize. Nevertheless, the information may be useful for understanding TAC in the real world, and therefore the results of effectiveness in 680 patients were included.

The effectiveness was evaluated by EULAR response criteria, physician's assessment (with three categorical treatment responses of good, moderate, and no response), and DAS28-CRP, which is based on the 28 joint counts, a general health assessment of a patient, and C-reactive protein (CRP). DAS28-CRP was divided into 4 categories: remission (≤2.6), low disease activity (>2.6 and ≤3.2), moderate disease activity (>3.2 and ≤5.1), and high disease activity (>5.1). The analysis for EULAR response criteria at week 24 was conducted using the last observation carried forward (LOCF) method in RA patients whose DAS28 scores were obtained both at the baseline and at least one follow-up visit after the first dose of TAC. Of 3,155 patients in the effectiveness population, 680 patients were evaluated by EULAR response criteria.

All statistical analyses were performed using SAS statistical software (BASE/SAS SAS/STAT Ver. 8.2; SAS Institute Inc., Cary, NC, USA).

In the statistical analysis, proportions were compared using Chi squared test or Fisher's exact test, as appropriate. Testing was 2-sided, and a significance level of 0.05 was used for each comparison. To determine risk factors for ADRs, candidate factors were identified using univariate analyses, followed by multivariate analyses with Cox proportional hazards models. The analyses were performed using a stepwise variable selection method (backward elimination), at a significance level of 0.05.

Results

The main patient characteristics of the safety population ($n = 3,172$) are shown in Table 1. Most patients were female (78.5 % of the overall patient population). Mean (±SD) age was 62.2 ± 12.0 years and 47.5 % of the patients were 65 years or older. Of all the patients, 64.0 % were in Steinbrocker's stage III or IV and 25.9 % in functional class 3 or 4. Mean disease duration was 11.1 years and 44.4 % of the patients had RA for 10 years or longer. Comorbidities were reported in 2,590 patients; the most common comorbidity was osteoporosis in 1,229 patients, followed by hypertension, dyslipidaemia, interstitial pneumonia, and diabetes mellitus. Just before the initiation of TAC treatment, 79.2 % of the patients used DMARDs, including mainly methotrexate (MTX), salazosulfapyridine, and bucillamine. Just before the initiation of TAC treatment, biological DMARDs, etanercept or infliximab, were used in 9.7 % of the patients. Mean CRP level was 3.3 mg/dl and 3.9 % of the patients had a CRP of 10 mg/dl or higher. Baseline mean DAS28-CRP was 5.1 and 48.7 % of the patients had a DAS28-CRP of 5.1 or higher. As for other characteristics, there were more outpatients than inpatients, with the former accounting for 89.6 % of the population. Corticosteroid was administered in 81.5 % of the safety population and the mean daily dose in these concomitant users was 6.8 mg (prednisolone [PSL] equivalent dose). The dose of corticosteroid was 10 mg/day or higher in 16.3 %. TAC was used as a monotherapy (i.e., without other non-biological DMARDs) in 52.5 % and the rest of the patients received non-biological DMARDs other than TAC; MTX was used in 28.9 %, salazosulfapyridine in 14.3 %, and bucillamine in 7.4 %. Biological DMARDs were administered in 7.1 %; etanercept was used in 4.4 % and infliximab in 3.0 %.

Among the patients included in the safety population, 69.8 % continued the treatment until week 24, 27.8 % discontinued the treatment before week 24, and 2.3 % were lost to follow-up. Mean (±SD) daily doses of TAC at week 0 were 1.6 ± 0.8 mg/day in nonelderly patients (<65 years) and 1.4 ± 0.6 mg/day in elderly patients (≥65 years). Mean (±SD) daily doses of TAC during the observation period were 1.9 ± 0.8 mg/day in nonelderly patients (<65 years) and 1.6 ± 0.6 mg/day in elderly patients (≥65 years).

Reasons for discontinuation were AEs in 14.3 %, lack of effectiveness in 7.2 %, patient's preference in 5.7 %, and improvement of signs and symptoms in 0.1 % (Table 2).

Of the 3,172 patients included in the safety population, 1,308 patients developed 2,292 AEs and 1,142 patients developed 1,855 ADRs; the incidences of AEs and ADRs were 41.2 and 36.0 %, respectively. The common system organ classification (SOC) categories for ADRs were abnormal laboratory values in 12.5 %, gastrointestinal disorders in 6.4 %, infections and infestations in 5.8 %, metabolism and nutrition disorders in 4.3 %, and renal and urinary disorders in 2.7 % (Table 3). The most frequently reported ADRs were pneumonia (1.0 %), diabetes mellitus (1.5 %), nausea (1.5 %), diarrhea (1.3 %), abnormal hepatic function (1.1 %), pruritus (1.0 %), renal impairment (1.2 %), elevation of white blood cell count (2.5 %), elevation of β-*N*-acetyl-D-glucosaminidase (2.1 %), elevation of blood urea (1.6 %), elevation of glycosylated hemoglobin (1.2 %), depletion of lymphocyte (1.2 %), elevation of blood creatinine (1.1 %), and elevation of urine β2 microglobulin (1.0 %). The overall incidence of ADRs was significantly higher in elderly patients compared to non-elderly patients (40.5 vs. 31.9 %, $p < 0.001$). The incidences of the following ADRs were higher in elderly than in nonelderly patients: abnormal laboratory values (14.5 vs. 10.7 %, $p = 0.001$), gastrointestinal disorders (7.4 vs. 5.5 %, $p = 0.035$), infections and infestations (6.9 vs. 4.9 %, $p = 0.015$), metabolism and nutrition disorders (5.8 vs. 2.9 %, $p < 0.001$), and renal and urinary disorders (3.8 vs. 1.6 %, $p < 0.001$) (Table 3).

Of the patients included in the safety population, 203 patients (6.4 %) developed 263 serious AEs and 157

Table 1 Patient characteristics of the safety population

Items All patients	Patients (%) 3,172
Sex	
Male	682 (21.5)
Female	2,490 (78.5)
Age (years)	
<20	9 (0.3)
20–29	40 (1.3)
30–39	103 (3.2)
40–49	258 (8.1)
50–64	1,256 (39.6)
65–74	1,088 (34.3)
≥75	418 (13.2)
Mean ± SD	62.2 ± 12.0
Inpatient/outpatient status	
Outpatient	2,843 (89.6)
Inpatient	329 (10.4)
Steinbrocker's stage classification (n = 3,134)	
I	256 (8.2)
II	873 (27.9)
III	1,010 (32.2)
IV	995 (31.7)
Steinbrocker's functional classification (n = 3,139)	
1	281 (9.0)
2	2,043 (65.1)
3	745 (23.7)
4	70 (2.2)
Disease duration (years) (n = 2,845)	
<3	576 (20.2)
≥3 to <5	339 (11.9)
≥5 to <10	666 (23.4)
≥10	1,264 (44.4)
Mean ± SD	11.06 ± 9.7
Comorbidity (n = 3163)	
No	573 (18.1)
Yes	2,590 (81.7)
Use of nonbiological DMARDs just before study entry*	
No	660 (20.8)
Yes	2,512 (79.2)
Use of nonbiological DMARDs just before study entry**	
Methotrexate	1,353 (42.7)
Salazosulfapyridine	809 (25.5)
Bucillamine	477 (15.0)
Use of biological DMARDs just before study entry*	
No	2,864 (90.3)
Yes	308 (9.7)
Use of biological DMARDs just before study entry**	
Etanercept	174 (5.5)
Infliximab	129 (4.1)

Table 1 continued

Items All patients	Patients (%) 3,172
CRP (mg/dl) (n = 2,374)	
<1.0	545 (23.0)
≥1.0 to <3.0	775 (32.6)
≥3.0 to <5.0	519 (21.9)
≥5.0 to <10.0	443 (18.7)
≥10.0	92 (3.9)
Mean ± SD	3.3 ± 3.0
DAS28-CRP (n = 680)	
≤3.2	13 (1.9)
>3.2 to ≤5.1	336 (49.4)
>5.1	331 (48.7)
Mean ± SD	5.1 ± 1.0
Concomitant corticosteroid***	
No	588 (18.5)
Yes	2,584 (81.5)
Dose of concomitant corticosteroid*** (prednisolone equivalent, mg/day) (n = 3,169)	
0 (non use)	588 (18.6)
0< to <5	621 (19.6)
≥5 to <7.5	1,077 (34.0)
≥7.5 to <10	365 (11.5)
≥10	518 (16.3)
Mean ± SD	6.8 ± 4.7
Concomitant nonbiological DMARD***	
No	1,665 (52.5)
Yes	1,507 (47.5)
Concomitant nonbiological DMARD**	
Methotrexate	916 (28.9)
Salazosulfapyridine	454 (14.3)
Bucillamine	236 (7.4)
Concomitant biological DMARD***	
No	2,947 (92.9)
Yes	225 (7.1)
Concomitant biological DMARD**	
Etanercept	140 (4.4)
Infliximab	94 (3.0)
Concomitant NSAID*** (n = 3,162)	
No	950 (30.0)
Yes	2,212 (70.0)

CRP C-reactive protein, *DAS28-CRP* disease activity score 28-CRP, *DMARD* disease modifying antirheumatic drug, *NSAID* non-steroidal anti-inflammatory drug

* Within 4 weeks of the start of treatment with TAC (For IFX, within 8 weeks); ** multiple response; *** drugs used before the date of onset of the first adverse drug reaction were included. In patients who did not develop adverse drug reactions, drugs which were used during the observation period were included

Table 2 Status of the treatment and reasons for discontinuation

Items	Patients (%)
Treatment status at week 24	
Continued	2,213 (69.8)
Discontinued before week 24	883 (27.8)
Lost to follow-up	74 (2.3)
Unknown	2 (0.1)
Reasons for discontinuation (multiple response)	
Adverse events	454 (14.3)
Lack of effectiveness	227 (7.2)
Patient's preference	181 (5.7)
Improvement of symptoms	3 (0.1)
Others	83 (2.6)

patients (4.9 %) developed 194 serious ADRs (Table 3). The most common SOC categories for serious ADRs were infections and infestations in 75 patients (2.4 %), followed by respiratory, thoracic and mediastinal disorders in 21 patients (0.7 %). Of 75 serious infections, 36 were pneumonia-related events (23 pneumonia, 4 *Pneumocystis jiroveci* pneumonia, 3 pneumonia bacterial, 2 bronchopneumonia, 2 pneumonia mycoplasmal, 1 pneumonia fungal, and 1 chlamydia pneumonia), and 5 were bronchitis. Tuberculosis was reported in 3 patients and two of them had been exposed to TNF inhibitors: one had used infliximab and etanercept prior to TAC administration and concomitantly received etanercept with TAC; the other had used infliximab prior to TAC administration. All patients were successfully treated with antibiotics. Serious impaired glucose tolerance-related ADR was reported in 9 patients (0.3 %) and serious renal impairment-related ADR was reported in 5 patients (0.2 %). Of 21 serious respiratory, thoracic and mediastinal disorders, 15 were interstitial pneumonia.

Almost half of the safety population was treated with other DMARDs, and 28.9 % were given MTX. Incidences of total ADRs and infection were 29.1 and 6.9 % in those with concomitant MTX and 38.8 and 6.8 % in those without MTX, respectively. Incidence of total ADRs and infection didn't increase in patients who used concomitant MTX. Incidence of total ADRs and infection in elderly patients didn't differ between those who concomitantly received MTX (34.3 and 7.8 %) and those who did not (42.2 and 7.5 %).

We identified risk factors of ADRs using multivariate Cox proportional hazards models (Table 4). The increased risk for overall ADRs was associated with the following patient characteristics at baseline: age ≥65 years, concurrent renal dysfunction, and concurrent diabetes mellitus. Risk factors were also explored for several important ADRs of TAC. For these analyses, we included 243 infectious events, 271 renal impairment events and 183 impaired glucose tolerance events. Definitions for these events are described in the legend of Table 4. Risk factors for infections were Steinbrocker's functional class 3 or 4, and dose of concomitant corticosteroids ≥10 mg. Risk factors for renal impairment were age ≥65 years, concurrent renal dysfunction, and concomitant use of NSAIDs. Risk factors for impaired glucose tolerance were concurrent diabetes mellitus and dose of concomitant corticosteroids ≥10 mg.

The response rate according to the EULAR criteria at week 24 was 65.4 % (good response in 28.1 % and moderate response in 37.4 %) in 680 patients, using the LOCF method (Fig. 1). Stratification of the patients revealed that elderly ($n = 373$) and nonelderly patients ($n = 307$) showed comparable response rates (66.5 vs. 64.2 %) and so did those with ($n = 178$) and without ($n = 502$) concomitant MTX at baseline (64.6 vs. 65.7 %). At baseline, 48.7, 49.4 and 1.9 % of the patients had high, moderate and low disease activity, respectively, whereas at week 24, the rate for high disease activity decreased to 17.8 % and that for low disease activity increased to 33.7 %, including remission in 19.3 % (Fig. 2). Mean (±SD) DAS28-CRP were 5.1 (±1.0) at baseline and decreased to 3.9 (±1.4) at week 24.

Discussion

This is the first report that describes the safety and effectiveness of treatment with TAC in clinical practice using data from a large prospective cohort of RA patients. Safety and effectiveness of TAC in this study exhibited similar profiles to those reported in clinical trial settings in RA patients who had shown insufficient response to conventional treatments [8, 9, 12].

As for drug safety, overall incidence of ADRs in the present study was 36.0 %, which was relatively lower than that reported in clinical trials (36.0–68.4 %) (unpublished data). The lower overall incidence of ADRs compared to clinical trials is mainly attributed to the lower rate of abnormal changes in laboratory test values such as renal functions and glucose tolerance in this study. Possible reasons for this difference include less stringent protocol of the PMS study compared to previous clinical trials in terms of frequency of laboratory examination and lack of direct monitoring by a pharmaceutical company, and lower average dose (1.8 mg/day) and lower starting dose (1.5 mg/day) of TAC.

Since TAC is frequently used in RA patients who had inadequate response to or were intolerant to MTX, we compared the results of this study with those from PMS studies for biological DMARDs in Japanese patients with

Table 3 Incidences of ADRs and serious ADRs by SOC classification

	ADRs	Serious ADRs	ADRs	
			Elderly (≥65 years) (n = 1,506)	Nonelderly (<65 years) (n = 1,666)
Number of patients with ADRs	1,142	157	610	532
Number of ADRs	1,855	194	1,018	837
Incidence of ADRs (%)	36.0	4.9	40.5	31.9
ADR types (system organ class)				
Infections and infestations	185 (5.8)	75 (2.4)	104 (6.9)	81 (4.9)
Bacteremia	2 (0.1)	2 (0.1)	2 (0.1)	0
Bronchopneumonia	3 (0.1)	2 (0.1)	2 (0.1)	1 (0.1)
Herpes zoster	12 (0.4)	2 (0.1)	6 (0.4)	6 (0.4)
Pneumonia	33 (1.0)	23 (0.7)	21 (1.4)	12 (0.7)
Pneumonia chlamydial	1 (0.0)	1 (0.0)	1 (0.1)	0
Pneumonia mycoplasmal	2 (0.1)	2 (0.1)	1 (0.1)	1 (0.1)
Pulmonary tuberculosis	3 (0.1)	3 (0.1)	1 (0.1)	2 (0.1)
Sepsis	4 (0.1)	4 (0.1)	2 (0.1)	2 (0.1)
Pneumonia bacterial	7 (0.2)	3 (0.1)	4 (0.3)	3 (0.2)
Pneumonia fungal	1 (0.0)	1 (0.0)	1 (0.1)	0
Pneumocystis jiroveci pneumonia	4 (0.1)	4 (0.1)	3 (0.2)	1 (0.1)
Neoplasms benign, malignant and unspecified (incl. cysts and polyps)	7 (0.2)	7 (0.2)	3 (0.2)	4 (0.2)
Blood and lymphatic system disorders	21 (0.7)	2 (0.1)	10 (0.7)	11 (0.7)
Immune system disorders	1 (0.0)	1 (0.0)	0	1 (0.1)
Metabolism and nutrition disorders	135 (4.3)	11 (0.4)	87 (5.8)	48 (2.9)
Psychiatric disorders	10 (0.3)	1 (0.0)	3 (0.2)	7 (0.4)
Nervous system disorders	81 (2.6)	14 (0.4)	47 (3.1)	34 (2.0)
Eye disorders	7 (0.2)	0	4 (0.3)	3 (0.2)
Ear and labyrinth disorders	5 (0.2)	2 (0.1)	2 (0.1)	3 (0.2)
Cardiac disorders	31 (1.0)	11 (0.4)	15 (1.0)	16 (1.0)
Vascular disorders	36 (1.1)	1 (0.0)	19 (1.3)	17 (1.0)
Respiratory, thoracic and mediastinal disorders	67 (2.1)	21 (0.7)	29 (1.9)	38 (2.3)
Interstitial pneumonia	17 (0.5)	15* (0.5)	9 (0.6)	8 (0.5)
Gastrointestinal disorders	203 (6.4)	9 (0.3)	111 (7.4)	92 (5.5)
Hepatobiliary disorders	49 (1.5)	4 (0.1)	19 (1.3)	30 (1.8)
Skin and subcutaneous tissue disorders	116 (3.7)	2 (0.1)	57 (3.8)	59 (3.5)
Musculoskeletal and connective tissue disorders	18 (0.6)	0	6 (0.4)	12 (0.7)
Renal and urinary disorders	84 (2.7)	4 (0.1)	57 (3.8)	27 (1.6)
Reproductive system and breast disorders	5 (0.2)	1 (0.0)	1 (0.1)	4 (0.2)
General disorders and administration site conditions	69 (2.2)	4 (0.1)	37 (2.5)	32 (1.9)
Laboratory test abnormal	397 (12.5)	8 (0.3)	219 (14.5)	178 (10.7)
Injury, poisoning and procedural complications	5 (0.2)	2 (0.1)	3 (0.2)	2 (0.1)

SOC system organ class

RA. The incidence rate for ADRs was 27.3 % for tocilizumab, 28.0 % for infliximab, 30.6 % for etanercept, and 35.5 % for adalimumab [13–16]. The incidence of serious ADRs in this study was 4.9 %, which didn't differ from the results of adalimumab (4.1 %), etanercept (5.7 %), infliximab (6.2 %) and tocilizumab (7.2 %) [13–16].

In the present study, metabolism and nutrition disorders, renal and urinary disorders, abnormal laboratory values, gastrointestinal disorders, and infections and infestations were frequently reported. These are known ADRs of TAC when used in transplant recipients [17–19]. Regarding safety in elderly RA patients aged 65 years or older, 1,018 ADRs were reported in 610 out of 1,506 patients (40.5 %). The common ADRs revealed in elderly patients in previous clinical trials of TAC included infections, renal impairment, gastrointestinal disorders, skin disorders and

Table 4 Patient characteristics at baseline as risk factors for ADRs

Factor	Hazard ratio	*p* value	95 % CI
Overall (ADRs)			
Age (≥65 vs. <65 years)	1.21	0.020	1.03–1.42
Concurrent renal dysfunction (presence vs. absence)	1.32	0.007	1.08–1.61
Concurrent diabetes mellitus (presence vs. absence)	1.60	<0.001	1.33–1.93
Infections			
Functional class (≥3 vs. ≤2)	1.45	0.042	1.01–2.08
Dose of concomitant corticosteroids (0–10 vs. 0 mg)	0.99	0.962	0.64–1.53
Dose of concomitant corticosteroids (≥10 vs. 0 mg)	1.68	0.047	1.01–2.80
Renal impairment			
Age (≥65 vs. <65 years)	1.59	0.004	1.16–2.17
Concurrent renal dysfunction (presence vs. absence)	1.90	<0.001	1.36–2.67
Concomitant NSAIDs (use vs. non use)	1.67	0.005	1.17–2.40
Impaired glucose tolerance			
Concurrent diabetes mellitus (presence vs. absence)	5.63	<0.001	3.85–8.21
Dose of concomitant corticosteroids (≥10 vs. 0 mg)	2.36	0.012	1.20–4.62

Infectious events (84 serious and 159 non-serious) for this analysis mainly included pneumonia (23 serious and 10 non-serious), upper respiratory tract infection (21 non-serious), nasopharyngitis (19 non-serious)

Renal impairment events (7 serious and 264 non-serious) for this analysis mainly included elevation of β-*N*-acetyl-D-glucosaminidase (68 non-serious), elevation of blood urea (2 serious and 50 non-serious), renal impairment (1 serious and 36 non-serious)

Impaired glucose tolerance events (9 serious and 174 non-serious) for this analysis mainly included diabetes mellitus (7 serious and 40 non-serious), elevation of glycosylated hemoglobin (39 non-serious), glucose tolerance impaired (1 serious and 29 non-serious), elevation of blood glucose (30 non-serious)

Concurrent renal dysfunction included membranous nephropathy (6 patients), interstitial nephritis (4 patients), IgA nephropathy (3 patients), lupus nephritis (2 patients), renal amyloidosis (2 patients) and other renal dysfunction (392 patients)

NSAID non-steroidal anti-inflammatory drug

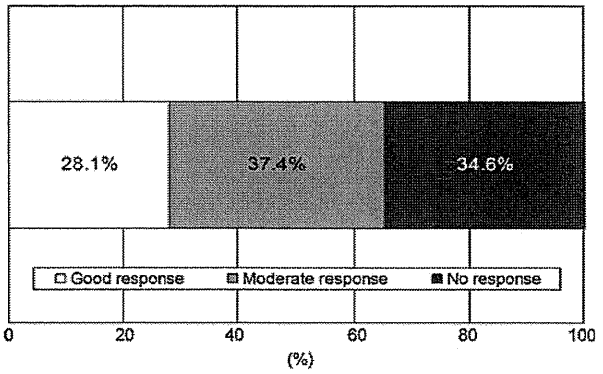

Fig. 1 Response to treatment according to the EULAR criteria ($n = 680$). The response rate was defined as the proportion of patients with good or moderate response

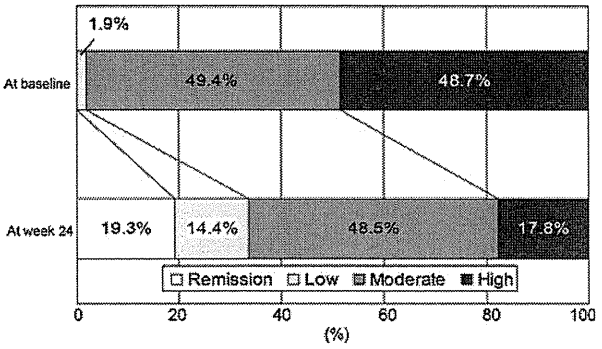

Fig. 2 Disease activity of rheumatoid arthritis at baseline and at the end of observation using the last observation carried forward (LOCF) method. Disease activity was defined using DAS28-CRP scores as follows: remission, DAS28-CRP < 2.6; low disease activity, 2.6 ≤ DAS28-CRP ≤ 3.2; moderate disease activity, 3.2 < DAS28-CRP ≤ 5.1; high disease activity, 5.1 < DAS28-CRP

abnormal glucose tolerance; these results are similar to those obtained in the present study.

Infection was the most frequently reported serious ADR in this study. Of 75 serious infectious events, 39 were pulmonary infections, including 23 pneumonia. It has been reported that pulmonary infection, especially pneumonia, is the major site-specific infection in RA [13–16, 20–26]; this is compatible with the results of this study. In this study, we identified advanced functional class and dosage of concomitant corticosteroid as risk factors for infections using multivariate analysis. Usage or dosage of corticosteroid are reported as risk factors for infections in various cohort studies for RA and in Japanese PMS studies for biological DMARDs as well [14–16, 21, 22, 27–31].

Risk factors for impaired glucose tolerance were concurrent diabetes mellitus and concomitant use of corticosteroids at doses of ≥10 mg (PSL equivalent). In the present study, 17.7 % of patients had diabetes mellitus at baseline and a higher percentage of these patients (17.6 %) reported impaired glucose tolerance as AE compared to those who did not have diabetes mellitus, suggesting that diabetes mellitus should be checked before starting TAC. In light of the influence on infection and diabetes mellitus, dose reduction of corticosteroids should be considered in patients with improved signs and symptoms of RA. The mean dose of corticosteroids used in this study was 6.8 mg/day at baseline and 6.1 mg/day at week 24. Furthermore, at week 24, 4.0 % ($n = 69$) of patients withdrew from corticosteroid therapy. The mean dose of corticosteroids in the