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Calcineurin inhibitors exert rapid reduction of inflammatory pain in rat adjuvant-induced arthritis

[1]Katsue Magari, *,[1]Susumu Miyata, [1]Yoshitaka Ohkubo, [1]Seitaro Mutoh & [1]Toshio Goto

[1]Medicinal Biology Research Laboratories, Fujisawa Pharmaceutical Co., Ltd, 2-1-6 Kashima, Yodogawa-ku, Osaka 532-8514, Japan

1 FK506 and cyclosporin A (CsA) are immunosuppressive drugs, that specifically inhibit T-cell activation *via* calcineurin inhibition. This study was undertaken to investigate whether calcineurin inhibitors exert analgesic actions in rat adjuvant-induced arthritis (AIA), an animal model of rheumatoid arthritis (RA).

2 AIA was induced in female Lewis rats. Single doses of FK506 and CsA were orally administered to arthritic rats 17 days after arthritis induction. Intensity of hyperalgesia was assessed by measuring the pain threshold of hind paws. Tumor necrosis factor (TNF)-α, IL-1β and PGE_2 levels in paw extracts were determined by ELISA. TNF activity was measured by L929 cell cytotoxicity assay. IL-1β and cyclooxygenase (COX) mRNA expression in arthritic paws were measured by RT–PCR.

3 Single doses of FK506 and CsA markedly reduced joint hyperalgesia 24 h after drug administration, without affecting inflammation in an advanced stage of AIA.

4 The calcineurin inhibitors partially reduced the elevated level of TNF-α in arthritic paws, however, the analgesic effects of these drugs were not associated with the reduction in TNF-α level.

5 Moreover, treatment with anti-rat TNF-α antibody did not affect the hyperalgesia, when TNF-α activity was suppressed in arthritic paws by that treatment.

6 Both calcineurin inhibitors reduced the elevated level of IL-1β in arthritic paws to a normal level, 24 h after drug administration.

7 FK506 reduced IL-1β and COX-2 mRNA expression and PGE_2 level in arthritic paws.

8 In conclusion, calcineurin inhibitors rapidly reduce joint hyperalgesia probably by downregulating IL-1β, but not TNF-α, in AIA. Our findings may provide a new strategy for the treatment of pain in RA.

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Keywords: Hyperalgesia; interleukin-1β; tumor necrosis factor-α; adjuvant-induced arthritis; rheumatoid arthritis; FK506; cyclosporin A

Abbreviations: AIA, adjuvant-induced arthritis; COX, cyclooxygenase; CsA, cyclosporin A; NSAID, nonsteroidal anti-inflammatory drug; RA, rheumatoid arthritis; TNF, tumor necrosis factor

Introduction

Rheumatoid arthritis (RA) is a chronic disease characterized by joint inflammation with concomitant destruction of cartilage and bone. RA patients suffer from joint pain that is not satisfactorily controlled with current therapies. Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of pain associated with RA as well as other immunological and nonimmunological diseases, although their use is strongly limited by gastrointestinal side effects (Lane, 1997). Recent evidence supports the hypothesis that T cells play a central role in initiating and perpetuating the chronic autoimmune responses associated with RA (Panayi *et al*., 1992). Additionally, inflammatory cytokines such as tumor necrosis factor (TNF)-α and IL-1β are reported to be responsible for the pathogenesis of RA. TNF-α has been characterized as a crucial cytokine in the pathogenesis of RA, based on the marked clinical efficacy of anti-TNF-α therapy (Maini & Taylor, 2000; Feldmann & Maini, 2001).

*Author for correspondence;
E-mail: susumu_miyata@po.fujisawa.co.jp

Adjuvant-induced arthritis (AIA) has been used in preclinical studies as a standard animal model of RA in humans. Hyperalgesia (inflammatory pain) is detected during development of AIA (Billingham, 1983; Sakuma *et al*., 2001). Prostaglandins formed by cyclooxygenase (COX)-2 cause inflammation and hyperalgesia in AIA (Anderson *et al*., 1996; Portanova *et al*., 1996). The mechanism of analgesic action of NSAIDs has been shown to result from a blockade of prostaglandin synthesis by inhibition of COX-2 (Hawkey, 1999). On the other hand, TNF-α and interleukin-1β (IL-1β) have been reported to cause hyperalgesia in animal models (Ferreira *et al*., 1988; Cunha *et al*., 1992; Safieh-Garabedian *et al*., 1995; Woolf *et al*., 1997; Martin, 1999). Local injection of IL-1β induces hyperalgesia *via* the release of secondary mediators such as prostaglandins or nerve growth factor (Ferreira *et al*., 1988; Safieh-Garabedian *et al*., 1995), while TNF-α produces hyperalgesic states by activating a cascade of multiple cytokine release, including IL-1β (Cunha *et al*., 1992; Woolf *et al*., 1997).

FK506 (tacrolimus) and cyclosporin A (CsA) are immunosuppressive drugs that specifically suppress T-cell activation

(Kino *et al*., 1987; Ho *et al*., 1996). These agents exert their immunosuppressive effects after binding to intracellular proteins termed immunophilins; FK506 binding proteins (FKBP) and cyclophilin, respectively. The drug–immunophilin complex inhibits calcineurin phosphatase, an enzyme involved in activation of transcription factor NF-AT, required for the expression of cytokine gene in T cells (Ho *et al*., 1996). We recently showed that these calcineurin inhibitors specifically suppress T-cell activation-mediated inflammatory cytokine production *in vitro* (Sakuma *et al*., 2000). It has been suggested that inflammatory cytokines are produced through activation of T cells and by subsequent interaction between the activated T cells and monocytes/macrophages in RA (Panayi *et al*., 1992). Therefore, we hypothesized that inhibition of T-cell activation and its triggered inflammatory cytokine production might be a potential treatment for pain in RA. Here, we report that the calcineurin inhibitors, FK506 and CsA, rapidly reduce hyperalgesic states probably by down-regulating IL-1β in an advanced stage of AIA.

Methods

Induction of arthritis

Female Lewis rats were obtained from Charles River Japan, Inc. (Kanagawa, Japan) and bred in a clean atmosphere. Arthritis was induced by injection of 0.5 mg of heat-killed *Mycobacterium tuberculosis* in 50 μl of liquid paraffin into the right hind footpad of Lewis rats aged 7 weeks. All experimental procedures were reviewed and approved by the Fujisawa Pharmaceutical Animal Experiment Committee.

Drug treatment

FK506 was suspended in distilled water (DW), CsA was dissolved in olive oil and indomethacin was suspended in 0.5% methylcellulose. Arthritic rats were randomized and grouped on the basis of pain threshold or paw swelling before drug administration on day 17 (17 days after arthritis induction) to exclude animals that have not responded appropriately to the pain stimulus. FK506 (10 mg kg^{-1}), CsA (30 mg kg^{-1}), indomethacin (1 mg kg^{-1}) or vehicle (DW for FK506 and indomethacin or olive oil for CsA) were orally administered after grouping.

Hyperalgesia measurement

The intensity of hyperalgesia was assessed using the method of Randall–Selitto (Randall & Selitto, 1957), with some modification. The mechanical pain threshold of the left hind paw was determined by compressing the ankle joint with a balance pressure apparatus (Ugo Basile, Varese, Italy). The threshold pressure of rats squeaking or struggling was expressed in grams. Observers were unaware of the drug treatment group in the measurement of hyperalgesia.

Evaluation of paw inflammation

Paw inflammation was quantified based on paw swelling and histological change. The volume of the left hind paw was measured before and after arthritis induction by a water displacement method using a plethysmometer for rats. Paw swelling was presented as a change in the hind paw volume. For histological assessment, the left hind paw was dissected above the ankle joint and fixed in 10% neutral buffered formalin. The samples were decalcified, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). Histological analysis was carried out on the basis of infiltration of inflammatory cells, edema and connective tissue hyperplasia. Lesion degree was classified into five grades. The sum of the three parameters (scored 0–4) was presented as the total histological score.

Preparation of paw sample

The left hind paw of each rat was dissected above the ankle joint, snap-frozen in liquid nitrogen and stored at -80°C until used. Before homogenization for each assay, the frozen paw containing bony tissue was weighed and broken into pieces on dry ice.

Determination of IL-1β, TNF-α and PGE_2 levels

For measurement of IL-1β and TNF-α levels, the paw tissues were added to 4 ml g tissue^{-1} of extraction buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 μg ml^{-1} aprotinin and 0.05% Tween-20 in phosphate-buffered saline (PBS). For measurement of PGE_2, the paw tissues were added to 4 ml g tissue^{-1} of lysis solution containing 80% methanol, 20% saline and 1 mM indomethacin. Tissues were homogenized on ice with a polytron and centrifuged at $5000 \times g$ for 15 min. The supernatants were stored at -80°C until analyzed. IL-1β and TNF-α levels in the supernatants were determined using ELISA kits specific for rat IL-1β and TNF-α. PGE_2 level was determined using a PGE_2 EIA kit. The sensitivities of the assays for IL-1β, TNF-α and PGE_2 were 12, 5 and 15 pg ml^{-1}, respectively. Percentage of reduction was calculated using the following formula: % reduction $= (1-B/A) \times 100$, where $A =$ vehicle treated $-$ normal, $B =$ drug treated $-$ normal.

Treatment of AIA with anti-TNF-α antibody and measurement of TNF activity

Polyclonal rabbit anti-TNF-α antibody (2 mg kg^{-1}) or PBS as vehicle control was injected intraperitoneally into arthritic rats 17 days after adjuvant injection. The pain threshold and TNF activity in the left hind paws were measured 3–24 h after antibody treatment. For measurement of TNF activity, the paw tissues were added to 4 ml g tissue^{-1} of saline. Tissues were homogenized on ice with a polytron and centrifuged at $5000 \times g$ for 15 min. Supernatants were stored at -80°C until analysis. TNF activity in the supernatants was determined by the mouse fibroblast L929 cell cytotoxicity assay, as previously reported (Smith-Oliver *et al*., 1993), with some modification. Briefly, L929 cells were suspended in media (DMEM supplemented with 10% FBS, 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin) containing the 1 μg ml^{-1} actinomycin D and plated at 1.5×10^4 cells 50 μl^{-1} in each well of a 96-well flat bottom plate. Media (50 μl^{-1}) containing the TNF sample was added to each well and incubated for 18 h followed by MTT assay. TNF activity was expressed as a concentration of standard rat TNF-α equivalent to the activity in a paw sample. TNF activity found in normal rat paw extracts was regarded as

nonspecific TNF-like activity, as the level of nonspecific TNF-like activity in paw samples was undetermined. Thus, specific TNF activity in arthritic control or anti-TNF-α-treated rats was calculated by subtracting TNF activity in normal rat samples from that in the measured samples.

RT–PCR

Total RNA was extracted from the paw sample using TRIzol, according to the manufacturer's instructions. Complementary DNA was prepared by reverse transcriptase. IL-1β, COX-1 and COX-2 transcripts were amplified by 26–30 cycles of PCR using the following primers; IL-1β, forward: 5′-CCAGGATGAGGACCCAAGCA-3′, reverse: 5′-TCCCGACCATTGCTGTTTCC-3′; COX-1, forward: 5′-TTTGCACAACACTTCACCCACCAG-3′, reverse: 5′-AAACACCTCCTGGGCCACAGCCAT-3′; COX-2, forward: 5′-ACTTGCTCACTTTGTTGAGTCATTC-3′, reverse: 5′-TTTGATTAGTACTGTAGGGTTAATG-3′. β-Actin primers were used as internal control to normalize the sample amounts. Agarose gels (2%) were stained with ethidium bromide and the band intensity was quantified using ATTO Densitograph software (Atto, Tokyo, Japan). IL-1β and COX mRNA expression levels were presented as a ratio relative to β-actin expression.

Materials

L929 cells were purchased from RIKEN Cell Bank (Ibaragi, Japan). A solid dispersion formulation of FK506 (Honbo *et al*., 1987) and CsA was prepared at Fujisawa Pharmaceutical Co., Ltd (Osaka, Japan). Heat-killed *M. tuberculosis* was purchased from Difco (Detroit, MI, U.S.A.), phenylmethylsulfonyl fluoride from Nacalai tesque (Kyoto, Japan), aprotinin from Sigma (St Louis, MO, U.S.A.), polyclonal rabbit anti-TNF-α antibody from R&D systems Inc. (Minneapolis, MN, U.S.A.), rat TNF-α from Pepro Tech EC Ltd (London, U.K.) and TRIzol from Life Technologies (Grand Island, NY, U.S.A.). ELISA kit for rat IL-1β was purchased from Endogen (Woburn, MA, U.S.A.), ELISA kit for rat TNF-α from Genzyme (Cambridge, MA, U.S.A.), PGE_2 EIA kit from Cayman (Ann Arbor, MI, U.S.A.) and Cell Proliferation kit I (MTT) from Roshe Diagnostic GmbH (Mannheim, Germany). All other chemicals were the highest grade commercially available.

Statistical analysis

Results are presented as mean ± s.e. Differences between vehicle and drug treatment groups were determined using Dunnett's multiple comparison test or two-tailed *t*-test. Differences before and after arthritis induction were determined using the two-tailed *t*-test. *P*-values <0.05 were considered statistically significant.

Results

Calcineurin inhibitors reduce joint hyperalgesia without affecting inflammation in an advanced stage of AIA

Hyperalgesia, as measured by the fall in pain threshold, was observed 10 days after arthritis induction, and was sustained through to day 21 during development of arthritis assessed by paw swelling (Figure 1). To investigate whether a calcineurin inhibitor, FK506, exerts direct analgesic effects on hyperalgesia in AIA, FK506 was administered to rats as a single dose on day 17, when the arthritis was fully established and hyperalgesia reached a plateau. The effect was compared with the NSAID, indomethacin. Indomethacin caused an increase in the pain threshold 3 h after drug administration but the analgesic effect disappeared by 24 h (Figure 2a). In contrast, FK506 did not affect the pain threshold up to 6 h after drug administration, however, it markedly reduced joint hyperalgesia 24 h after drug administration. The pain thresholds of FK506-treated rats were almost equal to those of indomethacin-treated or nonarthritic rats at this stage. FK506 did not affect the pain threshold of normal rats (data not shown). We further examined the effect of another calcineurin inhibitor, CsA, on hyperalgesia in this model. CsA caused an increase in the pain threshold in a similar fashion to FK506 and the analgesic effect at 24 h was almost equal to that of FK506 (Figure 2b).

To examine whether the analgesic effects of FK506 and CsA are secondary to suppression of inflammation, we assessed the degree of paw inflammation on the basis of histological change and paw swelling in AIA. The effect of agents on paw inflammation was assessed 24 h after drug administration, since the maximum suppression of hyperalgesia was observed at 24 h. Histological analysis showed that normal joints were clear of any signs of inflammation (Figure 3a). In contrast, the joints of arthritic controls had pronounced inflammation characterized by severe infiltration of inflammatory cells into the synovium and bone marrow, and synovial hyperplasia (Figure 3b). Neither FK506 nor CsA significantly affected histological scores for inflammation (Figure (3c and d) or paw swelling (Figure 3e).

Effects of calcineurin inhibitors on TNF-α level in arthritic paws

The effects of FK506 and CsA on the levels of TNF-α in arthritic paws were examined 3 and 24 h after drug administration, when the drugs showed no and maximum effect on hyperalgesia, respectively. FK506 caused a marked reduction

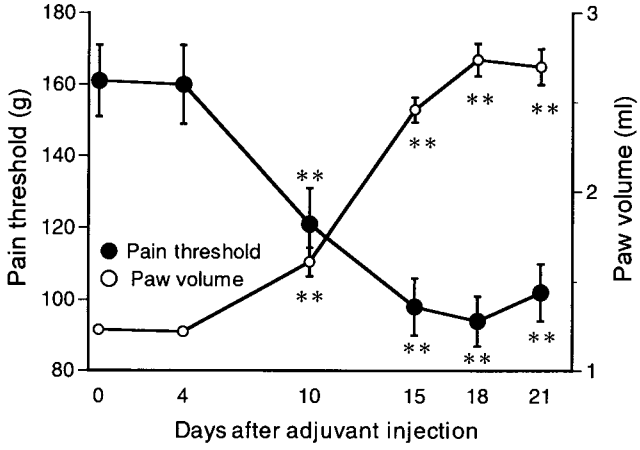

Figure 1 Change in pain threshold during development of AIA. Pain threshold in ankle joint and paw volume was measured before (day 0) and 4, 10, 15, 18, 21 days after arthritis induction. Data represent mean ± s.e. of 10 animals per group. $^{**}P<0.01$ compared to the group before adjuvant injection.

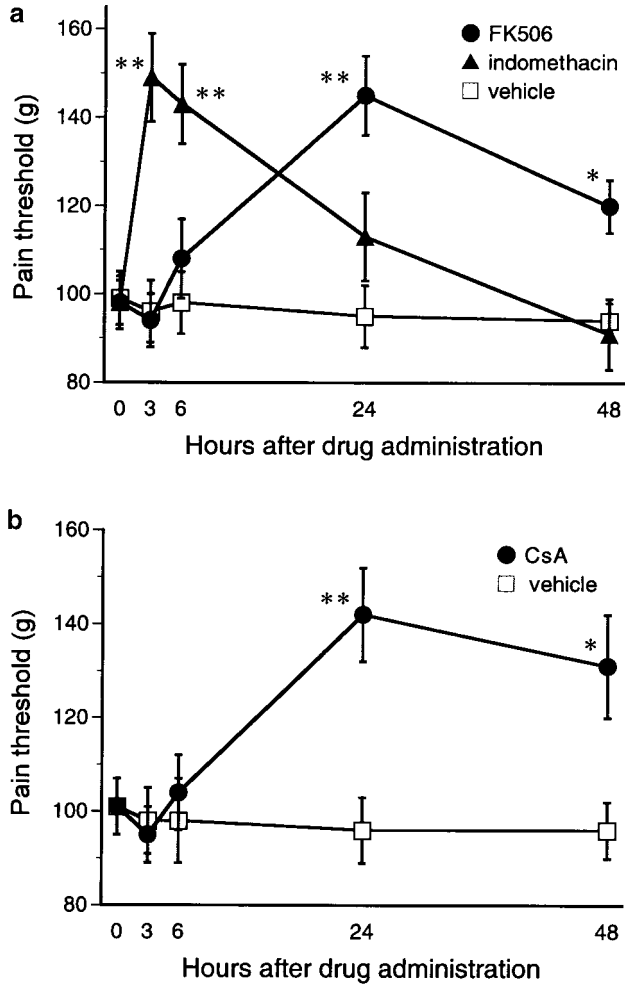

Figure 2 Single doses of calcineurin inhibitors reduce joint hyperalgesia in AIA. Arthritic rats were orally administered 10 mg kg^{-1} FK506, 1 mg kg^{-1} indomethacin or vehicle (a), and 30 mg kg^{-1} CsA or vehicle (b) on day 17. Pain thresholds were measured before and 3, 6, 24 and 48 h after drug administration. Averages of pain thresholds of normal nonarthritic rats were between 155 and 163 g in the experimental period. Data represent mean ± s.e. of 10 animals per group. $*P<0.05$, $**P<0.01$ compared to the vehicle controls.

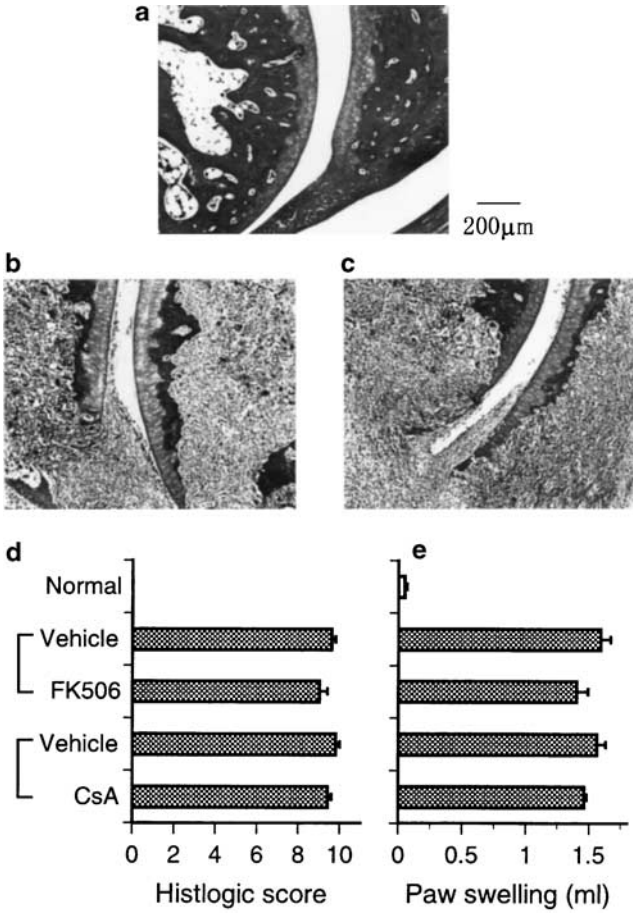

Figure 3 Effect of calcineurin inhibitors on paw inflammation in AIA. Arthritic rats were orally administered 10 mg kg^{-1} FK506, 30 mg kg^{-1} CsA or each vehicle on day 17. Joint histological change and paw swelling were evaluated 24 h after drug administration. Representative photomicrographs of H&E-stained ankle joints from normal rats (a), vehicle- (b) and FK506 (c) treated rats are shown. Severe infiltration of inflammatory cells into the synovium and bone marrow, and synovial hyperplasia were characteristics observed on days 17–18. FK506 did not reduce the inflammation in arthritic joints. Histological scores for inflammation (d) and paw swelling (e) are represented as mean ± s.e. of five animals per group.

of TNF-α level even at 3 h (64%), equivalent to that observed at 24 h (67%) (Figure 4a), although it did not affect the intensity of hyperalgesia 3 h after drug administration. CsA reduced TNF-α levels only by 41% at 24 h (Figure 4b) and the effect even at this time was less than that of FK506 at 3 h.

Effect of TNF-α blockade on joint hyperalgesia

The analgesic effects of calcineurin inhibitors were not associated with reduction of TNF-α level in arthritic paws. To investigate the involvement of TNF-α in hyperalgesia in AIA, we tested the effect of treatment with anti-rat TNF-α antibody on joint hyperalgesia in AIA. Intraperitoneal injection of polyclonal rabbit anti-rat TNF-α antibody (2 mg kg^{-1}) reduced TNF activity in arthritic paws by 82 and 59%, 3 and 24 h after drug administration, respectively (Figure 5a). However, it did not affect joint hyperalgesia between 3–24 h (Figure 5b). These results indicate that TNF-α blockade did not result in rapid reduction of hyperalgesia in AIA.

Calcineurin inhibitors potently reduce IL-1β level in arthritic paws

The effects of FK506 and CsA on the levels of IL-1β in arthritic paws were examined 3 and 24 h after drug administration. As shown in Figure 6a and b, IL-1β level of vehicle treated arthritic rats (> 2.0 ng g tissue^{-1}) was more than the 10-fold higher than the TNF-α level of those rats (< 0.2 ng g tissue^{-1}) (Figure 4a and b). Both FK506 and CsA weakly reduced the elevated level of IL-1β in arthritic paws at 3 h but completely reduced IL-1β to normal levels at 24 h (Figure 6a and b).

Effect of FK506 on COX mRNA expression and PGE_2 level in arthritic paws

IL-1β upregulates the expression of an inducible isoform of cyclooxygenase (COX-2), but does not affect the expression of the constitutive isoform (COX-1) (Crofford *et al.*, 1994). Prostaglandins formed by COX-2 cause inflammation and

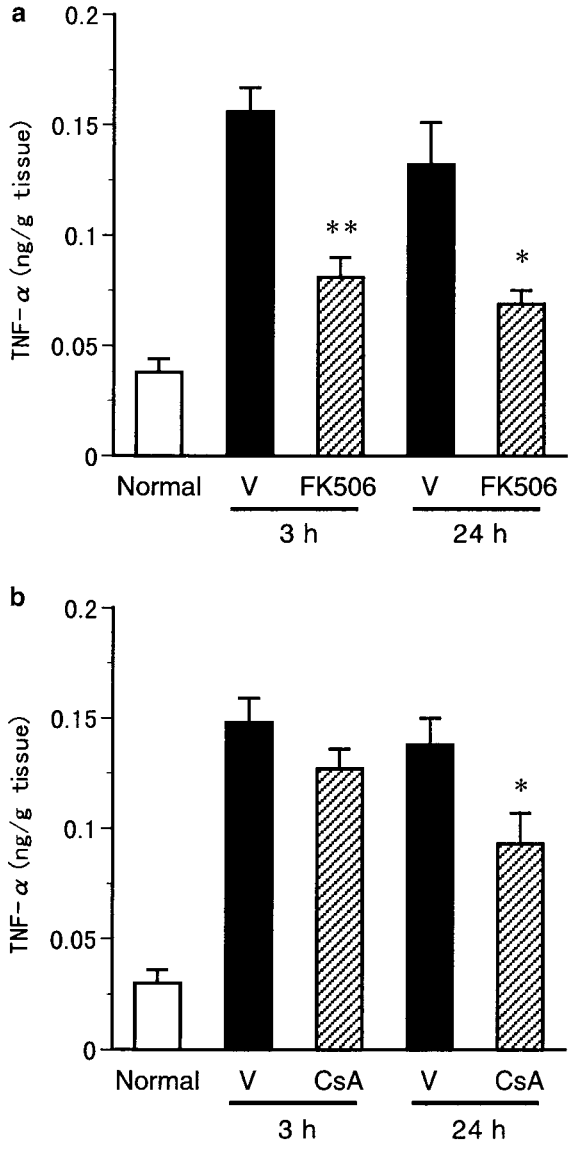

Figure 4 Effect of calcineurin inhibitors on TNF-α level in arthritic paws. Arthritic rats were orally administered 10 mg kg^{-1} FK506 (a), 30 mg kg^{-1} CsA (b) or each vehicle on day 17 after arthritis induction. Paw tissues were dissected 3 and 24 h after drug administration. TNF-α levels in the paw extracts were determined by ELISA. Data represent mean ± s.e.of five animals per group. $*P<0.05$, $**P<0.01$ compared to the vehicle controls (V) at the same hour after drug administration.

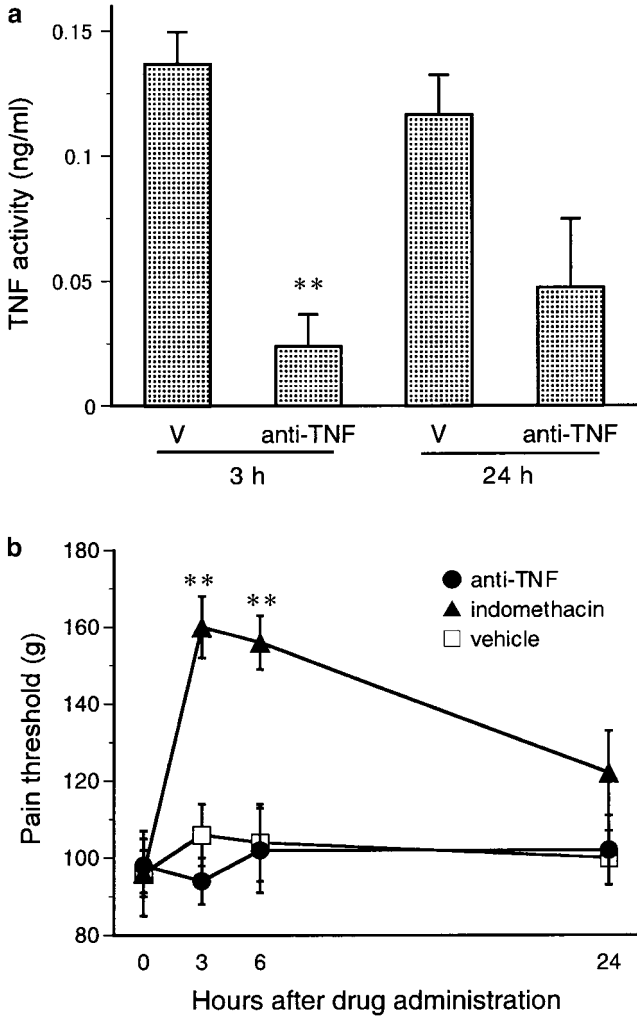

Figure 5 Effect of anti-TNFα antibody on joint hyperalgesia and TNF activity in arthritic paws. Arthritic rats were intraperitoneally administered 2 mg kg^{-1} polyclonal rabbit anti-rat TNFα antibody (anti-TNF), PBS as a vehicle control, or orally 1 mg kg^{-1} indomethacin on day 17. (a) TNF activities in paw extracts were measured 3 and 24 h after drug administration by L929 cytotoxicity assay. TNF activity was expressed as a concentration of standard rat TNF-α equivalent to the activity in a paw sample. Specific TNF activity in arthritic control or anti-TNF-α-treated rats was calculated by subtracting nonspecific TNF activity (0.142 ng ml^{-1}) in normal rat samples from that in the measured samples. (b) Pain thresholds were measured before and 3, 6 and 24 h after drug administration. Data represent mean ± s.e. of five animals per group. $*P<0.05$, $**P<0.01$ compared to the vehicle controls (V).

hyperalgesia in AIA (Anderson *et al*., 1996; Portanova *et al*., 1996). Therefore, we next examined the effect of FK506 on IL-1β and COX mRNA expression and prostaglandin E_2 (PGE_2) level in arthritic paws. FK506 suppressed expression of IL-1β and COX-2 but not COX-1 mRNA at 24 h (Figure 7a and b). Further, FK506 reduced the elevated level of PGE_2 at 24 h (Figure 7c), although the suppression was slightly less potent than that by indomethacin.

Discussion

Immunosuppressive drugs suppress the development of arthritis in long-term treatments by affecting differentiation and proliferation of immune cells. Suppression of joint inflammation results in reduction of inflammatory pain, hyperalgesia. In fact, our previous report (Sakuma *et al*., 2001) and a much earlier report on CsA (Francischi *et al*., 1997) showed that consecutive treatment with FK506 or CsA suppresses hyperalgesia in AIA when edema is suppressed by these agents. The present study revealed that immunosuppressive drugs, which inhibit T-cell activation, rapidly reduce inflammatory pain even in the presence of severe inflammation in an animal model of RA.

TNF-α and IL-1β have been demonstrated to cause hyperalgesia in animal models (Ferreira *et al*., 1988; Cunha *et al*., 1992; Safieh-Garabedian *et al*., 1995; Woolf *et al*., 1997; Martin, 1999). During T-cell activation, TNF-α is produced from both T cells and monocytes/macrophages (Sebbag *et al*.,

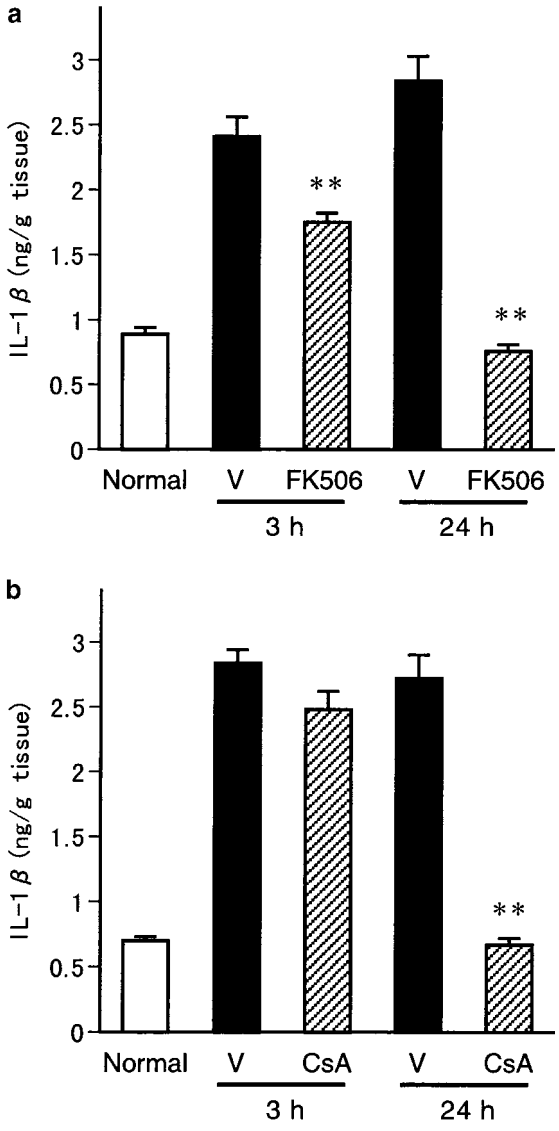

Figure 6 Calcineurin inhibitors potently reduce IL-1β level in arthritic paws. Arthritic rats were orally administered 10 mg kg^{-1} FK506 (a), 30 mg kg^{-1} CsA (b) or each vehicle on day 17 after arthritis induction. Paw tissues were dissected 3 and 24 h after drug administration. IL-1β levels in the paw extracts were determined by ELISA. Data represent mean ± s.e. of five animals per group. $^{**}P<0.01$ compared to the vehicle controls (V) at the same hour after drug administration.

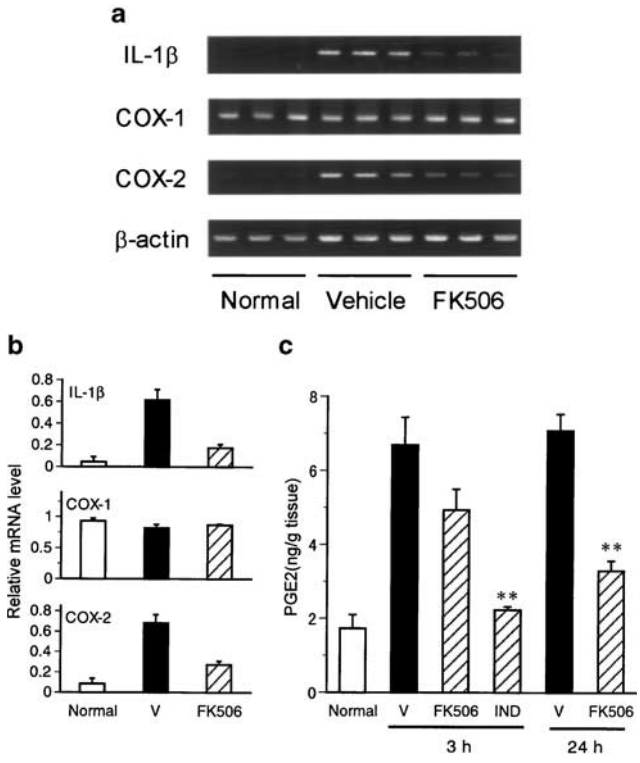

Figure 7 Effect of FK506 on COX mRNA expression and PGE_2 level in arthritic paws. (a) RT–PCR evaluation of COX-1, COX-2 and IL-1β mRNA in arthritic paws 24 h after administration of 10 mg kg^{-1} FK506. (b) mRNA expression levels were presented as a ratio relative to β-actin expression. Data represent mean ± s.e. of three animals per group. (c) PGE_2 levels in arthritic paws 3 and 24 h after administration of FK506, indomethacin (IND) or vehicle (V). PGE_2 levels in paw extracts were determined by ELISA. Data represent mean ± s.e. of five animals per group. $^{**}P<0.01$ compared to the vehicle controls at the same hour after drug administration.

1997), while IL-1β is produced from monocytes/macrophages through the interaction with activated T cells (Burger, 2000). We recently showed that FK506 and CsA specifically inhibit inflammatory cytokine production triggered by T-cell activation in an *in vitro* model, developed as a system reflecting activation states in RA (Sakuma *et al*., 2000). T-cell activation is involved in the pathogenesis of AIA as well as human RA (Panayi *et al*., 1992; Kong *et al*., 1999). TNF-α and IL-1β have also been reported to elevate in joints during development of AIA (Szekanecz *et al*., 2000). Therefore, we hypothesized that analgesic effects by these calcineurin inhibitors might be elicited via inhibition of TNF-α and IL-1β, produced through T-cell activation, in this arthritis model of RA. However, the analgesic effects of FK506 and CsA were not associated with reduction of TNF-α level in arthritic paws. Also, reduction of TNF-α level was only partial from treatment with these calcineurin inhibitors. In contrast, FK506 and CsA completely reduced the elevated level of IL-1β in arthritic paws to a normal level at 24 h. Thus, calcineurin inhibitors are thought to exert their analgesic effect by suppressing T-cell activation-triggered IL-1β production even after the arthritis is established in AIA, although other effects of the drugs than IL-1β inhibition might be responsible for the reduction in hyperalgesia.

Differences in maximal effects between IL-1β and TNF-α levels by both drugs, that is, complete reduction of IL-1β and partial reduction of TNF-α, suggest that the production of these cytokines may be regulated differently in AIA. FK506 and CsA inhibit TNF-α and IL-1β production triggered by T-cell activation, but do not affect the production of these cytokines induced by stimulants, such as lipopolysaccharide, which directly activates monocytes/macrophages (Sakuma *et al*., 2000). It is therefore conceivable that IL-1β is produced *via* T-cell activation, while TNF-α is produced through T-cell activation-dependent as well as independent mechanisms in AIA. Thus, TNF-α produced through a T-cell-independent pathway might not be affected by calcineurin inhibitors.

TNF-α has been focused on as a crucial cytokine in the pathogenesis of RA, based on the marked clinical efficacy of anti-TNF-α therapy (Maini & Taylor, 2000; Feldmann & Maini, 2001). However, our results indicate that TNF-α does

not play an important role in producing hyperalgesia in this disease model, as outlined below. (i) TNF-α level in arthritic paws was more than 10-fold lower than IL-1β level. Specific activity of TNF-α that induces hyperalgesia in rat is about five-fold less potent than IL-1β (Cunha *et al*., 1992). (ii) FK506 caused a marked reduction in TNF-α level 3 h after drug administration, but did not affect hyperalgesia at this time. (iii) CsA suppressed hyperalgesia without showing potent reduction of TNF-α level. (iv) Treatment with anti-rat TNF-α antibody did not affect hyperalgesia up to 24 h in this model, when TNF-α activity was suppressed in arthritic paws by the treatment. Taken together, our observations strongly suggest that TNF-α is not a major contributor to hyperalgesia and calcineurin inhibitors exert their analgesic effects by reducing in AIA IL-1β level.

Consecutive treatment with anti-TNF agents suppresses joint inflammation in adjuvant and collagen-induced arthritis (Williams *et al*., 1992; McComb *et al*., 1999). However, the effects of the agents on hyperalgesia in arthritis models have not been reported, although joint pain is a clinically important parameter in RA. It is thought that chronic treatment with anti-TNF agents reduces hyperalgesia by suppressing joint inflammation, although downregulation of TNF-α does not result in rapid reduction of hyperalgesia, as shown in the present study. Anti-IL-1 treatments can suppress bone and cartilage destruction without affecting joint inflammation in arthritis models (Bendele *et al*., 1999; Joosten *et al*., 1999). Downregulation of IL-1β may also reduce arthritic pain before the onset of the anti-inflammatory effects in AIA.

IL-1β upregulates the expression of COX-2 (Crofford *et al*., 1994). Prostaglandins formed by COX-2 cause inflammation and hyperalgesia in AIA (Anderson *et al*., 1996; Portanova *et al*., 1996). The mechanism of analgesic action of NSAIDs, such as indomethacin, has been shown to result from a blockade of prostaglandin synthesis by inhibition of COX-2 (Hawkey, 1999). FK506 did not show any inhibition of hyperalgesia within 6 h after drug administration, although indomethacin showed the maximum suppression at 3 h after drug administration. It is probable that FK506 exerts its analgesic effect on AIA by inhibiting IL-1β production and consequently decreasing COX-2 expression triggered by the cytokine. Thus, it takes a longer period for FK506 to suppress hyperalgesia in comparison with indomethacin, which directly inhibits COX-2 activity and prostaglandin production. It may also be possible that FK506 exerts its analgesic effect via suppression of release of not only PGE_2 but also IL-1-induced nociceptive mediators other than prostaglandins, such as nerve growth factor (Safieh-Garabedian *et al*., 1995).

In summary, the results of the present study strongly suggest that inhibitors of T-cell activation, FK506 and CsA, rapidly reduce hyperalgesic states probably by downregulating IL-1β, even in the presence of severe inflammation in an animal model of RA. This study is, to our knowledge, the first showing that immunosuppressive drugs possess analgesic actions on inflammatory pain. FK506 and CsA have already been used in the treatment of RA and shown to be effective in improving joint pain as well as other parameters of RA (Wells & Tugwell, 1993; Furst *et al*., 2002). In general, the efficacy of antirheumatic drugs has been evaluated based on application in chronic treatment. As demonstrated in AIA, analgesic effects may be found before the onset of antirheumatic or anti-inflammatory effects in clinical settings, if clinical studies are performed to focus on the immediate efficacy of FK506 or CsA. This may enable these calcineurin inhibitors to be differentiated from therapy with anti-TNF-α agents.

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