Abstract. Objective and design: To determine the effect of FK506 (tacrolimus) on paw inflammation, TNF-α expression in joint, and bone and cartilage destruction in type II collagen-induced arthritis (CIA) model in rats. Methods: CIA was induced by immunization of female Lewis rats with an emulsion of bovine type II collagen and incomplete Freund's adjuvant. Paw inflammation was assessed by the increase in paw volume. Tumor necrosis factor (TNF) -α expression in hind knee joint was assessed by immunohistochemical analysis. Lesions of bone and cartilage were assessed on the basis of histological change in knee joint, radiographic analysis in hind paw, bone mineral density in femora and proteoglycan contents in the cartilage of femoral heads. FK506 at doses of 1, 1.8 and 3.2 mg/kg or its placebo formulation was orally administered to rats for 28 days from the day after immunization (n = 10). Effect of FK506 was compared with that of vehicle (distilled water). Results: FK506 at a dose of 1.8 mg/kg significantly suppressed paw swelling (p < 0.01) and histological change in knee joint (p < 0.05). Tumor necrosis factor (TNF)-α was mainly expressed in the region with a marked infiltration of inflammatory cells in the hind knee joint. FK506 (3.2 mg/kg) markedly reduced TNF-α expression. FK506 at a dose of 1.8 mg/kg suppressed radiographic changes in hind paw (p < 0.05) and also recovered the decrease in bone mineral density in the femora (p < 0.05). Proteoglycan contents in the cartilage of femoral heads were determined to evaluate the cartilage destruction more quantitatively and found to significantly decrease in CIA rats. FK506 at a dose of 1.8 mg/kg recovered the loss of proteoglycan contents (p < 0.01). Conclusions: These results show that FK506 is effective in suppressing inflammation, TNF-α expression in joint, and damage to bone and cartilage in rat CIA, and may be useful in the treatment of rheumatoid arthritis.

Key words: Collagen-induced arthritis – Tumor necrosis factor-α – Bone – Cartilage – Proteoglycan – FK506

Introduction

Although the etiology of rheumatoid arthritis (RA) has not been completely characterized, T cells are thought to play an important role in initiating and perpetuating the chronic autoimmune responses associated with RA [1]. Additionally, inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, are reported to be responsible for the pathogenesis of RA [2]. Anti-cytokine therapy in clinical studies has also provided supporting evidence for the involvement of these cytokines in RA [3, 4].

FK506 (tacrolimus) is an immunosuppressive agent that specifically suppresses T cell activation [5–7]. FK506 exerts its immunosuppressive effects after binding to intracellular proteins, termed FK506 binding proteins (FKBPs). This complex inhibits calcineurin phosphatase, an enzyme involved in activation of transcription factor NF-AT, required for the expression of cytokine genes in T cells [8]. Recently, FK506 has been applied to the treatment of rheumatoid arthritis and shown to be effective in RA patients failing methotrexate (MTX) treatment [9]. We have investigated the mechanism of action of FK506 in vitro and found that FK506 potently suppresses TNF-α and IL-1β production through T cell activation in human peripheral blood mononuclear cells [10]. Therefore, specific inhibition of T cell activation and subsequent inflammatory cytokine production is thought to be the primary mode of action of FK506 in RA. We have already reported that FK506 is more effective and less toxic than MTX in treating established adjuvant-induced arthritis in rats [11]. Also, we have found that FK506 induces chondrogenic differentiation of clonal mouse embryonic carcinoma cells [12].

Rat type II collagen-induced arthritis (CIA) is an animal model of polyarthritis that can be induced in susceptible rats by immunization with native type II collagen [13]. The development of arthritis is associated with high levels of both cell-mediated and humoral immunity to type II collagen [14, 15] with the arthritic response appearing to be due to collagen immunity. Furthermore, this model has some of the clinical characteristics of RA; e.g. proliferation of synovial tissue, formation of subcutaneous nodules, and destruction of carti-
lage and bone. Although much is known about the pathology of cartilage damage in this model, quantitative evaluation is poorly defined. In the present study, the efficacy of FK506 on rat CIA was examined on the basis of paw inflammation, TNF-α expression in joint, and bone and cartilage destruction.

Materials and methods

Animals

Female Lewis rats (7 weeks) were obtained from Charles River Japan, Inc. (Kanagawa, Japan) and bred in a clean atmosphere. Animals were allowed to acclimate for 7 days prior to initiation of experiments. All experimental procedures were reviewed and approved by Fujisawa Pharmaceutical Animal Experiment Committee.

Induction of collagen-induced arthritis

For immunizations, bovine type II collagen (CII) was dissolved in 0.1 M acetic acid at a concentration of 2 mg/ml and emulsified in an equal volume of incomplete Freund’s adjuvant (Difco Labs, Detroit, MI, USA). Each rat was given intradermal (i.d.) injections of 500 μg CII in an emulsion volume of 500 μl at 2 tail sites and 10 sites on the back on day 0, then received booster injections at 2 tail sites (50 μl in divided doses) on day 7 and day 14. Normal non-treated rats were used as negative controls. After arthritis induction, the volume of both hind paws was measured by a water displacement method using a plethysmometer for rats. Paw swelling was presented as a change in the hind paw volume.

Drug treatment

A solid dispersion formulation of FK506 [16] and its placebo formulation were prepared at Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Animals were randomized and grouped (n = 10, except immunohistochemical analysis) for drug treatment, based on hind paw volume and body weight on day 0. FK506 (1, 1.8, 3.2 mg/kg), placebo equivalent to 3.2 mg/kg of FK506 and vehicle (distilled water) were orally administered once a day from day 1 to 28 after collagen immunization. FK506 (3.2 mg/kg) and vehicle (distilled water) were administered, in separate experiments, in the study of immunohistochemical analysis of TNF-α expression (n = 3).

Sample preparation

All immunized and normal rats were sacrificed on day 29. After exsanguination, both hindlimbs of each rat were dissected and heads of femora were separated and stored at −30°C until measurement of proteoglycan levels. Then, the left hindpaw of each rat was dissected above the ankle joint for radiographic evaluation, and the right hind knee joint was dissected and fixed in 10% neutral buffered formalin for histological evaluation. After removal of the hindpaw, the remainder of the left hindlimb was stored at −30°C until measurement of bone mineral density.

Histological evaluation

For histological evaluation, the right hind knee joints were decalcified, embedded in paraffin, sectioned longitudinally, stained with hematoxylin and eosin and evaluated under light microscopy. Histological analysis was carried out on the basis of infiltration of inflammatory cells, synovial hyperplasia, cartilage destruction and bone destruction. The severity of lesions was classified into four grades: 0 = normal; 1 = mild change; 2 = moderate change; 3 = severe change.

Immunohistochemical analysis of TNF-α in hind knee joint

Samples for immunohistochecmistry were prepared in a separate study. The right hind knee joint was dissected, embedded in Tissue-Tek OCT medium (Miles, Inc., Elkhart, IN, USA), immediately snap frozen in acetone prechilled on dry ice and kept at −70°C until sectioned. Sagittal cryostat sections of 5 μm thickness were incubated with 0.3% H2O2 for blocking endogenous peroxidase activity and blocked with 10% normal donkey serum. Rabbit anti-TNFα polyclonal antibody (Santa Cruz Biotechnology, Inc., CA, USA) was used as primary antibody. Peroxidase-conjugated donkey anti-rabbit IgG (Jackson Immuno-Research Laboratories, Inc., PA, USA) was used to detect bound primary antibody. The peroxidase reaction was developed with the substrate diaminobenzidine. Sections were then stained with hematoxylin and eosin, and evaluated under light microscopy.

Radiological evaluation

Radiographic analysis of the left hind paws was carried out on the basis of destruction of the calcaneus bone, destruction of the tarsal bone, destruction of the intertarsal joint and between the tarsal bone and talus bone, destruction of the metatarsal bone, and destruction of the distal tibia bone. The severity of destruction was classified into three grades: 0 = normal; 1 = moderate change; 2 = severe change. The sum of each score was recorded as the radiology score of bone destruction for each individual rat.

Determination of bone mineral density (BMD) in femora

To obtain the femur, the left hindlimb was autoclaved for 1 h at 100°C and flesh was removed. Bone minerals were measured using a dual-energy X-ray absorptiometer (DCS-600, Aloka, Tokyo, Japan). The mineralization profile for isolated femur was recorded as the monitoring image. Values for total bone mineral contents (BMC: mg/cm), bone width (cm) and BMD (mg/cm²) were obtained for each specimen.

Measurement of proteoglycan levels

Proteoglycan levels in the cartilage of both femoral heads were measured by a method described previously [17], with minor modifications. Briefly, femoral head cartilages were digested for 4 h at 65°C with papain solution (Sigma, St. Louis, MO, USA). Samples from the papain digest were assayed for glycosaminoglycan (GAG) as a measure of proteoglycan content. GAGs were assayed using the 1,9-dimethylmethylene blue binding assay, using chondroitin sulphate (Nacalai Tesque, Kyoto, Japan) as standard.

Statistical analysis

Results are presented as mean ± SE. Differences between arthritis control and FK506 treatment groups were determined by Dunnnett’s multiple comparison test. Differences between arthritis control and placebo treatment groups were determined by t-test. Differences between normal non-treated and the arthritis control group were determined by t-test. In the case of histological and radiological analysis, statistical analysis was performed using Kruskal-Wallis followed by Dunnnett’s multiple comparison or Wilcoxon’s rank sum test, respectively. Differences between groups were considered significant when the probability was less than 5% (p < 0.05).