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FK506 induces chondrogenic differentiation of clonal mouse embryonic carcinoma cells, ATDC5

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Abstract

FK506 (Tacrolimus) and cyclosporin A exert their immunosuppressive effects via a common mechanism, calcineurin inhibition, after binding to intracellular proteins termed immunophilins: FK506-binding protein (FKBP) and cyclophilin. In this study, FK506 was found to induce chondrogenic differentiation of ATDC5 cells (clonal mouse embryonal carcinoma cells) in a concentration-dependent manner (0.1–1000 ng/ml). Immunohistochemical staining showed that ATDC5 cells induced to differentiate by FK506 produced proteoglycan and type II collagen, main components of the extracellular matrix of cartilage. Rapamycin, an immunosuppressant that binds to FKBP, antagonized the effect of FK506. Cyclosporin A did not induce chondrogenesis at concentrations up to 1000 ng/ml. Taken together, these results suggest that FK506 induces chondrogenic differentiation of ATDC5 cells via a calcineurin-independent mechanism, after binding to FKBP.

Keywords: FK506; Cyclosporin A; ATDC5 cell; Chondrogenic differentiation; Calcineurin; Cartilage

1. Introduction

A clonal mouse chondrogenic cell line, ATDC5, was isolated from the feeder-independent teratocarcinoma stem cell line AT805, on the basis of chondrogenic potential in the presence of insulin (Atsumi et al., 1990). ATDC5 synchronously displays the sequential chondrogenic differentiation process that begins with the mesenchymal condensation stage of proliferating chondrocytes, proceeds through the nodule formation and hypertrophic stages, and ends with matrix mineralization in vitro (Akiyama et al., 1996, 1997; Shukunami et al., 1996, 1997; Ito et al., 1999). Thus, ATDC5 cells provide an excellent model to study the regulation of cartilage differentiation during endochondral bone formation in vitro.

FK506 (Tacrolimus) is an immunosuppressive agent with an increasing number of clinical applications. Recently, FK506 has been examined as a treatment for rheumatoid arthritis (Furst et al., 1999). The immunosuppressant drugs, FK506 and cyclosporin A, are thought to exert their therapeutic effects by binding to receptor proteins designated cyclophilins and FK506-binding proteins (FKBPs), respectively. When complexed to the immunosuppressant drugs, these binding proteins, designated immunophilins, bind to the Ca^{2+}-activated phosphatase calcineurin to inhibit its activity and increase levels of phosphorylated calcineurin substrate proteins. Both FK506 and cyclosporin A produce their immunosuppressive effects by inhibiting the calcineurin-mediated dephosphorylation of the nuclear factor of activated T cells (NFAT) in T cells, after binding FKBPs and cyclophilins, respectively. In a recent study, one of the NFAT family members, NFATp, was reported to be a repressor of chondrogenesis (Ranger et al., 2000). Therefore, it may be possible to induce differentiation of mesenchymal stem cells into cartilage by inhibiting calcineurin and activating NFAT. In this study, we investigated the effect of FK506 on chondrogenesis in vitro, using ATDC5 cells. The effect of FK506 on chondrogenic differentiation of ATDC5 cells was compared with cyclosporin A. Our results show that FK506, but not cyclosporin A, stimulates chondrogenic differentiation, indicating that the effect of chondrocytes by FK506 is not dependent on calcineurin.

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2. Materials and methods

2.1. Materials

FK506, recombinant human insulin-like growth factor 1 (rhIGF-1) and cyclosporin A were produced at Fujisawa Pharmaceutical (Osaka, Japan). Rapamycin was purchased from Sigma (St. Louis, MO). FK506, cyclosporin A and rapamycin were dissolved in ethanol. rhIGF-1 was dissolved in saline. These reagents were appropriately diluted in culture medium.

2.2. Cells and cell cultures

A chondrogenic mouse embryonic carcinoma cell line, ATDC5, was obtained from RIKEN cell bank (Tsukuba, Japan). Cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (D-MEM/F-12, Nikken Biomedical Laboratory, Kyoto, Japan) supplemented with 5% fetal bovine serum (Intergen, Purchase, NY) and penicillin (50 IU/ml)–streptomycin (50 μg/ml) (ICN Biomedicals, Aurora, OH). Cells were maintained at 37 °C in a humidified 5% CO_2/95% air atmosphere.

2.3. Measurement of chondrogenesis

Cells were plated at 1×10^5 cells/well in 12 multiwell plates. After cells attached to the plate, the medium was replaced with fresh medium containing agents of various concentrations or vehicle, and the culture was continued for 24 days with medium change three times per week. After culture, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) (−), fixed with methanol (2 min, −20 °C), rinsed once with distilled water and then stained overnight at room temperature with 0.1% Alcian blue (Alcian Blue 8 GX, Sigma) in 0.1 N HCl. Cells were rinsed three times with distilled water, and the amount of cell-associated dye was measured at 620 nm, after extraction with 6 M guanidine–HCl (300 μl/well, Sigma).

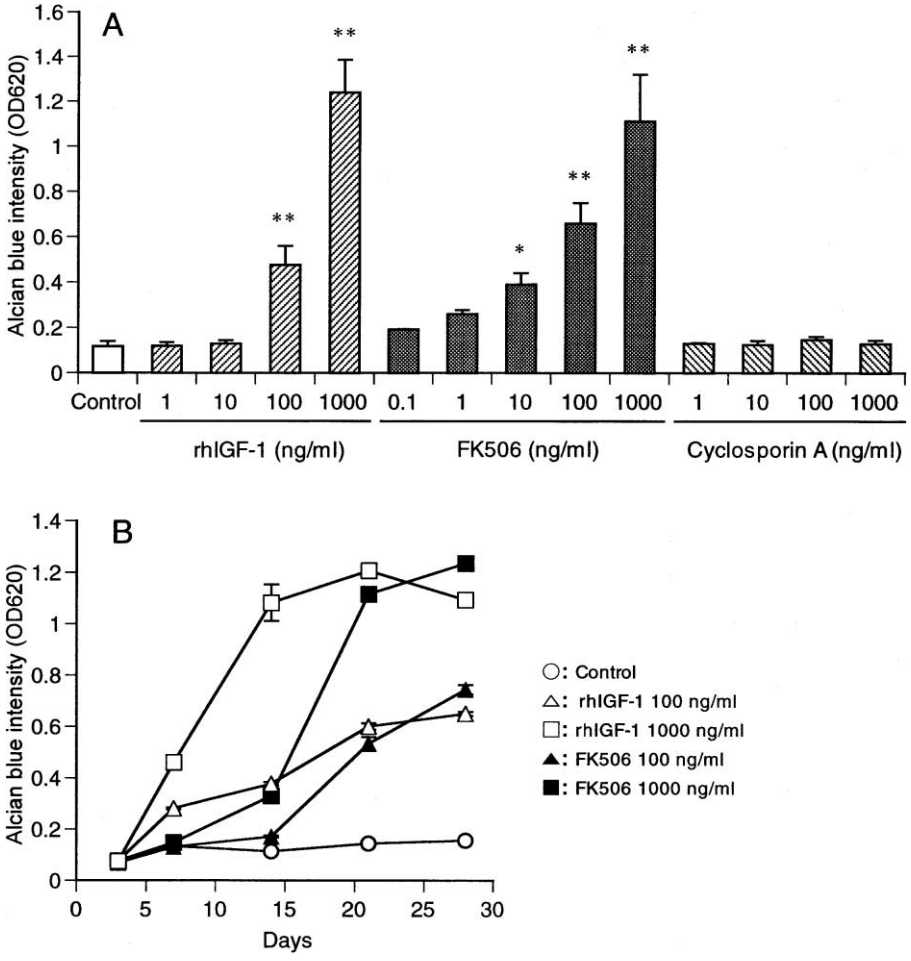

Fig. 1. Effects of FK506 and cyclosporin A on Alcian blue-stainable proteoglycan content. ATDC5 cells were cultured for 24 days with FK506, cyclosporin A or rhIGF-1. After culture, Alcian blue-stainable proteoglycan content was determined (A). The ATDC5 cells were cultured with FK506 or rhIGF-1, and Alcian blue staining was measured from day 3 to day 28 (B). The data represent mean ± S.E.M. of three separate experiments (A) or three wells (B). $^*P < 0.05$, $^{**}P < 0.01$ compared with Control.

2.4. Measurement of cell growth

Cells were plated at 1×10^5 cells/well in 12 multiwell plates. After cells attached to the plate, the medium was replaced with fresh medium containing agents of various concentrations or vehicle, and the culture was continued for 7 days with medium change three times. After addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (50 μl/well, 5 mg/ml in PBS (−), Sigma), the plates were incubated for 4 h. The medium was removed and the blue dye formed was dissolved in 300 μl of 0.04 N HCl in isopropanol. Absorbance was measured at 580 nm.

2.5. Immunohistochemical staining

Cells were plated at 3×10^4 cells/well in Lab-TekChamber Slide 8 well glass slides (Nunc, Roskilde, Denmark). After cells attached to the well, the medium was replaced with fresh medium containing FK506, rhIGF-1 or vehicle, and the culture was continued for 24 days with medium change three times per week. After incubation, cells were rinsed twice with ice-cold PBS (−) and fixed with methanol at 4 °C for 5 min. Cells were washed with PBS (−) two times and treated with PBS (−) containing 0.1% sodium azide and 0.3% H_2O_2 for 10 min at room temperature. After washing two times with PBS (−), the cells were treated with normal goat serum (Rockland, Gilbertsille, PA) for 10 min at room temperature. The cells were treated with anti-human adult cartilage proteoglycan mouse monoclonal antibody (Biogenesis, Poole, England) or anti-human cartilage collagen type II rabbit polyclonal antibody (quartett, Immunosiagnostikan und Biotechnologie, Berlin, Germany) in a humidified box at 4 °C overnight. After washing the cells, they were treated with fluorescent isothiocyanate (FITC)-conjugated AffiniPure goat anti-mouse immunoglobulin G (IgG) (H+L) (Jackson Immuno Research Laboratories, West Grove, PA) or FITC-conjugated AffiniPure donkey anti-rabbit IgG (H+L) (Jackson Immuno Research Laboratories) for 60 min at room temperature. After washing, the cells were treated with propiodium iodide solution (Sigma) for 2 min at room temperature.

2.6. Statistical analysis

Results are presented as mean ± S.E.M. or mean. Increase of Alcian blue intensity was analyzed using Dunnett's multiple comparison test following randomized block designed analysis of variance in some experiments.

3. Results

3.1. Effect of FK506 on chondrogenesis in ATDC5 cells

Atsumi et al. (1990) reported that a cell line, ATDC5, isolated from a differentiating culture of AT805 teratocarcinoma, could be induced to differentiate chondrocytes with the addition of insulin or IGF-1. These agents lead to the formation of nodular structures and the accumulation of sulfated glycosaminoglycan, an extracellular matrix component relevant to chondrogenesis, which is stainable with Alcian blue. We examined the effects of FK506 on chondrogenesis in ATDC5 cells. ATDC5 cells were cultured with FK506 or IGF-1 for 24 days and the intensity of Alcian blue staining was measured quantitatively. The intensity of Alcian blue staining was greatly enhanced by IGF-1 treatment. FK506 also enhanced the accumulation of the Alcian blue-stainable material in a concentration-dependent manner (Fig. 1A). ATDC5 cells exposed to 1 ng/ml of FK506 showed about a two-fold increase in Alcian blue staining. In contrast, cyclosporin A did not increase Alcian blue staining of the cells even at a high concentration of 1000 ng/ml.

For the first 7 days of treatment, IGF-1 induced chondrogenic differentiation of ATDC5 cells, but not FK506, as indicated by the intensity of Alcian blue staining (Fig. 1B). After 14 days of treatment, FK506 was also observed to increase the intensity of Alcian blue staining slightly. IGF-1 enhanced the growth of ATDC5 cells in a dose-dependent manner; however, FK506 did not enhance cell growth (Fig. 2).

Type II collagen and proteoglycan are main components of the extracellular matrix of cartilage. To examine the effects of FK506 on the production of these components of ATDC5 cells, cells were treated with 10 ng/ml FK506 or 100 ng/ml IGF-1 for 24 days and stained with anti-type II collagen or

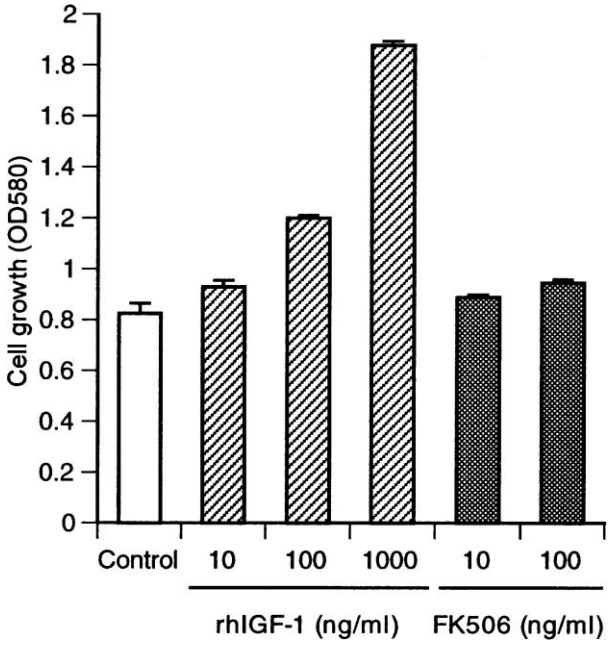

Fig. 2. Effects of FK506 and rhIGF-1 on growth of ATDC5 cells. ATDC5 cells were cultured for 7 days with FK506 or IGF-1, and the cell growth was measured by MTT assay. The data represent mean ± S.E.M. of three wells.

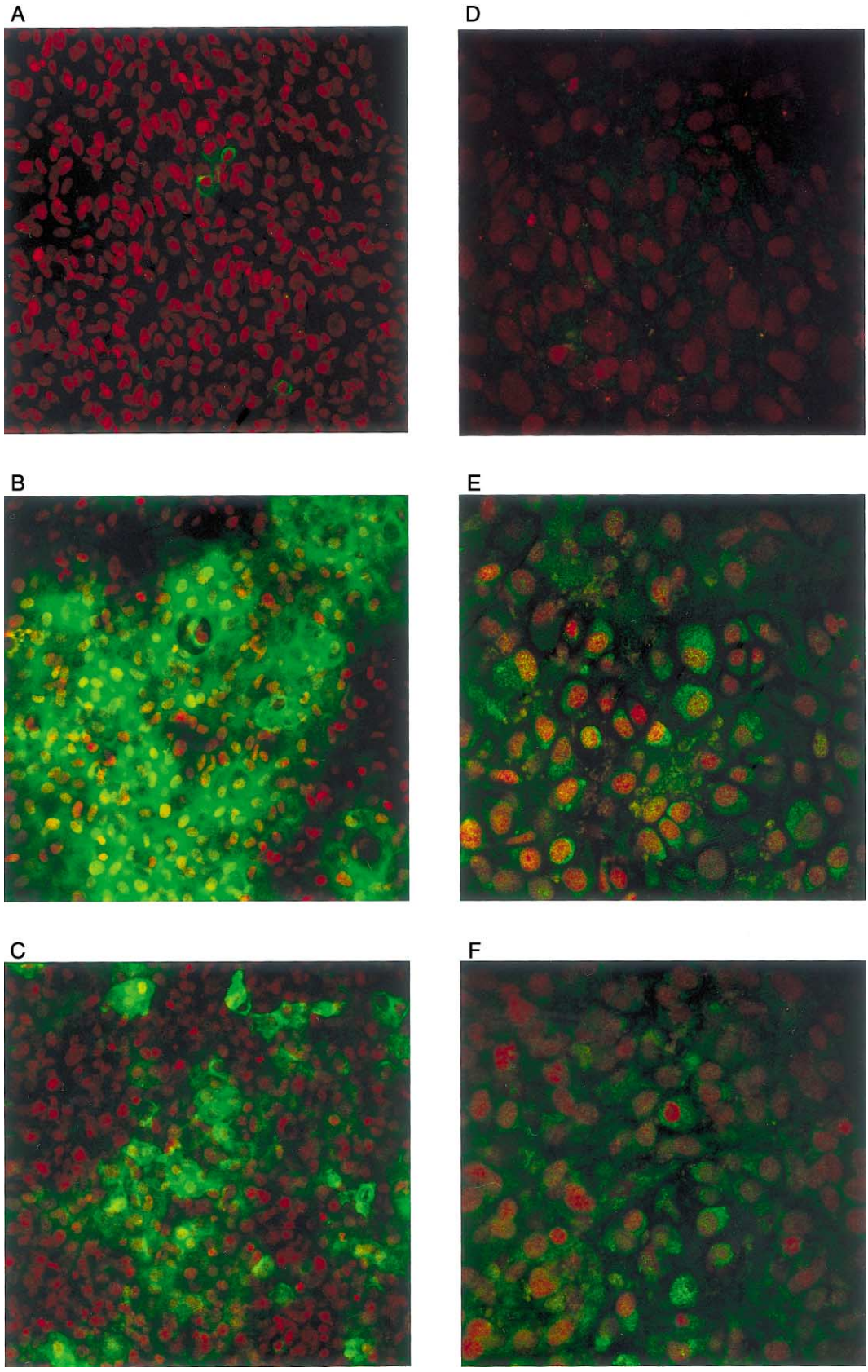

Fig. 3. Immunohistochemical staining of FK506 and rhIGF-1 treated ATDC5 cells. ATDC5 cells were cultured for 24 days with 10 ng/ml FK506 (B, E), 100 ng/ml rhIGF-1 (C, F) or medium (A, D). After culture, ATDC5 cells were stained with anti-human cartilage collagen type II rabbit polyclonal antibody (A–C) or anti-human adult cartilage proteoglycan mouse monoclonal antibody (D–F).

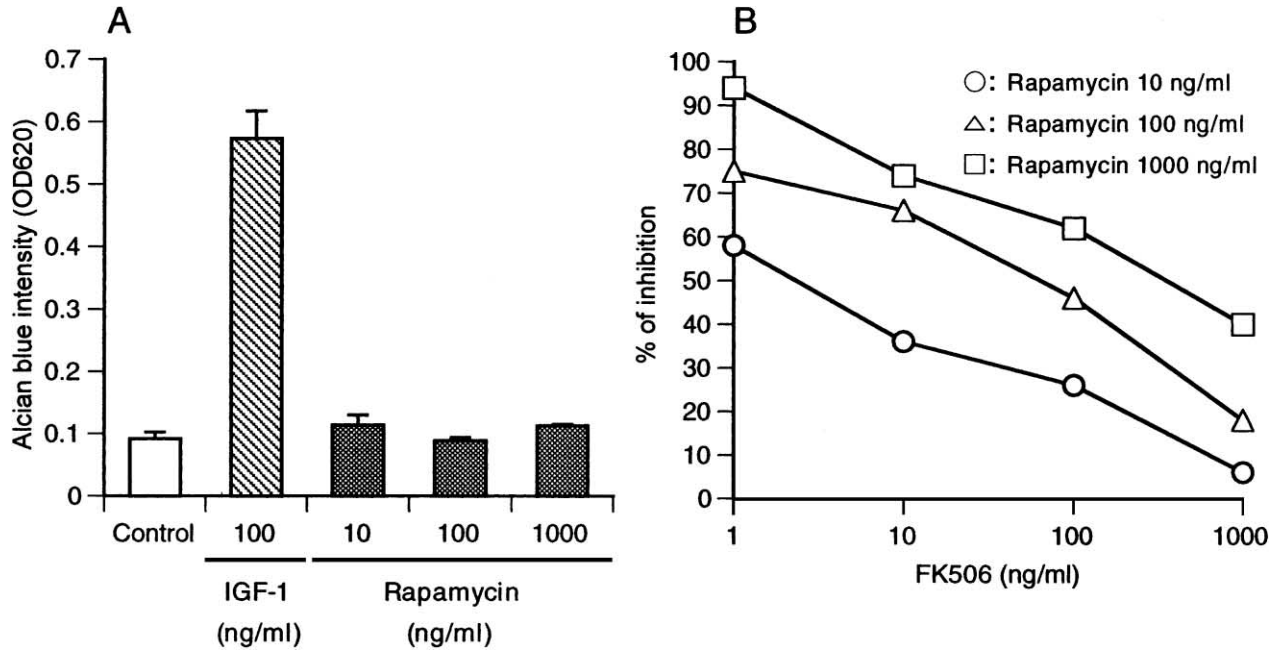

Fig. 4. Effect of rapamycin with and without FK506 on the Alcian blue-stainable proteoglycan content. ATDC5 cells were cultured for 24 days with rapamycin, and Alcian blue-stainable proteoglycan content was determined. The data represent mean ± S.E.M. of three wells (A). Cells were cultured with various concentrations of rapamycin in the presence of 1–1000 ng/ml of FK506. The data represent mean of percentage inhibition of three wells (B).

anti-proteoglycan antibodies. Untreated cells did not produce very much type II collagen or proteoglycan immunoreactivity (Fig. 3A,D). Both FK506 (Fig. 3B,E) and IGF-1 (Fig. 3C,F) clearly induced the synthesis of type II collagen and proteoglycan in differentiated ATDC5 cells. These data show that FK506 induces the chondrogenic differentiation of ATDC5 cells, a chondrogenic precursor cell line.

3.2. Effect of rapamycin on FK506-induced chondrogenic differentiation of ATDC5 cells

Rapamycin is known to block the actions of FK506 that occur through modulation of FKBP and calcineurin, by acting as an antagonist of FKBP (Bierer et al., 1990). Rapamycin, up to a dose of 1000 ng/ml, did not induce Alcian blue staining of ATDC5 cells after 24 days of culture (Fig. 4A). In the presence of rapamycin at concentrations of 10–1000 ng/ml, the effect of FK506 on chondrogenesis, as assessed by Alcian blue staining, was inhibited in a dose-dependent manner (Fig. 4B).

4. Discussion

It has been reported that FK506 induces favorable conditions that support osteoblastic differentiation of allogeneic rat marrow stromal stem cells on the surface of porous hydroxyapatite ceramics (Akahane et al., 1999). Ogawa et al. (1998) reported that FK506 induced osteoblastic differentiation of the rat osteoblast-like osteosarcoma cell line ROS cells. Voggnereiter et al. (2000) reported that FK506 caused a marked increase of bone formation in demineralized bone matrix in rats. Yoshikawa et al. (2000) reported that FK506 promoted bone formation in rats. However, the effect of FK506 on chondrocytes have not yet been determined. In this study, we found that FK506 induced chondrogenic differentiation of ATDC5 cells at a clinical dose. In immunohistochemistry studies, FK506 induced the production of type II collagen and proteoglycan in differentiated ATDC5 cells. FK506 may act as an agent to induce differentiation of both osteoblasts and chondrocytes.

In a recent study, it was reported that one of the NFAT family members, NFATp, is a repressor of chondrogenesis and therefore calcineurin inhibition may lead to the differentiation of mesenchymal stem cells into cartilage (Ranger et al., 2000). However, in this study, cyclosporin A, a calcineurin inhibitor like FK506, did not induce chondrogenic differentiation (Fig. 1A). It is unlikely that cyclosporin A fails to induce chondrogenic differentiation due to a potential nonspecific cytotoxicity at the concentration used, as the agent is reported not to affect normal cell proliferation up to 1000 ng/ml (Sakuma et al., 2000). It is also unlikely that lack of effect by cyclosporin A is due to the absence of cyclophilins in ATDC5 cells since cyclophilins distribute ubiquitously. It is therefore most probable that FK506-induced chondrogenic differentiation does not depend on calcineurin inhibition. Alternatively, it may be possible that the cyclosporin A–cyclophilin complex and FK506–FKBP complex each modulate different isotypes of either calcineurin or NFAT in this particular cell type. The effect of FK506 was antagonized by rapamycin, which competitively binds to FKBP (Fig. 4B). It may be possible that rapamycin

inhibits the chondrogenic differentiation via modulation of general cell cycling processes: via its known inhibition of p70S6 kinase (Sehgal, 1995). However, rapamycin did not lower the Alcian blue intensity of untreated ATDC5 cells as shown in Fig. 4A. Furthermore, inhibition of the differentiation by rapamycin was more potent at lower concentrations of FK506. It is therefore considered that rapamycin inhibits the effects of FK506 by competing for FKBP.

As it was reported that plasma IGF-1 levels were significantly elevated in FK506-treated rats (Inoue et al., 2000), we examined whether FK506-induced ATDC5 cell chondrogenic differentiation was mediated by IGF-1. IGF-1 not only induces chondrogenic differentiation of ATDC5, but also enhances the growth of ATDC5 cells (Fig. 2). However, FK506 did not induce the enhancement of cell growth of ATDC5 cells (Fig. 2), and no IGF-1 mRNA was detected in FK506-treated ATDC5 cells using the RT-PCR method (data not shown). IGF-1 protein was also not detected in the conditioned medium of FK506-treated ATDC5 cells (data not shown). Therefore, it is not likely that chondrogenic differentiation of FK506 on ATDC5 was induced as a result of FK506 inducing IGF-1 expression in ATDC5 cells.

In conclusion, the present study suggests that FK506 induce chondrogenic differentiation of ATDC5 cells via a calcineurin-independent mechanism after binding to FKBP. FK506 may be therapeutically useful not only in suppressing immune and inflammatory reactions but also in repairing articular cartilage lesions by inducing chondrogenesis in joint diseases such as rheumatoid arthritis.

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