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The immunosuppressant FK506 promotes development of the chondrogenic phenotype in human synovial stromal cells via modulation of the Smad signaling pathway

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Summary

Objective: To assess the effect of the immunosuppressant FK506 on chondrogenic differentiation of human synovial stromal cells (hSSCs).

Methods: hSSCs were isolated from synovium of the knee joint and 2×10^5 cells were subjected to pellet culture in chondrogenic culture medium for 3 weeks with or without growth factors [bone morphogenetic protein 2 (BMP2) or transforming growth factor β_1 (TGFβ_1)] and +/− addition of FK506 in chondrogenic culture media was evaluated. Chondrogenesis was assessed by the size of the pellet, the production of proteoglycans, and messenger RNA (mRNA) levels for chondrogenic markers. Furthermore, levels and intracellular location of phosphorylated Smad proteins related to BMP signaling and TGFβ signaling were evaluated following exposure to FK506.

Results: FK506 enhanced the differentiation of hSSCs toward a chondrogenic phenotype in a dose-dependent manner associated with increases in glycosaminoglycan synthesis and increased mRNA levels for chondrogenic marker genes. Additionally, FK506 further enhanced chondrogenesis of synovial stromal cells (SSCs) induced by BMP2 and TGFβ_1, also in a dose-dependent manner. Notably, phosphorylation of Smad1/5/8 and Smad3 was significantly increased by FK506. Also, the ratio of nuclear translocation to cytoplasmic levels of phosphorylated Smad1/5/8 and Smad3 were increased following exposure of SSCs to FK506. Moreover, inhibition of Smad signaling significantly abrogated FK506-induced chondrogenic differentiation of SSCs.

Conclusion: This study demonstrated that FK506 promotes chondrogenic differentiation of hSSCs likely via impact on Smad signaling pathways. With further optimization, FK506 could potentially be a unique therapeutic tool to promote cartilage repair in clinical situations, as well as enhance development of tissue engineered cartilage *in vitro*.

Key words: FK506, BMP, TGFβ, Synovium, Tissue engineering, Cartilage repair.

Introduction

It is well known that the healing potential of articular cartilage is limited, in part, due to its avascularity[1]. To compensate for such poor healing capacity, several cell-based approaches have been investigated to repair chondral lesions. Among them, autologous chondrocyte implantation (ACI) procedures have been widely performed[2]. Despite their promising clinical results[3,4], the limitations of this procedure may be related to the cell source and the *in vitro* expansion of the cells. Chondrocytes are often obtained from uninvolved cartilage tissue at minor load-bearing areas of the same joint. However, potential problems related to donor-site morbidity, which may increase the risk of developing osteoarthritis later in life remain unclear. In addition, it may be difficult to obtain a sufficient amount of healthy cartilage from joints in elderly patients due to the age-related development and progression of osteoarthritis. Chondrocyte expansion is likely another crucial factor for successful treatment. However, chondrocyte expansion in monolayer cultures usually results in dedifferentiation cells with increasing passage[5–7] and such loss of chondrogenic capacity may account for variable clinical results of ACI[3,8,9].

Mesenchymal stem cells (MSCs) were first identified in bone marrow and shown to have the ability to differentiate into a variety of connective tissue phenotypes including bone, cartilage, and adipose-tissue[10,11]. Recent studies have revealed that MSCs are also present in various tissues such as umbilical cord blood[12], muscle[13], adipose-tissue[14,15], tendon[16], and synovium[17,18]. Specifically, MSCs derived from synovium have been shown to have the ability to proliferate over many passages without losing their multipotency, and such expansion appears to be independent of donor age or cryopreservation[17]. In addition, a recent study has shown that MSCs derived from synovium are superior to those from bone marrow or adipose tissue in terms of chondrogenic differentiation[19] and it is a relatively easy and safe procedure to obtain cells from synovium with

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minimal donor-site morbidity. Taken together, synovium-derived MSCs could be a promising source for future cell-based cartilage repair.

FK506 (tacrolimus) is a widely used immunosuppressive agent with Food and Drug Administration (FDA) approval, usually used to prevent graft rejection. The immunosuppressive effect of FK506 is believed to be related to its ability to inhibit calcineurin, an enzyme involved in the activation of the nuclear factor of activated T cells (NFAT)[20]. Recent studies also suggest the involvement of NFAT in chondrogenesis[21,22]. Furthermore, it has been shown that FK506 can induce chondrogenic differentiation of clonal mouse embryonic carcinoma cells (ATDC5), although the molecular target for its effect on chondrogenesis remains unclear[23]. Such observations raise the possibility that FK506 might likewise promote chondrogenic differentiation of MSCs. To test this hypothesis, the effect of FK506 on chondrogenic differentiation of human synovial stromal cells (hSSCs) was investigated. Furthermore, the molecular mechanism(s) underlying the promotion of chondrogenic differentiation by FK506 were explored.

Materials and methods

ISOLATION AND EXPANSION OF SYNOVIAL STROMAL CELLS (SSCs)

SSCs were isolated from human synovium which was harvested from the knee joint of four patients during arthroscopic surgery in accordance with a protocol approved by the Osaka University Institutional Ethics Committee. The demographics of the patients who provided samples are summarized in Table I. Inflamed knees were excluded in this study. Synovium was digested with 0.4% collagenase (Worthington Bio. Chem., New Jersey, USA) for 2 h, and then the resultant cells were washed twice with phosphate buffered saline (PBS) and subsequently cultured in high glucose Dulbecco's modified Eagle's medium (HG-DMEM; Gibco BRL, Life Technologies Inc, Maryland, USA) containing 10% fetal bovine serum (FBS; HyClone, Utah, USA) and 1% Penicillin/Streptomycin (Gibco BRL, Life Technologies Inc) in humidified air with 5% CO_2 at 37°C. To expand the cultures, the cells were harvested, diluted at 1:3, and replated.

IN VITRO CHONDROGENESIS

To assess chondrogenic differentiation, a pellet culture system was used[24]. Approximately 2×10^5 cells (passages 3–5) were placed in a 15-ml polypropylene tube, and centrifuged at 500*g* for 10 min. The pellets were cultured at 37°C with 5% CO_2 in 500 μl of chondrogenic culture medium that contained HG-DMEM with 10% FBS, supplemented with 50 μg/ml ascorbate-2-phosphate, 100 μg/ml sodium pyruvate, and 50 mg/ml insulin, transferrin, and selenious acid (ITS) + Premix (BD Biosciences, California, USA), as described previously[25]. To assess the influence of FK506, the cell pellets were cultured in a chondrogenic culture medium supplemented with 0.1 or 1 μg/ml of FK506. Moreover, to evaluate the effect of FK506 with those of growth factors, 10 ng/ml transforming growth factor β_1 (TGFβ_1) (R&D Systems, Abingdon, UK) or 100 ng/ml recombinant human bone morphogenetic protein 2 (rhBMP2) (a generous gift from Wyeth, Massachusetts, USA) was added to the medium. The optimal concentration of bone morphogenetic protein 2 (BMP2) or TGFβ_1 was determined from preliminary *in vitro* optimization studies on chondrogenic differentiation of hSSCs in pellet cultures (unpublished data). The medium was replaced twice per week for 3 weeks.

Table I
Summary of patients

	Age	Sex	Sample volume (mg)	Diagnosis
Patient 1	19	M	230	Lt ACL injury
Patient 2	57	F	750	Lt OCD
Patient 3	23	F	450	Lt ACL injury
Patient 4	26	M	552	Rt ACL injury

The characteristics of patients involved in this study are indicated in the table. None of the patients was arthritic at the time of surgery. Patients underwent arthroscopic surgery for anterior cruciate ligament (ACL) injury or osteochondritis dissecans (OCD).

CELL PELLET SIZE ANALYSIS

After 3 weeks of culture, the cell pellets were harvested and macroscopic analysis was performed by stereomicroscopic procedures. The images were analyzed by WinROOF (Mitani, Osaka, Japan), and then the cross-sectional area (CSA) of pellets was calculated in two-dimensional (2-D) image and the CSA was evaluated as a parameter of pellet size.

HISTOLOGICAL ASSESSMENT

The cell pellet cultures were fixed in 4% paraformaldehyde, rinsed twice with PBS, embedded in paraffin, cut into 5 μm sections, and then stained for 2 h at room temperature with 1% Alcian blue (Alcian Blue 8 GX; Sigma, Missouri, USA) in 0.1 N HCl, and subsequently counter stained with kernechtrot.

MEASUREMENT OF GLYCOSAMINOGLYCAN (GAG) LEVELS

Proteoglycan levels in the pellets were measured by a color method as described previously[26], with minor modification. Briefly, synovial cell pellets were digested for 4 h at 65 °C with a papain solution (Sigma). Samples from the papain digest were subsequently assayed for GAG as a measure of proteoglycan content. GAGs were assayed using the 1,9-dimethylmethylene blue binding (DMMB) assay, using a chondroitin sulfate standard curve (Nacalai Tesque, Kyoto, Japan).

REAL-TIME REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was extracted from synovial cell pellets using the TRIzol Reagent (Invitrogen Life Technologies, California, USA), and 1 μg of total RNA was reverse transcribed into cDNA with Super Script III (Invitrogen Life Technologies) following the manufacturer's instructions. Real-time polymerase chain reaction (PCR) conditions were optimized for maximal PCR efficiency by adjustment of concentrations of the following PCR primers: Sox9, CAC ACA GCT CAC TCG ACC TT (forward) and CAA AGG GAA TTC TGG TTG GT (reverse); Collagen IIα1, TCT ACC CCA ATC CAG CAA AC (forward) and GTT GGG AGC CAG ATT GTC AT (reverse); Aggrecan, TGA GTC CTC AAG CCT CCT GT (forward) and TGG TCT GCA GCA GTT GAT TC (reverse); Collagen X, GGT TTT CCG GGA GAA ATG GGA CCA A (forward) and CTG GAG CCC CAG GGA GAC CTT TTG T (reverse); glyseraldehyde-3-phosphate dehydrogenase (GAPDH), AGC CAA AAG GGT CAT CAT CTC (forward) and GTT GTC ATG GAT GAC CTT GG (reverse). Real-time PCR was performed using a LightCycler System (Roche Applied Science, Mannheim, Germany).