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Sulforaphane–PLGA microspheres for the intra-articular treatment of osteoarthritis

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ABSTRACT

Sulforaphane (SFN) is a member of the isothiocyanate family that has anti-inflammatory action as well as anti-carcinogenic properties. The authors have devised an intra-articular injectable SFN–PLGA microsphere system that can be used for treating osteoarthritis (OA). The purpose of this study was to evaluate the *in vitro* and *in vivo* efficacy of the SFN–PLGA microsphere system. Articular chondrocytes were obtained from knee OA patients and were cultured in monolayers. The optimal concentration of SFN was obtained and the dose of SFN–PLGA microspheres was determined based on the concentration. The *in vitro* anti-inflammatory effect on markers such as cyclooxygenase (COX)-2, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-5, and matrix metalloproteinase (MMP)-2 was assessed by real-time PCR and Western blotting. The *in vivo* therapeutic effect of SFN–PLGA microspheres was investigated using surgically-induced rat OA model. Treatment with SFN–PLGA microspheres inhibited the mRNA and protein expression of COX-2, ADAMTS-5 and MMP-2 induced by LPS in articular chondrocytes. Intraarticular SFN–PLGA microspheres delayed the progression of surgically-induced osteoarthritis in rats. In conclusion, SFN–PLGA microspheres can be a useful injectable delivery system for treating osteoarthritis.

1. Introduction

Sulforaphane (SFN) is a member of the isothiocyanate family known to prevent, delay, or reverse carcinogenesis *in vitro* and *in vivo* [1–4]. The substance is found abundantly in cruciferous vegetables, such as broccoli, cauliflower, and radish [1]. SFN primarily modulates activities of phase II enzymes that convert carcinogens to inactive metabolites thereby preventing them from interacting with DNA [4]. SFN also traps free radicals and induces cell cycle arrest, which additionally prevents cancer [5,6]. Recently, several studies demonstrated that SFN also possesses anti-inflammatory activity. SFN down-regulates lipopolysaccharide (LPS)-stimulated inducible nitric-oxide synthase (iNOS), cyclooxygenase (COX-2) and tumor necrosis factor (TNF)-α expressions in RAW macrophages [7]. Likewise, SFN attenuates the LPS-induced production of inflammatory mediators such as interleukin (IL)-1β, TNF-α and IL-6 in primary co-cultures of rat microglial and astroglial cells [8]. SFN protects cells from hydrogen peroxide-induced toxicity and attenuates the production of reactive oxygen species (ROS) such as nitrogen oxide (NO) in response to LPS treatment of cells [9]. SFN also possesses anti-arthritic and immuno-regulatory activity, thereby inhibiting synovial hyperplasia and proliferation of activated T cells [10]. These findings raise the possibility that SFN may be an effective candidate drug for treating the most common arthritic disease, osteoarthritis.

Osteoarthritis (OA) is a group of diseases that involve the degeneration of articular cartilage and subchondral bone [11]. Breakdown of these tissues causes pain and stiffness of the joints, and eventually results in long-term disability in the patient [11]. Local therapy such as intra-articular injection of anti-inflammatory agents is one of the effective measures to alleviate the symptoms and prevent the progression of OA [12,13]. Conventionally, local intra-articular injection of anti-inflammatory agents has been effectively used for treatment of OA [12,14]. The rationale for intra-articular therapy is delivery of the drug to the site of action, thus minimizing the systemic toxic effect of the drug. However, the major limitation of intra-articular administration of soluble drug is its rapid clearance from the joint cavity, which necessitates the

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development of a prolonged, controlled release system for an injectable drug [13]. Therefore, several strategies including liposomes [15], serum albumin microspheres [16], gelatin microspheres [17] and polylactic acid/polyglycolic acid copolymer [18] have been employed in order to achieve a prolonged and sustained anti-inflammatory activity.

Poly(D,L-lactic-co-glycolic) acid (PLGA) is a biodegradable polymer that has been used for multiple medical purposes [18]. PLGA is broken down by nonenzymatic hydrolytic cleavage into lactic and glycolic acids in biologic fluids [19]. With a long-standing well-established safety record in humans [20], PLGA microspheres may be employed as an injectable carrier for the intra-articular therapeutic agent. Microspheres made of PLGA can be loaded with a variety of agents including chemotherapeutic drugs, anti-inflammatory agents, antibiotics, opioid antagonists, steroids, hormones, anesthetics [21].

Conventional local injectable drugs including hyaluronic acid and corticosteroids have been used for treating OA. The disadvantage of using hyaluronic acid as an intra-articular drug is the need for multiple injections, which is unavoidably associated with pain and non-compliance in the patients [22]. On the other hand, the important shortcoming of corticosteroids is the suppression of regeneration of articular cartilage. Corticosteroid use is therefore discouraged in early OA [23]. These limitations support the development of an alternative single-dose injectable drug that will relieve the symptoms and arrest the progression of OA. Intra-articular injection of paclitaxel and parathyroid hormone loaded in PLGA microsphere as a controlled release system has been reported to be successful in antigen-induced arthritis in the rabbits [24] and surgically-induced OA in the rats [25], respectively. Due to its proven anti-inflammatory effects, SFN can be also used as a local injectable agent in combination with a controlled release system for treating osteoarthritis.

The authors had devised an intra-articular injectable SFN–PLGA microsphere system that can be used for treating osteoarthritis. In this study, we first evaluated the *in vitro* effectiveness of the SFN–PLGA microsphere system in cultured human chondrocytes, and then confirmed the *in vivo* efficacy of the SFN–PLGA microsphere system in a surgically-induced rat OA model.

2. Materials and methods

2.1. Procurement of samples

Chondrocytes were isolated from the fragments of human articular cartilage (AC) which were obtained during total knee arthroplasty. The donors were three patients (age range 59–65 years, $n = 3$, one male and two females) who had advanced osteoarthritis of the knee joints. Informed consent was obtained from all donors. Chondrocytes were isolated from the areas which showed minimal osteoarthritic changes (usually lateral condyles) as described previously, and cultured to passage 3 [26].

2.2. Preparation and release testing of SFN–PLGA microspheres

10 mg of SFN (Sigma, St Louis, MO, USA) was dissolved in 1 ml of dimethyl sulfoxide (DMSO). 1 g of 75:25 PLGA (molecular mass 80 kDa, Birmingham Polymers, Birmingham, AL, USA) was dissolved in 10 ml of methylene chloride. Then these two solutions were mixed and homogenized using a PowerGen 700 homogenizer (IKA, Tokyo, Japan) at 20,000 rpm for 90 s. This homogenized solution was immediately poured into a beaker containing 200 ml of 3% (*w*/*v*) polyvinyl alcohol (molecular mass 30–70 kDa, Sigma) solution, then emulsified using an overhead propeller (LR-400A, Fisher Scientific, Pittsburgh, PA, USA) for 12 h at 700 rpm. The resultant microspheres were centrifuged, washed three times with deionized water, and then freeze-dried. The freeze-dried microspheres were placed in three 1.5 ml tubes, each tube containing 100 mg of microspheres. Then, 1.5 ml of sterile 4% PBS solution was added and set at 37 °C. For the detection of release kinetics of SFN from SFN–PLGA microspheres, total PBS (1.5 ml) was collected after centrifugation and replaced with the same volume of PBS. The release kinetics of SFN from SFN–PLGA microspheres was evaluated by isocratic reversed-phase high performance liquid chromatography (Ultimate 3000 series, Dionex, USA). The SFN–PLGA microspheres were imaged using a scanning electron microscope (SEM, JEM1010 JEOL, Tokyo, Japan). The sphere size distribution and morphology was analyzed using SEM pictures.

2.3. Cell culture and treatment

The human chondrocytes were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml penicillin and 50 μg/ml streptomycin in 5% CO_2 at 37 °C. After reaching confluence, the cells were passaged. Passage 3 chondrocytes were seeded in 6-well cell culture plastic plates (3.0×10^5 cells/well) in serum-free DMEM/F12 medium for one day. LPS (Sigma) was added at the concentration of 1 μg/ml for 6 h. Then, the medium was changed to DMEM/F12 medium supplemented with 5% FBS containing 0, 2.5, 5, 10 and 20 μM SFN respectively for 18 h. SFN stock solution was originally dissolved in DMSO and an equal volume of DMSO (final concentration < 0.1%) was added to the control cells.

For SFN–PLGA microsphere treatment, we used transwell inserts in 6-well cell culture plates to avoid direct contact between SFN–PLGA microspheres and chondrocytes. Chondrocytes were identically seeded in the lower compartment of 6-well plates (3.0×10^5 cells/well) and expanded for 1 day. Then 13 mg of SFN–PLGA which would give an SFN concentration of 5 μM at day 4 was added in the upper compartment of the transwell inserts and the chondrocytes were cultured for 4 days (Fig. 1B).

2.4. MTT assay for cell viability

Passage 3 chondrocytes were seeded in 96-well plates (1×10^4 cells/well). After incubation with LPS and SFN or SFN–PLGA microspheres, 50 μl of MTT solution (1 mg/ml, Sigma) was added to each of the well and the chondrocytes were cultured for 3 h. The reaction was stopped by removing the MTT solution. Then 150 μl of DMSO was added to each well for 10 min, and the absorbance was measured with an automated spectrophotometric microtiter plate reader (SpectraMax 340; Molecular Devices, Sunnyvale, CA), using a 570 nm filter.

2.5. Real-time PCR

RNA extraction was performed using Trizol (Invitrogen) according to the manufacturer's instructions. The yield and purity of the extracted RNA were determined using the Quant-I T™ RNA assay kit and Qubit Fluorometer system (Invitrogen). After the DNase I treatment, the total RNA was reverse-transcribed with Maxime RT preMix kit oligo (dT) primer (iNtRON Biotechnology, Gyunggido, Korea) in a 20 μl reaction volume, according to the manufacturer's instructions. All the PCR reactions were performed using a LightCycler 480 systemR (Roche Diagnostics, Mannheim, Germany) in standard 10 μl reactions. GAPDH was used as an internal control in PCR amplification and the relative normalization ratio of PCR products derived from each target gene was calculated using the LightCycler System software (Roche, Indianapolis, Indiana, USA). Expression of three inflammatory markers, COX-2, ADAMTS-5 and MMP-2 was tested. All these enzymes are known to be elevated in osteoarthritis [27–29]. All experiments were performed in triplicate.

The primer pairs used were as follows:

COX-2 forward: 5′-TGAGCATCTACGGTTTGCTC-3′
COX-2 reverse: 5′-AACTGCTCATCACCCCATTC-3′
ADAMTS-5 forward: 5′-CTTCACTGTGGCTCACGAAA-3′
ADAMTS-5 reverse: 5′-CATTTGGACCAGGGCTTAGA-3′
MMP-2 forward: 5′-ATGACAGCTGCACCACTGAG-3′
MMP-2 reverse: 5′-AGTTCCCACCAACAGTGGAC-3′
GAPDH forward: 5′-CACATGGCCTCCAAGGAGTAA-3′
GAPDH reverse: 5′-GTACATGACAAGGTGCGGCTC-3′

2.6. Western blot analysis

Proteins were extracted from cultured chondrocytes, electrophoresed by SDS–PAGE gel and transferred to a nitrocellulose membrane, as reported previously [30]. Proteins transferred to a membrane were incubated in 5% non-fat milk to block non-specific binding. The blot was probed with anti-rabbit COX-2 (1:1000; Abcam, Cambridge, UK), MMP-2 (1:1000; Cell Signaling Technology, Beverly, MA, USA), ADAMTS-5 (1:1000; Sigma), β-actin (1:5000; Abcam) followed by horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:2000; Cell Signaling Technology). The signals were visualized with enhanced chemiluminescence (ECL) Western blotting detection reagent (Amersham Biosciences, Piscataway, NJ). Immunoblot bands were analyzed using an image reader LAS-3000 (version 2.1; Fujifilm, Tokyo). This experiment was repeated in three samples, each from different donors.

2.7. In vivo effects of SFN–PLGA microspheres in surgically-induced osteoarthritis: the anterior cruciate ligament transection (ACLT) model

2.7.1. Anterior cruciate ligament transection (ACLT) model

The animal experiments conducted as a part of this study were approved by the Animal Research and Care Committee of our institution. 9-week-old male Sprague–Dawley rats were used in this study. The animals were anesthetized with zoletil