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Intra-articular administration of gelatin hydrogels incorporating rapamycin–micelles reduces the development of experimental osteoarthritis in a murine model

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ABSTRACT

Autophagy is a cellular homeostasis mechanism that may have a protective role against osteoarthritis (OA). The present study investigated the therapeutic effect of local administration of rapamycin, a potent activator of autophagy, against OA. To achieve controlled intra-articular administration of rapamycin, gelatin hydrogels incorporating rapamycin-micelles were created and the release profile was evaluated in vitro. The therapeutic effects of gelatin hydrogels incorporating rapamycin-micelles were then tested in a murine OA model. Mice were divided into four groups: Group 1, gelatin hydrogels alone; Group 2, single injection of 1 μg rapamycin; and Groups 3 and 4, gelatin hydrogels incorporating 100 ng or 1 μg rapamycin-micelles, respectively. Immunohistochemical analysis revealed that autophagic marker-positive chondrocytes were increased in the rapamycin-treated mice at 10 weeks after surgery. The histologic score was better in Groups 3 and 4 than in Groups 1 and 2, and Group 2 had a better score than Group 1. Delayed OA progression was maintained even at 16 weeks after surgery in Group 4. Microarray and real-time polymerase chain reaction analysis indicated that OA mediator genes were downregulated in the rapamycin-treated mice. Our novel system for intra-articular administration of rapamycin could be a novel therapeutic approach for treating patients with OA.

1. Introduction

Osteoarthritis (OA) is a degenerative disease of human articular cartilage associated with many pathologic conditions, including an imbalance between anabolic and catabolic activity of chondrocytes [1,2], increased production of cartilage-degrading enzymes [3], and increased apoptosis [4,5]. Several studies have aimed to find possible disease-modifying OA drugs. There is currently no convincing evidence, however, for the efficacy of many potential drugs, including those that inhibit cartilage-degrading enzymes [6], and no disease-modifying OA drugs have yet been developed [7]. Novel treatments against OA are thus in high demand and are the subject of the present study.

Autophagy is a cellular homeostasis mechanism for degrading macromolecules and organelles damaged by various stressors [8–10]. Autophagy dysfunction is associated with degenerative diseases and aging [9,11]. Several reports suggest that autophagy is also involved in the pathogenesis or development of OA. Murine OA models revealed the activation of autophagy in the superficial zone and middle zone of chondrocytes in the early stages of OA [12], and decreased autophagy in association with the progression of OA [15]. In human articular cartilage samples, increased expression of autophagic markers, such as microtubule-associated protein 1 light chain 3 type II (LC3-II), is observed in mild OA cartilage compared with normal cartilage, and decreased LC3-II expression is observed in severe OA cartilage [13]. Further, mechanical injury induces an increase in LC3-II expression 24 h after injury, followed by a decrease in bovine and human cartilage [14]. Thus, autophagy may be an important cell survival mechanism of cartilage under stress.

The mammalian target of rapamycin (mTOR) is an important regulator of cell survival, growth, and autophagy [15]. Inhibition of the mTOR pathway has beneficial effects, including life extension in various organisms, and is therefore a therapeutic target for aging-

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associated diseases [16,17]. Rapamycin, clinically widely used as an immunosuppressant [18], exerts various biologic effects, including induction of autophagy through the inhibition of mTOR pathways. We previously reported that autophagy regulates osteoarthritic gene expression of human chondrocytes, and rapamycin has a protective role against inflammatory stress [13]. Similarly, rapamycin induces autophagy and prevents chondrocyte cell death by mechanical injury in cartilage explants [14]. A recent study demonstrated that intraperitoneal injection of rapamycin reduces the development of experimental OA in a murine model [19]. These findings indicate that rapamycin could be a novel therapeutic drug against OA. Due to the side effects of systemic administration of rapamycin, such as immunosuppression and inhibition of bone growth [20], however, local administration would be more desirable.

Biodegradable hydrogels are a safe drug delivery system applicable to humans, and the effects of locally controlled release of other drugs using this system have been confirmed in previous studies [21–24]. In the present study, to investigate the therapeutic effects of intra-articular administration of rapamycin using biodegradable hydrogels in a murine OA model, we created gelatin hydrogels incorporating rapamycin-micelles.

2. Materials & methods

2.1. Gelatin hydrogels incorporating rapamycin–micelles

Rapamycin was provided by LC Laboratories (Woburn, MA, USA). Rapamycin-conjugated gelatin hydrogel was produced according to a previous report [25]. Briefly, in the first step, L-lactic acid oligomers (LAo) with a mean molecular weight of 1000 were synthesized from L-lactide monomers by ring-opening polymerization with stannous octoate as the catalyst and 1-dodecanol as an initiator. LAo-grafted gelatin was synthesized by activating the hydroxyl groups of LAo and mixing them with a gelatin solution. A rapamycin/ethanol solution was added to the LAo-grafted gelatin aqueous solution. The reaction mixture was freeze-dried to obtain rapamycin water-solubilized by LAo-grafted gelatin micelles (rapamycin–micelles). To create the desired amount of rapamycin, the rapamycin–micelle aqueous solution and gelatin hydrogel aqueous solution were mixed and adjusted, and 2-μl drops of mixed solution were placed on parafilm. Pellets of rapamycin-micelle conjugated gelatin hydrogels were prepared through dehydrothermal crosslinking (140 °C, 24 h) of gelatin and ethylene oxide gas sterilization. The obtained solid small pellets of gelatin hydrogels incorporating rapamycin–micelles were used in the study. The gelatin hydrogels were designed to biodegrade over a period of ~3 weeks under in vivo conditions [25]. Empty gelatin hydrogels without rapamycin were similarly prepared as controls.

2.2. Evaluation of rapamycin–micelle biologic activity

A bioassay of an in vitro cell culture was performed. Normal human knee articular chondrocytes (passage 6) were cultured at 37 °C in a 95% air–5% CO_2 atmosphere at a density of 10 × 10 cells/well with culture medium. Rapamycin–micelles at each concentration were added to the medium, and rapamycin-free micelles were used as the control. Cells were collected for Western blot analysis at 24 h after treatment.

2.3. Release test of rapamycin from gelatin hydrogels incorporating rapamycin–micelles and test of hydrogel degradation

To investigate the sustained release of rapamycin, gelatin hydrogels incorporating rapamycin–micelles were placed in 1 ml phosphate-buffered saline (PBS) with or without 5 μg/ml collagenase. The release test was performed at 37 °C and the PBS was exchanged at each time-point. The supernatant was collected and frozen at −80 °C until measurement. The amount of rapamycin in the supernatant was measured by high performance liquid chromatography. To investigate the sustained degradation of the gelatin hydrogel, gelatin hydrogels incorporating rapamycin–micelles were placed in 1 ml PBS with or without 5 μg/ml collagenase. The degradation test was performed under conditions similar to the rapamycin release test. The absorbance of the supernatant was measured at a wavelength of 280 nm and the gelatin concentration was determined based on a calibration curve, as previously described [25]. The released amount of rapamycin and gelatin remained constant over 24 h, and thus 5 μg/ml collagenase was added after 24 h in the release test and degradation test. After adding the collagenase, the supernatants were measured using the same procedure at each time-point.

2.4. Mice

We used 8-week-old C57BL6/J mice (wild-type; Charles River Japan, Yokohama, Japan), and OA was induced by destabilizing the medial meniscus [26]. The mice ($n = 56$) were divided into four groups. Group 1, the control group, was treated with gelatin hydrogels alone. Group 2 was treated with a single injection of 1 μg rapamycin dissolved in dimethyl sulfoxide. Group 3 was treated with gelatin hydrogels incorporating 100 ng rapamycin-micelles. Group 4 was treated with gelatin hydrogels incorporating 1 μg rapamycin-micelles. Gelatin hydrogels and single injections were administered intra-articularly at the time of surgery. Mice were killed at 10 weeks after surgery for histologic analysis. To investigate and confirm the long-term effect of gelatin hydrogels incorporating rapamycin-micelles, we created two additional groups (a control group, $n = 5$, and a group treated with gelatin hydrogels incorporating 1 μg rapamycin-micelles, $n = 7$), and mice were killed 16 weeks after surgery. The efficacy of the in vivo controlled release of rapamycin was examined by immunohistochemistry using an autophagic marker, LC3-II, in wild-type mice, and by fluorescence analysis of green fluorescent protein (GFP) fused LC3-II in transgenic mice that express GFP-LC3. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Kobe. The mice were maintained under pathogen-free conditions and were allowed free access to food, water, and activity.

2.5. Histologic analysis

To evaluate OA progression, the mice were killed, and the entire knee joints were fixed in 4% paraformaldehyde in 0.1 M PBS overnight at 4 °C, decalcified for 2 weeks with 10% EDTA, and embedded in paraffin wax. Each specimen was cut into 5-μm slices along the sagittal plane and stained with safranin O–fast green. Three slices were selected from each medial femoral condyle and medial tibial plateau, and microphotographs were obtained at a magnification of 40×. The histologic OA grade for each field was evaluated using the Osteoarthritis Research Society International (OARSI) cartilage OA histopathology grading system (grade × stage; 0–24) [27]. OA grading was assessed by a single observer who was blinded to the study.

2.6. Immunohistochemistry

After epitope retrieval, the sections were incubated with primary antibody, followed by secondary antibody. The signal was developed as a brown reaction product using the peroxidase substrate 3,3′-diaminobenzidine with methyl green counterstaining. All images were obtained under a microscope (Biozero, KEYENCE, Itasca, OH). The following antibodies were used: MMP-13 (Abcam, Cambridge, MA) and LC-3B (Cell Signaling Technology, Danvers, MA, USA).

2.7. Imaging of autophagosome formation in GFP-LC3 mice

The entire knee joints of GFP-LC3 transgenic mice were embedded in OCT compound (Sakura Finetek Co, Tokyo, Japan) and frozen in liquid nitrogen. Each specimen was cut into 7-μm slices along the sagittal plane using a cryostat, then air-dried and fixed in 4% paraformaldehyde for 10 min. Optical z-stack images were obtained under a LSM510 META confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

2.8. RNA isolation and real-time polymerase chain reaction

Articular cartilage was collected from the medial femoral condyle and medial tibial plateau of 3 mice at 10 weeks after surgery, and the obtained cartilage was pooled, as described previously [28]. Total RNA was extracted using TRIzol (Invitrogen, Burlington, ON, Canada), followed by RNeasy (Qiagen), according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed on the ABI Prism 7700 sequence detection system (Applied Biosystems) using the TaqMan probe. Real-time PCR for matrix metalloproteinase (MMP)-9, MMP-13, CCAAT/enhancer binding protein beta (C/EBPβ), Col2a1, mTOR, interleukin-1 beta (IL-1β), and IL-6 was also performed.

2.9. DNA microarray analysis

RNA samples isolated from the articular cartilage of three mice were pooled for analysis. The cDNA construction and microarray analysis were performed by the Dragon Genomics Center (Takara Bio Inc., Mie, Japan). Microarray analysis was performed using the Agilent SurePrint G3 Mouse GE 8x60K Array platform (Agilent, Santa Clara, CA, USA). Each sample of the control group (Group 1) and the group treated with gelatin hydrogels incorporating 1 μg rapamycin-micelles (Group 4) was placed on the microarray chip, and analyzed. The signal log ratio was calculated as the log2 ratio of the intensity of the rapamycin-treated cartilage to the intensity of the control-treated cartilage (log2 rapamycin/control). In the first step, all 55,821 probe sets on the Agilent arrays were filtered by signal intensity value (upper cut-off 100th percentile and lower cut-off 20th percentile) between Group 1 and Group 4. Next, 46,994 probe sets were filtered based on a two-fold change in expression (9502 probe sets). Subsequently, genes in the rapamycin-treated group that were downregulated to less than 50% of the expression level of the control group (log2 ratio less than −1.0) were selected (2760 probe sets) for further study. After filtering the fold changing data, genes related to keywords, including extracellular matrix