Periodic Knee Injections of BMP-7 Delay Cartilage Degeneration Induced by Excessive Running in Rats

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ABSTRACT: Strenuous running of rats enhances mechanical stress on the knee, thereby inducing degeneration of articular cartilage. Bone morphogenetic protein-7 (BMP-7) has an inhibitory effect on cartilage degeneration, suggesting its usefulness for human osteoarthritis patients. However, its mode of administration should be investigated. We examined whether weekly knee injections of BMP-7 delayed the progression of cartilage degeneration. Wistar rats were forced to run 30 km in 6 weeks on a rodent treadmill, and BMP-7 was injected weekly into the knee. Macroscopically and histologically, this strenuous running regimen induced cartilage degeneration. Weekly injections of 250 ng BMP-7 delayed the progression of cartilage degeneration. Immunohistochemically, in the control knee, type II collagen expression decreased, while BMP-7 expression in chondrocytes slightly increased. Interestingly, weekly injection of BMP-7 increased BMP-7 expression even 9 days after the final injection. Disulfate disaccharide keratan sulfate in serum transiently increased in the control group, while it remained at a low level in the BMP-7 group. Weekly BMP-7 injection increased BMP-7 expression in chondrocytes and its effect seemed to last more than 7 days. The effect of BMP-7 could be monitored by serum keratan sulfate concentration. Periodical injections of BMP-7 delayed progression of cartilage degeneration induced by excessive running in rats. © 2009 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 27:1088–1092, 2009

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Osteoarthritis (OA) in the knees constitutes an increasingly common medical problem for aging people.[1] Mechanical stress is one of the factors contributing to the progression of OA. Strenuous running of rats enhances mechanical stress on weight bearing joints, inducing OA of the knees.[2,3] This model requires no surgery or drugs, making it possible to detect subtle changes accompanying OA.

Bone morphogenetic proteins (BMPs) have a variety of biological effects including enhancement of cartilage repair.[4] Among BMPs, BMP-7 is especially attractive, because it is one of two BMPs already approved for clinical use in various applications by the FDA. Recent data from an anterior cruciate ligament transection model in rabbits demonstrated that continuous intra-articular infusion of BMP-7 had a protective effect on cartilage degeneration,[5] suggesting the possible utility of BMP-7 as a treatment for human OA patients. However, given the challenges associated with clinical delivery by continuous infusion, further consideration should be given to the mode of administration.

We speculated that periodic injections of BMP-7 into the knee joint might suppress the loss of cartilage matrix and consequently prevent OA progression. The purpose of this study was to examine whether weekly knee injections of BMP-7 delay development of OA and to investigate the possible mechanisms for this action in a strenuous running model of OA in rats.

MATERIALS AND METHODS

Strenuous Running of Rats

Wistar rats at 15–16 weeks of age (Sankyo Labo Service, Tokyo, Japan) were used for the experiments. For strenuous running, a rodent treadmill machine (MK-680R5; ME Service, Tokyo, Japan) was used with a 5% incline (Fig. 1A). After 10 min of "warm-up" at 12 m/min, rats were forced to run at 20 m/min for 50 min 5 days a week. Rats were forced to run 15 km in 3 weeks or 30 km in 6 weeks.[2,3] All experiments were conducted in accordance with our institutional guidelines for the care and use of experimental animals.

Intra-Articular Injection of BMP-7

rhBMP-7 lyophilized in 5% lactose buffer (Stryker Biotech, Hopkinton, MA) was dissolved in phosphate-buffered saline (PBS). BMP-7 (250 ng) in 100 μL PBS was injected into the right knee with a 27-gauge needle on a 1.0 mL syringe through the lateral infrapatellar area toward the intercondylar space of the femur in a deep knee flexed position. The injection was initially given 5 days after strenuous running, and repeated a total of five times at 5, 12, 19, 26, and 33 days under anesthesia of 10 mg sodium pentobarbital (Dainippon Sumitomo Pharma, Osaka, Japan) by intraperitoneal injections (Fig. 1B). For the control, the left knee was untreated. Neither saline nor PBS was injected into the left knee to avoid possible enhancement of articular cartilage damage.[6] Blood samples were collected 1 h after strenuous running at 0, 7, 14, 21, 28, and 35 days. The rats were sacrificed with an overdose of sodium pentobarbital.

Macroscopic Observation

Tibial condyles were carefully dissected separately without damaging the cartilage surface, and then stained with India ink to identify location, size, and severity of cartilage degeneration. Macroscopic pictures were taken using specifications of MPS-7 (Sugiura Laboratory Inc, Tokyo, Japan), a dedicated medical photography platform, and a Nikon Coolpix 4500 digital camera (Nikon, Tokyo, Japan).

Histology

Distal femur and proximal tibia were fixed in 4% paraformaldehyde at pH 7.4 for 3 days, decalcified in 20% ethylenediamino-tetraacetic acid (EDTA) solution at 4°C for 21 days, then embedded in paraffin wax. The specimens were sectioned in the sagittal plane at 5 μm and stained with safranin-O. Histological sections were visualized using an Olympus IX71

Additional supporting information may be found in the online version of this article.

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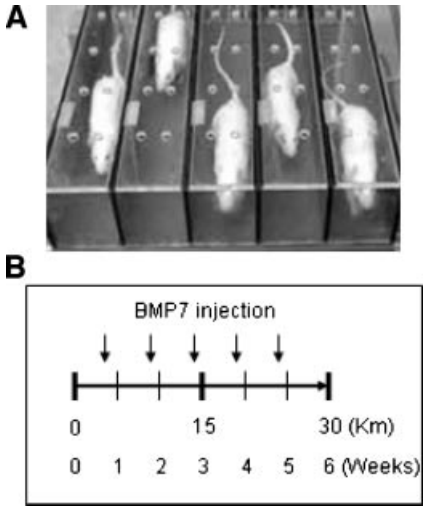

Figure 1. Outline for the study. (A) Treadmill for rats. (B) Schedule for running distance and BMP-7 injections.

microscope (Olympus, Tokyo, Japan). Each section was evaluated with the Mankin's histological grading system (Mankin's score: 0–14) for articular cartilage degeneration.[7]

Immunohistochemical Analysis

Sections were deparaffinized, washed in PBS, and pretreated with 0.4 mg/mL proteinase K (DAKO, Carpinteria, CA) in Tris-HCl buffer for 15 min at room temperature for optimal antigen retrieval. Endogenous peroxidases were quenched using 0.3% hydrogen peroxide in methanol for 20 min at room temperature. The sections were rinsed once in PBS and briefly blocked with 10% normal horse serum (Vector Laboratories, Burlingame, CA) to avoid nonspecific binding of the antibody. The tissue sections were then incubated in mouse monoclonal anti-BMP-7 antibody (12G3, 1:100 dilution; Stryker Biotech) or mouse monoclonal antihuman type II collagen (1:200 dilution; Daiichi Fine Chemical, Toyama, Japan) at 4°C overnight. After rinsing in PBS, the tissues were incubated with biotinylated horse antimouse IgG secondary antibody (Vector Laboratories) for 30 min at room temperature. Immunohistochemical staining was detected with Vectastain ABC reagent (Vector Laboratories), followed by DAB staining. For BMP-7, the sections were counterstained with methyl green.

Keratan Sulfate Concentration

Each serum, aliquots of 0.2 mL, was diluted in water (0.8 mL) and then digested with 0.1 mL of 2.0% Actinase E (Kaken Pharmaceutical, Tokyo, Japan) at 55°C for 24 h. The digest was then kept at 100°C for 10 min. The whole quantity of the solution was applied to Q Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden), and washed by 25 m*M* Tris-HCl buffer (pH 8.6) containing 0.15 *M* sodium chloride. After extraction with 50 m*M* Tris-HCl buffer (pH 8.6) containing 2 *M* sodium chloride, the extracted material was desalinated with PD-10 (Amersham Pharmacia Biotech) and dried. Then the material was dissolved again in 0.2 mL of distilled water containing 1 mU of Keratanase II (Seikagaku Corp.) After the addition of 0.04 mL of 100 m*M* sodium acetate buffer (pH 6.0), the mixture was incubated at 37°C for 3 h. The sample was ultrafiltered using an Ultrafree C3GC system (molecular size cut-off 10,000; Japan Millipore, Tokyo, Japan), and the filtrate, which contained mono-sulfate disaccharide and di-sulfate disaccharide derived from keratan sulfate, was analyzed by HPLC. The area of each peak corresponding to the monosulfate disaccharide and to disulfate disaccharide was calculated and converted to the amount of the corresponding disaccharides against the area of standard monosulfate disaccharide and disulfate disaccharide (Seikagaku Corp).[3]

Statistical Analysis

The StatView 5.0 program (SAS Institute, Cary, NC) was used for statistical analyses. The Wilcoxon signed rank was performed between BMP-7 treated and untreated knees in both femur and tibia. The Man-Whitney *U*-test was used for the disulfate disaccharide keratan sulfate concentration for the 30 km running groups between the BMP-7 injection group and the no injection group, and p values less than 0.05 were considered to be statistically significant.

RESULTS

Weekly BMP-7 Injection Delays Cartilage Degeneration

Strenuous running induced degeneration of cartilage in the untreated knees. Macroscopically, tibial surfaces of both lateral and medial condyles were irregular after 30 km of running (Fig. 2A). In contrast, cartilage surface remained smooth in BMP-7-injected knees. Histologically, in the untreated knees, 15 km of running slightly reduced safranin-O staining for femoral and tibial cartilages, and 30 km of running resulted in the loss of cartilage matrix in femoral cartilage and in the fissure formation in tibial cartilage (Fig. 2B). Though reduction of safranin-o staining for cartilage matrix was observed after 30 km of running in BMP-7-treated knees, quantitative analysis for histology demonstrated that the condition of the cartilage in BMP-7-treated knees was significantly better than that in untreated knees after 30 km of running in both femur and tibia (Fig. 2C). Histologies of the worst, representative, and best cartilages are shown in the Supplementary Material section.

Weekly BMP-7 Injection Increases Endogenous BMP-7 Expression

Type II collagen expression decreased after 30 km of running in untreated knees, while it was maintained in BMP-7-treated knees (Fig. 3). Our immunohistological analysis showed that normal rat cartilage before strenuous running hardly expressed BMP-7, but chondrocytes in untreated knees slightly expressed BMP-7 after 30 km of running. Interestingly, weekly BMP-7 injections increased BMP-7 expression at a higher level.

BMP-7's Effect Could Be Monitored by Serum Keratan Sulfate Concentration

In the control group, disulfate disaccharide keratan sulfate rapidly increased at 3 weeks, was maintained at a high level at 4 weeks, then decreased at 5 weeks (Fig. 4). Contrarily, in the BMP-7 group, disulfate disaccharide keratan sulfate remained at low levels

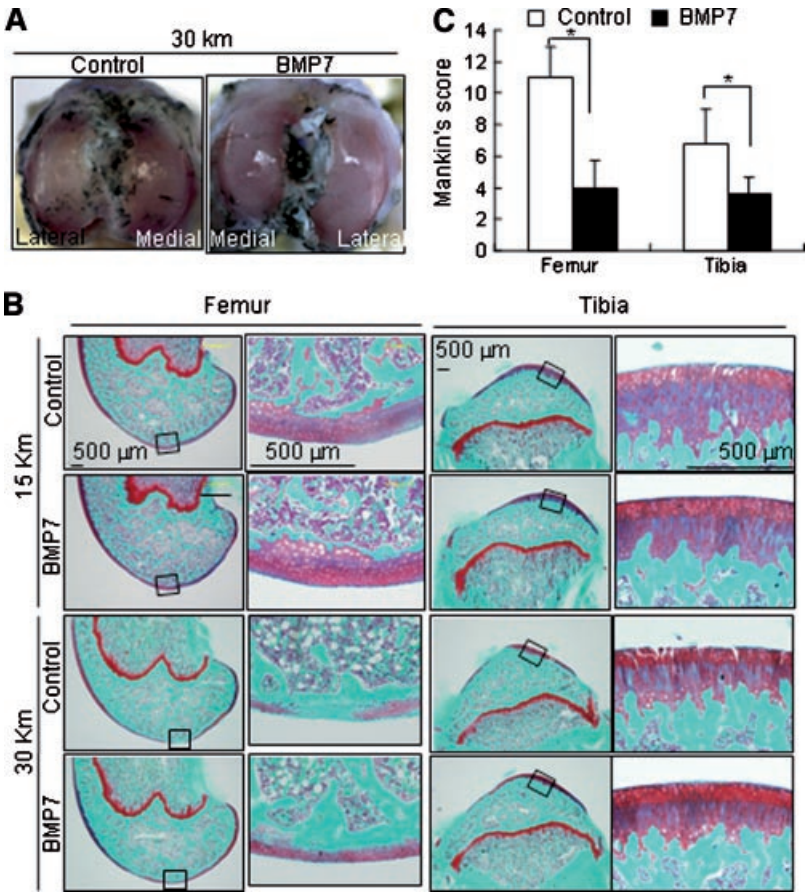

Figure 2. Analysis for articular cartilage of the knee. The right knee was injected with BMP-7 and the left knee was untreated. Paired analysis was performed. (A) Macroscopic observation of tibial articular cartilage stained with India ink. (B) Histologies of the lateral femoral and medial tibial cartilage stained with safranin-O. (C) Mankin's score for femoral and tibial cartilage lesions in 30 km running groups. Average values with standard deviations are shown ($n = 5$). $^*p < 0.05$ by Wilcoxon signed rank test.

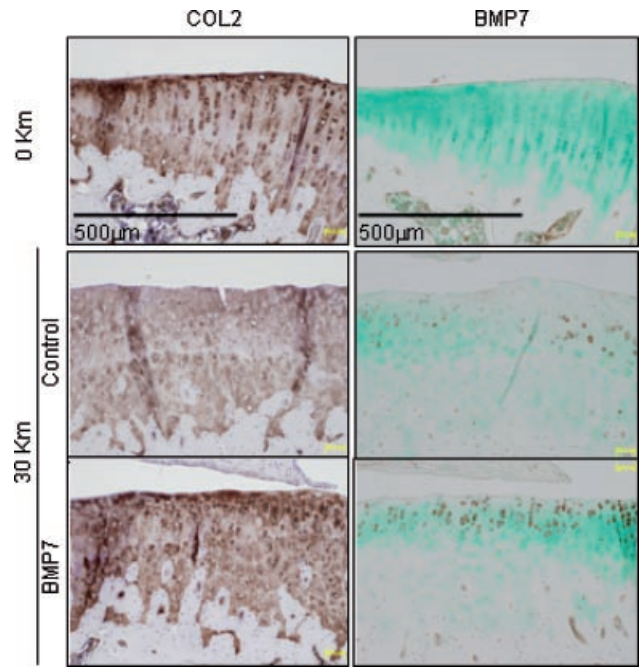

Figure 3. Immunohistochemical analysis for the medial tibial cartilage. For BMP-7, the sections were counterstained with methyl green.

over 5 weeks. Concentration of monosulfate disaccharide keratan sulfate in serum was stable in both control and BMP-7 groups.

DISCUSSION

In this study, we demonstrated that weekly injection of BMP-7 delays cartilage degeneration in a strenuous running model of rats. The effects of BMP-7 on cartilage can be explained by two different mechanisms: enhance-

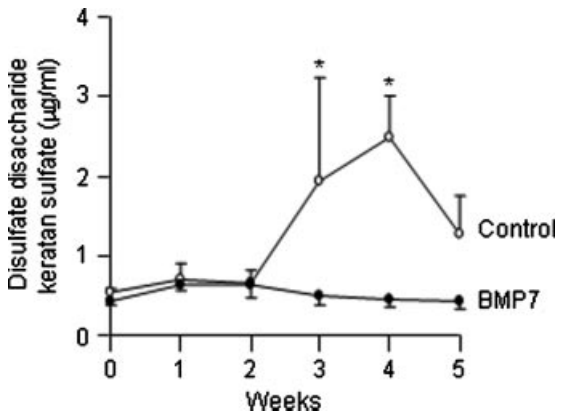

Figure 4. Serum concentration of disulfate disaccharide keratan sulfate. For BMP-7 group, BMP-7 was injected into both knees. For control group, both knees were untreated. Average values with standard deviations are shown ($n = 5$). $^*p < 0.01$ by Mann-Whitney U-test between BMP-7 and control groups at same periods.

ment of cartilage matrix synthesis, and inhibition of cartilage degeneration. Several *in vitro* studies indicated that BMP-7 promoted the production of type II collagen and proteoglycans in chondrocytes derived from normal[8] and osteoarthritic patients.[9] On the other hand, BMP-7 suppressed the IL-1-induced catabolism in explant culture of human articular cartilage[10] and aggrecanase in a rabbit model.[5]

For cartilage defect, implantation of a scaffold containing BMPs promoted cartilage repair in animal models.[11] However, progression of OA will not be inhibited by only a single administration of a BMP for a long period. Continuous administration of BMP-7 delivered by an osmotic pump delayed development of OA in a rabbit anterior cruciate ligament transection model.[5] However, from the standpoint of clinical availability, periodic knee injections of BMP-7 would be more attractive. To reduce frequency of injection, development of a slow release system for BMPs is required for clinical application.

Weekly BMP-7 injection enhanced BMP-7 expression in chondrocytes more than 7 days after the injection. We propose three possible mechanisms to explain what caused this. First, injected BMP-7 remained in the knee joint with activity for over 7 days. Second, exogenous BMP-7 induced endogenous BMP-7 expression in chondrocytes, and then the chondrocytes continued to express endogenous BMP-7 in an autocrine/paracrine manner. Third, synovial tissue absorbed injected BMP-7, and then synovial cells expressed endogenous BMP-7 to enhance endogenous BMP-7 expression in the chondrocytes.

In the control knee, BMP-7 expression in chondrocytes also increased after 30 km of running. One cause of this may be that endogenous BMP-7 expression increases as a protective response to cartilage degeneration. Chubinskaya et al.[12] reported that human OA patients showed higher BMP-7 mRNA expression in chondrocytes than normal patients. The other possibility is that the BMP-7 that was injected into the unilateral knee affected the contralateral knee via blood circulation. Simic et al.[13] demonstrated that ^{125}I-BMP-6 administered systemically accumulated in the skeleton and restored the quality of the skeleton in osteoporotic rats, though the concentration of BMP-6 they used was more than 10-fold higher than that in our study.

Keratan sulfate is a glycosaminoglycan specifically distributed in the extracellular matrix of the cartilage, cornea, and brain.[14] Wakitani et al. measured serum keratan sulfate using HPLC, which is more sensitive and more accurate than ELISA,[15] and demonstrated a higher value of serum keratan sulfate in patients with early-stage damage of the articular cartilage, which is undetectable by X-ray imaging.[16] During strenuous running of rats, serum keratan sulfate transiently increases in the early stage of OA with a decreasing of keratan sulfate in the affected cartilage.[3] Our study demonstrated that the effect of BMP-7 could be reflected by the concentration of keratan sulfate in serum.

We previously reported that intra-articular hyaluronan injection suppressed progression of cartilage degeneration in the same model of rat strenuous running.[3] Among drugs for the treatment of OA, intra-articular hyaluronan treatment is widely used due to the perceived benefits and the virtual absence of serious side effects. However, the effect of hyaluronan on prevention of OA seems to be limited according to several meta-analyses.[17]

Recently, novel approaches such as injection of caspase inhibitors,[18] treatment with basic fibroblast growth factor,[19] oral doxycycline,[20] and oral glucosamine [21] have been reported for OA prevention. In our results, BMP-7 reduced OA progression but did not block progression of OA completely. This suggests that BMP-7 is effective for delay of OA progression. If synthesis of cartilage matrix can be increased more by BMP-7, this treatment can be applied at the late stage of OA to regenerate cartilage. We advocate that periodic intra-articular injections of BMP-7 have potential as treatment for patients with OA.

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