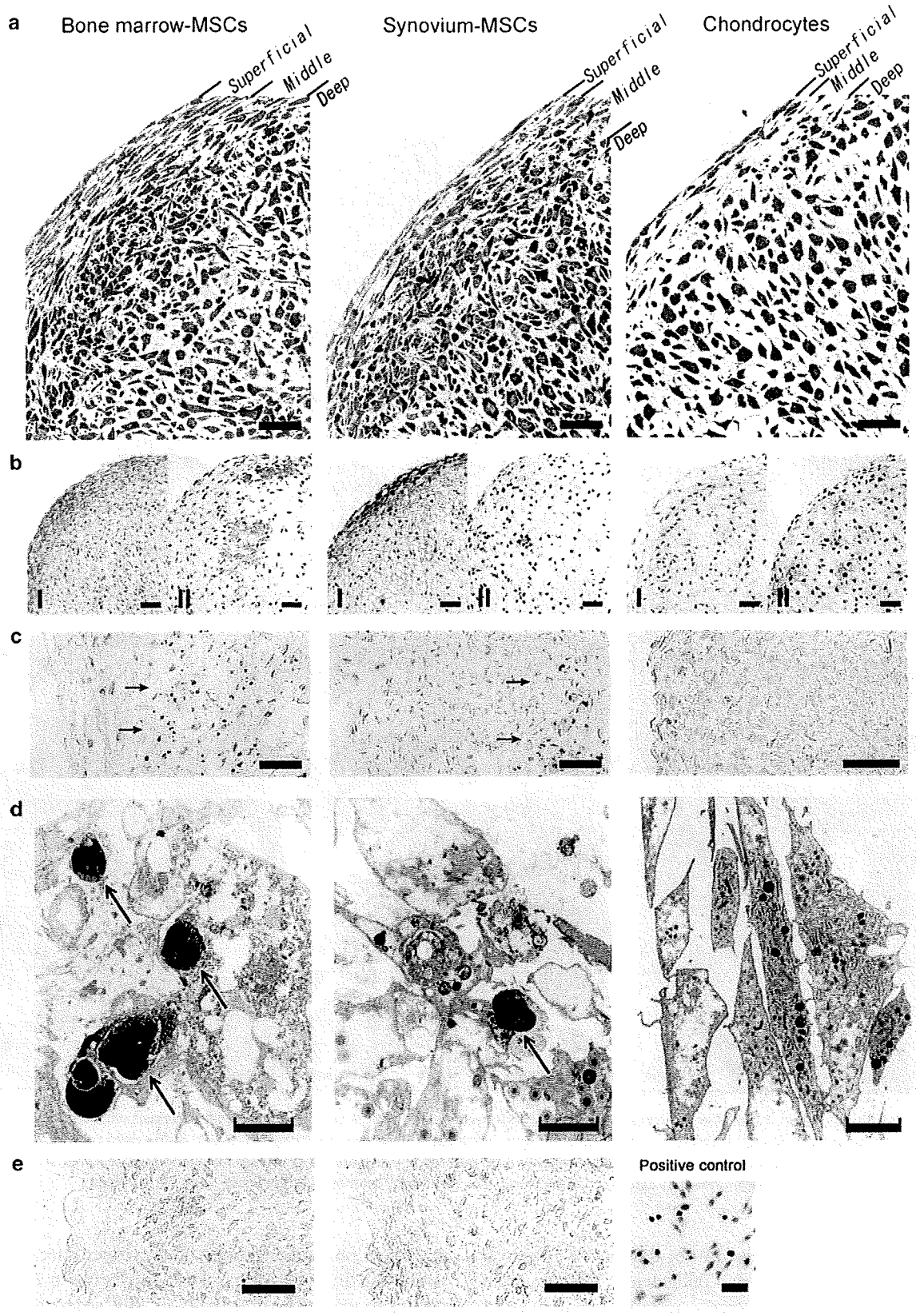

Figure 6 Morphology of pellets 7 days after induction of *in vitro* chondrogenesis. (**a**) Optical micrographs of pellets stained with toluidine blue. Scale bar = 50 μm. (**b**) Immunohistochemical staining for type I and II collagen. Scale bar = 50 μm. (**c**) TUNEL staining for apoptosis in the superficial and middle zone. TUNEL positive cells are shown as arrows. Scale bar = 50 μm. (**d**) TEM images in the middle zone. Apoptotic cells are shown as arrow. Scale bar = 5 μm. (**e**) Ki67 staining for proliferation in the superficial and middle zone. Hela cells are also shown as positive control. Scale bar = 50 μm.

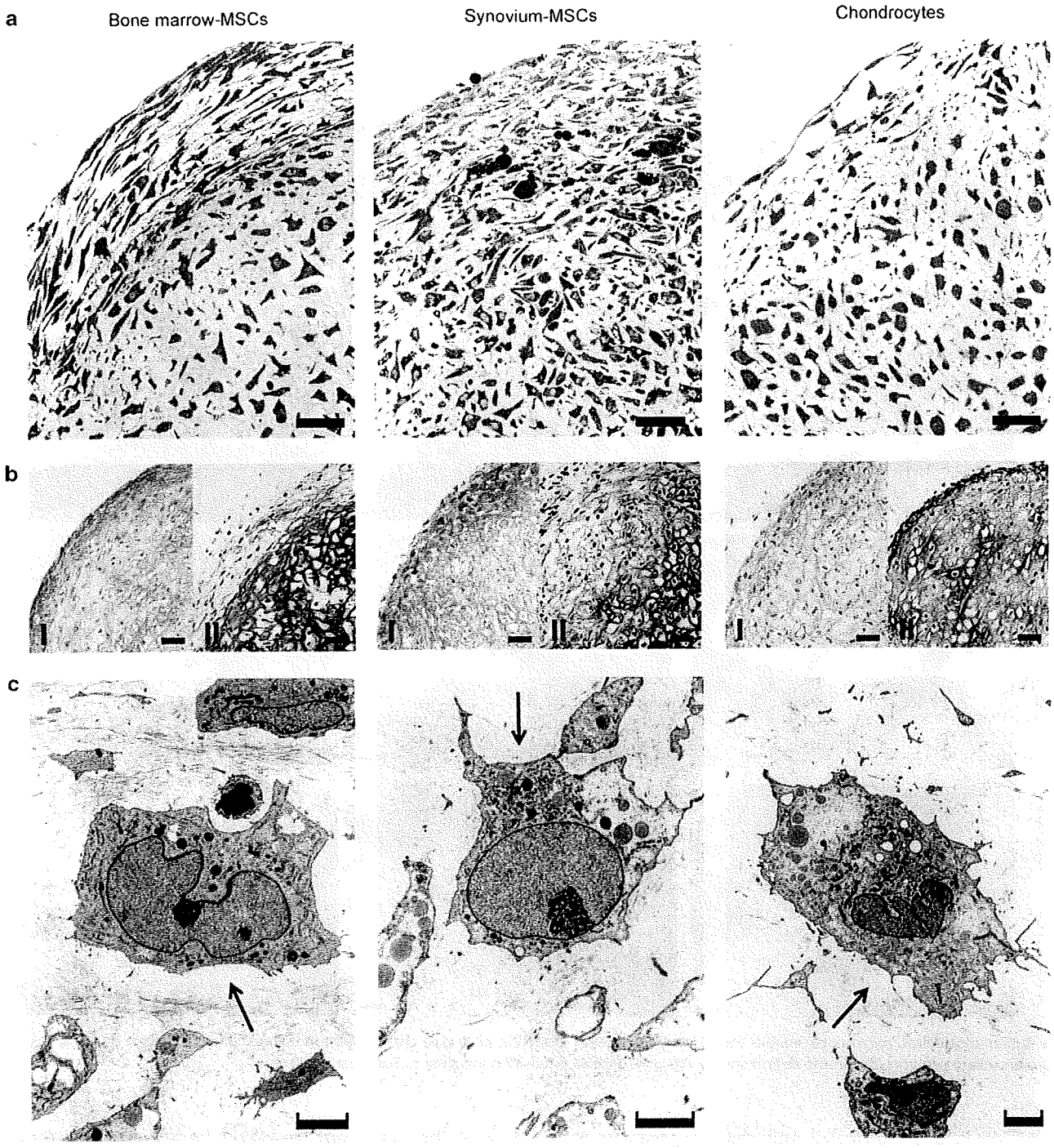

Figure 7 Morphology of pellets 21 days after induction of *in vitro* chondrogenesis. (**a**) Optical micrographs of pellets stained with toluidine blue. Scale bar = 50 μm. (**b**) Immunohistochemical staining for type I and II collagen. Scale bar = 50 μm. (**c**) TEM images in the deep zone. Mature chondrocyte-like cells are located at the center. Well-developed matrix fibers are shown as arrow. Scale bar = 5 μm.

largely within the first week and only slightly between 2 and 3 weeks because of apoptosis.

At the early phase of differentiation, pellets of bone marrow- and synovium-MSCs showed higher density of cells in the middle zone and demonstrated different features from that of chondrocytes. Was there any difference in cell proliferation between the cells? The answer is no. We examined Ki67 expressions for proliferation but did not observe any Ki67 expressions in the three populations. This indicates that different features between two populations of MSCs and chondrocytes in the middle zone were not due to cell proliferation. In our earlier report of bone

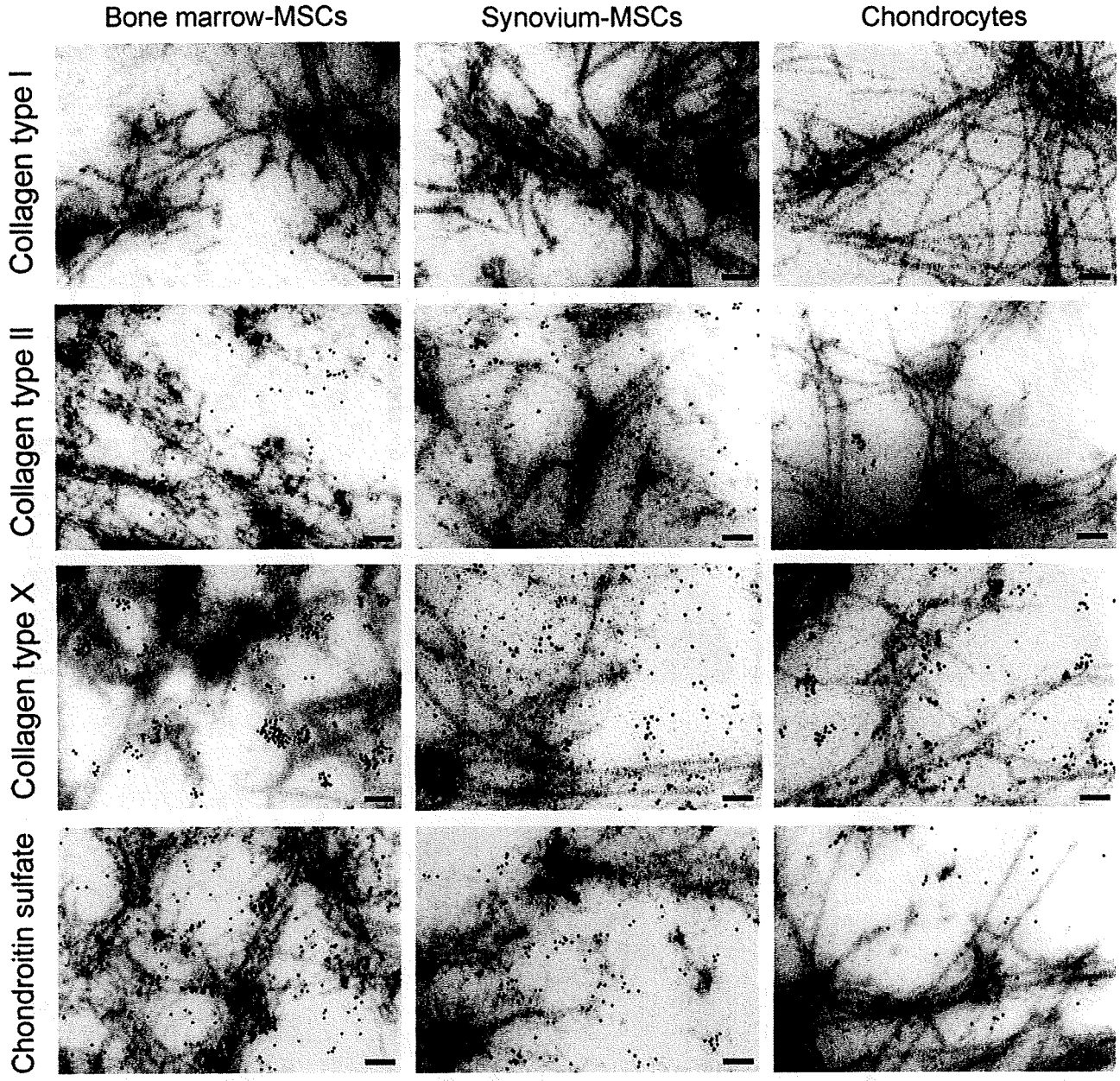

Figure 8 Immunoelectron microscopic images for pellets 21 days after induction of *in vitro* chondrogenesis. Expressions of collagen type I, II, X, and chondroitin sulfate proteoglycan in the deep zone by the gold particle deposition are shown. Scale bar = 100 nm.

marrow-MSCs, a pulse-labeled and chase experiment with [^{3}H] thymidine indicated that there was a 25% decrease in the specific activity of cellular DNA between day 0 and day 7, but thereafter the values remained constant,[17] demonstrating that bone marrow-MSCs did not divide at the early phase.

We performed all experiments in three donors with osteoarthritis, and similar results were obtained. To account for variances among donors and among procedures,[5] we harvested bone marrow, synovium, and cartilage simultaneously after total knee arthroplasty, performed the procedures, and analyzed the cells from bone marrow, synovium, and cartilage at the same time. It would be intriguing to examine whether similar results could be obtained in cells derived from young donors; however, it is not easy to harvest bone marrow, synovium, and especially cartilage from young donors simultaneously. The confirmation in young donors would be practically difficult.

Ectopic cartilage formation is one of the pathological conditions in articular joints.[14] Candidate cell sources for the ectopic cartilage formations are stem cells in bone marrow and synovium, in addition to chondrocytes. A comparison of the morphology of cartilage formation between *in vitro* chondrogenesis of these cells and the ectopic cartilage for-

Table 1 Critical differences of morphologies during *in vitro* chondrogenesis

Days	Zone	Bone marrow-MSCs	Synovium-MSCs	Chondrocytes
0		Round cells with a large number of processes at the cell surface		
1	Superficial zone		Spindle cells parallel to the surface	
	Deep zone	Round cells without intercellular space	Elongated cells with intermediate intercellular spaces	Oval and polygonal cells with intercellular spaces
		Intermediate junctions	Small desmosome	Small desmosome
7	Superficial zone		Spindle cells parallel to the surface	
	Middle zone	Higher cell density	Higher cell density	Lower cell density
		Apoptotic cells	Apoptotic cells	No apoptotic cells
	Deep zone	Intermediate intercellular space	Relatively narrow intercellular space	Large intercellular space
21	Superficial zone	Obvious	Unclear	Narrow
	Deep zone		Polygonal cells with pericellular matrix	

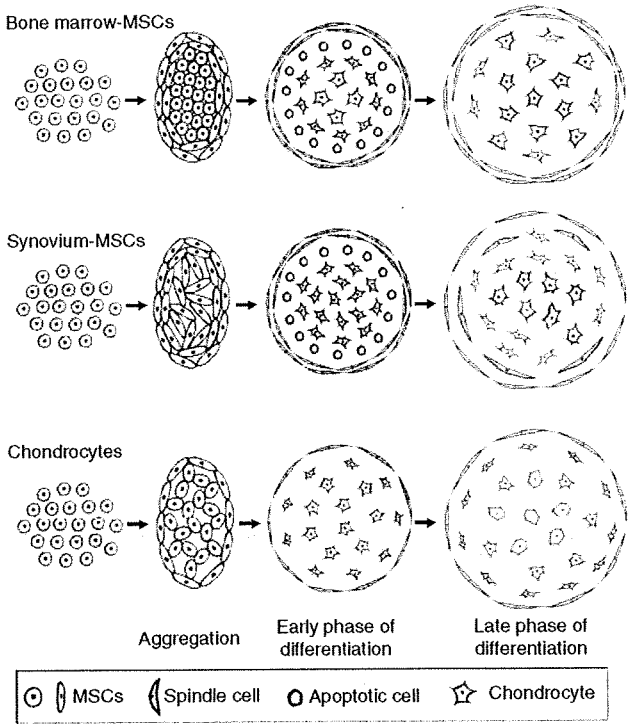

Figure 9 Scheme for morphological events during *in vitro* chondrogenesis of bone marrow-, synovium-MSCs, and chondrocytes. Explanations are summarized in Table 1.

mation could possibly clarify the cell source and the mechanisms of the ectopic cartilage formation.

The chondrogenic medium used in the study was previously demonstrated to be suitable for MSCs derived from bone marrow[16,18] and synovium.[23] This chondrogenic medium may not be optimal for chondrocytes. However, in this study, we demonstrated that colony-forming cells derived from cartilage produced cartilage matrix in the condition we used. If we had performed *in vitro* chondrogenesis of chondrocytes with a medium more suitable for chondrocytes, we could not have concluded that morphological differences are due to a different origin of the cells, because different culture medium may affect the morphology of the cells. Therefore, we used common chondrogenic medium for *in vitro* chondrogenesis of bone marrow-MSCs, synovium-MSCs, and chondrocytes.

There were some differences of histological patterns between toluidine blue and type II collagen staining, both of which were supposed to be correlated principally. We embedded histological samples in Epon for toluidine blue staining in order to enable detailed analysis, and in paraffin for immunohistology.[19] We did not use serial sections for toluidine blue and type II collagen staining, resulting in different patterns between in toluidine blue and type II collagen staining.

For treatment of cartilage defect, transplantation of MSCs is an effective strategy. Among a variety of MSC sources, bone marrow and synovium are useful for high chondrogenic potential of their MSCs. The two MSCs contain common features, but distinguishing properties dependent on their origin are emerging.[6–9,11,12] Colony-forming efficiency was higher in synovium-MSCs but colony size was larger in bone marrow-MSCs.[7] Expression of PDGF receptor-α was higher in synovium-MSCs based on flow cytometrical analysis.[12] In this study, we demonstrated morphological differences during *in vitro* chondrogenesis of bone marrow- and synovium-MSCs. Biologically, these differences are due to their different gene profiles. We previously compared gene profiles of bone

marrow- and synovium-MSCs. The expression levels of chitinase 3-like 1 (CHI3L1), aggrecan 1, WINT1-inducible signaling pathway protein 2 (WISP2), fibulin 1, and S100 calcium-binding protein were extremely low in bone marrow-MSCs and high in synovium-MSCs.[10]

Cytologically, differences of cell–cell junctions at the aggregation phase in the three populations were interesting. Wuchter *et al* recently reported novel type cell junctions. They demonstrated that bone marrow-MSCs under monolayer conditions were interconnected by special tentacle-like cytoplasmatic protrusions and invaginations.[24] Wagner and Ho[25] described that the frequency and morphology of these conjunction complexes were greatly affected by culture conditions. In our experiments, further detailed investigation would provide new insight into the nature of cell junctions in MSCs during the chondrogenesis.

Conclusions

In this study, we revealed morphological differences of bone marrow-MSCs, synovium-MSCs, and chondrocytes during *in vitro* chondrogenesis. The most obvious differences in the three populations were observed at the aggregation phase in the deep zone.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Original article

Atelocollagen-associated autologous chondrocyte implantation for the repair of chondral defects of the knee: a prospective multicenter clinical trial in Japan

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Abstract

Background. New tissue-engineering technology was developed to create a cartilage-like tissue in a three-dimensional culture using atelocollagen gel. The minimum 2-year follow-up outcome of transplanting autologous chondrocytes cultured in atelocollagen gel for the treatment of full-thickness defects of cartilage in knees was reported from the single institution. The present multicenter study was conducted to determine clinical and arthroscopic outcomes in patients who underwent atelocollagen-associated autologous chondrocyte implantation for the repair of chondral defects of the knees.

Methods. At six medical institutes in Japan, we prospectively evaluated the clinical and arthroscopic outcomes of transplanting autologous chondrocytes cultured in atelocollagen gel for the treatment of full-thickness defects of cartilage in 27 patients (27 knees) with cartilage lesions on a femoral condyle or on a patellar facet over 24 months.

Results. The Lysholm score significantly increased from 60.0 ± 13.7 points to 89.8 ± 9.5 points ($P = 0.001$). Concerning the ICRS grade for arthroscopic appearance, 6 knees (24%) were assessed as grade I (normal) and 17 knees (68%) as grade II (nearly normal). There were few adverse features, except for detachment of the graft in two cases.

Conclusions. We concluded that transplanting chondrocytes in a newly formed matrix of atelocollagen gel can promote restoration of the articular cartilage of the knee.

Introduction

Cartilage defects and subsequent osteoarthritis of the knee induce pain and dysfunction of the knee joint, leading to substantial lowering of patients' quality of life.[1,2] Numerous forms of treatment have been developed for cartilage defects in the knee joints,[3] although there is no superior procedure for all clinical situations with cartilage defects in the knee joints.[3,4] Based on the idea to use the patient's own chondrocytes for regeneration of the defect area, Brittberg et al.[5] treated large and deep cartilage defects with autologous chondrocyte implantation (ACI), a methodology first published in 1994. Over the past years, some concerns linked with ACI have become apparent.[6] The first concern is that monolayer cell cultures are used to proliferate chondrocytes before their implantation. It is known that chondrocytes in monolayer cultures alter their phenotype and dedifferentiate to fibroblast-like cells that no longer have the capacity to produce collagen type II and proteoglycans.[7–10] The second concern is the risk that chondrocytes may leak from the site of the graft after resumption of load-bearing because chondrocytes are transplanted in suspension.[11] In addition, there is the possibility that the transplanted chondrocytes in suspension accumulate on one side of the defect, mainly as a result of gravity, and are not evenly distributed. In response to these concerns, various scaffolds used as carriers for chondrocyte implantation are under investigation.[12]

To resolve these issues of ACI, matrix-induced autologous chondrocyte implantation (MACI) that utilizes the collagen matrix as a carrier has attracted attention.[13] Recently, Ochi et al.[14] developed new tissue-engineering technology to create a cartilage-like tissue in a three-dimensional culture using atelocollagen gel, from which telopeptides have been removed to eliminate the antigenic determinants of bovine type-I colla-

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gen. They investigated clinical, arthroscopic, and biomechanical outcomes of transplanting autologous chondrocytes cultured in atelocollagen gel for the treatment of full-thickness defects of cartilage in knees over a minimum period of 2 years.[15] As a result, they reported encouraging initial results at the single center. However, there is no multicenter evaluation of the effectiveness of the present new tissue-engineering implant. The purpose of the present study was to evaluate clinical and arthroscopic outcomes in patients undergoing atelocollagen-associated autologous chondrocyte implantation for the repair of chondral defects of the knees in a prospective multicenter clinical trial in Japan.

Materials and methods

Subjects

All patients who met predefined criteria were selected from the outpatients of six medical centers with a voluntary program designed to track the outcomes of patients who were treated using the tissue-engineered cartilage with autologous chondrocytes embedded in atelocollagen gel (ACC-01; Japan Tissue Engineering, Gamagori, Japan). The present clinical trial was approved by each institutional review board of the six participating medical institutions. All patients provided written informed consent according to the format of the Ethics Committee in each institution.

We defined the inclusion and exclusion study criteria before selecting patients. The patients were included in this study if (1) they were ≥20 years of age; (2) they had at least one knee full-thickness chondral lesion caused by trauma, osteochondritis dissecans, or osteoarthritis; (3) the chondral lesion had not been improved or was not expected to be improved by conventional treatments including arthroscopy, débridement, marrow stimulation technique, or autogenous osteochondral transplantation; (4) the area of their chondral defect was ≥1 cm^2. Patients were excluded from the study if (1) they had rheumatoid arthritis or other systemic joint disease; (2) they had undergone chemotherapy for malignant disease; (3) their general condition was considered to affect the healing process of the implanted cartilage (i.e., severe infection, impaired renal function, impaired liver function, severe diabetes); or (4) they had had an episode of anaphylactic shock or other allergic reaction to beef.

In accordance with these inclusion and exclusion study criteria, 31 patients were selected. Of these 31 patients, one was excluded due to erysipelas of the leg that occurred 3 weeks after the cartilage harvest. We lost three cases at the 24-month follow-up. We evaluated the remaining 27 cases (90%) at 3, 6, 12 and 24 months after the implantation surgery.

The causes of the osteochondral defect were trauma (19 knees), osteochondritis dissecans (3 knees), and osteoarthritis (5 knees). Concerning the radiographic stage of the osteoarthritic knees, three, one, and one were graded as Kellegren-Lawrence grades I, II, and III, respectively. The lesions were on the medial femoral condyle in 16 knees, the lateral femoral condyle in 5 knees, and the patella in 6 knees. The mean size of the lesion was 3.2 cm^2 (range 1.2–9.4 cm^2). Concerning previous surgical procedures, bone marrow stimulation procedure (arthroscopic drilling), open reduction and internal fixation for an osteochondral fracture, and anterior cruciate ligament (ACL) reconstruction had been performed in one, one, and six cases, respectively.

Isolation and culture of chondrocytes

We preoperatively confirmed that patients were not allergic to atelocollagen gel by skin tests. The patients underwent a two-stage procedure that included cartilage harvest and subsequent implantation of autologous chondrocytes embedded in atelocollagen gel.[14,15] The cartilage biopsy was sent to a single facility (Japan Tissue Engineering), where chondrocytes were isolated from the cartilage biopsy, the engineered cartilage was prepared, and the chondrocytes were cultured to expand the cell population. The cartilage biopsy was digested in collagenase (type XI; Sigma-Aldrich, St. Louis, MO, USA) solution, and chondrocytes were obtained. The chondrocytes were suspended in Dulbecco's modified medium (DMEM; GIBCO Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, St. Lenexa, KS, USA), and 20 mM HEPES (GIBCO Invitrogen). Four volumes of atelocollagen solution (3% type I collagen; Koken, Tokyo, Japan) were then added to one volume of cell suspension and mixed thoroughly. The mixture was placed onto culture dishes and gelated completely by incubation at 37°C. After 1 h, culture medium or DMEM supplemented with 10% FBS, and L-ascorbic acid phosphate magnesium salt 50 μg/ml (Nikko Chemicals, Tokyo, Japan), gentamicin sulfate 50 μg/ml (Schering-Plough, Munich, Germany), amphotericin B 0.25 μg/ml (Bristol-Myers Squibb, New York, NY, USA), and HEPES were added to the culture dishes. FBS was selected according to notification no. 210 of the Ministry of Health, Labor, and Welfare in Japan on the Standard for Biological Ingredients. Then, the tissue-engineered cartilage was incubated in an atmosphere of 5% carbon dioxide and 95% air at 37°C. The culture medium was changed every 3–4 days. During culturing, the culture medium was collected, and sterility testing was carried out. With the progress of cultivation, the atelocollagen gel, including chondro-

cytes, had become opaque and had acquired a jelly-like hardness.

Implantation of the tissue-engineered cartilage

The tissue-engineered cartilage was implanted 28 days after harvest of the cartilage. The manufacturer tested the sterility and cell viability according to strict operating procedures before shipping the tissue-engineered cartilage to the hospital for implantation. For all tissue-engineered cartilage, we then confirmed negative bacterial cultivation test of the medium, negative membrane filter sterility test, negative *Mycoplasma* screening test using the polymerase chain reaction (PCR), negative endotoxin test, more than 1.5-fold increase in the number of viable cells by microscopic examination determining cell number and viability with a hemocytometer and trypan blue staining, cellular outgrowth from the tissue-engineered cartilage, glycosaminoglycan content (>25 μg/cm^3), and bovine serum albumin content (<13 μg/cm^3) before shipping the tissue-engineered cartilage.

A medial or lateral parapatellar arthrotomy was carried out under tourniquet control. The chondral lesion was débrided as far as normal surrounding cartilage and until subchondral bone was visible. The defect was covered by a sutured periosteal flap taken from the proximal medial tibia. The flap was shaped and sutured to the surrounding rim of normal cartilage with interrupted 5-0 nylon and loosely tied 4-0 Vicryl sutures with the deep cambium layer facing the subchondral bone plate.[14,15] After suturing half of the border of the flap, the chondrocyte–telocollagen gel was placed in the defect, and the remaining border of the flap was sutured. The joint capsule, retinaculum, and skin were sutured in separate layers. The knee was supported by a lightweight brace for 2 weeks. If required, the ACL was reconstructed using hamstring tendons assisted by arthroscopy 4 weeks before transplantation, at the time of harvest of the cartilage. Two weeks after transplantation, continuous passive movement of the joint was begun. Partial weight bearing was introduced 3 weeks after surgery and was gradually increased to full weight bearing with muscle training during the first 8 weeks after surgery.

Evaluation

We evaluated the clinical outcome by our original knee function scale and the score described by Lysholm and Gillquist[16] at 3, 6, 9, 12, and 24 months after the implantation. Our original knee function scale was designed to evaluate specific knee symptoms that are considered to be indicative of deterioration by cartilage lesions (motion pain, rest pain, knee motion) (Table 1). The highest obtainable score is 100. We also performed arthroscopic evaluation for all cases at 12 months after the surgery. The hardness of the graft was tested with a probing hook, and the gross appearance was considered to be biologically acceptable if the transplanted cartilaginous tissue was in contact, as well as level, with the surrounding articular cartilage.

The arthroscopic results were graded according to the assessment scale of cartilage repair developed by the International Cartilage Repair Society (ICRS).[17] This 12-point scale awards up to four points each for the degree of repair of the defect, the degree of integration with the surrounding cartilage tissue, and macroscopic appearance. Grade I (12 points) is considered normal, grade II (8–11 points) nearly normal, grade III (4–7 points) abnormal, and grade IV (1–3 points) severely abnormal. Outcome scores at the postoperative periods were compared to the baseline scores by the one-sample Wilcoxon test. The Kruskal Wallis test and Mann-

Table 1. Original knee function scale

Description	Score
Knee motion pain	
No motion pain	50
Mild motion pain (rare, relieved)	35
Moderate motion pain (frequent, limiting)	20
Severe motion pain (constant, not relieved)	0
Rest knee pain	
No rest pain	25
Mild rest pain (rare, relieved)	15
Moderate or severe rest pain (frequent or constant)	0
Range of knee motion	
No loss of motion	25
Mild loss of motion (total arc ≥90°)	16
Moderate loss of motion (total arc <90°)	8
Ankylosis	0
Total	100

Whiteney U-test were used for comparison among the groups. The significance limit was set at $P = 0.05$.

Results

Clinical evaluation

Before the final follow-up, one patient required reimplantation of another tissue-engineered cartilage, which was described in the Treatment Failure and Subsequent Operations section. This case was excluded for the clinical evaluation. None of the cell cultures contained bacteria or fungi, and none of the patients had infections of the knee after transplantation. Clinically, pain, swelling, crepitus, and locking of the knee in all patients were relieved, and all of the patients had returned to normal activities.

Concerning our original scale, the total score and the scores for motion pain and rest pain were significantly higher at 3, 6, 12, and 24 months than the baseline values (Fig. 1a–c). The scores for knee motion at 12 months and 24 months were significantly higher than the baseline values, but there were no significant differences among the values at baseline, 3 months, and 6 months (Fig. 1d). Regarding the Lysholm scale, the total score 24 months after the implantation significantly increased from 60.0 ± 13.7 points to 89.8 ± 9.5 points. The scores at 3, 6, 12, and 24 months after the surgery were significantly higher than the baseline score (Fig. 2a).

Concerning the original cause of the cartilage defect, the increase in the score on the Lysholm scale from the preoperative period to the 24-month period were 26.6 ± 16.6 points in the cases of trauma, 37.3 ± 11.9 points in the cases of osteochondritis dissecans, and 36.6 ± 13.2 points in the cases of osteoarthritis; we could not find statistical differences in the increase of the Lysholm score among the cases with trauma, osteochondritis dissecans, and osteoarthritis (Fig. 2b). In comparison among the implantation locations, the increase in Lysholm scores from the preoperative period to the

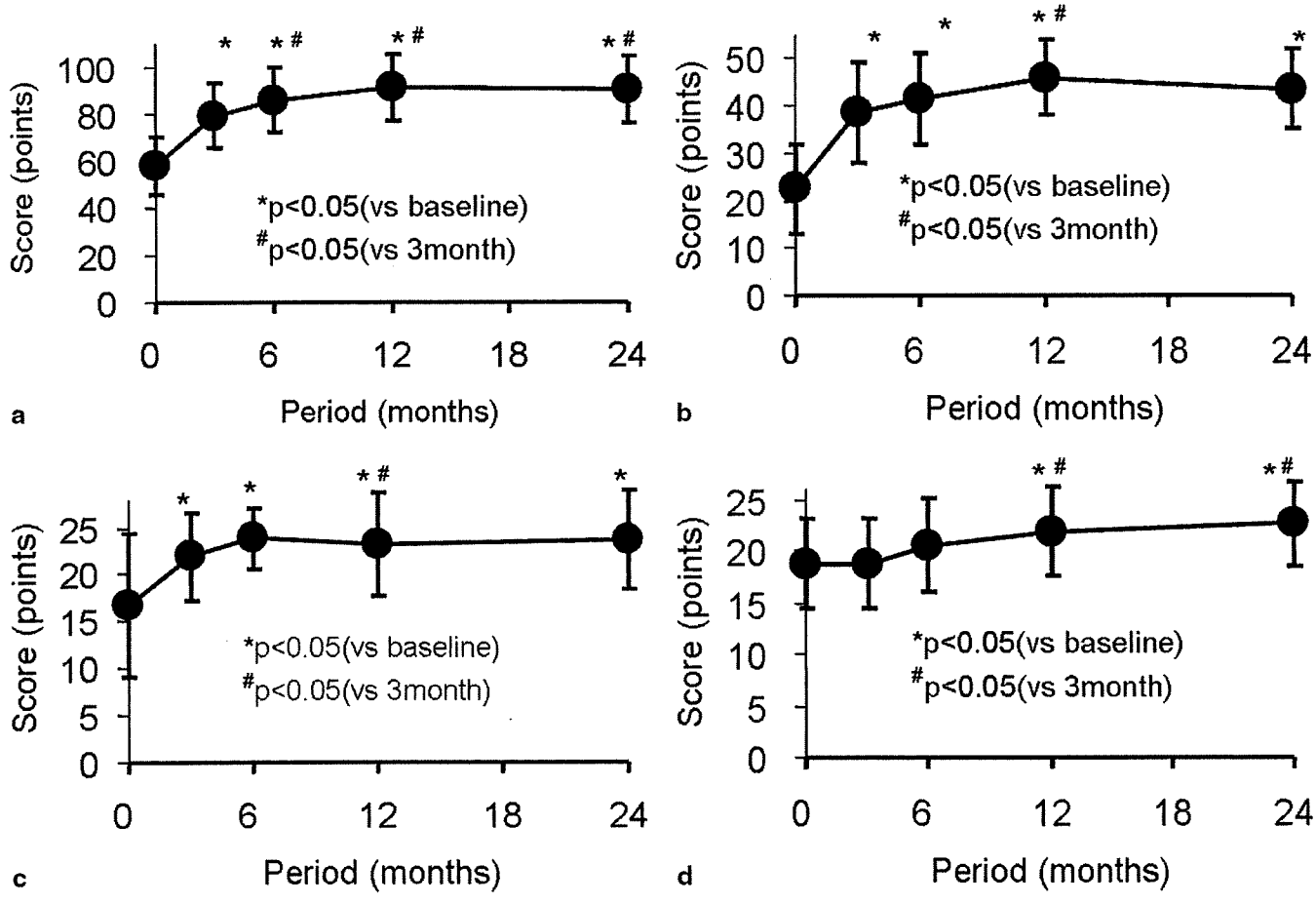

Fig. 1. Our original knee function scores: preoperatively (0) and at 3, 6, 12, and 24 months after the surgical procedure. **a** Total. **b** Motion pain. **c** Rest pain. **d** Knee motion

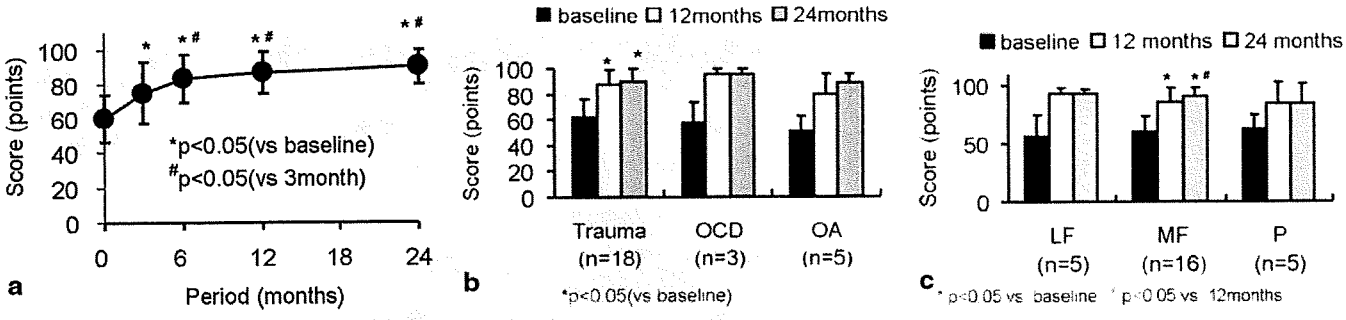

Fig. 2. Lysholm scores: preoperatively (0) and at 3, 6, 12, and 24 months after the surgical procedure. **a** Overall patients. **b** Comparison by the original cause of the cartilage defect. *OCD*, osteochondritis dissecans; *OA*, osteoarthritis. **c** Comparison by implantation location. *LF*, lateral femoral condyle; *MF*, medial femoral condyle; *P*, patella

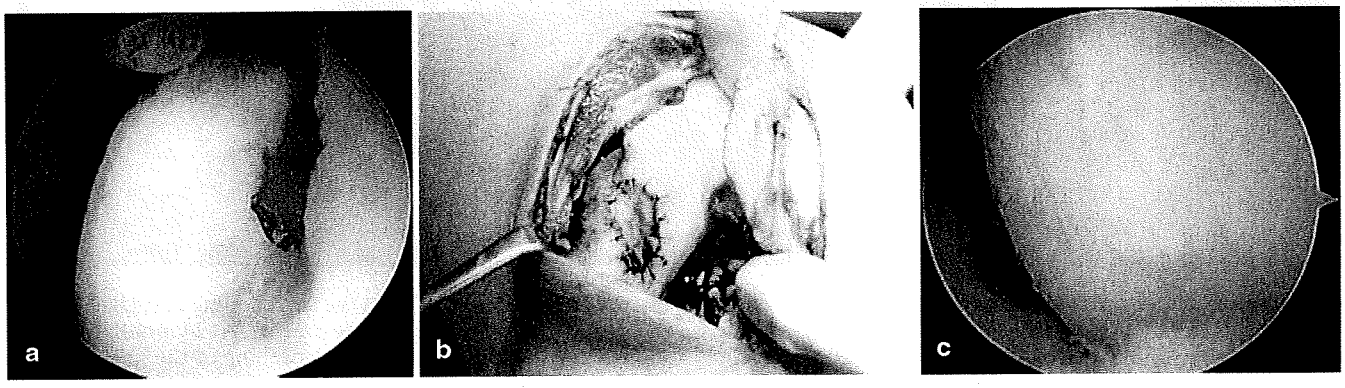

Fig. 3. Case 1. A 30-year-old man with a traumatic cartilage defect at the medial femoral condyle. **a** Arthroscopy showed a cartilage defect (10 × 20 mm) in the medial femoral condyle before transplantation. **b** Transplantation of autologous chondrocytes embedded in atelocollagen gel was performed. **c** Arthroscopic findings 12 months after transplantation showed grade I of the International Cartilage Repair Society (ICRS) cartilage repair assessment concerning repair of the defect, the degree of integration with the surrounding cartilage tissue, and macroscopic appearance. At 24 months, the patient was asymptomatic with a full range of knee flexion

24-month period were 36.4 ± 22.1 points in the lateral femoral condyle, 30.4 ± 13.2 points in the medial femoral condyle, and 21.0 ± 16.4 points in the patella; we could not find statistical differences in the scores among these three locations (Fig. 2c).

Arthroscopic evaluation

In two cases, the grafts were detached at 3 and 8 months after their implantation. In the remaining 25 cases, the arthroscopic evaluation was undertaken at 12 months after the operation. The transplants were congruous with the surrounding articular surface. They were white and slightly fibrillated but soft in both the central and marginal areas, whereas the marginal areas were harder than the central areas. Concerning the ICRS grade for arthroscopic appearance, 6 knees (24%) were assessed as grade I (normal) and 17 knees (68%) as grade II (nearly normal) (Fig. 3). One osteoarthritic knee was graded as grade III (abnormal) (Fig. 4). One case of osteochondritis dissecans was assessed as grade IV (severely abnormal). Concerning the degree of repair of the defect, the transplanted cartilage was healed in the level with surrounding cartilage in 22 cases. Seventeen cases obtained complete integration with surrounding cartilage. In addition, 11 cases showed normal smooth surfaces at the implanted sites.

Concerning the original cause of the cartilage defect, the arthroscopic score was 10.8 ± 1.2 points in cases of trauma, 7.8 ± 3.9 points in cases of osteochondritis dissecans, and 9.2 ± 2.6 points in the cases of osteoarthritis. The arthroscopic score of the cases with trauma was significantly higher that of osteochondritis dissecans cases, but we could not find statistical differences in the score between the cases of trauma and osteoarthritis or between the cases of osteochondritis dissecans and osteoarthritis. In comparison among the implantation locations, the arthroscopic scores were 10.6 ± 1.1 points in the lateral femoral condyle, 9.8 ± 2.7 points in the medial femoral condyle, and 10.5 ± 0.6 points in the

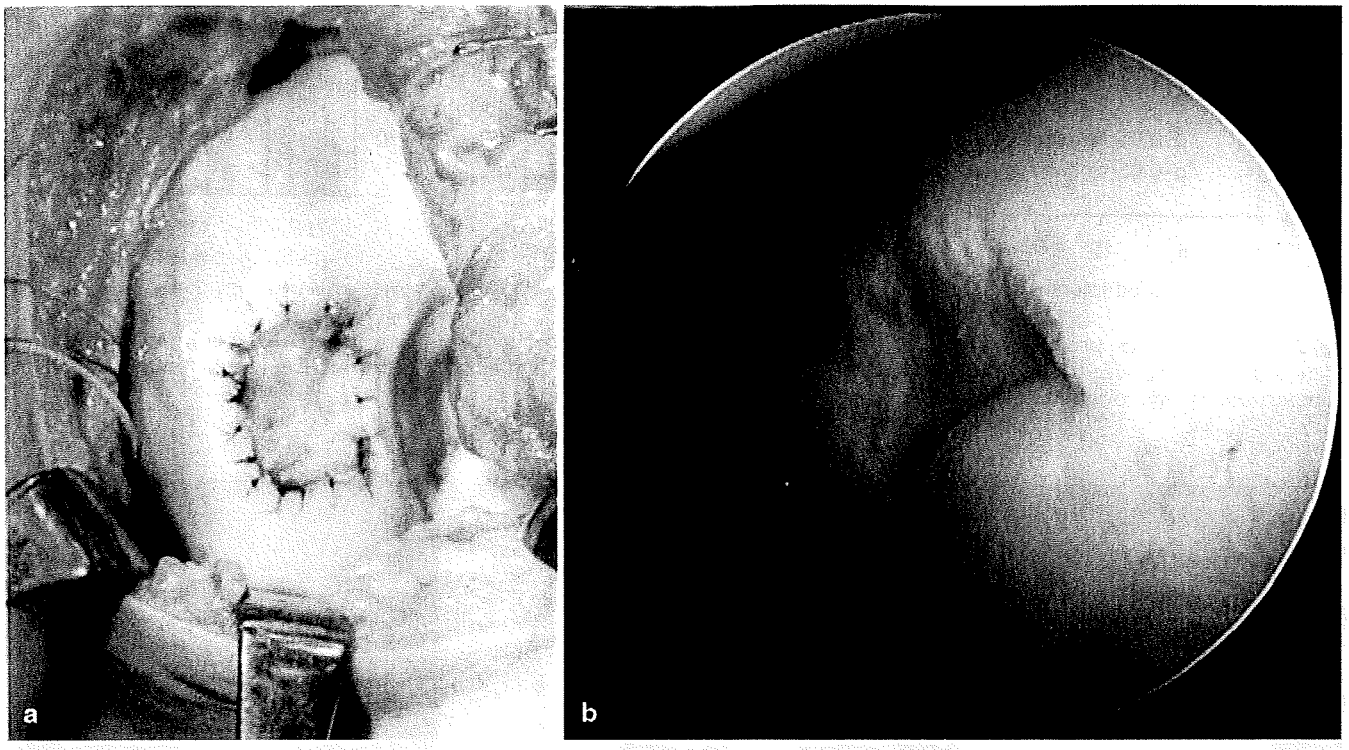

Fig. 4. Case 2. A 42-year-old man suffered waking pain caused by a cartilage defect at the left medial femoral condyle due to osteoarthritis. **a** Autologous chondrocytes embedded in atelocollagen gel were implanted at the cartilage defect (10 × 20 mm). **b** Arthroscopic findings at 12 months shows a large fissure between the graft and the surrounding cartilage (grade III of ICRS cartilage repair assessment). At 24 months, he has no walking pain despite pain and swelling of the knee during vigorous activity

patella. There were no significant differences in the arthroscopic scores among these three locations.

Transplant failure and subsequent surgery

Transplant failure was defined a priori as any subsequent procedure that violated the subchondral bone for the same defect, reimplantation with the tissue-engineered cartilage for the same defect, or delamination or the removal of the tissue-engineered cartilage. Based on the a priori definition of transplant failure, there were two (7.4%) failures. The treatment failures were subsequently treated with graft removal in one patient, who had a marked hypertrophic response at the grafted site and then detachment of approximately half of the graft (Fig. 5). Tissue-engineered cartilage was reimplanted in another patient who had knee pain after squatting at 3 months after the implantation and then had partial detachment of the graft 1 month later. In addition to these two cases, one case required manipulation under anesthesia 2 months after the implantation because the patient had obtained only 70° of knee flexion before the manipulation.

Discussion

We conducted the present multicenter study to evaluate the outcomes of atelocollagen-associated chondrocyte transplantation, which were originally evaluated only by the developers,[15] for the treatment of full-thickness defects of cartilage. As a result, we found that transplantation eliminated locking of the knee and reduced pain in all patients; moreover, the clinical scores based on Lysholm scale and our original knee-function scale improved significantly. In addition, arthroscopic assessment indicated that 92% of the present patients had a "normal" or "nearly normal" appearance. There were few transplant failures, except for detachment of the graft in two cases. Therefore, the findings of the present study suggest that transplanting chondrocytes in a newly formed matrix of atelocollagen gel promotes restoration of the articular cartilage of the knee.

In the present study, we used two kinds of bovine materials (i.e., injectable bovine collagen and fetal bovine serum). The use of these bovine materials may cause side effects and possible zoonotic infections. However, injectable bovine collagen has been used

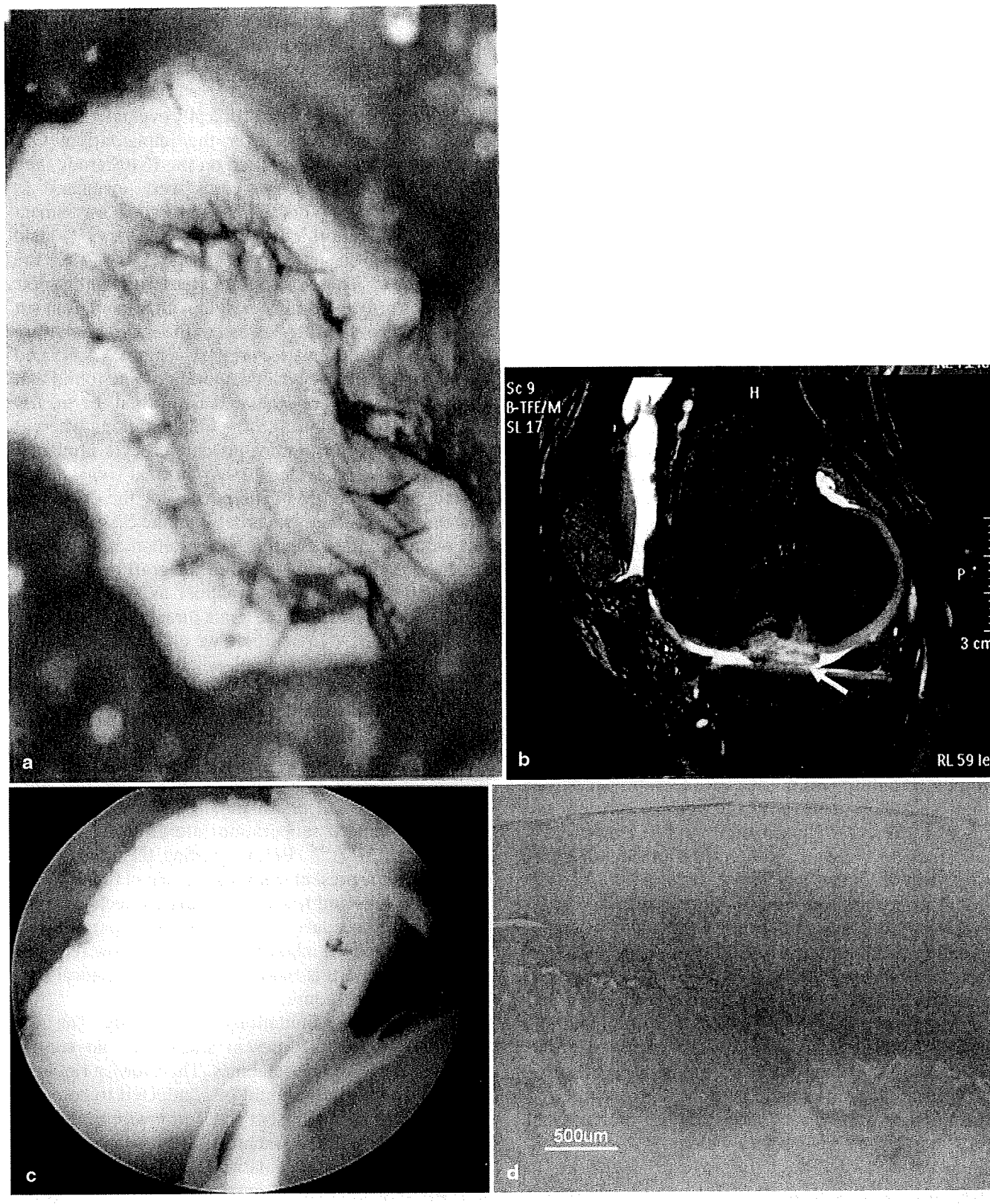

Figure 5. Case 3. A 29-year-old man with a cartilage defect at the left medial femoral condyle due to osteochondritis dissecans. **a** Autologous chondrocytes embedded in atelocollagen gel were implanted at the cartilage defect (15 × 20 mm). **b** Magnetic resonance imaging (MRI) 6 months after implantation shows a marked hypertrophic response (*arrow*) at the grafted site. **c** At 8 months, the patient suddenly could not extend his knee and underwent arthroscopy. Arthroscopic observation confirmed detachment of the graft from the implantation site. Approximately half of the graft was removed arthroscopically, after which restriction of knee extension disappeared. **d** Histology of the removed graft demonstrates the presence of proteoglycan production comparable to that of the native cartilage. Safranin-O stain

successfully for various applications — cosmetic and reconstructive — since the late 1970s.[18] Fetal bovine serum is utilized to manufacture Carticel (Genzyme, Cambridge, MA, USA), which is an autologous cultured chondrocyte product that has been approved by the U.S. Food and Drug Administration.[19] There is no report of adverse events related to zoonotic infections, and Carticel has been widely used in both the United States and Europe since 1997.[20] Therefore, the use of injectable bovine collagen and fetal bovine serum is clinically acceptable.

Concerning knee function, the present study showed that the average clinical score on the Lysholm scale significantly increased from 60 points to 90 points during the follow-up period. This average improvement of the score in the present study, 30 points, is similar to that in the original report, 26 points.[15] In the present study, we could not find any statistical difference among the cases caused by trauma, osteochondritis dissecans, or osteoarthritis or among the patella, lateral and medial femoral condyles. However, we should conduct a clinical trial with a large number of subjects to clarify the differences in the outcome of the present treatment among the original diseases and the location of the chondral defects of the knees.

In the present study, we modified the original method of atelocollagen-associated chondrocyte transplantation that was reported by the developers, Ochi et al.[14,15] First, we added 10% fetal bovine serum to the culture medium instead of 15% patient's serum. The recent study showed that monolayer cultured chondrocytes proliferated more rapidly in autologous human serum and pooled human serum than with fetal bovine serum supplementation.[21] However, before we started the present multicenter clinical trial, we had conducted a pilot study to compare proliferation of chondrocytes cultured in the same atelocollagen gel to the present study between human serum and fetal bovine serum supplementation and found that bovine serum supplementation showed approximately six-fold proliferation of chondrocytes cultured in the atelocollagen compared with adult human serum. Therefore, this change in the supplement of the culture medium from the patient's serum to 10% fetal bovine serum probably enhanced proliferation of chondrocytes during their culture. Second, we isolated chondrocytes from the harvested cartilage tissues 1 day after their harvest, whereas Ochi et al.[14,15] isolated chondrocytes within 2 h of collection. Therefore, we stored the harvested cartilage tissues in phosphate-buffered saline at 4°C overnight. The reason we isolated chondrocytes from the harvested cartilage tissues on the day after their harvest is that transportation of the harvested cartilage tissue usually takes several hours because we transported the tissues from six medical centers to the single facility where the tissue-engineered cartilage was prepared. However, our pilot study has confirmed that storage of the harvested cartilage tissue does not significantly affect the viability of chondrocytes.

The present study arthroscopically evaluated 25 of 30 cases (83%) at 1 year after the implantation. Our arthroscopic assessment based on the ICRS grade then showed that 92% of the cases were evaluated as "normal" or "nearly normal." Arthroscopic assessment in the original report indicated that 26 knees (93%) had a "normal" or "nearly normal" grade 2 years after the implantation.[15] These success rates based on arthroscopic evaluation are quite similar. On the other hand, Bartlett et al.[22] arthroscopically assessed the cases that were treated by a different technique — matrix-associated autologous chondrocyte implantation — and found that 67% of the cases were evaluated as "normal" or "nearly normal" 18 months after the implantation, whereas 79% of cases after autologous chondrocyte implantation (ACI) were evaluated as "normal" or "nearly normal" at 24 months.[23] The reason for the difference in arthroscopic success rates between the cases after our technique and the technique by Bartlett et al. is unclear. At the implantation, we covered the graft by a sutured periosteal flap in the same manner as ACI,[5] whereas Bartlett et al. attached the graft directly to the defect using fibrin glue.[22] In addition, we embedded chondrocytes in the atelocollagen gel, whereas Bartlett et al.[22] seeded chondrocytes on the surface of the collagen material. These technical differences might affect the arthroscopic success rate.

In the present study, 3 of 27 patients (11%) required further operation (one removal of the implant, one reimplantation, and one manipulation under anesthesia). The reported reoperation rate after ACI ranges from 5% to 57%.[23–27] Before starting this study, most surgeons in the present study visited one of the developers' institutions and learned the surgical techniques of the present procedure in detail. Such preparation for surgical techniques might contribute to a low reoperation rate despite this being a multicentric clinical trial on a surgical procedure.

There are some limitations to this study. The first limitation is that the present study used no control group to compare the outcomes. Therefore, a randomized controlled study should be conducted to compare the outcomes of the present procedure with those after conventional treatments including arthroscopic débridement, the marrow stimulation technique, and autogenous osteochondral transplantation.[3] The second limitation is that the minimum follow-up period of the present study was 24 months. Although we did not demonstrate significant differences in any clinical score between 12 months and 24 months, we need additional follow-up of the cases in the present study. The third

limitation is that we did not attempt to perform a biopsy during the arthroscopic examination 1 year after the implantation. Biopsies could show valuable scientific information about the maturation of our tissue-engineered cartilage after the implantation and its integration to the host. However, we did not include the biopsy in the protocol of the present study. Because we designed the present prospective multicenter clinical trial to evaluate all subjects in six medical centers of the preset study by the same protocol, it is considered impractical that all subjects undergo the biopsy during arthroscopic examination 1 year after the implantation. The fourth limitation is that we did not evaluate the mechanical characteristics of the grafted tissues at the follow-up.[15] Despite of these limitations, we believe that the present study has provided important information for the treatment of cartilage defects in the knee joint. Our study is the first report of a multicentric investigation on the clinical outcomes of matrix-associated chondrocyte transplantation.

Conclusions

The present multicenter clinical trial of atelocollagen-associated chondrocyte implantation showed a significant improvement in knee function, a high success rate of the arthroscopic appearance, and a low reoperation rate in the patients for repair of chondral defects of the knee. The technique offers several theoretical advantages compared to the conventional ACI procedure, including maintenance of the chondrocyte phenotype by a three-dimensional culture, prevention of chondrocyte leakage from the graft site, and even distribution in the three-dimensional matrix. Therefore, we conclude that atelocollagen-associated chondrocyte implantation can promote restoration of the articular cartilage of the knee.

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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

Keywords: BMP-7; articular cartilage; strenuous running; keratan sulfate; weekly injection

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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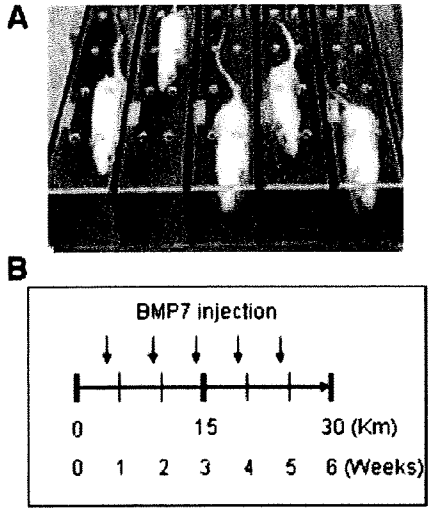

Figure 1. Outline for the study. (A) Treadmill for rats. (B) Schedule for running distance and BMP-7 injections.

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

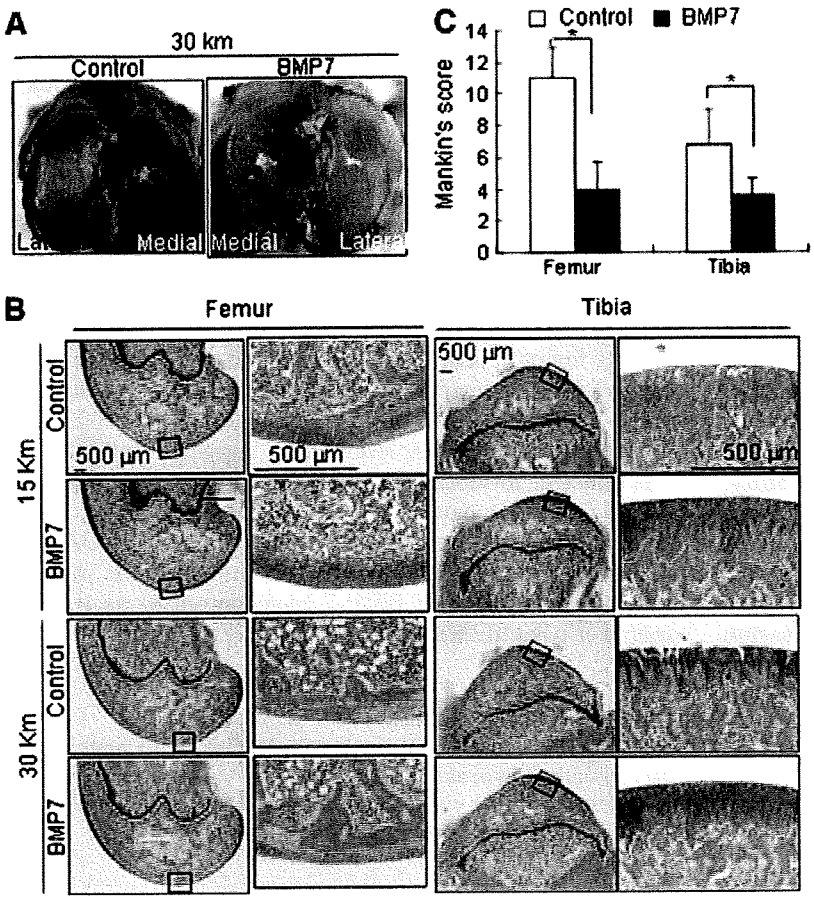

Figure 2. Analysis for articular cartilage of the knee. The right knee was injected with BMP-7 and the left knee was untreated. Paired analysis was performed. (A) Macroscopic observation of tibial articular cartilage stained with India ink. (B) Histologies of the lateral femoral and medial tibial cartilage 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.

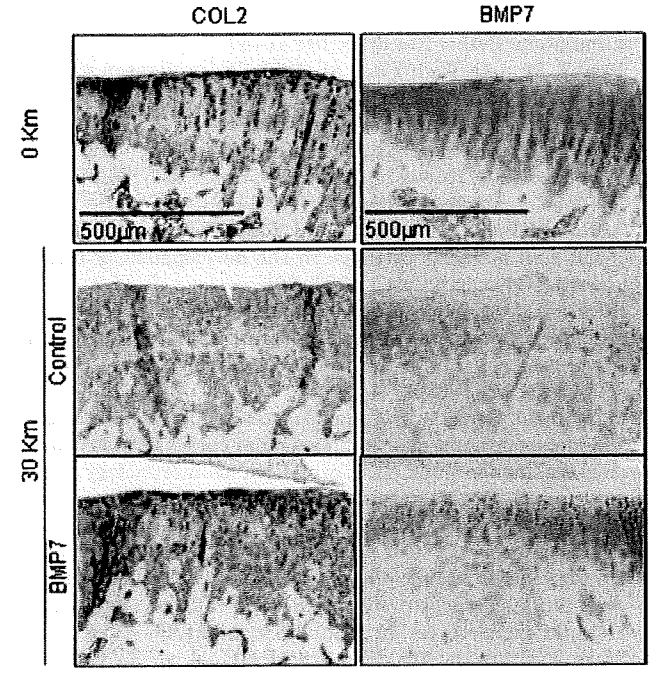

Figure 3. Immunohistochemical analysis for the medial tibial cartilage. For BMP-7, the sections were counterstained with methyl green.

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-

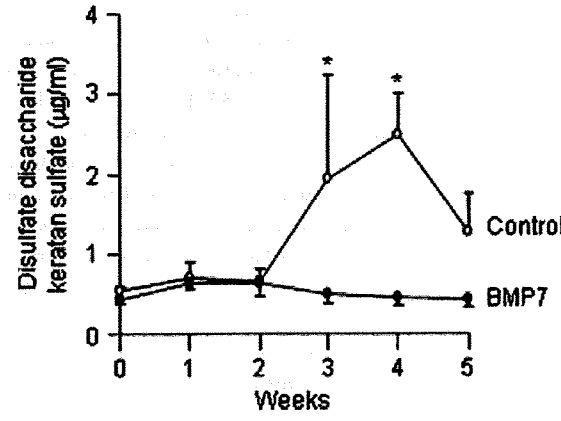

Figure 4. Serum concentration of disulfate disaccharide keratan sulfate. For BMP-7 group, BMP-7 was injected into both knees. For control group, both knees were untreated. Average values with standard deviations are shown ($n = 5$). $^*p < 0.01$ by Mann-Whitney U-test between BMP-7 and control groups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