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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 $\mu g/cm^3$), and bovine serum albumin content (<13 $\mu g/cm^3$) 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

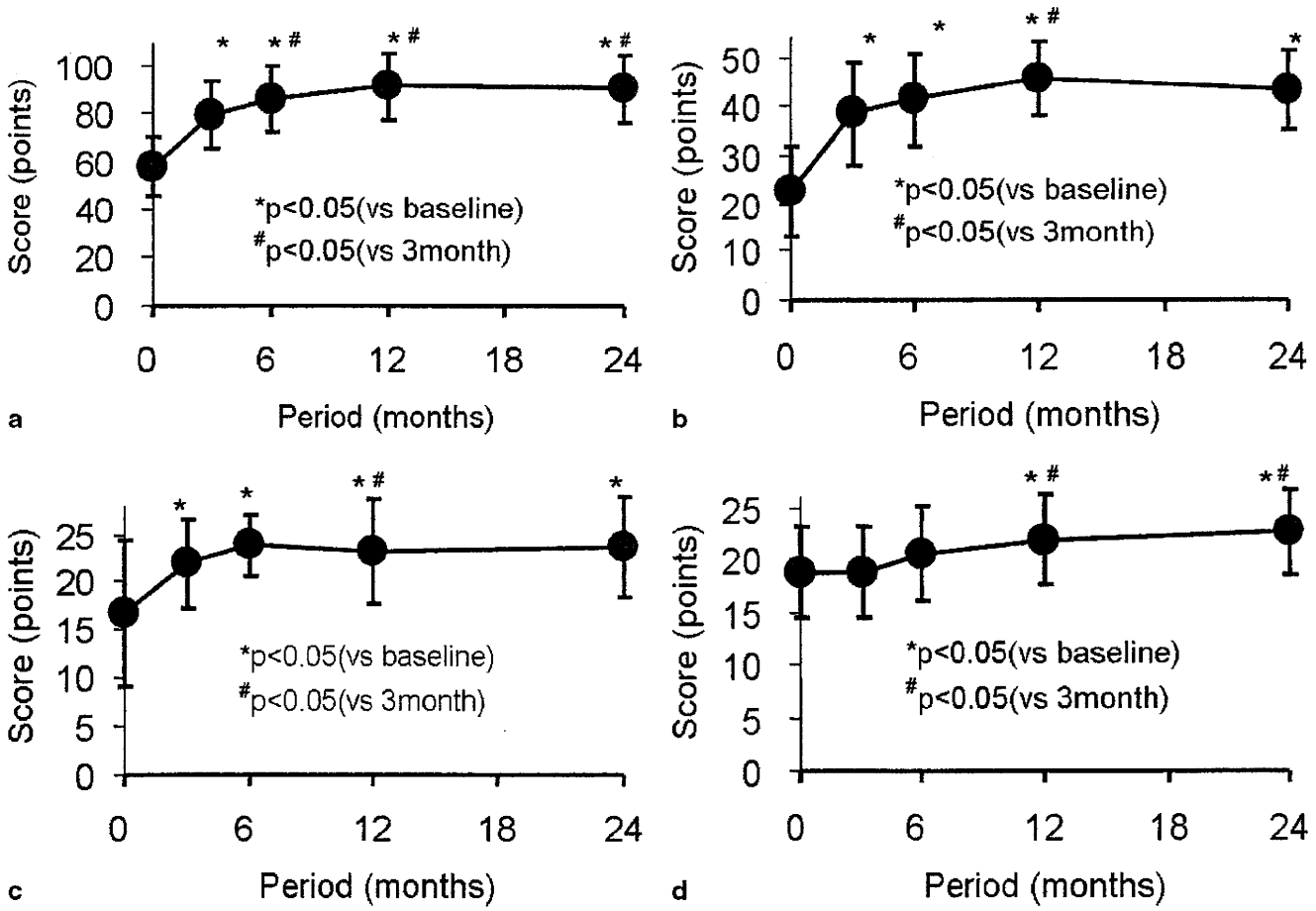

Fig. 1. Our original knee function scores: preoperatively (0) and at 3, 6, 12, and 24 months after the surgical procedure. **a** Total. **b** Motion pain. **c** Rest pain. **d** Knee motion

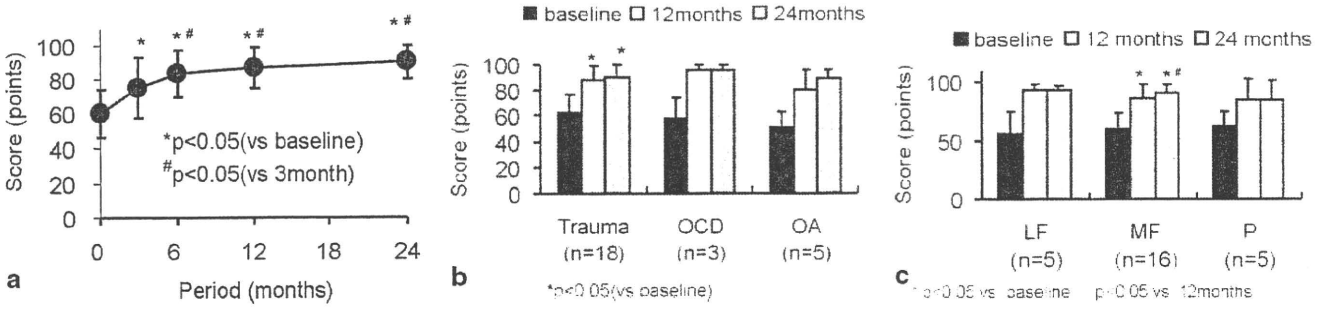

Fig. 2. Lysholm scores: preoperatively (0) and at 3, 6, 12, and 24 months after the surgical procedure. **a** Overall patients. **b** Comparison by the original cause of the cartilage defect. *OCD*, osteochondritis dissecans; *OA*, osteoarthritis. **c** Comparison by implantation location. *LF*, lateral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

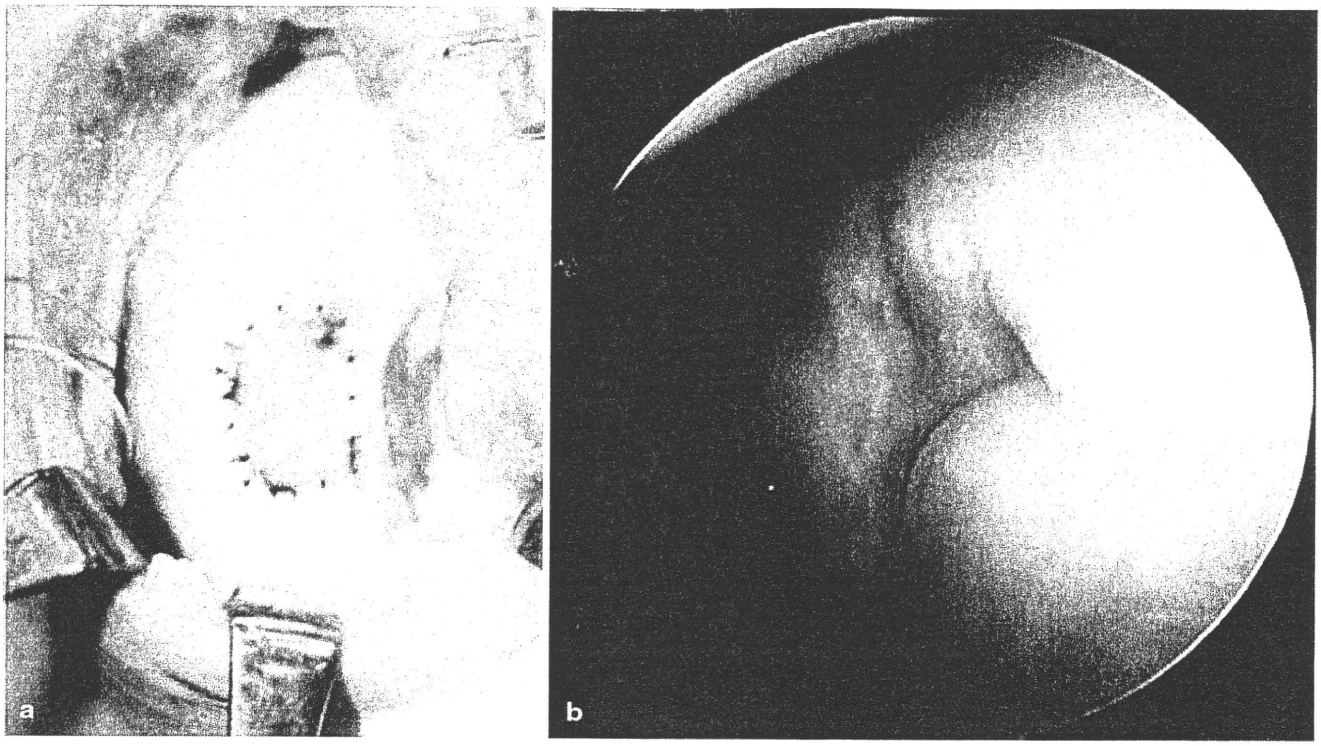

Fig. 4. Case 2. A 42-year-old man suffered waking pain caused by a cartilage defect at the left medial femoral condyle due to osteoarthritis. **a** Autologous chondrocytes embedded in atelocollagen gel were implanted at the cartilage defect (10 × 20 mm). **b** Arthroscopic findings at 12 months shows 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

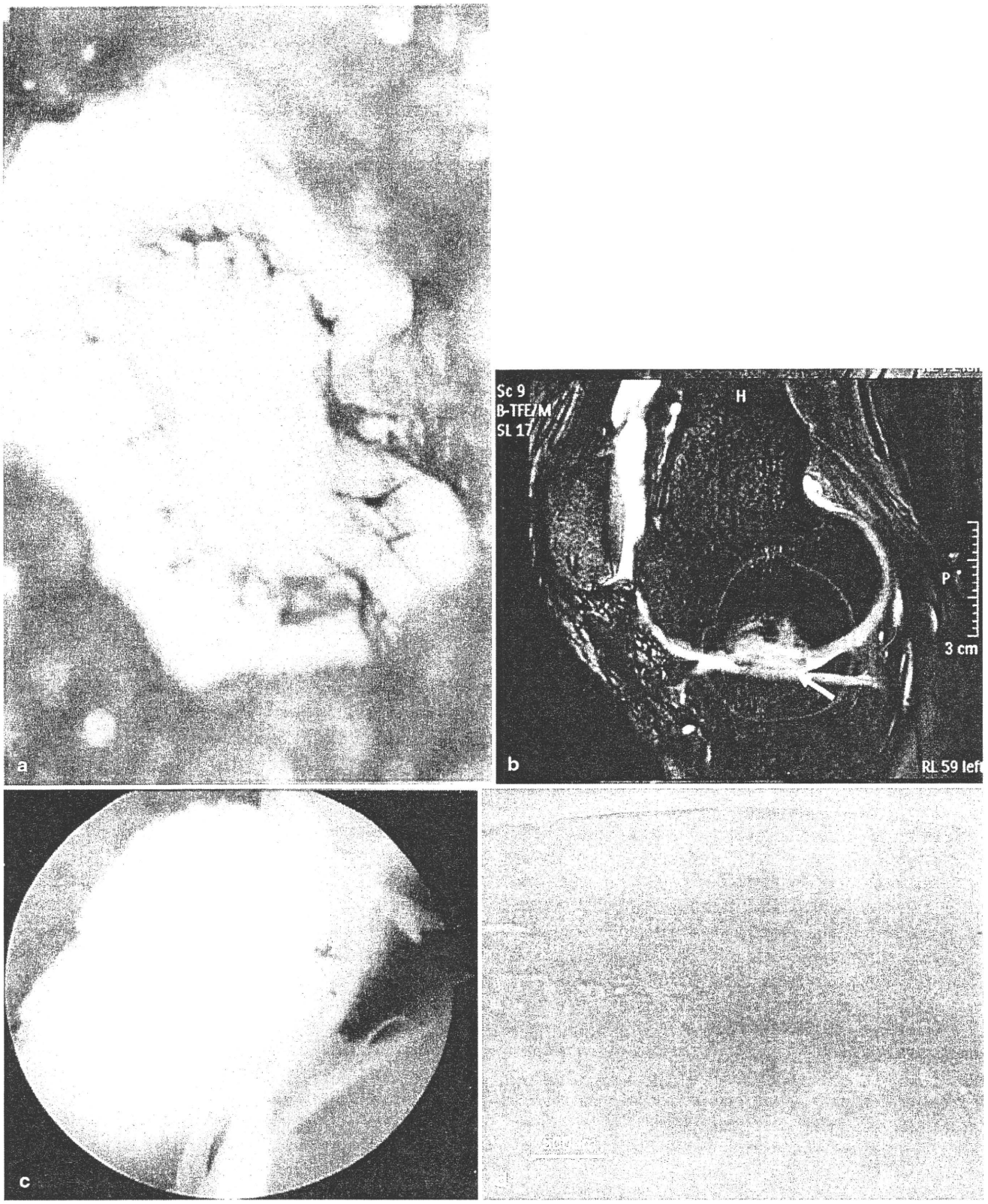

Figure 5. Case 3. A 29-year-old man with a cartilage defect at the left medial femoral condyle due to osteochondritis dissecans. **a** Autologous chondrocytes embedded in atelocollagen gel were implanted at the cartilage defect (15 × 20 mm). **b** Magnetic resonance imaging (MRI) 6 months after implantation 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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References

1. Buckwalter JA, Mankin HJ, Grodzinsky AJ. Articular cartilage and osteoarthritis. Instr Course Lect 2005;54:465–80.
2. O'Driscoll SW. The healing and regeneration of articular cartilage. J Bone Joint Surg Am. 1998;80:1795–812.
3. Smith GD, Knutsen G, Richardson JB. A clinical review of cartilage repair techniques. J Bone Joint Surg Br 2005;87:445–9.
4. Magnussen RA, Dunn WR, Carey JL, Spindler KP. Treatment of focal articular cartilage defects in the knee: a systematic review. Clin Orthop 2008;466:952–62.
5. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, P eterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994; 331:889–95.
6. Peterson L, Minas T, Brittberg M, Lindahl A. Treatment of osteochondritis dissecans of the knee with autologous chondrocyte transplantation: results at two to ten years. J Bone Joint Surg Am 2003;85(suppl 2):17–24.
7. von der Mark K, Gauss V, von der Mark H, Muller P. Relationship between cell shape and type of collagen synthesized as chondrocytes lose their cartilage phenotype in culture. Nature 1977;267: 531–2.
8. Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 1982;30:215–24.
9. Kimura T, Yasui N, Ohsawa S, Ono K. Chondrocytes embedded in collagen gels maintain cartilage phenotype during long-term cultures. Clin Orthop 1984;(186):231–9.
10. Uchio Y, Ochi M, Matsusaki M, Kurioka H, Katsube K. Human chondrocyte proliferation and matrix synthesis cultured in atelocollagen gel. J Biomed Mater Res 2000;50:138–43.
11. Katsube K, Ochi M, Uchio Y, Maniwa S, Matsusaki M, Tobita M, Iwasa J. Repair of articular cartilage defects with cultured chondrocytes in atelocollagen gel. Comparison with cultured chondrocytes in suspension. Arch Orthop Trauma Surg 2000;120: 121–7.
12. Frenkel SR, Di Cesare PE. Scaffolds for articular cartilage repair. Ann Biomed Eng. 2004;32:26–34.
13. Gikas PD, Aston WJ, Briggs TW. Autologous chondrocyte implantation: where do we stand now? J Orthop Sci 2008;13: 283–92.
14. Ochi M, Uchio Y, Tobita M, Kuriwaka M. Current concepts in tissue engineering technique for repair of cartilage defect. Artif Organs 2001;25:172–9.
15. Ochi M, Uchio Y, Kawasaki K, Wakitani S, Iwasa J. Transplantation of cartilage-like tissue made by tissue engineering in the treatment of cartilage defects of the knee. J Bone Joint Surg Br 2002;84:571–8.
16. Keefe J, Wauk L, Chu S, DeLustro F. Clinical use of injectable bovine collagen: a decade of experience. Clin Mater 1992;9: 155–62.
17. Revell CM, Athanasiou KA. Success rates and immunologic responses of autogenic, allogenic, and xenogenic treatments to repair articular cartilage defects. Tissue Eng Part B Rev 2009;15: 1–15.
18. Wood JJ, Malek MA, Frassica FJ, Polder JA, Mohan AK, Bloom ET, Braun MM, Coté TR. Autologous cultured chondrocytes: adverse events reported to the United States Food and Drug Administration. J Bone Joint Surg Am 2006;88:503–7.
19. Lysholm J, Gillquist J. Evaluation of knee ligament surgery results with special emphasis on use of a scoring scale. Am J Sports Med 1982;10:150–4.
20. Brittberg M, Winalski CS. Evaluation of cartilage injuries and repair. J Bone Joint Surg Am 2003;85(suppl 2):58–69.
21. Munirah S, Ruszymah BH, Samsudin OC, Badrul AH, Azmi B, Aminuddin BS. Autologous versus pooled human serum for articular chondrocyte growth. J Orthop Surg (Hong Kong) 2008: 16:220–9.
22. Bartlett W, Skinner JA, Gooding CR, Carrington RW, Flanagan AM, Briggs TW, Bentley G. Autologous chondrocyte implantation versus matrix-induced autologous chondrocyte implantation

for osteochondral defects of the knee: a prospective, randomised study. J Bone Joint Surg Br 2005;87:640–5.
23. Erggelet C, Browne JE, Fu F, Mandelbaum BR, Micheli LJ, Mosely JB. Autologous chondrocyte transplantation for treatment of cartilage defects of the knee joint. Clinical results. Zentralbl Chir 2000;125:516–22 (in German).
24. Browne JE, Anderson AF, Arciero R, Mandelbaum B, Moseley JB Jr, Micheli LJ, Fu F, Erggelet C. Clinical outcome of autologous chondrocyte implantation at 5 years in US subjects. Clin Orthop 2005;(436):237–45.
25. Henderson I, Tuy B, Oakes B. Reoperation after autologous chondrocyte implantation. Indications and findings. J Bone Joint Surg Br 2004;86:205–11.
26. Minas T. Autologous chondrocyte implantation for focal chondral defects of the knee. Clin Orthop 2001;(391, suppl):S349–61.
27. Peterson L, Minas T, Brittberg M, Nilsson A, Sjogren-Jansson E, Lindahl A. Two- to 9-year outcome after autologous chondrocyte transplantation of the knee. Clin Orthop 2000;(374):212–34.

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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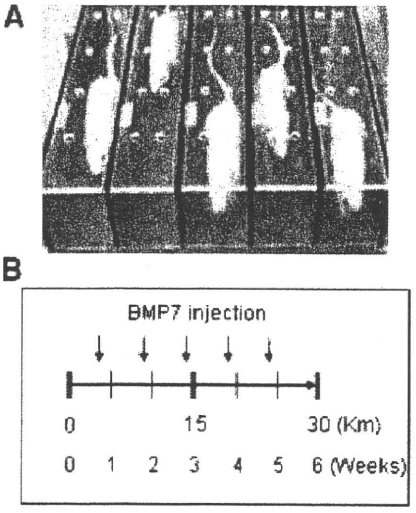

Figure 1. Outline for the study. (A) Treadmill for rats. (B) Schedule for running distance and BMP-7 injections.

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

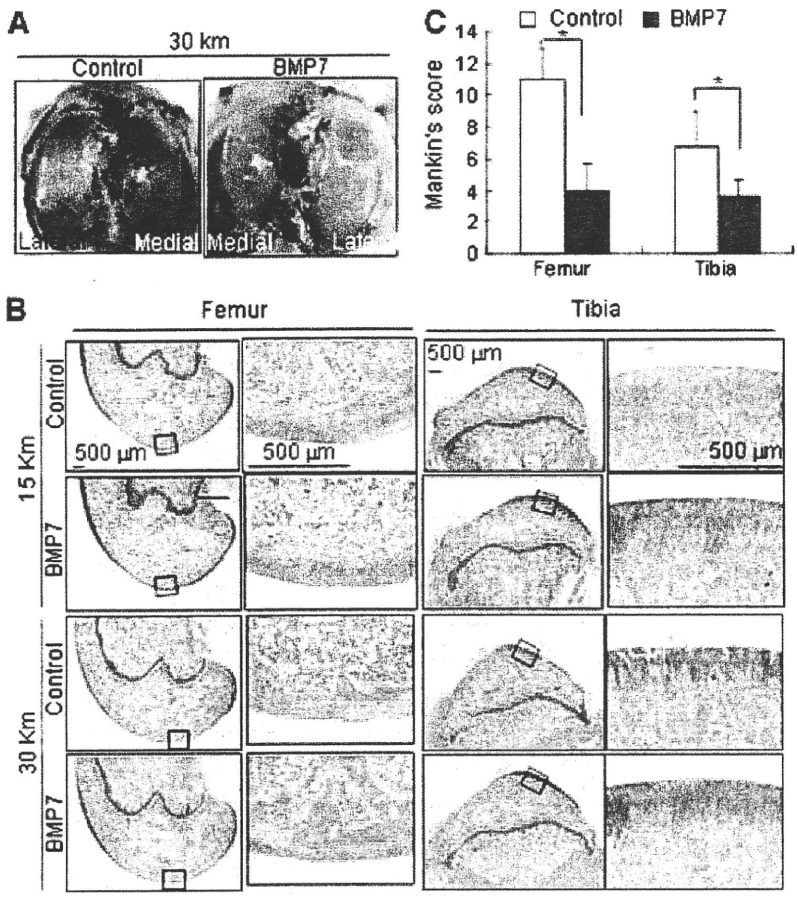

Figure 2. Analysis for articular cartilage of the knee. The right knee was injected with BMP-7 and the left knee was untreated. Paired analysis was performed. (A) Macroscopic observation of tibial articular cartilage stained with India ink. (B) Histologies of the lateral femoral and medial tibial cartilage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

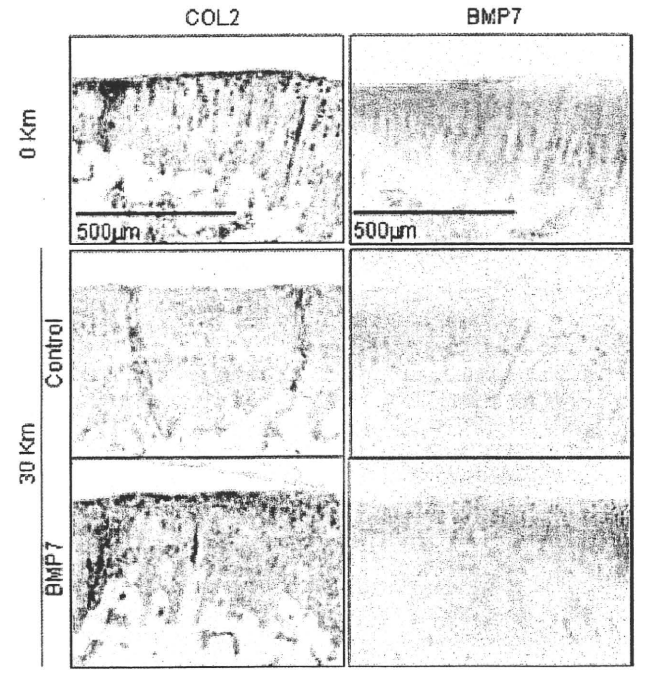

Figure 3. Immunohistochemical analysis for the medial tibial cartilage. For BMP-7, the sections were counterstained with methyl green.

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-

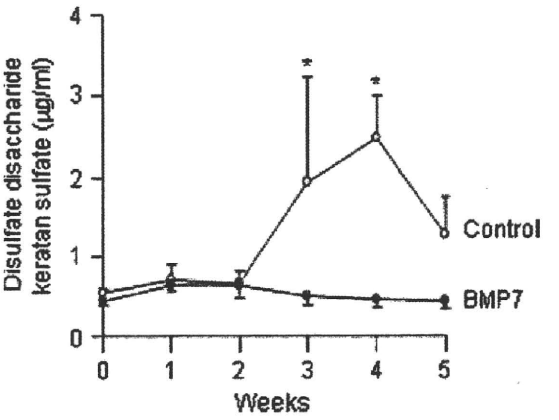

Figure 4. Serum concentration of disulfate disaccharide keratan sulfate. For BMP-7 group, BMP-7 was injected into both knees. For control group, both knees were untreated. Average values with standard deviations are shown ($n = 5$). $^*p < 0.01$ by Mann-Whitney U-test between BMP-7 and control groups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

1. Wieland HA, Michaelis M, Kirschbaum BJ, et al. 2005. Osteoarthritis—an untreatable disease? Nat Rev Drug Discov 4:331–344.
2. Pap G, Eberhardt R, Sturmer I, et al. 1998. Development of osteoarthritis in the knee joints of Wistar rats after strenuous running exercise in a running wheel by intracranial self-stimulation. Pathol Res Pract 194:41–47.
3. Tang T, Muneta T, Ju YJ, et al. 2008. Serum keratan sulfate transiently increases in the early stage of osteoarthritis during strenuous running of rats: protective effect of intra-articular hyaluronan injection. Arthritis Res Ther 10:R13.
4. Chubinskaya S, Hurtig M, Rueger DC. 2007. OP-1/BMP-7 in cartilage repair. Int Orthop 31:773–781.
5. Badlani N, Inoue A, Healey R, et al. 2008. The protective effect of OP-1 on articular cartilage in the development of osteoarthritis. Osteoarth Cart 16:600–606.
6. Hulmes DJ, Marsden ME, Strachan RK, et al. 2004. Intra-articular hyaluronate in experimental rabbit osteoarthritis can prevent changes in cartilage proteoglycan content. Osteoarth Cart 12:232–238.
7. Mankin HJ, Dorfman H, Lippiello L, et al. 1971. Biochemical and metabolic abnormalities in articular cartilage from osteoarthritic human hips. II. Correlation of morphology with biochemical and metabolic data. J Bone Joint Surg Am 53:523–537.
8. Flechtenmacher J, Huch K, Thonar EJ, et al. 1996. Recombinant human osteogenic protein 1 is a potent stimulator of the synthesis of cartilage proteoglycans and collagens by human articular chondrocytes. Arthritis Rheum 39:1896–1904.
9. Stove J, Schneider-Wald B, Scharf HP, et al. 2006. Bone morphogenetic protein 7 (bmp-7) stimulates proteoglycan synthesis in human osteoarthritic chondrocytes in vitro. Biomed Pharmacother 60:639–643.

10. Merrihew C, Soeder S, Rueger DC, et al. 2003. Modulation of endogenous osteogenic protein-1 (OP-1) by interleukin-1 in adult human articular cartilage. J Bone Joint Surg Am 85-A:67–74.
11. Cook SD, Patron LP, Salkeld SL, et al. 2003. Repair of articular cartilage defects with osteogenic protein-1 (BMP-7) in dogs. J Bone Joint Surg Am 85-A:116–123.
12. Chubinskaya S, Merrihew C, Cs-Szabo G, et al. 2000. Human articular chondrocytes express osteogenic protein-1. J Histochem Cytochem 48:239–250.
13. Simic P, Culej JB, Orlic I, et al. 2006. Systemically administered bone morphogenetic protein-6 restores bone in aged ovariectomized rats by increasing bone formation and suppressing bone resorption. J Biol Chem 281:25509–25521.
14. Funderburgh JL. 2000. Keratan sulfate: structure, biosynthesis, and function. Glycobiology 10:951–958.
15. Tomatsu S, Okamura K, Maeda H, et al. 2005. Keratan sulphate levels in mucopolysaccharidoses and mucolipidoses. J Inherit Metab Dis 28:187–202.
16. Wakitani S, Nawata M, Kawaguchi A, et al. 2007. Serum keratan sulfate is a promising marker of early articular cartilage breakdown. Rheumatology (Oxford) 46:1652–1656.
17. Divine JG, Zazulak BT, Hewett TE. 2007. Viscosupplementation for knee osteoarthritis: a systematic review. Clin Orthop Relat Res 455:113–122.
18. D'Lima D, Hermida J, Hashimoto S, et al. 2006. Caspase inhibitors reduce severity of cartilage lesions in experimental osteoarthritis. Arthritis Rheum 54:1814–1821.
19. Inoue A, Takahashi KA, Arai Y, et al. 2006. The therapeutic effects of basic fibroblast growth factor contained in gelatin hydrogel microspheres on experimental osteoarthritis in the rabbit knee. Arthritis Rheum 54:264–270.
20. Brandt KD, Mazzuca SA, Katz BP, et al. 2005. Effects of doxycycline on progression of osteoarthritis: results of a randomized, placebo-controlled, double-blind trial. Arthritis Rheum 52:2015–2025.
21. Tiraloche G, Girard C, Chouinard L, et al. 2005. Effect of oral glucosamine on cartilage degradation in a rabbit model of osteoarthritis. Arthritis Rheum 52:1118–1128.

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Differences in kinematics of single leg squatting between anterior cruciate ligament-injured patients and healthy controls

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Abstract Seventy to eighty percent of all anterior cruciate ligament (ACL) injuries are due to non-contact injury mechanisms. It has been reported that the majority of injuries due to single leg landing come from valgus positioning of the lower leg. Preventing valgus positioning during single leg landing is expected to help reduce the number of ACL injuries. We found that many ACL-deficient patients cannot perform stable single leg squatting. Therefore, we performed 3D motion analysis of the single-legged half squat for ACL-injured patients to evaluate its significance as a risk factor for ACL injuries. We evaluated the relative angles between the body, thigh, and lower leg using an electromagnetic device during single leg half squatting performed by 63 ACL-injured patients (32 males, 31 females) the day before ACL reconstruction and by 26 healthy control subjects with no knee problems. The uninjured leg of ACL-injured male subjects demonstrated significantly less external knee rotation than that of the dominant leg of the male control. The uninjured leg of ACL-injured female subjects demonstrated significantly more external hip rotation and knee flexion and less hip flexion than that of the dominant leg of the female control. Comparing injured and uninjured legs, the injured leg of male subjects demonstrated significantly less external knee and hip rotation, less knee flexion, and more knee varus than that of the uninjured leg of male subjects. The injured leg of female subjects demonstrated more knee varus than that of the uninjured leg of female subjects. Regarding gender differences, female subjects demonstrated significantly more external hip rotation and knee valgus than male subjects did in both the injured and uninjured legs ($P < 0.05$). The current kinematic study exhibited biomechanical characteristics of female ACL-injured subjects compared with that of control groups. Kinematic correction during single leg half squat would reduce ACL reinjury in female ACL-injured subjects.

Keywords Anterior cruciate ligament (ACL) · Single leg squat · Kinematic analysis · Injury prevention

Introduction

Several reports have examined the epidemiology of anterior cruciate ligament (ACL) injury. Seventy to eighty percent of all ACL injuries are due to non-contact injury mechanisms [1–3]. The ACL-injury rate of female athletes is 2–8 times higher than that of male athletes [4–6]. The ACL non-contact injury rate of female athletes is reportedly 2–4 times higher than that of male athletes in basketball and soccer [7]. The majority of ACL non-contact injuries occur in the valgus position of the lower leg during single leg landing and cutting maneuvers in sports activities [8, 9]. Therefore, preventing valgus positioning of the lower leg during single leg landing or cutting maneuvers is expected to reduce the number of ACL non-contact injuries. A number of biomechanical research studies examining jump landing have investigated the injury mechanism of the ACL. They have mainly reported on lower extremity

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kinematics during bilateral or unilateral jump landing or evaluated the effect of neuromuscular training [10–22].

A number of non-contact ACL injuries occur when one leg is overloaded during landing, cutting, and pivoting. If a single leg standing position could be maintained without collapsing during knee bending with weight-bearing during such decelerating maneuvers, ACL injuries could possibly be prevented. Therefore, we asked ACL-injured patients to perform a single leg squat and evaluated their performance pre- and postoperatively at an outpatient clinic.

We assumed that proper control during the single leg half squat depicts ability for safe landing for the ACL. This task is considered to be a simple and safe clinical examination to correct unstable valgus positioning of the lower leg in ACL-injured patients. However, based on clinical observations of ACL-injured patients, a number of female ACL-injured patients cannot perform a stable single leg half squat.

Therefore, we expected some kinematic differences to exist between female ACL-injured patients and healthy controls performing a single leg half squat. The ACL reinjury rate is speculated to decrease if a higher number of ACL-injured patients can perform a stable and correct single leg half squat. Therefore, this method could be a valuable screening tool for identifying predisposition for ACL rupture in healthy individuals. Even more, it could indicate the magnitude of functional disability in ACL-injured patients.

The main purpose of this study is to compare the kinematics of uninjured legs in ACL-injured patients and healthy controls during single leg squatting. Clarification of the characteristics of uninjured leg in ACL-injured patients might reveal a predisposing factor of ACL injury. To identify further details of the characteristics of ACL-injured patients, we evaluated the differences between ACL-injured and -uninjured knee in each male and female, and also gender differences in both injured and uninjured knee.

The primary hypothesis is that some characteristic differences occur between kinematics of ACL-injured patients and healthy controls performing a single leg squat for each gender. The secondary hypothesis is that some characteristic differences exist between the kinematics of ACL-injured knee and the uninjured knee in both males and females. The third hypothesis is that some characteristic differences exist between the kinematics of male and female ACL-injured patients in both injured and uninjured knee.

Materials and methods

A total of 63 ACL-injured patients (32 males, 31 females) participated in this study before ACL reconstruction. The mean age of the male patients was 26.4 years old (16–51) and 25.5 years old (14–47) for female patients. The period after ACL injury was 3.5 ± 1.8 months for male patients and 3.0 ± 1.7 months for female patients. Various kinds and levels of sports and injury mechanisms were included in this study; however, most patients participated in just recreational level sports. They had not undergone a special rehabilitation program before ACL reconstruction; however, they had sufficient conservative treatment to achieve daily living with no difficulty and knee motion with no remarkable limitation before surgery.

To be included in this study, subjects had to have no history of other lower extremity injuries. Examples of injuries that excluded participation were grade II or higher MCL injury, meniscal locking and tears with severe pain, bilateral ACL injury, and current symptoms of pain induced due to other reasons (visible joint effusions, patella instability, or lower extremity fractures). Subjects with a history of chronic conditions such as tendinitis or bursitis causing no pain at the time of evaluation were included in this study. As a control group, 26 healthy athletes were recruited to participate in this study. The demographic data for control subjects came from 14 male recreational level athletes (basketball $N = 4$, volleyball $N = 2$, skiing $N = 2$, and others) and 12 female competitive level athletes of volleyball. The mean age of the male control subjects was 26.2 years old (22–35) and 23.2 years old (19–33) for female control subjects. The subjects also had no history of any lower extremity injury, including ACL injury. Before starting this study, subjects were informed of the purpose and possible risk of the study and so forth and signed an informed consent form of the university.

The day before ACL reconstruction, each ACL-injured subject was asked to perform a single leg half squat as one of the preoperative clinical examinations. Subjects were instructed and given one opportunity to practice the single leg half squat maneuver. They were instructed to cross their arms over their chest and to perform a half squat while keeping proper balance, with the duration of the squat being 10 s or less. The depth of the squat was defined as the position for maintaining balance well during the 10 s maneuver (Fig. 1). Subjects performed two single leg half squats with both the injured and uninjured legs, while subjects of the control group performed squats with the dominant leg. All other data of the joint angles were extracted from data at maximum knee flexion during the single leg half squat. We measured these joint angles for two occurrences of single leg half squatting.

Instrumentation

3D motion analysis was performed using an electromagnetic device (FASTRACK; POLHEMUS, Colchester, VT),

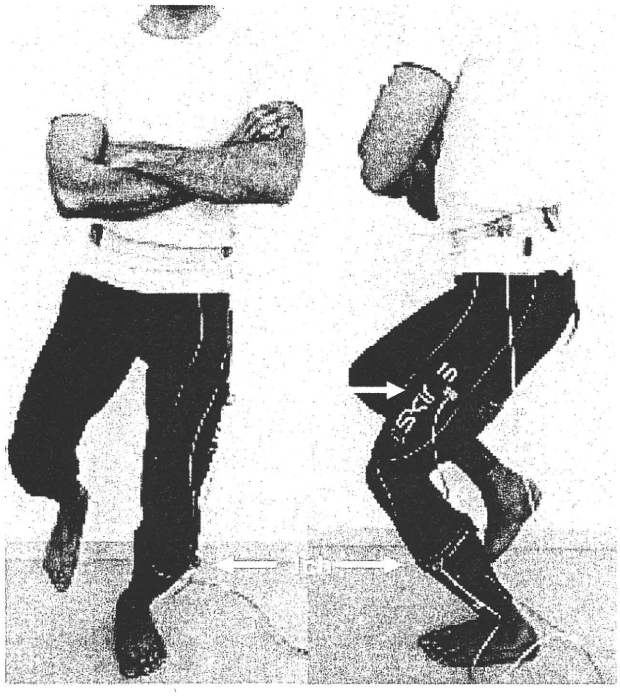

Fig. 1 Subjects were instructed to practice a single leg half squat maneuver. They were then instructed to cross their arms over their chest and to perform a half squat while maintaining proper balance. *Arrows* show receivers (1ch, 2ch, 3ch) attached to the anterior superior iliac spine, 10 cm above the patella on the lateralis of the thigh, and 15 cm below the patella on the lateral side of the lower leg

which can measure six degrees of freedom in a Cartesian coordinate system. This system's software can measure the 3D position of the receiver relative to a global coordinate system projected by the magnetic transmitter. The experiment was organized, so that there was no interference from magnetic materials. The accuracy of the cutaneous measurements was $1.6° \pm 1.3°$, the repeatability of the cutaneous measurements was $0.8° \pm 0.4°$, and that of the transosseous measurements was $1.0° \pm 0.5°$ [23]. This accurate and reproducible device provides a simple and noninvasive measurement. Therefore, it is well suited for biomechanical applications such as joint movement analysis.

Three of the receivers (1ch, 2ch, 3ch) were attached by a circumferential velcro strap placed on the anterior superior iliac spine, 10 cm above the patella on the lateral side of the thigh, and 15 cm below the patella on the lateral side of the lower leg (Fig. 1). This electromagnetic device tracks the relative position of the receivers attached to the anterior superior iliac spine, thigh, and lower leg. This system provides specified accuracy when receivers are located within 76 cm of the transmitter. The static accuracy of this system is 0.08 cm root mean square for the X, Y, and Z values of the receiver's position, and 0.15° root mean square for the receiver orientation.

Statistical analysis

The null hypothesis for this study was that there would be no differences in any kinematic data between the ACL-injured patients and the control group, nor between an ACL patient's injured and uninjured knee, nor a difference between the genders in the ACL-injured patients. Statistical means and standard deviations (SDs) for all measured variables were calculated for each subject group. Kinematic data were analyzed by StatView 5.0 (SAS, Cary, North Carolina) using one-way analysis of variance tests and Fisher's PLSD as a post hoc test. The level of significance was set at $P < 0.05$.

Results

Comparing the ACL-injured subjects to the healthy controls, the uninjured leg of male ACL-injured subjects demonstrated significantly less external knee rotation than the dominant leg of the male controls. The uninjured leg of female ACL-injured subjects demonstrated significantly more external hip rotation and knee flexion and less hip flexion than the dominant leg of the female controls (Table 1; Fig. 2).

Comparing the injured leg with the uninjured leg in ACL-injured subjects, the injured leg of male demonstrated significantly less knee and hip external rotation, less knee flexion, and more knee varus than the uninjured leg. The injured leg of female subjects demonstrated more knee varus than the uninjured leg (Table 2). There were definitive differences between the injured and uninjured legs from a coronal view of the single leg half squat (Fig. 3).

Comparing the gender differences in ACL-injured subjects, female subjects demonstrated significantly more external hip rotation and knee valgus in both the injured and uninjured legs than male subjects (Table 3). The characteristics of female ACL-injured subjects from the coronal view are shown in Fig. 4.

Discussion

The most important finding of the present study was the kinematic difference between the ACL-injured subjects and the healthy controls. The uninjured leg in female ACL-injured subjects exhibited less hip flexion and more external hip rotation and knee flexion compared with the dominant leg of the female controls. Less hip flexion in ACL-injured patients during single leg half squatting might suggest that they could perform more stable squatting by maintaining their hip in a more extended position.

Table 1 Comparison of mean joint angle at knee maximum flexion between control and uninjured knees of ACL-injured subjects during single leg squatting

Motion	Mean joint angle (°)					
	Male			Female		
	Control[a]	Subjects[b]	*P* values	Control[a]	Subjects[b]	*P* values
Knee						
Flexion	77.8 ± 11.7	74.3 ± 13.6	0.4783[n.s.]	66.2 ± 9.9	73.9 ± 13.3	0.0070[c]
Varus	16.9 ± 15.1	14.1 ± 11.5	0.6559[n.s.]	8.9 ± 8.2	3.7 ± 11.9	0.0814[n.s.]
External rotation	38.8 ± 12.6	18.9 ± 34.3	0.0090[c]	7.9 ± 49.3	14.1 ± 34.7	0.8147[n.s.]
Hip						
Flexion	35.7 ± 14.9	32.7 ± 17.3	0.3229[n.s.]	48.0 ± 11.3	29.9 ± 18.4	0.0000[c]
Varus	9.8 ± 12.0	12.0 ± 8.6	0.6166[n.s.]	8.3 ± 6.2	10.9 ± 8.7	0.1763[n.s.]
External rotation	5.5 ± 10.5	4.5 ± 8.6	0.3775[n.s.]	1.7 ± 6.1	9.1 ± 8.0	0.0000[c]

[a] Control group, dominant leg (14 males, 12 females)

[b] ACL deficient, uninjured leg (32 males, 31 females)

[c] Level of significance ($\alpha < 0.05$)

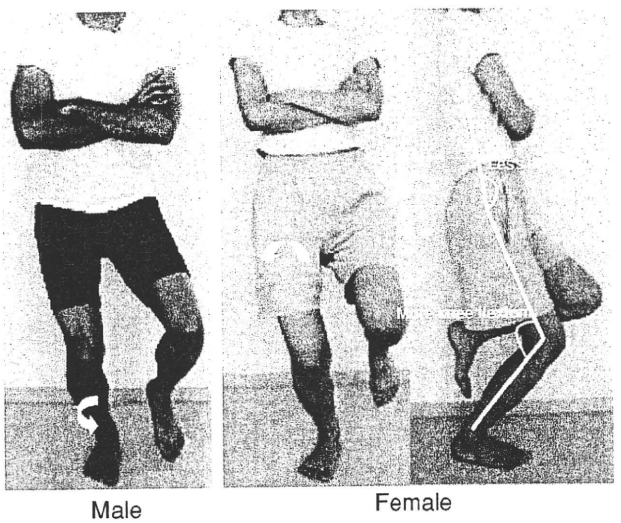

Fig. 2 Male ACL-injured subjects demonstrated less external knee rotation (*arrow*) than the control group. Female ACL-injured subjects demonstrated more external hip rotation (*arrow*), knee flexion, and less hip flexion than the control group

Several studies have examined the role of the hip joint as a potential risk factor in non-contact ACL injuries. Griffin et al. [8] reported the possibility of a decrease in hip-muscle activation influencing knee positioning. Zazulak et al. [24] showed that females exhibited a decrease in hip-muscle activity and an increase in rectus femoris muscle activity during landing maneuvers. According to Carcia et al. [25, 26], hip abductor fatigue altered frontal-plane lower extremity orientation; however, gluteus medius muscle activity was similar between genders during a drop jump. They also mentioned that gender differences in kinematics and electromyographic activity were present during the single leg squat for healthy volunteers and concluded with an emphasis on the hip as a risk factor for ACL injury in female athletes [27].

In the current study, the hip joint of an ACL-injured female is possibly more unstable due to the dysfunction of hip muscles and/or structural disability of the hip joint, which is similar to that of acetabular dysplasia. It is difficult to maintain balance of the hip joint without dynamic stability during single leg squatting, because it has a multidirectional range of motion (flexion/extension, abduction/adduction, external rotation/internal rotation) due to its ball and socket structure. Assuming that the center of gravity of the body exists at the center of the pelvis and the center of the hip joint performing the single leg squat exists outside the center of gravity. Therefore, if dysfunction of the hip due to structural abnormality and/or muscle weakness occurs, it might be difficult for ACL-injured patients to maintain stability due to the characteristics of the multidirectional mobility of the hip joint. Previous research suggests that the gluteus medius plays an important role in controlling hip joint kinematics [7, 28]. However, gender differences in gluteus medius activity have not been well investigated, while rectus femoris activity was reportedly higher in females during a single leg squat [27]. Youdas et al. [29] reported that women are quadriceps dominant while performing a single leg squat. Anterior tibial translation is often caused by force applied by the quadriceps muscles [13, 30, 31]. For this reason, the ratio of the hamstring muscle strength to the quadriceps muscle strength is reportedly important as a risk factor of ACL injury.

There was an apparent difference of physical activity between female ACL-injured subjects and female controls. The significance of the difference should be considered. The results showed that female controls demonstrated more

Table 2 Differences of mean joint angle at knee maximum flexion between uninjured and injured knees in ACL-injured subjects during single leg squatting

Motion	Mean joint angle (°)					
	Male			Female		
	Uninjured	Injured	*P* values	Uninjured	Injured	*P* values
Knee						
Flexion	74.3 ± 13.6	64.7 ± 19.0	0.0004[a]	73.9 ± 13.3	68.8 ± 13.3	0.0645[n.s.]
Varus	14.1 ± 11.5	19.8 ± 11.3	0.0068[a]	3.7 ± 11.9	8.2 ± 12.5	0.0336[a]
External rotation	18.9 ± 34.3	1.6 ± 35.2	0.0079[a]	14.1 ± 34.7	6.8 ± 36.7	0.1948[n.s.]
Hip						
Flexion	32.7 ± 17.3	33.7 ± 17.7	0.7489[n.s.]	29.9 ± 18.4	33.6 ± 15.9	0.2428[n.s.]
Varus	12.0 ± 8.6	11.2 ± 8.5	0.5629[n.s.]	10.9 ± 8.7	13.6 ± 6.4	0.0760[n.s.]
External rotation	4.5 ± 8.6	1.4 ± 8.9	0.0369[a]	9.1 ± 8.0	7.7 ± 7.7	0.3442[n.s.]

[a] Level of significance ($\alpha < 0.05$)

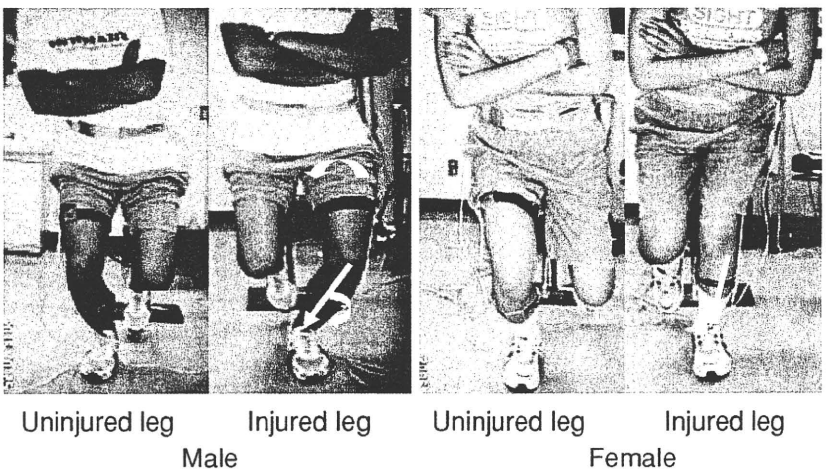

Fig. 3 The injured leg of the male subjects demonstrated more internal and varus knee and more external hip rotation than the uninjured leg. The injured leg of the female subjects demonstrated more knee varus than the uninjured leg from the coronal view

Table 3 Gender differences of mean joint angle at knee maximum flexion in ACL-injured subjects during single leg squatting

Motion	Mean joint angle (°)					
	Uninjured side			Injured side		
	Male	Female	*P* values	Male	Female	*P* values
Knee						
Flexion	74.3 ± 13.6	73.9 ± 13.3	0.8776[n.s.]	64.7 ± 19.0	68.8 ± 13.3	0.1265[n.s.]
Varus	14.1 ± 11.5	3.7 ± 11.9	0.0000[a]	19.8 ± 11.3	8.2 ± 12.5	0.0000[a]
External rotation	18.9 ± 34.3	14.1 ± 34.7	0.6674[n.s.]	1.6 ± 35.2	6.8 ± 36.7	0.4294[n.s.]
Hip						
Flexion	32.7 ± 17.3	29.9 ± 18.4	0.3744[n.s.]	33.7 ± 17.7	33.6 ± 15.9	0.9826[n.s.]
Varus	12.0 ± 8.6	10.9 ± 8.7	0.4381[n.s.]	11.2 ± 8.5	13.6 ± 6.4	0.1239[n.s.]
External rotation	4.5 ± 8.6	9.1 ± 8.0	0.0019[a]	1.4 ± 8.9	7.7 ± 7.7	0.0000[a]

[a] Level of significance ($\alpha < 0.05$)

hip flexion during the single leg squat, because they were well trained and might have participated in habitual squat exercise as physical training. The reason, the uninjured leg of female ACL-injured patients also demonstrated more hip extension compared with female controls, might have been due to the differences of the athletic level and kinds of