Table 1 Cell constituents of cystic lesions (in percent)

	Mononuclear cells	Osteoclasts	Vascular endothelial cells
MFC	78.7±8.36	1.20±1.21	21.2±8.33
LFC	77.2±8.00	0.6±1.10	22.4±7.79

MFC medial femoral condyle, *LFC* lateral femoral condyle

most of the affected subchondral plate is excised in TKA (debridement effect of TKA).

A discrepancy was found between X-ray image findings and pain severity in OA knee patients during daily, outpatient clinic evaluations. The X-ray image findings showed mild OA, but the patients' gonalgia was severe. This result might be accounted for by the expression levels of Cox-2, TNF-α, substance P, and nerve ingrowth, although in this study, we only examined cases that were painful enough to require TKA.

Cystic lesions

Positive immunoreactivities of TUJ1, substance P, Cox-2, and TNF-α were detected in cystic lesions that formed in the subchondral plate of the MFC. These cystic lesions have been called vascular channels, subchondral cracks, or bone resorption pits and are reported to be the result of an invasion from the bone marrow [12, 16, 31, 32].

Shibakawa et al. reported that the density of grade II bone resorption pits, which they defined as bone marrow tissue that infiltrated beyond the tidemark, was high in the medial tibial plateau in the medial-type OA knee [16]. This appeared to be consistent with our results showing that the density of cystic

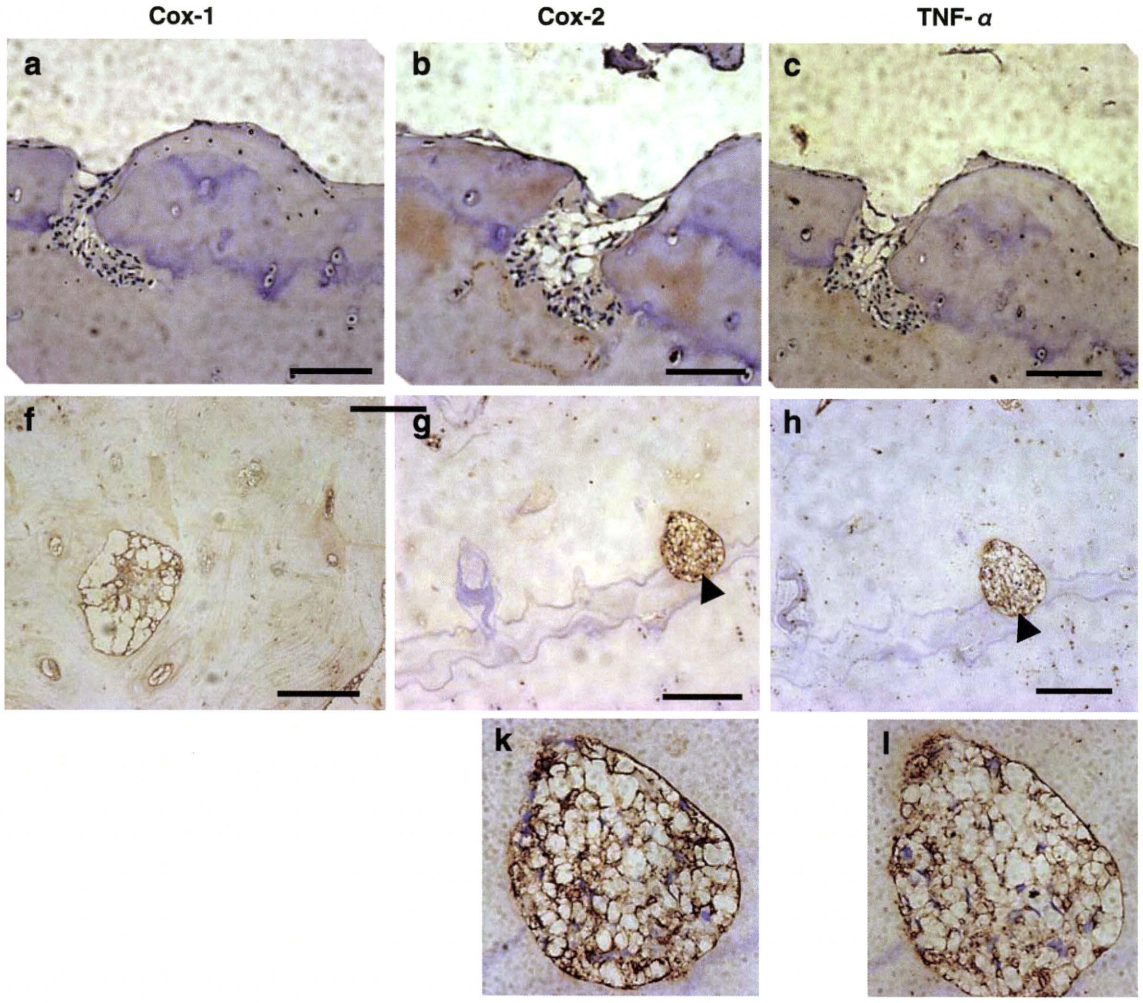

Fig. 4 Immunohistochemical analysis of subchondral bone from an osteoarthritic knee. The MFC and the LFC were stained with antibodies raised against Cox-1, Cox-2, TNF-α, substance P, and TUJ1. *Upper lanes* (**a**–**e**) show specimens from the LFC, and *middle lanes* are from the MFC (**f**–**j**). Cystic lesions in the MFC contained immunopositive cells or fibers (indicated by *arrowheads*), but those in the LFC did not. Scale bar denotes 100 μm. Higher magnification of cystic lesions were presented at the *lower lanes* (**k**–**n**). Cox-2, TNF-α, TUJ1, and substance P were all immunopositive in the cytoplasm as well as interstitial tissue but types of cells producing these molecules could not be identified

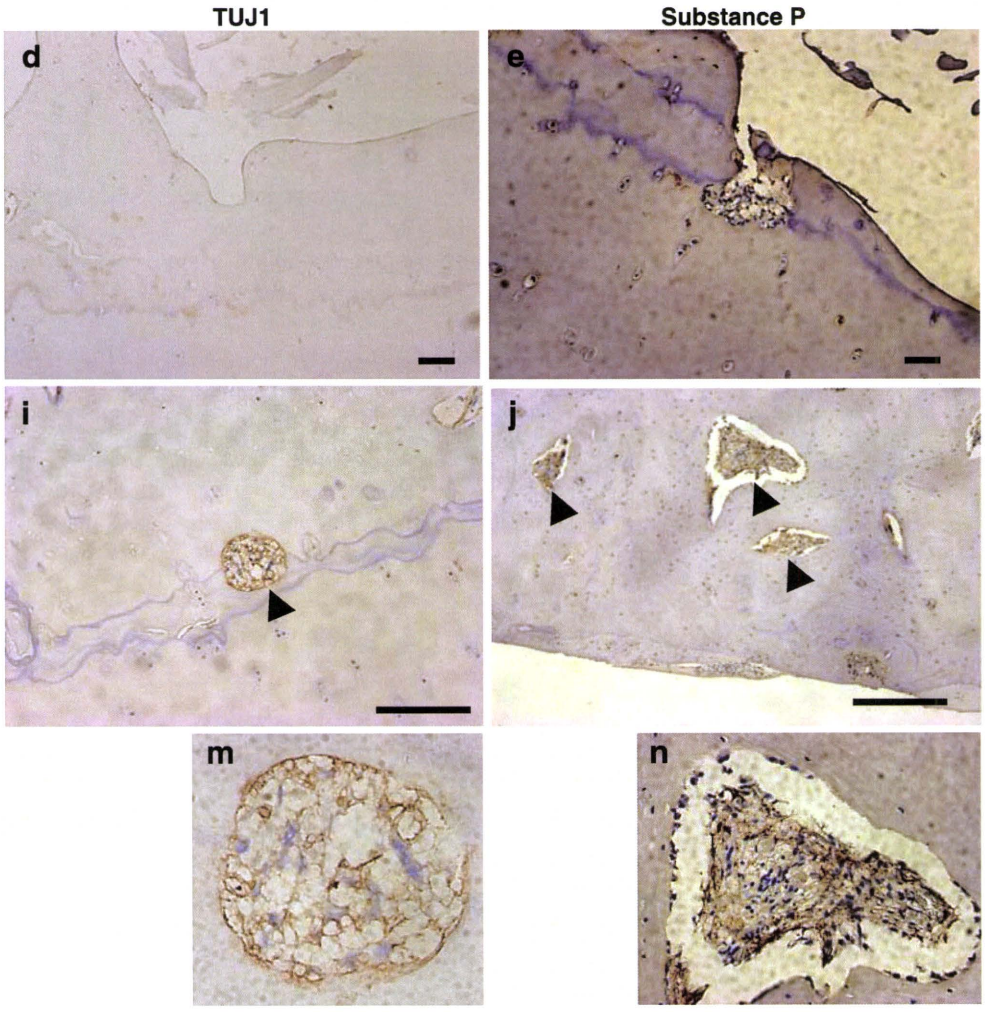

Fig. 4 (continued)

lesions was high in the MFC, but we did not distinguish them by their location because even when they were located within calcified cartilage as seen on a slide, they might have gone beyond the tidemark at other articular surfaces.

In this study, immunohistochemical analysis indicated that the cystic lesions were phenotypically different between the MFC and the LFC. Mononuclear cells, polynuclear cells, and vascular endothelial cells were all observed between the MFC and LFC and there was no difference regarding the cell constituents at the microscopic level. TRAP-positive polynuclear cells were considered to be osteoclasts (Fig. 3). Cells forming vessels were recognized as vascular endothelial cells because of their CD34-positive character (Fig. 3). Fibroblastic mononuclear cells were considered osteoblasts or preosteoblasts based on their morphology. Cells adjacent to bone tissue appeared as osteoblasts and cells farther away from the bone tissue appeared as preosteoblasts or stromal cells. However, they might have been attached to bone prior to the staining process. Alkaline phosphatase staining would be appropriate for differentiating osteoblasts, but this was difficult in the present study due to the use of decalcified specimens. Several stimuli are known to induce Cox-2 expression as well as TNF-α expression in osteoblasts or preosteoblasts [33–38]. Cox-2-positive or TNF-α-positive mononuclear cells in the cystic lesion did not contradict the notion that these were osteoblasts or preosteoblasts. Thus, osteoblasts or preosteoblasts, osteoclasts, and endothelial cells appeared to be the main constituents of the cystic lesions. Unidentified, specific cells may exist, but we can only state that cells in the MFC and LFC are morphologically the same even though they are phenotypically different.

Table 2 Proportion of positive specimens

	Cox-1	Cox-2	TNF-α	Substance P	TUJ1
MFC	4/15	15/15	13/15	15/15	15/15
LFC	0/15	0/15	0/15	0/15	0/15
Synovium	1/15	15/15	12/15	15/15	15/15

Bone remodeling

Cyclooxygenase is a major regulator of endogenous prostaglandin (PG) production in bone, and PGs have potent effects on bone metabolism. Raisz reported that Cox-2 is a major regulator of PG in bone [39], and Goldring reported that TNF-α induces differentiation of early osteoclast precursors [40]. Thus, these two cytokines also have a relationship regarding bone remodeling [41]. In the lateral compartment, where bone remodeling more or less occurs and where cystic lesions exist, we could not detect Cox-2-positive or TNF-α-positive cells. This finding suggests abnormally elevated expression of Cox-2 and TNF-α in the medial compartment, which has been previously described as abnormal bone remodeling [42, 43]. A larger number of observed cysts in the MFC might support the existence of abnormal remodeling. Several reports have stated that symptomatic OA knees showed BME on MRI [44–49]. Histological examination of BME lesions in the clinical setting is difficult, but Plenk reported that the vital bone trabeculae in BME regions showed continuous, partly osteoblast covered osteoid seams, and often the formation of irregular woven bone, which points to increased bone formation activity in BME syndrome of the hip [50]. This also implies that BME can occur from abnormal remodeling. In this study, the BME in the medial compartment that was seen in all of the patients might support elevated bone remodeling activity. Our hypothesis regarding knee pain is that the abnormal remodeling that occurs in the affected subchondral bone plate inevitably accompanies high levels of Cox-2 and TNF-α and that these cytokines cause knee pain, either by themselves or through nerve ingrowth into the subchondral plate that accompanies vascular invasion.

Limitations

There are several limitations to this study that should be pointed out. Firstly, only MFCs and LFCs from medial-type OA that required TKA were studied. Specimens from asymptomatic osteoarthritic knees (radiographic OA) would be ideal as control specimens, but we could not examine them due to the difficulties involved in obtaining them. Secondly, age-related changes that might affect the subchondral bone of both the MFC and the LFC were not counted, partly due to the lack of proper controls. Thirdly, we could not show quantitative data for the molecules of concern due to the nature of the immunohistochemical study. Lastly, we only examined small pieces of osteocartilaginous specimens and synovium removed at the time of surgery and other structures of the knee joint were not studied.

Conclusions

We demonstrated that pathological changes of the subchondral plate that occurred in the affected knee compartment can be a source of knee pain, since positive immunoreactivities of substance P, Cox-2, TNF-α, and TUJ1 were detected only in the subchondral bone of the affected compartment.

Acknowledgments This study was funded by the Comprehensive Research on Aging and Health, Health and Labor Sciences Research Grants of Japan.

Disclosures None.

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Augmentation of Degenerated Human Cartilage In Vitro Using Magnetically Labeled Mesenchymal Stem Cells and an External Magnetic Device

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Purpose: The purpose of this study was to investigate whether it is possible to regenerate degenerated human cartilage in vitro by use of magnetically labeled mesenchymal stem cells (MSCs) and an external magnetic device. **Methods:** MSCs from human bone marrow were cultured and magnetically labeled. Degenerated human cartilage was obtained during total knee arthroplasty. The osteochondral fragments were attached to the sidewall of tissue culture flasks, and magnetically labeled MSCs were injected into the flasks. By use of an external magnetic device, a magnetic force was applied for 6 hours to the direction of the cartilage, and then the degenerated cartilage was cultured in chondrogenic differentiation medium for 3 weeks. In the control group a magnetic force was not applied. The specimens were evaluated histologically. **Results:** A cell layer was formed on the degenerated cartilage as shown by H&E staining. The cell layer was also stained in toluidine blue and safranin O and with anti-collagen type II immunostaining, indicating that the cell layer contained an extracellular matrix. In the control group a cell layer was not observed on the cartilage. **Conclusions:** We were able to show that our system could deliver MSCs onto degenerated human cartilage and then form an extracellular matrix on the degenerated cartilage in vitro. **Clinical Relevance:** Our novel cell delivery system using magnetic force may lead toward a new treatment option for osteoarthritis. **Key Words:** Cell delivery system—Mesenchymal stem cell—Magnetic force—Regeneration—Degenerated cartilage.

Osteoarthritis of the knee is the most common joint disorder, and it accounts for more disability among elderly people than any other disease. In the United States it is estimated that 68% of individuals aged older than 55 years have radiographically observable osteoarthritis.[1] There are a number of treatment options for osteoarthritis, both nonoperative treatments and operative treatments, such as arthroscopic debridement, osteotomy, and total knee arthroplasty. One recent strategy for the treatment of osteoarthritis is by the implantation of mesenchymal stem cells (MSCs).[2-4] MSCs are the cell population of undifferentiated cells isolated from adult tissue that have the capacity to differentiate into mesodermal lineages, such as bone, cartilage, fat, muscle, and other tissues.[5,6] MSCs from bone marrow can be cultured and differentiated into the desired lineage in vitro with the application of specific growth factors or bioactive molecules. Intra-articular injection of too many MSCs, however, can generate free bodies of scar tissue.[7] We therefore developed a novel stem cell delivery system for

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cartilage repair using magnetically labeled MSCs and an external magnetic device to accumulate a relatively small number of MSCs to a desired area.[8] In a recent study we demonstrated the ability to deliver magnetically labeled MSCs to a desired place in rabbit and swine knee joints.[9] In the present study, using this device, we hypothesized that we could successfully regenerate a cartilage layer on degenerated human cartilage in vitro.

METHODS

Our research methods were reviewed and approved by the ethical committee of our university.

Cell Culture

The method of isolation and in vitro expansion of bone marrow–derived MSCs has been previously described.[9] In brief, 5 mL of bone marrow from the tibia of adult human donors was aspirated with 1 mL of heparin when they underwent anterior cruciate ligament reconstruction and was centrifuged for 5 minutes at 1,500 rpm, and the subsequent supernatant, including heparin sodium, was discarded. The extract was resuspended in 6 mL of culture medium composed of Dulbecco's modified Eagle's medium (Gibco BRL, Carlsbad, CA) with 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO) and 1% antibiotics (penicillin, streptomycin, and Fungizone; Bio-Whittaker, Walkersville, MD). Then 2 mL of the suspension was seeded onto 100-mm culture dishes, and 8 mL of culture medium was added to each dish. The dishes were incubated for 3 weeks under a humidified atmosphere and 5% carbon dioxide at 37°C. The medium was not changed for the first 7 days, and it was then changed every 3 days thereafter. When the cells had proliferated and reached confluence, about 2 weeks after seeding, they were harvested by treatment with 0.25% trypsin. To expand the cells, 2 to 3 $\times$ 10^5 of the harvested cells were seeded onto 100-mm culture dishes. On reaching confluence again, the cells were reseeded under the same conditions. We referred to these adherent cells as MSCs.

Magnetic Labeling of MSCs

Magnetic labeling of MSCs was performed as previously described.[9] In brief, MSCs were labeled overnight with 25 μg Fe/mL ferumoxides (Felidex; Tanabe Seiyaku, Osaka, Japan), a magnetic resonance contrast agent approved by the US Food and Drug Administration, and 375 ng/mL poly-L-lysine (poly-L-lysine hydrobromide; molecular weight, 388 kDa) (Sigma P-1524; Sigma, St Louis, MO) as a transfection agent. We confirmed that almost 100% of MSCs were stained blue with Prussian blue stain after magnetic labeling.

External Magnetic Device

The external magnetic device was used as previously described.[9] A variable direct-current electromagnet (model TM-SP12010SC-014; Tamagawa, Miyagi, Japan) was manufactured for the purpose of generating an external magnetic force. The magnetic field is directed to the center of the disk surface, and its magnitude decreases away from the surface. The magnitude of the magnetic field is increased by increasing the electric current through the electromagnet and is limited by the temperature of the coil. When a sample lies 8 cm from the center of the pole, the maximum magnetic field is 0.6 T.

Accumulation Capacity of Magnetically Labeled MSCs Under Influence of Magnetic Force

We used specially made flasks for this study (Fig 1). Two slide glasses were set into each flask, one on the bottom and the other on one of the long sidewalls. When 60 mL of culture medium was poured into the flask, the surface of the medium was at the same height as the upper side of the slide glass. Magnetically labeled MSCs (2×10^5/60 mL of culture medium) were injected into the flask. A magnetic force of 0.4 T or 0.6 T, to the direction of the slide on the sidewall of the flask, was then applied for 2 or 4 hours. After the treatment, the side and bottom slide glasses were fixed with 95% ethanol and were stained with H&E. After an adhesive sealing film with a grid was attached onto the side and bottom slide glasses, the cell numbers, expressed as a percentage of the total cell number on the side and bottom slide glasses, were counted.

Chondrogenesis Differentiation of Magnetically Labeled MSCs on Degenerated Human Cartilage

Degenerated Human Cartilage: Degenerated human cartilage was obtained during total knee arthroplasty from osteoarthritic patients with medial-type osteoarthritis. We chose osteochondral fragments of the lateral femoral condyle, classified as grade II according to Outerbridge,[10] which were then stored for 48 hours in Dulbecco's phosphate-buffered saline solution at 4°C.

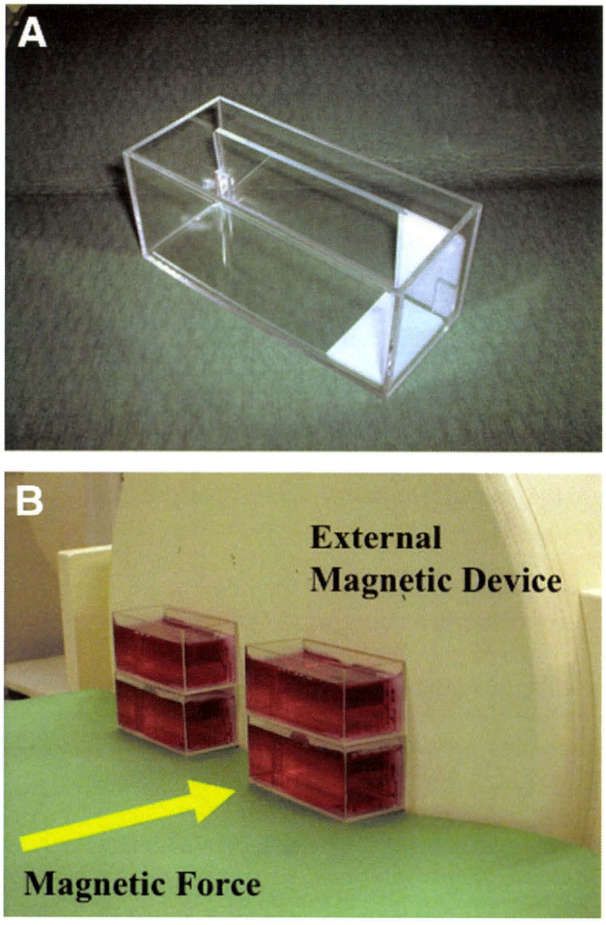

FIGURE 1. (A) Flasks specially made for use in study. The flasks could hold two slide glasses, one on the bottom and the other on the sidewall of the flask. Magnetically labeled MSCs (2×10^5/60 mL of culture medium) were injected into the flask. (B) A magnetic force of 0.4 or 0.6 T, to the direction of the slide on the sidewall of the flask, was then applied for 2 and 4 hours.

The fragments, which were cut to measure 1 cm × 1 cm, were allocated into 2 groups, a magnetic force group and a control group (Fig 2), and each experiment was performed in triplicate. In both groups 2 fragment pieces were attached to the sidewall of a tissue culture flask by use of a 2% agarose gel. Magnetically labeled MSCs (2×10^6 cells) in 10 mL of culture medium, composed of Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% antibiotics, were then poured into the flasks. In the magnetic force group a magnetic force of 0.6 T was applied for 6 hours to the direction of the fragment by use of an external magnetic device, whereas in the control group a magnetic force was not applied (Fig 2).

Chondrogenesis Differentiation: After the treatment, the fragments were cultured for 3 weeks under a humidified atmosphere and 5% carbon dioxide at 37°C in 3 mL of chondrogenesis differentiation medium (TMDFC-001; Toyobo, Osaka, Japan), which contained 3% fetal calf serum, 5 μg/mL of insulin, 5 μg/mL of transferrin, 5 ng/mL of transforming growth factor β1, 5 ng/mL of bone morphogenetic protein 2, and 0.1-μmol/L dexamethasone. The chondrogenic differentiation medium was changed every 3 days.

Evaluation: After the treatment, the specimens were fixed with 4% formalin for 2 days, decalcified with 10% ethylenediaminetetraacetic acid for 6 weeks, and then embedded in paraffin. Histologic sections were stained with H&E, Berlin blue, toluidine blue, and safranin O for microscopic analysis and also underwent immunostaining with polyclonal antibody against type II collagen (Chemicon, Temecula, CA).

Statistical Analysis

The statistical significance of differences in parameters was assessed by the Mann-Whitney *U* test. For all data collection, the experimenters were blinded to the group identities. $P < .05$ was regarded as statistically significant.

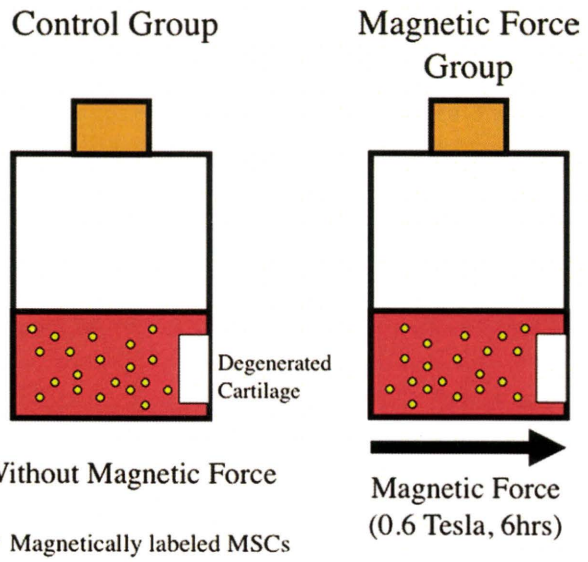

FIGURE 2. Degenerated cartilage was attached to the sidewall of a tissue culture flask, and magnetically labeled MSCs (2×10^6 cells) and culture medium were then poured into the flasks. In the magnetic force group a magnetic force of 0.6 T was applied for 6 hours by use of an external magnetic device. In the control group a magnetic force was not applied.

RESULTS

Accumulation Capacity of Magnetically Labeled MSCs Under Influence of Magnetic Force

With no magnetic field applied, no cells attached to the slide glass on the sidewall. In a magnetic field of 0.4 T, 56.3% of the cells had attached to the slide glass on the sidewall after 2 hours and 64.5% of the cells had attached after 4 hours. In a magnetic field of 0.6 T, 73.2% of the cells had attached to the slide glass on the sidewall after 2 hours and 68.2% of the cells had attached after 4 hours (Fig 3). The percentage of attached cells in the 0.4- and 0.6-T groups was significantly greater than that in the 0-T group after 2 and 4 hours. The percentage of attached cells in the 0.6-T group was significantly greater than that in the 0.4-T group after 2 hours but was not significantly different after 4 hours.

Chondrogenesis Differentiation of Magnetically Labeled MSCs on Degenerated Human Cartilage

Before the treatment, all specimens were graded 1 to 2 (slight reduction to moderate reduction) according to Mankin's histologic grading.[11] The surface of all specimens was not stained in safranin O.

In the control group, in 6 of 6 specimens, there was no cell layer on the degenerated cartilage (Fig 4). In the magnetic force group (Fig 5), a cell layer was formed on the degenerated cartilage as shown by H&E staining in all specimens. In 4 of 6 specimens in the magnetic force group, the cell layer was stained in toluidine blue, safranin O stain, and anti-collagen type II, indicating that the cell layer contained an extracellular matrix.

DISCUSSION

This study clearly showed that our system using magnetically labeled MSCs and an external magnetic device was able to deliver the MSCs in the direction of the magnetic force and that the MSCs were then able to form a cell layer that contained an extracellular matrix on the degenerated cartilage.

MSCs have the capacity to differentiate into mesodermal lineages, such as bone, cartilage, fat, muscle, and other tissues,[5,6] and large amounts of cells can be obtained by culturing the bone marrow cells in a monolayer culture system. Recently, many researchers have reported methods of transplanting MSCs to repair osteoarthritic cartilage defects.[2-4] Murphy et al.[2] reported that intra-articular injection of MSCs into an osteoarthritic knee stimulated regeneration of articular cartilage in goats. Nishimori et al.[3] reported that intra-articular injection of MSCs, along with a bone marrow–stimulation procedure, repaired chronic osteochondral defects in rats. In a clinical trial, Wakitani et al.[4] reported that the implantation of MSCs, together with high tibial osteotomy, was better than only high tibial osteotomy for the human osteoarthritic knee on the arthroscopic and histologic grading scale. On the other hand, Agung et al.[7] reported that injured cartilage was repaired after intra-articular injection of MSCs in rats, but the injection of too many MSCs generated free bodies of scar tissue in the joint. We therefore developed a novel stem cell delivery system

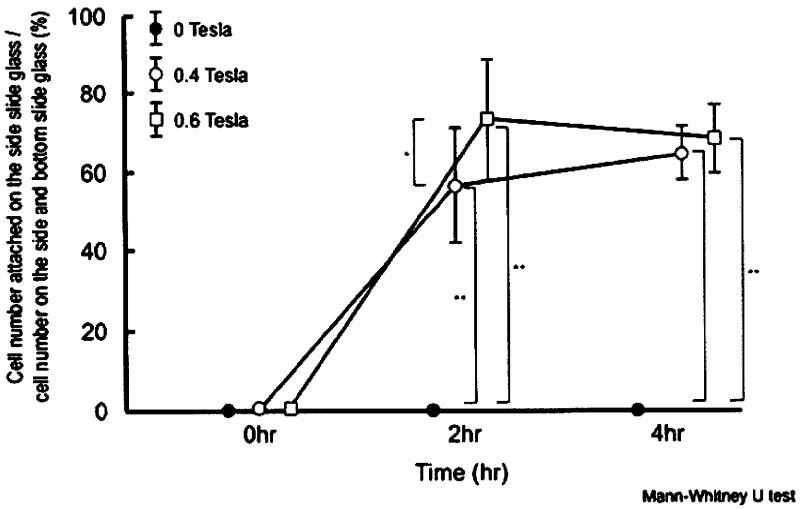

FIGURE 3. Percentage of attached cells on side and bottom slide glasses after application of magnetic force. The percentage of attached cells in the 0.4- and 0.6-T groups was significantly greater than that in the 0-T group after 2 and 4 hours. The percentage of attached cells in the 0.6-T group was significantly greater than that in the 0.4-T group after 2 hours but was not significantly different after 4 hours. (1 asterisk, $P < .05$; 2 asterisks, $P < .001$.)

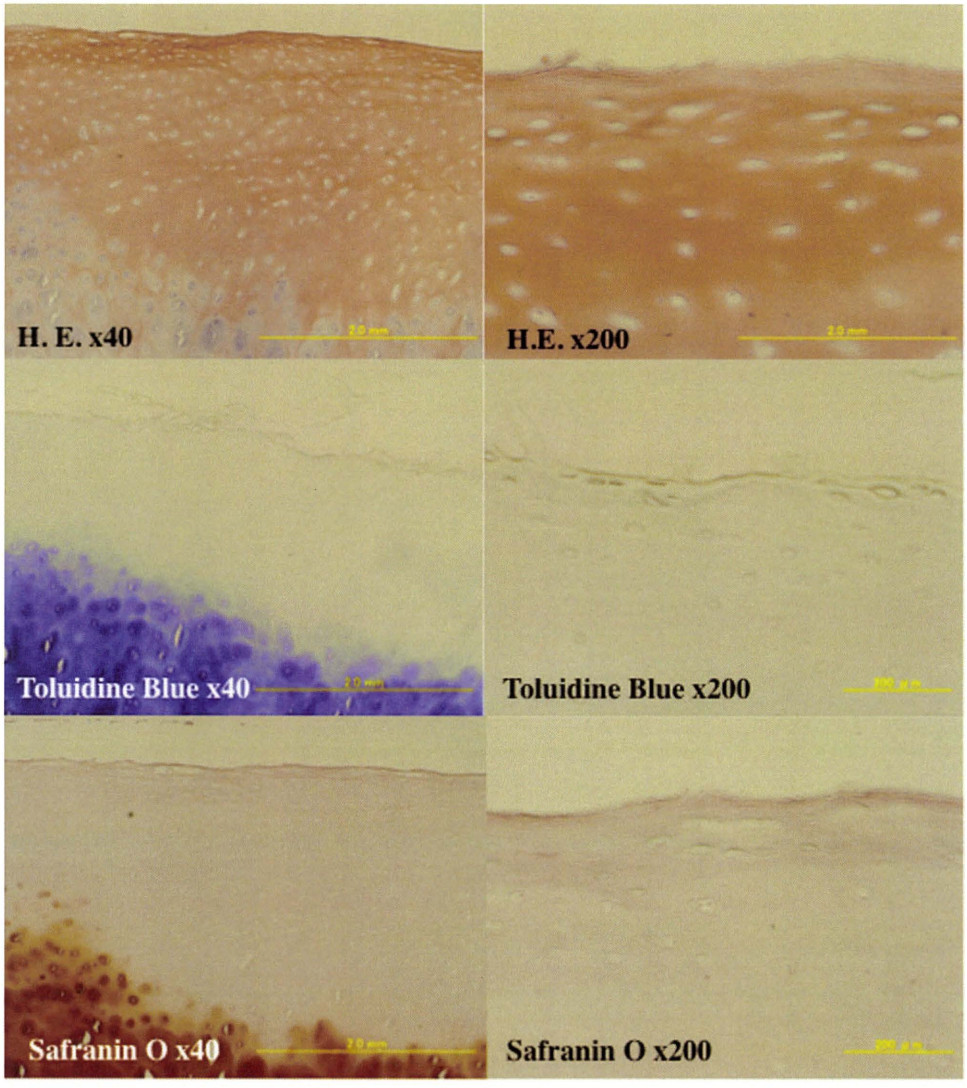

FIGURE 4. In the control group, in 6 of 6 specimens, there were no cells on the degenerated cartilage, and safranin O staining of the degenerated cartilage was grade 1 to 2 (slight reduction to moderate reduction) according to Mankin's histologic grading.[11] The surface of completely degenerated cartilage was not stained in toluidine blue and safranin O.

using magnetically labeled MSCs and an external magnetic device for cartilage repair with the aim of avoiding these side effects.[8,9,12,13] Using this system, we previously reported that magnetically labeled MSCs accumulated onto osteochondral defects of the patella in the knee joints of pigs and rabbits, as observed macroscopically, histologically, and arthroscopically, by controlling the direction of the magnetic force.[9] We also reported that this system was useful for spinal cord injuries[14-16] and bone regeneration.[17] In the present study we showed that our system using an external magnetic force was able to deliver magnetically labeled MSCs to degenerated human cartilage, and a cell layer that contained an extracellular matrix was formed on the cartilage. In clinical application, if expanded MSCs are injected into the osteoarthritic joint and an appropriate magnetic force is applied, call layers formed on the degenerated cartilage may contribute to cartilage regeneration. Therefore there is potential that this minimally invasive procedure can be a new treatment option for osteoarthritis.

There are some limitations to our study. First, this study was conducted in an in vitro setting, in which accumulated MSCs were cultured in chondrogenic differentiation medium. Therefore it is uncertain whether accumulated MSCs can differentiate into chondrocytes or contribute to cartilage repair in vivo. A long-term study in animals or humans is required to confirm whether magnetically labeled MSCs accumulated on degenerated cartilage by use of a magnetic force can contribute to cartilage regeneration in vivo, as well as to determine the minimal dose of cells necessary to

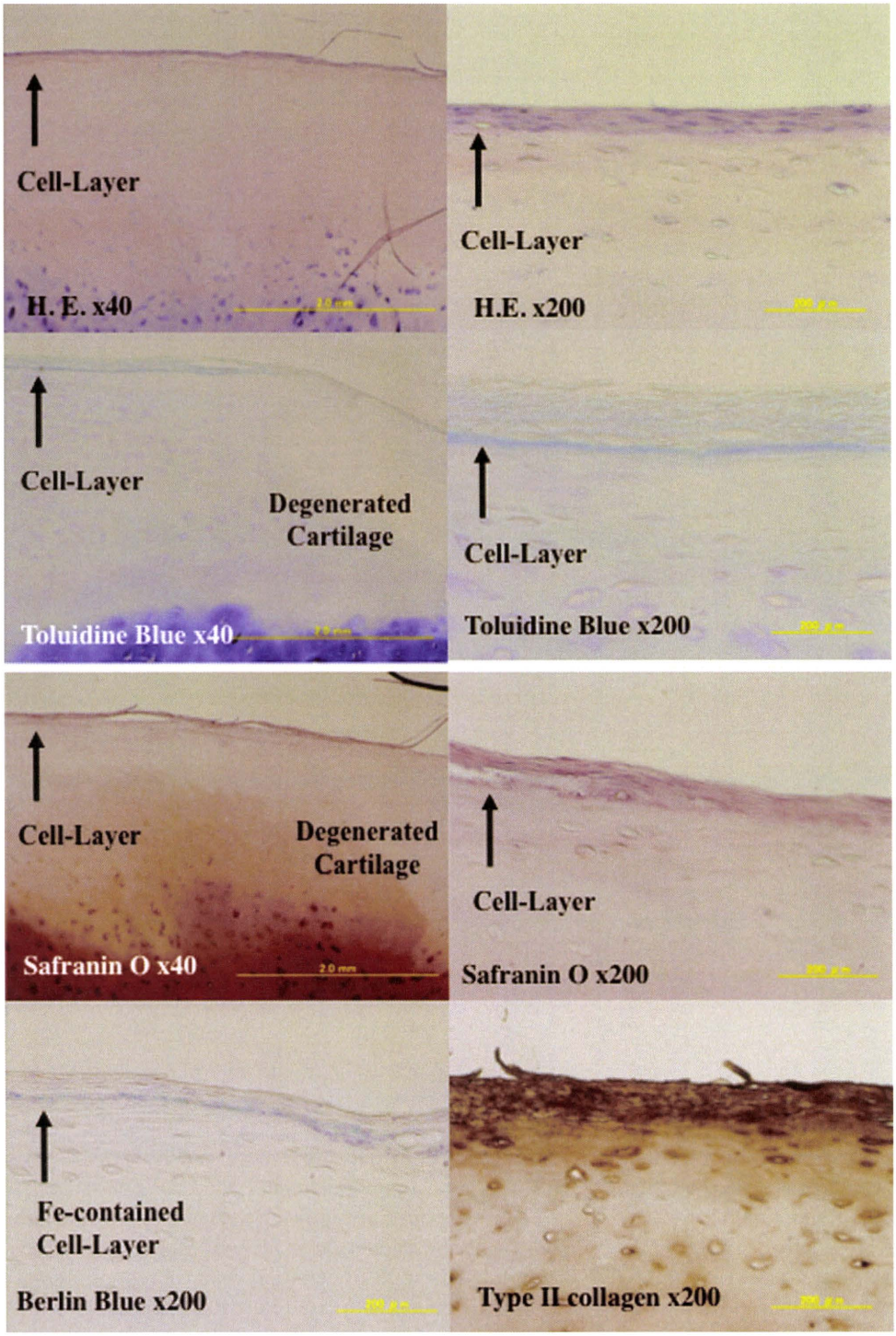

FIGURE 5. In the magnetic force group, cell layers were formed in all specimens. In 4 of 6 specimens a cell layer was formed on the degenerated cartilage as shown by H&E staining, and the cell layer was stained in toluidine blue and safranin O stains, as well as anti-collagen type II, indicating that the cell layer contained an abundant extracellular matrix.

achieve the desired effect without creation of loose bodies. Second, we used a magnetic field strength of 0.6 T for 6 hours in this in vitro study, but we have not determined the most effective strength for the magnetic field. We should therefore perform another study to optimize the field strength and the time of application of the magnetic field for effective cartilage repair with respect to the method's use in clinical applica-

tions. Third, we should study a control group in which osteochondral fragment pieces are attached to the bottom of the flask without a magnetic force applied.

Despite these limitations, we showed that our novel cell delivery system has the potential to be a new treatment option for osteoarthritis.

CONCLUSIONS

Using the external magnetic device and magnetically labeled MSCs, we showed that our system was able to form a cell layer that contained an extracellular matrix on degenerated human cartilage in vitro, indicating that the hypothesis of this study was supported.

Acknowledgment: The authors thank Paul Reay for valuable comments.

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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

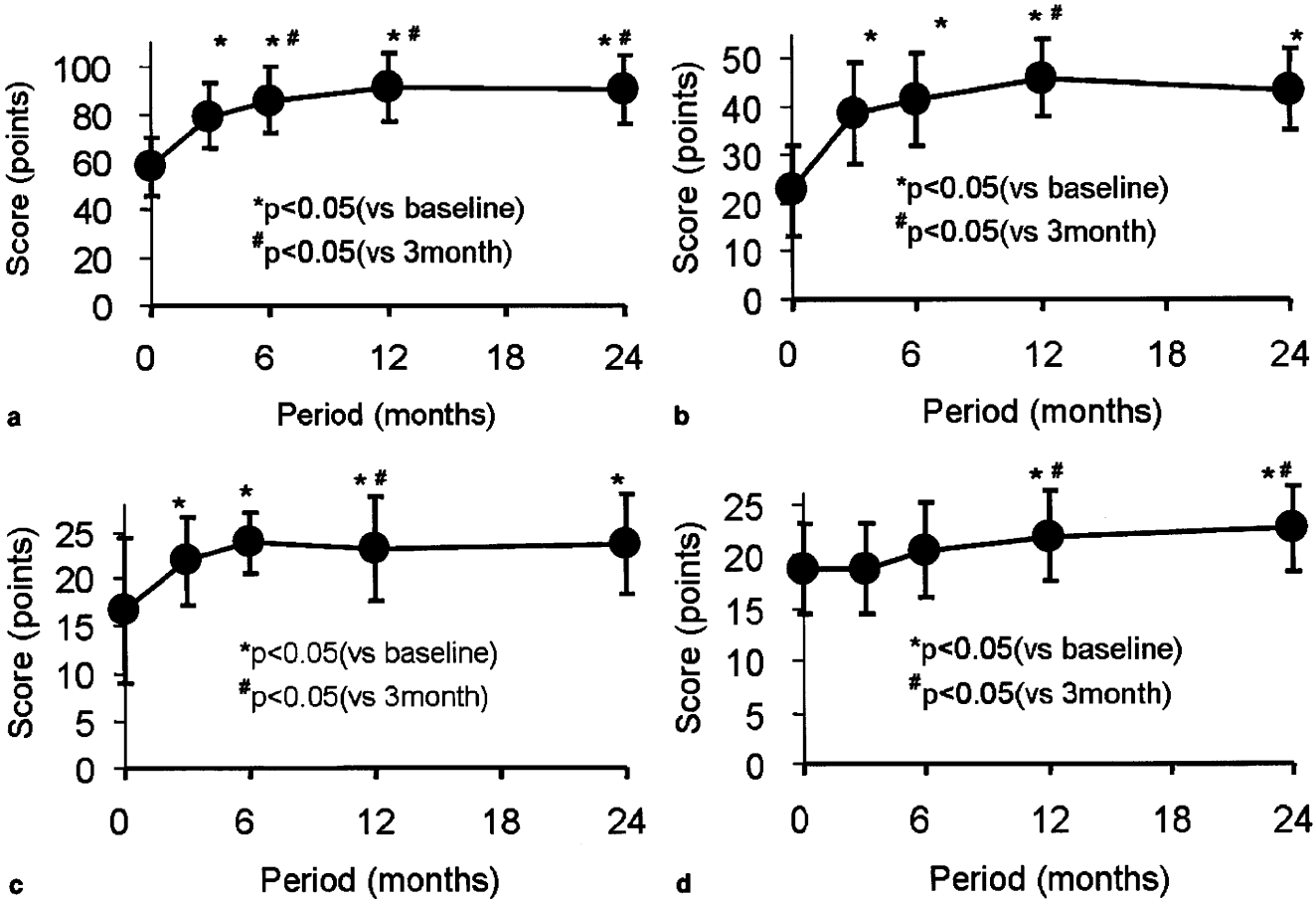

Fig. 1. Our original knee function scores: preoperatively (0) and at 3, 6, 12, and 24 months after the surgical procedure. **a** Total. **b** Motion pain. **c** Rest pain. **d** Knee motion

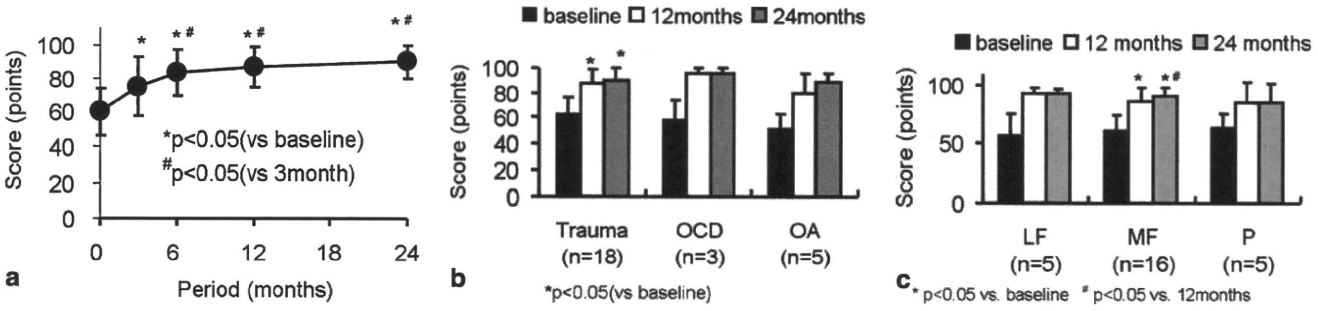

Fig. 2. Lysholm scores: preoperatively (0) and at 3, 6, 12, and 24 months after the surgical procedure. **a** Overall patients. **b** Comparison by the original cause of the cartilage defect. *OCD*, osteochondritis dissecans; *OA*, osteoarthritis. **c** Comparison by implantation location. *LF*, lateral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

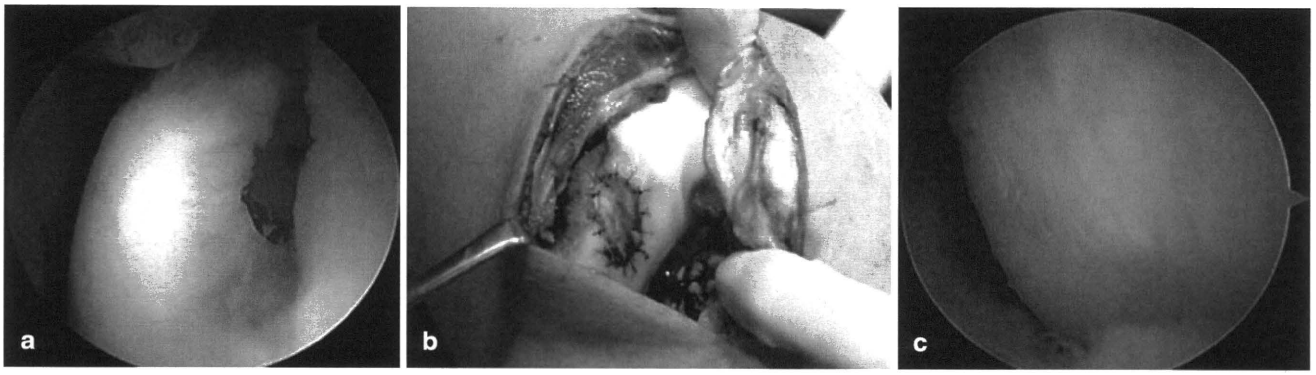

Fig. 3. Case 1. A 30-year-old man with a traumatic cartilage defect at the medial femoral condyle. **a** Arthroscopy showed a cartilage defect (10 × 20 mm) in the medial femoral condyle before transplantation. **b** Transplantation of autologous chondrocytes embedded in atelocollagen gel was performed. **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

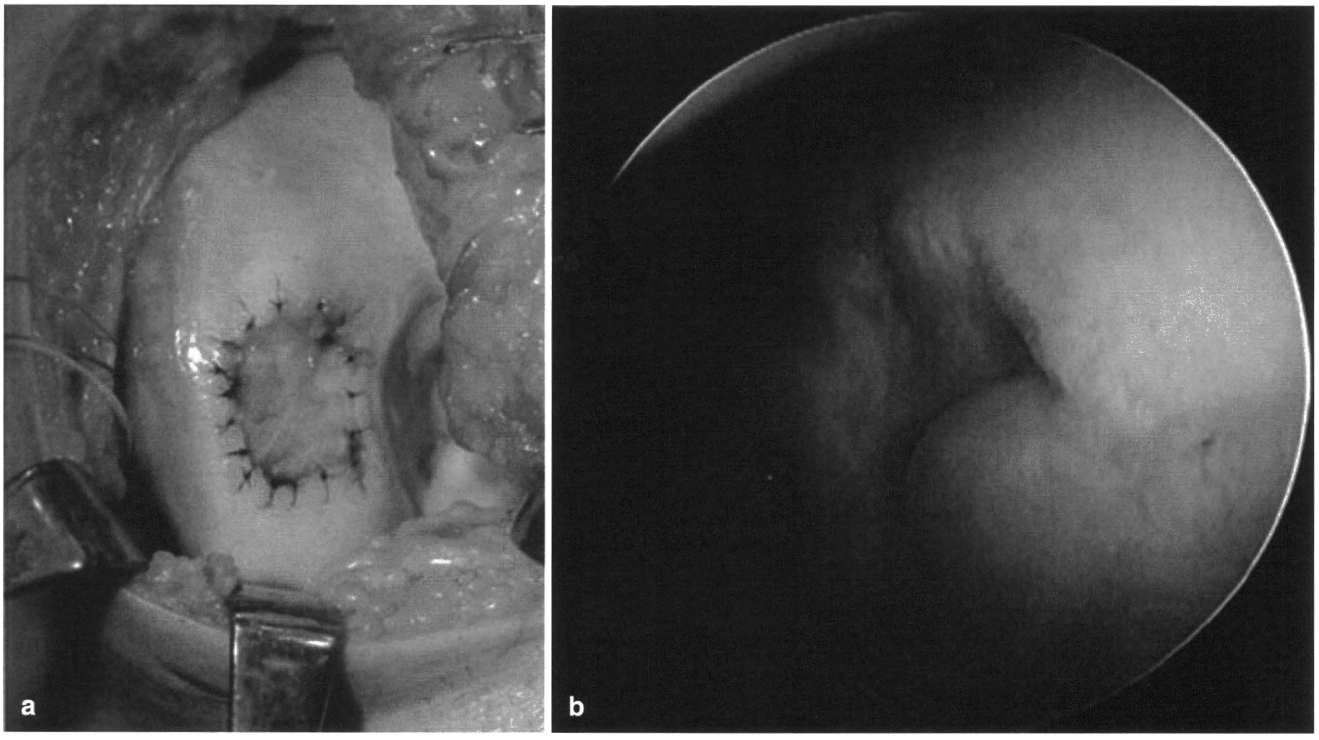

Fig. 4. Case 2. A 42-year-old man suffered waking pain caused by a cartilage defect at the left medial femoral condyle due to osteoarthritis. **a** Autologous chondrocytes embedded in atelocollagen gel were implanted at the cartilage defect (10 × 20 mm). **b** Arthroscopic findings at 12 months shows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

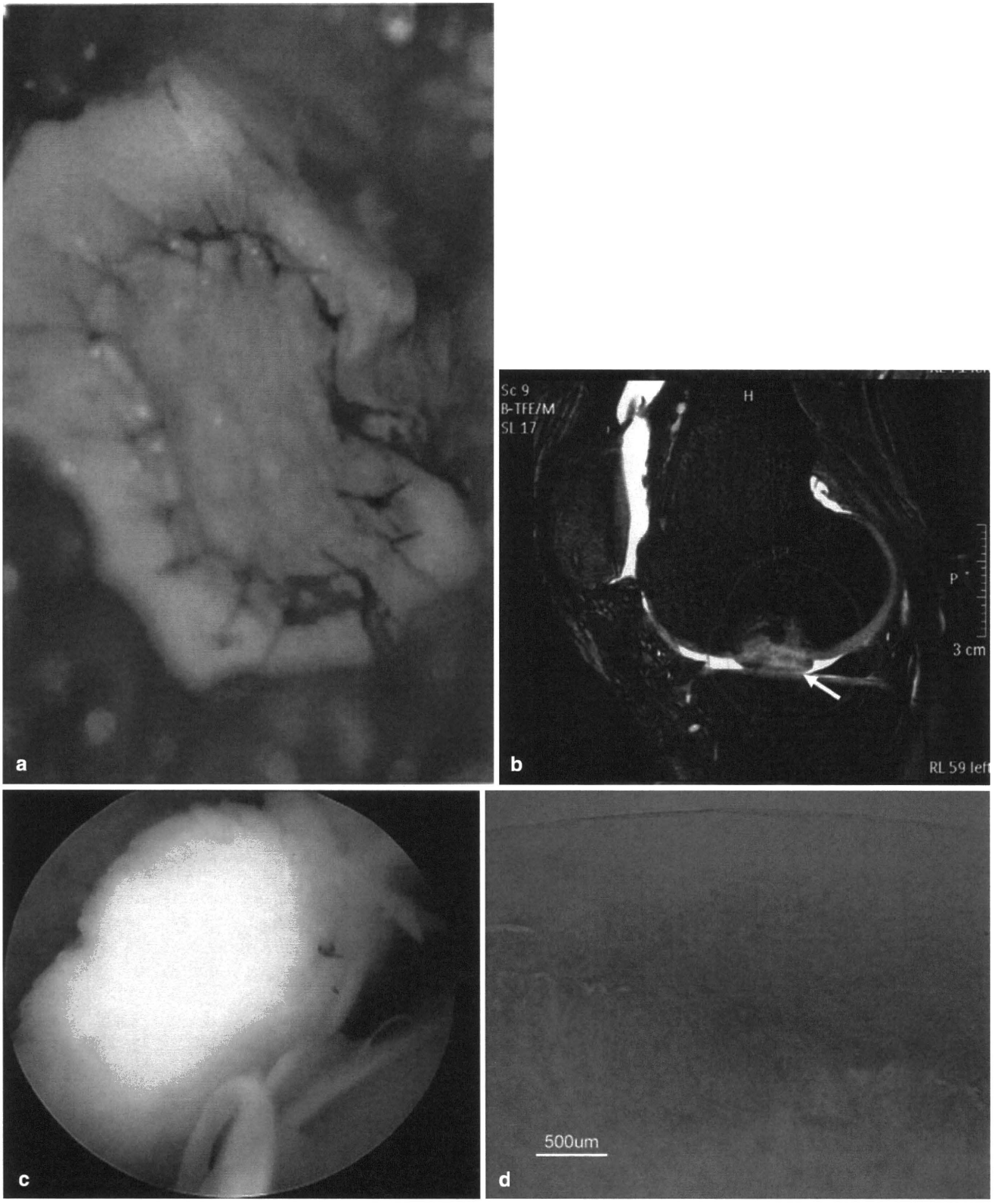

Figure 5. Case 3. A 29-year-old man with a cartilage defect at the left medial femoral condyle due to osteochondritis dissecans. **a** Autologous chondrocytes embedded in atelocollagen gel were implanted at the cartilage defect (15 × 20 mm). **b** Magnetic resonance imaging (MRI) 6 months after implantation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