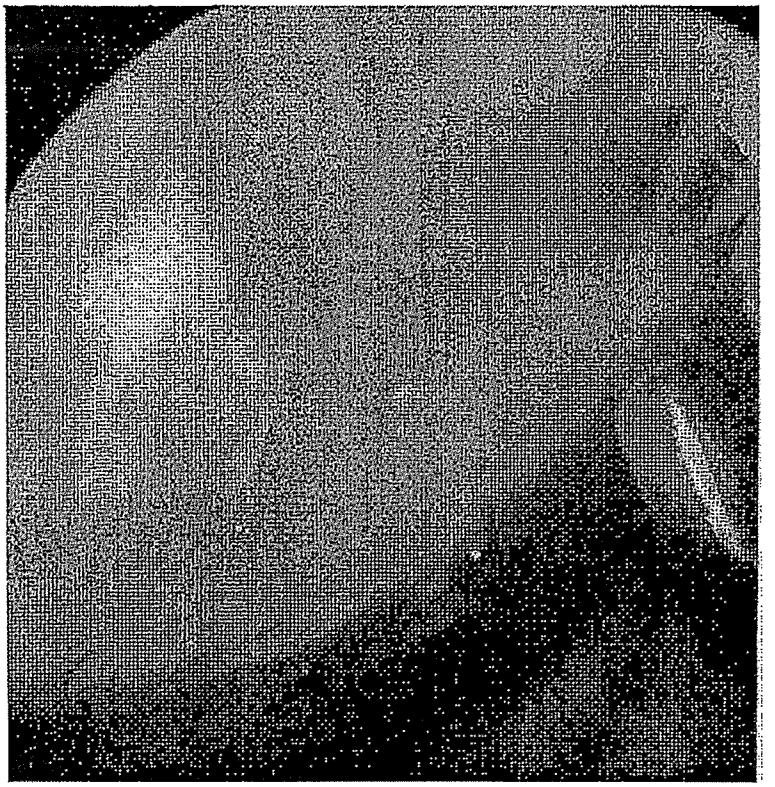

Figure 2. Arthroscopic appearance of the repair tissue 2 months after transplantation in the second patient. The defect was completely covered with tissue that was softer than the surrounding cartilage.

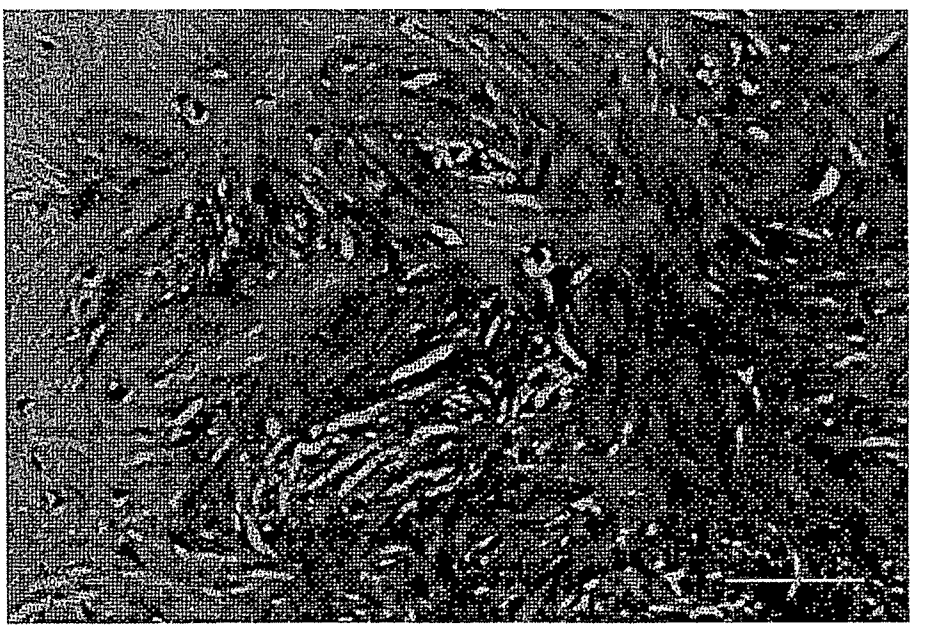

Figure 3. Microscopic appearance of the repair tissue 1 year after transplantation in the second patient. Toluidine blue staining (original magnification ×100). Scale bar indicates 100 μm. Intracellular matrix showed strong metachromatic staining but with a fibrous appearance. Fibroblastic cells with spindle-shaped nuclei and scattered chondrocytes with lacunae were also observed.

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Fibroblast growth factor-2 promotes the repair of partial thickness defects of articular cartilage in immature rabbits but not in mature rabbits

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Summary

Objective: To investigate cartilage response to fibroblast growth factor-2 (FGF-2) with increasing age *in vivo*, we examined the effect of FGF-2 on partial thickness defects of immature and mature rabbits.

Design: Sixty-nine Japanese white rabbits (34 immature rabbits, 35 mature rabbits) were examined. We made experimental partial thickness defects in articular cartilage of the knees. Then, we injected FGF-2 into the knees eight times, immediately after surgery and every 2 days for 2 weeks. A single dose of FGF-2 was 10 ng/0.1 ml or 100 ng/0.1 ml. In the control group, 0.1 ml saline was injected on the same time schedule. The rabbits were sacrificed at intervals following surgery that ranged from 2 to 48 weeks. The specimens were stained with toluidine blue and examined microscopically. We used a modified semiquantitative scale for evaluating the histological appearance of repair.

Results: In immature rabbits, the cartilage repair in the FGF-2 (100 ng)-treated group was significantly better than that of the other groups. The defects were almost completely repaired with chondrocytes that showed a round to polygonal morphology, and large amounts of extracellular matrix with intense metachromatic staining.

In mature rabbits, however, there was apparently no effect from FGF-2 in either group.

Conclusions: Application of FGF-2 facilitated cartilage repair in partial thickness defects in immature rabbits, but not in mature ones.

Key words: Age, Response, *In vivo*, Intra-articular injection.

Introduction

Articular cartilage has a limited capacity for repair. Partial thickness defects, which do not injure the subchondral bones, do not heal spontaneously. A short-lived tissue response has been reported, but it failed to provide sufficient cells and matrix to repair even small defects. In contrast, full thickness defects, which extend beyond the subchondral bone, receive an abundant source of mesenchymal cells for affecting cartilage repair through the subchondral bone[1]. However, the resulting repair tissue is predominantly of a fibrous nature, containing variable numbers of chondrocytes, fibrocytes and an unorganized matrix. This fibrocartilage does not resemble the original cartilage either biochemically or biomechanically and it ultimately deteriorates.

Several growth factors such as fibroblast growth factor-2 (FGF-2)[2–6], insulin-like growth factor-I (IGF-I)[7–9], hepatocyte growth factor[10], bone morphogenetic protein-2[11,12], and transforming growth factor-β[2,13,14], have been exogenously applied to articular cartilage defects to potentially enhance cartilage reconstitution. FGF-2 is widely distributed in mesenchymal structures, mesoderm- and neuro-ectoderm-derived cells. It is a member of a multi-lineage family that binds to heparin. It acts as a potent mitogen in a wide variety of cell types derived from mesoderm and neuro-ectoderm *in vitro*[15–17]. The importance of FGF-2 in cartilage repair is widely accepted.

In partial thickness defects, Cuevas *et al.* reported that FGF-2 stimulated the proliferation of chondrocytes in adolescent rabbits[4], while Hunziker and Rosenberg did not observe such effect in mature rabbits[19]. This difference is possibly due to the age of the rabbits they used. Guerne *et al.* showed age-related decline in chondrocyte response to FGF-2 *in vitro*[18]. However, cartilage response to FGF-2 *in vivo* with increasing age is unclear. The purpose of this study is to examine cartilage response to FGF-2 with increasing age *in vivo*, using partial thickness defects in immature and mature rabbits.

Materials and methods

Seventy-two Japanese white male rabbits (Japan Animals Co. Ltd, Osaka, Japan) were used (36 immature rabbits and 36 adult rabbits). The average age of the immature rabbits was approximately 12 weeks, and that of the mature rabbits

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was approximately 24 weeks. The average weight of the immature rabbits was approximately 2.3 kg, and that of the mature rabbits was approximately 3.6 kg. The rabbits were anesthetized by intramuscular injection of a mixture of ketamine (100 mg/ml, 0.60–0.70 ml/kg body weight, SANKYO Co. Ltd, Tokyo, Japan) and xylazine (20 mg/ml, 0.30 ml/kg body weight, BAYER Co. Ltd, Leverkusen, Germany).

The skin around the knee was shaved anteriorly and washed with 70% ethanol. A parapatellar medial approach was used to expose the knee joint. The patella was dislocated laterally. In principle, we made three longitudinal partial thickness defects in the articular cartilage of the mid-trochlear region of the femur with a round-shaped chisel. However, the area of the mid-trochlear region was so small that we could not occasionally avoid making only two longitudinal partial thickness defects in some cases. Then, the wound was closed. Partial thickness defects were made in both knees. All rabbits were returned to their cages after the operation and were allowed to move freely. No animal was observed to have an abnormal gait or impaired locomotion.

We injected human recombinant FGF-2 (KAKEN PHARM. Co. Ltd, Tokyo, Japan) into the articular cavity of the knees through the lateral side of the patella ligament at the level of the joint space. FGF-2 was injected eight times, immediately after surgery and every 2 days for 2 weeks. A single dose of FGF-2 was 10 ng/0.1 ml of saline, which was administered to the FGF-2 (10 ng)-treated group, or 100 ng/0.1 ml of saline, which was administered to the FGF-2 (100 ng)-treated group. In the control group, 0.1 ml saline was injected using the same time schedule.

At 2, 4, 8, 12, 24 and 48 weeks after surgery, respectively, six rabbits were scheduled for sacrifice. Two immature rabbits and one mature rabbit died before sacrifice. Therefore, five immature rabbits were sacrificed at 8 and 12 weeks, and five mature rabbits at 48 weeks. The rest of the rabbits were sacrificed as indicated in the time schedule. The distal part of the femur was fixed in 10% neutral-buffered formalin (Wako Pure Chemicals Industries Co. Ltd, Osaka, Japan) for 1 week, decalcified with 0.5 M ethylenediaminetetraacetic acid (SIGMA Co. Ltd, St. Louis, MO, USA) containing 0.1 M epsilon-amino-*n*-caproic acid (SIGMA Co. Ltd) and 0.005 M benzamidine (SIGMA Co. Ltd), and sectioned perpendicular to the defect. These sections were obtained from the center of the defect. The specimens were stained with toluidine blue and examined microscopically.

Partial thickness defects of articular cartilage are difficult to create. Although we confirmed no breakage of the subchondral bone in any histological sections, it is possible that it happened in other planes. We had trained in creation of partial thickness defects using a chisel, and no bleeding was observed for any defect. If the subchondral bone had been broken, the repair tissue in the defects would have been macroscopically detected in the broken area. We did not find such tissue in any sample at any time point.

These sections were examined blindly and scored independently by two of the authors, without knowledge of the group being examined, using a modified semiquantitative scale for evaluating the histological appearance of repair (Table I)[20]. The scale was composed of three categories with a score range from 0 (best) to 9 (worst) points: filling of the defect, matrix staining, and cell morphology. Whenever the scores differed, the scorers discussed them and came to an agreement.

The differences in the scores between the two FGF-treated groups and the control group at various post-operative times were tested using two-way factorial ANOVA, followed by the Mann–Whitney *U* test for comparison. Probability values less than 0.05 were considered significant. Stat View software (SAS Institute Inc., Cary, NC, USA) was used for analysis.

Table I
The semiquantitative scale for grading the healing of articular cartilage repair

(1) Filling of defect	
A continuous surface without depression	0
No or slight depression, but a non-continuous surface	1
Depression of less than 50% of the original defect	2
Depression of more than 50% of the original defect	3
(2) Matrix staining	
The same as that of the adjacent tissue	0
Slightly decreased	1
Moderately decreased	2
No repair tissue	3
(3) Cell morphology	
Chondrocytes of normal appearance and density	0
Chondrocytes that appeared normal but were hypocellular	1
Abnormal cells	2
An absence of cells	3

Results

IMMATURE RABBITS

Histology

In the control group, cartilage repair was poor at 2, 4 and 8 weeks after surgery. Proliferation of chondrocytes on the injured sites was observed slightly, but it was not adequate to provide sufficient cells and matrix to repair the defects. At 12, 24 and 48 weeks, however, cartilage repair was improved sequentially. In some cases, the repair tissue showed some intense metachromatic staining, but defects were filled only to half of the original depth in all cases (Fig. 1).

In the FGF-2 (10 ng)-treated group, regenerative response was poor at 2 weeks after surgery. At 4, 8 and 12 weeks, cartilage repair was superior to that of the control group. However, there was no advance in repair at 24 and 48 weeks. At 48 weeks, the defects were filled with chondrocytes, but the center of the defect was concave and metachromatic staining was less intense compared with the surrounding cartilage (Fig. 2).

In the FGF-2 (100 ng)-treated group, cartilage repair was poor at 2 weeks after surgery, similar to that of the other groups. At 4 weeks, on the surface of the injured cartilage, a few small masses of cells surrounded with intense metachromatic matrix were observed. Then, at 8, 12, 24 and 48 weeks, the repair was improved sequentially. At 48 weeks, the defects were almost completely repaired although the centers of the defects were slightly concave. The repair tissue consisted of clusters of chondrocytes and large amounts of extracellular matrix. The repair chondrocytes showed a round to polygonal morphology. Extracellular matrix showed slightly less intense metachromatic staining than the normal surrounding cartilage (Fig. 3).

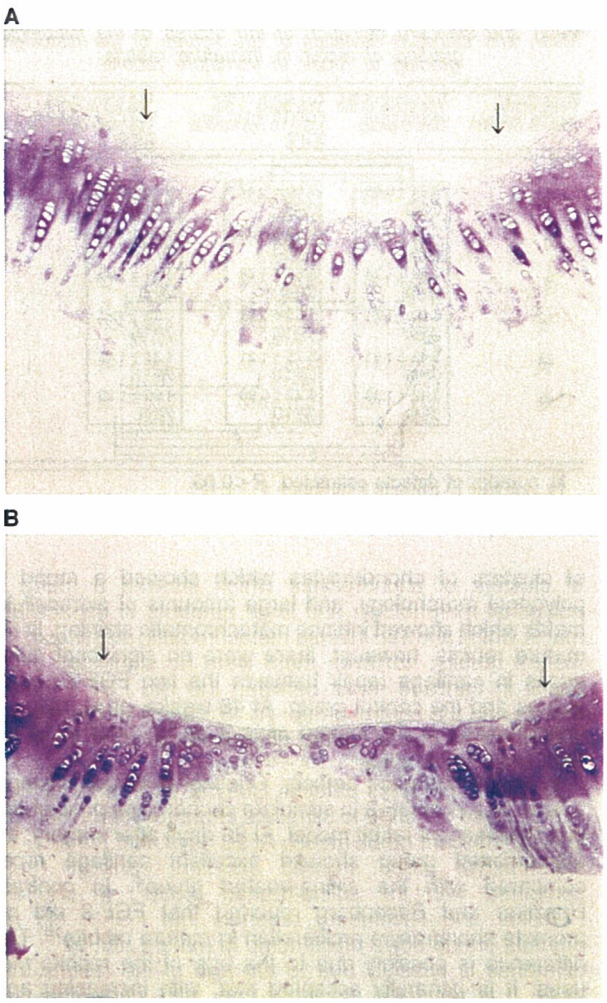

Fig. 1. Microscopic appearance of a perpendicular section of the articular cartilage defect of the control group of the immature rabbits. Toluidine blue staining, original magnification ×40. The arrows indicate the edges of the original defect. (A) The findings at 4 weeks; regenerative response rare. (B) The findings at 48 weeks; the defect is filled with repair tissue, but the center of the defect is concave and metachromatic staining is less intense compared with that of the surrounding cartilage.

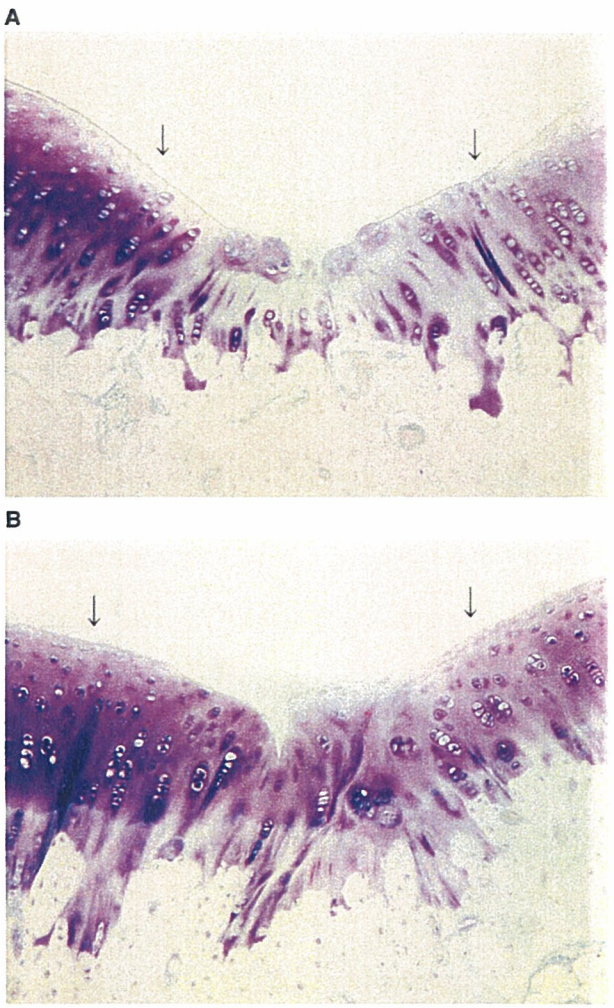

Fig. 2. Microscopic appearance of a perpendicular section of the articular cartilage defect of the FGF-2 (10 ng)-treated group of the immature rabbits. Toluidine blue staining, original magnification ×40. The arrows indicate the edges of the original defect. (A) The findings at 4 weeks; although a few small masses of cells surrounded with intense metachromatic matrix are observed, cartilage repair is poor. (B) The findings at 48 weeks; the defects are filled with repair tissue, but the center of the defect is concave and the articular surface is not continuous. Metachromatic staining is less intense compared with that of surrounding cartilage.

Histological score

The scores of the FGF-2 (100 ng)-treated group were significantly better than those of the control ($P = 0.002$) and FGF-2 (10 ng)-treated groups ($P = 0.009$).

At 2 weeks after surgery, the scores were poor in each group. At 4, 8, 12 weeks, the scores of the two FGF-2-treated groups were improved sequentially. The scores of the FGF-2 (100 ng)-treated group were significantly better than those of the control group at 8 weeks ($P = 0.028$), and better than those of the FGF-2 (10 ng)-treated group at 24 weeks ($P = 0.025$). Finally, the scores of the FGF-2 (100 ng)-treated group were significantly better than those of the control ($P = 0.018$) and the FGF-2 (10 ng)-treated groups ($P = 0.019$) at 48 weeks. However, there was no significant difference between the FGF-2 (10 ng)-treated group and the control group at each postoperative time point (**Table II**).

MATURE RABBITS

Histology

In all groups, cartilage regeneration at the injured sites was observed slightly, but regenerative response was too poor to repair defects at each postoperative time point. Some cases in the two FGF-2-treated groups showed a decrease in metachromatic staining in uninjured chondrocytes. Neither cartilage thickening nor osteophyte formation was seen (**Fig. 4**).

Histological score

Each group showed a smaller improvement of repair sequentially compared to that of the immature rabbits.

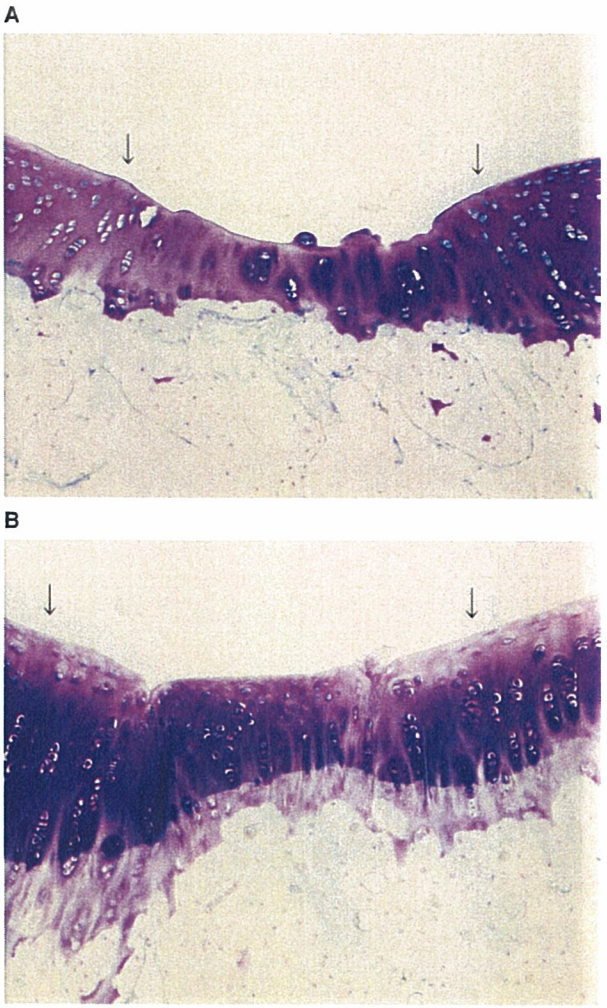

Fig. 3. Microscopic appearance of a perpendicular section of the articular cartilage defect of the FGF-2 (100 ng)-treated group of the immature rabbits. Toluidine blue staining, original magnification ×40. The arrows indicate the edges of the original defect. (A) The findings at 4 weeks; although a few small masses of cells surrounded with intense metachromatic matrix are observed, cartilage repair is poor. (B) The findings at 48 weeks; although the center of the defect is slightly concave, the defect is almost completely repaired. The repair tissue consists of clusters of chondrocytes and large amounts of extracellular matrix. The repair chondrocytes show a round to polygonal morphology. The extracellular matrix shows a slightly less intense metachromatic staining than that of the normal surrounding cartilage.

However, there were no significant differences in the scores between the two FGF-2-treated groups and the control group at each postoperative time point (Table III).

Discussion

The present study showed that the application of FGF-2 facilitated cartilage repair in partial thickness defects in immature rabbits, but not in mature rabbits. In the FGF-2 (100 ng)-treated group of immature rabbits, cartilage repair improved sequentially, and was significantly better than that of the other groups at 48 weeks. The repair tissue consisted

Table II
Mean and standard deviation of the scores of the histological grading of repair in immature rabbits

Potoperative periods (weeks)	The score of the control group	The score of the FGF(10 ng)-treated group	The score of the FGF(100 ng)-treated group
2	6.94 + 0.98 (*N*=9)	7.00 ⊥ 0.47 (*N*=8)	6.90 ± 0.96 (*N*=10)
4	7.18 ± 1.22 (*N*=8)	6.50 ± 1.225 (*N*=10)	6.00 + 1.22 (*N*=11)
8	7.40 ± 1.24 (*N*=8)	5.56 + 1.74 (*N*=8)	4.91 ± 1.93 (*N*=6)
12	5.60 ± 1.71 (*N*=5)	5.35 ± 0.88 (*N*=10)	4.68 + 1.27 (*N*=10)
24	5.42 ± 1.51 (*N*=8)	5.75 + 1.21 (*N*=12)	4.27 ± 1.42 (*N*=11)
48	5.42 ⁞ 1.09 (*N*=8)	5.27 ± 0.90 (*N*=11)	4.00 ± 1.28 (*N*=8)

N, number of defects estimated, $P<0.05$.

of clusters of chondrocytes which showed a round to polygonal morphology, and large amounts of extracellular matrix which showed intense metachromatic staining. In the mature rabbits, however, there were no significant differences in cartilage repair between the two FGF-2-treated groups and the control group. At 48 weeks, proliferation of chondrocytes at the injured sites was too poor to fill the defects in any groups.

In partial thickness defects, Cuevas *et al.* administered a large dose of FGF-2 to stimulate chondrocyte proliferation in an adolescent rabbit model. At 20 days after surgery, the experimental group showed excellent cartilage repair compared with the saline-treated group[4]. In contrast, Hunziker and Rosenberg reported that FGF-2 did not promote chondrocyte proliferation in mature rabbits[19]. This difference is possibly due to the age of the rabbits they used. It is generally accepted that, with increasing age, chondrocytes synthesize smaller, less uniform aggrecan molecules and less functional link protein, their mitotic and synthetic activity declines, and their response to anabolic mechanical stimuli and growth factors decreases. This was supported by Martin and Buckwalter, who measured cell senescence markers in human articular cartilage[21]. In addition, they also reported age-related decline in chondrocyte response to IGF-I *in vitro*[22]. Concerning FGF-2, Guerne *et al.* showed age-related decline in chondrocyte response to FGF-2 *in vitro*[18]. However, cartilage response to FGF-2 *in vivo* with increasing age is unclear. We demonstrated that, in immature rabbits, FGF-2 produced significantly better repair than that produced by the same treatment in mature rabbits. This result suggested that there was a difference in the response of cartilage to FGF-2 with age *in vivo*. Although the mechanism is unknown at present, we hypothesize that expression of FGF receptors might be stronger in immature rabbits or that the intense matrix around cells in mature rabbits might disturb the proliferation of cells.

In full thickness defects, mesenchymal cells, which are considered to be progenitor cells for chondrocytes in bone marrow, have an important role in cartilage repair[1,20]. It has been reported that FGF-2 stimulated cartilage repair in full thickness defects of articular cartilage in mature rabbits[5,6]. These results suggest that FGF-2 could influence not only the chondrocytes themselves, but also the mesenchymal cells, in full thickness defects of mature rabbits. However,

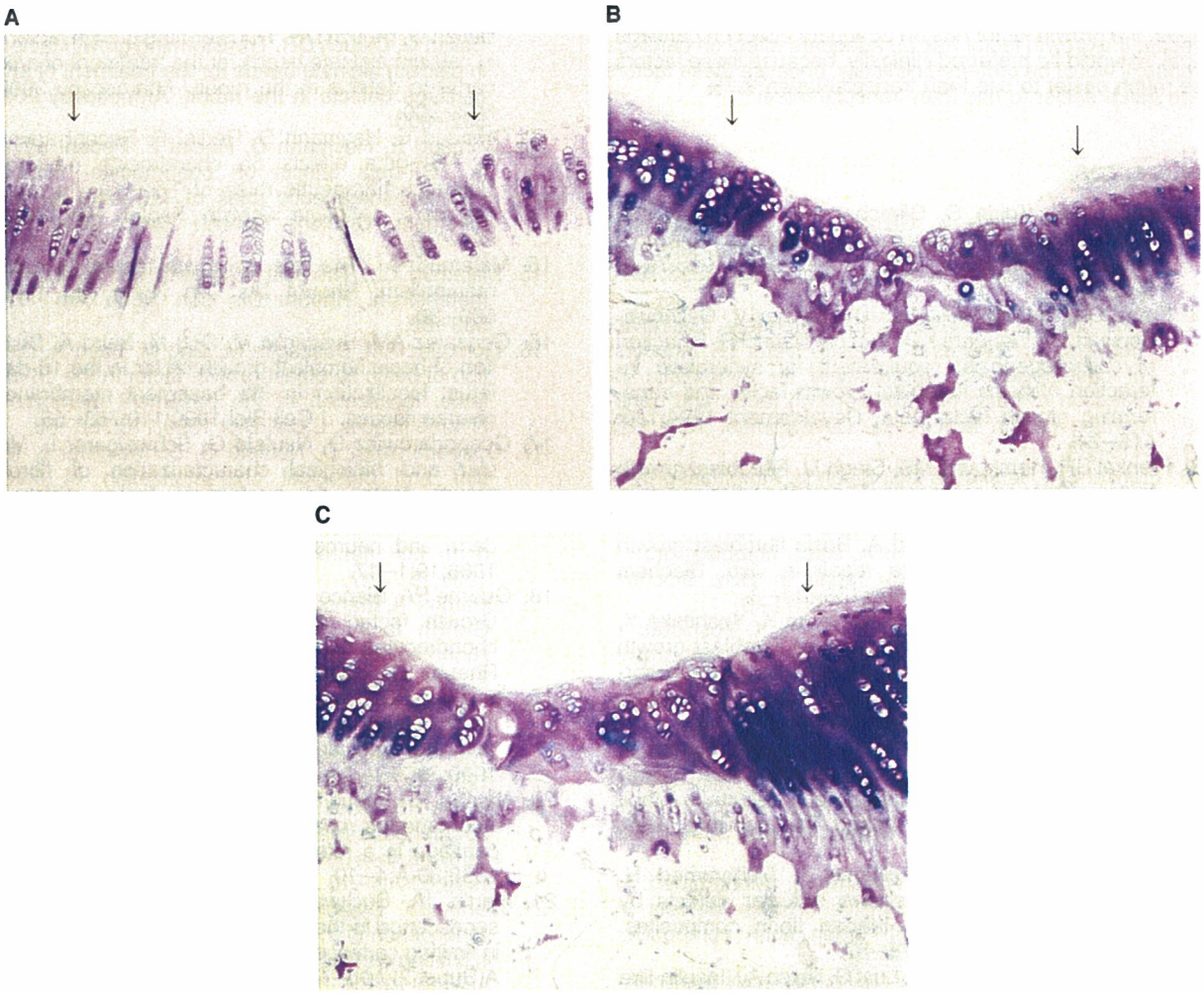

Fig. 4. Microscopic appearance of a perpendicular section of the articular cartilage defect at 48 weeks in mature rabbits. Toluidine blue staining ×40. The arrows indicate the edges of the original defect. (A) The control group, (B) the FGF-2 (10 ng)-treated group, and (C) the FGF-2 (100 ng)-treated group: proliferation of chondrocytes at the injured sites is observed, but the regenerative response is inadequate to provide sufficient cells and matrix to fill the defects.

as shown in this study, FGF-2 is capable of stimulating proliferation of chondrocytes, although this was confined to chondrocytes of immature rabbits.

Shida *et al.* reported that a single injection of FGF-2 into a rat joint stimulates articular cartilage and the subsequent osteophyte formation. They found that chondrocytes in the normal articular cartilage responded, but immature cells, such as periosteal cells at the edge of the femoral condyle, responded more and differentiated cartilage. Then, the thickened cartilage became bone[23]. Although we found no cartilage thickening, synovial proliferation or osteophyte formation, some cases in the FGF-2-treated groups showed a decrease in metachromatic staining in uninjured chondrocytes (data not shown). The acceptable dose should be identified in humans to prevent causing the degenerative change of cartilage.

Table III
Mean and standard deviation of the scores of the histological grading of repair in mature rabbits

Postoperative periods (weeks)	The score of the control group	The score of the FGF(10 ng)-treated group	The score of the FGF(100 ng)-treated group
2	8.36±0.50 ($N = 11$)	8.33±0.65 ($N = 12$)	7.81±0.98 ($N = 11$)
4	7.50±1.31 ($N = 12$)	8.20±0.78 ($N = 10$)	7.90±1.37 ($N = 11$)
8	7.44±1.33 ($N = 9$)	7.33±1.11 ($N = 9$)	7.45±0.82 ($N = 11$)
12	7.75±1.05 ($N = 12$)	7.25±1.42 ($N = 12$)	7.09±1.86 ($N = 11$)
24	7.00±2.17 ($N = 9$)	7.08±1.16 ($N = 12$)	7.25±1.21 ($N = 12$)
48	7.00±1.41 ($N = 6$)	6.66±1.43 ($N = 12$)	6.87±1.24 ($N = 8$)

N, number of defects estimated.

Many growth factors affect chondrogenesis and cartilage repair. If a growth factor has an adequate effect on cartilage repair, it would be preferred clinically, because these factors are much easier to use than transplantation[24–26].

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Repair of articular cartilage defects in rabbits using CDMP1 gene-transfected autologous mesenchymal cells derived from bone marrow

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Objective. **Cartilage-derived morphogenetic protein 1 (CDMP1), which is a member of the transforming growth factor-β superfamily, is an essential molecule for the aggregation of mesenchymal cells and acceleration of chondrocyte differentiation. In this study, we investigated whether CDMP1-transfected autologous bone marrow-derived mesenchymal cells (BMMCs) enhance *in vivo* cartilage repair in a rabbit model.**

Methods. **BMMCs, which had a fibroblastic morphology and pluripotency for differentiation, were isolated from bone marrow of the tibia of rabbits, grown in monolayer culture, and transfected with the CDMP1 gene or a control gene (GFP) by the lipofection method. The autologous cells were then implanted into full-thickness articular cartilage defects in the knee joints of each rabbit.**

Results. **During *in vivo* repair of full-thickness articular cartilage defects, cartilage regeneration was enhanced by the implantation of CDMP1-transfected autologous BMMCs. The defects were filled by hyaline cartilage and the deeper zone showed remodelling to subchondral bone over time. The repair and reconstitution of zones of hyaline articular cartilage was superior to simple BMMC implantation. The histological score of the CDMP1-transfected BMMC group was significantly better than those of the control BMMC group and the empty control group.**

Conclusion. **Modulation of BMMCs by factors such as CDMP1 allows enhanced repair and remodelling compatible with hyaline articular cartilage.**

KEY WORDS: Cartilage repair, Mesenchymal cell, Chondrogenic differentiation, CDMP1.

Articular cartilage is a highly differentiated avascular tissue with abundant extracellular matrix. Once damaged by various causes, such as trauma, osteoarthritis, articular cartilage often shows progressive deterioration without healing [1–3]. A number of methods have been developed to treat such damaged articular cartilage. These attempts can be categorized principally into restoration, replacement, relief, and resection of cartilage [4, 5; for review see 6]. Among them, biological resurfacing of cartilage is one of the methods that could restore joint function. In addition to tissue-based methods, such as osteochondral grafts [7, 8], development of cell therapy has aroused considerable interest. Human and experimental studies on the transplantation of cultured cells into areas of damage have shown promise in the repair of cartilage defects [9–13].

We and others have investigated the use of mesenchymal cells derived from bone marrow as a biological method for the repair of articular cartilage defects [14–17]. It is already established that bone marrow-derived mesenchymal cells (BMMCs) contain pluripotent cells that are capable of differentiating into various types of cells, including chondrocytes, osteoblasts and adipocytes [18–24]. Since BMMCs are easily isolated from the bone marrow and can be rapidly amplified, they are likely to be the most suitable cell type for the repair [17]. However, there are still arguments about the efficiency of chondrogenic differentiation, reconstitution of hyaline articular cartilage zone, the integration of the regenerated and surrounding tissues, and the long-term integrity of the repaired tissues. Although, culture-expanded and implanted BMMCs form cartilaginous tissue *in vivo*, the regeneration is sometimes limited to certain portion of the defect and the repair does not always result in reconstitution of the sustainable zones of articular cartilage [14]. Clearly, there is a need to further develop methods for the reliable repair of damaged cartilage using BMMCs.

Cartilage-derived morphogenetic protein 1 (CDMP1) is a member of the transforming growth factor β (TGF-β) superfamily and has been shown to be involved in chondrogenesis [25–29]. CDMP1 has been shown to promote aggregation of mesenchymal cells and enhance chondrocyte differentiation [30, 31]. These roles of CDMP1 during chondrogenesis from undifferentiated mesenchymal cells led us to hypothesize that the modulation of BMMCs with biologically active factor(s), such as CDMP1, could assist in the maintenance of cell viability and chondrogenic differentiation *in vivo*, and improve the repair of damaged cartilage. In the present study, we transfected autologous BMMCs with CDMP1, implanted them into full-thickness articular cartilage defects in rabbits.

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Materials and methods

Isolation and expansion of autologous BMMCs

Forty-six mature New Zealand White rabbits weighing 3.5 to ~4 kg were used. The rabbits were anaesthetized by intramuscular injection of ketamine hydrochloride (60–70 mg/kg) and xylazine (6 mg/kg). The BMMCs were obtained from the tibia as described previously [14]. Briefly, the aspirate from the tibia was washed, centrifuged and resuspended in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS) and antibiotics (100 U/ml of penicillin G and 100 μg/ml of streptomycin). Then the cells from the bone marrow were cultured in 100-mm plastic dishes containing the same medium at 37°C under 5% CO_2/95% air. One day after seeding, each culture dish was washed three times by mild agitation with new medium to remove non-adherent cells. When the adherent cells reached subconfluence, they were freed from the dish with 0.05% trypsin/0.02% EDTA and subcultured (passage 2). The cells were further subcultured at subconfluency (passage 3).

CDMP1 gene transfer into BMMCs

CDMP1 cDNA insert from the p742CDMP1Int vector [31] was used under the control of CMV-IE promoter (Clontech, Palo Alto, CA, USA). A green fluorescent protein (GFP) expression vector, pEGFP-C1 (Clontech), was used as the control vector. The passage-3 BMMCs from each rabbit were transfected with the CDMP1 or the control GFP gene by the lipofection method using FuGENE^{TM}6 (Roche, Indianapolis, IN, USA). Approximately 1×10^6 cells in a 100-mm culture dish were washed twice with Hanks' solution and covered with 6 ml of serum-free DMEM. Then the DNA-FuGENE^{TM}6 mixture (3 μg of the DNA mixed with 9 μl of FuGENE^{TM}6) was added to each dish, and the cells were incubated at 37°C for 6 h. Next, the medium was removed and replaced with a defined medium [22], consisting of DMEM with ITS+Premix; insulin 6.25 μg/ml, transferrin 6.25 μg/ml, selenous acid 6.25 μg/ml, linoleic acid 5.33 μg/ml, bovine serum albumin 1.25 mg/ml, pyruvate 1 mM, ascorbate 2-phosphate 0.17 mM, proline 0.35 mM, dexamethasone 0.1 μM, and recombinant human TGF-β3 10 ng/ml (No. 531-82501; Wako, Osaka, Japan). To confirm cell viability after gene transfer, the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide] assay was performed during culture as described previously [32].

Expression of CDMP1 and matrix genes in BMMCs

Total RNA from the transfected BMMCs after a 5-day culture was prepared using the modified acid guanidine–phenol–chloroform method [33]. Five micrograms of the RNA was converted to cDNA using the Super Script^{TM} First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed using an ABI prism 7000 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. The primers were as follows: CDMP1 forward primer, 5′-TCCAGACCCTGATGAACTCC-3′, CDMP1 reverse primer, 5′-TCCACGACCATGTCCTCATA-3′, CDMP1 TaqMan probe, 5′-CATTGACTCTGCCAACAACGTGGTGTATAA-3′; HPRT forward primer, 5′-GACCTTGCTTTCCTTGGTCA-3′, HPRT reverse primer, 5′-TCCAACAAAGTCTGGCCTGT-3′, HPRT TaqMan probe, 5′-CAGTATAATCCAAAGATGGTCAAGGT CGCA-3′. PCR was performed at 50° for 2 min, 95° for 10 min, and 50 cycles of 95° for 30 s and 60° for 1 min. Standardization was performed using RNA extracted from rabbit chondrocytes and quantitation was normalized to an endogenous control (HPRT). RT-PCR for matrix genes was performed with initial denaturation at 94° for 5 min, 30 cycles of 94° for 1 min, 57° for 1 min, 72° for 2 min, and final extension at 72° for 10 min. The primers were as follows: rabbit type II collagen (Col2a1) forward primer, 5′-CAACAACCAGATCGAGAGCA-3′, reverse primer, 5′-CCAGTAGTCACCGCTCTTCC-3′; rabbit aggrecan forward primer, 5′-TCTCCAAGGACAAGGAGGTG-3′, reverse primer, 5′-AGGCTCTGGATCTCCAAGGT-3′; rabbit type I collagen (Col1a2) forward primer, 5′-CAATCACGCCTCTCAGAACA-3′, reverse primer, 5′-TCGGCAACAAGTTCAACATC-3′.

Implantation of CDMP1-transfected autologous BMMCs for in vivo cartilage repair into full-thickness articular cartilage defect

Three days after CDMP1 and GFP gene transfer, BMMCs were freed from the culture dishes with trypsin/EDTA. Then 1×10^6 autologous cells were embedded in 200 μl of type-I collagen gel (at a final concentration of 0.15%; Nitta Gelatin, Osaka, Japan) and implanted into a large full-thickness articular cartilage defect. The defect (4 mm in diameter and 4 mm in depth) was created through the articular cartilage and into the subchondral bone of the patellar groove in 46 rabbits using an electric drill equipped with a 4-mm diameter drill bit. In 30 rabbits, the defects were implanted with individual autologous BMMCs; the defect in the right knee was filled with CDMP1-transfected BMMCs and the defect in the left knee was filled with control GFP-transfected BMMCs. In the remaining 16 rabbits, defects made in the right knees were not filled, as an empty control. The incision was closed using 4–0 Vicryl and all rabbits were allowed to move freely after surgery.

Histological examination of repair tissue

The animals were killed 2, 4 or 8 weeks after the operation. The distal part of each femur was removed, fixed in 4% paraformaldehyde, decalcified in 10% EDTA and embedded in paraffin. Then sections were cut through the centre of each defect, stained with safranin O/Fast Green, examined in a blinded manner by two evaluators, and were graded with use of a histological scale (see supplementary data at *Rheumatology* Online), which was a modification of those described by Wakitani *et al.* [14] and Pineda *et al.* [34]. The scale is composed of two categories. The first category evaluates surface layers (hyaline articular cartilage zone) repair and contains three parameters: cell morphology and matrix staining graded from 0 to 8 points, surface regularity graded from 0 to 3, integration of donor with host adjacent cartilage graded from 0 to 2. The second category evaluates filling and remodelling of the defect of the deeper zone, and contains two parameters: filling of defect graded from 0 to 4, reconstitution of subchondral bone and osseous connection graded from 0 to 3. Differences of the histological scores between three groups were analysed with the Kruskal–Wallis test, followed by the Scheffe method for multiple comparisons. Differences of the scores between two groups were analysed by the Mann–Whitney U test. A P value <0.05 was considered significant.

Immunohistochemistry

To investigate expression of the transgene *in vitro*, immunohistochemical staining for CDMP1 was performed using a goat polyclonal antibody specific for CDMP1 (N-17; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and standard procedures. Immunohistochemical analysis of the repaired tissue *in vivo* was also performed using antibodies specific for types I or II collagen (F-56, F57, Fuji Chemical, Takaoka, Japan). Immunoreactivity was detected using a biotinylated horse anti-mouse antibody and avidin–biotin reaction (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA).

Results

Rabbit BMMCs and CDMP1 gene transfer

Quantitative PCR analysis indicated that CDMP1-transfected BMMCs started expression of CDMP1 by day 5 (Fig. 1A). By immunostaining, approximately 20% of the cells reproducibly expressed the transgene (Fig. 1B) and the expression was maintained for at least 3 weeks in monolayer culture (not shown). CDMP1-transfected BMMCs showed enhanced expression of aggrecan and Col2a1 with decreased expression of Col1a2 during culture (Fig. 1C).

To analyse the possible adverse effect of CDMP1 gene transfer on BMMCs, the MTT assay was performed (see supplementary data at *Rheumatology* Online). There was an initial decrease in cell growth activity during culture in the defined medium. This was in accordance with the report of Sekiya *et al.* [35] which indicated loss of a portion of marrow stromal cell population during culture in defined medium, apparently through apoptosis. However, by comparing with control GFP gene transfer, CDMP1 alleviated the initial decline of cell growth and helped to maintain a higher level of activity thereafter.

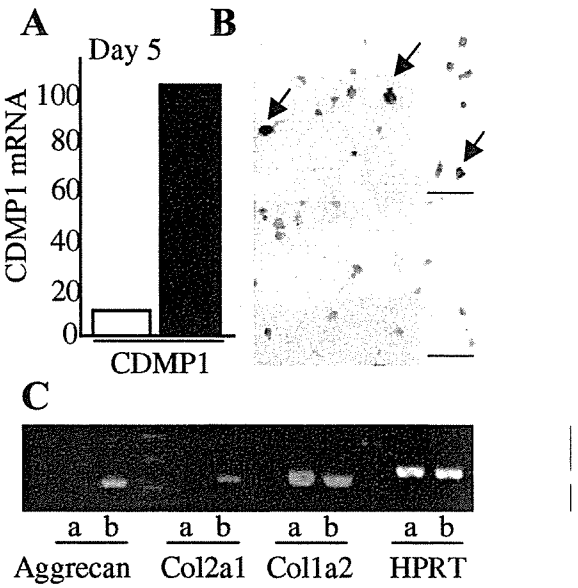

FIG. 1. Expression of CDMP1 and matrix genes. (A) CDMP1 expression in BMMCs was detected by real-time PCR analysis. There was a marked increase in the mRNA level for CDMP1 during CDMP1-transfected BMMC culture for 5 days. Results are the mean of three independent experiments. The value of each CDMP1 mRNA was normalized to the amount of HPRT mRNA. The standardized value of CDMP1-transfected BMMCs was arbitrarily set to 100. White bar indicates control BMMCs; black bar indicates CDMP1-transfected BMMCs. (B) Immunohistochemical staining for CDMP1. Arrows indicate CDMP1 expressing BMMCs in culture after transfection (upper panel). There were no CDMP1-positive cells in control BMMCs (lower panel). Scale bar is 100 μm. (C) RT-PCR analysis of matrix genes. The expression of aggrecan and Col2a1 was more prominent in the CDMP1-tranfected BMMCs after 5 days in culture. a, control BMMCs. b, CDMP1-transfected BMMCs. HPRT was used as internal control.

Repair of cartilage defects with autologous BMMCs

In the empty control group 2 weeks after the operation, the defect was incompletely filled and contained newly formed fibrous tissue as expected. On the other hand, the defects implanted with BMMCs were filled with repair tissue that contained hyaline cartilage-like elements. This hyaline repair was more obvious in the CDMP1-transfected BMMC group. Figure 2 shows the representative histological appearance of the defects at 4 weeks. In the empty control group, the defects were almost filled with fibrous tissue and cancellous bone at this stage. Although there was spotted safranin O staining in the deeper zone of the defects, cells in the surface zone of each defect were entirely non-chondrogenic (Fig. 2A–C). In the control BMMC-implanted rabbits (Fig. 2D–F), the defects were filled with repair tissue that contained hyaline cartilage. In most of the rabbits, the base of the defect was replaced by new bone. Although some knees showed repair by differentiated cartilage, safranin O staining tended to be more distinct in the deep zone of the regenerated tissue. The surface zone often showed a fibrous structure or had only moderate safranin O staining. Figure 2G–I shows autologous CDMP1-transfected BMMC-implanted right knees of the same animals shown in Fig. 2D–F respectively. In the CDMP1-transfected BMMC group, the defects were mostly filled with hyaline cartilage at 4 weeks. It was noteworthy that hyaline cartilage was formed up to the level of original articular surface and safranin O staining was intense throughout most of the regenerated articular surface zone.

Immunohistochemical staining indicated that regenerated cartilage after the implantation of CDMP1-transfected BMMCs showed intense staining for type II collagen (Fig. 2K), again supporting the differentiated hyaline cartilage nature of the repair tissue. Staining for type I collagen was mostly limited to the reconstituted subchondral bone (Fig. 2L).

Eight weeks after the autologous CDMP1-transfected BMMC implantation, the appearance of the repaired cartilage was comparable to differentiated hyaline cartilage, and the subchondral tissue was completely replaced by new bone of a thickness close to that of the host subchondral bone (see supplementary data at *Rheumatology* Online).

Histological score of the repair tissue

In comparison with the empty control group, the scores of the control autologous BMMC implantation were better (i.e. lower) at 2, 4 and 8 weeks (Table 1). However, not all joints behaved uniformly and the scores tended to become worse at 8 weeks, which was compatible with our previous observation after BMMC implantation [14]. On the other hand, the scores of the CDMP1-transfected autologous BMMC implantations were significantly better than those for the empty control. The scores were maintained at 8 weeks and were significantly better than those for control BMMC implantation and the empty control. The comparison of two categories, surface zone repair (A–C in Table 1) and deeper zone filling/remodelling (D–E in Table 1), indicates that CDMP1-transfected autologous BMMC implantation results in significantly better repair, especially in the surface layer (hyaline cartilage zone).

Discussion

The current investigation demonstrated that full-thickness articular cartilage defects were repaired with hyaline cartilage after implantation of autologous CDMP1-transfected BMMCs. The repair was superior to previously reported simple BMMC implantation, seemingly because of better surface zone repair and reconstitution.

Transplantation of cultured allogeneic or autologous chondrocytes into areas of cartilage damage has been shown to faithfully produce hyaline cartilage [10–12]. However, there remain questions about the fate of the transplanted cells, limits on the number of available cells and poor integration of the newly formed

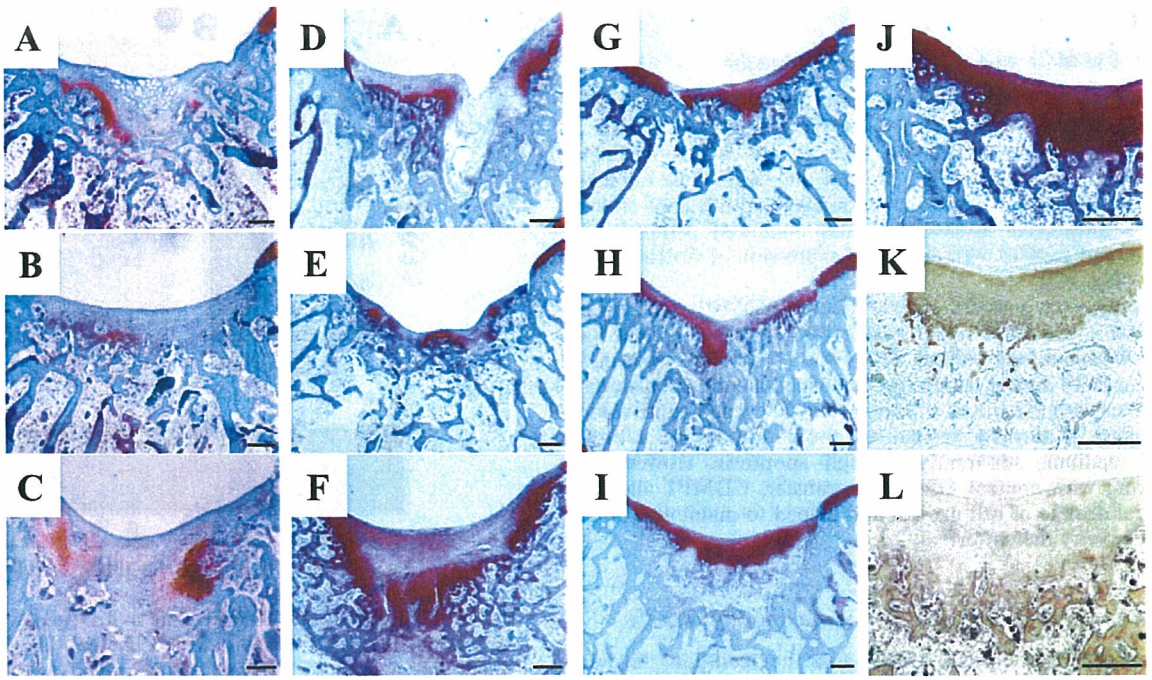

FIG. 2. Representative histological appearance of the defects after 4 weeks. (A, B, C) Empty control group. (D, E, F) Left knees of GFP-transfected BMMC group. (G, H, I) Right knees of CDMP1-transfected BMMC group. D and G, E and H, F and I show bilateral knee specimens from the same rabbit, respectively. J is a higher magnification of I. Safranin O/Fast Green staining. (K, L) Immunohistochemical staining specific for type II collagen and type I collagen, respectively. Scale bar is 500 μm.

TABLE 1. Results of histological grading scale[1]

		Grade (points)							
		A	B	C		D	E		
Interval until animals were killed (weeks)	No.	Cell morphology and matrix staining	Surface regularity	Integration	Subtotal (A C)	Filling of defect	Reconstitution of subchondral and osseous connection	Subtotal (D E)	Total
CDMP1- transfected BMMCs									
2	10	6.2	0.7‡	1.2	8.1‡	0.8	2.6‡	3.4‡	11.5‡
4	10	4.4	0.1‡	0.7	5.2‡	0.7	1.5	2.2	7.4
8	10	4.6	0.3‡	1.0	6.0†,‡	0.7	1.1	1.8‡	7.8†,‡
GFP-transfected BMMCs									
2	10	7.0	1.2	1.4	9.6	1.2	2.6	3.8	13.4
4	10	6.2	0.9	0.9	8.0	1.1	1.6	1.7	10.7
8	10	6.8	1.5	1.0	9.3	1.5	1.9	3.4	12.7
Empty control									
2	2	8.0	3.0	2.0	13.0	2.5	3.0	5.5	18.5
4	7	6.6	1.1	1.6	9.3	1.6	1.0	3.4	12.7
8	7	7.4	1.6	1.0	10.0	1.6	2.0	3.6	13.6

[1]The scale has two categories assigning a total score ranging from 0 (best) to 20 (worst). A C evaluate surface layers and D and E evaluating filling and remodelling of the defect. A is graded from 0 to 8, B from 0 to 3, C from 0 to 2, D from 0 to 4, and E from 0 to 3. (See supplementary data at *Rheumatology* Online).

†$P < 0.05$ compared with the GFP group at corresponding time (Mann Whitney U test).

‡$P < 0.05$ compared with the empty control at the corresponding time (Scheffe test for multiple comparisons).

cartilage plug with host cartilage, and doubts about the ability of dedifferentiated cells to form hyaline cartilage. To overcome these potential drawbacks of chondrocyte-based cell therapy, we attempted to employ BMMCs for cartilage repair [14, 17]. In these experiments, however, we also noticed that the repair of articular cartilage after BMMC implantation was not yet satisfactory. Although the regeneration of cartilage after BMMC implantation was impressive, the articular surface was not always repaired by a layer of hyaline cartilage in the case of larger defects. Such insufficient hyaline repair often fails to reconstitute well-remodelled cartilage surface zone and tends to become deteriorated with time [14]. The problem of insufficient hyaline repair by BMMCs can be explained in two ways. First, the number of BMMCs used to repair the cartilage defect may be too low relative to the defect size. This is partly supported by the fact that small defects show spontaneous repair by regenerating cartilage through the migration of relatively sufficient mesenchymal progenitor cells from the bone marrow [36, 37].

Secondly, not all of the BMMCs may differentiate into chondrocytes within the cartilage defect after implantation. For

in vitro chondrogenesis from mesenchymal stem cells, TGF-β and dexamethasone are reported to be essential [20–22], and addition of other factors, such as bone morphogenetic proteins (BMCs), could improve differentiation. During *in vivo* repair after BMMC or mesenchymal stem cell implantation, these bioactive factors may be supplied at the site of the chondro-osseous defect from the host tissues and initiate cells into the chondrogenic lineage. However, the availability of such bioactive factor(s) may not be always sufficient to achieve chondrogenesis. In order to overcome these obstacles to BMMC-based repair, it seems likely that engineered BMMCs expressing soluble factor(s), such as BMP2, recently reported by Gelse *et al.* [38], should be useful. Use of cells that have already been engineered to enter chondrogenic lineage may also have therapeutic potential.

The CDMP1 (GDF5) gene, which we used in the present study, has been shown to be involved in commitment of mesenchymal cells to the chondrogenic lineage and acceleration of chondrocyte differentiation [25–31]. Taking advantage of such an *in vivo* role of CDMP1 during chondrogenesis from mesenchymal cells, we used engineered CDMP1-transfected BMMCs for cartilage repair in the present study. Although the repair was not perfect, implantation of CDMP1-transfected BMMCs resulted in better surface zone repair as well as deeper zone remodelling. There is no doubt that reconstitution of hyaline articular cartilage zone and its superficial layers is a prerequisite for the prolonged integrity of the repaired tissue. Why, then, did the CDMP1 transfection result in better surface zone repair with hyaline cartilage? It is possible that CDMP1-transfection helped to maintain cell growth activity, as indicated in the *in vitro* study (see supplementary data at *Rheumatology* Online). Knowledge from previous studies [30, 31] and the present *in vitro* study also suggests that CDMP1 helped the implanted BMMCs to enter chondrogenic lineage in the defect.

If cells with differentiated chondrogenic phenotype are desired for transplantation, use of further differentiated BMMCs or chondrocytes could be suitable. Such cells should enable immediate synthesis and formation of hyaline cartilage matrix in the defect. In our experience, transplantation of already differentiated cells or chondrocytes forms a good cartilage plug in the defect, but often fails to show the necessary remodelling and integration in the surface zone and is unable to reconstitute a good subchondral structure [10]. We speculate that use of BMMCs committed to the chondrogenic lineage, rather than already well-differentiated chondrocytes, should promote better remodelling and integration of the regenerated cartilage.

The use of engineered autologous BMMCs in future *in vivo* studies may enable us to regenerate extensive defects of articular tissues. However, therapeutic application in humans may pose several problems. The use of transient transfection by lipofection, as in the present study, should help to avoid possible toxicity, the provocation of an inflammatory response and technical complexity, although transfection efficiency is relatively low. Transfection of cells to express bioactive proteins, as well as other factors that are important for differentiation, cell viability or matrix synthesis, may eventually provide the basis for effective BMMC-based repair of damaged articular cartilage.

Rheumatology	Key messages
	• Modulation of bone marrow-derived mesenchymal cells by factors such as CDMP1 could enhance the repair and remodelling of damaged articular cartilage.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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Use of Bone Morphogenetic Protein 2 and Diffusion Chambers to Engineer Cartilage Tissue for the Repair of Defects in Articular Cartilage

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Objective. **To examine the ability of cartilage-like tissue, generated ectopically in a diffusion chamber using recombinant human bone morphogenetic protein 2 (rHuBMP-2), to repair cartilage defects in rats.**

Methods. **Muscle-derived mesenchymal cells were prepared by dissecting thigh muscles of 19-day postcoital rat embryos. Cells were propagated in vitro in monolayer culture for 10 days and packed within diffusion chambers (10^6/chamber) together with type I collagen (CI) and 0, 1, or 10 μg rHuBMP-2, and implanted into abdominal subfascial pockets of adult rats. Tissue pellets were harvested from the diffusion chambers at 2 days to 6 weeks after implantation, and examined by histology, by reverse transcription–polymerase chain reaction (PCR) for aggrecan, CII, CIX, CX, and CXI, MyoD1, and core binding factor a1/runt-related gene 2, and by real-time PCR for CII. Tissue pellets generated in the chamber 5 weeks after implantation were transplanted into a full-thickness cartilage defect made in the patellar groove of the same strain of adult rat.**

Results. **In the presence of 10 μg rHuBMP-2, muscle-derived mesenchymal cells expressed CII messenger RNA at 4 days after transplantation, and a mature cartilage mass was formed 5 weeks after transplantation in the diffusion chamber. Cartilage was not formed in the presence of 1 μg rHuBMP-2 or in the absence of rHuBMP-2. Defects receiving cartilage engineered with 10 μg rHuBMP-2 were repaired and restored to normal morphologic condition within 6 months after transplantation.**

Conclusion. **This method of tissue engineering for repair of articular defects may preclude the need to harvest cartilage tissue prior to mosaic arthroplasty or autologous chondrocyte implantation. Further studies in large animals will be necessary to validate this technique for application in clinical practice.**

Regeneration of articular cartilage is a challenging subject for research on joint surgery (1), and several methods have been devised and attempted in clinical practice to repair focal defects in articular cartilage, especially in young patients (2–5). Currently, mosaic arthroplasty (6), a procedure in which pieces of autogeneic chondro-osseous mass are procured from peripheral parts of the joint surface and transplanted into the focal cartilage defects, is often used with success in the knee joint (7). However, a number of limitations persist, and these include the limited source of the autogeneic osteochondral tissue mass and the potential risk of progression to osteoarthritis due to the injury caused by procurement of graft tissue from the normal joint surface. In addition, the functional durability of the repaired cartilage and the limited application of the approach to small joints are further areas of concern.

Recently, technologies have been developed in order to fabricate tissues for the repair of skeletal defects. The transplantation of chondrocytes of auto- or allogeneic origin has been demonstrated in both experimental (8–11) and clinical (12) situations. In these

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cases, cells are dissociated from pieces of articular cartilage, propagated (or left unpropagated) on dishes in ex vivo conditions to expand the cell population, and then transplanted with or without scaffolding carrier materials into the cartilage defect of the recipient. Although these methods can repair cartilage defects, some difficulties persist. Allogeneic transplantation has the inherent risks of disease transmission and rejection; autologous transplantation causes damage to the donor site.

In an effort to address the limitations of existing approaches, we attempted to generate cartilage tissue by inducing the differentiation of muscle-derived cells into the chondrocytic lineage in an in vivo environment with recombinant human bone morphogenetic protein 2 (rHuBMP-2). Articular defects in rat joints that received the induced cartilage-like tissue were repaired and restored to normal condition. The present report provides evidence to support this approach for the successful treatment of articular cartilage defects.

MATERIALS AND METHODS

Preparation of muscle-derived mesenchymal cells and diffusion chambers. Mesenchymal cells were obtained from the thigh muscles of 19-day, postcoital, F344 rat embryos (purchased from Japan SLC, Hamamatsu, Japan). The muscle tissues were minced with scissors and digested in 0.25% trypsin with 1 m*M* EDTA-Na_4 (Invitrogen, Carlsbad, CA). The dissociated cells were propagated on plastic culture dishes (10 cm in diameter) in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (volume/volume) fetal calf serum (Invitrogen) and antibiotics (mixture of 5 mg/ml penicillin G, 5 mg/ml streptomycin, 10 mg/ml neomycin; Invitrogen) and passaged under routine culture conditions for 10 days. At the end of this period, the cells were detached from the dishes with 0.25% trypsin with 1 m*M* EDTA-Na_4 and packed within diffusion chambers (10^6 cells/chamber).

In order to construct a diffusion chamber for cell transplantation, a diffusion chamber kit (Millipore, Billerica, MA), consisting of a plastic ring (14 mm in outer diameter and 10 mm in inner diameter), a membrane filter (comprising a mixture of cellulose acetate and cellulose nitrate [0.45 μm in pore size]), and adhesive sealant, was utilized. The inner diameter of the ring was reduced to 5 mm by inserting another plastic ring. Only one side of the larger plastic ring was initially sealed with a membrane filter and adhesive sealant. For the next step, 40 μl of 0.3% (weight/weight) pig type I collagen (Cellmatrix LA; Nitta Gelatin, Osaka, Japan) and 0, 1, or 10 μg of rHuBMP-2 (Yamanouchi Pharmaceutical, Tokyo, Japan) were introduced into the diffusion chamber. The chamber was then freeze-dried and sterilized with ethylene oxide gas.

After these processes were completed, 10^6 cells suspended in 40 μl of serum-free culture medium containing 0.3% (w/w) pig type I collagen (Cellmatrix I-A; Nitta Gelatin) were introduced into the diffusion chamber, and another open side of the chamber was sealed with a filter and adhesive sealant. Sixty-two chambers (42 for histologic examination, 8 for reverse transcription–polymerase chain reaction [RT-PCR] analysis, and 12 for real-time PCR analysis) with 10 μg of rHuBMP-2 (group B10), 10 chambers (all for histologic examination) with 1 μg of rHuBMP-2 (group B1), and 46 chambers (26 for histologic examination, 8 for RT-PCR analysis, and 12 for real-time PCR analysis) without rHuBMP-2 (group B0) were prepared for analysis and implantation.

Transplantation of the diffusion chamber into the abdominal pocket of rats. Immediately after loading the cells into the diffusion chambers, each chamber was surgically inserted into a pocket in the abdominal muscles of 8-week-old F344 rats under diethyl ether anesthesia. After surgery, the rats were housed in cages and were given free access to standard chalk-like food and water. At 2, 4, 6, 8, 14, 21, 28, 35, and 42 days after implantation, the animals were killed in due order and the diffusion chambers were harvested (Table 1) for histologic examination. For RT-PCR analysis, 2 chambers were harvested at 2-, 4-, 7-, and 14-day intervals after implantation. For real-time PCR analysis, 2 chambers were harvested at 2-, 4-, 6-, 14-, 28-, and 42-day intervals after implantation.

Harvested tissue pellets within the chambers were inspected for vascular invasion caused by seal failure or breakage of the filter membranes. When vascular invasion was noted, the tissue was excluded from the transplantation into the cartilage defect and from PCR analysis. The tissue pellets for histologic examination were radiographed and fixed in 20% neutral buffered formalin solution, prior to processing for histologic examination. Some parts of the tissue pellet from the 5-week-old sample were used for transplantation into the rat-knee defect. Tissue pellets for RT-PCR or real-time PCR were frozen in liquid nitrogen immediately after harvesting.

Transplantation of tissue pellets from diffusion chambers into osteochondral defects of rats. Some portions of the tissue pellet removed from the diffusion chambers at 5 weeks after implantation were transplanted into cartilage defects generated on the patellar grooves of the knee joints of 7 (4 from group B10, 3 from group B0) mature, same-strain rats (a quarter tissue pellet/animal). The transplantation procedure was performed with the rats under anesthesia, using an intramuscular injection of a mixture of ketamin (100 mg/ml, 0.6

Table 1. Cartilage formation in diffusion chamber*

	rHuBMP-2			Area of cartilage tissue in cross-section
	0 μg	1 μg	10 μg	
2 days	0/2	–	0/2	–
4 days	0/2	–	0/2	–
6 days	0/2	–	0/2	–
8 days	0/2	–	0/2	–
14 days	0/2	–	0/2	–
21 days	0/4	–	4/6	1/4
28 days	0/4	0/4	9/10	1/3
35 days	0/4	0/6	9/10	Almost all
42 days	0/4	–	6/6	Almost all

* Except where indicated otherwise, values are the number of samples with cartilage formation/number of experiments. rHuBMP-2 = recombinant human bone morphogenetic protein 2.

ml/kg body weight; Sankyo, Tokyo, Japan) and xylazine (20 mg/ml, 0.3 ml/kg body weight; Bayel, Osaka, Japan). Pellets were transplanted into the left knees, and defects made on the right knees did not receive the implants.

In order to generate an osteochondral defect on the patellar groove of the distal femur of the rats, a longitudinal skin incision was made in the midline of the knee and the patellar groove was exposed by medial parapatellar arthrotomy and lateral dislocation of the patella. The osteochondral defect was made by drilling in 2 mm in depth and 2 mm in diameter, vertically to the patellar groove. The tissue pellet was detached from the inner surface of the membrane filters of the diffusion chamber and press-fitted into the defect. The knee joint was then closed with sutures. After surgery, the rats were fed in cages and killed at 24 weeks after surgery. The knee joints were excised and processed for histologic examination.

Histologic examination. Diffusion chambers and distal femurs with an articular cartilage defect were removed from the animals at 24 weeks after implantation and fixed in 20% buffered formalin. The harvested chambers were radiographed with a soft x-ray apparatus (Sofron, Tokyo, Japan) and visualized on radiographic films (Fuji Photo Film, Tokyo, Japan). The harvested chambers with calcified tissue and the distal ends of femurs with articular defects were decalcified in 4% EDTA solution, and then dehydrated with a gradient ethanol series, embedded in paraffin, sectioned in 5-μm thickness, and stained with hematoxylin and eosin or toluidine blue. Results of the histologic examination were evaluated using the scoring system described by Wakitani et al (13) for histologic grading of a cartilage defect (Wakitani's score; a lower score indicates improvement).

RT-PCR analysis. In order to detect changes in the expression of cartilage matrix–specific molecules in cells from the harvested diffusion chambers, RT-PCR analyses for aggrecan, types II, IX, X, and XI collagens, MyoD1, and core binding factor a1 (Cbfa1)/runt-related gene 2 (Runx2) were performed with the tissue pellets from the B10 and B0 groups. Frozen tissue pellets were ground down to powder with liquid nitrogen in a mortar on dry ice, and total messenger RNA (mRNA) was extracted from the tissue using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. After treating samples with RNase-free deoxyribonuclease I (Takara Bio, Otsu, Japan), 500 ng of total mRNA from each sample was reverse transcribed using SuperScript II (Invitrogen). The reaction time was 60 minutes at 42°C. Thereafter, 1 μl of each reaction product was amplified in a 15-μl PCR mixture containing 0.5 units TaKaRa EX Taq (Takara Bio) and 10 pmoles of each primer to detect mRNA specific to each molecule.

Amplifications were performed in a Program Temp Control System (DNA Engine PTC-200; MJ Research, Waltham, MA) for 35 cycles after an initial denaturation step at 95°C for 3 minutes, denaturation at 95°C for 30 seconds, annealing for 30 seconds at 60°C, and extension at 72°C for 30 seconds, with a final extension at 72°C for 3 minutes. The PCR products (10 μl) were electrophoresed in a 3% agarose gel and detected by ethidium bromide staining. The nucleotide sequences of the primers for each of these genes are as follows: for AGC1, 5′-TCCAAACCAACCCGACAAT-3′ (forward) and 5′-TTCTGCCCAAGGGTTCTG-3′ (reverse); for Col2A1, 5′-GCTCGAGGAGACACTGGTG-3′ (forward) and 5′-ACCTGGGGGACCATCAGA-3′ (reverse); for Col9A1, 5′-GGTCCTCCGGGGAAGCCT-3′ (forward) and 5′-CCAACCTCTCCCGGCGGT-3′ (reverse); for Col10A1, 5′-CGAGGTCTTGTTGGCCCTAC-3′ (forward) and 5′-CCTGGGTCTCTGTCCGCT-3′ (reverse); for Col11A1, 5′-ATTGCCACCAGTCAACTGCT-3′ (forward) and 5′-TTGGACTGTGCCTCCGTC-3′ (reverse); for MyoD1, 5′-ACTACAGCGGCGACTCAGAC-3′ (forward) and 5′-GTGGAGATGCGCTCCACTAT-3′ (reverse); and for Cbfa1/Runx2, 5′-TGCTTCATTCGCCTCACAAAC-3′ (forward) and 5′-TAGAACTTGTGCCCTCTGTTG-3′ (reverse).

Real-time quantitative RT-PCR. Quantitative RT-PCR assay for type II collagen was carried out with the use of gene-specific expression–labeled fluorescent probes and sets of specific primers in an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). On the basis of the published sequence of rat type II collagen, specific primer pair and probe sets were designed with the aid of Primer Express software, version 2.0 (Applied Biosystems). The sequences of the primers were 5′-AGGCGCTTCTGGTAACCCA-3′ (forward) and 5′-GACCAGTTGCACCTTGAGGAC-3′ (reverse), and the probe was 5′-TTCCCGGAGCCAAAGGATCTGCTG-3′. We used 6-carboxyfluorescein for type II collagen as the 5′ fluorescent reporter for the probe, while we added 6-carboxy-tetramethylrhodamine (Tamura Pharmaceutical, Osaka, Japan) to the 3′ end as a quencher.

Standard curves were constructed with the use of dilutions of accurately determined pCR2.1 plasmid vector (Invitrogen) containing complementary DNA (cDNA) products of type II collagen. A relative standard curve representing 10-fold dilutions of a rat type II collagen cDNA ranging from 2×10 to 2×10^5 copies/μl was used for linear regression analysis of the samples. PCR was carried out in 50 μl of reaction mixture containing 3 μl of the RT reaction, 1× Universal Master Mixture (Applied Biosystems), 500 n*M* of each primer, and 200 n*M* of the Taqman probe purchased from Applied Biosystems.

To compensate for the differences in cell number and/or RNA recovery, the copy number of type II collagen mRNA was determined relative to 18S ribosomal RNA (rRNA) (Applied Biosystems), which was also analyzed quantitatively. Thus, a partial cDNA of 18S rRNA was amplified from rat bone and cartilage samples using a specific primer set for 18S rRNA, and then subcloned into pCR2.1 (Invitrogen). Ten-fold dilutions of the resultant vector, pCR2.1-18S rRNA, ranging from 2×10 to 2×10^5 copies/μl, were used to construct a relative standard curve for 18S rRNA. The PCR mixture was basically the same as that for type II collagen, except for 200 n*M* of an 18S rRNA–specific Taqman probe set carrying a 5′-VIC reporter label and 3′-TAMURA quencher group, and 500 n*M* of the specific primer for 18S rRNA that was purchased from Applied Biosystems. These samples were placed in the ABI PRISM 7700 Sequence Analyzer and preheated at 95°C for 10 minutes, then amplified for 50 cycles of 95°C for 15 seconds, followed by 60°C for 1 minute. These experimental protocols were in compliance with the guidelines established by the Institutional Committee for Animal Care and Experiments of Shinshu University.

Statistical analysis. The histologic score was statistically analyzed using the SPSS software package (SPSS Japan, Tokyo, Japan). The Kruskal-Wallis H test followed by the

Mann-Whitney U test was used to determine differences between the groups.

RESULTS

Cartilage induction in diffusion chambers by rHuBMP-2. The tissue mass harvested from group B10 chambers (those receiving 10 μg rHuBMP-2) had a gelatinous appearance, with no histologic features characteristic of cartilage until 2 weeks after implantation. At 3 and 4 weeks after implantation, the tissue had a pale, opaque gelatinous appearance and revealed some cartilaginous characteristics along the inner surface of the filter membranes of the chamber on histologic examination (Figures 1A–H).

At 5 and 6 weeks postimplantation (Figures 1I–P), the cells of group B10 formed an elastic tissue mass with opaque appearance and no evidence of calcification on radiography (Figure 2B). Histologic examination of the opaque tissue mass in the chambers indicated normal features of cartilage, with round chondrocytic cells enclosed in a metachromatic matrix, as revealed by toluidine blue staining (Figures 1L and P). Small amounts of osseous tissue were found on the outer or host-side surfaces of the membrane filter of those samples. In one chamber with an accidental "hole" on the membrane filter, containing 5-week postimplantation tissue of group B10, the tissue became a hard mass with a reddish appearance; on radiography, the tissue was highly calcified (Figure 2C) and showed a normal histologic appearance of bone with hematopoietic marrow (Figure 2A). In contrast, the tissue of groups B0 (Figure 1) and B1 (chambers without rHuBMP-2 or with 1 μg rHuBMP-2, respectively) showed a gelatinous appearance with no histologic evidence of cartilage formation throughout the experimental period.

PCR findings. PCR analysis of the tissue in the diffusion chambers revealed a consistent expression of types X and XI collagen (Figure 3). Expression of type X collagen gradually increased in group B10. The expression of type II collagen was detected at low levels 2 days after implantation in group B10 (Figure 3). After 4 days, the expression of type II collagen was clearly detected in group B10. The expression of Cbfa1/Runx2 was clearly detected after 96

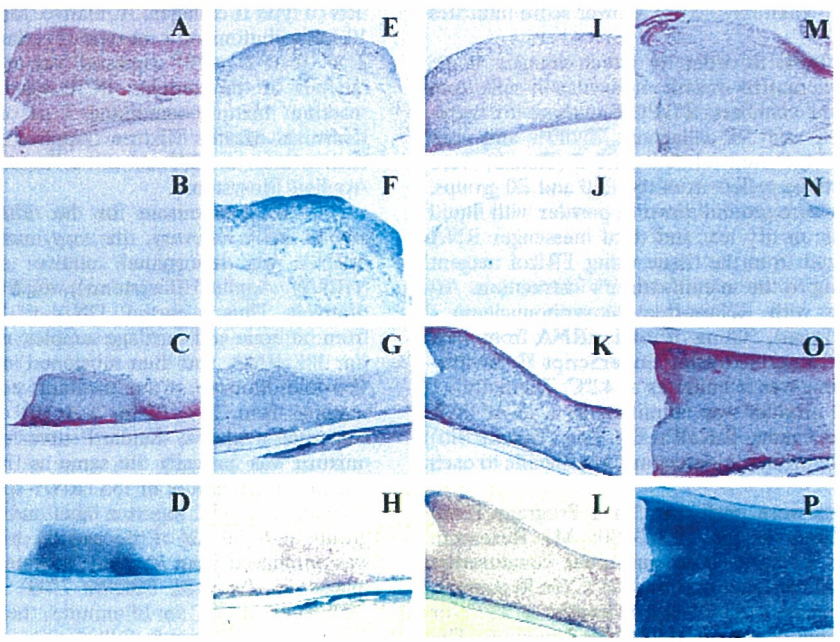

Figure 1. Cartilage formation in the diffusion chamber. Tissue pellets in diffusion chambers were examined at 3 weeks (**A–D**), 4 weeks (**E–H**), 5 weeks (**I–L**), and 6 weeks (**M–P**) postimplantation, in group B0 (without recombinant human bone morphogenetic protein 2 [rHuBMP-2]) (**A, B, E, F, I, J, M,** and **N**) compared with group B10 (with 10 μg rHuBMP-2) (**C, D, G, H, K, L, O,** and **P**). (Stained with hematoxylin and eosin in **A, C, E, G, I, K, M,** and **O**, with toluidine blue in **B, D, F, H, J, L, N,** and **P**; original magnification × 40.)

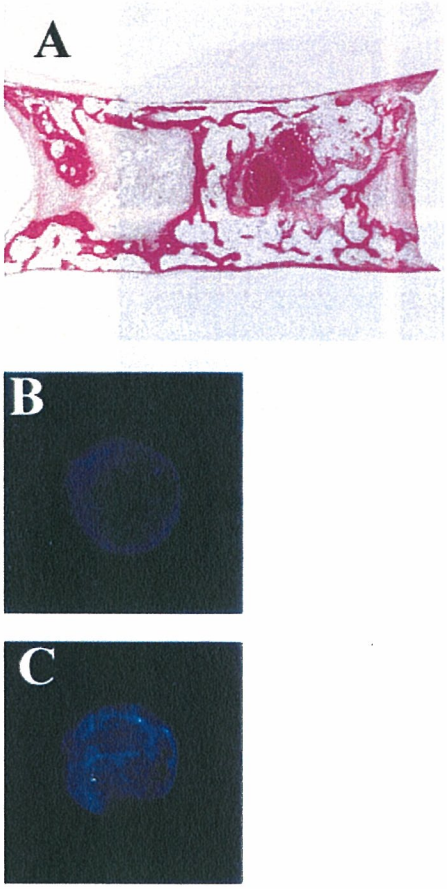

Figure 2. Histologic and radiologic evaluations of engineered cartilage tissue. For the tissue pellet in the diffusion chamber with an accidental hole on the filter (at 5 weeks posttransplantation; obtained from group B10), the normal histologic appearance of bone is clearly visible (stained with hematoxylin and eosin; original magnification ×20) (**A**), and the soft radiographic view shows bone trabeculae (**C**). Another soft radiographic view of group B10 tissue (same sample as in Figures **1K** and **L**) shows no calcification (**B**).

hours in group B10 only (Figure 3). The expression of MyoD1 was not detected in either group at any time point.

Real-time PCR revealed that the expression of type II collagen increased markedly at 4 days after implantation (Figure 4). A high level of aggrecan was seen in group B10 after 2 days. Type IX collagen was weakly expressed in group B10 after 4 days, but increased significantly after 1 week. Low expression levels of aggrecan and type II collagen were detected in all groups at later time points in the study.

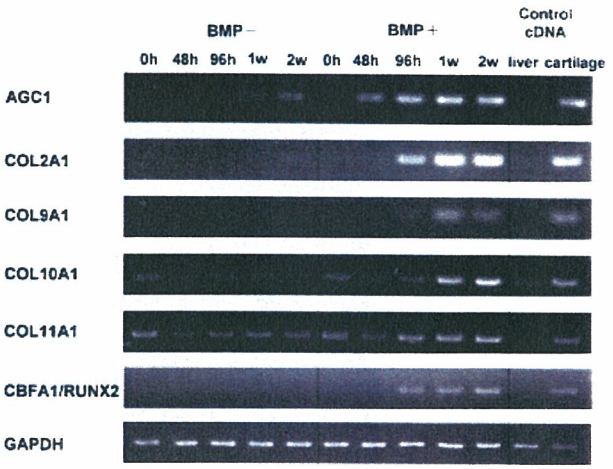

Figure 3. Reverse transcription–polymerase chain reaction analysis. Expression of types X and XI collagen (COL10A1 and COL11A1, respectively) was detected consistently in both groups (with 10 μg recombinant human bone morphogenetic protein 2 [BMP+; group B10] and without [BMP−]) throughout the experimental period. Expression of type IX collagen (COL9A1) was detected after 96 hours, indicating that effective cartilage matrix synthesis begins 3 or 4 days after implantation. Expression of type II collagen (COL2A1) was detected at low levels after 2 days in group B10 only, and after 4 days, it became more prominent. The expression of core binding factor a1/runt-related gene 2 (CBFA1/RUNX2) was clearly detected after 96 hours in group B10 only. AGC1 = aggrecan.

Repair of cartilage defects by transplantation of the engineered cartilage. The osteochondral defects that received the cartilaginous tissue mass, which was generated for 5 weeks in diffusion chambers containing tissue from group B10, were restored to a normal appearance at 24 weeks after transplantation. Upon examination, the site of the defects had a smooth surface and no

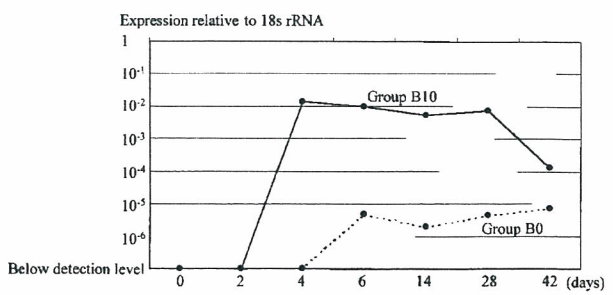

Figure 4. Real-time polymerase chain reaction analysis for type II collagen mRNA. After 4 days, expression of type II collagen mRNA was markedly increased in group B10 (with 10 μg recombinant human bone morphogenetic protein 2 [rHuBMP-2]). Group B0 = without rHuBMP-2; rRNA = ribosomal RNA.

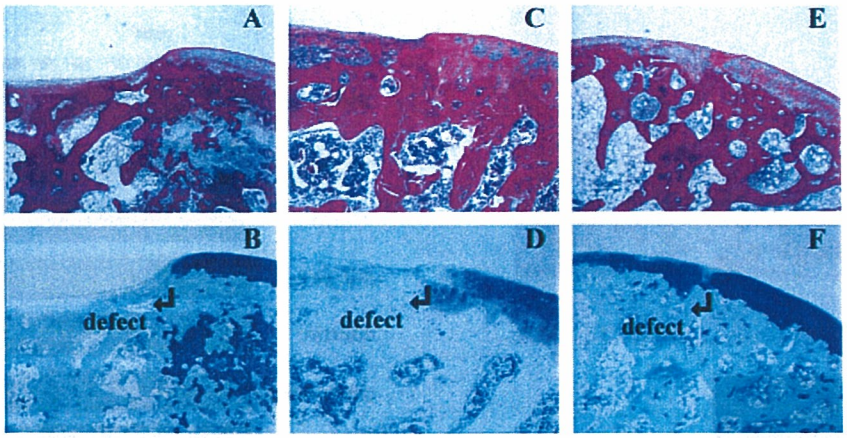

Figure 5. Osteochondral defects of a rat knee repaired with tissue pellets generated in diffusion chambers 24 weeks after transplantation. **A** and **B,** Defect with no implant. **C** and **D,** Defect implanted with tissue pellet generated in the chamber of group B0 (without recombinant human bone morphogenetic protein 2 [rHuBMP-2]). **E** and **F,** Defect implanted with tissue pellet generated in the chamber of group B10 (10 μg rHuBMP-2). (Stained with hematoxylin and eosin in **A, C,** and **E,** with toluidine blue in **B, D,** and **F**; original magnification × 40.)

obvious border with the surrounding normal articular cartilage (Figures 5E and F). The defects were filled with a layer of cartilage exhibiting subchondral cancellous bone connecting to the original subchondral bone. Although the architecture of the repaired articular cartilage was similar to that of normal cartilage with regard to cell arrangement, differences were noted. A tidemark was visible at the base of the cartilage layer adjacent to the subchondral bone, and the thickness of the regenerated cartilage was slightly less than that of the neighboring normal articular cartilage.

In contrast, the defects transplanted with tissue mass from group B0 were partially repaired, with a depressed surface visible at the defect site (Figures 5C and D). Histologic assessment of the defects that received either the tissue from group B0 or no implant revealed a small amount of fibrocartilage, with slightly positive metachromatic staining at the periphery of the defects and dominant fibrous tissue in the defect space.

Upon histologic evaluation of the knee cartilage after repair, the average histologic score (Wakitani's score) was 4.25 for group B10, 11.67 for group B0, and 14.00 for the defect-only group. The score for group B10 was significantly better than that for group B0 (P = 0.032) and the defect-only group (P = 0.002).

DISCUSSION

The experimental data presented herein indicate the capacity of rHuBMP-2 to induce the differentiation of young muscle-derived mesenchymal cells into chondrocytes within diffusion chambers in in vivo conditions. The resultant heterotopic cartilage formation represents a significant volume of induced tissue mass derived from these cells.

In order to induce the cartilage tissue, the diffusion chamber system was essential. When vascular invasion into the chamber occurred as a result of membrane seal failure, new bone with hematopoietic marrow was seen in the chambers harvested at 5 weeks after transplantation. Budenz and Bernard have reported similar findings (14). This bone was likely formed through the process of endochondral ossification, as deduced from classic reports describing the actions of BMP (15) and from comparison with the process of direct ossification (16,17). In the process of BMP-induced endochondral bone formation, cartilage is formed in the early phase of the bone-forming process. The cartilage tissue is then absorbed by invading vascular connective tissue and replaced by newly formed bone, as seen in embryonic osteogenesis (18) and in callus in fracture repair (19). During the process of ectopic bone formation elicited by