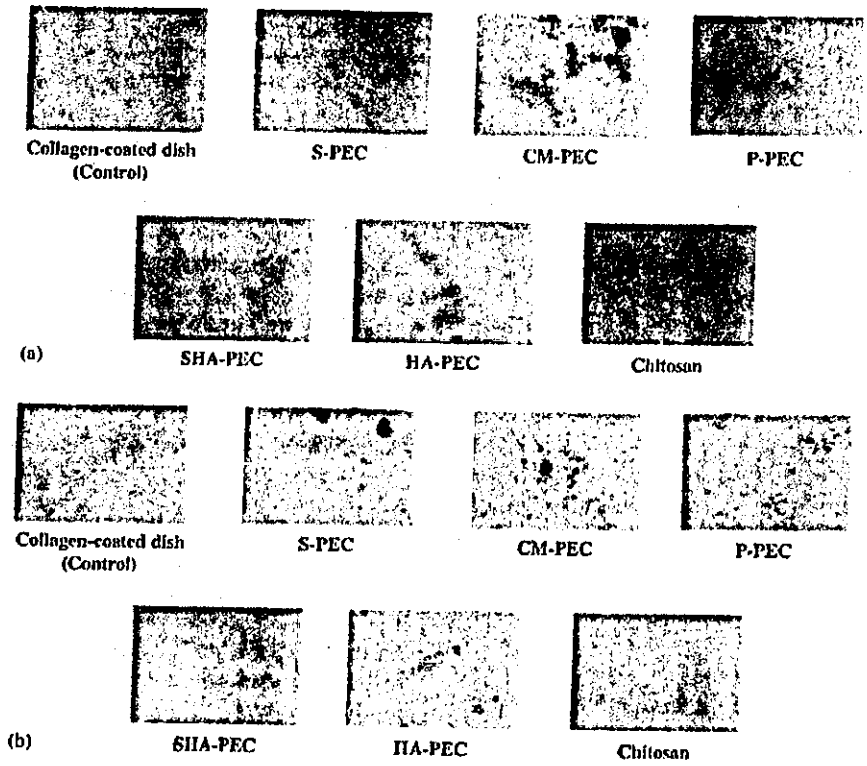

Fig. 2. Light micrographs of normal human osteoblasts (NHOst) on various PEC films after a 2-day incubation: (a) and 1-week incubation, (b). (Original magnification: × 100).

After 2-day incubation, the NHOst on PEC composed of chitosan and either sulfated chitin (S-PEC) or sulfated hyaluronan (SHA-PEC) showed morphologies similar to those on a normal culture plate. When cells were cultured on PEC of chitosan and phosphated chitin (P-PEC), some of them formed small aggregates, while the rest showed morphologies similar to those on S-PEC and SHA-PEC. On the other hand, NHOst cultured on PEC from chitosan and either carboxymethyl chitin (CM-PEC) or hyaluronan (HA-PEC) did not adhere well and showed aggregation. Similar morphologies of the cells on the PEC were observed after 1 day of incubation (data not shown). Even after 1 week of incubation, the morphologies and attachment of the cells on the PEC films did not change (Fig. 2). Only cells grown on cationic polysaccharide chitosan-coated culture dishes preserved morphology of very similar to NHOst grown on collagen-coated cultured dishes, indicating that these morphological differences are ascribable to differences in the anionic polysaccharides of which the PEC is composed.

It has been reported that cell attachment, morphology, and response are influenced by physico-chemical properties of the material surface [23,26]. To clarify what properties of PEC control the attachment and morphology of the cell, the contact angle and zeta potential of PEC films were estimated. Although their compositions are different, large differences in their contact angles were not observed (Fig. 3). On the other hand, a measurement of zeta potentials of the PEC showed interesting results (Table 1). The measurement revealed that S-PEC and SHA-PEC have negative zeta potentials, whereas PEC films made of polysaccharides

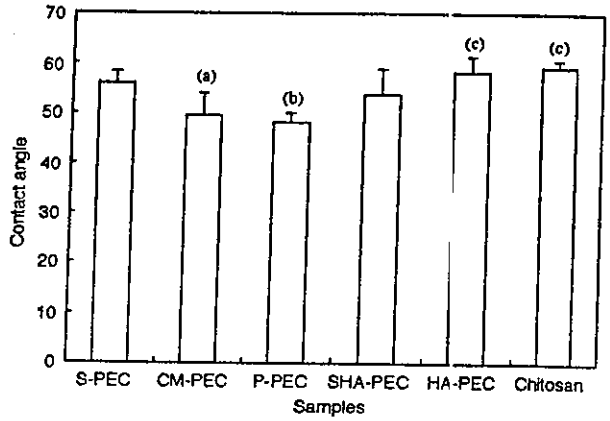

Fig. 3. Contact angles of PEC films studied: (a) $p<0.05$ against S-PEC, (b) $p<0.01$ against S-PEC, (c) $p<0.01$ against both CM-PEC and P-PEC.

Table 1
Zeta potentials of various PEC prepared on a culture dish

	Culture	S-PEC	CM-PEC	P-PEC	SHA-PEC	HA-PEC
Zeta potential (mV)	−58.7	−28.0	34.5	24.9	−5.7	29.5

Table 2
The cell number and differentiation of NHOst cultured on various PEC films after 1 week

Samples	The cell number (percent against control)	$\frac{\text{ALP activity}}{\text{The cell number}}$ (ratio)	$\frac{\text{Ca amount}}{\text{The cell number}}$ (μg/ratio)
Collagen-coated dish	100.0±17.0	1.00±0.15	3.4±0.5
S-PEC	82.2±6.1	0.98±0.11	10.7±3.6
CM-PEC	6.0±2.6*	0.05±0.08*	27.4±3.0*
P-PEC	130.4±6.3	0.02±0.01*	2.5±0.8
SHA-PEC	71.4±22.1	1.35±0.48	2.1±1.0
HA-PEC	8.1±3.0*	0.52±0.31	38.3±12.3*
Chitosan	79.5±25.0	0.93±0.13	2.7±2.0

*$p<0.01$ against collagen-coated dish.

with a carboxyl group, such as HA-PEC and CM-PEC, showed positive zeta potentials. In addition, P-PEC showed a positive potential less than that of HA-PEC. These data indicate that attachment of NHOst on surfaces with positive zeta potentials is reduced, suggesting the zeta potential of a PEC film partially controls cell attachment and morphology. Although all PEC were prepared by mixing anionic and cationic polysaccharides to neutralize their charge, zeta potential of each PEC film was ranged from −30 to 35 mV as shown in the table. This might indicate that not all anionic and cationic chemical groups were interacted to make PEC and their main chain composition and type of chemical groups may influence their side chain mobility, resulting in different surface zeta potential of each PEC. Details of surface properties of PEC films and their relationship to cell attachment will be reported in the near future.

After 1-week of incubation on various PEC films, the differentiation level of NHOst was estimated by measuring proliferation, alkaline phosphatase (ALP) activity and the amounts of calcium deposited. Table 2 shows the proliferation and ALP activity of NHOst cultured on various PEC films as well as the amounts of calcium deposited on the PEC. The proliferation of NHOst on the PEC is expressed as a percentage of proliferation of NHOst on a normal culture dish. The ALP activity was also calculated as a percentage of the control and normalized using the results of proliferation. In addition, the amount of calcium detected was normalized using the proliferation results as well. After a 1-week incubation, many dark spots, presumably calcium deposits, were observed on the collagen-coated dish and other PEC films (Fig. 2). When NHOst were cultured on CM-PEC or HA-PEC, it was observed that the NHOst aggregates were covered by the calcium deposits. It was reported that a surface with carboxyl group could induce calcium deposition after its incubation in simulated body fluid [27]. However, when the PEC were incubated in the medium without NHOst, no calcium deposition was detected. In addition, zeta potential estimation suggests less carboxyl groups are appeared on a surface of the PEC. These indicate that calcium deposition occurred only on aggregated NHOst but not on surfaces lacking NHOst. Therefore, normalization is necessary to estimate the capacity of PEC films to induce NHOst differentiation, although the raw values of deposited calcium or ALP activity are low. In fact, CM-PEC or HA-PEC films show a capacity to induce NHOst differentiation comparable to the collagen-coated dish and other PEC films, judging from the normalized values of deposited calcium shown in the table, even though the ratio of NHOst number on them was only 6–8% of that on a collagen-coated dish. Their ALP activities were, however, much lower than those on the collagen-coated dish. Incubation of the PEC films without NHOst for 1 week resulted in no calcium deposition, irrespective of their composition, suggesting that the PEC films themselves had no effect on calcium deposition. Thus, enhancement of calcium deposition on the PEC films may be ascribed to enhancement of NHOst functions related to their differentiation even though their ALP activity was suppressed. The reason for this inconsistency observed between calcium deposition and ALP activity must be investigated further.

When sulfated polysaccharides were used to prepare PEC films, proliferation of NHOst on the PEC films was 70–80% of that on a collagen-coated dish, and ALP

activity was very similar to that on the collagen-coated dish. This suggests that sulfated polysaccharide PEC does not affect NHOst functions. Actually, there were no statistical differences in the amounts of calcium deposited between NHOst on the PEC and the collagen-coated dish although NHOst on S-PEC showed higher average calcium deposition. Thus, it is suggested that the PEC films made from sulfated polysaccharides are comparable substrates to a collagen-coated dish for cell culture. When compared to a normal culture dish, it has been reported that S-PEC can induce aggregation of cultured human fibroblasts and enhance their DNA synthesis in an earlier stage of cell culture by activation of the ERK pathway [28]. Since we used a collagen-coated dish as a control in this study, it is expected that the pathway of NHOst on the dish may be already activated through integrin molecules on the NHOst membrane. Therefore, the results in this study suggest the PEC from sulfated polysaccharides have a potential to proliferate and differentiate NHOst very similar to that of collagen.

To assess the effects of PEC films on cell function, gap junctional intercellular communication (GJIC), which is an important function of cells for maintenance of homeostasis [17], of NHOst on the films were measured. As shown in Fig. 4, GJIC of NHOst on PEC films did not show statistically significant differences compared to those grown on a collagen-coated dish. Although the GJIC of NHOst on CM-PEC showed a decrease after 1 day of incubation, it had recovered after 1 week. This result suggests that most PEC films have the potential to maintain homeostasis of attached cells although they showed different influences on the number and the differentiation of NHOst. On the other hand, NHOst on chitosan, which was used as the polycation for all PEC, showed suppression of GJIC after 1 week. This suggests that chitosan disturbs homeostasis maintenance of NHOst, but improve its biocompatibility by forming PEC films with other anionic polysaccharides. Therefore, PEC might be used as a biocompatible material for medical devices and tissue engineering scaffolds.

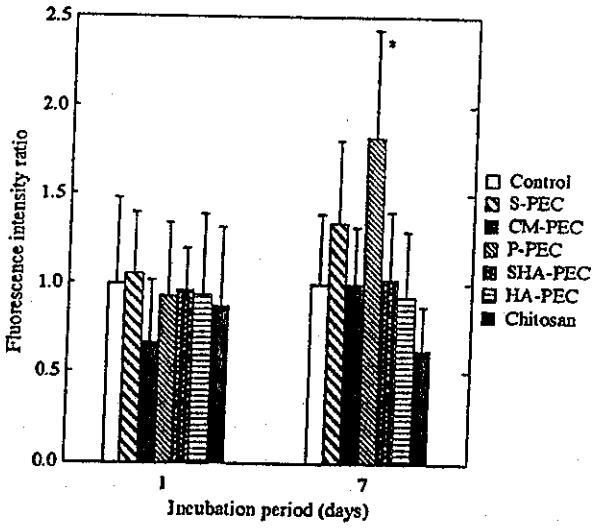

Fig. 4. Gap junctional intercellular communication activity of NHOst on various PEC films estimated by FRAP analysis technique. (*$p<0.01$ against control).

4. Conclusion

PEC films composed of various polysaccharides were prepared, and their effects on NHOst functions were evaluated. Attachment, morphology, growth and differentiation of NHOst were influenced by the composition of the PEC on which they were grown. NHOst attachment decreased and their aggregates were observed on PEC prepared from polysaccharides containing a carboxyl group (CM- and HA-PEC). ALP activity of NHOst was suppressed on these PEC films although calcium deposition was observed more frequently than on other PEC films. In addition, these PEC films strongly suppressed proliferation of NHOst. PEC prepared from phosphated chitin and chitosan (P-PEC) showed low ALP activity and calcium deposition, although the number of NHOst was highest after 1-week incubation. These indicate unsuitability of these three PEC for usage in tissue engineering. On the other hand, NHOst adhered to and proliferated well on PEC films when sulfated polysaccharides were used as the polyanion (S- and SHA-PEC). Moreover, these PEC films showed almost the same suitability as the collagen-coated dish in all cell functions studied, indicating that these PEC films, especially S-PEC can be used as a scaffold for bone regeneration. Further studies, especially in vivo studies, are needed to clarify the usefulness of PEC films for tissue engineering.

Acknowledgements

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Embryonic Stem Cells Form Articular Cartilage, not Teratomas, in Osteochondral Defects of Rat Joints

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Embryonic stem (ES) cells are considered to be a potential tool for repairing articular cartilage defects, but so far it has been impossible to cause these cells to differentiate into chondrocytes exclusively, either in vivo or in vitro. To explore a potential new cell source of cell transplantation for articular cartilage defects, we transplanted ES cells into articular cartilage defects in immunosuppressed rats. ES cells (AB2.2 or CCE cells) were transplanted into articular cartilage defects in the patellar groove of immunosuppressed rats treated with cyclosporine. The cells were histologically observed until 8 weeks after transplantation. To determine whether the repair tissue in the defect in the AB2.2-transplanted group was derived from the transplanted cells, the neomycin-resistant gene, which had been transfected into AB2.2 cells but does not exist in rat cells, was used for detection. The cells produced cartilage, resulting in repair of the defects from 4 weeks until 8 weeks after the transplantation without forming any teratomas. The neomycin-resistant gene was detected in every sample, demonstrating that the repair tissue in the AB2.2-transplanted group was derived from the transplanted AB2.2 cells. The environment of osteochondral defects is chondrogenic for ES cells. ES cells may thus be a potential tool for repairing articular cartilage defects.

Key words: Chondrogenic lineage; Hyaline cartilage; Regeneration; Local environment

INTRODUCTION

Articular cartilage covers the surface of moving joints, and its main function is to reduce impact and friction. Because defects of articular cartilage are not completely restored, many attempts have been made to repair articular cartilage defects, but no widely accepted methods have been developed (5).

Embryonic stem (ES) cells (7,17) are one of the most promising tools for tissue repair, because of their unlimited proliferative capacity and ability to differentiate into any tissue or cell (1,2,9,10,13,16). ES cells are thus a potential tool for repairing articular cartilage defects, but so far it has been impossible to cause these cells to differentiate into chondrocytes exclusively, either in vivo or in vitro (12). Because the local environment is important for differentiation into specific tissues or cells (14), we first transplanted ES cells into the joints of immunodeficient mice, but the cells formed teratomas and subsequently destroyed the joints (20). In our next attempt, reported here, we transplanted ES cells into osteochondral defects in the patellar groove of immunosuppressed rats treated with cyclosporine.

MATERIALS AND METHODS

ES Cell Preparation

AB2.2 prime ES cell kits were purchased from Lexicon Genetics Inc. (Houston, TX). They were cultured according to the manufacturer's instructions on ESQ feeder cells that were supplied with the kit.

CCE ES cells were kindly provided by Dr. Elizabeth J. Robertson and cultured by us on feeder cells (RJ) inactivated by mitomycin C.

Both ES cell lines were obtained from 129/Sv/Ev mice. The ES cells were embedded in a collagen solution (type I collagen obtained from porcine Achilles tendon; Nitta Gelatin, Osaka, Japan) at 4°C at a density of 10^7 cells/ml, and were gelated in 15-μl aliquots at 37°C.

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Surgery

Twenty-four rats (F344/DuCrj, Charles River Japan Inc., Yokohama, Japan) weighing about 330–350 g were anesthetized by intraperitoneal injection of ketamin (10 mg/100 g, Sankyo Co., Ltd., Tokyo, Japan) and xylasine (1 mg/100 g, Bayer Co., Ltd., Tokyo, Japan). A hole (2 mm in diameter and 2 mm in depth) was made in the patellar groove of the femur with a hand drill and 15 μl of gel was inserted into the hole. The calculated volume of the defect was 6.28 μl. Because some of the gel protruded from the hole, water was removed from the gel with gauze to make sure it was confined to the hole. Twelve rats received gel with CCE cells transplanted into the right knees and gel without cells into the left knees, while the other 12 rats received gel with AB2.2 cells in the right knees and gel without cells in the left knees.

Immediately after surgery, the recipient animals received daily subcutaneous injections of cyclosporine (14 mg/kg, Novartis Pharma AG, Basel, Switzerland).

We also transplanted the cells embedded in collagen gels into the subcutaneous tissue of severe complex of immunodeficient mice ($n = 2$ for AB2.2 cells and $n = 4$ for CCE cells) and observed them for 8 weeks to determine whether the ES cells formed teratomas.

Histological Evaluation

At 1, 2, 4, and 8 weeks after surgery, three rats from the two groups transplanted with different ES cells were sacrificed each time. The distal femurs were collected, fixed in 10% buffered formalin, and the tissues decalcified and sectioned. Staining was performed with hematoxylin/eosin and safranin-O. The digital images were prepared with Photograb (Fujifilm Co., Tokyo, Japan) and microscopy.

Each sample was graded according to the histological scale described by Wakitani et al. (18). The scale consisted of five categories: cell morphology, matrix staining, surface regularity, thickness of cartilage, and integration of donor with host cartilage. The scores ranged from 0 (normal articular cartilage) to 14 (no cartilaginous tissue).

Two-way factorial ANOVA was used for statistical comparative analysis of the overall scores for the four time points between the cell-transplanted groups and the cell-free group. Stat View software (SAS Institute Inc., Cary, NC) was used for analysis, and probability values less than 0.05 were considered significant.

DNA Analysis

DNA was extracted with DEXPAD (TaKaRa, Kyoto, Japan) from a small sample of the repair tissue obtained at each time point. Samples with histologically optimal repair were selected for each postoperative period. We then amplified DNA by means of polymerase chain reaction to detect the neomycin-resistant gene that had been transfected into AB2.2 cells. The primers for the neomycin-resistant gene were neo p4 (5′-AGGATCTC GTCGTGACCCATG-3′) and neo int2 (5′-TCAGAAG AACTCGTCAAGAAGGC-3′), and the size of the reaction product was 250 base pairs. To detect rat cells, we used two other primers: M14103F (5′-GTGGAATGAC GTTCCTTGCT-3′) and M14103R (5′-TGGTAACCAA TGTTGAATTGC-3′). The size of the reaction product was 180 base pairs. The primers for the glyceraldehyde 3-phosphate dehydrogenase (GAPD) gene were GAPD-F (5′-AGAAATCCCCTGGAGCTCTATAGGG-3′) and GAPD-R (5′-CCGGAATGCCATTCCTGTTAGCTTC-3′), and the size of the reaction product was 250 base pairs.

This study was approved by our Institutional Review Board.

RESULTS

Four weeks after transplantation, osteochondral defects in the patellar groove of the femur were covered by histologically hyaline-like cartilage in three out of three rats transplanted with CCE cells and two out of three transplanted with AB2.2 cells. This result was also observed in two out of three rats with CCE cells and in one out of three rats with AB2.2 cells 8 weeks after the transplantation, whereas rats without ES cell transplantation showed coverage by fibrous tissue or fibro-cartilage (Figs. 1 and 2).

One week after transplantation, ES cells had collected to form numerous small clumps, corresponding to embryoid bodies, in the repair tissue. Two weeks after transplantation, these clumps had disappeared and the cells were distributed more evenly. Cartilaginous tissue was first observed at 4 weeks after transplantation; at this time, thick cartilaginous tissue covered the defect. The cells in the repair cartilage were round and the matrix showed strong metachromasia, indicating that this was hyaline cartilage. Eight weeks after transplantation, the repair tissue still resembled hyaline cartilage, and no cell masses suggestive of teratoma were found in any sample at any time of observation (Fig. 1).

As for the collagen gel without cell implantation (control group), amorphous acellular material, assumed to be the implanted collagen gel, was observed at 1 week. By 2 weeks after implantation, the defects were filled with fibrous tissues, and 4 weeks after collagen gel implantation, metachromasia was observed adjacent to the joint spaces in the fibrous tissue filling the defects, but the cellular morphology and matrix staining indicated that this was fibro-cartilage. Eight weeks after implantation, the repair tissue was still fibro-cartilaginous (Fig. 1).

Histological grading scores were determined and used

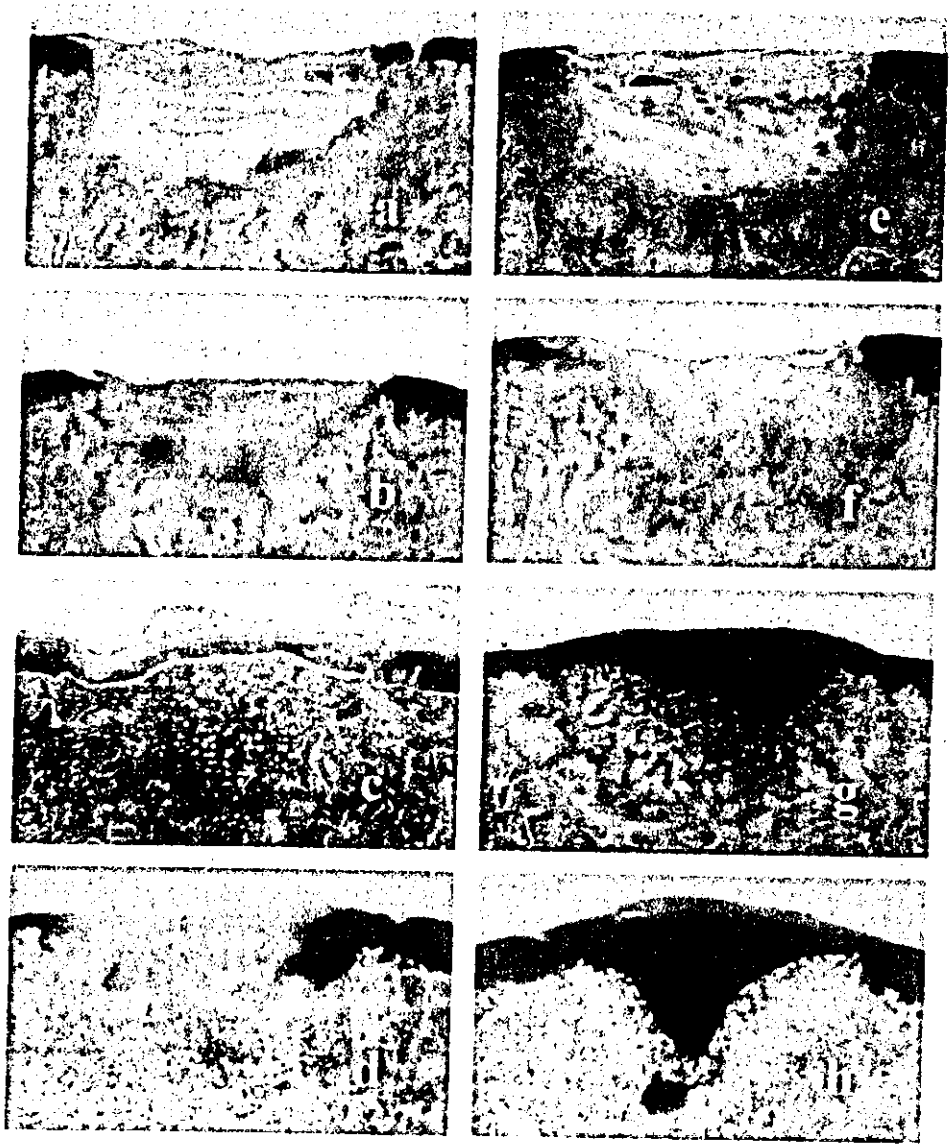

Figure 1. Photomicrographs of sagittal section of defects at 1 (a, e), 2 (b, f), 4 (c, g), and 8 weeks (d, h) after transplantation of CCE cells (e–h) and cell-free controls (a–d). We selected histologically best repair at each postoperative period. The size of cartilage defects was almost 2 mm in diameter. Safranin-O staining, original magnification ×20.

to compare the repair tissues (Table 1). The differences in the overall scores for the four time points between the cell-transplanted groups and the cell-free group were analyzed with two-way factorial ANOVA. The scores of the CCE cell-transplanted group were significantly better than those of the cell-free control group ($p = 0.0478$), whereas there was no significant difference between those of the AB2.2 cell-transplanted and control groups ($p = 0.0910$).

The two types of cells were also transplanted into the subcutaneous tissue of severe complex of immunodeficient mice. After 8 weeks, we found teratomas in two out of two mice transplanted with AB2.2 cells and three out of four mice transplanted with CCE cells.

To determine whether the repair tissue in the defect was in fact derived from the transplanted cells, we checked the tissue for the presence of the neomycin-resistant gene, which had been transfected into AB2.2 cells but does not exist in rat cells. The gene was detected in every sample, demonstrating that the repair tissue was derived from the transplanted AB2.2 cells (Fig. 3). We also tried to detect a gene that is present in rats, but not in mice. This gene was detected in one sample, in which the neomycin-resistant gene was also detected,

Figure 2. Photomicrograph of sagittal section of defects at 4 weeks after transplantation of AB2.2 cells. The size of cartilage defects was almost 2 mm in diameter. Safranin-O staining, original magnification ×30. The repair by AB2.2 cell transplantation was inferior to that by CCE cell transplantation.

which meant that the tissue contained both transplanted cells and host cells.

DISCUSSION

To the best of our knowledge, this is the first report of articular cartilage repair by ES cells. Interestingly, no sign of tumor growth or nonchondrocyte tissue was observed in the transplant recipients, and the defects were repaired with hyaline-like cartilage. The repair cartilage was thicker than the adjacent normal cartilage at 4 and 8 weeks. The repair cartilage was thought to fill all areas of the defect at first, and then be replaced by the host bone over time, as reported previously (18). ES cells transplanted into the osteochondral defects appeared to differentiate exclusively into chondrocytes, while those transplanted into subcutaneous tissue generated teratomas. Thus, the environment of the osteochondral defect in knee joint was chondrogenic for ES cells.

We have previously reported that AB2.2 cells injected into knee joint spaces of immunodeficient mice formed teratomas (20), whereas in the study presented here they did not generate teratomas in osteochondral defects in rats immunosuppressed with cyclosporine.

Table 1. Histologic Grading Scores for the Repair Tissues

	1 Week	2 Weeks	4 Weeks	8 Weeks
CCE*	14, 14, 14	14, 14, 14	0, 3, 6	1, 6, 14
Control	14, 14, 14	12, 14, 14	12, 12, 14	11, 11, 14
AB2.2	14, 14, 14	14, 14, 14	4, 7, 14	5, 10, 10
Control	14, 14, 14	14, 14, 14	8, 14, 14	13, 13, 14

Scores ranged from 0 = best to 14 = worst.
*Significantly better than the control group.

Because different species of animals were used for the two experiments, it was possible that ES cells would not generate teratomas in immunosuppressed rats. However, we confirmed that ES cells do produce teratomas in immunosuppressed rats in another experiment (data not shown). One reason may be that nutrition was different. Cells in joint spaces are fed by joint fluid not by blood, whereas those in osteochondral defects are fed by both joint fluid and blood in osteochondral defects. It is thus conceivable that some growth factors are released from bone marrow and promote the chondrogenesis of ES cells. Another reason may be that the mechanical stress was different. In osteochondral defects, cells are confined to the defect and pressed down by the patella, so that they cannot produce a large mass that grows out of the defect, whereas cells in joint spaces can expand substantially. Mechanical stress on tissue in the defect may also play an important role in promoting chondrogenesis.

The histological grading scores of the cell-transplanted groups at 4 and 8 weeks differed, and the reason for these differences is thought to be due to technical difficulties in transplantation surgery or differences in immunological reaction of individual rats. We transplanted two kinds of ES cells (AB2.2 and CCE) and both promoted repair of the articular cartilage defect, although that resulting from AB2.2 cell transplantation was not significant, which was probably due to these differences. To confirm the efficacy of ES cell transplantation for cartilage repair, further experiments may be necessary.

The repair tissue in the defect in the AB2.2-transplanted group was proven to be derived from the transplanted cells because the neomycin-resistant gene, which had been transfected into AB2.2 cells but does not exist in rat cells, was detected in the repair tissue. We also detected a gene that is present in rats but not in mice in one of the four samples, in which neomycin-resistant gene was also detected. We may have collected the underlining host tissue in this case or it is possible that the repair tissue consisted of both transplanted cells and host cells. In the other three cases, we concluded that the repair tissue was derived from the transplanted cells, not from the host cells. The transplanted AB2.2 cells differentiated into chondrocytes and formed repair cartilage.

It has been difficult to use ES cells for tissue repair because we could not target the cells exclusively to differentiate into a particular tissue in vitro or in vivo. To obtain homogenous cell populations, it has been possible to induce ES cells with differentiation factors [i.e., neurons and skeletal muscles (4) and glial precursors (6)] or transfect them with vectors that make selective differentiation into cardiomyocytes possible (11). It has also been reported that in vitro differentiation of ES cells into

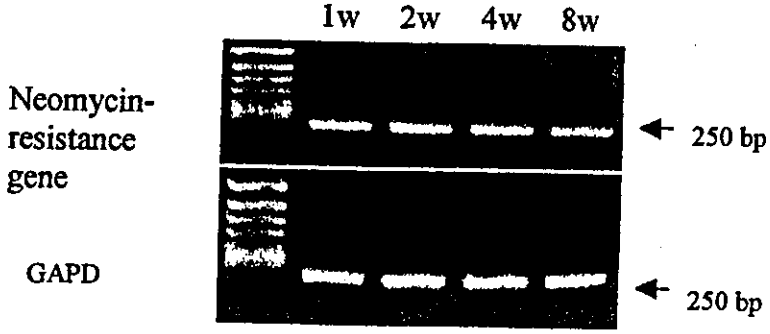

Figure 3. Gel electrophoresis of DNA amplified by the polymerase chain reaction. DNA was extracted from repair tissue at 1, 2, 4, and 8 weeks after transplantation. Neomycin-resistant gene was detected in each sample.

chondrocytes can be promoted by bone morphogenetic protein-2 or -4, but the cell aggregates thus formed contained a variety of cells and not only chondrocytes (12). When ES cells are transplanted into cartilage defects, the formation of cells other than chondrocytes may interfere with the repair process. Although long-term studies will be necessary to comprehensively address the safety of ES cell transplantation, the possibility of using these cells for tissue repair has been expanded by the results of our study. Further optimization of the donor ES cells such as overexpression of factors promoting differentiation may help to meet the challenges involved in clinical application.

We used an immunosuppressive agent in this study because ES cells are xenogeneic. In the case of human application of ES cells, such cells will be allogeneic. Further investigation is required to assess the immune effects of ES cell transplantation on cartilage defects. Various strategies, such as autologous nucleus transplantation into ES cells or HLA-matched ES cell transplantation, may be required to render the clinical use of ES cells feasible.

Recently, autologous cultured chondrocyte transplantation (3,15) and mosaic plasty (autologous osteochondral grafting) (8) have been proven to alleviate symptoms and achieve a certain degree of repair. However, these methods involve the collection of autologous cartilage, which creates new cartilage defects in the peripheral areas. Thus, new cell sources have been investigated, and stem cells, such as mesenchymal stem cells (19) and ES cells, are thought to be among the most promising sources for use in tissue repair because these cells are capable of both proliferation and differentiation.

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Autologous Bone Marrow Stromal Cell Transplantation for Repair of Full-Thickness Articular Cartilage Defects in Human Patellae: Two Case Reports

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This study assessed the effectiveness of autologous bone marrow stromal cell transplantation for the repair of full-thickness articular cartilage defects in the patellae of a 26-year-old female and a 44-year-old male. These two patients presented in our clinic because their knee pain prevented them from walking normally. After thorough examination, we concluded that the knee pain was due to the injured articular cartilage and decided to repair the defect with bone marrow stromal cell transplantation. Three weeks before transplantation, bone marrow was aspirated from the iliac crest of each patient. After erythrocytes had been removed by use of dextran, the remaining nucleated cells were placed in culture. When the attached cells reached subconfluence, they were passaged to expand in culture. Adherent cells were subsequently collected, embedded in a collagen gel, transplanted into the articular cartilage defect in the patellae, and covered with autologous periosteum. Six months after transplantation, clinical symptoms (pain and walking ability) had improved significantly and the improvement has remained in effect (5 years and 9 months posttransplantation in one case, and 4 years in the other), and both patients have been satisfied with the outcome. As early as 2 months after transplantation, the defects were covered with tissue that showed slight metachromatic staining. Two years after the first and 1 year after the second transplantation, arthroscopy was performed and the defects were repaired with fibrocartilage. Results indicate autologous bone marrow stromal cell transplantation is an effective approach in promoting the repair of articular cartilage defects.

Key words: Chondro-progenitor cell; Cartilage regeneration; Resurfacing; Fibrocartilage

INTRODUCTION

Articular cartilage defects have a weak potential for self-repair because of the reduced mitotic capacity of chondrocytes in vivo (2). Because some patients with articular cartilage defects may progress to osteoarthritis as described previously, such defects need to be repaired even though their exact natural course remains obscure (6,8,12).

Traditional methods for repair, such as multiple perforations (9), abrasion arthroplasty (3), and microfracture (4), have not produced consistent satisfactory long-term clinical results. Studies using animal models showed that the repair tissue produced with these methods is mainly fibrocartilage, not hyaline cartilage, and is subject to subsequent degradation (5,10,13). Exploration of the feasibility of autologous chondrocyte implantations has started recently (1), but some problems remain unsolved. These include difficulties in obtaining sufficient chondrocytes, defects associated with autologous cartilage tissue collection, and insufficient histological repair (7). Multiple autologous osteochondral grafting (mosaic plasty) can result in the repair of articular cartilage defects through the formation of hyaline-like cartilage. This procedure is also limited by defects in donor sites, insufficient repair between the grafts, and technical difficulties in resurfacing the original curvature of the joint surface (7,11).

We reported previously that transplantation of autologous bone marrow stromal cells (MSC) expanded in culture could result in the repair of articular cartilage defects in human osteoarthritic knees (16). This method is clinically straightforward to perform because autologous cells can be readily harvested and expanded in culture

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without losing their capacity to differentiate into chondrocytes.

The purpose of this study was to evaluate the clinical results obtained with autologous MSC expanded in culture for the treatment of full-thickness chondral defects in human patellae.

CASE 1

The first case involved a 31-year-old female patient. At 23 years of age, the articular cartilage in her left patella had been treated with arthroscopic shaving at another institute because of chondromalacia in her patellae. However, following this surgical procedure, her left knee pain did not diminish and she had to walk with a crutch. She came to our hospital when she was 25 years old because the pain prevented her from walking normally. Physical examination established patello-femoral crepitation and anterior knee pain. The range of motion of the left knee was restricted to between 0° and 130°. The X-ray findings demonstrated narrowing of the patello-femoral joint space and magnetic resonance imaging deficiency of the patellar articular cartilage. After thorough examination of her left knee, we concluded that the other parts of the knee were normal and that the knee pain was due to the injured articular cartilage of the patella. A few months of conservative treatment, such as muscle training, thermotherapy, medication with painkillers, and intra-articular injection of hyaluronic acid, did not result in improvement in the pain. We decided to perform autologous transplantation of MSC expanded in culture to repair the articular cartilage defect in the patella with the informed consent of the patient.

Ten milliliters of heparinized bone marrow was aspirated from the left iliac crest. After erythrocytes had been removed with dextran, the remaining nucleated cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum. After 3 days of culture, attachment of cells was observed and when the medium was changed, nonadherent cells were removed with the medium. After approximately 10 days, the attached cells had achieved subconfluence and were then passaged to expand in culture. After another 10 days, on the day before surgery, 1.8×10^7 cells were collected and embedded (5×10^6 cells/ml) in 0.25% type I acid-soluble collagen from porcine tendon (Nitta Gelatin Inc., Osaka, Japan) and gelated. This gel–cell composite was then cultured overnight in DMEM supplemented with 15% autologous serum.

The transplantation surgery took place on January 27, 1998. After a medial parapatellar approach, all of the fibrous tissue covering the surface of the patella was removed, the subchondral bone was exposed, and multiple perforations using K-wire with a 1.5-mm diameter were made to facilitate bleeding. The area of the full-thickness cartilage defects in the left patella was about 12.0 cm^2. Because the volume of the collagen with cells was about 3.6 ml, the gel–cell composite was formed with a thickness of 2 mm. The composite was then put in place and covered with autologous periosteum taken from the anterior surface of the tibia with the cambium layer facing the bone marrow. The flap was sutured to the surrounding rim of the normal cartilage or soft tissue with interrupted absorbable sutures. Continuous passive motion was started 3 days after surgery but otherwise the knee was immobilized with a brace for 3 weeks. Partial weight bearing was started 3 weeks and full weight bearing 6 weeks after the operation. The pain in the knee diminished and the patient was able to walk without crutches or a cane 6 months after the operation. The knee range of motion remained somewhat limited from 0° to 120°.

Arthroscopic surgery on March 17, 1998 (7 weeks postoperatively) showed that the patellar articular surface was completely covered with tissue that exhibited slight metachromatic staining upon histological examination.

Arthroscopic surgery on October 25, 2000 (2 years postoperatively) showed that the patellar articular surface was completely covered with cartilage-like tissue, which appeared to have a smooth surface and a firm consistency as established by probing. Histological examination of a tissue specimen taken from the surface of the articular cartilage in the graft area demonstrated the presence of a matrix with strong metachromatic staining but with a fibrous appearance, leading us to conclude that this was fibrocartilaginous repair tissue. At the time of writing this report (5 years and 9 months after transplantation) the patient could walk, run without ambulatory support, do housework without knee pain, and was satisfied with the outcome of the transplantation.

CASE 2

The second case involved a 48-year-old male patient. The articular cartilage of his right patella had been injured in a traffic accident when he was 44 years old, and he came to our hospital 4 months after the injury. He had been suffering from right knee pain that caused him to limp and made stair climbing difficult. Physical examination showed neither patello-femoral maltracking nor ligamentous instability, but retropatellar crepitation accompanied by pain. Computed tomography demonstrated a partial depression in the patella, and arthroscopy showed that the medial area of the patella's articular cartilage was fibrillated.

A careful examination of the knee led to the conclusion that the knee pain was due to the injured articular cartilage of the patella. As for the first case, conservative treatment was initiated but the pain also did not diminish for this second patient. We therefore decided on autolo-

gous transplantation of MSC expanded in culture to repair the articular cartilage defect in the patella with the patient's informed consent.

MSCs (1.4×10^7) were prepared in the same manner as for the Case 1 patient. They were embedded in collagen solution with the same cell density (5×10^6 cells/ml), but in this case they were put on a collagen sheet (Koken Inc., Tokyo, Japan) and gelated. The collagen sheet containing the cells was transplanted. The transplantation surgery was performed on November 12, 1999. The medial part of the articular cartilage was found to be damaged (Fig. 1). The injured articular cartilage was removed, the subchondral bone was exposed, and multiple perforations were made to facilitate bleeding from the bone marrow. The area of the full-thickness cartilage defect in the right patella was about 4.5 cm^2. Because the sheet measured about 10 cm^2, almost half of the cells were used. The sheet was put in place and covered with autologous periosteum with the cambium layer facing the bone marrow (Fig. 1). The postoperative physiotherapy program was the same as for the first case except for the omission of immobilization.

Arthroscopic surgery on January 11, 2000 (2 months postoperatively) revealed that the patellar articular surface was completely covered with tissue that was softer than the surrounding normal articular cartilage (Fig. 2).

Six months after the operation, the patient no longer experienced his initial symptoms during daily activities, although the knee range of motion was limited from 0° to 130°.

One year after transplantation, arthroscopy and needle biopsy were performed. Arthroscopy showed that the color and hardness in the graft area were similar to those of the surrounding normal cartilage. Histological examination showed that the repair tissue was fibrocartilaginous (Fig. 3). Fibroblastic cells with spindle-shaped nuclei and scattered chondrocytes with lacunae were also observed. There was no evidence of inflammatory cells or vascular proliferation.

At present (4 years after transplantation), the patient can work without knee pain as a truck driver in the same manner as before the injury and is satisfied with the outcome of the transplantation.

This study was performed in accordance with the ethical standards of the hospital committee on human experimentation and of the World Medical Association Declaration of Helsinki.

DISCUSSION

This article describes autologous MSC expanded in culture transplantation procedures used to treat two patients with large patella articular cartilage defects. Histological examination showed that the defects of the patellar cartilage of both patients were repaired with fibrocartilage and not with complete hyaline cartilage. However, the repair was much faster than natural repair or repair with conventional marrow stimulation techniques. As early as 2 months after transplantation, the defects were covered with cartilaginous tissue. More importantly, the clinical symptoms were reduced dramatically and remained good for a long time, although both patients continued to show slightly limited knee motion.

It is conceivable that the repair in these cases was to some extent due to multiple perforations or the periosteal flap used simultaneously with the cell transplantation. However, we concluded that cell transplantation was the key contributor to the repair seen in the two cases reported here because of the following reasons. Firstly, it is possible that periosteal transplantation after skeletal maturity may not result in the repair of articular cartilage defects because the chondrogenic potential of periosteum reportedly declines significantly with age (14). Secondly, the clinical outcome has persisted for almost 6 years, although tissues repaired with multiple perforations are reported to result in tissue degradation over time (5,9,10,13).

We previously reported that MSC transplantation for the repair of articular cartilage defects was effective in humans because the arthroscopic and histological repair in the cell-transplanted group was superior to that in the cell-free group (16). In that report, we transplanted MSCs into the articular cartilage defects in the medial femoral condyle of osteoarthritic knees in the same manner used in the cases studies described in this article. Tissue repair was judged to be of higher quality in our precious studies, although the observation period was shorter. Differences in the final location of transplanted materials may also influence the outcome of this approach.

Another procedure for cartilage repair by cell transplantation is autologous chondrocyte implantation, which has been widely performed in the United States and Europe. However, the outcome of repair by means of cell transplantation remains controversial. With autologous chondrocyte implantation, clinical symptoms improved but histological examination showed that repair is insufficient (1,7). Furthermore, it has been reported that with this procedure, repair in the patella was substantially inferior to that in the femoral condyle (1). It is possible that repair by cell transplantation in the patella is inferior to that in the femoral condyle because of certain mechanical properties or the small amount of bone marrow in the patella. Therefore, we concluded that the outcomes of our procedure are not inferior to those of autologous chondrocyte implantation, although the repair in our cases was accomplished with fibrocartilage, not hyaline cartilage.

Moreover, autologous MSC transplantation is clinically much easier to perform because there are no side effects associated with cell collection, which can be per-

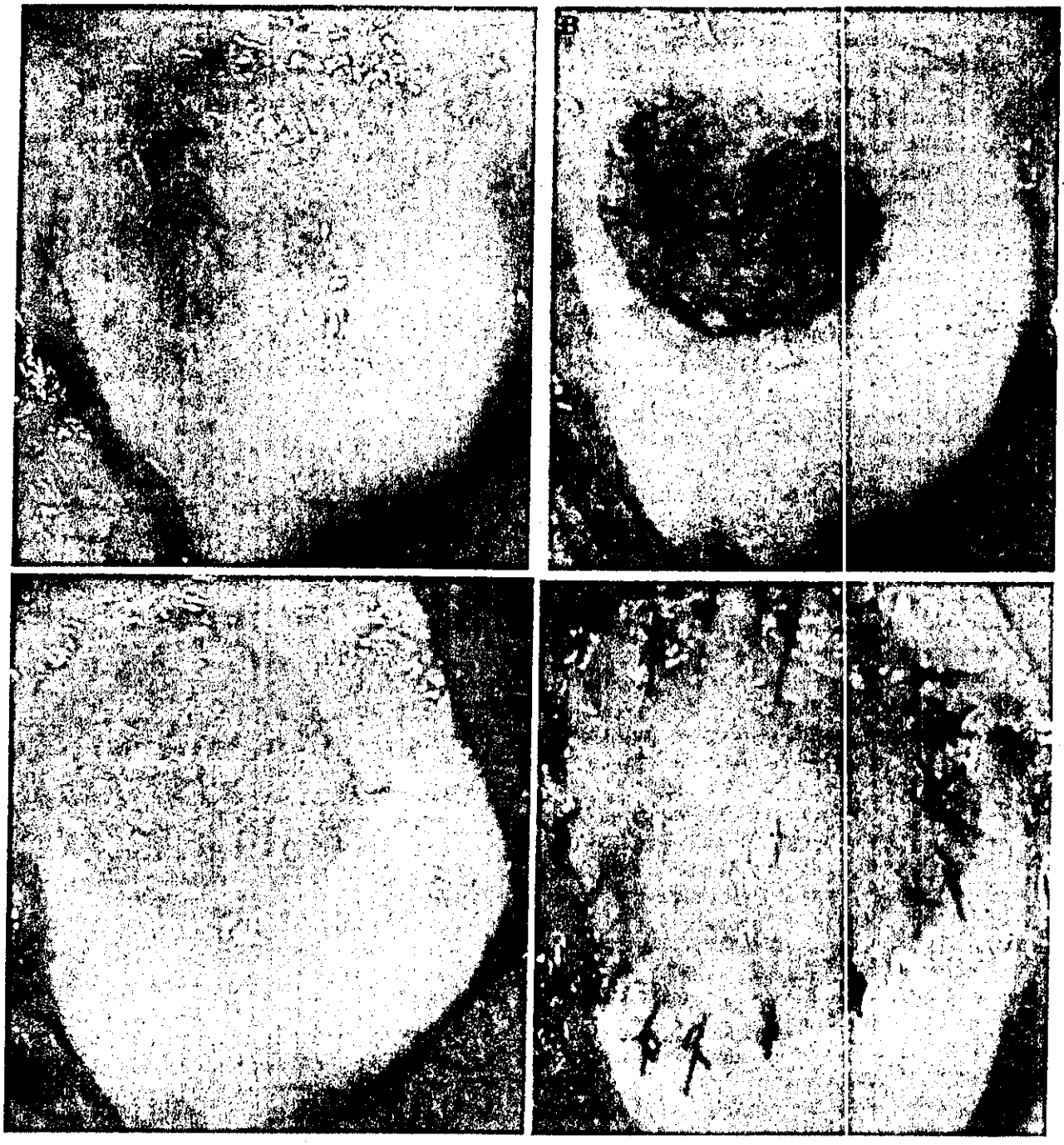

Figure 1. Macroscopic appearances of the patella from the second patient. (A) Damaged articular cartilage. (B) Following removal of damage cartilage. (C) Following implantation of gel–cell composite. (D) Following suturing of autologous periosteum.

formed under partial aesthesia in the outpatient clinic. This also means that surgery is required only once. Furthermore, no bone or cartilage defects remain after collection of autologous cells, and MSCs can proliferate without losing their capacity to differentiate (15).

Cells embedded in collagen gel can be firmly secured in the defect. For the first patient, we used collagen gel only, which was too soft and brittle. For the second patient, we used collagen gel together with a collagen sheet, which was much easier to handle. However, the eventual outcomes of these two patients were very similar.

For these reasons, autologous MSC transplantation can be considered to be a highly promising method for the repair of articular cartilage defects.

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.
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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).

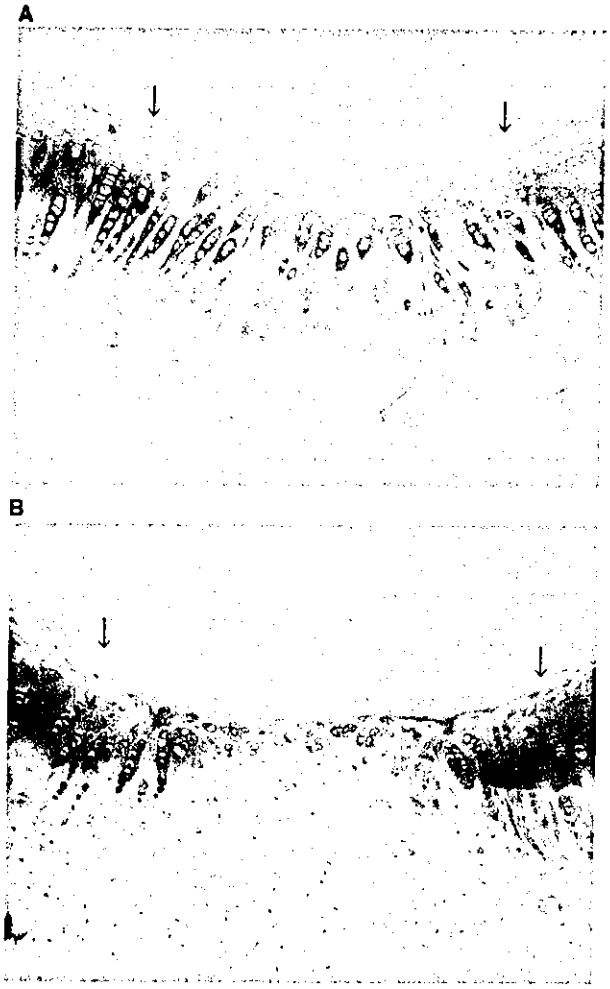

Fig. 1. Microscopic appearance of a perpendicular section of the articular cartilage defect of the control group of the immature rabbits. Toluidine blue staining, original magnification ×40. The arrows indicate the edges of the original defect. (A) The findings at 4 weeks; regenerative response rare. (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.

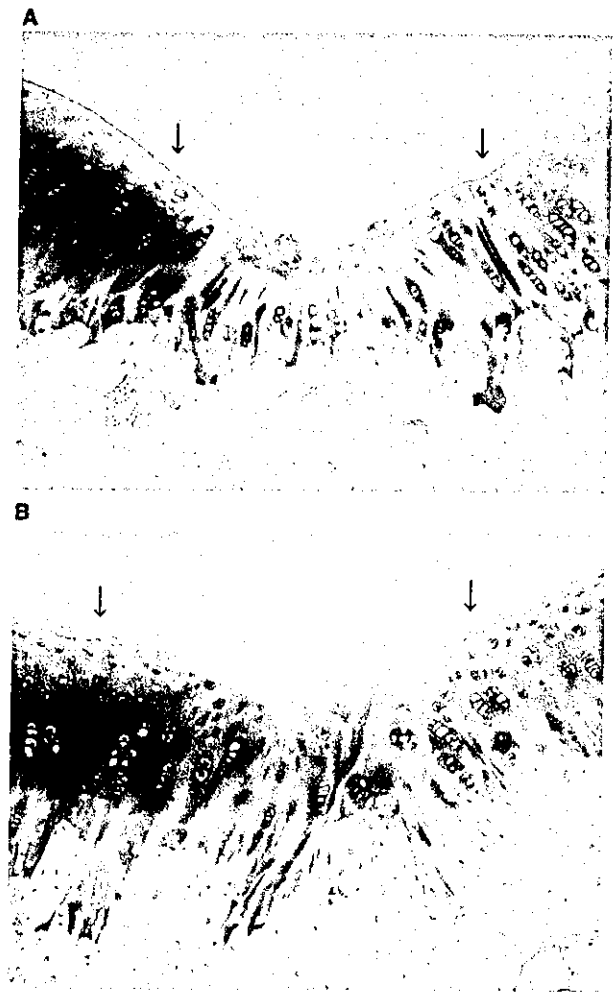

Fig. 2. Microscopic appearance of a perpendicular section of the articular cartilage defect of the FGF-2 (10 ng)-treated group of the immature rabbits. Toluidine blue staining, original magnification ×40. The arrows indicate the edges of the original defect. (A) The findings at 4 weeks; although a few 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.

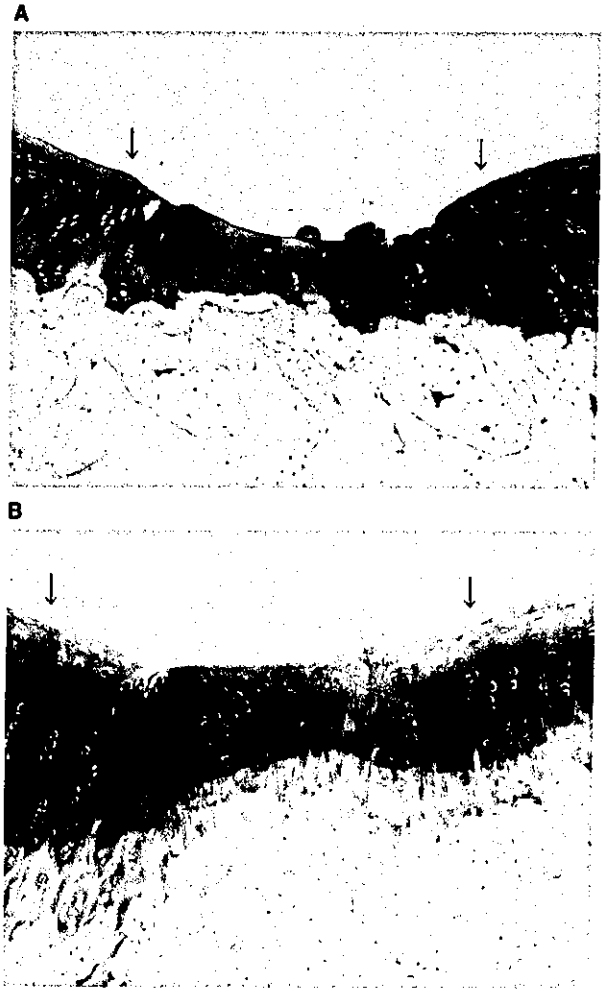

Fig. 3. Microscopic appearance of a perpendicular section of the articular cartilage defect of the FGF-2 (100 ng)-treated group of the immature rabbits. Toluidine blue staining, original magnification ×40. The arrows indicate the edges of the original defect. (A) The findings at 4 weeks; although a few 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)	5.58 ± 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,