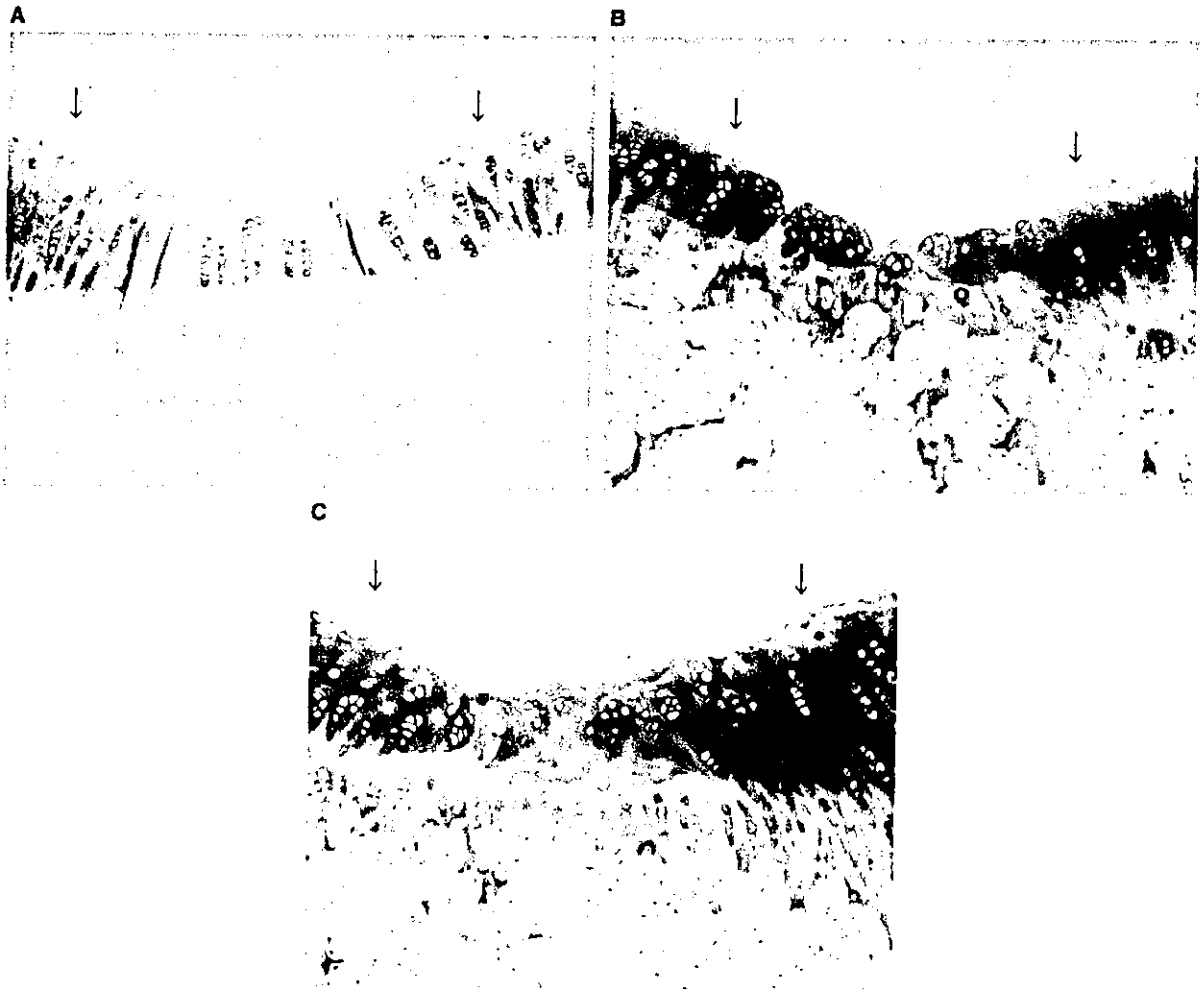

Fig. 4. Microscopic appearance of a perpendicular section of the articular cartilage defect at 48 weeks in mature rabbits. Toluidine blue staining ×40. The arrows indicate the edges of the original defect. (A) The control group, (B) the FGF-2 (10 ng)-treated group, and (C) the FGF-2 (100 ng)-treated 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′-TCTCCAAGGACAAGGAGGTC-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.

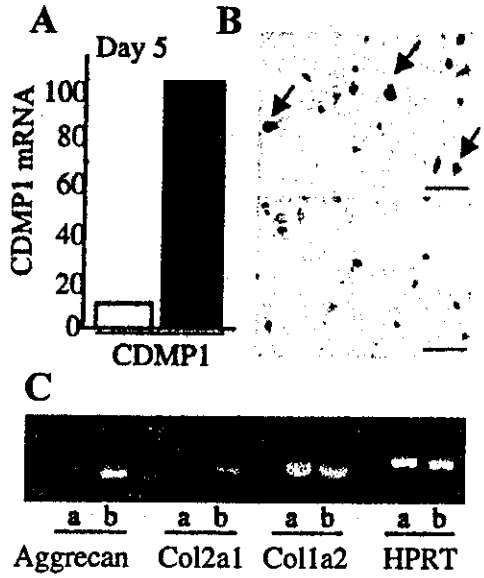

FIG. 1. Expression of CDMP1 and matrix genes. (A) CDMP1 expression in BMMCs was detected by real-time PCR analysis. There was a marked increase in the mRNA level for CDMP1 during CDMP1-transfected BMMC culture for 5 days. Results are the mean of three independent experiments. The value of each CDMP1 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

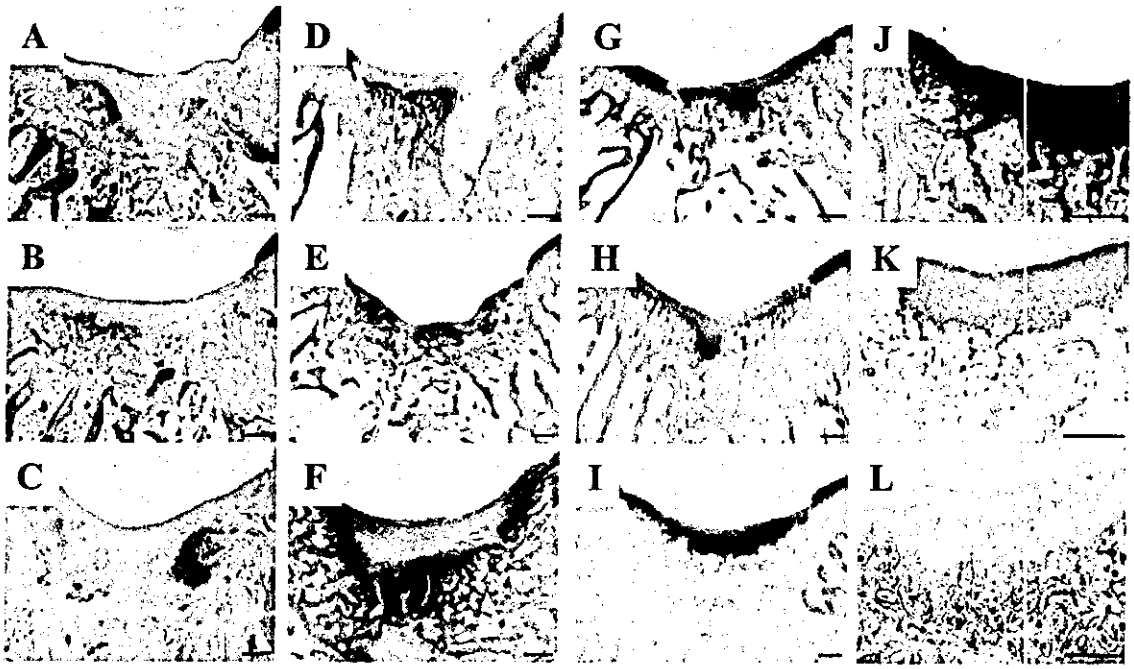

FIG. 2. Representative histological appearance of the defects after 4 weeks. (A, B, C) Empty control group. (D, E, F) Left knees of GFP-transfected BMMC group. (G, H, I) Right knees of CDMP1-transfected BMMC group. D and G, E and H, F and I show bilateral knee specimens from the same rabbit, respectively. 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)							
Interval until animals were killed (weeks)	No.	A Cell morphology and matrix staining	B Surface regularity	C Integration	Subtotal (A–C)	D Filling of defect	E 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.

Acknowledgements

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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Effect of Poly DL-Lactic-Co-Glycolic Acid Mesh on a Three-Dimensional Culture of Chondrocytes

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Collagen gel and a copolymer mesh of polylactate and polyglucuronic acid (PLGA) were combined for a three-dimensional (3D) culture of chondrocyte cells having both uniform cell distribution and mechanical strength. Although the 3D culture in 96-multi-wells caused decreases in the glucose consumption rate and cell density in the latter stages of cultivation, transfer of the culture gel from a 96-multi-well plate to a 24-multi-well plate and an increase in medium volume effectively increased the glucose consumption rate and the accumulation of glycosaminoglycan (GAG) in the gel. The reason for the decrease in glucose consumption rate in a 96-multi-well plate was not the depletion of glucose or the accumulation of lactate in the gel, but the accumulation of degradation products of PLGA.

[**Key words:** chondrocyte, three-dimension, collagen, poly DL-lactic-co-glycolic acid]

There is a standard cultivation method of pellet culture for obtaining three-dimensional (3D) chondrocyte tissues, in which chondrocytes prepicitate after centrifugation and are cultured in a centrifuge tube as it is. However, the size of the pellet is less than 1 mm, while the size of the cartilage tissue to be repaired is more than 5 mm in diameter. This is largely the reason why several 3D cultivation methods for primary chondrocyte cells such as gel-embedded cultures and cultures with porous carriers were developed. We have reported that the transplantation of allogeneic chondrocytes embedded in collagen gels (1, 2), allogeneic chondrocytes cultured in collagen gels (3), or autologous culture-expanded bone mesenchymal stem cells (4), can repair articular cartilage defects in a rabbit model.

Although collagen has a potential advantage in specific cell interactions and is able to obtain a uniform cell distribution in 3D space, it offers limited versatility in designing a scaffold with specific physical properties such as mechanical strength. The shrinkage of gels such as collagen and alginate during culture disturbs the harvesting of a cartilage tissue of the desired size. On the other hand, scaffolds made from synthetic biodegradable materials (5–8) such as polyglycolic acid (PGA), poly L-lactic acid (PLLA) (9, 10), and a copolymer of poly DL-lactic-co-glycolic acid (PLGA) (11) lack cell-recognition signals and uniform cell distribution, but provide a macrostructure with mechanical properties and degradation time that can easily be controlled and manipulated. A combination of a collagen sponge and PLGA may not satisfy the requirement for uniform cell distribution (12).

In the present study, a 3D culture method combining a collagen gel-embedded culture and a PLGA mesh with both uniform cell distribution and mechanical strength without shrinkage during culture was employed for chondrocytes and the effect of the PLGA mesh on the 3D culture was studied.

MATERIALS AND METHODS

Cells Articular cartilage was harvested aseptically from the femoropatellar grooves of knee joints of pigs. Chondrocytes were isolated after digestion of cartilage by type II collagenase and resuspended in MEM medium containing 10% FCS.

Media The culture medium consisted of MEM (Gibco, NY, USA), 10% FCS, 2500 U/l penicillin, 2.5 mg/l streptomycin, and 50 μg/ml L-ascorbic acid 2-phosphate (Wako Pure Chemicals, Osaka). The gelling medium contained 30.5 g/l MEM, 35.7% FCS, 8930 U/l penicillin, 8.93 mg/l streptomycin, and 179 μg/ml L-ascorbic acid 2-phosphate.

Pellet cultivation Pellet culture with high cell density was initiated by centrifugation (500×*g* for 5 min) of 5×10^5 cells suspended in 0.5 ml of the culture medium in 15-ml conical tubes. The pellet was incubated with the supernatant (0.5 ml) for 2 to 4 weeks at 37°C in 5% CO_2 with an initial cell density of 1×10^6 cells/ml-culture, during which the medium was changed weekly.

3D cultivation Eight pieces of PLGA mesh (6 mmϕ, 0.25 mm thickness, interval between bundle of fibers; approximately 400 μm, Vicryl Mesh 910™; Johnson & Johnson, Tokyo) were laid in a 96-multi-well plate for suspension culture (Sumilon, Osaka) (Fig. 1). Cell suspension (3.57×10^6, 3.57×10^7 cells/ml) in the gelling medium was mixed with 2.57 aliquot volume of type I collagen (0.5%, pH 3, Kokencellgen I-PC™; Koken, Tokyo) employing a vortex mixer on an ice bath. Although the major collagen in

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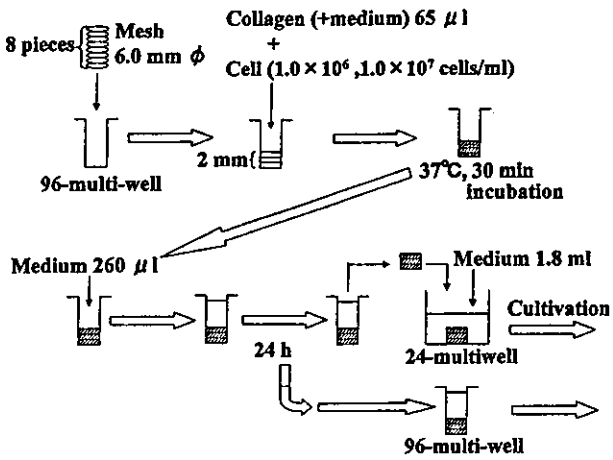

FIG. 1. Schematic start-up flow of the three-dimensional culture combining collagen gel and PLGA mesh.

cartilage is not type 1 but type 2, type 1 collagen was employed here because it is difficult to form a gel with type 2 collagen. After the cell mixture in collagen (65 μl) was poured into the 96-multi-well plate with or without PLGA mesh (the open spaces of the PLGA mesh were just filled with the cell mixture), and incubated at 37°C for 30 min, the culture medium (260 μl) was added and the gel was incubated for 24 h at 37°C in 5% CO_2. Then, the gel (7 mm in diameter) was transferred into a 24-multi-well plate and incubated for 4 weeks at 37°C in 5% CO_2 together with 1.8 ml of culture medium, during which the medium was changed several times in order to avoid glucose depletion. In the case of culture without this transfer, the culture was continued in the medium (260 μl) and initial cell densities were 0.5×10^6 and 0.5×10^7 cells/ml-culture.

Media pretreated with the PLGA mesh were prepared as follows. The medium (260 μl) was placed in a 96-multi-well plate with or without eight pieces of PLGA mesh and incubated for 2 or 7 d at 37°C in 5% CO_2 without cell inoculation. Thereafter, the supernatant in the wells was harvested as the pretreated media.

Cell number analysis The pellet was hydrolyzed at 37°C for 40 min with 5 g/l trypsin (Sigma), 5 g/l type 2 collagenase (Worthington Biochemistry, Lakewood, NJ, USA), and 5 g/l type 1 collagenase (Wako). The 3D gel culture was hydrolyzed at 37°C for 3 h using 2.5 g/l collagenase. Cell concentration was determined by the trypan blue method after hydrolysis.

Staining The pellet and collagen-gel culture were rinsed twice with PBS, fixed in 20% formalin, dehydrated through a graded series of ethanol, infiltrated with isoamyl alcohol, and embedded in paraffin. Sections of 3 μm thickness were cut through the center of the pellets and gel, and were stained with 1% Safranin O in 1% sodium borate.

Glucose and lactate concentrations The glucose and lactate concentrations in the culture medium were determined by the glucose oxidase-peroxidase and lactic acid oxidase-peroxidase methods (Biochemistry Analyzer 2700; YSI Inc., Yellow Springs, OH, USA), respectively. Glucose consumption rate was calculated as an index of cell density and activity. All experiments were replicated two to three times and the reproducibility of the results was confirmed.

RESULTS

Effect of PLGA mesh on cell activity In order to study the influence of PLGA mesh on cell activity, 3D cultures with or without the PLGA mesh in a 96-multi-well plate were performed employing inoculum cell densities of 1.0×10^6 and 1.0×10^7 cells/ml of gel together with pellet culture. Microscopic observation of fixed sections of cultured gel showed uniform cell distribution (data not shown). There was almost no change in volumetric glucose consumption rate throughout the entire period of pellet culture (Fig. 2). The glucose consumption rate in the 3D culture without the PLGA mesh was almost constant except for the initial increase. On the other hand, the volumetric glucose consumption rate in the 3D culture containing the PLGA mesh markedly decreased after 200 h. Cell density was determined at 590 h (Table 1). Cell densities in the 3D culture with the PLGA mesh were apparently lower than those without the PLGA mesh for both inoculum sizes.

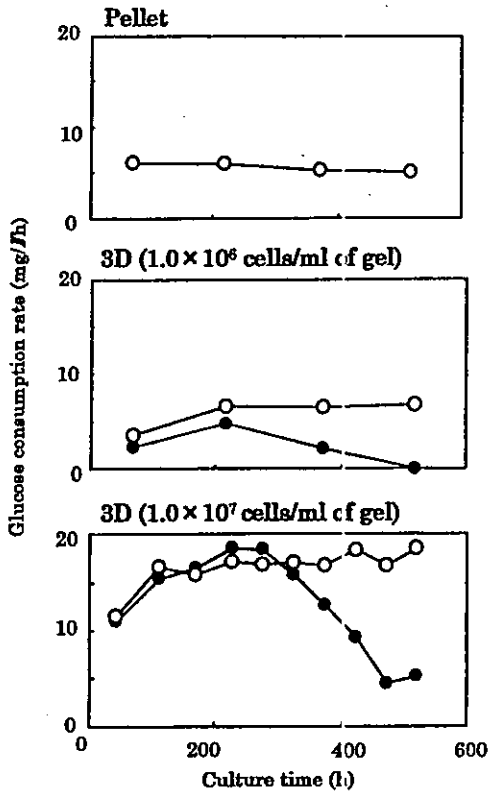

FIG. 2. Influence of PLGA mesh on glucose consumption rate during 3D cultures. Porcine chondrocytes were cultivated in pellets or 3D cultures with (closed circles) or without (open circles) PLGA mesh. 3D cultures were performed in 96-multi-well plates throughout the entire period employing two inoculum cell densities of 1.0×10^6 and 1.0×10^7 cells/ml of gel. The mean of duplicate cultures is shown.

Effect of volume of culture medium on cell activity To confirm the effect of the volume of culture medium on cell activity, 3D cultures with the PLGA mesh were initiated

TABLE 1. Influence of PLGA mesh on cell density

PLGA mesh	Inoculum cell density (cells/ml of gel)	
	1.00×10^6	1.00×10^7
−	1.62×10^6	0.75×10^7
+	0.59×10^6	0.48×10^7

Cell density was determined at 590 h during 3D cultures with or without PLGA mesh as shown in Fig. 2. The average of two cultures is shown.

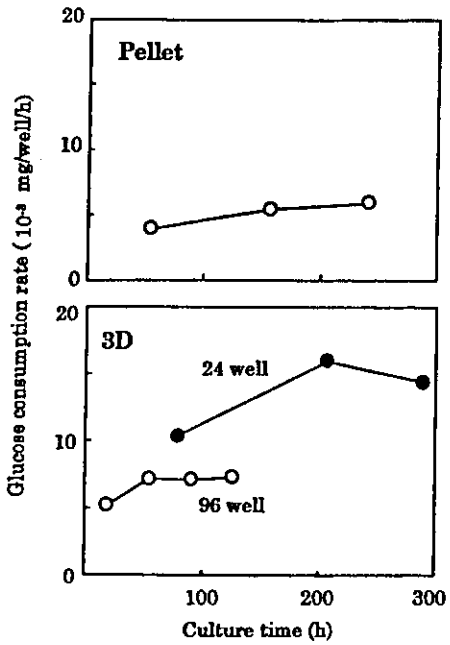

FIG. 3. Effect of medium volume on glucose consumption rate in 3D culture. 3D cultures with PLGA mesh were initiated with an inoculum cell density of 1.0×10^7 cells/ml gel in a 96-multi-well plate with 260 µl medium, and some gels (closed circles) were transferred to 24-multi-well plates with 1.8 ml medium at 24 h. The mean of duplicate cultures is shown.

in a 96-multi-well plate with culture medium of 260 µl and some of the cultures were transferred to a 24-multi-well plate with culture medium of 1.8 ml at 24 h. The glucose consumption rate in the 24-multi-well increased even after 100 h and reached approximately twice that in the 96-multi-well plate at 200 h, while the rate in the 3D culture in the 96-multi-well was almost constant after 50 h (Fig. 3). Sections of the pellet and 3D cultures at 290 h were stained with Safranin O (Fig. 4). The section of the pellet culture was the darkest. The section of the 3D culture in the 24-multi-well plate was visibly darker not only in the center area but also in the peripheral area, compared with the same areas in the 96-multi-well plate.

Effects of glucose and lactate concentrations on cell activity To confirm whether glucose and lactate concentrations in the medium affect the cell activity in the 3D culture, 3D cultures in a 96-multi-well plate were performed employing medium with a normal or higher glucose concentration, and 3D cultures in a 24-multi-well plate were carried out using medium with a normal or higher lactate concentration (Fig. 5). The higher glucose concentration in the 3D cultures in the 96-multi-well plate and the higher lactate concentration in the 3D cultures in the 24-multi-well plate did not affect the glucose consumption rate.

Influence of degradation products of PLGA on cell activity To confirm the influence of degradation products of PLGA on cell activity, 3D cultures in a 24-multi-well plate were performed employing the pretreated media, which was prepared by the method outlined in Materials and Methods. While the glucose consumption rate in the culture with the normal medium increased during the culture period and the use of the medium pretreated for 2 d in the absence of the PLGA mesh slightly influenced the glucose consumption rate, the glucose consumption rate in the culture with the medium pretreated with PLGA for 2 d was markedly low (Fig. 6). Moreover, there was almost no increase in the consumption rate during the culture with the medium pretreated with PLGA for 7 d.

DISCUSSION

The 3D culture with PLGA mesh showed no shrinkage, while the culture without the mesh shrank markedly (data

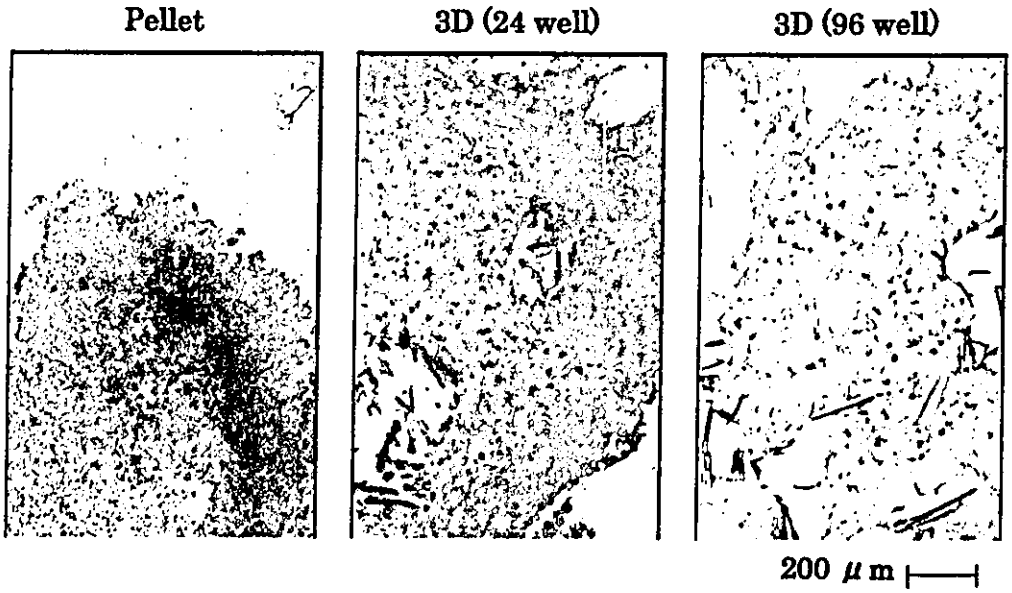

FIG. 4. Effect of medium volume on GAG accumulation in 3D culture. The sections of pellet and 3D cultures shown in Fig. 3 at 290 h were stained with Safranin O.

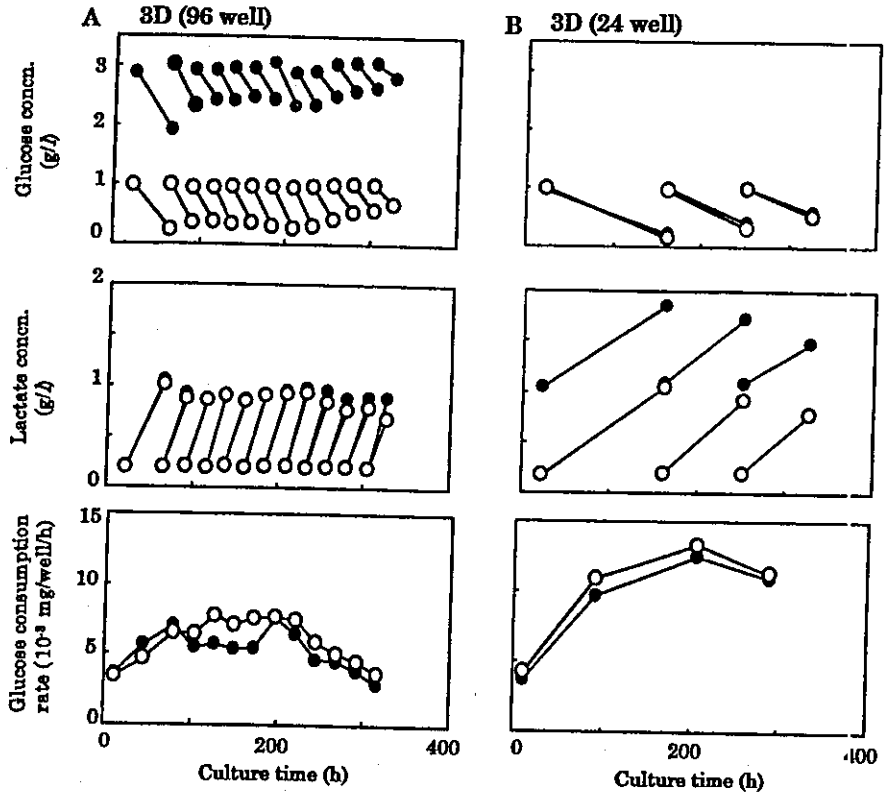

FIG. 5. Influences of glucose and lactate concentrations on 3D culture of chondrocytes. (A) Normal- (1 g of glucose/*l*; open circles) and high-glucose-concentration media (3 g/*l*; closed circles) were employed in 3D cultures in a 96-multi-well plate. (B) Normal-glucose-concentration medium without lactate supplementation (open circles) and high-lactate-concentration medium (1 g of lactate/*l*; closed circles) were used in 3D cultures, which were initiated in a 96-multi-well plate and transferred to a 24-multi-well plate after 24 h.

not shown). Tweezers could be used to hold the 3D culture with the mesh while the culture without the mesh could not be held due to its softness. These results showed the improvement in mechanical strength due to the PLGA mesh.

There was no marked increase in cell density during cultures of chondrocytes (Table 1), which might not be conflicting with the objective of the culture of chondrocytes with matrix accumulation. The degradation products of PLGA

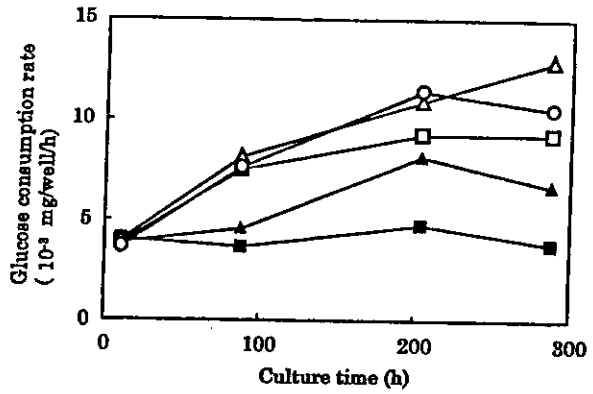

FIG. 6. Influence of degradation products of PLGA on glucose consumption rate in 3D culture. Normal medium (open circles), pre-incubated media without PLGA at 37°C for 2 (open triangles) or 7 d (open squares), and media pre-incubated with PLGA at 37°C for 2 (closed triangles) or 7 d (closed squares) were used for 3D culture with PLGA mesh in a 24-multi-well plate.

mesh may be the reason the cell density in the presence of the PLGA mesh was markedly lower than that in its absence. The ratio of glucose consumption rate to cell density at 590 h in the culture with the PLGA mesh was lower than that without the PLGA mesh (Fig. 2, Table 1). This may also be due to the degradation products of PLGA.

The glucose consumption rate was almost constant throughout the culture without the mesh (Fig. 2). On the other hand, the 3D culture with the PLGA mesh could not maintain the glucose consumption rate.

Employing a larger medium volume and a 24-multi-well plate as shown in Figs. 3 and 4 reduced this problem in the 3D culture with the PLGA mesh, because this modification could maintain a high glucose consumption rate and showed high glycosaminoglycan (GAG) accumulation. Movement of the gel from the 96- to 24-multi-well plate was necessary because gels of an appropriate size for transplantation cannot be formed in 24-multi-well plates.

The reason why the increase in medium volume improved cell activity was investigated. Frequent changing of the medium avoided glucose depletion in the culture supernatant even in the 96-multi-well plate, as shown in Fig. 5. It was suggested by the result in Fig. 5 that there might be no glucose depletion and lactate accumulation inside the gel. On the other hand, there might be inhibition by the accumulation of the degradation products of PLGA inside the gel because the degradation products apparently lowered the glucose consumption rate in the 3D culture (Fig. 6). The

transfer from the 96- to 24-multi-well plate resulted in an increase in not only the medium volume but also the interface area between the gel and medium. While the increase in the interface area might also contribute to the increase in the glucose consumption rate, the increase in diffusion rate of the degradation products may also contribute. The stimulation of the diffusion of the degradation products from inside to outside of the gel may be the reason why the increase in medium volume from 260 μl to 1.8 ml and well size improved the glucose consumption rate and GAG accumulation, while the increase in the glucose consumption rate may involve both an increase in cell density and the cell-specific glucose consumption rate.

Consequently, a 3D culture of chondrocytes was successfully performed without shrinkage by combining a collagen gel-embedded culture with the PLGA mesh. Increasing the medium volume and well size resulted in a high glucose consumption rate and high accumulation of GAG.

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特集　骨・軟骨破壊の分子機構から修復再生に向けて

関節軟骨再生の現状と展望

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修復能力の弱い関節軟骨を再生するためにここ数十年は骨髄刺激方法がおこなわれてきた．修復組織は線維軟骨であると考えられてきたが，短期臨床成績は良好であり，現在でも広くおこなわれている．自己骨軟骨移植の改良型であるモザイクプラスティーはいくつかの問題点があるが，適応を厳選し，うまくおこなえば良好な成績が得られる方法がある．組織工学の進歩により開発された自己軟骨細胞移植あるいは骨髄間葉系細胞移植でもいくつかの問題がある．それらを解決するために，成長因子，遺伝子導入，力学的刺激などを加えて改良する研究がおこなわれている．確実に硝子軟骨で修復でき，特殊な技術を必要とせずどの医療機関でもおこなえ，患者に対する侵襲が低い方法の開発が望まれる．

はじめに

『損傷した関節軟骨は再生しない』と一般的には考えられており，関節軟骨の再生はわれわれ整形外科医にとって長いあいだの夢であった．近年，核磁気共鳴画像診断装置（magnetic resonance imaging：MRI）などの診断技術の進歩により関節軟骨欠損の自然経過を追うことが可能になると弱いながらも修復能力があることが明らかになり，さらに組織工学の進歩により細胞移植という方法が開発され，夢が現実のものとなりつつある．

KEY WORDS

骨髄刺激法
モザイクプラスティー
自己軟骨細胞移植
骨髄間葉系細胞移植
遺伝子導入

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1 関節軟骨の自己修復能力

教科書的には，関節軟骨層内にとどまる浅い損傷（部分欠損）の場合は修復されず，関節軟骨下骨も損傷する深い損傷（骨軟骨欠損）の場合は線維軟骨（本来の関節軟骨は組織学的に硝子軟骨）で修復されると考えられている．しかしながら，関節軟骨の自己修復能力については諸説があり，未だに真実が不明である．これは，関節軟骨の自然修復が，動物種，年齢，損傷の性状（部位，大きさ，深さ），その部分にかかる荷重の大きさなどにより異なるためである．ヒトでは家兎などの動物とくらべて修復が悪い．

関節軟骨欠損を放置した場合の詳細も不明である．軟骨欠損を10～20年の長期間放置すると変形性関節症性変化が生じるとの報告があり[1]，少しずつ解明されてきた．

自然経過が明らかでないために手術適応も確立されていないが，少なくとも，若年者の荷重部の大きな関節軟骨欠損は将来的には変形性関節症になる可能性が高く，修復すべきであろう．しかしながら，どうやって修復す

るかが大きな問題である．

(2) 現在の関節軟骨修復法

1．骨髄刺激法 (marrow stimulation technique)

従来からある関節軟骨欠損修復法は，関節軟骨欠損部の軟骨下骨の連続性を断ち，骨髄から出血させ軟骨前駆細胞および成長因子を供給する方法で，骨髄刺激法とよばれる．これらの方法は約50年前から報告されているが，その修復は硝子軟骨ではなく線維軟骨であると考えられている．線維軟骨による修復の是非については論争中であるが，少なくとも短期成績には問題がないと考えられる．この方法は，短期臨床成績は良好であること，特別な道具を必要とせず簡単で廉価であり，誰でも何処ででもおこなえ，侵襲が小さいという利点があるために，世界中で広くおこなわれており，細胞移植などをする前にまずおこなってみる価値のある方法である．

2．骨軟骨移植

同種骨軟骨移植は，わが国では tissue banking system がなく死体からの骨軟骨片の採取が困難であるために，ほとんどおこなわれない．同種骨軟骨片の採取が可能な国々でも，感染，拒絶反応の問題があり，施行しにくくなりつつある．

自己骨軟骨移植は，自己組織採取による欠損が生じるために，ほとんどおこなわれなかった．しかし，小さな自己組織を関節の周辺部から複数個採取し，それを骨軟骨欠損部にモザイク状に移植するモザイクプラスティーが開発されてから，以前より頻繁におこなわれるようになった．この方法の利点は，組織学的修復が良好であること，あるいは骨軟骨片を特別な道具なしで確実に固定できることである．欠点は，関節全体の曲率を保つよう移植片をあわせるのが技術的に困難であること，関節周辺部であるとはいえ関節軟骨欠損をつくってしまうこと，あるいは修復できる大きさに限界があることなどである．いくつかの問題点はあるが，適応を厳選し，うまくおこなえば良好な成績が得られる方法である．

3．組織再生工学

組織再生工学の発達により，細胞移植による関節軟骨欠損修復がおこなわれるようになった．

a）同種軟骨細胞移植

最初に報告されたのは家兎での同種軟骨細胞移植である．酵素処理で軟骨細胞に分離し，血清に浮遊させて移植したが，十分な成績が得られなかった．われわれは，軟骨細胞をコラーゲン・ゲルに包埋して移植すると修復成績が改善され[2]，コラーゲン・ゲルに包埋して培養し軟骨片にして移植すると成績がさらに改善されることを報告した[3]．同種軟骨細胞移植は実験動物では良好な成績をあげられたものの，臨床応用にあたっては，組織採取，感染症，あるいは拒絶反応の問題があり，実用化が困難であった．米国では，わが国と違って死体からの組織採取が容易であるため，ヒトでの同種軟骨移植の臨床応用が試みられた．Advance Tissue Science 社により商品化に向けた研究がおこなわれていたが，同社の倒産により，その開発は大きく遅れている．

b）自己軟骨細胞移植

自己軟骨細胞移植は，1989年 Grande ら[4]が家兎の実験系で報告し，1994年 Brittberg ら[5]がヒトに応用した．自己細胞移植であるので免疫反応あるいは感染症が問題とならない．この方法は Genzyme Biosurgery 社により Carticel という名で商品化され，米国 FDA で認可された．整形外科の分野で世界で最初に商業化された組織細胞工学を利用した修復法である．

まず関節鏡で荷重にあまり関与しない周辺部の自己関節軟骨を少量採取し，会社の細胞培養施設に送る．そこで軟骨細胞に分離し，増殖させ，病院に送り返す．病院で患者に血清に浮遊させて移植し骨膜で覆う．この方法では良好な臨床成績が得られ，欧米ではすでに約10,000例におこなわれているが，いくつかの問題点が残る．自己組織採取のために別に1回手術が必要であり，さらに関節軟骨欠損が生じること，少量の細胞しか採取できず増殖させる必要があるが軟骨細胞を増殖させるとその分化した形質を失い軟骨細胞としての機能が低下することなどである．これらの方法による修復が，本当に自然修復あるいは骨髄刺激法の修復よりすぐれているのか，一定の結論を得ていない．その原因としては，軟骨欠損の病態や症状がさまざまであること，自然経過がよくわかっていないこと，治療成績の評価法が確立していない

こと，治療成績の比較研究がなされていないこと，長期成績が不明なこと，などがあげられる．最近，自己軟骨細胞移植と骨髄刺激法の1つであるmircofracture法を比較したrandomized controlled trialの成績が報告された[6]．これによると，2年の経過で，臨床成績および組織修復ともこの2つの方法には有意差がなかった．

Ochiら[7]は，自己軟骨細胞をコラーゲン・ゲル内で培養した後，移植して，良好な成績を報告している．

c）軟骨細胞移植の課題

軟骨細胞を二次元培養で，ある程度以上に増殖させると，脱分化して線維芽細胞状となり，軟骨基質の産生を停止する．少量しか採取できない軟骨細胞を，軟骨細胞としての機能を維持したまま増殖させることができれば有用であることから，三次元培養や成長因子の添加による方法が試みられている．また，単層培養で増殖させ脱分化した軟骨細胞を，大量浮遊培養法，アルギン酸ビーズ内培養など，*in vitro*で再分化させる方法も試みられている．

このように，培養方法，成長因子，あるいは力学的刺激により増殖させた軟骨細胞を，*in vitro*で軟骨に分化させ軟骨様組織を作成して移植することが可能となれば，軟骨細胞移植はさらに広くおこなわれるようになると考えられる．

③ 将来の関節軟骨修復法

1．間葉系幹細胞

幹細胞とは，増殖能と分化能をともに備えた細胞である．間葉系幹細胞とは，胎生期の中胚葉に由来する組織（骨，軟骨，筋肉，脂肪，靭帯，腱など）の幹細胞である．これらの組織は整形外科の対象器官であるため，間葉系幹細胞は整形外科分野での組織再生のための細胞として注目されている．

間葉系幹細胞を識別するためにさまざまな研究がおこなわれた．この細胞を特異的に認識する抗体として作成されたSH-2，3は，その後の研究でそれぞれCD 105，CD 106であり，骨髄間葉系幹細胞に特異的ではないことが明らかになった[8]．一方，未分化な造血系幹細胞として報告されたSP細胞（side population cells）は，骨髄のみならず，脳，肝臓，膵臓，腎臓，心臓などほとんどすべての組織に存在し，臓器幹細胞である可能性が示されている．この細胞はHoechst 33342というDNA結合色素を細胞外に運び出す能力が高いために，この色素に染らない細胞である．2001年，間葉系幹細胞の特異的マーカーとして報告されたBcrp 1/ABCG 2は，色素の細胞外へのくみ出しに関与していると考えられる[9]．このように，この細胞の独自の表面抗原は同定されておらず，この細胞を識別するにはいくつかの表面抗原を組み合わせるしか方法はない．CD 29（インテグリンβ1），CD 44（トアルロン酸），CD 105（エンドグリン），CD 106（血管接着因子-1）などは陽性で，造血マーカーであるCD 14，CD 34，白血球マーカーであるCD 45などは陰性である[8]．

2．骨髄間葉系細胞

骨髄血中の有核細胞はそのほとんどが血液系の細胞であり培養すると浮遊する．しかし，ごく一部の細胞は接着，増殖する．この接着細胞を継代培養すると紡錘型の細胞がほとんどを占めるようになり，骨髄間葉系細胞とよばれる．この細胞から骨，軟骨，筋肉，脂肪などの間葉系の細胞が分化誘導されることから，間葉系幹細胞とよばれることもある[8]．しかしながら，骨髄間葉系細胞は増殖能に限界があること，すべての間葉系組織への分化は証明されていないこと，あるいは不均一な細胞集団であることなどから，前述の幹細胞の定義とは完全には合致せず，間葉系幹細胞とよぶのは不適切であるとわれわれは考える．

1999年，骨髄間葉系細胞から，内胚葉由来である肝細胞[10]，外胚葉由来である神経細胞[11]が分化誘導されること（分化転換）が報告され，間葉系のみならず，あらゆる組織再生の細胞源として注目されている．

この細胞の利点は，自己細胞を採取するのが容易であること，および細胞の分化能を維持したまま*in vitro*で細胞を増殖させることが可能であることである．そのため臨床応用が容易である．骨，軟骨の再生のみならず，皮下組織，神経，大血管の内皮，心筋梗塞部の再生，末梢循環障害時の小血管再生促進など，さまざまな応用が考えられている．

3．骨髄間葉系細胞移植による関節軟骨修復

われわれは，この自己骨髄間葉系細胞を骨軟骨欠損部に移植すると，修復が促進されることを家兎の実験系で報告した[12]．この結果をふまえ，2例のヒト膝蓋骨軟骨欠損に移植した[15]．この2症例とも臨床症状の改善は顕著であったが，それが欠損修復によるためという証拠はない．臨床症状の改善が組織の再生のためか，あるいは手術につづく安静（一般的に関節症状は関節を使わなければ軽快することが多い）のために改善されたのかわからない．細胞移植なしでそれ以外は同じ手術を施行したコントロール群をつくって比較しなければ，臨床症状で治療成績を正確に判断できない．

そこで，われわれは，内側型変形性膝関節症に対し高位脛骨骨切り術を受ける患者24症例を対象に，細胞移植群と非移植群の2群を作成し比較することにより，骨髄間葉系細胞移植の有効性を検討した[14]．手術時平均年齢は63歳であった．チャンレー型創外固定を用いた高位脛骨骨切り術をおこない，半数の12例に対しては同時にコラーゲン・ゲルに包埋した自己骨髄間葉系細胞を移植し自己骨膜で覆い，残りの12例では細胞なしのコラーゲン・ゲルを入れ同様の手術を施行しコントロール群とした．臨床成績は，細胞移植群，コントロール群とも術前にくらべて有意に改善したが，両群間で改善度に有意差はなかった．抜釘時，同意が得られた症例で関節鏡を施行し，鏡視下および組織学的に修復組織を点数化し評価した．移植後7週，42週，いずれにおいても細胞移植群が有意に良好な修復であった．今後，さらに経過を追い，長期の臨床成績を評価する必要がある．

4．骨髄間葉系細胞による修復の問題点

骨髄間葉系細胞移植で欠損修復は促進されたが，完全な硝子軟骨による修復を得られなかった．今回，われわれは培養骨髄間葉系細胞には何も処置を加えずそのまま移植し，移植部位での分化促進効果にゆだねたため，分化が不十分であったと考えられる．骨髄間葉系細胞を軟骨細胞に積極的に分化誘導する方法が成績改善に有効である可能性がある．現在，*in vitro* で小さな細胞塊しか軟骨に分化させることはできず，またその場合でも中心部が石灰化するという問題が残る[8]．

骨髄間葉系細胞の培養中に成長因子を投与，あるいは遺伝子を導入して軟骨への分化を促進する方法が研究されている．Sekiya ら[16]は骨形成蛋白（bone morphogenetic protein：BMP）-6を加えると，大きな軟骨片が形成されることを報告した．軟骨増殖あるいは分化促進の作用のあるトランスフォーミング増殖因子-β（transforming growth factor-β：TGF-β），BMP，線維芽細胞増殖因子-2，インスリン様成長因子-1（insulin like growth factor-1：IGF-1）などの，培養細胞への投与が実験的におこなわれている．

またこれらの成長因子の関節内への直接の投与実験も多くおこなわれてきたが，単一で十分な組織再生を得られるものは報告されていない．この方法はペプチドの投与であるために臨床応用が容易であるという利点がある．欠点としては，関節内に投与されたこれらのペプチドは急速に関節内から排除されることである．徐放のために osmotic pump や担体の研究がおこなわれている．

ペプチドの遺伝子を関節内に導入すると，持続的しかも長期間にわたるペプチドの供給が可能になり，理想的なペプチド供給法となる．遺伝子導入のうち，*in vivo* 法では関節内の滑膜あるいは軟骨細胞に遺伝子が導入されることが報告されている．*ex vivo* 法の際の遺伝子導入細胞として，骨髄間葉系細胞は最も有望なものの1つである．遺伝子を導入された骨髄間葉系細胞が移植部位でそれらの遺伝子を発現し，自らが目的組織に分化する可能性（オートクライン）もあるが，周辺細胞にそれらの因子を供給し分化に影響すること（パラクライン）も考えられる．導入する成長因子の遺伝子としては TGF-β，BMP-2，-4，-7，cartilage derived matrix protein-1，IGF-1 などが報告されている[16]．それら以外にも，軟骨の初期分化に関与する Sry-related HMG box（Sox）-5，6，9，PTH/PTHrP，ヘッジホッグファミリー，成長因子の構成的活性型レセプター，細胞内情報伝達物質（BMP における Smads など），あるいは増殖能力を維持するためにテロメラーゼなど，さまざまな遺伝子の導入が試みられている．

また，不均一な細胞の集まりである骨髄間葉系細胞から，本当の骨髄間葉系幹細胞を選択する試みもおこなわれている[17]．

5．他の細胞による関節軟骨修復

同種細胞としては，胚性幹細胞[18]，羊膜細胞などを使った研究がおこなわれている．免疫反応を避けるために，自己核の導入（therapeutic cloning）や，HLAを合わせるためにさまざまなHLAをもった細胞プールなどが必要で，また倫理的問題もあり，実用化には時間がかかると考えられる．

自己細胞としては，末梢血幹細胞，筋肉サテライト細胞，脂肪細胞などさまざまな細胞が研究されている．ヒトの骨髄細胞から誘導され，胚性幹細胞とほぼ同じ性質をもつと報告されたmultipotent adult progenitor cells[19]は，夢の細胞と期待されたが，再現がむずかしく，実用化には更なる研究が必要である．

④ 臨床応用における問題点

実際に細胞移植をおこなう際にいくつかの問題がある．

ヒト移植用細胞を培養するのにはGood Manufacturing Practice基準に適合した培養室が必要であるが，このような施設を各病院に作成するには経済的にむずかしい．細胞培養センターに患者の検体を送り，センターで細胞を分離し増殖させ，病院に送り返すシステムが必要であろうが，そのためには治験が必要であり，まだまだ時間がかかる．

人畜共通感染症の問題がある．そのため牛胎児血清の使用がむずかしくなりつつあるが，これは自己血清で，ある程度は代替可能である．細胞担体としてのコラーゲンも問題である．動物由来ではない，人工のマトリックスを使えるようになることが望ましい．

軟骨再生の手術は侵襲が少ないほうがよい．そのために関節鏡視下のおこなう手術がいくつか報告されている．今後，それらの方法の有用性が明らかにされるとともに，さらに有用な方法の開発が望まれる．

おわりに

確実に硝子軟骨で修復できる方法で，特殊な技術を必要とせずどの医療機関でもおこなうことができ，患者に対する侵襲が低い方法の開発が望まれる．さらには関節リウマチや変形性関節症などの大きな関節軟骨欠損も修復できるように，研究が進むことを期待する．

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骨髄間葉系細胞移植

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はじめに

再生医療における大きな問題の1つに，移植用細胞を増殖させるのが困難であることがあげられる。細胞の増殖と分化は通常は相反するものであるために，増殖させると本来の分化した機能を失ってしまうことが多い。移植用細胞が十分量採取できない自己移植の場合には，細胞を増殖させる必要がある場合が多いためとくに大きな問題である。軟骨再生においても，軟骨細胞を増殖させると脱分化するために，増殖させるのに限界がある。さまざまな研究が行われているが，いまだに解決していない。

幹細胞とは，増殖能と分化能を維持した細胞である。したがって増殖させたあとに必要とする細胞に分化させることができれば，再生医療においては非常に有用性が高い細胞であると考えられている。生体の発生は全能性幹細胞である受精卵から始まり，発生の進行とともに多分化能を失うが，成人の組織のなかにも，それぞれの組織へと分化する前駆細胞（組織幹細胞）が存在すると考えられている（図1）。組織幹細胞としては，間葉系幹細胞，神経幹細胞，造血系幹細胞，消化器幹細胞，皮膚幹細胞，網膜幹細胞などがある。

骨髄血より得られる間葉系細胞は，間葉系幹細胞に近い細胞といわれ，しかも，自己細胞の採取が容易であり，臨床応用されやすい細胞源として注目されている。

骨髄間葉系細胞

骨髄は造血の主要組織であり，骨髄中の細胞は将来血球に分化する造血細胞が中心である。これらの細胞を支持するものの1つに骨髄間質細胞があり，細胞増殖因子やサイトカインを分泌することにより，造血細胞の分化・増殖のサポートを行っている。その骨髄間質細胞のなかの1つに，骨髄間葉系細胞が含まれており，骨・軟骨・脂肪・筋肉などのさまざまな中胚葉系の細胞に分化誘導される能力があることが報告された[1]。さらに，骨髄間葉系細胞から胚葉を超え

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