Articular cartilage repair

Rabbit experiments with a collagen gel-biomatrix and chondrocytes cultured in it

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To repair a full-thickness articular cartilage defect in rabbit knees, we developed a technique of using a collagen gel hardened by cultured allogeneic chondrocytes in it. The gel-chondrocyte composite accumulated an intense metachromatic matrix, and had elasticity and stiffness enough to be shaped easily after 2 weeks' culture in vitro. It was implanted into full-thickness articular cartilage defects. Histologic evaluation was performed up to 6 months after surgery, using a histologic grading scale composed of 5 categories.

In the gel-chondrocyte composite implanted group, good repair was observed from as early as 1 day up to 6 months. On the other hand, in the empty control group, no repair was observed 1 day to 2 weeks after the defects were made. At 4 weeks, some repair occurred, but even at 6 months the repair was not good.

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Isolated chondrocyte implantation is a promising method for the repair of articular defects (Chesterman and Smith 1968, Bentley and Greer 1971, Aston and Bentley 1986). We found up to 80% improvement in the success rate, through the use of implanted rabbit allogeneic articular chondrocytes in a delivery vehicle composed of a type I collagen gel (Wakitani et al. 1989). Brittberg et al. (1994) reported autologous articular chondrocyte implantation into osteochondral defects using a cover of autologous periosteum in humans. Although their overall results were stated to be excellent, a few patients developed severe central wear of the implants that seemed to be related to the poor fixation of the implanted chondrocytes.

Chondrocytes maintain their differentiated characteristics when cultured in collagen gels (Kimura et al. 1984). This is an advantage over the conventional monolayer culture, where the ability of chondrocytes to form cartilage matrix is poorer (Benya et al. 1977). When maintained in culture in this condition for 2 weeks, the collagen gel acquires a higher elastic resistance, without apparent alterations in chondrogenic phenotype. This hardened gel-chondrocyte composite is easy to manipulate and shape and could serve as an appropriate vehicle for chondrogenic cell-mediated repair of cartilage defects. To extend our original study and to continue to develop this technique of using allogeneic chondrocytes for repair of full-thickness cartilage defects (Wakitani et al. 1989), we describe collagen gels hardened by cultured chondrocytes and subsequently implanted into full-thickness cartilage defects. The evaluation of these implants involved use of macroscopic and histologic criteria and continued up to 6 months after surgery for articular cartilage defects.

Material and methods

Chondrocyte preparation

Articular cartilage slices were taken from the proximal humerus, distal femur and proximal tibia of 4-week-old Japanese white rabbits (Nihon Dobutsu Inc., Osaka, Japan), cleaned of adherent connective tissue and cut into small pieces, following death by intravenous injection of 5 mL of Nembutal. Chondrocytes were isolated by enzymatic digestion, as previously reported (Wakitani et al. 1989). Briefly, the cartilage slices were digested for 30 minutes at 37 °C with 0.25% trypsin in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, NY, USA) containing antibiotics (penicillin G, 100 U/

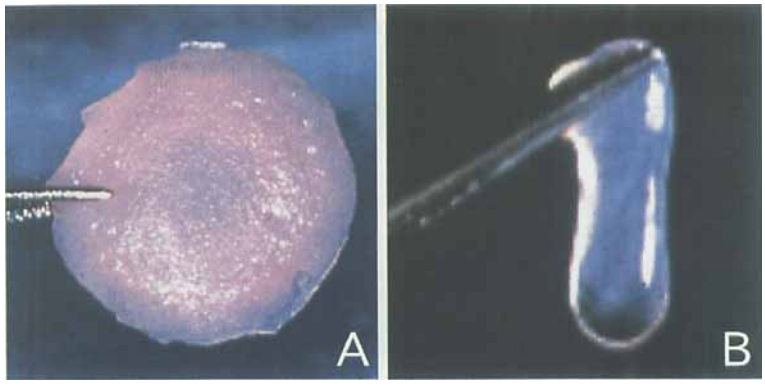

Figure 1. Macroscopic appearance of a collagen gel containing chondrocytes and cultured for 2 weeks (A) and just after gelation (B). The cultured gel-chondrocyte composite, 5 mm in diameter, is hardened and holds its shape even when held with forceps.

mL; streptomycin, 100 µg/mL; amphotericin B, 0.25 µg/mL). After rinsing twice with phosphate-buffered saline (PBS), pH 7.4, and centrifuging at 180 xg for 5 minutes, the slices were digested for 8 hours at 37 °C with 0.25% collagenase (CLS 2, 247 U/mg; Worthington Biochemical Co., Freehold, NJ, USA) in DMEM containing 10% fetal calf serum. The liberated cells were passed through a 110-µm Nitex filter to obtain a single cell suspension. An aliquot of the suspension was exposed to trypan blue and the number of viable cells determined.

Chondrocytes were then introduced into the collagen gel delivery vehicle (Cellmatrix type I-A; 0.3% acid-soluble type I collagen obtained from porcine tendon; Nitta Gelatin, Osaka, Japan). Briefly, 1×10^6 previously pelletted cells were suspended in 100 µL of 2× concentrated DMEM, and 100 µL of collagen solution was added, with gentle agitation at 4 °C. The 200 µL of the collagen-DMEM-cell mixture (final concentration 5×10^6 cells/mL, 0.15% type I collagen) was gelled in a CO_2 incubator at 37 °C within 10 minutes. This gel containing articular chondrocytes was cultured in DMEM containing 10% FCS and 50 µg/mL L-ascorbate (Wako Pure Chemical Industry, Osaka, Japan) for 2 weeks, during which gradual hardening was observed. The medium was changed twice a week, and freshly-passed L-ascorbate was added to the medium every day.

Implantation surgery for osteochondral defects

24 Japanese white rabbits (Nihon Dobutsu Inc) weighing 2.5 kg were used in this study. All the animals were males, and 6 months old, which meant that their growth plates were closed. Rabbits were anesthetized by intramuscular injection of a mixture of Ketamine HCl and Xylazine at the time of implantation.

A full-thickness defect (4 mm in diameter, 4 mm in depth) through the articular cartilage and into the subchondral bone was made with an electric drill applied to the patellar groove on both knees. The implant, which has a shiny appearance and an elastic resistance when palpated manually (Figure 1), was shaped and pushed into the defect, where it remained secure, without any additional fixation. 12 rabbits received the gel-chondrocyte composite implantation in the right knees while defects in the left knees were left empty. They were observed for up to 24 weeks, and were compared at different times. 6 rabbits received the gel-chondrocyte composite implantation in both knees and the other 6 rabbits were left with empty defects in both knees, and observed for 1 day until 2 weeks. All rabbits were returned to their cages and were allowed to move freely after surgery; none had an abnormal gait or mobility difficulties.

Evaluation of cartilage defects

The rabbits were killed 1 day and 1, 2, 4, 12 and 24 weeks after surgery. The entire knees were dissected, examined macroscopically, and photographed, and the distal femurs were fixed for 1 week in 10% buffered formalin. Each specimen was decalcified with a 0.5M EDTA solution containing 0.1M ß-amino-n-caproic acid and 0.005M benzamidine and then embedded in paraffin and sectioned perpendicularly through the center of the defect. Each section was stained with toluidine blue.

The sections were independently scored by at least 3 investigators, without knowledge of the study group being examined. Specimens were scored according to a histologic grading scale (Wakitani et al. 1994), composed of 5 categories with a total score range from 0 to 14 points (Table 1).

When the scores differed between the scorers, they discussed and came to consensus. This scoring was performed again by the same scorers more than one week later, and the repeatability of the scoring was ascertained.

Table 1. Histologic grading scale for defect cartilage

A. Cell morphology	0	hyaline cartilage
	1	mostly hyaline cartilage
	2	mostly fibrocartilage
	3	mostly non-cartilage
	4	non-cartilage only
B. Matrix staining (metachromasia)	0	normal (compared to host)
	1	slightly reduced
	2	significantly reduced
	3	no metachromatic stain
C. Surface regularity	0	smooth (>3/4 [a])
	1	moderate (1/2< <3/4 [a])
	2	irregular (1/4< <1/2 [a])
	3	severely irregular (<1/4 [a])
D. Thickness of cartilage	0	>2/3 [b]
	1	1/3< <2/3 [b]
	2	1/3 [b]
E. Integration of donor to host adjacent cartilage	0	both edges integrated
	1	one edge integrated
	2	both edges not integrated
Total maximum: A–E	14	

[a] Total smooth area of reparative cartilage compared to the whole area of the cartilage defect.
[b] Average thickness of reparative cartilage compared to that of surrounding cartilage.

Statistics

The difference in scores between the gel-chondrocyte composite implanted group and the empty control group at different postoperative times (4 weeks, 12 weeks and 24 weeks after surgery) was tested, using two-way factorial ANOVA, followed by the Mann-Whitney U-test for comparison. P-values less than 0.05 were considered significant.

Results

Collagen gels in which chondrocytes were cultured

Initially, collagen gels containing chondrocytes were very soft and drooped when held. However, when they were maintained in culture for 2 weeks, they gained elasticity (i.e., hardened) and kept their shape when they were grasped with forceps (Figure 1). Histology of the gels cultured for 2 weeks showed abundant metachromatic matrix, which suggests the active accumulation of cartilage proteoglycans and other matrix macromolecules (Figure 2). The cellular morphology at this time is that of a cartilage tissue comparable to that of in vivo preparations containing well differentiated, biosynthetically active chondrocytes surrounded by a typical cartilage-like metachromatic matrix.

Macroscopic observations of cartilage defects

At all times following implantation, no signs of arthrosis (such as osteophytes, cartilage erosion or synovial proliferation) were observed in any operated knees, including the control knees.

At 1 day after implantation, the defects were filled with a dull white material that was easily distinguishable from the surrounding host articular cartilage. At 2 weeks, the findings were similar. At 24 weeks, the implants resembled the surrounding articular cartilage, although the color of the implant was still white and the margin of the defect was still discernible (Figure 3).

In the control group, at 1 day after surgery, the defect was filled with a pink or dark red blood clot hav-

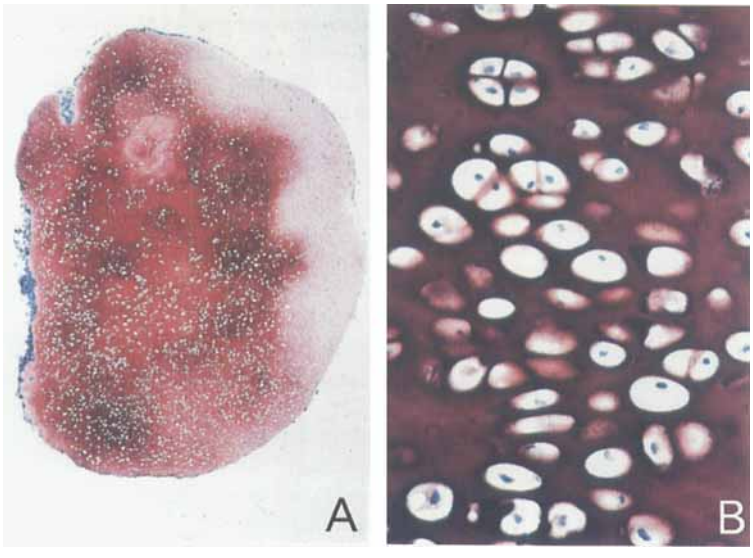

Figure 2. Photomicrographs of a section of chondrocytes embedded in a collagen gel and cultured for 2 weeks prior to in vivo implantation. The sample was fixed with formalin and stained with toluidine blue, × 20 (A) and × 160 (B).

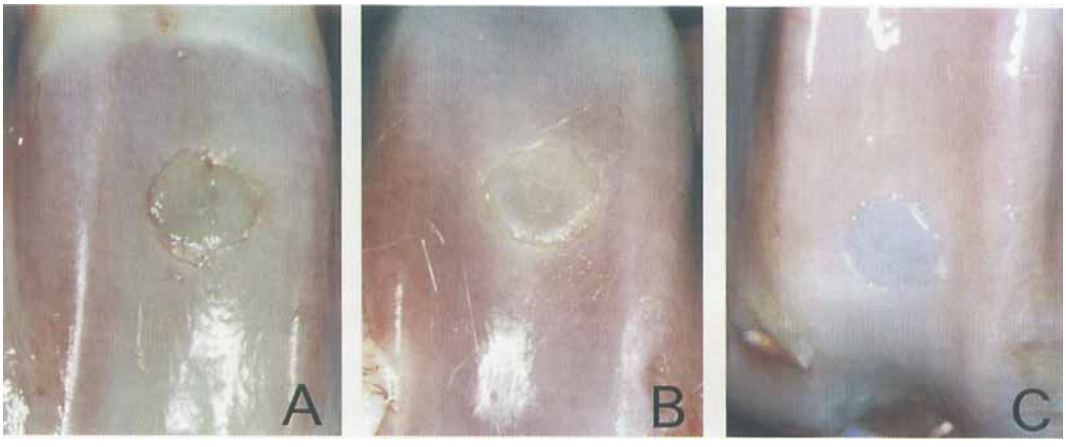

Figure 3. Macroscopic appearance of a defect in the femoral patellar groove of a rabbit knee at 1 day following implantation of hardened gels with chondrocytes (A), at 2 weeks (B) and at 24 weeks (C). Defects are 4 mm in diameter.

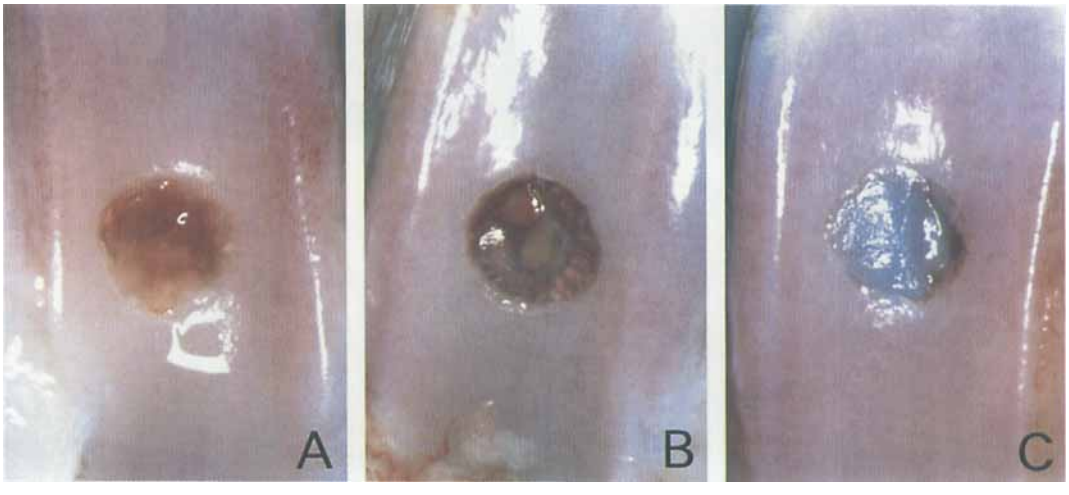

Figure 4. Macroscopic appearance of a control defect in the femoral patellar groove of a rabbit knee at 1 day (A), at 2 weeks (B) and at 24 weeks (C) after the defect was made. Defects are 4 mm in diameter.

ing an irregular surface. At 2 weeks, the color was still pink or red and the surface was irregular. By 24 weeks, the color of tissues filled into the defect was white; however, the surface was irregular and the defect margin was still easily recognized (Figure 4).

Histologic observations of cartilage defect

No signs of arthrosis (such as fibrillation, erosion, cracks, claster formation) were observed histologically in any articular cartilage, except the reparative tissue in the control group.

At 1 day, the repair tissue had filled all of the original defects and exhibited an intensely staining metachromatic matrix. The cellular morphology of the predominant tissue resembled that produced by well differentiated chondrocytes. However, there was poor integration of the implant into the surrounding bone and cartilage. At 2 weeks, metachromasia of the superficial layer was decreased and, in some cases, a central depression was observed and thin perichondrial-like tissue covered the surface of the composites. At 12 weeks, the implanted tissue had the morphology of hyaline cartilage and a smoother surface; the previously observed central depression was not found. Substantial integration of the donor tissue to adjacent host cartilage was observed in most cases. The decrease in metachromasia of the surface layer of the composite was maintained in some cases. Even 24 weeks after implantation, the repair cartilage remained in the original defect without a decrease in thickness; this well differentiated defect cartilage was not invaded by vessels or replaced by bone at 24 weeks after implantation. The surface of the implant was smooth, and integration of implant tissue into the

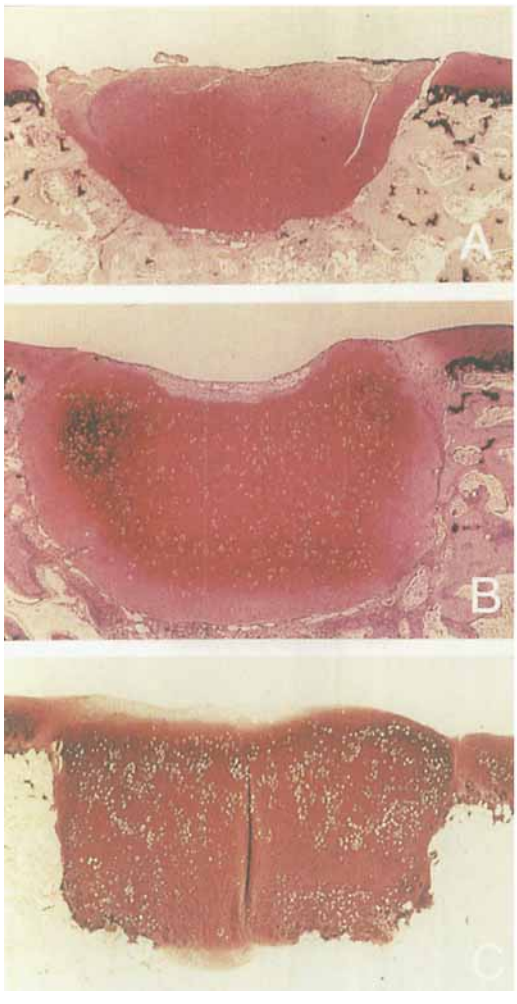

Figure 5. Photomicrographs of sagittal sections of the femoral patellar groove at different healing periods following gel-chondrocyte implantation: (A) 1 day, (B) 2 weeks and (C) 24 weeks after surgery. Toluidine blue staining, × 15.

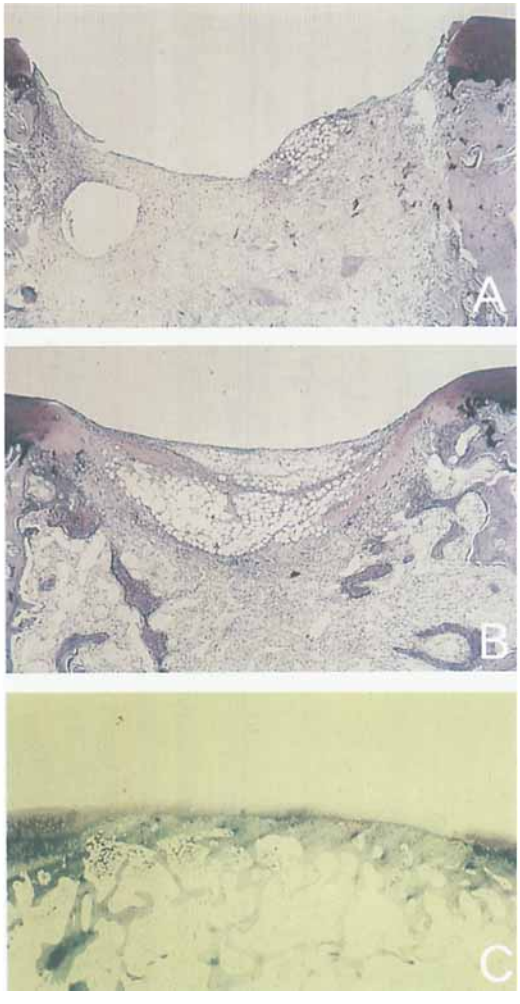

Figure 6. Photomicrographs of sagittal sections of the femoral patellar groove at different healing periods after the defect was made: (A) 1 day, (B) 2 weeks and (C) 24 weeks after surgery. Toluidine blue staining, × 15.

original cartilage was seen. The intense metachromasia of the composite was comparable to that of adjacent normal articular cartilage in most of the implants except in small areas close to the joint space (Figure 5).

In the empty control group, the defect was not filled completely until 4 weeks after surgery. A faint or dilute metachromatic staining was observed 4 weeks after surgery, and it became stronger with time. At 24 weeks after surgery, although subchondral bone remodeled and extended to the level of the host bone, the articular cartilage zone was covered with thin metaplastic cartilage having a faintly metachromatic matrix (Figure 6).

Histologic scoring of the repair tissue (Table 2)

The scores for the implanted gel-chondrocyte composites were impressive from as early as 1 day after implantation up to 24 weeks.

In the empty control group, no repair was observed 1 day to 2 weeks after the defects were made. At 4 weeks, some repair was observed (mean 7 points), but even at 24 weeks the repair was not good (mean 9 points).

In comparison with the empty control group, the total scores of the gel-chondrocyte composite implanted group were better at 4 weeks until 24 weeks after surgery (two-way factorial ANOVA, $p < 0.0001$). The total scores of the gel-chondrocyte composite implanted group were better than those of

Table 2. Results of histologic grading scale, mean score (range)

	Time after surgery	Scores on histologic grading scale					
		A	B	C	D	E	Total [a]
Early periods (from 1 day to 2 weeks)							
Chondrocyte-	1 d	1.0 (1–2)	1.0 (1)	1.0 (0–2)	0.0 (0)	2.0 (2)	5.0 (3–6)
implanted	1 w	0.5 (0–1)	0.5 (0–1)	1.0 (0–2)	0.0 (0)	2.0 (2)	4.0 (2–6)
	2 w	0.8 (0–1)	0.8 (0–1)	0.8 (0–1)	0.0 (0)	1.5 (1–2)	3.8 (1–6)
Empty	1 d	4.0 (4)	3.0 (3)	3.0 (3)	2.0 (2)	2.0 (2)	14.0 (14)
control	1 w	4.0 (4)	3.0 (3)	3.0 (3)	2.0 (2)	2.0 (2)	14.0 (14)
	2 w	4.0 (4)	3.0 (3)	3.0 (3)	2.0 (2)	2.0 (2)	14.0 (14)
Late periods (from 4 weeks to 24 weeks and showing statistical comparisons)							
Chondrocyte-	4 w	0.3 (0–1)	1.0 (0–2)	1.3 (0–2)	0.0 (0)	1.5 (1–2)	4.0 (2–6) [b]
implanted	12 w	0.5 (0–1)	0.5 (0–1)	0.3 (0–1)	0.0 (0)	1.3 (1–2)	2.5 (1–4) [b]
	24 w	0.8 (0–1)	0.5 (0–1)	0.5 (0–1)	0.0 (0)	1.0 (0–2)	2.8 (0–5) [b]
Empty	4 w	2.0 (2)	1.8 (1–2)	1.3 (1–2)	0.5 (0–1)	1.8 (1–2)	7.3 (6–9)
control	12 w	1.3 (0–2)	2.0 (2)	1.8 (1–2)	1.5 (1–2)	0.8 (0–2)	7.3 (4–10)
	24 w	1.8 (1–3)	1.5 (1–2)	2.0 (1–3)	1.8 (1–2)	1.8 (1–2)	8.8 (6–12)

[a] Total is the average of totals of each case.
[b] $p < 0.05$ when compared to the empty control at the same time after surgery.

the empty control group at 4, 12 and 24 weeks after surgery (Mann-Whitney U-test, $p = 0.04$, 0.03 and 0.02, respectively).

The score for surface regularity (category C) also showed a tendency to improvement with time, although not statistically significant. The thickness of the implants (category D) was maintained throughout all sampling times and was especially impressive at 24 weeks. The integration of the implant (category E) was poor until 1 week, but from 2 weeks after the implantation, the repair tissue had an improved score; after 12 weeks the score was significantly improved, compared that of earlier sampling times.

Discussion

Our 6-month investigation of implanted gel-chondrocyte composites cultured for 2 weeks prior to implantation shows that full-thickness articular cartilage defects can be repaired with hyaline cartilage from as early as 1 day. Although the observation period is short, the repaired cartilage is histologically one of the most impressive hyaline-like cartilage ever reported (Aston and Bentley 1986, O'Driscoll et al. 1988, Grande et al. 1989, Noguchi et al. 1994). Moreover, the very early success on 1 day is remarkable.

It is generally accepted that immature articular cartilage has greater repair capacity than a mature one and that the capacity subsides with age (Buckwalter et al. 1987). Thus, articular cartilage of adolescent rabbits used in our study is thought to have greater repair capacity than that of aged rabbits. In comparison with the good spontaneous repair of the adolescent rabbits, the repair using gel-chondrocyte composite implantation was significantly better.

The use of chondrocytes embedded in a suitable matrix allows the rapid circumferential accumulation of newly synthesized matrix, just as in normal articular cartilage. By allowing the cartilage matrix to accumulate while culturing the gel-chondrocyte composite, protection against mechanical pressure or metabolic changes experienced at implantation is established. In previous studies (Wakitani et al. 1989), we used chondrocytes embedded in collagen gels without culturing the composite; thus, the chondrocytes were not surrounded with their own matrices and were vulnerable to environmental influences at implantation. We now suggest that this lack of surrounding matrix compromises the success rate.

The incorporation of the implant into surrounding bone and cartilage was poor until 2 weeks. Thereafter, bone was observed around the undersurface of the implant, and the incorporation of donor into surrounding host articular cartilage was improved. However, because this was not complete, even 6 months after implantation, we must attempt to improve this incorporation of the repair cartilage into the host, perhaps by using enzymatic predigestion of the defect (Mochizuki et al. 1993).

At all study times following implantation, the repair cartilage maintained its thickness into the full depth of the original defect; the tissue derived from the implanted chondrocytes was not invaded by vessels or replaced by subchondral bone. The cartilage was probably protected from vascular invasion by anti-angiogenesis factors (Moses et al. 1990) or by its dense cartilage matrix. The repair tissue in the depth

of the defect obviously did not progress to the hypertrophic stage, which would not be replaced by subchondral bone, as shown in our previous report (Wakitani et al. 1989). Although overt indications of arthrosis were not observed 6 months after implantation, it is possible that the lack of regeneration of a proper subchondral bony base in this repair tissue will contribute to failure of this construct at later times.

We suggest that our approach is of value for clinical use. The hardened gel we used has elastic resistance and is easy to handle and shape. In previous reports with unhardened collagen gels, fixation of the gels into the defects was difficult and thus limited their utility (Wakitani et al. 1989). However, using these cell-hardened gels, we can manipulate the composite into any shape and can place well formed tissue into a variety of defects. In a small defect, the implant can be lodged without fixation, because the gel-chondrocyte composite is elastic. With large defects, staples or sutures can be used to fix the gels, because the composite is hard and can, therefore, be immobilized.

The source of chondrocytes is of clinical relevance. Allogeneic chondrocytes can be isolated and multiplied in vitro, without loss of their differentiated phenotype, by using three-dimensional gel culture conditions (Kimura et al. 1984) and/or specific growth factors (Kato et al. 1987, Morales and Roberts 1988, Wakitani et al. 1997). The results of a graft of chondrocytes in the hardened gel format we describe appear to be as good as those for isogeneic grafts (Noguchi et al. 1994). In this regard, chondrocytes can be collected from amputated human limbs or from joint arthroplasties, or autologous chondrocytes can be collected from non-weight-bearing surfaces. Moreover, these chondrocytes can be stored frozen until needed when they can be thawed and embedded into a collagen gel and the composite can be cultured for 2 weeks.

Acknowledgments

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