Hiroshima J. Med. Sci.
Vol. 50, No. 4, 87~96, December, 2001
HIJM 50–14

Repair of Articular Cartilage on the Surface of Heat-treated Bone by Transplantation of Cultured Chondrocytes

Seigo NAKAMURA, Takashi SUGITA, Yu MOCHIZUKI, Yuji YASUNAGA, Kazuhiko KIKUGAWA and Yoshikazu IKUTA

Department of Orthopaedic Surgery, Hiroshima University Faculty of Medicine, 1–2–3 Kasumi, Minami-ku, Hiroshima 734–8551, Japan

ABSTRACT

The present study addresses clinical problems associated with the degeneration of articular cartilage, which occurs when heat-treated bone with articular cartilage is used for re-implantation after resection of malignant bone tumors adjacent to the joints. We therefore evaluated the effect of transplantation of chondrocytes embedded in collagen gel on the surface of heat-treated bone.

A cylindrical complex of bone and articular cartilage 6 mm in diameter was resected from rabbits' patellar grooves and treated in saline at 60°C for 30 min. In Group A, articular cartilage was resected from the complex and the remaining bone was returned to the patellar groove. Then, autologous chondrocytes cultured in collagen gel were transplanted and covered with periosteum. As controls, the original complex of heat-treated bone and articular cartilage (Group B) and heat-treated bone directly covered with periosteum (Group C) was returned to the patellar groove.

In Group A, histological study showed that round cells were mainly observed and the matrix was well stained with Safranin O in the repair tissue after 24 weeks. The repair tissue was as thick as the adjacent normal cartilage. Immunohistological study detected type-II collagen and chondroitin-6-sulphate (3B3+) in the matrix of the repair tissue, but not type-I collagen. The repair tissue was consequently cartilaginous in Group A. The repair tissue was not cartilaginous or was degenerative in the control groups.

We believe that this modality of heat-treated joints will contribute to limb salvage reconstruction after resection of malignant bone tumors adjacent to the joints.

Key words: *Articular cartilage, Heat-treated bone, Autologous chondrocyte, Malignant bone tumor*

Re-implantation of resected bone treated at 60°C for 30 min is a means of providing not only tumor cell necrosis but also skeletal reconstruction and is currently used in operations on malignant bone tumors. Even after attempting to reconstruct joints with heat-treated bone, total joint replacement with artificial materials is indicated for patients who acquire tumors adjacent to the joints. However, total joint replacement does not usually survive throughout the patient's lifetime. Total joint replacement is thus a burden especially for young patients. On the other hand, re-implantation of resected and heat-treated joints has been attempted in several cases but has resulted in the degeneration of articular cartilage because chondrocytes are unable to survive such heat-treatment[5].

Some authors have reported that transplantation of cultured chondrocytes was able to repair defects in the articular cartilage and restore articular cartilage[1,2,11]. Manabe et al[6] also described how bone heat-treated at 60°C for 30 min was gradually replaced with viable bone in a manner similar to that of fresh bone grafts and that its mechanical strength was similar to that of fresh bone. In view of these findings, heat-treated bone has the potential of supporting articular cartilage and providing cytokines, such as bone morphogenic protein (BMP). The present study was therefore conducted to confirm whether cultured chondrocytes in collagen gel could repair cartilaginous tissue on the surface of heat-treated bone after transplantation.

MATERIALS AND METHODS

117 Japanese White Rabbits, comprising 40 rab-

tel: +81822575233, fax: +81822575234

bits weighing 1,000 g and 77 rabbits weighing 2,000 g, were prepared for the examination. Articular cartilage slices were obtained from the left knee of 40 rabbits weighing 1,000 g and cut into small pieces. Chondrocytes were isolated by enzymic digestion. After culturing in Dulbecco's Modified Eagle's Medium (DMEM, Nacalai Tesque Inc., Kyoto Japan) containing 10% Fetal Bovine Serum (FBS,Walkersville, Maryland) for 10 days, chondrocytes were suspended in a 0.2% type-I collagen-DMEM mixture (KOKEN, Tokyo Japan) and adjusted to 5×10^6 cells/ml as previously described[12]. After culturing for 3 weeks in collagen gel, the chondrocytes were transplanted into the right knees of the same rabbits. The rabbits, which had weighed 1,000 g when the cartilage slices were obtained, weighed 2,270 g (1,950–2,830 g) on average at the time of transplantation. Before transplantation, a cylindrical complex of articular cartilage and subchondral bone 6 mm in diameter and 8 mm in height was removed from the patellar groove of the right femur and heat-treated in saline at 60°C for 30 min. Articular cartilage was resected from the cylindrical complex and the remaining bone of the complex was returned to the patellar groove. Chondrocytes in collagen gel were transplanted onto the surface of the heat-treated bone and covered with periosteum (Group A: 40 rabbits)(Fig. 1). As controls, cylindrical complexes of bone and articular cartilage were removed from the patellar groove of 77 rabbits weighing 2,000 g, and heat-treated in saline at 60°C for 30 min. In 38 of these rabbits, the heat-treated complex of articular cartilage and bone was returned to the patellar groove (Group B). In the remaining 39 rabbits, the articular cartilage was resected from the cylindrical complex and the remaining bone was returned to the patellar groove and covered with periosteum (Group C). The weight of the rabbits in Group A was equal to or heavier than that of the other groups at the time of transplantation.

The rabbits were allowed to move their knees freely in cages after the procedures. They were subsequently sacrificed at 3, 6, 12, and 24 weeks. The specimens were fixed with 4% formaldehyde for 96 hours, decalcified with EDTA for 4 weeks and embedded in paraffin. Specimen slices were stained with hematoxylin and eosin (H.E.) and Safranin O-fast green and evaluated histologically by three members of the study team following the method of Sellers[10]. We adopted the grading scale designed by Sellers et al[10] because this grading scale includes an evaluation not only of the repair tissue itself but also of the subchondral bone and tidemark, which were important factors in this study. Safranin O-fast green staining was used to identify the presence of sulfated glycosaminogly-

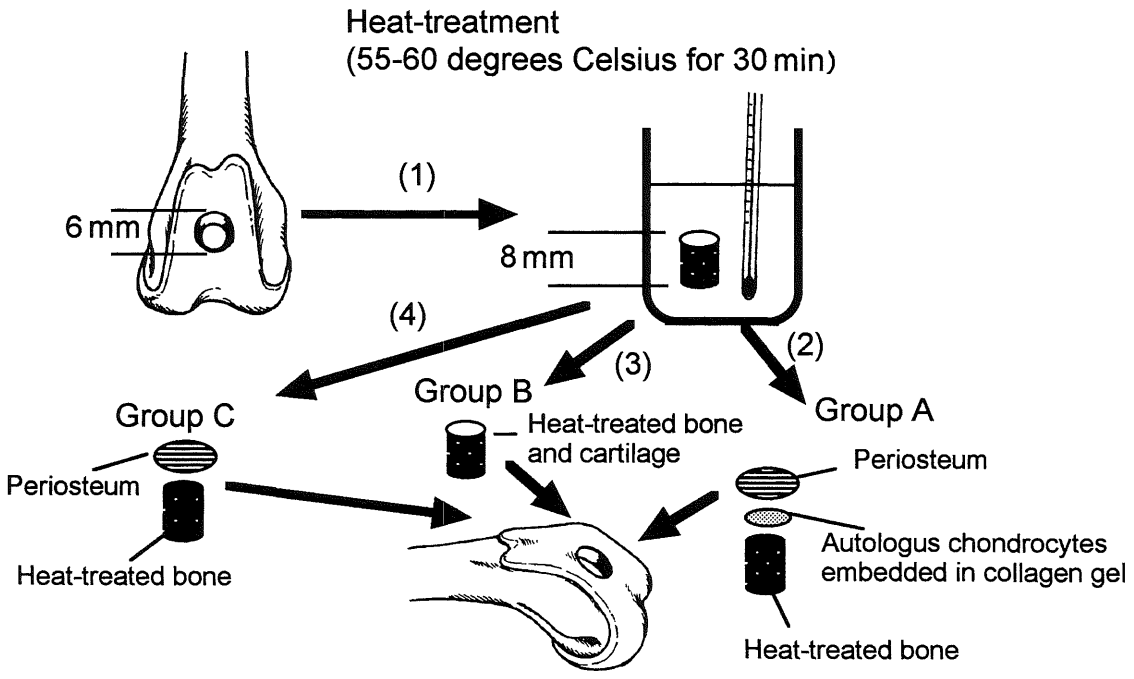

Fig. 1. Graft procedures.
(1) A cylindrical complex of bone and articular cartilage with a width of 6mm and height of 8 mm was removed, and heat-treated at 55–60°C for 30 min. (2) Articular cartilage was removed from the heat-treated complex and the remaining bone was returned to the patellar groove. Chondrocytes cultured and embedded in collagen gel were transplanted on the surface of heat-treated bone, and everything was covered with periosteum (Group A). (3) The heat-treated cylindrical complex of articular cartilage and bone was returned to the patellar groove (Group B). (4) Articular cartilage was removed from the heat-treated complex and the bone was returned to the patellar groove. The bone was directly covered with periosteum (Group C).

Table 1. Modified histological grading scale for articular cartilage[10]

Category	Score (Points)
1: Fillings of defect relative to surface of normal adjacent cartilage	
111–125%	1
91–110%	0
76–90%	1
51–75%	2
26–50%	3
< 25%	4
2: Integration of repair tissue with surrounding articular cartilage	
Normal continuity and integration	0
Decreased cellularity	1
Gap or lack of continuity on one side	2
Gap or lack of continuity on two sides	3
3: Matrix staining with safranin O	
Normal	0
Slightly reduced	1
Moderately reduced	2
Substantially reduced	3
None	4
4: Cellular morphology	
Normal	0
Mostly round cells with the morphology of chondrocytes	
> 75% of tissue with columns in radial zone	0
25–75% of tissue with columns in radial zone	1
< 25% of tissue with columns in radial zone (disorganized)	2
50% of round cells with the morphology of chondrocytes	
> 75% of tissue with columns in radial zone	3
< 25% of tissue with columns in radial zone (disorganized)	4
Mostly spindle-shape (fibroblast-like) cells	5
5: Architecture within entire defect (not including margins)	
Normal	0
1–3 small voids	1
1–3 large voids	2
> 3 large voids	3
Clefts or fibrillations	4
6: Architecture of surface	
Normal	0
Slight fibrillation or irregularity	1
Moderately fibrillation or irregularity	2
Severe fibrillation or irregularity	3
7: Percentage of new subchondral bone	
if new bone is below original tidemark	
90–100%	0
75–89%	1
50–74%	2
25–74%	3
< 25%	4
if new bone is above original tidemark	
(average percent of original thickness of repair articular cartilage)	
90–100%	0
75–89%	1
50–74%	2
25–74%	3
< 25%	4
8: Formation of tidemark	
Complete	0
75–99%	1
50–74%	2
25–49%	3
< 25%	4

cans, which were stained with Safranin O. The other constituents of the tissue were stained with fast green. In the category of "filling of defects", the percentage was calculated relative to a depth of 3 mm (18 square millimeters for the present study) following Sellers[10]. In the category of "subchondral bone", new subchondral formation was interpreted as an incorporation of repair tissue with subchondral bone (Table 1). The average score and standard deviation were calculated from the values obtained for each category and the total. Statistical analyses of total scores were performed by Kruskal-Wallis non-parametric analysis of variance. The level of significance was defined as $p < 0.05$ for all analyses.

The specimens were also evaluated immunohistochemically for type-I and II collagen and chondroitin-6-sulphate (3B3+). The sections were deparaffinized in xylene, passed through decreasing gradations of ethanol, washed in phosphate-buffered saline (PBS), pretreated with trypsin (Wako Pure Chemical, Osaka) 0.25%w/v in tris-HCl buffer for 30 min and chondroitinase ABC (0.25IU/ml; Seikagaku, Tokyo) for one hour and submerged for 30 min in methanol with 0.3% hydrogen peroxide to remove endogenous peroxidase activity. The sections were washed again in PBS and incubated in a blocking buffer. The sections were incubated in primary monoclonal mouse anti-human type-II collagen IgG (Daiichi Fine Chemical, Takaoka,Toyama) in a dilution of 1:100, monoclonal mouse anti-human type-I collagen IgG (Daiichi Fine Chemical, Takaoka,Toyama) in a dilution of 1:100 and monoclonal mouse anti-human chondroitin-6-sulphate IgM (Seikagaku, Tokyo) in a dilution of 1:50 for 48 hours at 4°C. The sections were washed in PBS solution, and biotin-conjugated anti-mouse IgG+IgA+IgM (Nichirei, Tokyo) was applied for 3 hours. After another wash in PBS, the sections were incubated for 30 min with a streptoavidin-biotin-peroxidase reagent (Nichirei, Tokyo) and then washed again. The sections were soaked in 0.02% diaminobezidine tetrachloride in PBS containing 0.05% H_2O_2 for colorization. Sections, which were to serve as controls, were treated with goat serum in blocking buffer as a substitute for the primary antibodies under the same protocol.

RESULTS

Macroscopic Findings

Group A: The repair tissue was white and more elastic than the adjacent cartilage at 3 and 6 weeks. At 12 weeks, the repair tissue was still white, regular and as elastic as the adjacent cartilage. At 24 weeks, the repair tissue was consistent with the adjacent cartilage in color and elasticity, but was somewhat whiter than the adjacent cartilage. Continuity of the repair tissue with adjacent tissue was evident after 12 weeks.

Group B: The repair tissue was irregular, and the surface color was brown and white at 3 weeks. The irregularity became severe at 6 and 12 weeks, and the level of the surface was lower than the adjacent cartilage. At 24 weeks, the repair tissue was irregular, but its level was not as low as at 6 or 12 weeks. The surface color was still brown and white.

Group C: The repair tissue was white and more elastic than the adjacent cartilage at 3 and 6 weeks as in Group A. However, the color became browner than Group A, and the surface was harder than Group A at 12 weeks. The repair tissue was hard and somewhat brown at 24 weeks.

Histological Findings

Group A: At 3 weeks after surgery, the heat-treated bone was necrotic and was surrounded by mesenchymal cells and multinuclear giant cells (Fig. 2-A). The subchondral bone formation was poor (33% on average). The repair tissue was almost discontinuous with the adjacent normal cartilage. The matrix of regenerative repair tissue was moderately or intensely stained with Safranin O. The repair tissue filled the area where cultured chondrocytes had been transplanted, over the level of the adjacent normal cartilage surface (118%). The repair tissue was thicker than the adjacent normal cartilage. In the repair tissue, collagen fibers were observed in a clear and homogenous matrix, where many lacunae were observed. The lacunae contained round cells suggesting chondrocytes. At 6 weeks, small fragments of the heat-treated bone still remained, but new bone formation was observed close to the heat-treated bone (Fig. 2-B). Incorporation of the repair tissue with the subchondral bone remained poor (43%). The repair tissue was still thick. Gaps or discontinuities between the repair tissue and the adjacent normal cartilage had decreased in number and size. The repair tissue was basophilic by H.E. staining. The matrix was intensely stained with Safranin O and was covered with thin fibrous tissue (Fig. 2-B). Numerous lacunae were formed in the matrix. The lacunae contained hypertrophic round cells, most of which were clustered. Cells were partly arranged in columns (Fig. 3-A). At 12 weeks, the heat-treated bone had almost been absorbed and new subchondral bone had formed simultaneously (Fig. 2-C). Incorporation of the repair tissue with subchondral bone had improved (65%), but tidemark formation remained poor (43%). The surface level of the repair tissue was even or slightly higher than that of the adjacent normal cartilage (110%) and the repair tissue was still thicker than the adjacent normal cartilage. In the repair tissue, basophilic and homogenous matrix was observed by H.E. staining. The matrix was intensely stained with Safranin O (Fig. 2-C). Cells were round-shaped, were contained in lacunae and were partly arranged in columns. At 24

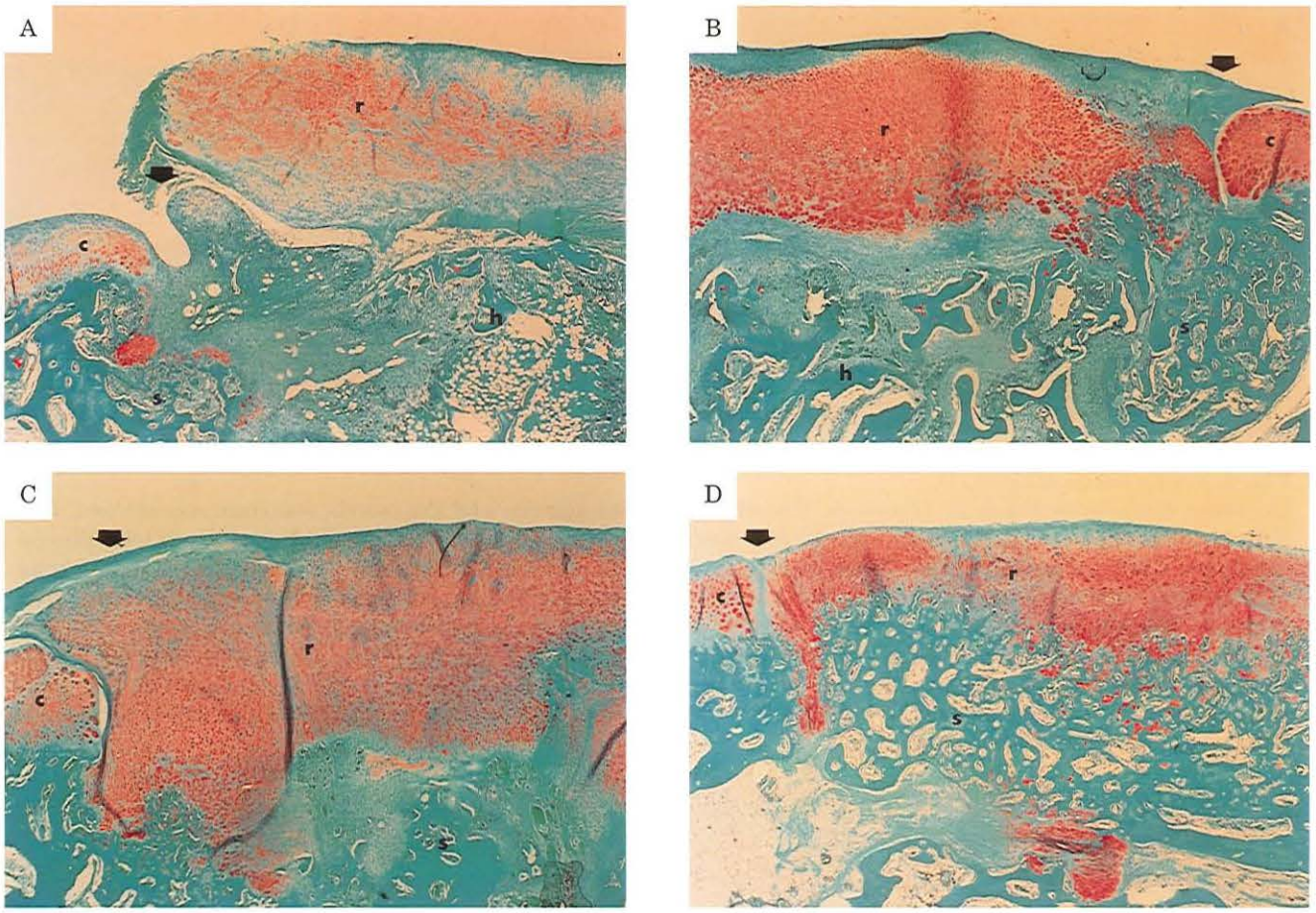

Fig. 2. Repair tissue of Group A.
(A) Three weeks after the graft procedure. Round cells were observed in the repair tissue, and the matrix was intensely stained with Safranin O. (B) At 6 weeks, the heart-treated bone still remained. Hypertrophic round cells were partly arranged in columns in the Safranin O-stained matrix in the repair tissue. (C) At 12weeks, the subchondral bone was well formed but the repair tissue was still thicker than the adjacent normal cartilage. (D) At 24 weeks, the heat-treated bone had been completely replaced with new subchondral bone, but the repair tissue remained thicker than the adjacent normal cartilage. In the repair tissue, round cells were partly arranged in columns. (r = repair tissue, c = normal adjacent cartilage, h = heat-treated bone, s = new subchondral bone. The arrows denote the margins between normal adjacent cartilage and repair tissue. Safranin O-fast green, × 10).

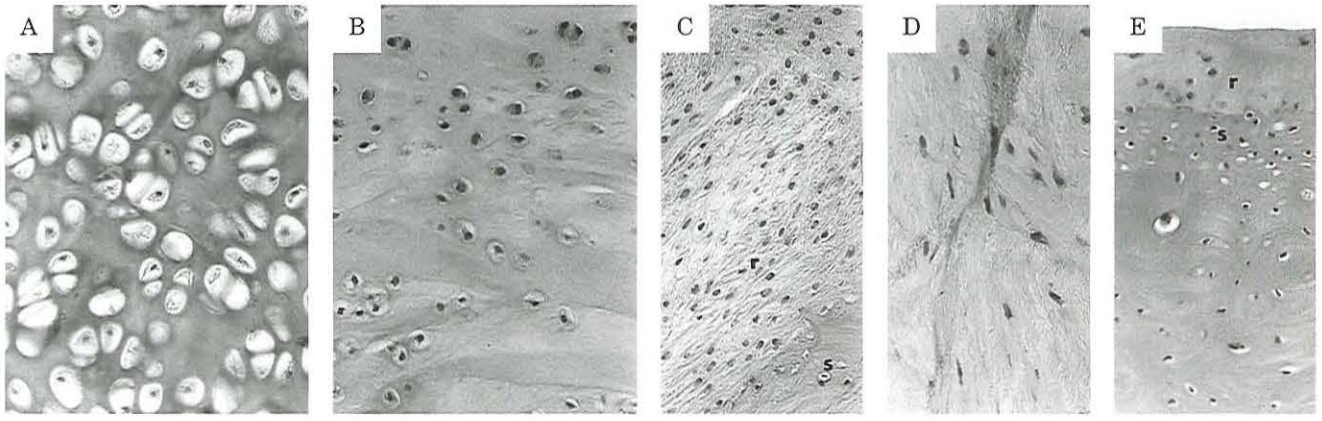

Fig. 3. Histologic sections from the center of repair tissue stained with H.E.
(A) Group A at 6 weeks after the graft procedure. In basophilic and homogenous matrix, numerous lacunae were observed. The lacunae contained hypertrophic round cells. These histological features resemble normal hyaline cartilage. (B) Group A at 24 weeks. The matrix was still basophilic and homogenous. The lacunae contained round cells. The cells were not so hypertrophic as those of 6 weeks. (C) Group B at 24 weeks. Only spindle cells were observed in the thin fibrous repair tissue. (D) Group C at 6 weeks. Spindle cells were mainly observed in the basophilic matrix. (E) Group C at 24 weeks. A very small number of round cells were observed in the thin repair tissue. (r = repair tissue, s = new subchondral bone. H.E., × 200).

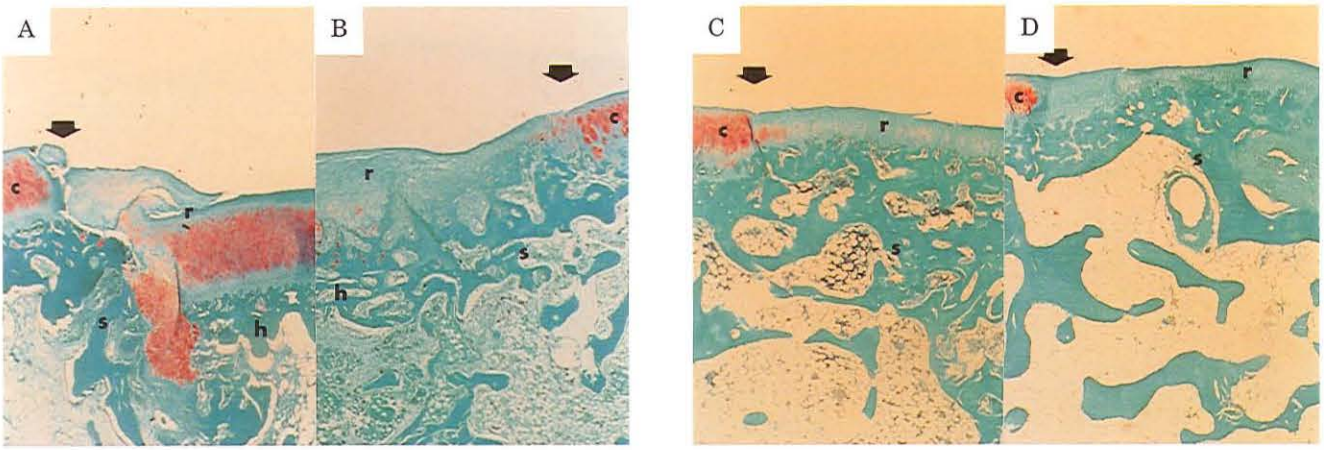

Fig. 4. Repair tissue of Group B.
(A) Three weeks after the graft procedure. Chondrocytes were necrotic and the matrix was intensely stained with Safranin O in the heat-treated cartilage. (B) At 6 weeks, the heat-treated cartilage had been replaced with fibrous tissue and the matrix of repair tissue was poorly stained with Safranin O. (C) At 12 weeks, the heat-treated bone had been replaced with new subchondral bone and the repair tissue had become thinner than the adjacent normal cartilage. The matrix was not stained with Safranin O. (D) At 24 weeks, the heat-treated bone had been completely replaced with new subchondral bone and the repair tissue had become very thin. The matrix was not stained with Safranin O. The heat-treated cartilage had disappeared. (r = repair tissue, c = normal adjacent cartilage, h = heat-treated bone, s = new subchondral bone. The arrows denote the margins between normal adjacent cartilage and repair tissue. Safranin O-fast green, × 10).

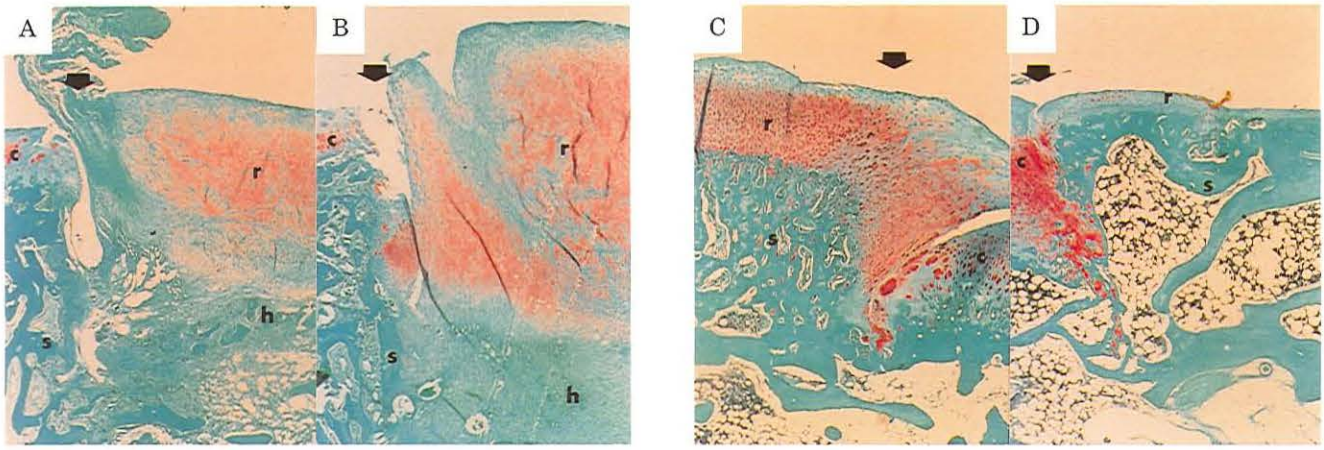

Fig. 5. Repair tissue of Group C.
(A) Three weeks after the graft procedure. The repair tissue was thick on the heat-treated bone. The matrix of the repair tissue was moderately stained with Safranin O. (B) At 6 weeks, the heat-treated bone still remained, and the repair tissue was still thick. The matrix was moderately stained with Safranin O. (C) At 12 weeks, the heat-treated bone had been replaced with new subchondral bone, and the repair tissue had become thinner than the adjacent normal cartilage. The matrix was moderately stained with Safranin O in the repair tissue. (D) At 24 weeks, the subchondral bone was well formed, and the repair tissue was remarkably thin above the new subchondral bone. The matrix of the repair tissue was very poorly stained with Safranin O. (r = repair tissue, c = normal adjacent cartilage, h = heat-treated bone, s = new subchondral bone. The arrows denote the margins between normal adjacent cartilage and repair tissue. Safranin O-fast green, × 10).

weeks, the heat-treated bone had been completely replaced with new subchondral bone, and incorporation of the repair tissue with subchondral bone (83%) and tidemark formation beneath the repair tissue (70%) were apparent (Fig. 2-D). The amount of repair tissue was smaller than that at earlier periods, but still thicker than the adjacent normal cartilage (108%). Many lacunae were observed in the basophilic and homogenous matrix, and contained round cells, which did not cluster. The matrix was intensely stained with Safranin O. The cells were partly arranged in columns (Fig. 3-B). These findings were comparable with hyaline cartilage. The surface and architecture of the repair tissue were stable at all time points.

Group B: At 3 weeks after surgery, the heat-treated articular cartilage still showed basophilic and homogenous matrix by H.E. staining. However, the chondrocytes in the heat-treated cartilage were necrotic, and the nuclei were picnotic

or karyotic. The matrix of the heat-treated cartilage was moderately stained with Safanin O (Fig. 4-A). Heat-treated cartilage and bone were surrounded by fibrous tissue. The surface level of the treated area was even or lower than that of the adjacent cartilage (89%). At 6 weeks, the heat-treated cartilage had been gradually absorbed and was surrounded by fibrous tissue (Fig. 4-B). In the newly formed fibrous tissue, small number of round cells appeared in the small areas of homogenous matrix. The matrix of the repair tissue was very poorly stained with Safranin O. The heat-treated bone still remained and was surrounded by numerous multinuclear giant cells. At 12 weeks, the heat-treated bone had almost been replaced with new subchondral bone (Fig. 4-C). Discontinuity between the repair tissue and the adjacent normal cartilage had become rare. The repair tissue was thinner than the adjacent normal cartilage. In the repair tissue, the heat-treated cartilage had almost been absorbed, and fibrous tissue was predominantly observed. The matrix of the repair tissue was not stained with Safranin O. At 24 weeks, the heat-treated bone had been completely replaced with subchondral bone (Fig. 4-D). The heat-treated cartilage was completely replaced with very thin fibrous tissue where cells were predominantly spindle-shaped (Fig. 3-C). The matrix of the repair tissue was not stained with Safranin O. These findings were comparable with fibrous tissue or fibrocartilage.

Group C: At 3 weeks after surgery, the heat-treated bone was necrotic and surrounded by multinuclear giant cells. Repair tissue appeared under the periosteum (Fig. 5-A). The repair tissue contained a large amount of fibrous tissue and was thicker than the adjacent normal cartilage. The repair tissue was partially discontinuous with the adjacent normal cartilage. The matrix of the repair tissue was weakly stained with Safranin O. At 6 weeks, the heat-treated bone had been partly absorbed, and new subchondral bone had partly formed (50%). The repair tissue was still thicker than the adjacent normal cartilage. The matrix was moderately stained with Safranin O (Fig.5-B). In the H.E.-stained repair tissue, basophilic and homogenous matrix was observed with spindle shaped cells, suggesting the proliferation of fibroblasts (Fig. 3-D). At 12 weeks, the heat-treated bone was completely replaced with newly formed subchondral bone, which was above the original tidemark (Fig. 5-C). In addition, the repair tissue had become thinner than the adjacent normal cartilage. The matrix was weakly or moderately stained with Safranin O. In the H.E.-stained repair tissue, the matrix was basophilic and homogenous, containing round cells. At 24 weeks, the new subchondral bone was so remarkably formed that the level of subchondral bone was conspicuously higher than the original tidemark (Fig. 5-D). Therefore, the average percentage of new subchondral bone was evaluated to be lower (27%) than that at earlier periods. The repair tissue had become markedly thin. The matrix of the repair tissue was very poorly stained with Safranin O. In the thin layer of repair tissue, the matrix was homogenous and contained a small number of round cells (Fig. 3-E).

The histological grading scale was evaluated. The scores of all groups were similar at 3 weeks (group A: 18.2, group B: 17.0, group C: 19.1 at 3 weeks respectively), but Group A scores were sig-

Table 2. Scores on the modified histological grading scale

	Category								
	Filling of Defects	Integration at Margin	Staining of Matrix	Cellular Morphology	Architecture within	Architecture of Surface	New-Bone Formation	Formation of Tidemark	Total
Group A									
3W (n = 10)	0.8	2.1	1.9	2.9	2.3	1.2	3.3	3.8	18.2 ± 2.3
6W (n = 9)	0.6	1.7	1.4	2.4	1.3	1.1	2.4	3.1	14.1 ± 3.8*
12W (n = 10)	0.5	1.6	1.3	1.8	1.3	1.2	1.5	2.1	11.4 ± 3.5**
24W (n = 11)	0.4	1.3	1.6	1.6	1.5	1.6	1.0	1.7	10.6 ± 3.3***
Group B									
3W (n = 9)	0.6	1.8	2.7	4.0	2.9	1.6	1.6	1.9	17.0 ± 2.5
6W (n = 9)	0.7	1.6	3.0	4.6	2.1	1.8	1.9	3.3	18.9 ± 3.1*
12W (n = 10)	0.9	1.7	3.7	4.7	1.9	2.3	3.3	3.1	21.6 ± 3.9**
24W (n = 10)	0.5	1.7	3.9	4.9	2.3	1.8	3.0	3.1	21.2 ± 3.4***
Group C									
3W (n = 9)	0.6	2.3	1.8	3.7	2.3	1.4	3.4	3.7	19.1 ± 3.1
6W (n = 10)	0.7	2.1	2.3	3.8	1.7	1.9	2.4	3.5	18.3 ± 3.9*
12W (n = 10)	0.6	1.7	2.1	3.6	1.9	1.9	2.6	2.4	16.9 ± 4.1**
24W (n = 10)	0.5	1.4	3.3	3.2	0.8	1.7	3.2	2.0	16.1 ± 3.5***

The total score of Group A was significantly different from those of Group B and C at 6 weeks (*, $p < 0.05$). The total scores of Groups A, B and C were significantly different from one another at 12 (**, $p < 0.05$) and 24 weeks (***, $p < 0.01$).

nificantly better than the other two at 6, 12 and 24 weeks ($p < 0.05$). Group C scores were significantly better after 12 and 24 weeks than Group B scores (Group A: 10.8, Group B: 21.2, Group C: 16.1 at 24 weeks, respectively, $p < 0.01$). In Group A, the staining scores with Safranin O, cellular morphology and percentage of new subchondral bone were superior to those of Group C at 24 weeks (Table 2).

Immunohistochemical analysis

Type-II collagen

In Group A, immunostaining for type-II collagen was poor at 3 weeks, but improved at 6 weeks. Immunostaining became evident throughout the matrix of repair tissue at 12 and 24 weeks, and the staining was almost comparable with the adjacent normal cartilage (Fig. 6-A). In Group B, immunostaining for type-II collagen was evident but found only in the matrix of heat-treated cartilage at 3 weeks. It degenerated at 6 weeks, and became very weak at 12 and 24 weeks (Fig. 6-B). In Group C, immunostaining for type-II collagen was weak at 3 and 6 weeks, but improved at 12 and 24 weeks (Fig. 6-C), as in Group A. However, the immunostaining was observed only in the surface layer of the repair tissue.

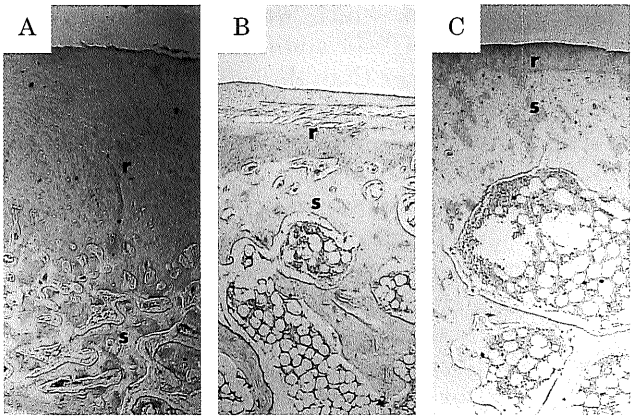

Fig. 6. Immunostaining of type-II collagen in the repair tissue at 24 weeks after the graft procedure. (A) Group A. Immunostaining was observed throughout the matrix in the repair tissue. (B) Group B. Immunostaining was very poor in the repair tissue. (C) Group C. Immunostaining was observed in the remarkably thin layer of repair tissue. (r = repair tissue, s = new subchondral bone. × 20).

Type-I collagen

In Group A, immunostaining for type-I collagen was weak and partial in the matrix of the repair tissue at 3, 6 and 12 weeks. Type-I collagen was finally almost undetectable by immunostaining in the matrix of repair tissue and was clearly bordered by subchondral bone which was well stained with type-I collagen at 24 weeks (Fig. 7-A). In Group B, the immunostaining for type-I collagen was weak in the heat-treated cartilage at 3 weeks, became stronger at 6 and 12 weeks and became evident in the matrix of the repair tissue at 24 weeks (Fig. 7-B). In Group C, immunostaining for type-I collagen was observed in almost all the matrix of repair tissue, but was weak at 3, 6 and 12 weeks. Type-I collagen was almost undetectable by immunostaining in the markedly thin layer of repair tissue at 24 weeks (Fig. 7-C). Immunostaining for type-I collagen was evident in the subchondral bone in every group.

Chondroitin-6-sulphate (3B3+)

Immunostaining for chondroitin-6-sulphate

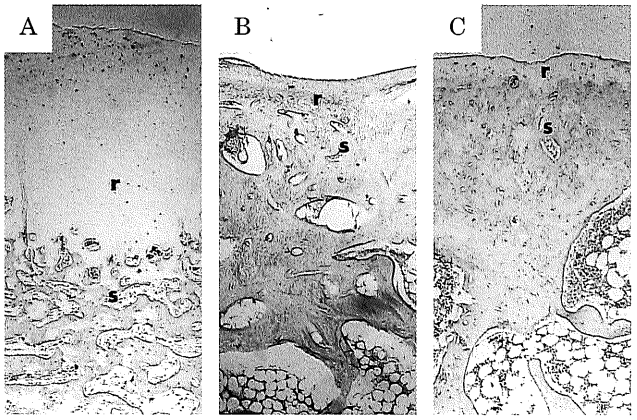

Fig. 7. Immunostaining of type-I collagen in the repair tissue at 24 weeks after the graft procedure. (A) Group A. Immunostaining was not observed or was very poor in the repair tissue and was clearly bordered by subchondral bone, which was well stained. (B) Group B. Immunostaining was observed in the matrix of repair tissue similar to that in subchondral bone. (C) Group C. Immunostaining was not observed or was very poor in the thin layer of repair tissue. The subchondral bone was well stained. (r = repair tissue, s = new subchondral bone. × 20).

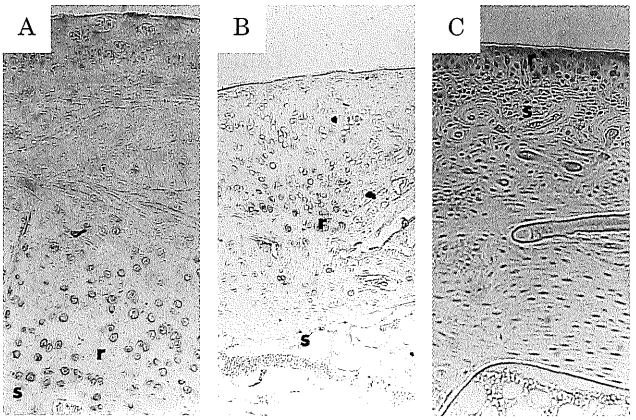

Fig. 8. Immunostaining of chondroitin-6-sulphate (3B3+) in the repair tissue at 24 weeks after the graft procedure.
(A) Group A. Immunostaining was observed throughout the matrix in the upper zone and pericellularly in the deeper zone of the repair tissue. (B) Group B. Immunostaining was very poor and was restricted to remnants of the heat-treated cartilage. (C) Group C. Immunostaining was restricted to the thin layer of the repair tissue. (r = repair tissue, s = new subchondral bone. × 40).

(3B3+) was seen throughout the matrix of the repair tissue, but was poor at 3 weeks in Group A and C. In Group A, immunostaining in the matrix improved at 6, 12 and 24 weeks, and immunostaining of the upper zone of the repair tissue was observed throughout the matrix, while immunostaining of the deeper zone was observed pericellularly at 12 and 24 weeks (Fig. 8-A). In Group B, immunostaining for chondroitin-6-sulphate was observed in the heat-treated cartilage at 3 weeks, but deteriorated as the heat-treated cartilage was replaced with repair tissue; the immunostaining was restricted to small fibrocartilaginous areas of the repair tissue and was rarely observed after 6 weeks (Fig. 8-B). In Group C, the immunostaining for chondroitin-6-sulphate (3B3+) was observed in the matrix of repair tissue at 12 and 24 weeks, but it was restricted to the thin layer of repair tissue, and the subchondral bone was close to it. In the thin layer, the immunostaining was throughout the matrix (Fig. 8-C).

DISCUSSION

We demonstrated that transplantation of cultured chondrocytes in collagen gel covered with periosteum added to the surface of bone heat-treated at 60°C for 30 min improved the repair of articular cartilage defects. The repair tissue had all the characteristics of hyaline cartilage, including adequate filling, round cells partly arranged in columns, and matrix staining with Safranin O. Histologically, the repair tissue was comparable to the normal adjacent articular cartilage. The histological grading scale score was significantly excellent when the cultured chondrocytes were transplanted and covered with periosteum.

Concerning the matrix of articular cartilage which is hyaline cartilage histologically, type-II collagen is proved to be one of the main constituents, and is well observed in normal articular cartilage[3]. Type-I collagen is not observed in hyaline cartilage but well observed in mature bone, tendon, skin, and other coarsely fibered tissues[3]. Chondroitin-6-sulphate (3B3+) is observed pericellularly in hyaline cartilage, but throughout the matrix in fibrocartilaginous tissue[9]. Typical fibrocartilage is found in the semilunar meniscus of the knee[3]. Type-II collagen, type-I collagen, and chondroitin-6-sulphate (3B3+) can be identified by immunohistochemical staining. When the cultured chondrocytes were transplanted and covered with periosteum, type-II collagen was well stained in the repair tissue. Type-I collagen was not stained in the repair tissue and was clearly bordered by subchondral bone, which was well stained with type-I collagen. Chondroitin-6-sulphate (3B3+) was stained throughout the matrix of repair tissue in the upper zone and was stained pericellularly in the deeper zone like adjacent articular cartilage. These immunohistological findings indicate that the repair tissue was predominantly composed of matrix components similar to those of normal articular cartilage, especially in the deeper zone.

We used immature joints (rabbits weighing 2,270g on average at time of transplantation and rabbits weighing 2,000g) because reconstruction of articular cartilage on heat-treated bone is mainly used for the treatment of young patients. It is indicated that the nutrition of the articular cartilage in immature joints comes from the synovial fluid and subchondral bone[4]. The nutrition of articular cartilage did not come through the subchondral bone unlike normal immature joints after the procedure. The heat-treated bone had no blood vessels at least until new subchondral bone was formed. However, the nutrition of articular cartilage from the synovial fluid is preserved even on the heat-treated bone. Therefore, the chondrocytes in repair tissue could survive even on the surface of heat-treated bone.

Transplantation of isolated chondrocytes on the surface of living subchondral bone has provided histological evidence that the repair tissue maintains the differentiated characteristics of hyaline cartilage[1,2,11]. In the present study, the isolated and cultured chondrocytes were transplanted on the surface of heat-treated bone, in which all the cells are killed by heat-treatment and blood vessels are absent. We found that the transplanted chondrocytes could survive on the surface of heat-treated bone and propose that heat-treated bone is able to support repair tissue.

Heat-treated bone is gradually replaced with viable bone in a manner similar to that of fresh bone grafts, and its mechanical strength was similar to that of fresh bone[6]. In addition, heat-treated bone is able to preserve BMP activity and maintain bone inductive ability[7]. Therefore, heat-treated bone has the ability not only to support articular cartilage but also to provide cytokines, such as BMP, which can differentiate chondrocytes until new subchondral bone is present effectively.

Incorporation of repair tissue with subchondral bone was subtle and weak until 12 weeks in the present study. The repair tissue may be vulnerable during this period. Firm fixation of periosteum to adjacent articular bone and cartilage was necessary to protect transplanted chondrocytes until new subchondral bone was formed. As long as the periosteum was fixed firmly, the repair tissue obtained with transplanted chondrocytes covered with periosteum provided histological findings comparable to the adjacent normal articular cartilage.

On the contrary, heat-treated articular cartilage degenerated soon after re-implantation, presumably due to the death of chondrocytes. Round cells were not observed and the matrix was not stained with Safranin O. The main histological findings

were spindle-shaped cells in thin repair tissue, and the tissue completely degenerated at 24 weeks. We confirmed the report of Manabe et al[5] that the degeneration of heat-treated articular cartilage was inevitable. Therefore, heat-treated articular cartilage cannot be used for re-implantation.

We also evaluated the effects of periosteum coverage because periosteum can reconstruct cartilaginous repair tissue on the surface of naked cancellous bone[8]. The repair tissue of the chondrocyte transplants covered with periosteum was evaluated to be significantly better than that of periosteum coverage without transplanted chondrocytes. When the defect was covered with periosteum only, the repair tissue was definitely different from the adjacent articular cartilage and the cartilaginous matrix was also immunohistologically restricted to the surface of the repair tissue. The effect of transplanted chondrocytes covered with periosteum in reconstructing cartilaginous repair tissue on the surface of heat-treated bone was more significant than that of periosteum alone.

Clinically, after resection of malignant bone tumors adjacent to joints, total joint replacement with artificial materials is a good modality for reconstructing joints for a short period. However, it constitutes a burden to child, adolescent and young adult patients who are likely to survive for a long period. Some other modalities are desired to reconstruct joints after resection of malignant bone tumors adjacent to joints. Re-implanted heat-treated joint cannot avoid necrosis of articular cartilage so heat-treated bone is re-implanted without its articular cartilage at present. If it is possible to reconstruct the articular cartilage of heat-treated bone, re-implantation of a heat-treated joint may be a useful modality for reconstruction after resection of malignant bone tumors. Our study demonstrated the possibility of reconstruction of articular cartilage on the surface of heat-treated bone.

In conclusion, it can be said that transplantation of chondrocytes cultured in collagen gel covered with periosteum could be one of the best ways to repair cartilaginous tissue on the surface of heat-treated bone. We believe that this method will contribute to the reconstruction of heat-treated joints after resection of malignant bone tumors.

ACKNOWLEDGEMENTS

The authors express their appreciation to H. Kajiwara M.D., PhD, and to Mrs Hidaka for their assistance.

No benefits in any form have been received or will be received from any commercial party related directly or indirectly to the subject of this article.

(Received July 6, 2001)
(Accepted November 12, 2001)

REFERENCES

1. **Aston, J.E. and Bentley, G.** 1986. Repair of articular surfaces by allograft of articular and growth-plate cartilage. J. Bone Joint Surg. (Br.) **68:** 29–35.
2. **Brittberg, M., Lindahl, A., Nilsson, A., Ohlsson, C., Isaksson, O. and Peterson, L.** 1994. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N. Engl. J. Med. **331:** 889–895.
3. **Furukawa, T., Eyre, D.R., Koide, S. and Glimcher, M.J.** 1980. Biochemical studies on repair cartilage resurfacing experimental defects in the rabbit knee. J. Bone Joint Surg. (Am.) **62:** 79–89.
4. **Honner, R. and Thompson, A.R.** 1971. The nutritional pathways of articular cartilage. An autoradiographic study in rabbits using 35S injected intravenously. J. Bone Joint Surg. (Am.) **53:** 742–748.
5. **Manabe, J., Kawaguchi, T., Matsumoto, S., Kuroda, K., Tanikawa, H., Sawaizumi, M., Takakura, Y., Furuya, K. and Isobe, Y.** 1993. A clinical study of pasteurized bone grafts. Zasshi Seikeigeka **44:** 1838–1845. (In Japanese)
6. **Manabe, J.** 1993. Experimental studies on pasteurized autogenous bone graft. Nippon Seikeigeka Gakkai Zasshi **67:** 255–266. (In Japanese)
7. **Nakanishi, K., Sato, K., Sato, T., Takahashi, M., Fukaya, N. and Miura, T.** 1992. Preservation of bone morphogenic protein in heat-treated bone. Nippon Seikeigeka Gakkai Zasshi **66:** 77–83.
8. **O'Driscoll, S.W., Keeley, F.W. and Salter, R.B.** 1986. The chondrogenic potential of free autologous periosteal grafts for biological resurfacing of major full-thickness defects in joint surfaces under the influence of continuous passive motion. J. Bone Joint Surg. (Am.) **68:** 1017–1035.
9. **Richardson, J.B., Caterson, B., Evans, E.H., Ashton, B.A. and Roberts, S.** 1999. Repair of human articular cartilage after implantation of autologous chondrocytes. J. Bone Joint Surg. (Br.) **81:** 1064–1068.
10. **Sellers, R.S., Peluso, D. and Morris, E.A.** 1997. The effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on the healing of full thickness defects of articular cartilage. J. Bone Joint Surg. (Am.) **79:** 1452–1463.
11. **Wakitani, S., Kimura, T., Hirooka, A., Ochi, T., Yoneda, M., Yasui, N., Owaki, H. and Ono, K.** 1989. Repair of rabbit articular surfaces with allograft chondrosytes embedded in collagen gel. J. Bone Joint Surg. (Br.) **71:** 74–80.
12. **Yasui, N., Osawa, S., Ochi, T., Nakashima, H. and Ono, K.** 1982. Primary culture of chondrocytes embedded in collagen gel. Expl. Cell Biol. **50:** 92–100.