Mesenchymal Cell-Based Repair of Large, Full-Thickness Defects of Articular Cartilage*

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ABSTRACT: Osteochondral progenitor cells were used to repair large, full-thickness defects of the articular cartilage that had been created in the knees of rabbits. Adherent cells from bone marrow, or cells from the periosteum that had been liberated from connective tissue by collagenase digestion, were grown in culture, dispersed in a type-I collagen gel, and transplanted into a large (three-by-six-millimeter), full-thickness (three-millimeter) defect in the weight-bearing surface of the medial femoral condyle. The contralateral knee served as a control: either the defect in that knee was left empty or a cell-free collagen gel was implanted.

The periosteal and the bone-marrow-derived cells showed similar patterns of differentiation into articular cartilage and subchondral bone. Specimens of reparative tissue were analyzed with use of a semiquantitative histological grading system and by mechanical testing with employment of a porous indenter to measure the compliance of the tissue at intervals until twenty-four weeks after the operation. There was no apparent difference between the results obtained with the cells from the bone marrow and those from the periosteum. As early as two weeks after transplantation, the autologous osteochondral progenitor cells had uniformly differentiated into chondrocytes throughout the defects. This repair cartilage was subsequently replaced with bone in a proximal-to-distal direction, until, at twenty-four weeks after transplantation, the subchondral bone was completely repaired, without loss of overlying articular cartilage. The mechanical testing data were a useful index of the quality of the long-term repair. Twenty-four weeks after transplantation, the reparative tissue of both the bone-marrow and the periosteal cells was stiffer and less compliant than the tissue derived from the empty defects but less stiff and more compliant than normal cartilage.

CLINICAL RELEVANCE: The current modalities for the repair of defects of the articular cartilage have many disadvantages. The transplantation of progenitor cells that will form cartilage and bone offers a possible alternative to these methods. As demonstrated in this report, autologous, bone-marrow-derived, osteochondral progenitor cells can be isolated and grown *in vitro* without the loss of their capacity to differentiate into cartilage or bone. Sufficient autologous cells can be generated to initiate the repair of articular cartilage and the reformation of subchondral bone. The repair tissues appear to undergo the same developmental transitions that originally led to the formation of articular tissue in the embryo. This approach to the repair of defects of the articular cartilage may have useful applications in the repair of large, full-thickness defects of joint surfaces.

It is well known that the capacity of articular cartilage for repair is limited. Injuries of the articular cartilage that do not penetrate the subchondral bone do not heal and usually progress to the degeneration of the articular surface; a short-lived tissue response has been observed, but it fails to provide sufficient cells and matrix to repair even small defects[18,20,32,36]. Injuries that penetrate the subchondral bone undergo repair through the formation of tissues usually characterized as fibrous, fibrocartilaginous, or hyaline-like cartilaginous, depending on the species, the age of the animal, and the location and size of the injury[14,16,37,48]. However, these reparative tissues, even those that resemble hyaline cartilage histologically, differ from normal hyaline cartilage both biochemically and biomechanically, and by six months, fibrillation, fissuring, and extensive degenerative changes occur in the reparative tissues of approximately half of the full-thickness defects[13,19]. Similarly, the degenerated cartilage seen in

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osteoarthrosis does not usually undergo repair but progressively deteriorates. There have been a number of attempts to develop clinically useful procedures to repair damaged articular cartilage, but these have not proved totally successful. Currently, prosthetic joint replacement is the usual clinical approach for the treatment of severe degeneration of the cartilage, but it has short-term and long-term problems, including infection and loosening of the components.

Biological resurfacing is an alternative technology that could result in long-term successful regeneration. Historically, biological resurfacing has involved joint, osteochondral, or cartilage-shell transplantation[21,25,29,30,45,55]. Experimental studies have used isolated chondrocytes[1,3,12,24,27,59] or periosteal membrane[43,44]. However, these methods have limited clinical application in the repair of defects of the articular cartilage because of the limited availability of donor material. Clearly, there is a need to develop alternative technologies for the repair of cartilage in order to provide a clinically useful procedure.

At our laboratory, we have investigated the use of osteochondral progenitor cells from the periosteum or bone marrow as a biological method for the repair of defects of the articular cartilage. The presence of osteochondral progenitor cells in periosteal membrane and bone marrow has been well documented[2,17,28,57]. We recently reported the isolation of osteochondral progenitor cells (which we referred to as mesenchymal stem cells[22,23,40-42]) from either bone marrow or the periosteum; these cells proliferate (grow) in culture without loss of the ability to form bone or cartilage. The presumed advantage of the use of mesenchymal stem cells[8,9,11], as opposed to articular chondrocytes[1,24,58,59], is that the stem cells are capable of a broad range of chondrogenic expression, and they may recapitulate the embryonic lineage transitions originally involved in the formation of joint tissue[53]. These cells, when transplanted into cartilage defects, also provide a practical source of autologous cells with chondrogenic potential. Because collagen gels have been used successfully as delivery vehicles in the transplantation of cells and are of low antigenicity[59], we embedded autologous osteochondral progenitor cells from the periosteal membrane or bone marrow of long bones into a type-I collagen gel. These cellular grafts were then transplanted into large (three by six by three-millimeter), full-thickness defects in the weight-bearing articular surface of the knee in rabbits.

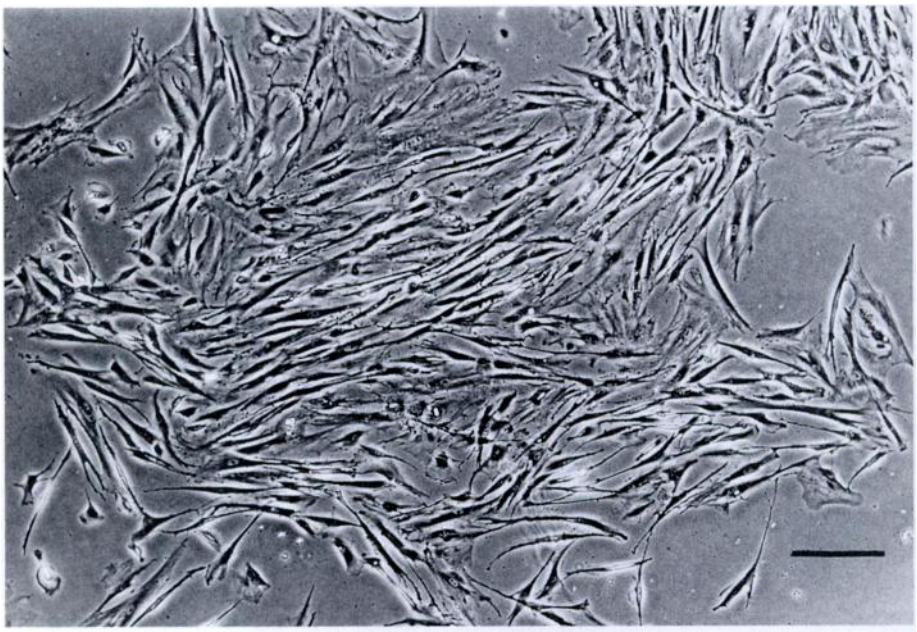

FIG. 1

Phase micrograph showing a living culture of the bone-marrow-derived mesenchymal cells, released by harvesting with trypsin-EDTA and then implanted into the condylar defect (scale bar = fifty micrometers).

The sequential repair of these full-thickness defects was studied by histological and biomechanical evaluation from two to twenty-four weeks after transplantation of the osteochondral progenitor cells. This analysis demonstrated the uniform differentiation of the implanted cells into chondrocytes. The subchondral plate reformed by means of endochondral replacement of the basal cartilage.

Materials and Methods

Sixty-eight three to four-month-old New Zealand White rabbits (Hazelton Research Products, Denver, Pennsylvania), weighing an average of 2.5 kilograms, were used in this study. The rabbits were anesthetized by intramuscular injection of a mixture of ketamine hydrochloride (100 milligrams per milliliter and 0.60 to

0.70 milliliter per kilogram of body weight) and xylazine (twenty milligrams per milliliter and 0.30 milliliter per kilogram of body weight) at the time of harvesting of the bone marrow or periosteum and at the time of transplantation. The two operative procedures were accomplished within a two-week period.

Preparation of the Bone-Marrow-Derived Mesenchymal Cells

The proximal-medial surface of each tibia was exposed through a small incision. The soft tissue was retracted, and two milliliters of blood was aspirated from the bone marrow with use of an 18-gauge needle that was fastened to a five-milliliter syringe containing 0.1 milliliter of heparin (3000 units per milliliter). The aspirate was washed twice with Tyrode balanced solution (Sigma Chemical, St. Louis, Missouri) and centrifuged at 180 times gravity for five minutes. After resuspension in Ham F12 medium (Gibco Laboratories, Grand Island, New York), containing selected lots of 10 per cent fetal calf serum (JR Scientific, Woodland, California) and antibiotics (penicillin G, 100 units per milliliter; streptomycin, 100 micrograms per milliliter; and amphotericin B, 0.25 microgram per milliliter), a small aliquot was removed, and the red blood cells were disrupted by treatment with 4 per cent acetic acid before the number of cells was determined with a hemocytometer. Usually, 10^6 to 10^7 nucleated marrow cells were obtained from each rabbit; the cells were cultured in a 100-millimeter plastic dish in complete medium (10 per cent fetal calf serum) at 37 degrees Celsius in a humidified atmosphere of 5 per cent carbon dioxide. The medium was first changed five days after seeding and was completely replaced every other day thereafter. The change of medium at five days removed almost all of the cells that had not adhered to the culture dish. The typical appearance of the bone-marrow-derived cells was fibroblastic at ten days after seeding (Fig. 1). These cells grew as a monolayer in nests that spread into neighboring nests. Approximately ten to fourteen days after seeding, the adherent cells were near confluence, at which time the cells were released from the dish by a five-minute exposure to 0.25 per cent trypsin, one-millimolar EDTA, and were counted in a hemocytometer before incorporation into the collagen. Careful notation and handling were required to ensure the introduction of the cultured cells into the same rabbit from which they had been taken.

To prepare the collagen solution for the delivery vehicle, Pancogene S (0.3 per cent acid-soluble type-I collagen, obtained from calf skin) (Gatefossé, St. Priest, France) was dialyzed against hydrochloric acid, pH 3, for one week at 4 degrees Celsius, and the solution was sterilized on ice with ultraviolet light for one hour. To neutralize the hydrochloric acid, 100 microliters of two-times concentrated Ham F12 medium was added to 100 microliters of collagen solution with gentle agitation at a temperature of 0 degrees Celsius. The cells were collected by centrifugation after removal from the culture dish and were suspended at 1×10^6 per 200 microliters of the collagen-Ham F12 mixture (final concentrations, 5×10^6 cells per milliliter, 0.15 per cent type-I collagen). The mixture was allowed to gel in a carbon-dioxide incubator at 37 degrees Celsius for ten minutes. This gel that contained bone-marrow mesenchymal cells was transplanted into the cartilage defects within two hours.

Preparation of the Periosteum-Derived Mesenchymal Cells

Periosteum was harvested from the anteromedial surface of the tibia of each rabbit and incubated with 0.25 per cent collagenase (CLS 2, 247 units per milligram) (Worthington Biochemical, Freehold, New Jersey) in Ham F12 medium for three hours at 37 degrees Celsius with intermittent agitation. The tissue was disrupted by vortexing, and the liberated cells were passed through a 100-micrometer Nitex filter (Tetko, Briarcliff Manor, New York), washed twice with Tyrode balanced solution, and collected by centrifugation. The cells were then counted in a hemocytometer, seeded, and cultured in complete medium. The medium was replaced every other day. After the adherent cells had become nearly confluent, they were harvested with trypsin-EDTA, suspended in the collagen-Ham F12 solution in the same manner as the bone-marrow-derived mesenchymal cells, and transplanted autologously.

Repair of the Defects, and Study Groups

A full-thickness defect (six millimeters long, three millimeters wide, and three millimeters deep) was created, through the articular cartilage and into the subchondral bone of the weight-bearing surface of the medial femoral condyle, by the drilling of two connecting three-millimeter holes, each three millimeters deep. In one knee, the defect was filled with the collagen gel containing the bone-marrow or the periosteum-derived mesenchymal cells. In the other knee, the defect either was filled with a collagen gel without cells or was left empty. Of the 136 knees in the study, thirty-one received bone-marrow-derived mesenchymal cells, thirty-seven received periosteum-derived mesenchymal cells, forty-nine served as control knees in which the defect was left empty, and nineteen served as control knees in which the cell-free collagen gel was implanted.

All rabbits were returned to their cages after the operation and were allowed to move freely. No animal was observed to have an abnormal gait or impaired locomotion.

Histological Evaluation

The rabbits were killed at two, four, twelve, or twenty-four weeks after the operation by placement in a carbon-dioxide chamber. The entire knee was dis-

TABLE I
HISTOLOGICAL GRADING SCALE FOR THE DEFECTS OF CARTILAGE*

Category	Points
Cell morphology	
Hyaline cartilage	0
Mostly hyaline cartilage	1
Mostly fibrocartilage	2
Mostly non-cartilage	3
Non-cartilage only	4
Matrix-staining (metachromasia)	
Normal (compared with host adjacent cartilage)	0
Slightly reduced	1
Markedly reduced	2
No metachromatic stain	3
Surface regularity†	
Smooth (>3/4)	0
Moderate (>1/2-3/4)	1
Irregular (1/4-1/2)	2
Severely irregular (<1/4)	3
Thickness of cartilage‡	
>2/3	0
1/3-2/3	1
<1/3	2
Integration of donor with host adjacent cartilage	
Both edges integrated	0
One edge integrated	1
Neither edge integrated	2
Total maximum	14

*Modified from the scale described by Pineda et al.[46].
†Total smooth area of the reparative cartilage compared with the entire area of the cartilage defect.
‡Average thickness of the reparative cartilage compared with that of the surrounding cartilage.

sected, examined macroscopically, and photographed. The distal part of the femur was then fixed with 10 per cent buffered formalin for one week. Each specimen was decalcified with 0.5-molar EDTA containing 0.1-molar epsilon-amino-n-caproic acid and 0.005-molar benzamidine and was sectioned sagittally, perpendicular to the defect. The sections were stained with toluidine blue O. These sections were obtained from the center of the defect, and as many as ten such sections were prepared from each knee. The sections from each animal were examined and scored independently by three of us (S. W., S. J. P., and V. M. G.) who had no knowledge of the study group from which they had been obtained.

Each sample was graded with use of a histological scale, which is a modification of that described by Pineda et al.[46]. The scale is composed of five categories and assigns a score ranging from 0 to 14 points (Table I). The cell morphology was graded from 0 (for tissue that was normal, compared with the adjacent, uninjured cartilage) to 4 points (when cartilaginous tissue was absent). Matrix-staining, or the degree of metachromatic staining with toluidine blue, was graded from 0 (for tissue that was normal, compared with the adjacent, uninjured cartilage) to 3 points (no metachromatic staining). Surface regularity, or the proportion of the surface of the defect that appears smooth compared with the entire surface, was graded from 0 (when more than three-quarters of the surface was smooth) to 3 points (when less than one-quarter was smooth). The thickness of the cartilage, or the average thickness of the cartilage in the defect compared with that of the surrounding cartilage, was graded from 0 (when the average thickness of the cartilage in the defect was more than two-thirds that of the surrounding cartilage) to 2 points (when the average thickness was less than one-third that of the surrounding cartilage). Integration of the donor cartilage with the host adjacent cartilage was graded from 0 (no gap between the donor and the host cartilage) to 2 points (a complete lack of integration, which we refer to as dissociation).

Mechanical Properties of the Repair Tissue

Microindentation was performed on the repair tissues from six defects that had been treated with bone-marrow-derived mesenchymal cells, five defects that had been treated with periosteum-derived mesenchymal cells, and five spontaneously healing, unfilled defects. The tissues were retrieved twenty-four weeks after the operation. The lateral, untreated condyles served as normal controls. The thickness of the cartilage and the depth of the indentation were measured with use of procedures developed by Mow et al.[39] and refined by Matsuura et al.[33-35] to estimate the compliance of the reparative cartilage.

A needle-penetration technique was used to determine the thickness. The force on the needle and its displacement were measured simultaneously. The position of the needle at the surface of the cartilage and at the zone of calcified bone was identified by a change in the force[33-35], as displayed on a strip-recorder.

In the indentation test, a 1.5-millimeter porous indenter tip was used. An initial preload of two grams was applied to ensure uniform contact between the tip of the indenter and the cartilage. The normal, control cartilage slowly deformed under this preload and reached an equilibrium displacement after approximately one minute. In some of the defects, more time was required to reach equilibrium displacement because the cartilage was softer than normal cartilage. An additional test load of two grams was then applied. This resulted in an increased deformation of the cartilage, which again came to an equilibrium. The displacement under the test load and the thickness of the cartilage were used to estimate the compliance of the cartilage on the basis of the equation: compliance = 1/stiffness = k × indentation depth/cartilage thickness, where k is an instrument constant that was the same for all tests.

The compliance values give a relative measure of the mechanical properties of the tissue, with emphasis on the inherent resistance of the tissue to compression. Small numbers (usually single digits) imply normal, low compliance (high stiffness), while large numbers (ap-

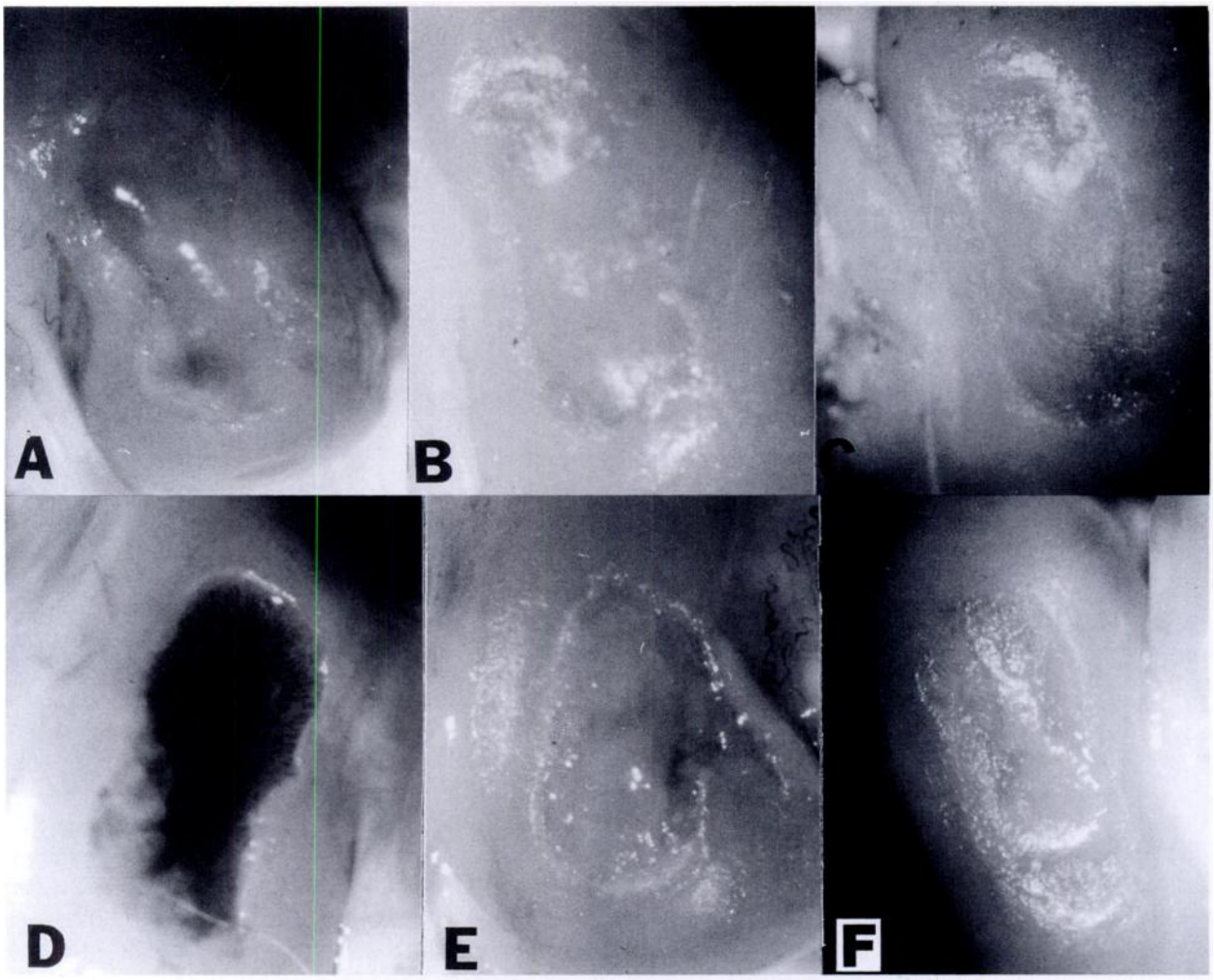

FIG. 2

Macroscopic appearance of defects at different intervals of healing, after implantation with bone-marrow-derived mesenchymal cells in collagen and evaluation at two weeks *(A)*, four weeks *(B)*, and twenty-four weeks *(C)*. Also shown are control defects that were left empty and evaluated at two weeks *(D)*, four weeks *(E)*, and twenty-four weeks *(F)*.

proximately twenty) indicate that the tissue is softer and more compliant than normal articular cartilage in the rabbit.

Results

Macroscopic Observation

No signs of osteoarthrosis, such as osteophytes, erosion of cartilage, or synovial proliferation, were observed in any of the knees at any sampling interval. This observation is important in light of the large proportion of the weight-bearing surface that was occupied by the defect.

There was no difference in the appearance of the defects that had been treated with the bone-marrow-derived mesenchymal cells compared with those that had been treated with the periosteum-derived mesenchymal cells. At two weeks after implantation, the defects were filled with shiny, smooth, white, semitransparent tissue that resembled articular cartilage; the margins of the defects were always recognizable and grossly indicated the lack of continuity between the host and the donor tissue in most regions of the defects (Fig. 2, *A*). At four weeks, the reparative tissue became opaque and well incorporated. The margins of the defects were unclear; it was generally difficult to discern the boundary between the host and the reparative tissue (Fig. 2, *B*). At twenty-four weeks, the reparative tissue had changed little in gross appearance and was quite similar to the surrounding articular cartilage (Fig. 2, *C*).

In the control group in which the defects were left empty, at two weeks the defects were filled with red, semitransparent tissue, with the margins sharply defined and the edges completely discernible (Fig. 2, *D*). At four weeks, the defects were filled with white, shiny tissue, with the margins clearly defined (Fig. 2, *E*). At twenty-four weeks, the surface of the reparative tissue appeared very irregular, and the margins of the defects were still clearly distinguishable (Fig. 2, *F*). This reparative tissue was not cartilage-like in appearance but nonetheless filled the defects.

In the cell-free-collagen-gel control group, the defects were filled with white, gel-like material at two

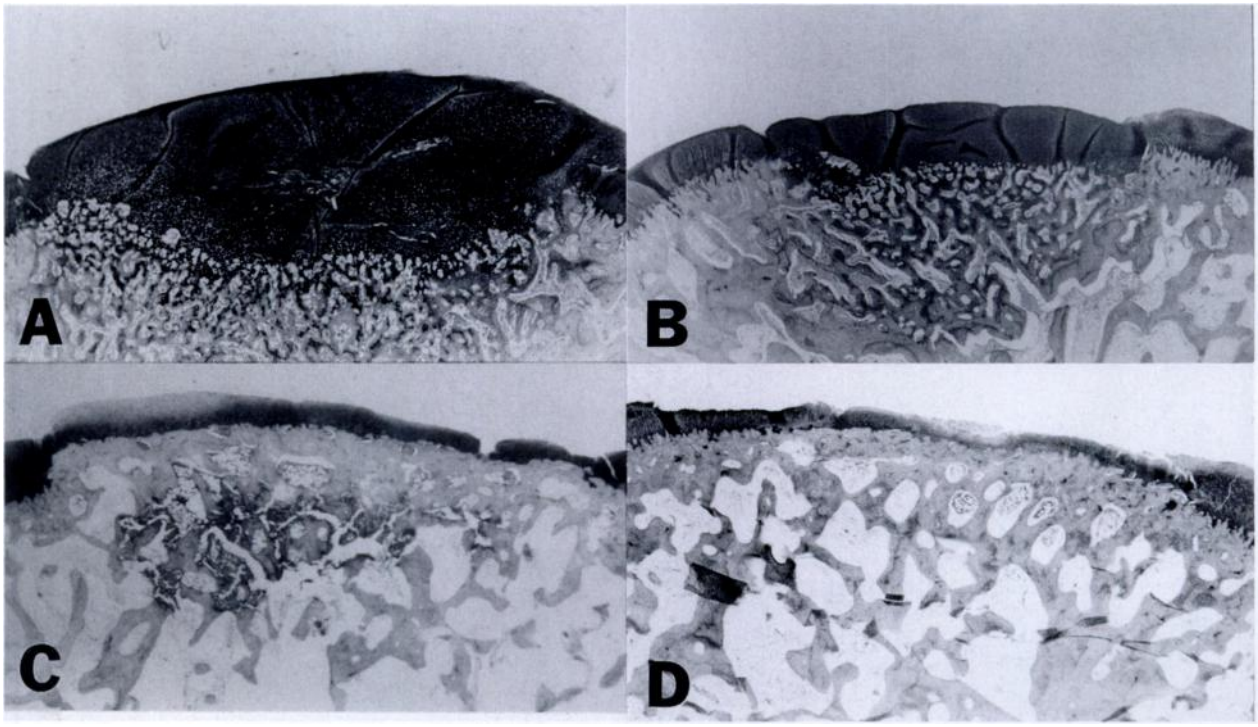

FIG. 3

Photomicrographs of defects after transplantation of bone-marrow-derived mesenchymal cells and evaluation at two weeks *(A)*, four weeks *(B)*, twelve weeks *(C)*, and twenty-four weeks *(D)* (toluidine blue; original magnification × 20).

weeks. At four and twenty-four weeks, no substantial differences were detected between this group and the empty-defect control group. The macroscopic appearance of the periosteal and the bone-marrow-derived mesenchymal-cell grafts indicated markedly improved filling of the defects compared with that in the cell-free-collagen-gel and the empty-defect control groups. The appearance of the reparative tissue formed from these grafts was similar to that of hyaline cartilage, and, in some specimens, showed impressive integration of tissue at the margins of the defects as early as four weeks after the operation. By twenty-four weeks, surface irregularities were observed in some specimens.

Histological Observations

Two Weeks after the Operation

The defects containing the bone-marrow-derived mesenchymal cells were filled with reparative tissue that resembled hyaline cartilage (Fig. 3, *A*). The reparative tissue that filled the superficial half of the defects had intensely staining, metachromatic matrix. At higher magnification, the cells resembled well differentiated chondrocytes and were surrounded by metachromatic matrix (Fig. 4). The deeper half of the defects was filled with newly formed cancellous bone, which had replaced the repair cartilage. Some unresorbed cartilage was observed in this newly formed bone in most specimens.

At the base of the defects, cartilage had been replaced by bone. At the margins of the defects, well differentiated chondrocytes and cancellous bone were seen in apposition to each other (Fig. 5, *A*). The cartilage was eroded, and host cells with associated host vasculature had begun to deposit bone matrix into the spaces previously occupied by the eroded repair cartilage.

Defects that had been filled with the periosteum-derived mesenchymal cells contained thicker cartilage (Fig. 6, *A*) than those that had been filled with the bone-

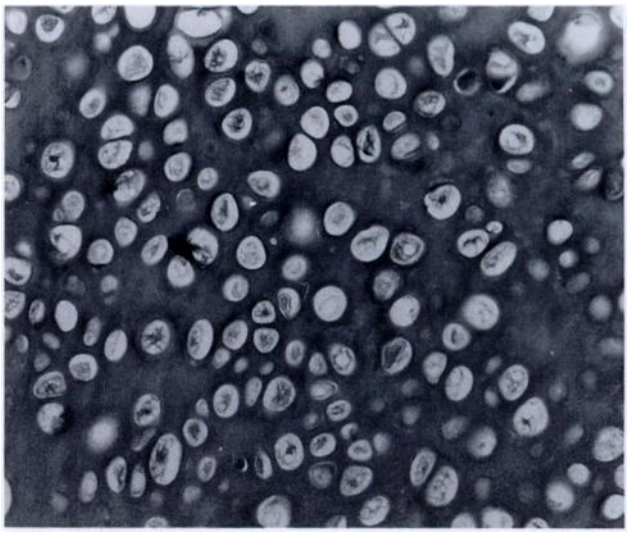

FIG. 4

Higher magnification of reparative tissue two weeks after transplantation of bone-marrow-derived mesenchymal cells (toluidine blue; original magnification × 200).

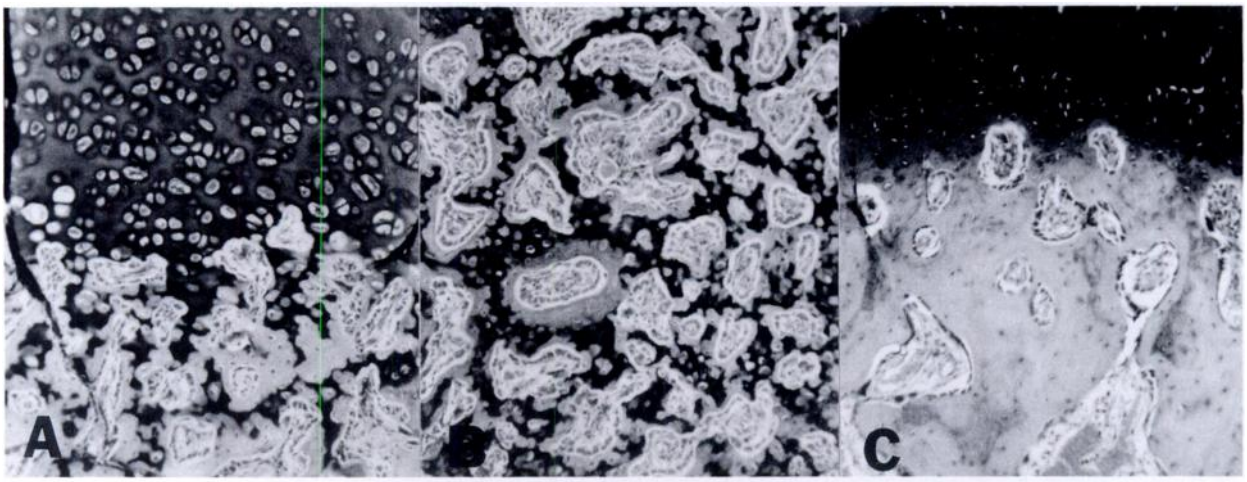

FIG. 5

Photomicrographs showing the transition from cartilage to bone in the reparative tissue at the base of the defects that were treated with bone-marrow-derived mesenchymal cells and evaluated at two weeks *(A)*, four weeks *(B)*, and twelve weeks *(C)* (toluidine blue; original magnification × 200).

marrow-derived mesenchymal cells. The cartilage occupied almost the full thickness of the original defects and had an appearance of hyaline cartilage comparable with that of the defects in the bone-marrow-derived mesenchymal-cell group.

No discernible differences were observed between the cell-free-collagen-gel and the empty-defect control groups; therefore, these groups are described together. Initially, the defects were not filled completely (Fig. 7, *A*). The deeper half of the defects was filled with newly formed, thin cancellous bone, and the superficial regions were filled with fibrous tissue.

Four Weeks after the Operation

The thickness of the cartilage was reduced proximally, and new bone filled this region of the defects in the bone-marrow-derived mesenchymal-cell group (Fig. 3, *B*). Chondrocytes below the subchondral-bone line had been replaced by dense, highly vascularized new bone beneath the reparative cartilage (Fig. 5, *B*).

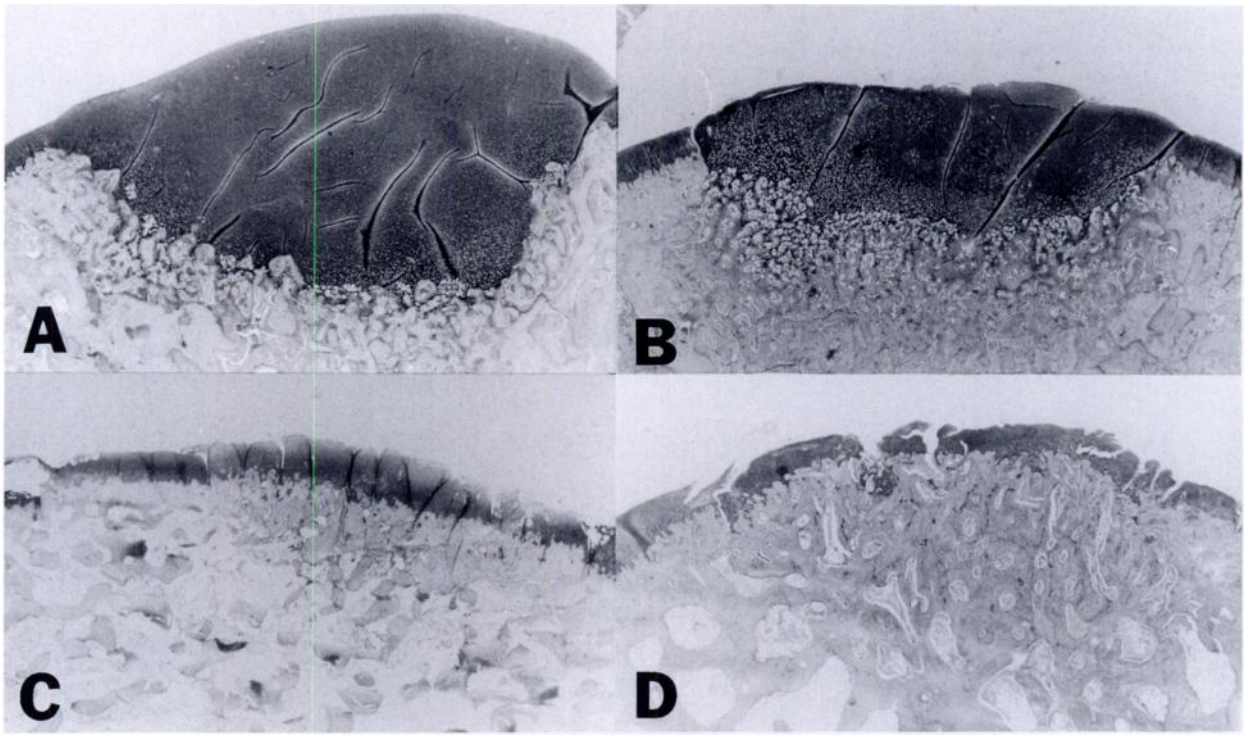

FIG. 6

Photomicrographs of sagittal sections of defects that were treated with periosteum-derived mesenchymal cells and evaluated at two weeks *(A)*, four weeks *(B)*, twelve weeks *(C)*, and twenty-four weeks *(D)* (toluidine blue; original magnification × 20).

TABLE II

RESULTS OF THE HISTOLOGICAL GRADING SCALE

Group	Interval until Animals Were Killed (Wks.)	No. of Specimens	Grade* (Points)					
			Cell Morphol.	Matrix-Staining	Surface Regular.	Thickness of Cartilage	Integ. of Donor with Adjac. Host Cartilage	Total
Bone-marrow-derived mesenchymal cells	2	7	2.6	2.2	1.8	1.6	1.6	9.8
	4	8	1.3	1.3	1.3	0.6	1.1	5.6
	12	9	1.9	1.7	1.8	1.4	1.1	7.9
	24	7	1.9	1.7	2.0	1.6	1.2	8.4
Periosteum-derived mesenchymal cells	2	6	2.5	2.0	2.2	1.1	1.5	9.3
	4	17	1.4	1.3	1.6	0.6	1.2	6.0
	12	8	1.7	1.6	2.3	1.0	1.5	8.1
	24	6	2.0	1.6	2.4	1.3	1.2	8.4
Empty defects (control)	2	11	2.9	2.2	2.6	2.0	1.9	11.6
	4	19	1.7	1.6	1.8	0.8	0.9	6.9
	12	12	1.8	1.7	2.2	1.4	1.6	8.7
	24	7	2.2	2.0	2.2	1.3	1.2	8.9
Cell-free collagen gel (control)	2	5	3.2	2.2	2.6	1.9	1.9	11.8
	4	7	1.9	1.8	2.6	0.9	1.8	9.0
	12	4	2.2	1.8	2.6	1.4	1.3	9.2
	24	3	2.4	1.9	2.5	1.4	1.4	9.5

*The values are the average of each specimen that was assessed with the histological grading scale and scored by three different investigators. Statistical analysis is not provided because the grading system is not linear or comparably equivalent between the five categories.

The articular cartilage was thicker than the surrounding host cartilage and was similar in appearance to hyaline cartilage.

In the defects that had been repaired with the periosteum-derived cells, the thickness of the cartilage was reduced, compared with that in the defects that had been treated with the bone-marrow-derived mesenchymal cells. Bone had replaced the cartilage at the base of the defects, as was observed in the bone-marrow-derived mesenchymal-cell group. However, the

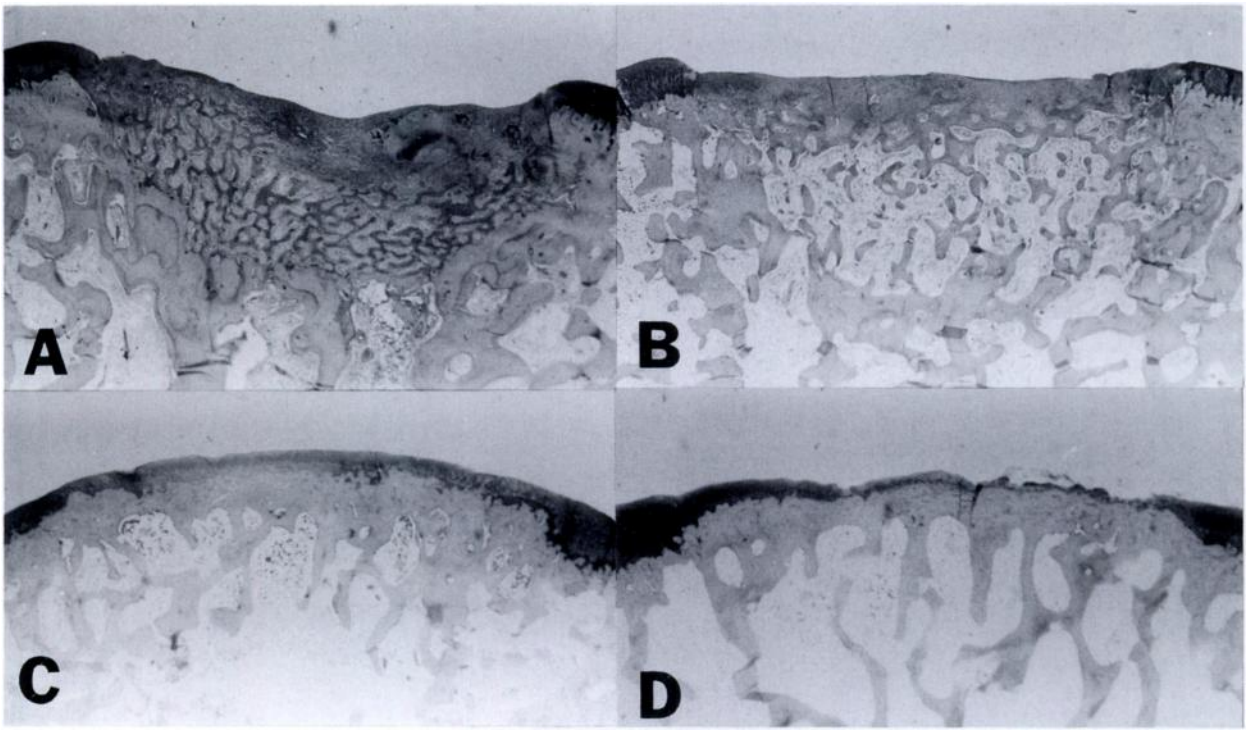

FIG. 7

Photomicrographs of sagittal sections of defects that were left empty and evaluated at two weeks *(A)*, four weeks *(B)*, twelve weeks *(C)*, and twenty-four weeks *(D)* (toluidine blue; original magnification × 20).

articular cartilage was still thicker than that of the adjacent, normal cartilage (Fig. 6, *B*). The appearance of the cartilage was grossly comparable with that of hyaline cartilage, although the surface was slightly irregular in some specimens.

The defects in the cell-free-collagen-gel and the empty-defect control groups were filled with thick, *de novo* bone in the proximal half and with fibrous tissue extending to the distal edge of the host margin (Fig. 7, *B*). We consistently observed faint metachromatic staining in the matrix of the fibrous tissue covering the articular surface.

Twelve Weeks after the Operation

The cartilage in the defects of the bone-marrow-derived mesenchymal-cell group was thinner than that of the adjacent host cartilage (Fig. 3, *C*). The appearance of the cartilage was still comparable with that of hyaline cartilage, but in some areas metachromatic staining was abnormally faint or absent. The subchondral cartilage was completely replaced by new bone of the same thickness as that of the host subchondral bone (Fig. 5, *C*).

The cartilage in the defects of the periosteum-derived mesenchymal-cell group also appeared thinner than that of the surrounding articular cartilage (Fig. 6, *C*). Although most of the chondrocytes had an appearance comparable with that of the chondrocytes of hyaline cartilage, the articular surface of the cartilage appeared to be more irregular.

In the cell-free-collagen-gel and the empty-defect control groups, the metachromatic staining in the fibrous tissue appeared more intense than at four weeks but was patchy and uneven, and the articular tissue was thinner than the adjacent host cartilage (Fig. 7, *C*).

In all groups, subchondral bone had been completely reformed.

Twenty-four Weeks after the Operation

The cartilage that formed where the bone-marrow-derived mesenchymal cells had been placed in the defects was slightly thinner than at twelve weeks (Fig. 3, *D*). Metachromatic staining was not observed in some regions, but the repaired surface remained smooth. The cartilage that formed where the periosteum-derived mesenchymal cells had been placed in the defects appeared quite thin, metachromatic staining was absent in some regions, and the surface was more irregular than in the twelve-week specimens (Fig. 6, *D*). In the cell-free-collagen-gel and the empty-defect control groups, the tissue was very thin, with an irregular surface, and appeared comparable with tissue in the final stages of an osteoarthrotic lesion (Fig. 5, *D*). In all groups, subchondral bone had been completely reformed.

Histological Grading of the Repair Tissue

The scores of both the bone-marrow and the periosteum-derived mesenchymal-cell groups were improved at four weeks compared with those at two weeks, but at twelve and twenty-four weeks after implantation, the scores were inferior to the four-week scores (Table II). These results were in accord with those of macroscopic and histological observations. The scores of the two mesenchymal-cell groups were similar, except for that for surface regularity. The surface of the cartilage that formed where the periosteum-derived mesenchymal cells had been placed in the defects became progressively more irregular compared with the cartilage surface in the bone-marrow-derived mesenchymal-cell group.

In the cell-free-collagen-gel and the empty-defect control groups, the histological scores indicated markedly inferior repair compared with that in both of the cell-implanted groups, at each interval of sampling.

TABLE III

COMPLIANCE VALUES FOR THE TISSUE OF THE DEFECTS AT TWENTY-FOUR WEEKS AFTER THE OPERATION

Group	No. of Specimens	Compliance Values*		
			Defect Cartilage	
		Normal Cartilage	Post. Portion	Ant. Portion
Bone-marrow-derived mesenchymal cells	6	5.1	18.4	24.0
		2.7	6.1	18.4
		5.4	5.2	5.2
		5.3	12.8	16.5
		8.8	13.1	11.9
		4.0	13.1	14.7
Average		5.2	11.5	15.1
Periosteum-derived mesenchymal cells	5	4.7	7.4	9.1
		4.5	4.1	26.7
		2.8	9.6	12.0
		3.1	12.4	27.2
		3.7	11.1	18.1
Average		3.8	8.9	18.8
Empty defects (control)	5	3.5	†	41.2
		3.9	21.0	†
		3.5	20.0	†
		5.9	21.0	†
		4.2	20.7	41.2
Average		4.2	>21.7	>41.2

*Compliance was measured for the normal cartilage of the lateral condyle in the operatively treated knees and for the posterior and anterior portions of the repaired tissue of the same knees. Since all values for all specimens are provided, statistical analysis of the averages is not given.

†The compliance value could not be determined because the cartilage was too soft.

Mechanical Properties of the Repair Tissue

Compliance values were determined for the repair cartilage and the normal cartilage, from the same knees, in the groups that had been treated with the bone-marrow and the periosteum-derived cells and in the empty-defect control group (Table III). The compliance values for the bone-marrow and the periosteum-derived-cell groups indicated that the repair tissue was less compliant (more stiff) than that in the empty-defect control knees. These values also indicated that the repair tissue was more compliant (less stiff) than normal

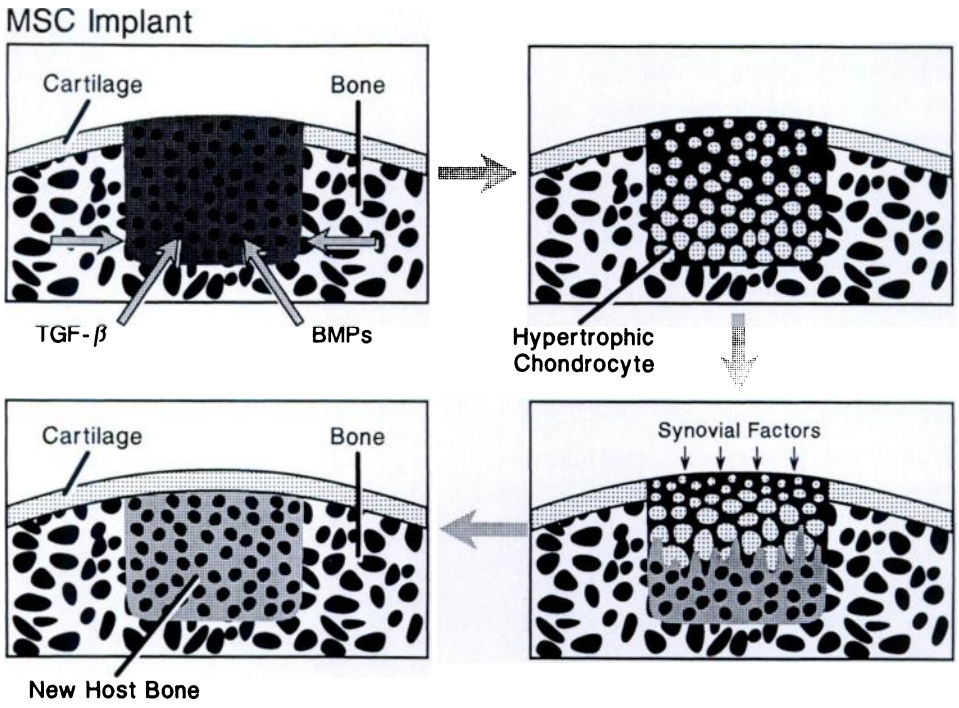

FIG. 8

Schematic drawings showing the sequence of events during the first four weeks, from the time of implantation of mesenchymal stem cells in a collagen gel (upper-left panel) to the time of replacement of the resulting cartilage by donor host bone covered by articular cartilage. MSC = mensenchymal stem cell, TGF-β = transforming growth factor-beta, and BMPs = bone morphogenetic proteins.

cartilage. Within a defect, the posterior portion of the repair cartilage was less compliant than the anterior portion. This was probably due to the kinematics of the rabbit knee and the different forces exerted on the posterior compared with the anterior aspect of the femoral condyle.

Discussion

The current investigation demonstrates that large, full-thickness defects of the weight-bearing region of the articular cartilage were repaired with hyaline-like cartilage after implantation of autologous osteochondral progenitor cells that had been isolated from bone-marrow or periosteal tissue and grown in cell culture. As early as two weeks after transplantation, the osteochondral progenitor cells had differentiated into chondrocytes; the repair cartilage in the deeper portion of the defects was then replaced with vascularized bone. By four weeks after transplantation, the deeper portion of the defects had been almost completely replaced by bone, and by twelve weeks, subchondral bone had been completely repaired without loss or alteration of the overlying articular cartilage. In some specimens, the articular cartilage remained separated by a gap from the surrounding host cartilage, but the underlying bone was always completely united with that of the host. This is the first report, to our knowledge, of the implantation of purified, cultured, autologous osteochondral progenitor cells into full-thickness defects, and of the differentiation of these mesenchymal cells into chondrocytes that were subsequently replaced by bone up to the bone-articular cartilage junction. These events resulted in the formation of articular cartilage on subchondral bone, which, in effect, resulted in the resurfacing of the condyle.

Previous reports from our laboratory have noted that bone-marrow or periosteum-derived cells, which we have called mesenchymal stem cells[8], have both osteogenic and chondral potential when tested in either *in vivo* or *in vitro* systems[22,23,26,40-42]. Although these progenitor cells are scarce, we have been able to grow them in culture from marrow harvested from rats, chickens, humans, mice, dogs, goats, and, as described here, rabbits. These mesenchymal-stem-cell preparations rapidly differentiated into chondrocytes in defects of the distal femoral condyle of the rabbit. We hypothesize that these donor chondrocytes and the cartilage tissue that they form is replaced by host-derived vascular and bone-forming cells up to the bone-articular cartilage junction. Without suitable experimental marking of these donor cells, this theory cannot be verified; however, the speed with which the cartilage is replaced by vascularized bone is impressive and is comparable with the process of formation of endochondral bone, which we have described previously[8]. With this in mind, we propose the following hypothetical sequence of events, which forms a basis for our discussion of the observations presented here (Fig. 8).

First, the creation of the large defect of the distal femoral condyle establishes an osseous receptacle into

which host-bone-derived bioactive factors, such as transforming growth factor-beta and bone morphogenetic proteins, are released. In addition, we suspect that the post-injury response serves to infuse the implanted collagenous delivery vehicle with bioactive agents, provided both from the site of the wound itself and systemically. These agents initiate the immediate and rapid entrance of the donor mesenchymal stem cells into the chondrogenic pathway, which results in the rapid fabrication of embryonic-like cartilage tissue.

Second, the continually changing milieu of the osseous receptacle causes the chondrocytes in the defect to change rapidly until the most proximal cells in the repair tissue become hypertrophic, and this tissue, in turn, elicits chondroclast-mediated erosion and vascular infiltration. The resorption in the cartilage of the defect and the invasion by vascular tissue draw host-derived mesenchymal stem cells from the marrow or perivascular-associated cells into this region. The host-derived cells in these highly vascularized sites enter the osteogenic pathway and rapidly fabricate osseous tissue, which replaces the repair cartilage.

Third, the process of erosion and replacement rapidly changes the repair cartilage into bone up to the natural junction of the host cartilage and the subchondral bone. The distal repair articular cartilage is not resorbed because it has differentiated in a manner quite unlike that of the cartilage in the defect, which has been in contact with the osseous receptacle. This distal cartilage at the articular boundary is influenced by the milieu of the synovial cavity and the molecules found in the synovial fluid. Because the rabbits were allowed to move freely after the operation, the joint cartilage developed rapidly into load-bearing tissue, which is intrinsically different from the proximal repair tissue and, thus, can neither be resorbed nor infiltrated by the vasculature.

There are several important aspects of our hypotheses for which substantial support is already available in the literature. Injured bone rapidly disburses and attracts potent bioactive factors, the most often studied of which are the transforming growth-factor-beta family or the bone morphogenetic proteins[4,51,60,61]. These factors function to convert osteochondral progenitor cells into chondrocytes; indeed, the bioassay developed by Urist et al.[57] and others[49,50,52,54] for the purification of these factors involves either the *in vivo* or the *in vitro* conversion of progenitor mesenchymal cells into chondrocytes. It must be stressed that the target of these bioactive factors is mesenchymal stem cells, not differentiated chondrocytes; differentiated chondrocytes respond to these factors in a negative manner[15,38]. Thus, when articular chondrocytes are transplanted into a full-thickness condylar defect, we would expect these cells to resist resorption substantially and to be unresponsive to the bioactive factors released from the osseous defect. This prediction is borne out by the observation that heterologous articular chondrocytes are retained in the depths of full-thickness defects for months and replacement by bone, when it occurs, is an extremely slow process[58,59].

Others have shown that when articular chondrocytes are introduced into cell culture or exposed to bioactive agents, the quantity of phenotype-specific macromolecules is affected, but the identity or chemical characteristics of these entities is not[31,56]. In particular, developing cartilage exhibits a changing pattern in the composition of the synthesized proteoglycans, specifically aggrecan[6,10,53]. In contrast to comminuted chondrocytes, when articular cartilage chondrocytes are placed under identical *in vitro* circumstances, they continue to synthesize the aggrecan species that were previously synthesized *in vivo*[56]. In contrast, mesenchymal stem cells that enter the chondrogenic lineage can rapidly progress through this lineage to the hypertrophic state, even in culture[42]. The *in vivo* hypertrophic cartilage is then the target for erosion and vascular invasion[8]. The presence of vasculature and new, host-derived mesenchymal stem cells will enhance and contribute to the replacement of that cartilage by bone. The sequence of the replacement events (endochondral myelopoiesis) is identical to that observed in the development of the embryonic joint, at the growth plate in growing animals, at sites of heterotopic implantation of demineralized bone chips, or at *in vitro* or *in vivo* sites of either native or recombinant bone-morphogenetic-protein release[7-9,11].

The size of the defects that were created in the medial femoral condyle in the current study was three millimeters wide, six millimeters long, and three millimeters deep. These defects, which composed 40 to 50 per cent of the weight-bearing surface of the condyle, are among the largest ever reported in repair studies in rabbits. Although small defects in articular cartilage have been noted to undergo repair either spontaneously or under conditions of tissue or cell augmentation[5], defects as large as those in our study have not been described previously, to our knowledge, as having undergone either spontaneous healing or substantial repair. The observed repair, although not perfect, is a major accomplishment.

Two aspects of this repair require additional experimentation and improvement. The first involves the progressive thinning of the repair tissue, which was discerned clearly by twenty-four weeks after transplantation. The mesenchymal-stem-cell preparation used for the periosteal-cell group showed some differences compared with the mesenchymal cells derived from the bone marrow. These differences involved the smoothness of the surface of the repair cartilage at the latest sampling intervals. With respect to the periosteal-cell group at twenty-four weeks, although not all joints behaved uniformly, there was a tendency for the repaired surface to split and fibrillate. With respect to the bone-marrow-cell group, the repair cartilage remained smooth, although the cartilage became thin. The second area for improvement involves the integration of the

repair cartilage with the cartilage of the host. Although substantial integration occurred around the edges of the defects, we observed incomplete integration of the repair and host cartilage at some locations. Systematic experimentation is required to develop techniques for the integration of these surfaces.

We realize that a detailed biochemical, immunohistochemical, and molecular biological analysis, including *in situ* hybridization, is required to define the phenotypic character of the tissue that was formed and the sequence of events that occurred at the repair site. However, these studies will be conducted after we have optimized the repair of the defect by measurement of the macroscopic, histological, and biomechanical parameters reported here.

We used mechanical testing to evaluate the qualities of the repair tissue. The indentation data clearly indicate that the mechanical properties of the repair tissue at six months approached those of the host tissue. Differences between the anterior and posterior aspects of the repair tissue probably reflect the mechanical environment of the rabbit knee, which is substantially different in these two locations. These mechanical differences are due to the gait and motion of the rabbit joint, which suggest that the posterior cartilage is subjected to more force than the anterior cartilage. It should be stressed that the rabbits were returned to full activity in their cages immediately after the operation. In humans, gradual weight-bearing, coupled with constant passive motion[47], would probably be the protocol of choice. O'Driscoll et al.[43,44] believed that constant passive motion may be an important factor in the successful repair of full-thickness defects of the cartilage.

The procedures to elicit repair that are described in this report may lead to a clinically useful method of treatment for defects of the articular cartilage. A small number of autologous progenitor cells may be isolated, grown *in vitro*, and then introduced into massive full-thickness defects. These cells, when implanted in such defects, appear capable both of differentiating into articular cartilage and of inducing the formation of subchondral bone. The procedures have considerable relevance to the treatment of defects in the cartilage of humans and provide the basis for the development of a repair technology that is capable of regenerating large areas of articular cartilage.

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