Repair of large full-thickness articular cartilage defects in the rabbit

THE EFFECTS OF JOINT DISTRACTION AND AUTOLOGOUS BONE-MARROW-DERIVED MESENCHYMAL CELL TRANSPLANTATION

We produced large full-thickness articular cartilage defects in 33 rabbits in order to evaluate the effect of joint distraction and autologous culture-expanded bone-marrow-derived mesenchymal cell transplantation (ACBMT) at 12 weeks. After fixing the knee on a hinged external fixator, we resected the entire surface of the tibial plateau. We studied three groups: 1) with and without joint distraction; 2) with joint distraction and collagen gel, and 3) with joint distraction and ACBMT and collagen gel.

The histological scores were significantly higher in the groups with ACBMT collagen gel (p < 0.05). The area of regenerated soft tissue was smaller in the group allowed to bear weight (p < 0.05). These findings suggest that the repair of large defects of cartilage can be enhanced by joint distraction, collagen gel and ACBMT.

Lesions of articular cartilage do not heal well, and usually only partially. They are often associated with pain, stiffness and altered function of the joint and often progress to secondary osteoarthritis.1 Mosaicplasty2 and autologous cultured chondrocyte implantation3 are effective for the repair of partial articular defects. The size of the defect which can be repaired is limited and for large defects, such as occur in osteoarthritis or rheumatoid arthritis, no methods of repair are available, although techniques such as total joint replacement, osteotomy or arthrodesis can be performed for elderly patients.

There are few clinical reports of the repair of large defects of the articular cartilage. In 1978, Judet and Judet4 reported satisfactory results of the repair of large defects mainly in osteoarthritis of the ankle. They used a hinged external fixator which allowed both joint distraction and movement.

Joint distraction reduces the contact pressure on the weight-bearing area and provides a mechanical stimulus which is important for the nutrition of articular cartilage.5,6 After high tibial osteotomy Koshino et al7 found that a medial femoral condyle which had been exposed to a high contact pressure and had a bare sclerotic bony surface, became covered by regenerated cartilage as a consequence of the alterations in contact pressure created by surgical realignment.

Movement of the joint is advantageous for the repair of articular cartilage whereas immobilisation results in its degeneration. Salter et al8,9 observed both clinically and experimentally that continuous passive motion of a synovial joint was more effective in repairing articular cartilage than intermittent movement or immobilisation. They hypothesised that continuous passive motion enhanced the nutrition and metabolic activity of articular cartilage and stimulated pluripotent mesenchymal cells to differentiate into it. Recently, the effects of mechanically-controlled movement on the regeneration of cartilage have been investigated using in vivo models.10,11 Harada et al10 demonstrated that a higher sliding displacement with a continuous bending movement stimulated partial regeneration of articular cartilage in an osteotomised vertebra model in the rat.

Spontaneous repair of articular cartilage does not occur unless the defects penetrate the bone marrow,12,13 because repair cells are thought to originate from undifferentiated mesenchymal cells in the underlying marrow.14 Drilling, microfracture, and spongialisation15 can be used clinically for the treatment of partial defects in order to induce undifferentiated mesenchymal cells in the bone marrow. However, reparative tissues are usually biochemically and biomechanically different from normal hyaline cartilage, and extensive degenerative changes can occur in the long term.16-18 To produce cartilage of better quality, as yet unidentified favourable conditions must be created.

Although undifferentiated mesenchymal cells from the bone marrow do not undergo
chondrogenesis in monolayer culture, their differentiation can be induced in aggregate culture in the presence of a specific growth factor such as transforming growth factor-beta (TGF-β).\textsuperscript{19,20} A high density of mesenchymal cells is a requirement for this, which suggests that cell interactions are very important for chondrogenesis. So-called \textit{in vivo} culture, within a joint cavity may also provide better conditions than those found \textit{in vitro} because the movements of the joint provide mechanical stimulation and the synovial fluid contains many growth factors.

In our study, we established an animal model of large full-thickness articular cartilage defects in the knee of rabbits using a hinged fixator, which allowed the animals to move the joint freely. We then studied the effects of autologous culture-expanded bone-marrow-derived mesenchymal cell transplantation (ACBMT) at a high cell density. For partial defects the transplanted cells are usually held in place under a periosteal flap which covers the defects by being sutured to the rim of normal peripheral cartilage.\textsuperscript{3,21-23} In our large-defect model, however, there was no normal cartilage around the defect, and it was therefore difficult to hold the transplanted cells in place even when using a scaffold or a periosteal flap. ACBMT was therefore performed by joint injection in the expectation that the transplanted cells would adhere to the site of the defect and differentiate into articular cartilage or secrete growth factors in the same way as undifferentiated mesenchymal cells from the bone marrow.

\textbf{Materials and Methods}

We used 33 Japanese white rabbits, aged from four to six months with a mean weight of 3.47 ± 0.47 kg. The study was approved by the University Committee for Animal Experimentation.

All the operations were performed on the left knee of the rabbits under general anaesthesia induced by an intravenous injection of pentobarbital sodium (Somnopentyl, Schering-Plough Animal Health, Union, New Jersey). An incision was made over the medial aspect of the knee. A smooth wire, 2 mm in diameter, was passed through the distal femur at the bone at the origin of the lateral collateral ligament and was attached to an external fixator at the centre of hinge joints. Two threaded half pins 2 mm in diameter were drilled into the femur and tibia and were attached to half rings (Fig. 1a). The hinged fixator allowed concentric circular movements.

After external fixation, a lateral parapatellar incision was made and the patella everted. The cruciate and collateral ligaments were divided and the menisci resected. We then formed a full-thickness defect on the articular cartilage of the entire surface of the tibial plateau with an oscillating saw (Fig. 1a). The size of the defect was more than 10 x 20 mm (Fig. 1b). A space was formed in the femorotibial joint by the resected meniscus and osteochondral defect, resulting in joint distraction (Fig. 1).

Four groups of rabbits were used, groups C, W, T and V. In group C (control group, n = 15) the fixators were distracted and the rabbits were killed at 1 (n = 3), 3 (n = 3), 6 (n = 3) and 12 (n = 6) weeks after external fixation. Group W (weight-bearing group) contained six rabbits whose fixators were not distracted. These were killed at 12 weeks. In group T (transplantation group, n = 6), the joints were distracted and ACBMT with atelocollagen gel (Koken Inc, Tokyo, Japan) was injected into the knee using an 18-gauge}
CO (Gibco BRL) and antibiotics (Gibco BRL) at 37˚C in 5% 

- CO

A method similar to that of both Wakitani et al

- A method

The aspirate was washed twice with phosphate-buffered

- Phosphate

syringe containing 0.3 ml of heparin (1000 units per ml).

- Heparin

2 ml of blood were aspirated from the bone

- Bone

exposed and 2 ml of blood were aspirated from the bone

- Bone

marrow using an 18-gauge needle attached to a 5 ml

- Syringe

injection. Collagen gel was used to prevent the

- Collagen

injected material. Collagen gel was used to prevent the

- Collagen

leakage of the newly-formed bony tibial plateau.

- Bone

three weeks after operation. The rabbits were killed

- Bone

at 12 weeks. In group V (vehicle control, n = 6), the rabbits

- Rabbit

were injected with atelocollagen gel only and were killed at

- Rabbit

weeks. In groups V and T, the joints were distracted by

- Joints

2 mm at the time of injection to provide space for the

- Space

injected material. Collagen gel was used to prevent the

- Collagen

transplanted cells leaking out of the joint cavity.

- Cells

Cell culture and preparation of cell-seeded implants. We used a method similar to that of both Wakitani et al24 and Im et al.25 The intercondylar notch of the left distal femur was exposed and 2 ml of blood were aspirated from the bone

- Blood

marrow using an 18-gauge needle attached to a 5 ml

- Syringe

aspirate was washed twice with phosphate-buffered saline and centrifuged at 180 x g for five minutes. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, Grand Island, New York) in a 75 

- DMEM

cm² culture flask, containing 10% fetal bovine serum (Gibco BRL) and antibiotics (Gibco BRL) at 37°C in 5% 

- CO₂

The medium was changed on the fourth day of culture and then twice per week thereafter. Non-adherent cells were then removed. After three weeks of culture, the adherent cells were almost confluent and were removed, treated with 0.05% trypsin and 0.001 M ethylene diamine tetra acetic acid (EDTA) and counted in a haemocytometer before incorporation into the atelocollagen gel (0.5% acid-soluble type-I collagen, obtained from bovine skin) containing the culture medium. The final cell density was adjusted to 5.0 x 10⁶ cells/ml in the atelocollagen gel-DMEM mixture (0.3% type I collagen). The mixture was allowed to gel at 37°C in carbon dioxide for ten minutes. This gel containing bone-marrow-derived mesenchymal cells was transplanted into the defect within two hours.

- Bone

Histological and histochemical analyses of specimens. The rabbits were killed by injecting a lethal dose of pentobarbital sodium. The defects were examined and photographed.

- Specimens

The proximal tibia was resected en bloc, fixed in 10% neutral buffered formalin for at least one week and decalcified with 0.5 M EDTA solution. Sagittal sections of the midportion of the medial and lateral tibial plateaux were cut, embedded in paraffin and microtomed into sections 5 μm thick. These were stained with haematoxylin and eosin, Safranin-O/Fast Green, a monoclonal antibody for type II collagen (Oncogene, Darmstadt, Germany) and a monoclonal antibody for type-I collagen (Sigma, St Louis, Missouri).

- Section

For immunohistochemical staining, sections were deparaffinised with xylene and rehydrated with decreasing concentrations of ethanol solutions. They were incubated with 3% H₂O₂ for ten minutes and proteinase K (Dako, Glostrup, Denmark) for ten minutes to block the activity of endogenous peroxidase. They were then incubated with monoclonal mouse antibodies for type I and type II collagen at the optimal dilution in 0.05 M tris buffered saline for one hour. The sections were rinsed with 0.05 M tris buffered saline three times for five minutes each and then incubated with Envision (Dako) for 30 minutes. They were then rinsed three times with tris buffered saline and then incubated with DAB solution (Dako) for a few minutes.

- Envision

Histological and histochemical assessments. Using the sections taken at 12 weeks, we assessed the central one-third of regenerated tissue and scored it blind using a grading scale modified from the International Cartilage Repair Society (ICRS) visual histological assessment scale.26 The original ICRS criteria were: the regularity of the surface, matrix morphology, cell distribution, cell population viability, subchondral bone and mineralisation of cartilage (Table I). To these we added two more namely, staining for type I and for type II collagen (Table I).

- ICRS

Histomorphometry. The defect was repaired by regenerating bone and soft tissue, including fibrous tissue, fibrocartilage and hyaline-like cartilage. We obtained digital images of the sections and defined the regenerated articular surface of the newly-formed bony tibial plateau. We drew a line C between the edges of the anterior (point A) and posterior (point B) bony tibial plateau. Lines D and E were constructed perpendicular to line C, intersecting at points A

<table>
<thead>
<tr>
<th>Feature</th>
<th>Score</th>
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<tbody>
<tr>
<td>I Surface</td>
<td></td>
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<tr>
<td>Smooth/continuous</td>
<td>3</td>
</tr>
<tr>
<td>Irregularities discontinuous</td>
<td>0</td>
</tr>
<tr>
<td>II Matrix</td>
<td></td>
</tr>
<tr>
<td>Hyaline</td>
<td>3</td>
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<tr>
<td>Mixture: hyaline/fibrocartilage</td>
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<tr>
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<td>1</td>
</tr>
<tr>
<td>Fibrous tissue</td>
<td>0</td>
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<tr>
<td>III Cell distribution</td>
<td></td>
</tr>
<tr>
<td>Columnar</td>
<td>3</td>
</tr>
<tr>
<td>Mixed/columnar-clusters</td>
<td>2</td>
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<tr>
<td>Clusters</td>
<td>1</td>
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<tr>
<td>Individual cells/disorganised</td>
<td>0</td>
</tr>
<tr>
<td>IV Cell population viability</td>
<td></td>
</tr>
<tr>
<td>Predominantly viable</td>
<td>3</td>
</tr>
<tr>
<td>Partially viable</td>
<td>1</td>
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<tr>
<td>&lt; 10% viable</td>
<td>0</td>
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<tr>
<td>V Subchondral bone</td>
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<tr>
<td>Normal</td>
<td>3</td>
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<tr>
<td>Increased remodelling</td>
<td>2</td>
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<tr>
<td>Bone necrosis/granulation tissue</td>
<td>1</td>
</tr>
<tr>
<td>Detached/fracture/callus at base</td>
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<tr>
<td>VI Cartilage mineralisation</td>
<td></td>
</tr>
<tr>
<td>Calcified cartilage</td>
<td>3</td>
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<tr>
<td>Normal</td>
<td>0</td>
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<tr>
<td>Abnormal/inappropriate location</td>
<td>0</td>
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<tr>
<td>VII Type I collagen staining of the matrix</td>
<td>2</td>
</tr>
<tr>
<td>Normal or nearly normal</td>
<td>3</td>
</tr>
<tr>
<td>Slight</td>
<td>2</td>
</tr>
<tr>
<td>Moderate</td>
<td>1</td>
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<tr>
<td>Abundant</td>
<td>0</td>
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<tr>
<td>VIII Type II collagen staining of the matrix</td>
<td>2</td>
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<tr>
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<td>Moderate</td>
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<tr>
<td>Slight</td>
<td>1</td>
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<tr>
<td>None</td>
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- *ICRS, International Cartilage Repair Society²⁶

Table I. The modified ICRS* visual histological assessment scale
and B (Fig. 2). We measured the area of regenerated soft tissue and that stained by Safranin-O and anti-type-II collagen antibody within the range of lines D and E by the public domain programme NIH image (National Institutes of Health, Bethesda, Maryland), and calculated the percentage of each area. In the regenerated soft tissue, the areas of fibrous tissue, synovial tissue and meniscus-like thick fibrous tissue attached to the articular capsule were excluded from the measurements (Fig. 2). We also measured the length of the surface of the bony tibial plateau (point A to point B) and the lengths of the exposed bony and articular surfaces, and calculated the percentages.

**Statistical analysis.** This was performed using an analysis of variance, differences between the groups being calculated. The post-hoc test according to Fisher’s protected least significant difference allowed the assessment of significance for individual differences to be made. We considered a p value of less than 0.05 to be significant.

**Results**

**Gross morphological findings.** In most of the 33 rabbits, when the joint was exposed the synovial tissue and fluid appeared to be grossly normal, although there was evidence of hypertrophy in some joints. In each group, at 12 weeks, a glossy regenerating tissue was generally observed at the centre of each regenerated tibial plateau facing the femoral condyle. There was evidence of a meniscus-like thick fibrous tissue peripherally and a cruciate-like massive fibrous tissue in the intercondylar notch. Formation of ectopic bone was not observed in any of the specimens. Ulceration of cartilage was sometimes observed in the articular surface of the femoral condyle.

**Histological findings**

**Control group (Fig. 3).** Immediately after forming the defect, the trabecular bone was exposed to the joint space (Fig. 3a). Seven days later, the articular surface was smooth and remodelled (Fig. 3b). Spindle-shaped mesenchymal cells were observed on the surface of the defect. At three weeks, there was synthesis of the cartilaginous extracellular matrix, as indicated by the staining of the matrix by Safranin-O and anti-type I and type II collagen antibodies. The matrix was synthesised between the superficial fibrous stroma and the deeper area of a newly-formed woven bone (Fig. 3c). At six weeks, the regenerated cartilage tissue had reached the surface of some areas and the cartilage matrix stained strongly with Safranin-O (Fig. 3d). The flat bone surface of the tibial plateau was remodelled into a concavity which adapted to the shape of each femoral condyle. At 12 weeks, the cells had a columnar distribution (Fig. 3e), and were covered with a surface layer containing flat fibrocartilaginous cells. In many sections, a meniscus-like tissue was observed in addition to the regenerated articular cartilage tissue. At six and 12 weeks, type II collagen was observed in the chondrocytes and in the matrix; and type I
Photomicrographs showing repair of the cartilage with time in the control group (a to e), and after 12 weeks in each study group (f, group W; g, group V; h, group T) (Safranin-O/Fast Green, a, b, c and f x40; d, e, g and h, x100).
collagen was less intense compared with type II collagen (Figs 4a and 4b).

**Treatment groups.** Regeneration of cartilage was observed in all the treatment groups to some degree. In group W, there was little regenerated tissue on the contact surface with exposed bone on the articular surface (Fig. 3f). Hyaline-like cartilage was observed only peripherally in some sections. In group V, most of the regenerated tissues in the superficial and middle zones seemed to be fibrocartilage, which was strongly stained by the anti-type I collagen antibody (Figs 3g and 4c). The hyaline-like cartilage tended to be localised in the deeper zone. In group T, hyaline-like cartilage was observed immediately below the superficial zone, which was strongly stained by the anti-type I collagen and anti-type II collagen antibody (Figs 3g, 4c and 4d).

**Histological and histochemical assessments.** The mean scores are shown in Table II. Groups C and T were significantly better than group V in terms of their matrix morphology.
Those which exceed a critical size do not repair spontaneously. In our study, we formed full-thickness defects of the articular cartilage by resecting the entire articular cartilage from the weight-bearing area of the tibial plateau, the size of the defect being more than 10 mm in diameter. In rabbits, defects of 3 mm in diameter healed spontaneously with a repair tissue that was well developed at three weeks, true hyaline cartilage appeared at four to eight weeks and the chondrocyte layer was well developed at 12 to 24 weeks. In our study, mesenchymal cells adhered to the surface of the defect seven days after its formation while the chondrocyte layer was observed at three weeks and became well developed by 12 weeks. Our observations resembled a gradual, partial repair of a cartilage defect and suggest that even a large articular cartilage defect can repair by the same mechanism when ideal environmental conditions are applied to the mesenchymal cells.

Clinically, joint distraction has been proposed as a method of treatment for osteoarthritis in order to reduce pain and delay the time until an arthrodesis, usually of the ankle, become necessary. In vivo, it has been shown that intermittent fluid pressure, in combination with the absence of mechanical stress, is beneficial for osteoarthritic cartilage and can diminish inflammation. Animal studies have shown that joint distraction can change chondrocyte activity beneficially. In our study, the regenerated soft-tissue area significantly increased in the joint-distracted group compared with the weight-bearing group. In the latter, the regenerated cartilage was present mostly in the non-contact area. Therefore, joint distraction was considered to be favourable for the regeneration of articular cartilage.

In recent clinical trials, the transplantation of mononuclear cells isolated from bone marrow by simple injections has been used in ischaemic heart disease and in ischaemia of the limbs, with beneficial effects such as an improvement in cardiac function or increased blood flow. In vivo, mononuclear cells from bone marrow can differentiate to form various tissues according to their environment.

In our results, the vehicle-treated group was significantly inferior to the control group for the following reasons. First, in the vehicle-treated groups we had to perform one more injection at three weeks and joint distraction of about 2 mm to form a new space. Secondly, we injected collagen gel in the vehicle-treated groups. Both of these may variably affect the spontaneous regeneration of articular cartilage. When articular defects penetrate the bone marrow, the defect occupied by a fibrin matrix containing releasable growth factors, other proteins, glycoproteins, platelets, and white blood cells as well as bone-marrow mesenchymal

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<th>Table II. Histological mean (sd) scores of the four groups according to the ICRS* scale26 (see Table I)</th>
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<tr>
<td>Group†</td>
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<tr>
<td>C</td>
</tr>
<tr>
<td>W</td>
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<td>V</td>
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<td>T</td>
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* ICRS, International Cartilage Repair Society
† C, control; W, weight-bearing; V, vehicle; T, transplantation
‡ significantly superior compared with group V
§ significantly inferior than other groups

Discussion
The size and location of defects formed in our study present the most challenging repair problem. Spontaneous repair of articular cartilage depends upon both a defect which penetrates the bone and bone-marrow spaces and its size. Those which exceed a critical size do not repair spontaneously. In a study of defects in the distal aspect of horse femora, reported that in a goat model small defects of 3 mm in diameter underwent complete repair. Convery, Akeson and Keown, reported that in a goat model small defects of 3 mm in diameter underwent complete repair. Butnariu-Ephrat et al28 reported that in a goat model small defects of 3 mm in diameter underwent complete repair. In vivo, it has been shown that intermittent fluid pressure, in combination with the absence of mechanical stress, is beneficial for osteoarthritic cartilage and can diminish inflammation. Animal studies have shown that joint distraction can change chondrocyte activity beneficially.4 In our study, the regenerated soft-tissue area significantly increased in the joint-distracted group compared with the weight-bearing group. In the latter, the regenerated cartilage was present mostly in the non-contact area. Therefore, joint distraction was considered to be favourable for the regeneration of articular cartilage.

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(p = 0.03 and p = 0.008, respectively) and cell distribution (p = 0.005 and p = 0.01, respectively). In terms of cell distribution, group W was also better than group V (p = 0.03). The area of regenerated soft tissue was significantly smaller in group W than in the other groups (Fig. 5a). The Safranin-O-stained area was significantly smaller in groups W and V than in group T (Fig. 5b). The percentages of exposed bone to articular surface were 10.6% (± 12.6%) in group C, 25% (± 24.0%) in group W, 0% (± 0%) in group V, and 0.8% (± 2.7%) in group T. There were no statistical differences as regards to the type II collagen-positive area (Fig. 5c). The percentage was significantly larger in group W than in the other groups (p < 0.02). The percentage of the area of type II collagen to that of the regenerated soft tissue was significantly larger in group W than in the other groups (Fig. 6a) and also significantly larger in group C than in group V (Fig. 6b).

(Continued on next page)
cells, which promote the regeneration of cartilage. Collagen gel injected into the joint may interfere with the development of a fibrin matrix and may also induce inflammation. Clinically and experimentally, collagen gel was used as the delivery vehicle when the cells were transplanted into a partial defect. In our model, however, collagen gel may be unfavourable for regeneration of cartilage.

In the ACBMT group, the morphology of the matrix, cell distribution and the area of regenerative cartilage, indicated by staining with Safranin-O, were superior to the vehicle-treated group, although a significant difference was not seen when compared with the control group. The ACBMT and vehicle-treated groups had the same procedure, including injection of collagen gel with or without the cells and joint distraction at three weeks. The better results in the ACBMT group compared with the vehicle-treated group and the absence of the formation of ectopic bone or cartilage in experimental joints indicated that the
injection of ACBMT was effective. However, there was no evidence that these transplanted cells stayed in the joint cavity and adhered to the defect, or that newly-formed cartilage was host-derived. To prove this, suitable experimental marking of these donor cells is needed.

Our model has several advantages for the spontaneous regeneration of large defects of articular cartilage. Joint distraction using a hinged fixator reduces contact pressure on the regenerated area and results in the formation of a space for cell proliferation and differentiation. The intermittent movements of the joint provide the mechanical environment of hydrostatic pressure, which promotes the proliferation of chondrocytes,\(^\text{40,41}\) produces lubrication which allows the permeation of growth factors, and suppresses angiogenesis. These are optimal conditions for \textit{in vivo} culture. If the defects are too large for the spontaneous repair of cartilage, ACBMT provides the undifferentiated mesenchymal cells at a high cell density.

Our study has considerable relevance to the treatment of large defects of articular cartilage and provides the basis for the development of repair technology for the regeneration of such large defects.

No benefits in any form have been received or will be received from a commer-

References

2. Matussy Y, Yamamuro T, Hama H. Arthroscopic multiple osteochondral transplantation to the chondral defect in the knee associated with anterior cruciate liga-