Repair of chronic osteochondral defects in the rat

A BONE MARROW-STIMULATING PROCEDURE ENHANCED BY CULTURED ALLOGENIC BONE MARROW MESENCHYMAL STROMAL CELLS

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Bone marrow mesenchymal stromal cells were aspirated from immature male green fluorescent protein transgenic rats and cultured in a monolayer. Four weeks after the creation of the osteochondral defect, the rats were divided into three groups of 18: the control group, treated with an intra-articular injection of phosphate-buffered saline only; the drilling group, treated with an intra-articular injection of phosphate-buffered saline with a bone marrow-stimulating procedure; and the bone marrow mesenchymal stromal cells group, treated with an intra-articular injection of bone marrow mesenchymal stromal cells plus a bone marrow-stimulating procedure. The rats were then killed at 4, 8 and 12 weeks after treatment and examined.

The histological scores were significantly better in the bone marrow mesenchymal stromal cells group than in the control and drilling groups at all time points ($p < 0.05$). The fluorescence of the green fluorescent protein-positive cells could be observed in specimens four weeks after treatment.

The capacity of articular cartilage for self-repair is limited. Operations to repair articular cartilage defects for limited chondral and osteochondral lesions, autologous chondrocyte implantation or autologous osteochondral mosaicplasty have been widely performed.¹⁻⁶ However, these procedures may not be suitable for extensive chondral and osteochondral lesions. Procedures that stimulate the bone marrow such as drilling, abrasion and microfracture, have been generally accepted.⁷⁻¹² Such procedures are thought to promote chondrogenesis with the formation of fibrous tissue, fibrocartilage and/or hyaline cartilage, thereby inducing bone marrow mesenchymal stromal cells (MSCs) from the subchondral bone by bleeding. However, in experimental studies they have resulted in the formation of fibrocartilaginous tissues.^{12,13}

There are several possible reasons why the bone marrow-stimulating procedure does not always induce satisfactory results. One explanation is that the fragile repair tissues induced from the bone marrow may be damaged by overloading at an early stage after microfracture or drilling. Kajiura et al¹⁴ reported that joint distraction with movement in addition to the bone marrow-stimulating procedure was effective for repairing an osteochondral defect, in contrast to the bone marrow-stimulating procedure used alone. They dem-

onstrated that adequate loading of an osteochondral lesion treated with the bone marrow-stimulating procedure accelerated the regeneration of the articular cartilage better than did the bone marrow-stimulating procedure alone.

Another explanation is that the number of bone marrow MSCs was not adequate to repair the lesion. We hypothesised that the addition of cultured bone marrow MSCs to the bone marrow-stimulating procedure would accelerate the regeneration of the articular cartilage in the defects better than the bone marrow-stimulating procedure alone.

Several types of cell are used for articular cartilage repair.¹⁵⁻¹⁹ There are also reports describing the use of growth factors for the repair of articular cartilage defects.²⁰⁻²³ However, there are no reports describing the use of these cellular therapies for chronic osteochondral defects. The objective of this study was to determine whether the delivery of cultured bone marrow MSCs plus the bone marrow-stimulating procedure to an osteochondral defect in the knee would enhance its repair.

Materials and Methods

All procedures were performed with approval from the Guide for Animal Experimentation, Hiroshima University, and the Committee of Research Facilities for Laboratory Animal

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©2006 British Editorial Society of Bone and Joint Surgery
doi:10.1302/0301-620X.88B9.17810 \$2.00

J Bone Joint Surg [Br]
2006;88-B:1236-44.

Received 24 February 2006;
Accepted after revision 17 May 2006

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Green rat. The Green rat (CZ-004 and SD TgN (act-EGFP) OsbCZ-004) developed by the Osaka University Genome Information Research Center was used in this study. All of the cells of the Green rat contain green fluorescent protein, and the luminescence of these cells can be observed with a fluorescence microscope for as long as the cells survive.

These cells can easily be distinguished from wild-type rat-derived cells. Some studies have used these cells in other situations, for example in a meniscal injury model,²⁴ a skeletal muscle injury model²⁵ and an osteochondral defect model.^{26,27}

Isolation and culture of allogenic bone marrow stromal cells. A heparinised bone marrow aspirate was obtained from the tibiae of green fluorescent protein-positive rats ($n = 6$). Under anaesthesia induced by an intraperitoneal administration of pentobarbital sodium (Nembutal, Dainippon Pharmaceutical Co. Ltd., Osaka, Japan; 0.5 mg/kg body weight), the rear right knee was shaved and disinfected. A 1 cm incision was made on the medial aspect of the proximal tibia, centred between the anterior and medial borders, beginning at the level of the joint line and extending inferiorly. The bone marrow was aspirated with a 21-gauge needle on a 10 ml syringe containing 1 ml of heparin (3000 U/ml). The aspirate was suspended in medium (Dulbecco's Modified Eagle's Medium, DMEM; Hyclone, Logan, Utah) containing 10% fetal bovine serum (Sigma-Aldrich, St Louis, Missouri) and penicillin-streptomycin-fungizone (Bio-Whittaker, Walkersville, Maryland). The cells were seeded on to 100 mm culture dishes and incubated in a humidified 5% CO₂/95% air atmosphere at 37°C. The medium remained unchanged for the first five days, and thereafter was changed at three-day intervals. Two weeks after seeding, the cells had usually proliferated to confluence. They were then harvested after treatment with 0.25% trypsin and 0.02% ethylene diamine tetracetic acid (EDTA), rinsed twice with culture medium and counted using a haemocytometer. Finally, the cells were suspended at 6.0×10^6 cells/ml in culture medium. The appearance of bone marrow MSCs, as detected by fluorescence from the enhanced green fluorescent protein, was recorded before transplantation. Thereafter, we referred to these cells as MSCs.

Animals and surgical technique. We used 60 skeletally-immature Sprague-Dawley rats between ten and 12 weeks old. Their mean weight was 0.5 kg. Before surgery, the animals were anaesthetised with an intraperitoneal injection of 1 ml/kg pentobarbital sodium. The patella was everted through a medial approach. We created an osteochondral defect of 2 mm x 2.5 mm and 1 mm deep on the articular cartilage of the patellar groove of the distal femur using a power drill. After this the knees were thoroughly irrigated with normal saline. The arthrotomy was closed with interrupted 6-0 nylon sutures, and the skin was closed with continuous 6-0 nylon sutures.

We allocated the rats ($n = 54$) randomly into three groups and the treatments were performed four weeks after the creation of the defects, when we considered that the change in growth factors and cytokines caused by the initial injury would have returned to normal.²⁸ The remaining six rats were allocated to the chronic osteochondral defect model.

Control group. The control group of 18 animals received an injection of phosphate-buffered saline (PBS) (50 µl) only into the operated joint. This group represented the natural course of healing of the osteochondral defect when exposed to the carrier solution.

Drilling group. A 1 cm medial parapatellar incision was made over the knees of 18 animals and the patella everted. We identified the defect in the patellar groove and made five drill holes into it, using a hand drill, until blood emerged from the bone. The diameter of the drill was 0.2 mm and the depth was 0.8 mm. The knee was then thoroughly irrigated with normal saline and the soft tissues were closed. PBS (50 µl) was subsequently injected into the joint.

Bone marrow MSC group. The bone marrow MSC group of 18 animals was treated in an identical way except that after closure of the joint the animals received a single intra-articular injection of 1.0×10^6 allogenic bone stromal cells from the green fluorescent protein rats as a suspension in vehicle (PBS, 50 µl).

After all of the interventions the rats were returned to their cages and were free to exercise.

Histological and immunological evaluation

Evaluation of the chronic osteochondral defect. Four weeks after the creation of the chronic osteochondral defects, the rats in the chronic osteochondral defect model ($n = 6$) were killed by intraperitoneal injection of a lethal dose of pentobarbital sodium. The knee joints were first evaluated macroscopically. The distal femora were resected *en bloc* and fixed in 4% paraformaldehyde for 24 hours. They were then decalcified with a 0.5 M EDTA solution. Next, the specimens were embedded in plastic resin (Technovit 8100, Okenshoji, Tokyo, Japan) and cut into 5 µm sections serially along the axial plane that included the centre of the defect and the femoral trochlea.

For histological evaluation, the sections were stained with safranin-O/fast green and toluidine blue (Sigma, St. Louis, Missouri). For immunohistological evaluation, the sections were mounted on to poly-L-lysine-coated glass slides and immersed in 0.3% H₂O₂ to block endogenous peroxidase activity. The sections were then blocked with normal horse serum and incubated with a mouse monoclonal antibody directed against type II collagen (Fuji Chemipha, Toyama, Japan). The reaction for visualisation was performed using an avidin-biotin peroxidase system (Vecstain Elite ABC kit; Vector Laboratories, Burlingame, California), and the sections were coloured with a freshly-prepared diaminobenzidine solution.

Evaluation of the chronic osteochondral defect after treatment. At 4, 8 and 12 weeks after treatment the rats in the

three groups ($n = 54$) were killed as described. The knee joints were first evaluated macroscopically. The distal femora were resected *en bloc* and evaluated both histologically and immunohistologically. The fluorescence of the enhanced green fluorescent protein, which had a wavelength of 508 nm was evaluated on explants activated by 489 nm wavelength light under a fluorescent microscope (Leica Microsystems, Wetzlar, Germany), which was also used to detect the green fluorescent protein-positive cells derived from the donor cells.

The specimens were graded semi-quantitatively by one observer (AK) who was not aware of the source of cartilage. The grading scale was based on the filling of the defect (0 to 4 points), any reconstitution of the osteochondral junction (0 to 2 points), matrix staining (0 to 4 points) and cell morphology (0 to 4 points) as described by Pineda et al²⁹ (Table I). Four elements of repair were assessed. According to this scale, a maximum score of 14 points was possible, with better-quality repair having fewer points.

Statistical analysis. A two-way analysis of variance test was used to compare the histological scores between the groups and between the various times. When a significant p value was found, the *post hoc* Scheffe test was used to identify significant differences among the groups. A p value of < 0.05 was considered to be statistically significant. All statistical analyses were performed on a personal computer using the statistical package StatView version 5.0 (Abacus Concepts, Berkeley, California).

Results

Gross findings. There were no signs of infection and/or immunological rejection in the knees at any time point after surgery in all three groups of rats.

In the osteochondral defect model, the defect margins were clearly recognisable. The defect surfaces were significantly depressed but were covered with a semi-transparent white tissue.

In the control group, the edges of the defects were clearly seen after treatment and at 4, 8 and 12 weeks. At four weeks the defect surfaces were still covered slightly in the peripheral areas. At 12 weeks after treatment the defect surfaces were still depressed but were covered significantly better than at four weeks (Figs 1a to 1c).

In the drilling group, at four weeks after treatment the surfaces of the defect were slightly depressed and were covered with a semi-transparent, glistening white tissue but the edges were clearly defined. Similarly, at 8 and 12 weeks the margins of the defects could still be differentiated from the surrounding normal cartilage (Figs 1d to 1f).

In the bone marrow MSC group, at four weeks after treatment the surfaces of the defect were slightly depressed and covered with a semi-transparent, glistening white tissue, but the edges were recognisable. Similarly, at eight weeks the margins of the defects could still be differentiated from the surrounding normal cartilage. At 12 weeks, the

Table I. Histologic grading of the osteochondral defects (Pineda et al²⁹)*

Filling of defect in percentage	
125	1
100	0
75	1
50	2
25	3
0	4
Reconstitution of osteochondral junction	
Yes	0
Almost	1
Not close	2
Matrix staining	
Normal	0
Reduced	1
Significantly reduced	2
Faint	3
None	4
Cell morphology	
Normal	0
Mostly hyaline and fibrocartilage	1
Mostly fibrocartilage	2
Some fibrocartilage but most non-chondrocytic cells	3
Non-chondrocytic cells only	4

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margins of the defects were still discernible but indistinct (Figs 1g to 1i).

Histological findings. The defect site was also evaluated after safranin-O/fast green and toluidine blue staining of the matrix from serial sections for microscopic evaluation.

In the osteochondral defect model, at four weeks after creation of the defect but before treatment, the degree of repair was poor. The mean histological score was 9.7 (SD 0.5) (Fig. 2).

In the control group, the margins of the defects were clearly recognisable and their surfaces were slightly irregular at four weeks after treatment. They were filled with fibrous tissue which failed to stain with either safranin-O/fast green or toluidine blue. No osseous tissue was observed within the bony portion of the defects. The mean histological score was 10.7 (SD 0.8) (Fig. 3).

Likewise at eight and 12 weeks the margins of the defects were clearly recognisable, and their surfaces were slightly irregular. They were still filled with fibrous tissue. Neither cartilage nor bone was observed. The mean histological scores were 9.8 (SD 0.8) (results not shown) and 8.5 (SD 0.5), respectively (Fig. 4).

In the drilling group the margins of the defects were clearly recognisable four weeks after treatment, and their surfaces were slightly irregular. They were filled with fibrous tissue, which was well integrated with the surrounding bone in the deeper regions. The tissue was stained faintly with safranin-O/fast green, and with toluidine blue. The mean histological score was 8.5 (SD 1.6) (Fig. 3c).

At eight weeks, the margins of the defects were still recognisable and their surfaces were slightly irregular. The

Fig. 1

Gross appearance of the osteochondral defect four weeks after treatment (a, d, g), eight weeks after treatment (b, e, h) and 12 weeks after treatment (c, f, i). The arrowheads point to the edges of the defect.

repair cartilage was mainly composed of fibrous tissue which stained faintly with safranin-O/fast green and with toluidine blue. The mean histological score was 7.7 (SD 0.8) (results not shown).

At 12 weeks, the margins of the defects were still recognisable and their surfaces were less irregular than at eight weeks. By 12 weeks, the defect surfaces were smooth. The matrix of the repair cartilage was slightly stained with

Fig. 2

Typical histological results using light microscopy four weeks after the creation of the osteochondral defect a) toluidine blue staining, b) safranin-O/fast green staining, and c) immunohistochemical staining for type-II collagen (original magnification $\times 50$).

safranin-O/fast green staining and toluidine blue. The mean histological score was 7.2 (SD 1.7) (Fig. 4c).

In the bone marrow MSC group, the repaired cartilage-like tissue was good in almost all joints at every stage. The tissue was strongly stained with safranin-O/fast green and toluidine blue, and was covered with a mixture of cartilage-like tissue and fibrous tissue. The well-repaired cartilage had a relatively smooth surface with no depressions. The thickness and appearance were comparable to those of normal cartilage but there were more cells than in the surrounding articular cartilage. The adjacent normal cartilage showed few degenerative changes but the border with the regenerated cartilage was clearly distinguishable. The mean histological scores were 5.2 (SD 1.3) at four weeks (Fig. 3e), 4.7 (SD 1.0) at eight weeks (results not shown), and 4.0 (SD 1.5) at 12 weeks, respectively (Fig. 4e).

The semi-quantitative histological scores were significantly better in the bone marrow MSC group than in either the control or the drilling group at all time points after treatment ($p < 0.05$) (Fig. 5).

Immunological staining for type-II collagen. In the bone marrow MSC group the regenerated cartilage had a similar staining pattern as the surrounding articular cartilage, with clear positive staining of the extracellular matrix around the chondrocyte-like cells at the site of the articular defect (Figs 3f and 4f). The intensity of staining of type-II collagen in the regenerated tissues increased with time. In the drilling and control groups the cells were also positive for type-II collagen, especially at the junction with the normal cartilage, but the matrix had less intense staining than the normal cartilage (Figs 3b, 3d, 4b and 4d).

Histochemical evaluation. Fluorescence from the green fluorescent protein could be observed in the specimens at four weeks after the treatment (Fig. 6), but could not be detected in the post-treatment specimens at eight and 12 weeks. Green fluorescent protein-positive cells were observed only at the injured site, and lay in the interstitial area of the osteochondral defect. Judging from the localisation, this would suggest that the injected bone marrow MSCs derived from the green fluorescent protein

Fig. 3

Typical light microscopy histological results four weeks after treatment. The arrows indicate the junction between the normal articular cartilage and the artificial defect (a, b, control model; c, d, drilling model; e, f, bone marrow mesenchymal stromal cells (MSC) model; a, c, e, safranin-O/fast green staining; b, d, f, immunohistochemical staining for type-II collagen. Original magnification x 100).

Fig. 4

Typical light microscopy histological results from the normal cartilage 12 weeks after treatment. The arrows indicate the junction between the normal articular cartilage and the artificial defect (a, b, control model; c, d, drilling model; e, f, bone marrow mesenchymal stromal cells (MSC) model; a, c, e, safranin-O/fast green staining; and b, d, f, immunohistochemical staining for type-II collagen. Original magnification x 100).

positive rats might mobilise and differentiate into chondrocytes.

Discussion

This study demonstrated that the bone marrow-stimulating procedure together with injections of cultured bone marrow MSCs into the joint was more effective for repairing a chronic osteochondral defect than the bone marrow-stimulating procedure alone. This is the first report to evaluate the performance of injected cultured bone marrow MSCs in conjunction with a bone marrow-stimulating procedure for the treatment of chronic osteochondral defects in a rat model.

The drilling procedure for damaged articular cartilage as a bone marrow-stimulating procedure was first reported by Pridie in 1959,¹¹ and is often performed as treatment for osteochondral lesions.^{7-10,12} The microfracture technique, which was elaborated by Steadman et al,⁹ represents no more than a modification of the Pridie drilling procedure and thus relies on the same biological principles. The rationale behind these concepts is to stimulate a spontaneous repair reaction. In experimental studies, the repaired cartilage induced by these techniques included both hyaline cartilage and fibrocartilage with fibrocartilage predominating.³⁰⁻³² It was hypothesised in a previous study³³ that bone marrow stimulation allows pluripotent mesenchymal cells and growth factors from the marrow space to access the defect. This procedure led to greater tissue volume in animal defects than in the untreated osteochondral defects, as well as good attachment to the adjacent cartilage and underlying bone. However, the bone marrow-stimulating procedure has a limited role, especially in the treatment of extensive chondral and osteochondral lesions such as in

Fig. 5

Histological grading score of the regenerated tissue in the repaired osteochondral defect. Results from the control, drilling and bone marrow mesenchymal stromal cells (MSC) groups are shown; n = 6 for each group.

osteoarthritis, and has been shown to result in a poor quality of repaired tissue.^{8,13}

Recently, many investigators have examined bone marrow MSCs as an attractive cell source for cartilage engi-

Fig. 6

Findings in the bone marrow mesenchymal stromal cells group; a and b are from a fluorescence microscope, and c is from a normal microscope of the immunohistological staining for type-II collagen. The sections were obtained four weeks after treatment. The open arrows indicate the artificial defect area, and the closed arrow indicates the normal cartilage area (original magnification, a and c x 100; b x 200).

earing because of their availability and ability to differentiate into different cell lineages. Bone marrow MSCs are undifferentiated cells which have been isolated from various mesenchymal tissues in adults, including synovial membranes and hepatic tissues.³⁴⁻³⁶ They are found in small numbers in the bone marrow and have the ability to differentiate into a variety of connective tissue cells,³⁷⁻⁴⁰ including bone, cartilage, tendon, muscle, and adipose tissue.^{39,41-46} Owing to their multipotential nature and capacity for self-renewal, adult stem cells may represent units of active regeneration for tissues damaged as a result of trauma or disease.⁴⁷ These cells are able to re-establish chondrogenesis during the developmental process when implanted into osteochondral defects and when cultured under appropriate *in vitro* conditions.⁴⁸⁻⁵¹ Wakitani et al¹⁵ first reported the successful repair of a large, full-thickness defect in the rabbit by the implantation of cultured mesenchymal osteochondral progenitor cells isolated from the periosteum and bone marrow. They indicated that the local delivery of mesenchymal stem cells might therefore enhance the repair of articular cartilage defects. The potential of bone marrow MSCs to form cartilage and bone is well known.^{39,48,51-53} However, what is not known is the best technique to induce the bone marrow MSCs to differentiate into a chondrogenesis lineage in both *in vivo* and *in vitro* cultures.⁵³⁻⁵⁵

In this study model, we waited for 28 days from the creation of the osteochondral defect to treat it. We considered that growth factors and cytokines from the initial injury had no lasting effects after this period of time.²⁸ Using the bone marrow-stimulating procedure on the osteochondral defect, we speculated that the growth factors are induced from the bone marrow and might be attributed to the injected bone marrow MSCs adhering to the defect and might prevent the cells from escaping and differentiating into chondrocytes. Evidence of this is that the green fluorescent protein-positive cells could be observed in the specimens four weeks after treatment.

Oshima et al²⁶ reported the behaviour of transplanted mesenchymal cells obtained from green fluorescent protein-transgenic rats. They created dense mesenchymal cell masses by hanging-drop culture and transplanted them with fibrin glue into the osteochondral defects of wild-type rats. They reported that the defects were repaired with hyaline-like cartilage and subchondral bone. Although green fluorescent protein-positive cells decreased in number with time, they were observed in the regenerated tissues for 24 weeks.

In this study, we found that the green fluorescent protein-positive cells from the donor animals did not persist beyond four weeks. We speculated that there may be two possible explanations for this. First, the fluorescent activity that produces luminescence in the green fluorescent protein cells could decrease with time. Another explanation is that the injected bone marrow MSCs derived from the green fluorescent protein-positive rats might mobilise and differentiate into chondrocytes. However, we also speculated that the

injected bone marrow MSCs could survive and release various kinds of cytokine for cartilage formation.

The advantage of using cultured bone marrow MSCs in a clinical context is that they can be obtained by minimally-invasive means. A further advantage of the bone marrow-stimulating procedure is that it can also be performed by a minimally-invasive arthroscopic approach that requires no costly instrumentation and causes only minimal iatrogenic tissue damage. It is questionable whether the procedure would be an appropriate treatment for individuals whose spontaneous tissue repair potential is compromised, such as in osteoporosis.^{48,56,57} However, combining the bone marrow-stimulating procedure with an injection of cultured bone marrow MSCs as a therapeutic approach eliminates the problem of osteoporosis in which the potential for spontaneous tissue repair might be attenuated.

Our results confirmed that the bone marrow MSCs group had significantly better histological scores than either the control group or the drilling group at all time points after treatment, but the histological appearance of the regenerated cartilage indicated that the repair process was not perfect. Long-term studies are necessary to evaluate the ultimate fate of the injured cells with respect to their viability and dedifferentiation. In conclusion, these findings suggest that the injection of cultured bone marrow MSCs promotes, but does not guarantee, cartilaginous healing.

This study was supported in part by grants-in-aid to MO from the Japan Ministry of Education, Culture, Sports, Science and Technology (No. 16209045).

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

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