patients with limb ischemia[5,6] In addition, the therapy is safe and feasible[5,6] It is well known that bone fracture healing requires a blood supply, so BMMNC implantation might be useful not only for revascularization, but also for subsequent bone regeneration. Indeed, in the present study clinical and functional healing of bone fracture was achieved after BMMNC implantation.

Some possible mechanisms by which BMMNC implantation induces angiogenesis and bone regeneration are postulated. BMMNC include endothelial progenitor cells and other cell populations, including osteoblasts. We did the following experiment to confirm that the BMMNC differentiated into osteocytes in the present patient. The BMC, including erythrocytes, were seeded at a density of 1×10^6 cells per 35-mm tissue culture dish (Corning, Nagog Park Acton, MA, USA) and maintained in 10 ml Dulbecco's modified minimum essential medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 100 U/ml penicillin G (Sigma), and 100 μg/ml streptomycin (Sigma) at 37°C in a 5% CO_2 incubator. After 4 days in culture, non-adherent cells were removed and fresh medium was added. Attached cells were fed with fresh medium every 3 days. Passages were performed when the cells were approaching confluence[7] Osteogenic conversion of these cells from the 6th passage culture was identified as previously described[8] For osteogenic differentiation, cells were seeded at 4×10^4 cells/16-mm well (2.3×10^4 cells/cm^2) and maintained in DMEM supplemented with 10% FBS, 10 mmol/L glycerophosphate (Tokyo Kasei Kogyo, Tokyo, Japan), 100 nmol/L dexamethasone (Sigma), and 50 μg/ml ascorbic acid-2-phosphate (Sigma, osteogenic induction medium). Cultures maintained in the osteogenic induction medium or in the control medium (DMEM supplemented with 10% FBS) were stained with alizarin red on day 21. Mineralized matrix (alizarin red staining) was observed in the osteogenic induction medium but not in the control medium (Fig 4). After BMMNC implantation, endothelial progenitor cells and osteoblasts will differentiate to mature endothelial cells and bone. In addition, the implanted cells might release several angiogenic factors and cytokines, such as fibroblast growth factor, vascular endothelial growth factor, hepatocyte growth factor, and insulin-like growth factor-1, leading to an increase in blood supply and bioactivity in the process of bone regeneration[9,10] Interestingly, these cytokines promote bone healing by activation of osteogenesis, resulting in union of the fracture. We speculate that the process of bone healing in this case was mainly by bone regeneration after angiogenesis. However, we cannot rule out the possibility that angiogenesis and bone regeneration occurred at the same time after BMMNC implantation.

A non-union bone fracture is defined as one that has not healed after a certain period of time. If non-union is still evident at 6 months post-injury, the bone fracture will remain unhealed. In the present case because the tibial fracture had not healed at 6 months after onset, it is unlikely that the course of bone healing was a natural course. We believe that the union of the bone fracture was largely because of the BMMNC implantation therapy.

In conclusion, it is obvious that increased blood flow helped maintain the structural and functional integrity of peripheral tissue and facilitated bone regeneration in this case. Angiogenesis in cartilage might contribute to bone repair, as well as the increase in blood flow. BMMNC implantation therapy might be a novel treatment for patients with compartment syndrome.

References

1. van den Dolder J, Farber E, Spauwen PH, Jansen JA. Bone tissue reconstruction using titanium fiber mesh combined with rat bone marrow stromal cells. *Biomaterials* 2003; **24:** 1745–1750.
2. Kawaguchi H, Nakamura K, Tabata Y, Ikada Y, Aoyama I, Anzai J, et al. Acceleration of fracture healing in non-human primates by fibroblast growth factor-2. *J Clin Endocrinol Metab* 2001; **86:** 875–880.
3. Hisatome T, Yasunaga Y, Yanada S, Tabata Y, Ikada Y, Ochi M. Neovascularization and bone regeneration by implantation of autologous bone marrow mononuclear cells. *Biomaterials* 2005; **26:** 4550–4556.
4. Shintani S, Murohara T, Ikeda H, Ueno T, Sasaki K, Duan J, et al. Augmentation of postnatal neovascularization with autologus bone marrow transplantation. *Circulation* 2001; **103:** 897–895.
5. Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, et al; Therapeutic Angiogenesis using Cell Transplantation (TACT) Study Investigators. Theraputic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: A pilot study and a randomized controlled trial. *Lancet* 2002; **360:** 427–435.
6. Higashi Y, Kimura M, Hara K, Noma K, Jitsuiki D, Nakagawa K, et al. Autologous bone-marrow mononuclear cell implantation improves endothelium-dependent vasodilation in patients with limb ischemia. *Circulation* 2004; **109:** 1215–1218.
7. Tsutsumi S, Shimizu A, Miyazaki K, Pan H, Koike C, Yoshida E, et al. Retention of multilineage differentiation potential of mesenchymal cells during proliferation. *Biochem Biophys Res Commun* 2001; **288:** 413–419.
8. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas S, Mosca JD, et al. Multilinage potential of adult human mesenchymal stem cells. *Science* 1999; **284:** 143–147.
9. Kinnard T, Stabile E, Burnet MS, Lee CW, Barr S, Fuchs S, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ Res* 2004; **94:** 678–685.
10. Globus RK, Plouet P, Gospodarowicz D. Cultured bovine bone cells synthesize basic fibroblast growth factor and store it in their extracellular matrix. *Endocrinology* 1989; **124:** 1539–1547.

International Society for Cellular Therapy ISCT

Cytotherapy (2006) Vol. 8, No. 4, 343–353

Taylor & Francis
Taylor & Francis Group

Transplantation of autologous rabbit BM-derived mesenchymal stromal cells embedded in hyaluronic acid gel sponge into osteochondral defects of the knee

M Kayakabe[1], S Tsutsumi[1], H Watanabe[2], Y Kato[3] and K Takagishi[1]

[1]Department of Orthopaedic Surgery, Gunma University Graduate School of Medicine, Maebashi, Japan, [2]Department of Physical Therapy, Gunma University School of Health Science, Maebashi, Japan and [3]Department of Dental and Medical Biochemistry, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan

Background

Mesenchymal stromal cells (MSC) have the potential to differentiate into distinct mesenchymal tissues including cartilage, suggesting that these cells are an attractive cell source for cartilage tissue engineering approaches. Various methods, such as using hyaluronan-based materials, have been employed to improve transplantation for repair. Our objective was to study the effects of autologous transplantation of rabbit MSC with hyaluronic acid gel sponges into full-thickness osteochondral defects of the knee.

Methods

Rabbit BM-derived MSC were cultured and expanded with fibroblast growth factor (FGF). Specimens were harvested at 4 and 12 weeks after implantation, examined histologically for morphologic features, and stained immunohistochemically for type II collagen and CD44.

Results

The regenerated area after autologous transplantation of hyaluronic acid gel sponge loaded with MSC into the osteochondral defect at 12 weeks after surgery showed well-repaired cartilage tissue, resembling the articular cartilage of the surrounding structure, of which the histologic score was significantly better than that of the untreated osteochondral defect. In the regenerated cartilage, type II collagen was found in the pericellular matrix of regenerative chondrocytes, while CD44 expression in the regenerative tissue could not be revealed.

Discussion

These data suggest that the autologous transplantation of MSC embedded in hyaluronan-based material may support chondrogenic differentiation and be useful for osteochondral defect repair.

Keywords

autologous cell transplantation, fibroblast growth factor (FGF), hyaluronic acid gel sponge, mesenchymal stem cells.

Introduction

Full-thickness lesions of articular cartilage are capable of being repaired in young animals [1,2]. In injured joint cartilage, progenitor cells from the BM can proliferate and differentiate within the injured osteochondral defects [3]. Adult cartilaginous tissues contain populations of stem cells that have the capacity for renewal after trauma, disease and aging [4]. However, the capacity of articular cartilage for repair is limited, and frequently is insufficient for full-thickness repair. Then the untreated injuries of articular cartilage do not heal, with inferior tissue-possessing mechanical properties resulting in osteoarthritis of the joint [5–8].

Mesenchymal stromal cells (MSC) are undifferentiated pluripotential cells capable of differentiating into many cell types and may be a suitable autogenous cell source for

Correspondence to: **Masatomo Kayakabe**, MD, Department of Orthopaedic Surgery, Gunma University Graduate School of Medicine, 3-39-22 Showa, Maebashi, Gunma 371-8511 Japan. E-mail kayakabe@kg8.so-net.ne.jp

DOI: 10.1080/14653240600845070

articular cartilage repair [4,9]. MSC obtained from adult human contribute to the regeneration of mesenchymal tissues such as bone, cartilage, fat, muscle, tendon and marrow stroma [4]. MSC thus have been used for re-establishing chondrogenesis by being implanted in osteochondral defects of rabbit [5,6,10]. However, regeneration of cartilage tissue is so far not ideal because the homing of the cell in the required area has not been sufficient. Recently, several studies have approached the problem of articular cartilage repair by providing the repair site with BM-derived progenitor cells combined with biocompatible carrier materials [5,6,11,12].

Among biomaterial carriers as scaffolds onto which cells are seeded are fibrin, polymers of polyglycolic and polylactic acids, alginate, collagen gels and Gelfoam (a purified gelatin sponge), which have been constructed as three-dimensional matrices and tested in different animal models [13–15]. A collagen gel used as the delivery vehicle for the BM-derived progenitor cells led to incomplete integration of the *de novo* differentiated tissue with subsequent deterioration of the repair site [11]. A hyaluronan-based scaffold allows not only the expression of specific extracellular matrix molecules by human chondrocytes grown onto them, but also a down-regulation of degenerative cartilage molecules in chondrocytes [16]. Hyaluronan-based polymers, such as ACP sponge made of cross-linked hyaluronan or HYAFF-11 made of benzylated hyaluronan, have the ability to enhance the natural healing response. Hyaluronan-based polymers placed into defects provide an appropriate scaffolding and favorable microenvironment for the reparative process [11,13,17,18]. Hyaluronan-based scaffolds support the chondrogenic differentiation of BM-derived MSC. A marrow-loaded matrix has been developed for the repair of articular cartilage defects [11,12].

Our previous report showed that MSC can maintain their multilineage differentiation potential even after many cycles of cell division in the presence of fibroblast growth factor-2 (FGF-2) [19]. FGF-2 increased the growth rate and the life span of rabbit MSC in monolayer culture [19]. In the present study, a hyaluronic acid (HA) gel sponge, which was freeze-dried hyaluronic acid gel, was used to fill the cartilaginous region of the defect. The useful effects of this high molecular weight hyaluronan, with an average molecular weight of 1900 kDa, on cartilage degeneration have been investigated in a rabbit model of osteoarthritis [20,21]. This study examined the value of BM-derived MSC expanded *in vitro* in the presence of FGF-2 and HA gel sponge as a scaffold for the repair of osteochondral defects of the articular cartilage of the rabbit. Three months later, MSC expanded in the presence of FGF-2 embedded in the high-molecule HA gel sponge exhibited a significant regenerative effect in histologic assessments.

Methods

Isolation of MSC

MSC were isolated from the ilium of 12–14-week-old Japanese White rabbits (NIBS, Tokyo, Japan), weighing an average of 2.3 kg. All operations were performed under general anesthesia. The rabbits were anesthetized by an intramuscular injection of xylazine (20 mg/kg of body weight) and ketamine hydrochloride (30 mg/kg of body weight). BM with blood of the ilium was aspirated by myelocentesis. After the BM samples (0.5 mL/100-mm dish) were centrifuged to remove heparin, cells were seeded on 100-mm tissue culture dishes and maintained at 37°C under 5% CO_2 in air in 10 mL DMEM supplemented with 10% FBS and antibiotics (medium A). Three days after seeding, floating cells were removed and the medium was replaced by fresh medium A. Thereafter, attached cells were fed with fresh medium A every 3 days. The cultures were divided into two groups: supplementation with or without 1.0 ng FGF-2/mL. Passages were performed twice at 5×10^3 cells/cm^2 in 100-mm dishes when cells were approaching semi-confluence. After two passages of the cells, rabbit MSC were suspended into a freezing medium, Cell banker (Nippon Zenyaku Kogyo Co. Ltd, Fukushima, Japan), frozen and stored at −80°C for 6 months. After 6 months of cryopreservation, cells were recovered and cultured in the same medium as that used before cryopreservation. Once the culture reached semi-confluence, the cells were subjected to autologous transplantation. MSC were harvested with 0.05% trypsin-EDTA and absorbed into HA gel sponge or atelocollagen gel at 1×10^6 cells/100 μL DMEM. These were incubated overnight at 37°C under 5% CO_2 in air in 1 mL DMEM in 16-mm culture dishes before transplantation.

Implant

Two different delivery vehicles were used to fabricate an implant for repair of the full-thickness osteochondral defects. One implant was HA gel sponge. The degradable

hyaluronic acid material used was freeze-dried hyaluronate solution. Hyaluronic acid (an average molecular weight of 1900 kDa) solution was freeze-dried to prepare the HA gel sponge (Denki Kagaku Kogyo KK, Tokyo, Japan). The HA gel sponge was a column (5 mm in diameter) and became soluble 2 weeks after incubation in medium A. The other delivery vehicle was 3% atelocollagen gel (Koken, Tokyo, Japan), used for comparison with the HA gel sponge. To prepare the three-dimensional collagenous material for the delivery vehicle, atelocollagen containing 1×10^6 MSC was incubated in a glass ring 5-mm in diameter at 37°C under 5% CO_2 in air overnight. HA gel sponge materials without MSC underwent the same *in vitro* processing as those loaded with MSC.

Surgical procedure and autologous implantation of MSC on the osteochondral defect

Bilateral surgery was performed under sterile conditions on the 36–38-week-old rabbits, according to the Institutional Animal Care and Use Committee-approved protocol. The rabbits were anesthetized by the same procedure as used for BM harvesting. A medial parapatellar approach was made and the patella was luxated laterally to expose the femoral articular surface of the patellofemoral joint of rabbits. A full-thickness defect of diameter 5 mm and depth 2–3 mm on the articular cartilage of the patellar groove of the distal femur was made with a hand drill. All debris was removed from the defect with a curette. Before transplantation, the joints were rinsed with sterile saline thoroughly. The HA gel sponge, with or without loading of autologous MSC, was randomly implanted into the left or right knee. For comparison with the HA gel sponge, a three-dimensional collagenous material with loading of autologous MSC was implanted. The animals were divided into five groups, as shown in Table 1. In seven rabbits, empty delivery vehicles were used and the animals also underwent sham operations as a negative control (group 1, control). In nine rabbits, the HA gel sponge without loading of MSC was inserted into the defect (group 2, HS). In seven rabbits, the HA gel sponge loaded with autologous MSC grown without FGF-2 was inserted into the defect (group 3, HS + MSC − FGF). In 17 rabbits, the HA gel sponge loaded with autologous MSC grown with FGF-2 was inserted into the defect (group 4, HS + MSC + FGF). For comparison with the HA gel sponge, three-dimensional collagenous material with loading of autologous MSC grown with FGF-2 was implanted in 14 animals (group 5, collagen + MSC + FGF). The patella was repositioned, and the capsule, deep fascia, subcutaneous tissues and skin were then repaired with 4–0 nylon sutures. Postoperatively, all rabbits were allowed to move freely in the cage. Severe infection, requirement of additional medication, wound breakdown or premature death were defined as individual stopping rules [22].

Table 1. Study design

Group	Treatment
1 (control)	Empty delivery vehicles also underwent sham operations as a negative control
2 (HS)	HA gel sponge without loading of MSC was inserted into the defect
3 (HS + MSC − FGF)	HA gel sponge loaded with autologous MSC grown without FGF-2 was inserted into the defect
4 (HS + MSC + FGF)	HA gel sponge loaded with autologous MSC grown with FGF-2 was inserted into the defect
5 (collagen + MSC + FGF)	Three-dimensional collagenous material with loading of autologous MSC grown with FGF-2 was inserted into the defect

Approval for the animal study was obtained from the ethics committee of Gunma University (Maebashi, Japan) before starting the experiment.

Histologic evaluation

The rabbits were killed at 4 and 12 weeks after the operation. Tissue samples of the distal femora were thawed and fixed in 10% buffered paraformaldehyde. After decalcification with buffered 10% EDTA, the samples were dehydrated and embedded in paraffin. Sections were cut sagitally at a thickness of 5 μm and stained with toluidine blue, safranin-O and hematoxylin and eosin. Histomorphologic findings for each section were evaluated according to the histologic grading scale for defective cartilage, described by Wakitani *et al.* [5]. Assessment was performed by a blinded assessor based on the following parameters: cell morphology, matrix-staining, surface regularity, thickness of cartilage and integration of donor with host. The total score ranged from 0 to 14 points, with decreasing points indicating better repair of tissues.

The histologic scores were compared with one-factor analysis of variance (ANOVA) on ranks, followed by Newman-Keuls all-pairwise multiple comparison procedure to identify differences among groups [11]. All statistical tests were performed with the Statmate software package (ATMS Co., Ltd., Tokyo, Japan). Statistical significance corresponded to $P < 0.05$.

Immunohistochemical analyzes

For phenotypic characterization, immunohistologic stains were performed on 5-μm paraffin sections. For immunohistochemistry, serial sections were used for detection of type II collagen and CD44. Sections were deparaffinized with xylene, rehydrated with decreasing solutions of ethanol and rinsed three times with PBS, and pretreated with 1% BSA for 30 min to block any non-specific reaction. Serial sections were used for the detection of type II collagen with a specific Ab. Type II collagen was immunolocalized with a MAb to mouse anti-human collagen type II (1:100; Daiichi Fine Chemical, Toyama, Japan). After incubation overnight with the primary Ab at 4°C, sections were rinsed five times in PBS and then incubated with biotinylated anti-mouse Ig for secondary Ab. They were rinsed three times with PBS, and treated for 60 min with streptavidin solution (Dako, Hamburg, Germany). Visualization was performed with diaminobenzidine and counterstaining with Mayer's hematoxylin. For negative controls, the primary Ab was omitted according to the immunohistochemical protocol.

For immunohistochemical staining of CD44, the sections were incubated with proteinase K (Dako) to expose certain epitopes masked during fixation, followed by a washing procedure [23]. Endogenous peroxidase activity was quenched by treating tissue sections with 3% H_2O_2 for 30 min. After washing, the sections were incubated with 3% BSA in PBS to block non-specific binding. After rinsing three times with PBS, the sections were incubated overnight at 4°C with a 1:200 dilution of a monoclonal mouse anti-rabbit CD44v6 Ab (BMS125; Bender MedSystems, Vienna, Austria). The sections were washed three times with PBS and then treated with anti-mouse IgG for 30 min at room temperature. To visualize Ab binding, after sections were washed three times with PBS, the staining was developed by incubation with aminoethylcarbazole chromogen substrate for 30 min, and the reaction was stopped by rinsing in distilled water [23]. They were rinsed three times with PBS and treated for 60 min with avidin biotin complex solution (VECTASTAIN ABC KIT; Vector Laboratories Inc., Burlingame, CA, USA). Visualization was performed in the same way as that performed for collagen type II staining. For positive controls, sections were made from femoral cartilage slices obtained from the osteoarthritic knee at surgery for total knee replacement.

Results

After three passages of culturing, the MSC grown with FGF-2 became more spindle-shaped than MSC grown without FGF-2. FGF-2 also increased the growth rate of MSC in monolayer cultures. After three passages of culturing, the MSC grown with FGF-2 were approximately 10^2 times as great as without FGF-2. Expanded MSC had *in vitro* differentiation potential: chondrogenic, osteogenic and adipogenic potentials, as evaluated by the protocol of Pittenger *et al.* [4] These results were same as those described previously by Tsutsumi *et al.* [19] (data not shown).

Macroscopic findings

At 4 weeks after surgery, the edges of the defects could be determined in all the knees. Among the groups, few differences were revealed. The representative gross appearance of the repair tissue at 12 weeks after surgery is shown in Figure 1. Debris of either the HA gel sponge or atelocollagen gel material was not observed in the knee joint, and there were no signs of synovitis or infection in the tissues surrounding the implants. For all procedures, the osteochondral defect was filled by repair tissue. The regenerated area of the control group (group 1) was red or dark brown. Irregular tissue with depression was noted and the margin of the defect was clearly differentiated from surrounding normal cartilage (Figure 1A). Grafted tissue of group 2 was smoother and more obvious with pale tissue than that of group 1. At 12 weeks after surgery in the groups containing MSC treated with and without FGF (groups 3, 4 and 5), the regenerated area showed smooth, consistent, glistening white tissue that resembled articular cartilage, although the color incorporation into surrounding normal tissue in group 4 was most evident (Figure 1).

Histologic evaluation

In the control group (group 1), at both 4 and 12 weeks the lesions were covered with connective tissue (Figure 2A and

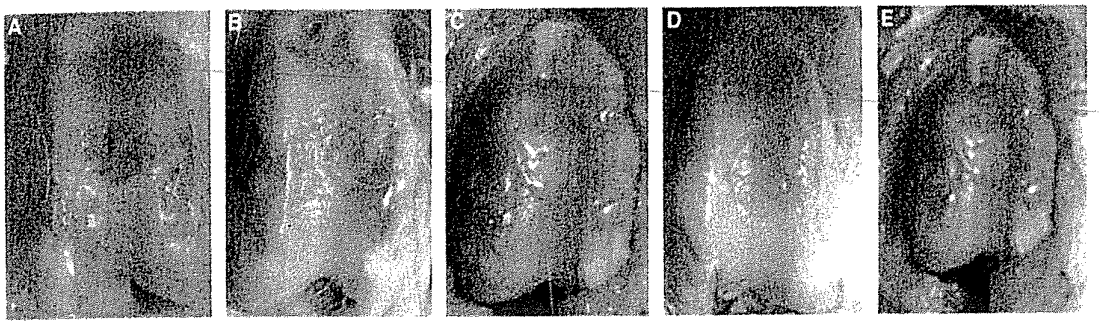


Figure 1. Macroscopic appearance of the rabbit medial femoral condyle at 12 weeks after surgery, showing the control defect without transplantation (A), HA gel sponge alone (B), HA gel sponge loaded with autologous MSC grown in the absence (C) or presence (D) of FGF-2, and atelocollagen gel sponge loaded with autologous MSC grown in the presence of FGF-2 (E).

3A). None of the untreated defects healed completely with hyaline cartilage. Adjacent cartilage tissue showed degenerative changes, including clustering and reduced cellularity at 4 and 12 weeks after surgery. At 12 weeks after surgery, the surface of the regenerative tissue varied from fibrous to fibrocartilagenous tissue (Figure 4A and Figure 5A). There was little metachromatic staining. The subchondral area showed reactive vascular formation, suggesting a regenerative reaction.

In the transplanted groups, HS or collagenous material was replaced with autologous reactive tissues in all rabbits. In contrast to the control group, cartilage-like tissue, similar to adjacent normal cartilage, appeared and replaced the defect. Compared with the surrounding articular cartilage, there were more cells and the arrangement seemed to be more irregular. The adjacent normal cartilage showed little degenerative change, as seen in the control group. The border of the regenerated cartilage

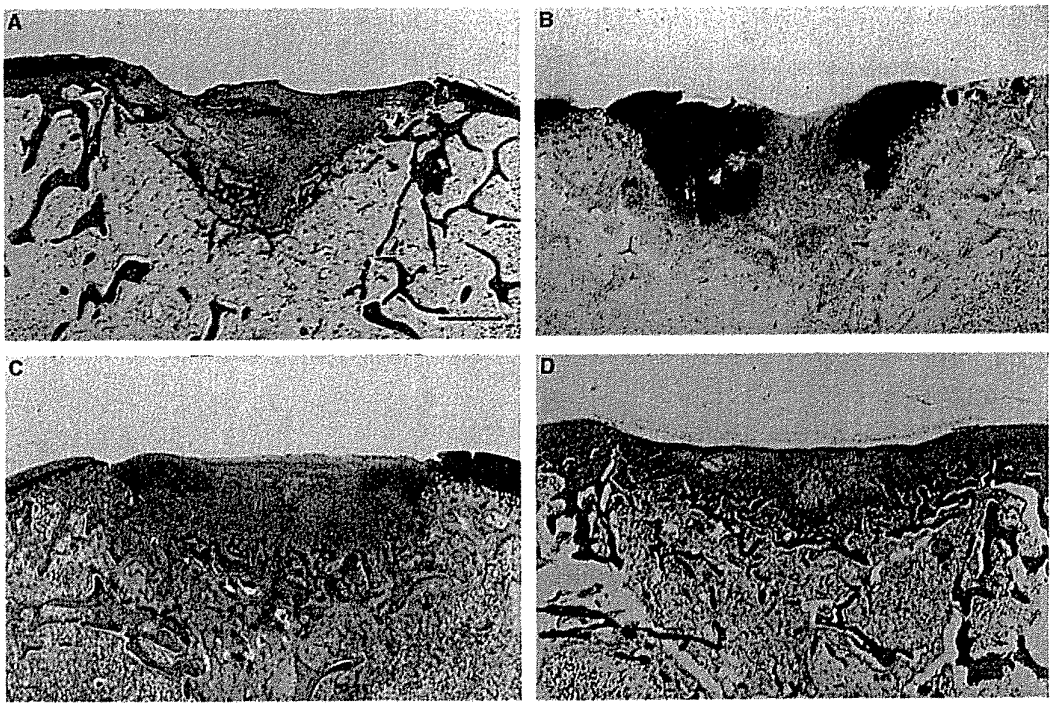


Figure 2. Histologic findings of the articular cartilage of adult rabbits at 4 weeks after surgery showing the control defect without transplantation (A), HA gel sponge alone (B) and HA gel sponge loaded with autologous MSC grown in the absence (C) or presence (D) of FGF-2. Bar indicates 1 mm.

was clearly distinguishable. At 4 weeks, all groups showed the regenerative tissue with a rim of hypertrophic chondrogenic cells (Figure 2B–D) and few characteristic differences were detected among the groups. At 12 weeks, among the four transplanted groups no significant differences were observed qualitatively; they showed more clusters of cartilage occupying the defect,with reduced thickness compared with normal tissue (Figure 3B–D). The defects of groups 3 and 4 at 12 weeks had a rim of chondrogenic cells at the interface with the host tissue. The central portion of the repair tissue had a low density of the cells. The top layer varied, and consisted of either hyaline-like cartilage or fibrocartilage tissues. At a higher magnification, chondroid tissue seemed to be more often observed in the repair tissue when the HS material was loaded with MSC than in repair tissue without MSC loading (Figure 4B–D). The well-repaired cartilage showed a relatively smooth surface without depression in group 4 at 12 weeks. At a higher magnification, the synthesis of extracellular matrix was revealed by toluidine blue and safranin-O (Figure 4D and 5B). Metachromatic staining was distinct in the deep zone of regenerated cartilage, compared with the superficial zone. The subchondral plate was thicker or similar to that of surrounding normal cartilage. Histologic findings in group 5, in which cells were embedded in collagen, seemed to be similar to those observed in group 4, while matrix staining was less evident than group 4.

Results according to the histologic grading scale described by Wakitani *et al.* [5] are shown in Table 2. As shown in Figure 6, there was no significant difference in the total points among groups at 4 weeks. In contrast, at 12 weeks the mean total histologic score was reduced in treated groups. In particular, the score of group 4, which was treated with transplanted HA gel sponge loaded with autologous MSC grown with FGF-2, 4.0 ± 1.4, was significantly improved compared with that of group 1, 8.5 ± 1.3 ($P < 0.05$). This may have been because of the improvement of the category cell morphology, in which there was a significant difference between groups 1 and 4 ($P < 0.01$; Table 2).

Immunohistochemical analyzes

Because the effect of hyaluronic acid transplantation or the regeneration of cartilage tissue was demonstratec 12 weeks after transplantation on histology, immunohisto-

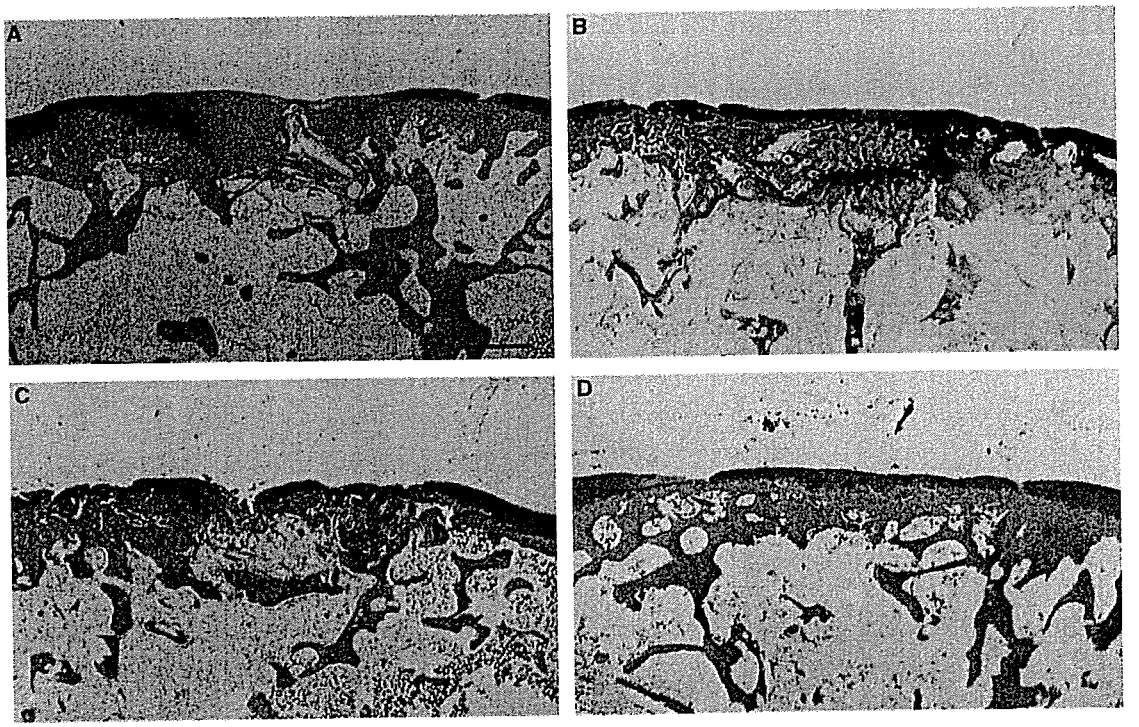


Figure 3. Histologic findings of the articular cartilage of adult rabbits at 12 weeks after surgery showing the control defect without transplantati (A), HA gel sponge alone (B) and HA gel sponge loaded with autologous MSC grown in the absence (C) or presence (D) of FGF-2. Bar indicat 1 mm.

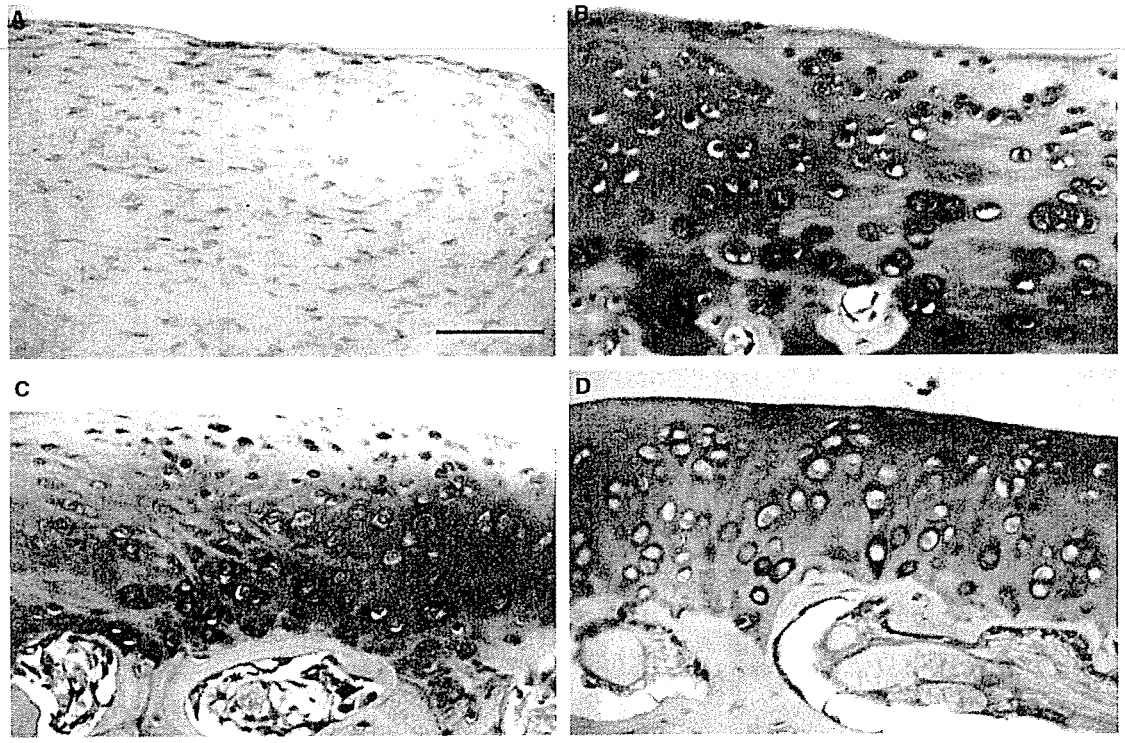


Figure 4. High-magnitude histologic findings of the articular cartilage of adult rabbits at 12 weeks after surgery showing the control defect without transplantation (A), HA gel sponge alone (B) and HA gel sponge loaded with autologous MSC grown in the absence (C) or presence (D) of FGF-2. Bar indicates 100 μm.

chemical analyzes were performed for the tissue specimen obtained at that time. In the regenerative area of the control group, little type II collagen was detected (Figure 7A). In contrast, type II collagen was accumulated in the pericellular and extracellular matrix cartilage in groups 2, 3 and 4, while staining for type II collagen was more intense on chondrocytes and the matrix around lacunae in normal cartilage (Figure 7B). The staining was denser in the matrix than in surrounding articular cartilage.

In order to investigate the role of CD44, a receptor for hyaluronan on the cell surface, in the chondrogenic cells embedded in hyaluronate gel, the expression of the receptor was evaluated by immunohistochemistry. Unexpectedly, in both the control and experimental groups the CD44 protein was not detected (Figure 8B). To examine

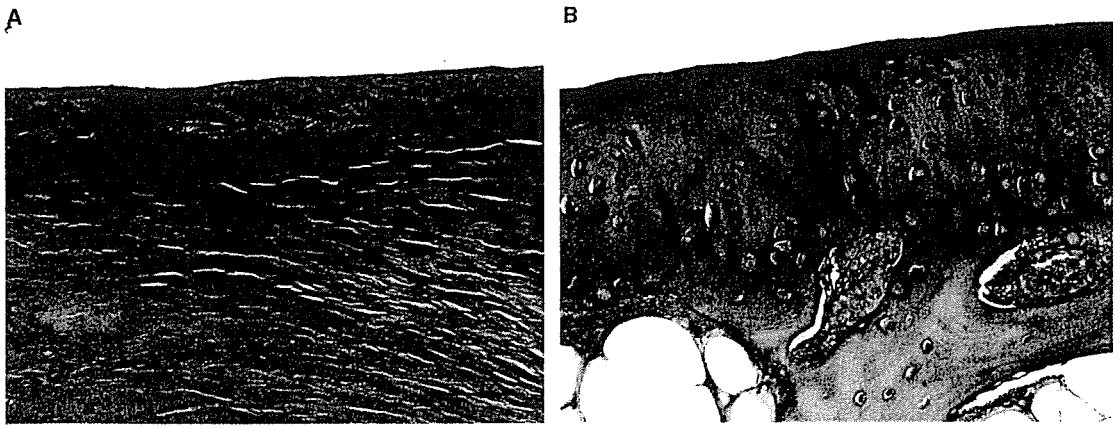


Figure 5. Safranin-O staining of hyaline cartilage of rabbits in the control group (A) and the group with autologous transplantation of HA gel sponge with rabbit MSC treated with FGF-2 (B) at 12 weeks after surgery. Bar indicates 100 μm.

Table 2. Results of the histologic grading scale

Group	Average of score (SD)													
	4 weeks						12 weeks							
	1	2		3		4		1	2		3		4	
Category	($n=3$)	($n=6$)	P^*	($n=3$)	P^*	($n=9$)	P^*	($n=4$)	($n=3$)	P^*	($n=4$)	P^*	($n=8$)	P^*
Cell morphology	2.7 (0.6)	2.3 (0.8)	NS	2.0 (0.0)	NS	2.4 (1.3)	NS	2.8 (0.5)	1.3 (0.6)	NS	1.5 (0.6)	NS	0.9 (0.6)	<0.01
Matrix staining	2.7 (0.6)	2.0 (0.6)	NS	1.3 (0.6)	NS	2.2 (1.0)	NS	2.0 (0.0)	1.0 (0.0)	NS	1.3 (0.5)	NS	0.5 (0.8)	NS
Surface regularity	1.7 (1.2)	2.3 (0.8)	NS	1.3 (0.6)	NS	1.8 (0.7)	NS	1.0 (0.8)	1.3 (0.6)	NS	1.0 (0.8)	NS	0.8 (0.5)	NS
Thickness of cartilage	1.3 (0.6)	1.7 (0.4)	NS	1.0 (0.0)	NS	1.2 (0.7)	NS	1.8 (0.5)	1.0 (0.0)	NS	0.8 (0.5)	NS	0.9 (0.4)	NS
Integration of donor with host	1.3 (0.6)	1.3 (0.5)	NS	2.0 (0.0)	NS	1.0 (1.0)	NS	1.0 (0.0)	1.7 (0.6)	NS	1.3 (1.0)	NS	1.0 (0.5)	NS
Total	9.7 (2.5)	9.2 (2.5)	NS	7.7 (0.6)	NS	8.7 (3.1)	NS	8.5 (1.3)	6.3 (1.2)	NS	5.8 (1.5)	NS	4.0 (1.4)	<0.05

NS, no significant difference.
**No probability of significant difference from the control group according to Newman-Keuls all-pairwise multiple comparison procedure.*

Figure 6. Total histologic grading score of repaired tissues at the rabbit knee joint. Asterisk indicates a significant difference according to Newman-Keuls all-pairwise multiple comparison procedure ($P < 0.05$*). Bars indicate SD.*

whether the lack of staining was the result of the absence of the protein or a technical failure, human cartilage tissue obtained from osteoarthritic knee was used as a positive control, because chondrocytes in the osteoarthritic cartilage clearly expressed CD44. Immunohistochemical staining of CD44 showed marked staining of the cell membrane and, in many cases, weaker staining of the cytoplasm (Figure 8A), suggesting that hyaluronan may not induce CD44 expression in the stem cells differentiating chondrocytes embedded in hyaluronic acid.

Discussion

In the present study, full-thickness repair of cartilage tissue of the knee was not sufficient with no treatment for the defect. Transplantation of hyaluronic acid alone did not induce a significant effect on the regeneration of the full-thickness lesion either. Only in rabbits treated with FGF-2-expanded autologous stem cells embedded within hyaluronic acid gel was full-thickness repair of cartilage tissue obtained sufficiently.

The effect of hyaluronic acid alone on regeneration of cartilage tissue as a scaffold was not significantly evident in the histologic scoring system used in the present study. In a previous report, hyaluronic acid-based biomaterials have been used as scaffolds for defects of articular cartilage, and the histologic score was significantly better than that obtained for untreated defects [11]. The reason why hyaluronic acid gel alone did not have a significant effect in the present study is uncertain at present. Possibly differences in the materials of the hyaluronic acid may be related to the insufficient regenerative effect. However, the

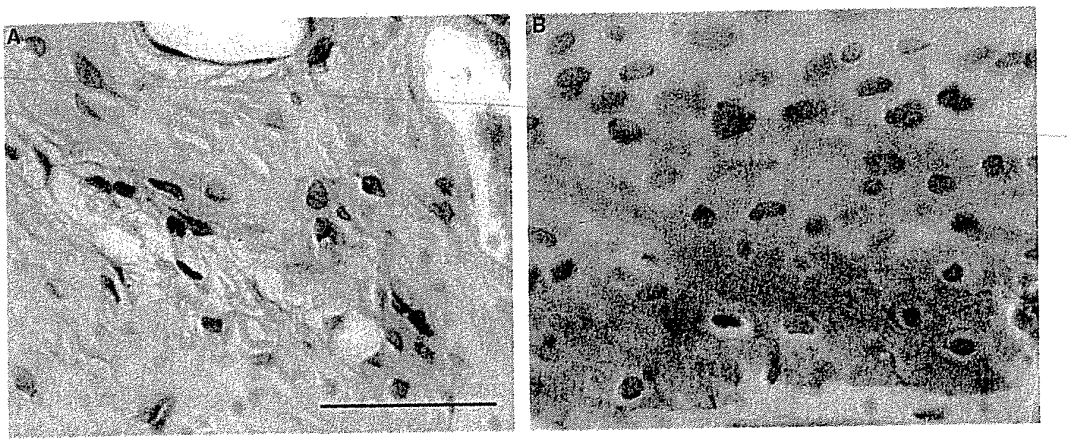


Figure 7. Immunologic localization of type II collagen in repaired tissues in the control group (A) and the group with autologous transplantation of HA gel sponge with rabbit MSC treated with FGF-2 (B) at 12 weeks after the operation. Bar indicates 100 μm.

useful effects of the present high molecular weight hyaluronic acid on cartilage degeneration have been shown in a rabbit osteoarthritis model [20,21]. Alternatively, defect size, which was larger in the present study than the other investigations, may have lead to an insufficiency in the hyaluronic acid-regenerative effect. Shapiro *et al.* [1] described small (three-millimeter-diameter) full-thickness lesions in very young animals as fully capable of repair. In contrast, large defects, with either partial thickness penetrating the cartilage alone or full thickness accessing the subchondral bone, are generally incapable of natural repair. In the present study, the defect size created was larger (5-mm diameter) than previous reports. It is therefore suggested that the capability of hyaluronic acid-assisted regeneration of cartilage tissue may not be enough for sufficient recovery.

In the present study, the regenerated area after autologous transplantation of HA gel sponge loaded with MSC into the osteochondral defect showed effective repair that resembled articular cartilage at the macroscopic and microscopic level. Solchaga *et al.* and Gao *et al.* [11,12] demonstrated that hyaluronic acid-based scaffolds support osteogenic and chondrogenic differentiation of BM-derived MSC; this marrow-loaded matrix was then developed for the repair of articular cartilage defects. Hyaluronic acid-based biomaterials used for cartilage repair allow not only the expression of specific extracellular matrix molecules by human chondrocytes grown onto them, but also a down-regulation of some catabolic factors as matrix metalloproteinases, MMP-1 and MMP-13 and nitric oxide [16,24]. Chondrocyte apoptosis is reduced during the growth of the cells onto the biomaterial

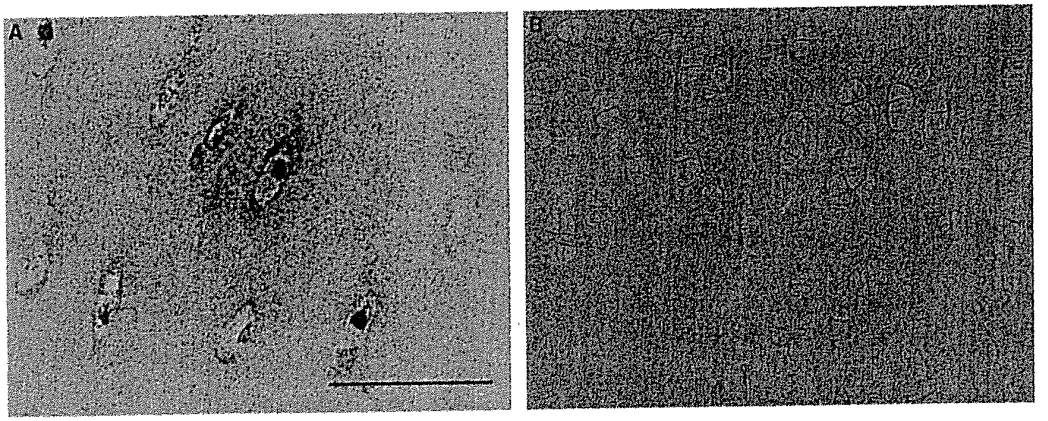


Figure 8. Immunostaining for CD44 in repaired tissues in the control group (A) and the group with autologous transplantation of HA gel sponge with rabbit MSC treated with FGF-2 (B) at 12 weeks after the operation. Bar indicates 100 μm.

[16]. Wakitani *et al.* [5] demonstrated that MSC transplantation with the collagen gel effectively repaired the defect of articular cartilage, in which histologic recovery was dominant in the cell morphology. Similarly in the present study, autologous MSC grown with FGF-2 transplantation with the use of HA gel sponge significantly improved the reparative capability of articular cartilage, as measured by histologic analyzes, especially for cell morphology. It is suggested that the HA sponge may enable MSC to induce an ability to differentiate into cartilaginous tissue and supply appropriate conditions for cartilage regeneration.

CD44 has been identified as the principal cell surface receptor for hyaluronic acid [25]. The hyaluronan receptor CD44 has been implicated in cell adhesion molecules, and serves as the critical link for the retention of hyaluronan–proteoglycan aggregates with the chondrocyte cell surface [26–28]. In several investigations, CD44 was expressed in chondrocytes from normal and osteoarthritic tissue in human cartilage [28]. Tibesku *et al.* [23] reported that chondrocytes in rabbit normal articular cartilage did not express CD44, or expressed it only at a very low percentage, and suggested that CD44 did not play an essential role in normal cartilage. In contrast, Ostergaard *et al.* [27] analyzed human femoral heads and found significantly up-regulated expression of CD44 in the deep zone of osteoarthritis cartilage. In the experiments, over the time–course of osteoarthritis of the rabbit, significantly more chondrocytes expressed CD44, suggesting that the augmentation of CD44 expression is related to the pathogenesis of osteoarthritis [23]. The present results, showing clear expression of the receptor in the cartilage chondrocytes in the osteoarthritic knee, corresponded well to these previous reports. On the other hand, the induction of receptor expression in response to hyaluronic acid in the regenerative cartilage is controversial. Addition of hyaluronic acid in biomaterial favors the adhesion of chondrocytes to their extracellular matrix, and results in a significant increase in $CD44^+$ cell number compared with in the absence of hyaluronic acid [29]. In contrast, however, the findings that the mRNA level of CD44 is unaffected by treatment with hyaluronan oligosaccharides was confirmed by flow cytometry analysis [26]. Grigolo *et al.* [16] reported that chondrocytes grown onto a hyaluronic acid-based scaffold redifferentiate and express their original phenotype, but reduce the production and expression of many factors including CD44, which are involved in cartilage degrada tion. In the present study, expression of CD44 in th regenerative tissue could not be determined. Thes results suggest that CD44, possessing a harmful role i regenerative cartilage tissue, may not be associated wit communication between regenerative chondrocytes an the surrounding hyaluronic acid. Instead, other receptor such as RHAMM, may be involved in the regenerativ process performed by MSC.

In a previous study, we showed the retention of th chondrogenic and osteogenic potentials of FGF-expose MSC during proliferation *in vitro*. Also, FGF stimulate the growth rate and life span of rabbit and human MSC i monolayer cultures [19]. The velocity of chondrocyt migration was accelerated in the presence of FGF and was not affected by the addition of hyaluronic acid alon However, simultaneous administration of hyaluronic aci and FGF showed augmented effects. On the other hanc the velocity of synovial cell migration was enhanced b hyaluronic acid but not by FGF. Hyaluronic acid had chemokinetic effect on synovial cells and FGF had th same effect on chondrocytes [30]. The results of ou studies showed that autologous MSC grown with FG transplantation with the use of HA gel sponge significantl improved the reparative capability of articular cartilag as measured by histologic analyzes. It is suggested tha FGF may stimulate the MSC motility necessary fc osteochondral regeneration occurring at defects fille with hyaluronic acid gel.

In addition to its bionic lubricating function, hyalurona has various biochemical actions, including anti-inflamma tion by suppressing proliferation of synovial cells an lymphocytes, synthesis of prostaglandin E_2, and protectio of the cartilage matrix from degradation. Thus intra articular administration of hyaluronic acid is often use clinically for patients with osteoarthritis or rheumatoi arthritis, and the effects are well documented [20]. Th present finding that autologous transplantation of rabb BM-derived MSC with HA gel sponge can effectivel regenerate osteochondral defects has merit in cell therap and regenerative therapy for the restoration of damaged c diseased articular cartilage.

Acknowledgements

The authors thank Teruzo Miyoshi PhD and Yasukaz Himeda PhD (Denka Advanced Research Department c Biochemistry Research Center, Tokyo, Japan) for thei

technical assistance in the production of hyaluronic acid gel sponge. This work was supported in part by a Grant-in-Aid for scientific research (C) 16591472 (H Watanabe) from the Japan Society for the Promotion of Science.

References

1 Shapiro F, Koide S, Glimcher MJ. Cell origin and differentiation in the repair of full-thickness defects of articular cartilage. *J Bone Joint Surg [Am]* 1993;75A:532–53.

2 Huibregtse BA, Johnstone B, Caplan AI *et al.* Natural repair of full-thickness osteochondrial defects in growing and mature rabbits. *Trans Orthop Res Soc* 1998;23:796.

3 Solchaga LA, Dennis JE, Goldberg VM *et al.* Hyaluronic acid-based polymers as cell carriers for tissue-engineered repair of bone and cartilage. *J Orthop Res* 1999;17:205–13.

4 Pittenger MF, Mackay AM, Beck SC *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;284:143–7.

5 Wakitani S, Goto T, Pineda SJ *et al.* Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. *J Bone Joint Surg [Am]* 1994;76A:579–92.

6 Gao J, Dennis JE, Solchaga LA *et al.* Repair of osteochondral defect with tissue-engineered two-phase composite material of injectable calcium phosphate and hyaluronan sponge. *Tissue Eng* 2002;8:827–37.

7 Mankin HJ. The reaction of articular cartilage to injury and osteoarthritis. *N Engl J Med* 1974;291:1285–92.

8 Mankin HJ. The response of articular cartilage to mesenchymal injury. *J Bone Joint Surg [Am]* 1982;64-A:460–6.

9 Caplan A I. Mesenchymal stem cells. *J Orthop Res* 1991;9: 641–50.

10 Im G, Kim D, Shin J *et al.* Repair of cartilage defect in the rabbit with cultured mesenchymal stem cells from bone marrow. *J Bone Joint Surg [Br]* 2001;83B:289–94.

11 Solchaga LA, Yoo JU, Lundberg M *et al.* Hyaluronan-based polymers in the treatment of osteochondral defects. *J Orthop Res* 2000;18:773–80.

12 Gao J, Dennis JE, Solchaga LA *et al.* Tissue-engineered fabrication of an osteochondral composite graft using rat bone marrow-derived mesenchymal stem cells. *Tissue Eng* 2001;7:363–71.

13 Grigolo B, Roseti L, Fiorini M *et al.* Transplantation of chondrocytes seeded on a hyaluronan derivative (Hyaff-11) into cartilage defects in rabbits. *Biomaterials* 2001;22:2417–24.

14 Nehrer S, Breinan HA, Ramappa A *et al.* Chondrocyte-seeded collagen matrices implanted in a chondral defect in a canine model. *Biomaterials* 1998;19:2313–28.

15 Wakitani S, Goto T, Yonug RG *et al.* Repair of large full-thickness articular cartilage defects with allograft articular chondrocytes embedded in a collage gel. *Tissue Eng* 1998;4:429–44.

16 Grigolo B, Franceschi LD, Roseti L *et al.* Down regulation of degenerative cartilage molecules in chondrocytes grown on a hyaluronan-based scaffold. *Biomaterials* 2005;26:5668–76.

17 Goldberg VM, Solchaga LA, Lundberg M *et al.* Regenerative cell-based repair of osteochondral defects of articular cartilage. *Sem Arthroplasty* 1999;10:30–6.

18 Aigner J, Tegeler J, Hutzer P *et al.* Cartilage tissue engineering with novel nonwoven structured biomaterial based on hyaluronic acid benzyl ester. *J Biomed Mater Res* 1998;42:172–81.

19 Tsutsumi S, Shimazu A, Miyazaki K *et al.* Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. *Biochem Biophys Res Commun* 2001;288:413–9.

20 Kikuchi T, Yamada H, Shimmei M. Effect of high molecular weight hyaluronan on cartilage degeneration in a rabbit model of osteoarthritis. *Osteoarth Cart* 1996;4:99–110.

21 Himeda Y, Yanagi S, Takema T *et al.* Adhesion preventive effect of a novel hyaluronic acid gel film in rats. *J Int Med Res* 2003;31:509–16.

22 Sabo D, Brocai DRC, Eble M *et al.* Influence of extracorporeal irradiation on the reintegration of autologous grafts of bone and joint. *J Bone Joint Surg [Br]* 2000;82B:276–82.

23 Tibesku CO, Szuwart T, Ocken SA *et al.* Increase in the expression of the transmembrane surface receptor CD44v6 on chondrocytes in animals with osteoarthritis. *Arthritis Rheum* 2005;52:810–7.

24 Hashimoto S, Takahashi S, Amiel D *et al.* Chondrocyte apoptosis and nitric oxide production during experimentally induced osteoarthritis. *Arthritis Rheum* 1998;41:1266–74.

25 Aruffo A, Stamenkovic I, Melnick M *et al.* CD44 is the principal cell surface receptor for hyaluronate. *Cell* 1990;61:1303–13.

26 Knudson CB, Knudson W. Hyaluronan and CD44. *Clin Orthop Related Res* 2004;427S:S152–62.

27 Ostergaard K, Salter DM, Andersen CB *et al.* CD44 expression is up-regulated in the deep zone of osteoarthritic cartilage from human femoral heads. *Histopathology* 1997;31:451–9.

28 Salter DM, Godolphin JL, Gourlay MS *et al.* Analysis of human articular chondrocyte CD44 isoform expression and function in health and disease. *J Pathol* 1996;179:396–402.

29 Gigant-Huselstein C, Hubert P, Dumas D *et al.* Expression of adhesion molecules and collagen on rat chondrocytes seeded into alginate and hyaluronate based 3D biosystems. Influence of mechanical stresses. *Biorheology* 2004;41:423–31.

30 Maniwa S, Ochi M, Motomura T. Effects of hyaluronic acid and basic fibroblast growth factor on motility of chondrocytes and synovial cells in culture. *Acta Orthop Scand* 2001;72:299–303.

特集　いますぐ使えるヒト細胞最新活用法

ヒト間葉系幹細胞
～培養と品質管理のストラテジー

加藤幸夫，五十嵐晃，金輪真佐美

最近，ヒト骨髄や脂肪由来の間葉系幹細胞（mesenchymal stem cells：MSC）株が入手可能となった（各種MSC株の入手のためのアドレスを稿末に示した）．われわれも理研の細胞バンクに多数のヒト骨髄MSC株を寄託している．ここではヒトMSC株の活用方法のほかに，その規格，均質性（純度），遺伝子マーカーによる品質検査などについて述べる．

はじめに
～ヒトMSC株の有用性

軟骨細胞や骨芽細胞は，軟骨／骨の再生に有用であるものの，これらを採取するには健常部の損傷や疼痛を伴う．一方，MSCは骨髄から少ない苦痛で採取できる．したがって変形性関節症，骨欠損，骨形成不全に対して，MSCの細胞治療が行われている．さらにMSC移植は，歯周病[1]，心筋梗塞，難治性皮膚潰瘍に対しても有効であった．

また骨髄MSCは造血細胞の増殖と分化を支持しており，骨髄，臍帯血，造血幹細胞の移植後の *in vivo* での造血促進作用や造血幹細胞の増殖を促進する作用が報告されている[2]．

またMSCは異種リンパ球混合反応でのリンパ球の活性化を抑制するなど，同種アロ細胞*1への拒絶反応つまりGVHD（graft-vs-host disease）を強く抑制した．したがってアロの皮膚移植後の壊死と脱落をMSCが抑制した．臨床試験でもMSCは同種アロ細胞*1への拒絶反応を抑制した[3]．この免疫修飾能を利用して他家MSCによる再生医療が米国のオサイリス社で開発が進められている．心筋梗塞や脳梗塞では，自家MSCの増幅が間に合わないので，他家MSCの利用が考えられている．他家MSCの認可はハードルが高いものの，生命にかかわる疾患に対して認可される可能性がある．

興味深いことにMSCは創傷部位に選択的に遊走するので，ドラッグデリバリー担体としても注目されている．例えばインターフェロン発現ベクターやBMP発現ベクターを組み込んだMSCを移植する治療が模索されている．

さらにヒト骨芽細胞，軟骨細胞，筋肉細胞，肝臓細胞は入手が困難であるが，ヒトMSCはこれらの細胞へと *in vitro* で分化できるので，創薬のスクリーニング系での活用が考えられる．

当然ながら，ヒトMSCは幹細胞の基礎研究で有用である．ヒトMSCには個体差や年齢差があるので，基礎／臨床研究のために，多様なMSC株をバンクする意義は大きい．

1．MSC株の安定的増殖

MSCの大量増幅は困難であったが，われわれの超増幅法でそれが可能になった．すなわち線維芽細胞増殖因子2（fibroblast growth factor-2：FGF-2）あるいは基底膜細胞外基質でコートした培養皿を用いることで，多分化能を維持したままMSCを500億倍に増やすことができるようになった．またウサギの関節全層欠損モデルにおいて，従来法で調製したMSCではなく，超増幅したMSCを移植した場合のみが対照に比較して軟骨の修復が有意に亢進した[1]．しかもヒト血清でもFGF-2を添加すればMSCの培養が可能となった．つまり自家MSCを自家血清で培養できるようになった．なお自家血清を安全に閉鎖系で調製するための血液バックは，JMS社，ツーセ

＊1：アロ細胞
他家移植に用いる細胞．移植の際，自己の組織，細胞を自己の他の場所に移し変える場合は自家移植と呼ばれ，ドナーとレシピエントは同一であるが，これに対して自己以外の組織，細胞を他人に移し変える場合は他家移植と呼ばれ，ドナーとレシピエントは異なる．他家移植には人間の組織，細胞を用いる同種移植（allotransplantation）と人間以外の組織，細胞を用いる異種移植（xenotransplantation）に分けられる．

ル社，広島大学の共同研究で開発されている．

ヒトMSCの増殖には，血清のドナーおよび細胞ドナーの個体差と年齢差の影響がでるが，FGF-2を使用することでそれらを減少できる．ウシ胎児血清にはFGFなどの成長因子が高濃度に存在するが，ヒトやウマ血清では濃度が低く個体差が大きい．したがってFGF-2のMSC増殖促進作用は，ウシ胎児血清含有培地で見られるよりも，ヒトやウマ血清の場合でより明確に現れる．

2．ドナーの年齢の影響

ドナーの年齢が，MSCの骨，脂肪，軟骨分化能に影響するかを検討することは，再生医療で重要である．MSCの増殖や骨分化に対する年齢の影響について多くの研究があるが，一致した結論は得られていない．われわれの経験では，ドナーの加齢は腸骨由来MSCの増殖能を低下させるようである．つまり同様な培養条件で若いMSCはより速い増殖を示した．また50歳を過ぎると顎骨MSCの分離培養の成功率が低下した[5]．

一方，大串らは，高齢者からの腸骨MSCが骨再建のための移植用細胞として十分有効であると報告している．加齢や骨粗鬆症が骨髄MSCの数と増殖能に影響しないという報告もある．われわれの研究では，加齢は腸骨MSCの骨分化および脂肪分化能にほとんど影響しなかったが，軟骨分化能を低下させた．加齢の影響は骨と軟骨分化能で異なるかもしれない．

3．MSCの採取部位の影響

MSCは，骨髄以外にも，脂肪，滑膜，骨膜，臍帯血などからも分離できる．しかし採取部位がMSCの増殖，分化能にどのように影響するのかについては研究が少ない．一般には，脂肪由来のMSCは脂肪になりやすいものの骨，軟骨分化能が低く，滑膜由来のMSCは骨髄MSCと類似しているが軟骨になりやすいといわれている[6]．

おなじ骨髄でも，腸骨，顎骨，大腿骨と脛骨ではMSCの性質は異なるかもしれない．実際，歯槽骨／顎骨MSCの骨分化能は腸骨MSCとほぼ同等であるものの，脂肪，軟骨分化能が腸骨MSCよりも明らかに低かった[5]．一方，大腿骨と脛骨のMSCは腸骨MSCと同等の骨，軟骨，脂肪分化能を示した．顎骨は外胚葉の神経冠細胞由来であるのに対して，腸骨，大腿骨と脛骨は中胚葉の体節由来である．この発生の由来の違いが顎骨MSCの異なる性質に関係しているかもしれない．以上の知見はMSCの採取部位が，再生医療に影響しうる要因の1つであることを示唆している．

4．移植細胞が目的の細胞であることの証明

移植細胞が目的の細胞であることを示すためには，MSCの規格化が必要である．われわれは，MSCが以下の図1のような遺伝子発現パターンを示すことを見出した．このパターンは線維芽細胞と異なり（図1），骨芽細胞，脂肪細胞，軟骨細胞とも異なっていた．すなわち遺伝子発現パターンによってMSCを規格化できることが判明した．

5．MSCの純度

われわれは歯周病患者へのMSC移植の前に，線維芽細胞が混入していないことを2種類の方法で検査している．1つはMSCマーカー遺伝子を利用する方法である[7]．もう1つは活性型ビタミンDに対するオステオカルシン応答を利用する方法である．

しかしより正確にMSCの純度を検定するには，目的の細胞集団から数十個の細胞を無差別によりだしてクローニングし，すべてのクローンがいずれも母集団と同一の遺伝子発現パターンを示すことを証明する必要がある．われわれはこの方法で，超増幅したMSCが均質な細胞集団であることを明らかにした．すなわち骨髄液から分離した培養皿接着細胞は，老化の程度に関して不均質であったが，FGF-2存在下では若いMSCのみが選択的に増殖して増えるため1～2回の継代後は均質な細胞集団となった．また

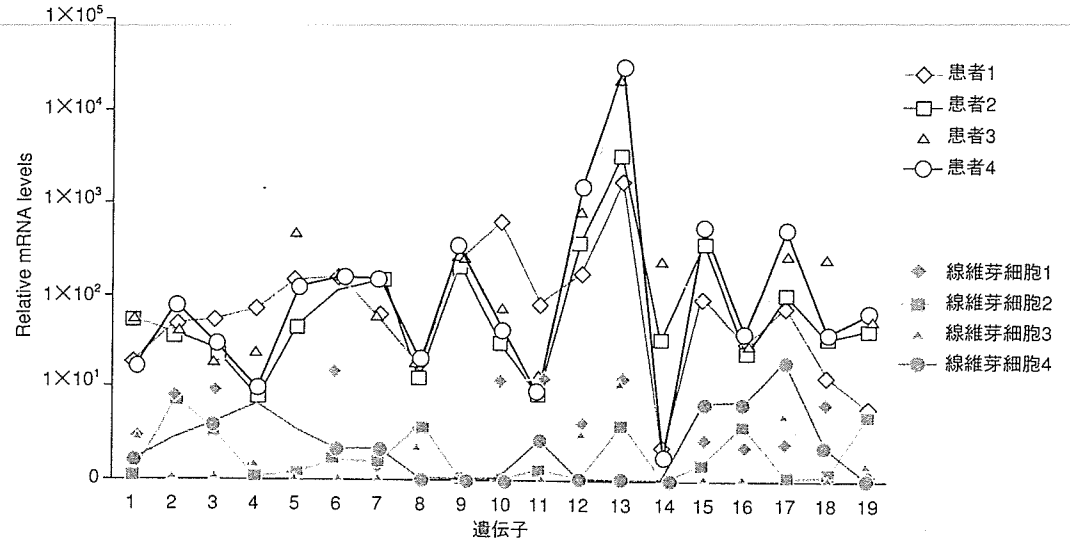


図1 ●ヒトMSCの規格化

4名の遺伝子発現レベルはQ-PCR法で測定した．ヒトMSC株の遺伝子発現プロファイルをヒト線維芽細胞と比較した

従来法では，MSCが次第に多分化能を失い不均質になるのに対して，FGF-2存在下ではすべての細胞が多分化能を維持するので継代後は均質な細胞集団となった（図2）．

6．MSCの病的変化

正常ドナー，歯周病ドナー，バージチャー病ドナー由来のMSCの分化能力を比較したが，例数は少ないものの，これらの病気はMSCの多分化能に大きな影響を及ぼさなかった．一方，脂肪由来のMSCを1年間近く培養すると癌化したという報告が1つあるので[8]，長期間培養したMSCについては癌化の有無を判定する必要がある．

7．MSC治療の安全性

MSCを移植するには，安全性を保証する必要がある．そのための検査の項目を表に列挙した．なおMSCと倫理[9]，MSCの「stemness」[10]およびMSCの基本的性質[11]については，最近のわれわれの総説を参照していただきたい．

おわりに

将来，ヒトMSCによる再生医療が自家および他家細胞を利用して行われるようになる．他家MSCは心筋梗塞，脳梗塞などの急性かつ生死にかかわる病気や造血支持のためおよび遺伝性骨不全[12]や代謝病[13]へ適応され，自家MSCは，慢性だがQOLを著しく障害する歯周病，骨疾患，関節症などの治療に用いられる．皮膚の再生医療でも自家と他家細胞移植の両者があり長い歴史もあるが，ビジネスとしての拡大は遅かった．これは細胞治療そのものが悪いのでなく，皮膚再生では異種動物細胞を培養のフィーダー細胞*2として用いる点や熱傷の患者数（＜10,000人）が少ないことが障害となっていた．その点MSCの培養には異種動物細胞は不必要であるし，MSC治療の対象疾患と患者数は非常に多い（＞4,000万人）．したがって，MSCによる再生医療

表 ●MSCの検査項目

①細胞の感染	細菌，真菌，ウイルスの否定
②血清の感染	プリオン，ウイルスなどの否定
③細胞の同定	目的の細胞であること MSC規格に一致すること
④純度	線維芽細胞否定試験 クローン分析など
⑤分化能	骨，軟骨，脂肪分化能など
⑥病的変化	癌化／炎症の否定

＊2：フィーダー細胞
増殖や分化を起こさせようとする目的の細胞の培養条件を整えるために用いる，補助役を果たす他の細胞種を示す言葉．通常フィーダー細胞は増殖しないようにあらかじめガンマ線照射や抗生物質によって処理しておく．ES細胞の場合は，マウス胎仔由来の線維芽細胞が用いられ，この細胞の未知の液性因子などがES細胞培養に必要だと考えられている．実験の目的や細胞によってさまざまな細胞種がフィーダー細胞として用いられる．

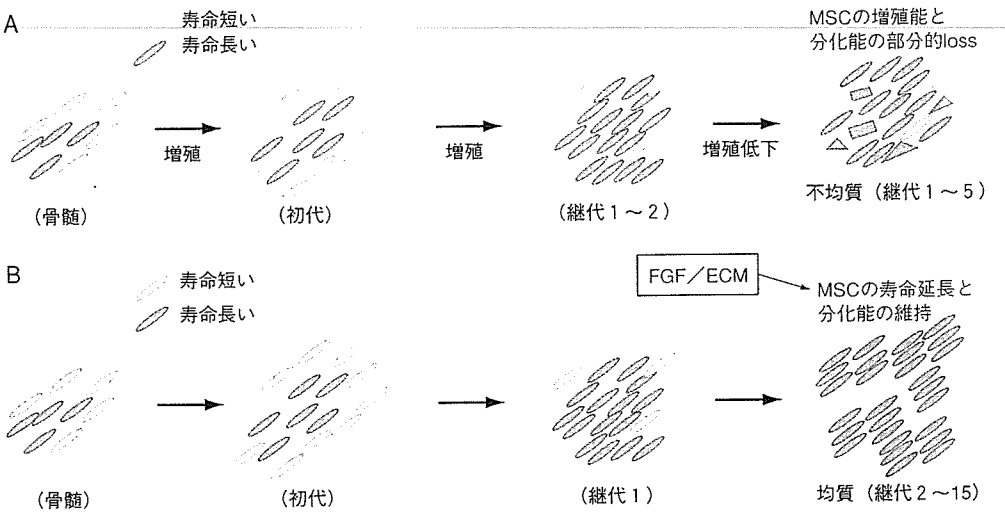


図2 ●超増幅法による均質MSC集団の大量培養（従来法と超増幅法の比較）

A）試験管内でのMSCの増殖（従来法）．MSC細胞群の増殖寿命は短い．また，MSCは細胞ごとにさまざまな速度で，潜在的分化能／増殖能を失うので継代しても不均質な細胞集団のままである．B）超増幅法．MSC以外の不均質な細胞群の増殖寿命が短いため，継代培養の1～2代以後にはMSCだけのほぼ均質な細胞集団となる．しかもMSCの分化能／増殖能は細胞分裂中も維持されるので最後まで，均質な細胞集団として増幅される

は，いったん認可が成立すれば大きく広がるものと予想され，それに向けて基礎／臨床研究で証拠を積み重ねることが今日求められている．

文献

1）Kawaguchi, H. et al.：J. Periodontol., 75：1281-1287, 2005
2）Robinson, S. N. et al.：Bone Marrow Transplant., 37：359-366, 2006
3）Blanc, L. et al.：Lancet, 363：1439-1441, 2004
4）Kayakabe, M. et al.：Cytotherapy, 8：in press
5）Matsubara, T. et al.：J. Bone Mineral Res., 20：399-409, 2005
6）Sakaguchi, Y. et al.：Arthritis Rheum., 52：2521-2529, 2005
7）Ishii, M. et al.：BBRC, 332：297-303, 2005（トップ10ダウンロード優秀論文）
8）Rubio, D. et al.：Cancer Res., 65：3035-3039, 2005
9）加藤幸夫 & 辻紘一郎：デンタルダイヤモンド，31：70-73, 2006
10）加藤幸夫 ほか：腎と骨代謝，印刷中
11）加藤幸夫 ほか：関節外科，25：63-69, 2006
12）Horwitz, E. M.：Proc. Natl. Acad. Sci. USA, 99：8932-8935, 2002
13）Koc, O. N. et al.：Bone Marrow Transplant., 30：215-222, 2002

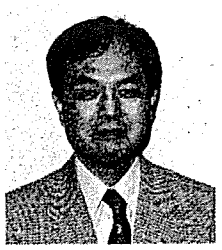
■MSC株などの入手のためのアドレス■

●ヒト骨髄由来MSC
理研バイオリソースセンター；
http://www.brc.riken.jp/index.shtml
http://www2.brc.riken.jp/lab/cell/search_hms.cgi

●ラット骨髄由来MSC
大日本住友製薬株式会社；http://labopro.ds-pharma.co.jp/htm/search/category/category2.html

●ヒト脂肪由来MSC
Zen-bio, Inc；http://www.zen-bio.com/

●ヒト骨髄由来MSC
Cell Applications, Inc；http://www.cellapplications.com/HMSC.htm#top



加藤幸夫（Yukio Kato）

広島大学大学院医歯薬学総合研究科教授．株式会社ツーセル取締役．
1977年大阪大学大学院歯学研究科修了後，カリフォルニア大学サンフランシスコ校とロックフェラー大学へ留学した（1982～1984年）．1991年より現職．
現在，幹細胞生物学と時間生物学に興味をもっている．

Behavior of Transplanted Bone Marrow–Derived Mesenchymal Stem Cells in Periodontal Defects

Naohiko Hasegawa,* Hiroyuki Kawaguchi,* Akio Hirachi,* Katsuhiro Takeda,* Noriyoshi Mizuno,* Masahiro Nishimura,† Chika Koike,‡ Koichiro Tsuji,§ Hideo Iba,‖ Yukio Kato,‡§ and Hidemi Kurihara*

Background: Recently, there have been an increased number of basic and clinical reports indicating the superior potential of bone marrow–derived mesenchymal stem cells (MSCs) for tissue regeneration. In periodontal treatment, previous animal studies indicated that autotransplantation of bone marrow MSCs into experimental periodontal defects enhanced periodontal tissue regeneration. However, mechanisms for periodontal tissue regeneration with MSCs are still unclear. The purpose of this study was to elucidate the behavior of transplanted MSCs in periodontal defects.

Methods: Bone marrow MSCs were isolated from beagle dogs, labeled with green fluorescent protein (GFP), and expanded in vitro. The expanded MSCs were mixed with atelocollagen (2% type I collagen) at final concentrations of 2×10^7 cells/ml and transplanted into experimental Class III periodontal defects. Localizations of GFP and proliferating cell nuclear antigen (PCNA)-positive cells were evaluated by immunohistochemical analysis.

Results: Four weeks after transplantation, the periodontal defects were almost regenerated with periodontal tissue. Cementoblasts, osteoblasts, osteocytes, and fibroblasts of the regenerated periodontal tissue were positive with GFP. PCNA-positive cells were present in regenerating connective tissue.

Conclusion: These findings suggest that transplanted mesenchymal stem cells could survive and differentiate into periodontal tissue cells, resulting in enhancement of periodontal tissue regeneration. *J Periodontol 2006;77:1003-1007.*

KEY WORDS

Atelocollagen; bone marrow cells; mesenchymal stem cells; regeneration.

* Department of Periodontal Medicine, Division of Frontier Medical Science, Hiroshima University, Hiroshima, Japan.
† Department of Prosthetic Dentistry, Graduate School of Biomedical Sciences, Hiroshima University.
‡ Department of Dental and Medical Biochemistry, Division of Molecular Medical Science, Graduate School of Biomedical Sciences, Hiroshima University.
§ Two Cells, Hiroshima, Japan.
‖ Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan.

Tissue regeneration requires three key elements: cells, scaffolds, and signaling molecules.[1] Considering current regenerative surgeries of periodontal tissue, scaffolds and signaling molecules are already used; however, the other key element, cells, has not yet been well established.

Bone marrow mesenchymal stem cells (MSCs) can easily be obtained and differentiated into osteoblasts, chondrocytes, tenocytes, adipocytes, muscle cells, or nerve cells in vitro and in vivo.[2-8] Thus, transplantation of bone marrow MSCs may provide a new method for treatment of osteoporosis, arthritis, cardiac diseases, and degenerative nerve diseases. Taking all of these findings into consideration, it is conceivable that MSCs might be useful for periodontal tissue regeneration.

We have focused on bone marrow MSCs and already succeeded in developing a new culture system with fibroblast growth factor-2 (FGF-2) to expand MSCs with multilineage differentiation potential.[8] To investigate the possibility of using MSCs in periodontal therapy, our previous animal study revealed that transplantation of bone marrow MSCs enhanced periodontal tissue regeneration.[9] Although it has been speculated that transplanted MSCs participate in periodontal tissue regeneration, the precise mechanism by which MSC transplantation enhances periodontal tissue

doi: 10.1902/jop.2006.050341

regeneration is unknown. To elucidate the performance of the MSCs after transplantation, we transduced MSCs with DNA encoding for green fluorescent protein (GFP). GFP is widely used as a tracer and is well tolerated both in vitro and in vivo. The purpose of this study was to evaluate whether the resulting periodontal tissue regeneration originated from the transplanted MSCs.

MATERIALS AND METHODS

After approval was obtained from the Committee of Research Facilities for Laboratory Animal Science, Hiroshima University School of Medicine, six female beagle dogs weighing 10 to 14 kg and aged 12 to 20 months were used in this study. Good oral health was established by scaling and mechanical toothbrushing.

Isolation and Development of Bone Marrow MSCs

Bone marrow aspirates of 1 ml were taken from the iliac crest of animals under sodium pentobarbital (40 mg/kg) anesthesia. Cell culture was performed in accordance with the technique described by Tsutsumi et al.[8] The cells were seeded at 2×10^8 cells/100-mm tissue culture dish and maintained in 10 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS),¶ 0.05 U/ml penicillin,# and 0.05 mg/ml streptomycin.** Three days after seeding, floating cells were removed, and the medium was replaced by fresh medium. Passages were performed when cells were approaching confluence. Cells were seeded at 5×10^3 cells/cm^2 in 100-mm dishes and maintained in a medium containing 3 ng/ml FGF-2†† for 2 weeks. The cells were harvested with trypsin plus EDTA, washed with phosphate-buffered saline, and stored in liquid nitrogen until use.

Transduction of MSCs With a Retrovirus Carrying GFP cDNA

PtG-S2-pMZGFPIP (enhanced GFP packaging cells) and the adenovirus to transduce Cre recombinase were used.

Preparation of Vesicular Stomatitis Virus Glycoprotein (VSV-G) Pseudotype Retrovirus

DMEM (high glucose) supplemented with 10% FBS and penicillin/streptomycin was used for retrovirus vector preparation. The VSV-G-pseudotyped vector was harvested every 24 hours from PtG-S2-pMZGFPIP at 3 to 7 days after introduction of Cre recombinase, as described previously.[10] The VSV-G-pseudotyped vector was concentrated by centrifugation at 6,000 × g for 16 hours at 4°C, and the pellet was suspended in DMEM without FBS (final ×100-fold concentration).

Retrovirus Transduction and Determination of Its Efficiency and Selection

All transduction was carried out in the presence of 8 μg/ml polybrene‡‡ after a 1-day passage. Three days after transduction, the titer of MSCs was determined by counting the numbers of clones with green fluorescence in cultures transduced with the vector in 10-fold serial dilutions made by adding fresh culture medium. We determined that the 1:40-fold dilution of the concentrated vector solution could transduce the MSCs effectively. To select the transduced cells, 4 μg/ml puromycin was added to eliminate any non-transduced cells. The transduced cells (>90% GFP-positive) obtained from cultures at passages 3 through 5 were used for transplants.

Creation of Class III Furcation Defects, Transplantation of MSCs, and Tissue Preparation

Creation of Class III furcation defects and tissue preparation were performed according to the technique reported by Kawaguchi et al.[9] Mandibular second, third, and fourth premolars (P2, P3, and P4) in each dog were selected for experimentation. The MSCs cultured for 2 weeks were suspended, and then an MSC collagen gel material was prepared. A total of 2×10^7 MSCs were mixed with 1 ml atelocollagen (2% type I collagen, extracted from bovine calf skin by pepsin digestion).§§ These MSCs in atelocollagen or atelocollagen only were transplanted. During the postoperative observation periods, good oral hygiene was maintained by brushing and swabbing with 0.2% povidone iodine.‖‖

Four weeks after transplantation, anesthetized animals were sacrificed by vascular perfusion with 4% paraformaldehyde in a sodium cacodylate buffer containing 0.05% calcium chloride (pH 7.3). The mandibles were dissected and decalcificated with 10% EDTA for 2 weeks. They were dehydrated through graded ethanol, cleared with xylene, and embedded in paraffin. Serial sections (5 μm) were cut in the mesial-distal plane throughout the buccal-lingal extension of the tooth. The sections were stained with hematoxylin and eosin (H&E) and observed using a light microscope.

Immunohistochemical Procedures

Av peptide antibody¶¶ and mouse monoclonal anti-cell nuclear antigen## (proliferating cell nuclear antigen [PCNA]) were used as the primary antibodies. Av peptide antibody is a mixture of several rabbit

¶ Hyclone, South Logan, UT.
# Invitrogen, Carlsbad, CA.
** Invitrogen.
†† PeproTech EC, Rocky Hill, NJ.
‡‡ Sigma, St. Louis, MO.
§§ Koken, Tokyo, Japan.
‖‖ Meiji-seika, Tokyo, Japan.
¶¶ Living Colors, BD Biosciences, Palo Alto, CA.
DAKO, Carpinteria, CA.

anti-GFP antibodies. Sections were deparaffined with xylene, rehydrated through a descending ethanol series, and washed in distilled water. Endogenous peroxidase was blocked by incubating the sections with 3% hydrogen peroxide. After washing sections with Tris-buffered saline (TBS) (pH 7.2), the sections were treated with 0.1% bovine serum albumin*** to prevent non-specific binding, and the following incubations were performed. The primary antibodies were diluted in antibody diluent††† and incubated for ~1 hour at room temperature. The primary antibody dilutions were GFP (1:200) and PCNA (1:200). After incubation with the primary antibodies, sections were rinsed with TBS and incubated with an alkaline phosphatase-dextran complex‡‡‡ for 30 minutes in a moist chamber. These slides were rinsed in TBS. Antibody complexes were visualized with 3,3′-diaminobenzidine (DAB) substrate, washed in distilled water, and counterstained with hematoxylin. As controls, some sections were treated in the same way but without incubation with the primary antibodies. The differential labeling patterns obtained with various antibodies also served as internal controls.

RESULTS

Morphological and Immunohistochemical Findings

Morphological findings at 4 weeks after transplantation were almost the same as in the previous report. Most experimental specimens showed a significant amount of new bone, and an adequate width of periodontal ligament was observed (Fig. 1A).

The denuded root surface was almost completely covered with new cementum, and the regenerated periodontal ligament separated the new bone from cementum. However, complete alveolar bone reconstruction was not yet obtained (Fig. 1A). In contrast, periodontal tissue regeneration was insufficient in the control group compared to the experimental group (Fig. 2). Epithelial cells invaded into the top of the furcation, and cementum regeneration was not observed in the area (Fig. 2).

Immunohistochemical study showed that GFP-positive cells were present in the whole area of the defect. In the top area of the defect, abundant GFP-positive cells were observed in regenerating soft connective tissue and on the surface of regenerating alveolar bone (Figs. 1B and 1D).

Cementoblasts arranged along the denuded surface, osteoblasts and osteocytes of regenerated bone, and fibroblasts of the regenerated periodontal ligament were immunoreactive for GFP (Figs. 1C and 1E). In all control incubations, immunoreaction was not found over the tissue sections.

PCNA-positive cells were present in regenerating soft connective tissue (Figs. 1F and 1G). Cementoblasts, fibroblasts, osteoblasts, and osteocytes of regenerated periodontal tissue showed weak staining with PCNA (Fig. 1G).

DISCUSSION

To elucidate the survivability and behavior of transplanted MSCs in periodontal defects, we used GFP-transduced MSCs and examined their localization in a regenerative procedure using an immunohistochemical method. Initially, we observed with a fluorescence microscope but could not detect any GFP-positive cells. Decalcification of the tissue during tissue preparation might influence GFP fluorescence. Therefore, GFP was visualized using anti-GFP monoclonal antibody immunostaining in this study.

Our histological analysis showed that the defects were almost regenerated with cementum, periodontal ligament, and alveolar bone 4 weeks after MSC transplantation. These results were consistent with our previous report.[9] Moreover, this study showed that osteoblasts and osteocytes in regenerated alveolar bone, cementoblasts on the denuded root surface, and fibroblasts in the soft connective periodontal tissue were GFP-positive. These anti-GFP monoclonal antibody immunostaining findings confirmed that some transplanted MSCs survived in periodontal defects at least 4 weeks after transplantation and differentiated into periodontal tissue-composing cells. It is also speculated that transplanted MSCs survive, differentiate into periodontal tissue cells, and release various kind of cytokines, all of which promote periodontal tissue regeneration.

The exact mechanism by which MSCs differentiate into cementoblasts, osteoblasts, and periodontal ligament fibroblasts is unknown. Previous in vivo studies revealed that host factors influence transplanted MSCs to differentiate into various connective tissue cells.[11,12] It has also been reported that the microenvironment and surrounding tissue provide the nutrients, growth factors, and extracellular matrices to support MSC differentiation.[13] It appears that when stem cells are removed to different locations, they undergo reprogramming of gene expression and cross lineage boundaries. For instance, brain stem cells give rise to hematopoietic cells, and bone marrow cells give rise to epithelial cells after transplantation.[14,15] In the periodontal field, bone marrow MSCs gain characteristics of periodontal ligament cells after co-culturing with periodontal ligament–derived cells.[16] The periodontal environment seemed to stimulate the transplanted MSCs to differentiate into specific periodontal tissue cells.

With regard to MSCs differentiated into cementoblasts, transplanted MSCs may promote their

*** Sigma.
††† DAKO.
‡‡‡ ENVISION, DAKO.

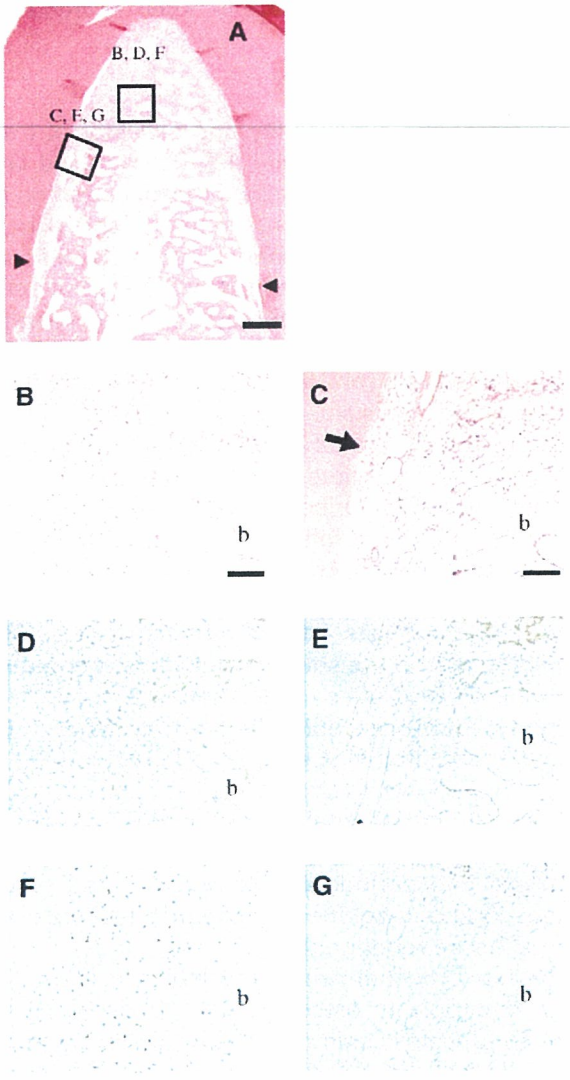


Figure 1.
Four weeks after transplantation. ***A)*** *New bone and an adequate width of periodontal ligament are observed in the experimental group. New cementum almost completely covers the denuded root surface. Regenerated periodontal ligament separates the new bone from the cementum. Arrowheads indicate the apical border of the denuded root surface.* ***B through G)*** *Higher magnification of square areas of A. B and D) In the top area of the defect, osteoblasts on regenerated bone (b) and fibroblasts in the surrounding connective tissue are GFP-positive. C and E) In the middle area of the defect, cementoblasts, fibroblasts, osteoblasts, and osteocytes of regenerated periodontal tissue are GFP-positive. F) Cells in connective tissue are PCNA-positive. G) Cementoblasts, fibroblasts, osteoblasts, and osteocytes show weak staining with PCNA. (B and C: H&E staining; D and E: immunohistochemical staining of GFP; F and G: immunohistochemical staining of PCNA; scale bars: A = 200 μm; B and C = 20 μm.)*

differentiation after attachment to the denuded dentin surface. Our in vitro study involving reverse transcription–polymerase chain reaction of MSCs showed that MSCs expressed non-collagenous bone

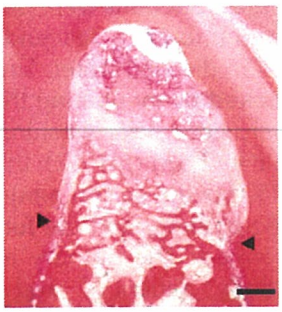


Figure 2.
Four weeks after implantation of atelocollagen alone (control group). Epithelial cells invaded into the top of the furcation, and no cementum regeneration was observed in the area. Note less periodontal regeneration compared to Figure 1A. Arrowheads indicate the apical border of the denuded root surface. Bar = 200 μm.

proteins such as osteocalcin, osteopontin, and bone sialoprotein when MSCs were cultured on dentin blocks (data not shown). These findings suggest that MSCs attached on the root surface differentiate into cementoblasts in the early stage of the process. A variety of chemotactic factors, adhesion molecules, growth factors, and extracellular matrix macromolecules could participate together in inducing differentiation of MSCs into cementoblasts.[17] Once MSCs along the root surface differentiate into cementoblasts, these cells could release various kinds of cytokines, leading to a subsequent process of periodontal tissue regeneration.

The present study indicates that up to 4 weeks after transplantation, regenerating and regenerated periodontal tissue was derived largely from transplanted MSC cells. Furthermore, results of an immunohistochemical PCNA study suggest that MSCs are present at various differentiation stages. Nevertheless, the results in the control group showed that some of the defects were repaired by the invasion of cells from the surrounding tissue: regenerated periodontal tissue originates, at least partly, from host cells. It is not clear how long transplanted MSCs survive and take part in tissue formation. Transplanted MSCs in chondral defects showed that they decreased in number with time.[18] Although the cells might have lost the ability to produce GFP, their disappearance is more likely.[19] Other groups reported that transplanted cells survived for 10 days to 8 weeks in chondral defects.[20,21] Additional studies with short- and long-term observations will extend our knowledge on the exact fate of the transplanted MSCs.

CONCLUSION

This study indicates that transplanted MSCs can survive and differentiate into periodontal tissue–composing