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Meniscal repair using bone marrow-derived mesenchymal stem cells: experimental study using green fluorescent protein transgenic rats

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Abstract

Meniscal tears in the avascular zone have very limited potential to heal because of a poor blood supply. Although there have been many attempts to promote the healing potential of the torn meniscus, no established treatments have achieved sufficient meniscal healing. In this study, we evaluated the efficacy of mesenchymal stem cell transplantation as a cell source to promote meniscal healing, using cells from the green fluorescent protein (GFP) transgenic rat and organ culture model. Mesenchymal stem cells from bone marrow were isolated and expanded in monolayer culture. They were embedded in fibrin glue and were transplanted into the meniscal defects of Sprague–Dawley rats. In the control groups, the defects remained untreated, or only fibrin glue without cells was transplanted. The GFP-positive cells enabled us to detect the transplanted cells from recipient cells easily. As a result, transplanted mesenchymal stem cells could survive and proliferate in the meniscal defects in the organ culture model. They also could produce an abundant extracellular matrix stained by toluidine blue around the cells which contributed to meniscal healing in the avascular status. We could detect transplanted GFP cells under a fluorescent microscope until 8 weeks after transplantation. In a clinical situation, mesenchymal stem cell transplantation is a promising new clinical strategy for the treatment of meniscal tears in the avascular zone.

Keywords: Meniscus; Mesenchymal stem cell; Organ culture; Green fluorescent protein; Transgenic rat

1. Introduction

Menisci have important biomechanical roles for knee joint stability, shock absorption, or load distribution [1–5]. Although total or partial meniscectomy is widely indicated for the treatment of symptomatic meniscal tears, some basic studies have demonstrated that articular contact pressure of the knee joint significantly increases following even a partial meniscectomy [1,6]. This incremental load on the articular cartilage can lead to cartilage damage or regeneration. As a matter of fact, many clinical studies have shown early osteoarthritic change of the knee joint after meniscectomy [3–5,7,8]. Therefore, meniscal repair has recently strongly recommended that the meniscal tear located in the outer third of the vascular area of the meniscus should preserve the normal meniscal structure. However, despite recent progress in meniscal repair technique, the indications of meniscal repair for meniscal tears in the avascular area are still limited, and we are usually obliged to resect the torn meniscus in those cases.

Although there have been many attempts to accelerate the healing process of the torn meniscus in the avascular area using a fibrin clot, synovial tissue, small intestine mucosa, or several methods making vascular channels to the lesion, none of these methods have demonstrated reproducible healing of the meniscus [9–14]. Recently, a regenerative medicine using a tissue-engineering technique for meniscal repair has been the focus of attention in the

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orthopaedic field. Tissue engineering technique consists of three major parts: transplanted cells, scaffold for cell proliferation, and signalling molecules. Transplanted cells from mesenchymal stem cells in the bone marrow are one promising option for tissue repair. It is well known that in the appropriate conditions, they can differentiate into several lineages such as osteogenic, chondrogenic, or adipogenic lineages [15–17].

The purpose of this study was to evaluate the efficacy of mesenchymal stem cell transplantation for meniscal repair in the avascular state using an organ culture model. The cellular origin of the repaired tissue was also investigated using mesenchymal stem cells from green fluorescent protein (GFP) transgenic rats.

2. Materials and methods

The research protocol of this experiment was reviewed and approved by the ethical committee of our university.

2.1. Preparation of mesenchymal stem cells

The GFP rats were purchased from Japan SLC (Hamamatsu, Japan). Four 12-week-old male GFP rats were anesthetized by an intraperitoneal injection of sodium pentobarbital. The proximal medial surface of each tibia was exposed through a small incision. The soft tissue was retracted, and 2 ml of the bone marrow cells were aspirated from each tibia with a 24-gauge needle that was fastened to a 10-ml syringe containing 0.2 ml of heparin (1000 units/ml). The aspirate was washed with Dulbecco's phosphate-buffered saline (PBS; Gibco, Grand Island, NY) and centrifuged at 1500 rpm for 5 min. The cells were suspended in culture media consisting of Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and penicillin–streptomycin–fungizone (Bio-Whittaker, Maryland, USA), seeded on 100 mm culture dishes, and incubated in a humidified 5% CO_2–95% air atmosphere at 37 °C. The medium remained unchanged for the first 5 days. Then, the medium was changed at intervals of 3 days. Two weeks after incubation, the cells usually proliferated up to confluence (Fig. 1). Then the cells were harvested after treatment with 0.25% trypsin and 0.02% EDTA, rinsed with culture medium twice, and counted using a hemocytometer. Thereafter, we referred to these cells as mesenchymal stem cells. Finally, the cells were suspended at 6.0×10^6 cells/ml in culture media.

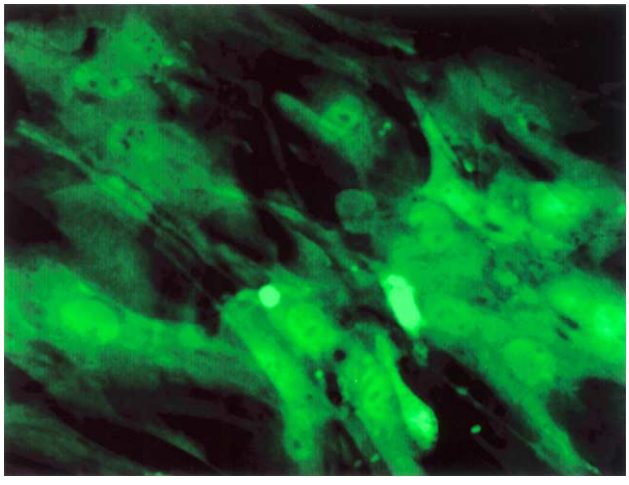

Fig. 1. Fluorescent microscopic appearance of expanded mesenchymal stem cells.

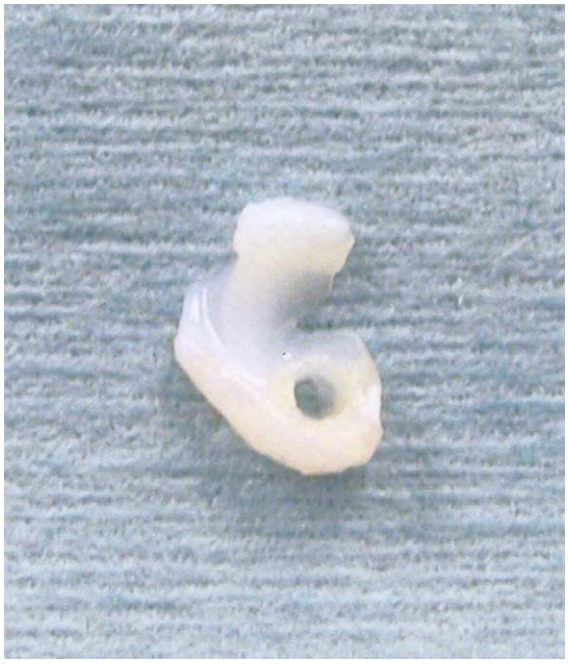

Fig. 2. Harvested meniscus of a Sprague–Dawley rat. A full-thickness circular defect is created, 1.2 mm in diameter, vertically penetrating the anterior portion.

2.2. Creation of meniscal defects

Eighteen 12-week-old male Sprague–Dawley rats were sacrificed by an overdose of an intraperitoneal injection of sodium pentobarbital. Both knee joints were exposed under sterile conditions. A full-thickness circular defect, 1.2 mm in diameter, penetrating vertically the anterior portion of the lateral and medial menisci, was produced using a drill (Fig. 2). The lateral and medial menisci were removed carefully from the adherent articular capsules or synovium and harvested aseptically from both knee joints. The meniscal specimens were washed with PBS.

2.3. Organ culture

Fibrin glue (Bohheal™; Fujisawa Pharmaceutical, Osaka, Japan) was used as a scaffold for mesenchymal stem cells. The fibrin adhesive systems were composed of solutions A and B. Solution A consisted of dense

fibrinogen, aprotinin, and factor X III and solution B contained thrombin and CaCl2. Mixing solutions A and B made fibrin glue. The meniscal defects were treated in one of the following three ways: in the M group, the defect was filled with 1.0×10^6 cells/ml of mesenchymal stem cell in fibrin glue; in the F group, the defect was filled with fibrin glue without cells; and in the C group, the defect was left empty without cells and fibrin glue. Each of the meniscal explants was placed in a well on a 24-well tissue culture plate (Cellstar, Greiner Japan, Tokyo, Japan) and cultured at 37 °C in 5% CO_2 in DMEM containing 10% FBS and 1% antibiotics. The medium was changed every three days. The explants were transferred to a new plate every week in a standard manner so that the cells did not reach confluence in the wells.

After culturing the explants for 2, 4, and 12 weeks, eight explants each time point were examined histologically. Each meniscal explant was fixed in 4% paraformaldehyde for 24 h and decalcified in EDTA. The specimens were embedded in plastic legin (Technovit 8100®, Okenshoji, Tokyo, Japan) and cut into 5 μm sections along a horizontal plane. The horizontal plane was designed to contain the graft. For histological evaluation, the sections were stained with hematoxylin and eosin and toluidine blue. The fluorescence of enhanced GFP, which had a 508 nm wavelength, was evaluated on explants activated by a 489 nm wavelength ray under a fluorescent microscope (Leica Microsystems, Germany). A fluorescent microscope was used to detect the GFP-positive cells derived from the donor cells in the repaired tissues.

3. Results

3.1. Histological evaluation

In the M group, after 2 weeks of culture, fibroblastic cells in a monolayer were observed at the edge of the meniscal defect (Fig. 3). The round cells were found in the fibrin glue, but the production of a newly formed extracellular matrix was not observed around the cells. After 4 weeks of culture, round cell proliferation was found in the fibrin glue with new production of an extracellular matrix around the cells in six of the eight specimens. After 12 weeks of culture, the reparative tissue with many round cells surrounded by an abundant extracellular matrix stained by toluidine blue was observed in six of the eight specimens. Cartilage-like tissue around the several-layer fusiform cells could be observed in the meniscal defect.

In the F group, after 2 weeks of culture, a monolayer of fibroblastic cells was observed in a monolayer at the edge of the meniscal defect (Fig. 4). The cells were rarely found

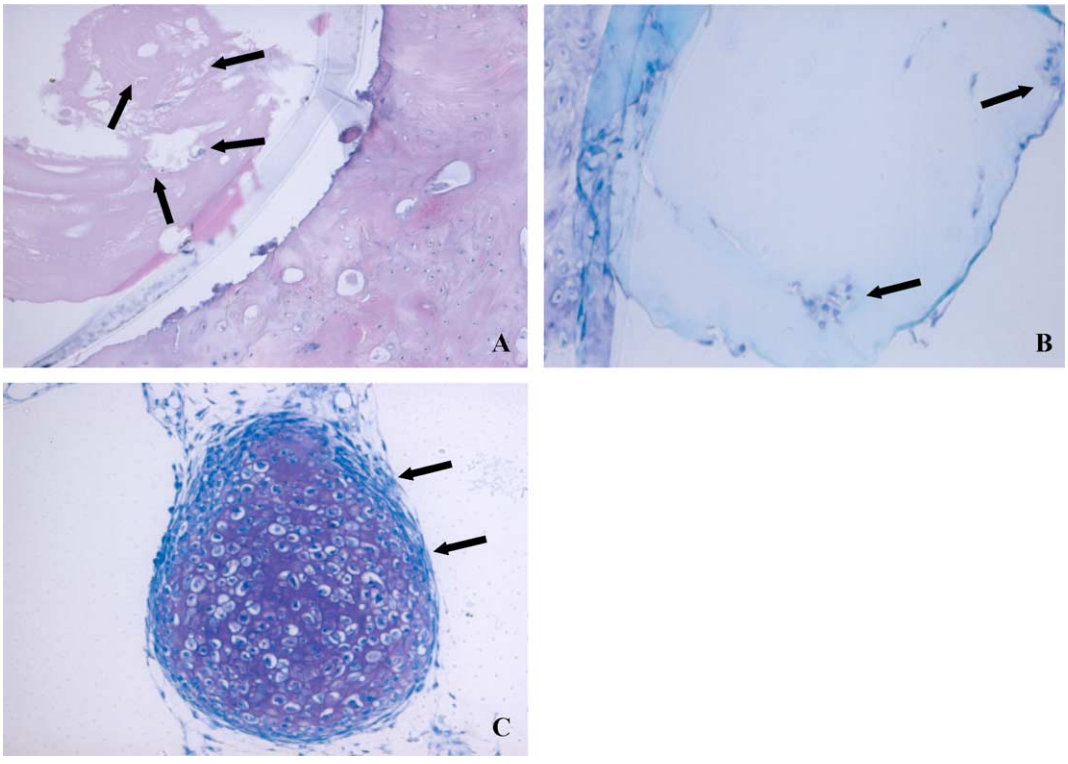

Fig. 3. Typical microscopic appearance in the M group. (A) After 2 weeks of culture, fibroblastic cells (arrows) in a monolayer are observed at the edge of the meniscal defect. The round cells are found in the fibrin glue, but the production of newly formed an extracellular matrix was not observed around the cells. (HE staining, ×100). (B) After 4 weeks of culture, round cell proliferation (arrows) is found in the fibrin glue with new production of extracellular matrix around the cells. (Toluidine blue staining, ×100). (C) After 12 weeks of culture, the reparative tissue with many round cells was observed, surrounded by an abundant extracellular matrix (arrows) stained by toluidine blue. (Toluidine blue staining, ×100).

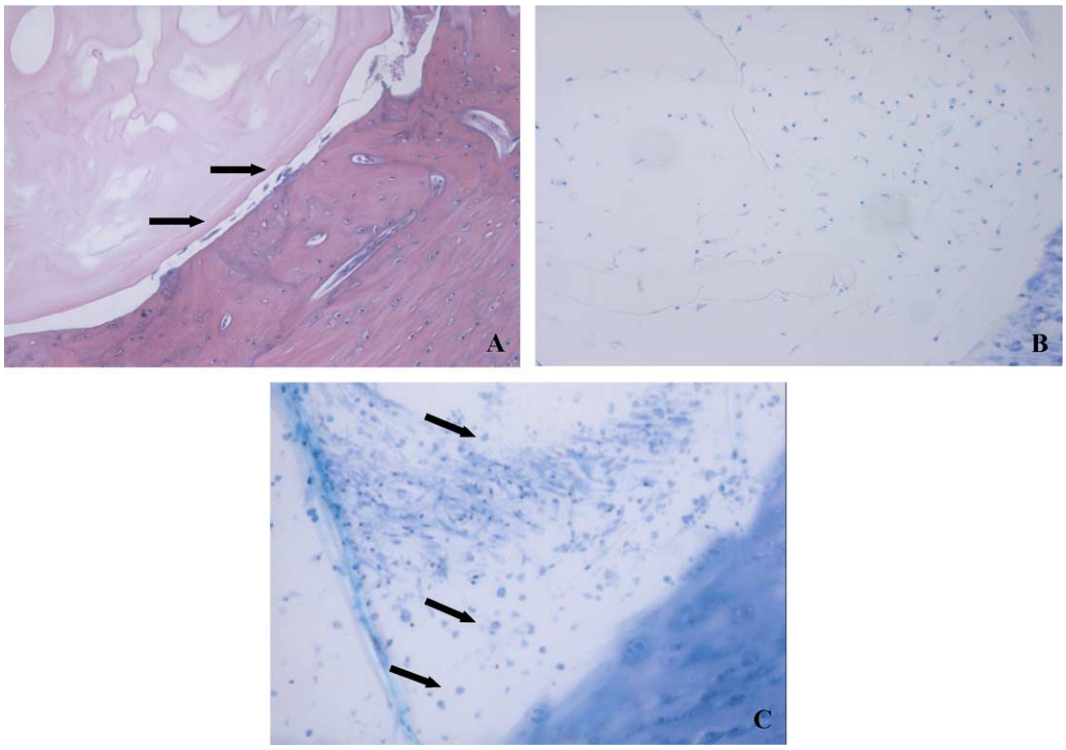

Fig. 4. Typical microscopic appearance in the F group. (A) After 2 weeks of culture, fibroblastic cells are observed in a monolayer at the edge of the meniscal defect. The cells were rarely found to migrate into the fibrin glue from the edge of the defect. (HE staining, ×100). (B) After 4 weeks of culture, polygonal cells had invaded the fibrin glue from the edge of the meniscal defect. However, production of a newly formed extracellular matrix is not seen in any specimens. (Toluidine blue staining, ×100). (C) After 12 weeks of culture, many small round cells had invaded the fibrin glue. A small amount of extracellular matrix stained by toluidine blue is observed. (Toluidine blue staining, ×100).

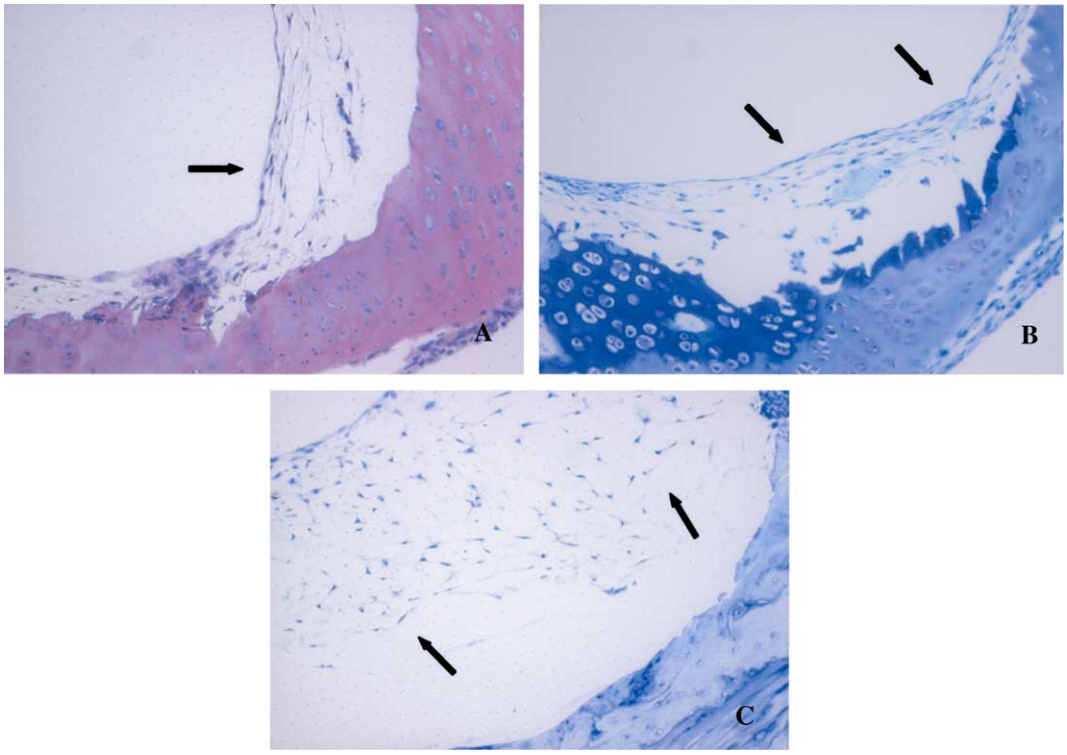

Fig. 5. Typical microscopic appearance in the C group. (A) After 2 weeks of culture, a small number of fibroblastic cells are found at the edge of the meniscus. (HE staining, ×100). (B) After 4 weeks of culture, the number of fibroblastic cells at the edge of the meniscus increased. (Toluidine blue staining, ×100). (C) After 12 weeks of culture, many polygonal cells are observed in the defect. However, an extracellular matrix stained by toluidine blue is not observed. (Toluidine blue staining, ×100).

to migrate into the fibrin glue from the edge of the defect. After 4 weeks of culture, polygonal cells had invaded into the fibrin glue from the edge of the meniscal defect. However, production of a newly formed extracellular matrix was not seen in any specimens. After 12 weeks of culture, many small round cells had invaded into the fibrin glue. A small amount of extracellular matrix stained by toluidine blue was observed in two of the eight specimens.

In the C group, after 2 weeks of culture, a small number of fibroblastic cells was found at the edge of the meniscus (Fig. 5). After 4 weeks of culture, the number of fibroblastic cells at the edge of the meniscus increased. The cells located at the superficial area were fusiform, and the remaining cells were polygonal. After 12 weeks of culture, many polygonal cells were observed in the defect. However, an extracellular matrix stained by toluidine blue was not observed in any specimens.

3.2. Fluorescent microscopic evaluation

The typical fluorescent microscopic findings in the M group are shown in Fig. 6. In the M group after 2 and 4 weeks of culture, almost all cells in the fibrin glue in all specimens were GFP-positive cells derived from the donor mesenchymal stem cells. However, at 12 weeks after cultivation, GFP-positive cells in the meniscal defect decreased significantly.

4. Discussion

Our study clearly demonstrated that the isolated mesenchymal stem cells embedded in fibrin glue could survive and proliferate in the meniscal defect in the avascular status, and they also could contribute to the promotion of meniscal healing by producing an extracellular matrix around the cells.

Although there have been many attempts to accelerate the healing process of the torn meniscus in the avascular area such as by using fibrin clots, synovial tissue, small intestine mucosa, or several methods making vascular channels to the lesion, none of these methods demonstrated reproducible healing of the meniscus [9–14]. One strategy to promote meniscal healing in the avascular zone has been cell transplantation.

The isolated meniscal cells should be used as a cell option to promote meniscal healing, because we can obtain meniscal cells from the resected torn meniscus in a clinical situation. However, the number of these cells is usually very small and the quality of the cells may be compromised by trauma. As for other cell sources, in 2004, Peretti et al. [18] transplanted autologous chondrocytes harvested from articular cartilage to the meniscal tear in the avascular portions in the pig model. They demonstrated the ability of seeded chondrocytes to heal a meniscal tear. However, we are concerned that the extracellular matrix of the articular cartilage is different from that in the meniscus. Moreover,

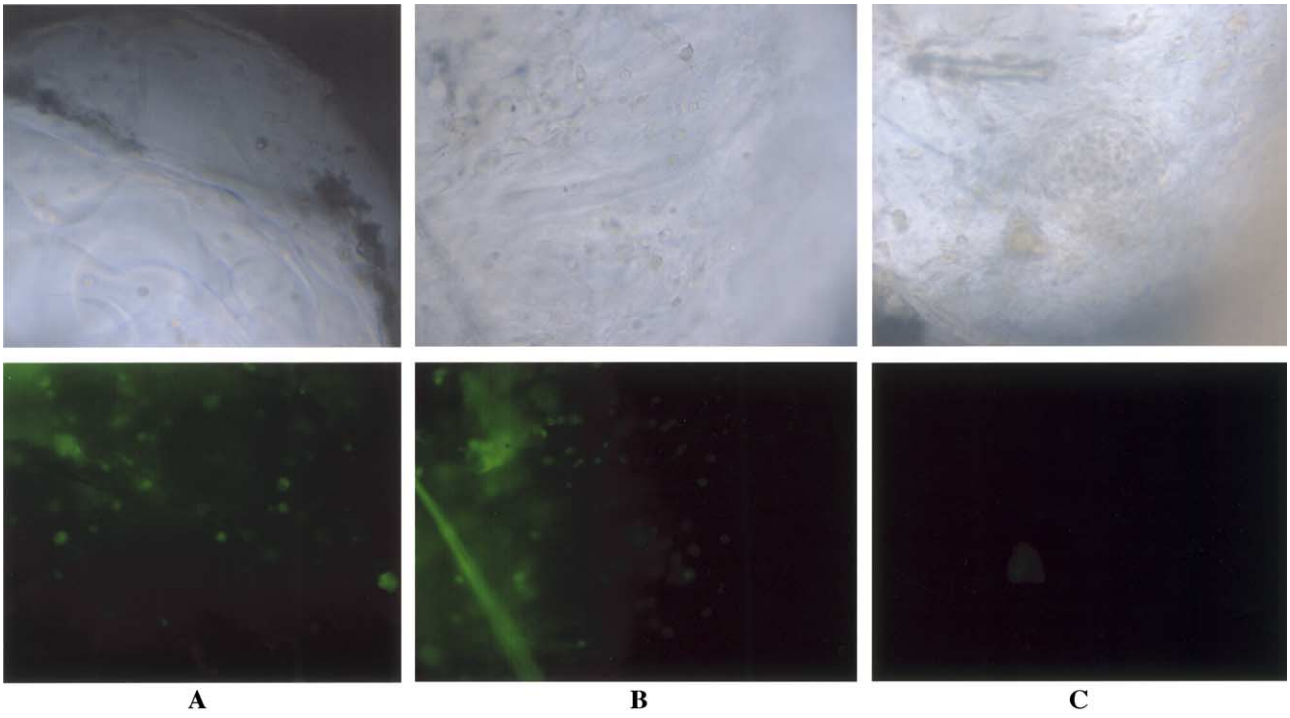

Fig. 6. The typical fluorescent microscopic findings in the M group. The upper figures are from a normal microscope and the lower figures are from a fluorescent microscope. (A, B) After 2 and 4 weeks of culture, almost all cells in the fibrin glue in all specimens are GFP-positive cells derived from the donor mesenchymal stem cells. (HE staining, ×100). (C) At 12 weeks after cultivation, GFP-positive cells in the meniscal defect decreased significantly.

harvesting chondrocytes from articular cartilage is a more invasive procedure. Recently, mesenchymal stem cells derived from bone marrow has been a focus due to its potential as a cell source for tissue repair, because they can differentiate into several mesodermal lineages such as bone, cartilage, fat, or other tissue with the proper addition of differentiation molecules [15–17]. Ishimura et al. [19] made an experimental meniscal defect in the avascular area of the rabbit meniscus and transplanted bone marrow cells with fibrin glue as a carrier of the cells. They demonstrated that earlier histological maturation and more rapid healing than with those treated with fibrin glue alone. They also stated that bone marrow cells contains pluripotent stromal stem cells and may contribute to enhanced healing of the meniscus. However, it has been reported that in the bone marrow cells, mesenchymal stem cells are supposed to be in a small percentage (estimated at about 0.001% to 0.01%). If the transplantation of the bone marrow cells could enhance meniscal healing in the avascular area, isolated mesenchymal stem cells may have greater potential for meniscal healing. In 2003, Murphy et al. [20] injected the isolated mesenchymal stem cells from bone marrow to the osteoarthritic knee joints which had been induced by a complete resection of the medial meniscus and anterior cruciate ligament. They demonstrated that the implanted cells were detected in the regenerated meniscus and stated that the transplanted mesenchymal stem cells stimulated the regeneration of meniscal tissue. Although a simple intraarticular injection of the mesenchymal stem cells is an attractive procedure, regeneration of a new meniscus after total meniscectomy is not likely in humans. Especially for the treatment of meniscal tears in the avascular zone, scaffolds can reside in the cells and may promote meniscal healing. We transplanted mesenchymal stem cells isolated from the bone marrow with fibrin glue as a delivery vehicle. Our study clearly demonstrated that mesenchymal stem cells could proliferate in the fibrin glue in the meniscal defects in the avascular organ culture model and they contributed to meniscal healing by producing an abundant extracellular matrix around the cells. This fact means that there is a possibility that mesenchymal stem cells can be transplanted to a meniscal tear in the avascular zone. The mesenchymal stem cells have several potential advantages for tissue repair. (1) They can be isolated from bone marrow with aspiration and expanded in the usual culture system. (2) We can avoid an immunoreaction from the host tissue, because we can use autologous cells. (3) There are fewer ethical problems than with embryonic stem (ES) cells. (4) They can be easily transduced by several vectors. We believe that these mesenchymal stem cells from bone marrow are the most clinically promising stem cells for many tissues including the meniscus.

It is very important and essential to know the cellular origin of the repaired tissue, because both transplanted cells and recipient cells can contribute to meniscal healing. In this experiment, we used mesenchymal stem cells harvested from the bone marrow of GFP rats and transplanted them to full-thickness meniscal defects in normal SD rats. In this cell transplantation system, it was quite easy to detect the GFP-positive cells derived from the donor mesenchymal cells in repaired tissue using a fluorescent microscope without special immunostaining or in situ hybridization. This simple technique to detect the transplanted cells decreased false-positive and false-negative phenomena. Although we could detect the GFP-positive cells in the fibrin glue in the M group until 4 weeks after culture, GFP-positive cells decreased significantly after 12 weeks of culture. One explanation for this significant decrease of GFP-positive cells in the reparative tissue was the fact that the fluorescent activity of GFP cells could decrease with time. To the best of our knowledge, this is the first study in which isolated mesenchymal stem cells derived from the bone marrow of GFP rats were used to promote meniscal healing using an organ culture model.

Organ culture provides a useful model to assess the meniscal healing potential without the influence of a blood supply or the synovium. Webber et al. [21,22] demonstrated the ability of meniscal fibrochodrocytes from intact rabbit menisci to extricate themselves from the surrounding matrix and to migrate to an exogenous, purified fibrin clot in the organ culture model. In our study, the degree of cell migration into the fibrin glue from the meniscal edge in the F group seemed to be less than that into the fibrin clot as reported by Webber et al. This may be due to the fact that fibrin glue contains fewer growth factors than a fibrin clot because of the heat treatment used to destroy virus as such as the human immunodeficiency virus or hepatitis virus. Even in the avascular status of the organ culture model, transplanted mesenchymal stem cells embedded in fibrin glue produced a more abundant extracellular matrix. Although we only performed a histological evaluation to assess the meniscal healing potential and did not evaluated biochemical and biomechanical factors which are very important aspects of the meniscus, with further in vivo studies we are confident that mesenchymal stem cell transplantation will become a new clinical strategy for the treatment of meniscal tears in the avascular zone.

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