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Ex vivo cartilage defect model for the evaluation of cartilage regeneration using mesenchymal stem cells

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An *ex vivo* cartilage defect model for the evaluation of cartilage regeneration using mesenchymal stem cells (MSCs) was developed. Porcine chondrocytes and human MSCs were transplanted into cartilage defects created on the porcine osteochondral and chondral discs and cultivated for 3 weeks. Although the regeneration of cartilage-like tissues was observed after the transplantation of chondrocytes to defects on both of the osteochondral and chondral discs, the transplanted MSCs formed cartilage-like tissues only in the defect on the chondral disc, in which an *in vivo* cartilage-like structure was partly observed, and a degraded tissue was observed in the defect on the osteochondral disc. The effects of medium additives such as serum, transforming growth factor-β3 (TGF-β3), and fibroblast growth factor-2, and the transfection of TGF-β3 gene to MSCs could be clearly estimated using the cartilage defect model. In conclusion, a chondral disc with defects is useful for evaluating cartilage regeneration using MSCs.

[**Key words:** Cartilage regeneration; Human mesenchymal stem cell; Explant culture; Cytokine; Transfection]

Human mesenchymal stem cells (MSCs) give rise to multiple mesodermal tissue types, including bone, tendon, and cartilage, and can be easily isolated from adult bone marrow aspirates (1). Therefore, they are an attractive cell source for cartilage regenerative medicine (2), and some clinical applications of the transplantation of undifferentiated MSCs into articular cartilage defects in humans have been reported (3). However, their use results in fibrocartilage tissue formation at defect sites, and thus the generation of a stable hyaline-rich cartilage tissue using MSCs is desired (4).

Several growth factors such as bone morphogenetic proteins (BMPs), basic fibroblast growth factor (FGF-2), insulin-like growth factor-1 (IGF-1) and transforming growth factor-β (TGF-β) have been reported to induce the chondrogenesis of MSCs, and thus the transplantation of MSCs together with these growth factors may be effective (5–7). Moreover, the transplantation of MSCs transfected with these growth factor genes has been considered to increase the amounts of these transgene products in the transplantation site, resulting in the induction of the hyaline-rich chondrogenic differentiation of transplanted MSCs by the autocrine or paracrine effects (4).

The evaluation of the efficacy and safety of infused growth factors and transplanted MSCs using animal experimental models might be essential to realize the previously mentioned clinical applications. However, there are several problems with the use of animal experimental models, such as animal protection, individual differences between the animals and the high cost of large animals (8). Therefore, the development of *in vitro* evaluation systems that enable preliminary screening before animal experiments is important.

MSCs transfected with multiple chondrogenic genes using a recombinant adenovirus show efficient chondrogenesis of MSCs in aggregate culture (9,10). This aggregate culture system of MSCs may be the most useful system for evaluating multiple chondrogenic genes because of its ease of use and small scale. However, the microenvironment of this culture system contains not cartilage tissue but only MSCs and this system cannot determine whether transgene products can promote the differentiation of MSCs in cartilage defects, because the growth factors produced by transplanted MSCs may act on not only MSCs but also cartilage tissues in cartilage defects. Therefore, the development of an *in vitro* evaluation model of MSCs including cartilage tissue (*ex vivo* model) is necessary to evaluate the effects of growth factors and transfected MSCs on cartilage regeneration.

Several studies of *ex vivo* cartilage explant models for the evaluation of chondrocytes or MSCs have been reported (8,11–13). For example, IGF-1 gene transfected chondrocytes were seeded on the surface of a cartilage explant, which resulted in the formation of a new tissue layer on the surface of the cartilage explant (11). However, in this report, the size of the cartilage explant and the number of chondrocytes (more than 8.0×10^5 cells) required for one assay were too large to perform multiple screening of growth factors or their genes.

Therefore, in this study, we establish a culture system of a cartilage explant containing multiple defects (Fig. 1) and show the usefulness of the system in the evaluation of the effects of growth factors and gene-transfected MSCs on cartilage regeneration.

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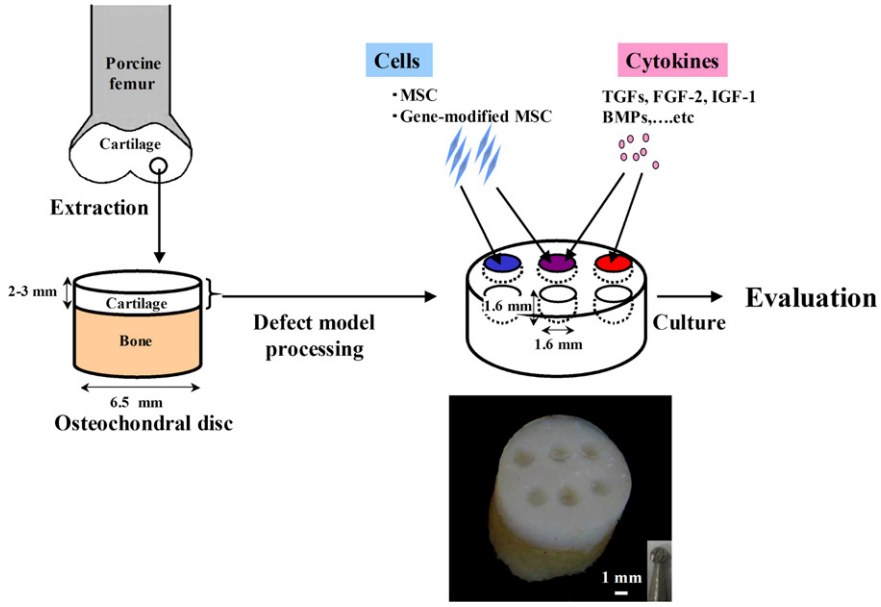

FIG. 1. Preparation of *ex vivo* cartilage defect model. An osteochondral disc is harvested from porcine femoral cartilage and multiple defects are created on the surface of the cartilage. Chondrocytes, MSCs and gene-modified MSCs are transplanted into the defects and cultivated in different media containing chondrogenic inductive cytokines. After *ex vivo* cultivation, the tissues are evaluated.

MATERIALS AND METHODS

Isolation of human MSCs and porcine chondrocytes Human MSCs were isolated from a bone marrow aspirate obtained by routine iliac crest aspiration from a human donor (19-year-old male), as previously reported (14). All the subjects enrolled in this study were approved by our institutional committee on human research, as required by the study protocol. Briefly, bone marrow cells were plated on a dish (55 cm^2; Corning, Tokyo) at a concentration of 6.0×10^5 nucleated cells/cm^2 using DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS (Invitrogen), 2500 U/l penicillin + 2.5 mg/l streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and cultured at 37°C in a humidified atmosphere containing 5% CO_2. After two weeks, adherent cells were detached using trypsin-EDTA (Sigma), and subcultivated at a cell density of 0.15×10^4 cells/cm^2. When the culture reached near confluence, the cells were harvested and subcultivated again at an initial cell density of 0.15×10^4 cells/cm^2.

Articular cartilage was harvested aseptically from femoropatellar grooves of pig knee joints purchased from a butcher (15) and peripheral blood was collected from muscle tissue covering the pig knee joints. Chondrocytes were isolated after the digestion of cartilage using type II collagenase and used for a single experiment.

Transfection MSCs (6.0×10^5 cells) were transfected with 1.0 pmol (4.0–5.0 μg) of the pcDNA3.1 control vector (Invitrogen) as a mock gene and the pCMVE-Col1A2p/hTGF-β3 vector encoding the human TGF-β3 gene and CMVE-Col1A2 promoter as in our previous study (16). Briefly, MSCs (6.0×10^6 cells/ml) in 100 μl of human MSC Nucleofector® solution (Lonza, Köln, Germany) were mixed with vectors (4.0–5.0 μg) in an amaxa electroporation cuvette (Lonza). Immediately after electroporation using the NucleofectorII® (Lonza) in accordance with the manufacturer's protocol (U-23 program), MSCs were inoculated onto a 6-well plate (Corning) at a cell density of 6.5×10^4 cells/cm^2 using DMEM supplemented with 20% FCS. After adhesion of transfected MSCs in a 6-well plate for 24 h, the MSCs harvested by trypsinization were transplanted into cartilage defect. MSCs used in this study showed approximately 60% of transfection efficiency (% of transgene (GFP)-expressing population) (data not shown).

Cartilage defect model and transplantation Osteochondral discs (diameter: 6.5 mm; height: 7 mm) were harvested from medial condyles of the porcine femur using a mosaicplasty trephine (tubular chisel, 8.5 mm; Smith Nephew Inc., MA, USA). Chondral discs were obtained by removing the subchondral bone from osteochondral discs using a scalpel. Hemispherical partial defects (radius: 0.8 mm, depth: 1.6 mm) were generated on the surface of the osteochondral and chondral discs using a manual grinder (Pentype tool PT-α; 13,000 rpm, Toyo Associates, Tokyo, Japan) and a spherical reamer (radius: 0.8 mm) with six cutting edges.

Transplantation of cells into defect model The osteochondral or chondral discs were placed in a well (24-well plate) with the growth medium (500 μl or 200 μl) (DMEM supplemented with 10% FCS, 2500 U/l penicillin, 2.5 mg/l streptomycin, and 50 μg/ml ascorbate 2-phosphate (Wako Pure Chemicals, Osaka, Japan)), and the upper surface of the discs containing defects was not soaked in the medium. A suspension (1 μl) of porcine chondrocytes and human MSCs in PBS (2.0×10^8 cells/ml) was poured into the defects, and the discs were incubated for 6 h at 37°C in a humidified atmosphere containing 5% CO_2. Then, the medium was replaced with 1.5 ml of different media including the growth medium (GM), chondrogenic differentiation medium (DM: DMEM containing 2500 U/l penicillin, 2.5 mg/l streptomycin, 100 μg/ml pyruvate (Wako), 50 μg/ml ascorbate 2-phosphate, 40 μg/ml proline (Wako), 39 ng/ml dexamethasone (ICN Biomedicals, Irvine, CA, USA), 100 ng/ml human insulin-like growth factor-1 (IGF-1; Peprotech, Rocky Hill, NJ, USA), 1% ITS™-Premix (BD Biosciences, MA, USA), and human TGF-β3 (10 ng/ml, Peprotech)). The discs were then cultivated for 3 weeks with medium changes every 3 days.

Histological and immunohistochemical analyses The subchondral bone in the osteochondral disc was removed using a scalpel. The discs were fixed in 10% buffered formalin, dehydrated in graded alcohols, embedded in paraffin, and cut into 4 μm-thick sections. The sections were deparaffinized, rehydrated, and stained by routine techniques with hematoxylin and eosin (H E; Wako), alcian blue (Wako) or propidium iodide (PI; Beckman Coulter, CA, USA) for histological analysis.

The fragmentation of DNA in the deparaffinized sections was examined using a TdT-mediated dUTP nick end-labeling (TUNEL) assay kit (Promega, Madison, WI, USA), in which the 3′ OH ends of DNA fragments were labeled with a fluorescein isothiocyanate-conjugated probe.

The deparaffinized sections were incubated with 4 mg/ml hyaluronidase (Wako) and 250 mU/ml chondroitinase ABC (Seikagaku Corp., Tokyo) in PBS at 37°C for 30 min, and blocked with 1% BSA in PBS at room temperature for 30 min. Then, the sections were incubated with rabbit anti-human collagen type I or II polyclonal antibodies (Millipore, Billerica, MA, USA) at 37°C for 2 h. The sections were washed three times with PBS and incubated with goat anti-rabbit IgG fluorescein conjugate (Millipore) at 37°C for 1 h and analyzed by fluorescence microscopy. For negative controls, the sections were incubated with a nonimmune serum instead of the primary antibody. Four defects for each groups were analyzed in two independent experiments.

Observation of suspended cells during the transplantation The osteochondral and chondral discs were placed in a well (24-well plate) with 1.5 ml of GM and incubated for 4 days at 37°C, during which the conditioned medium (50 μl) was harvested every day. The cells in the medium were counted using a hemocytometer and stained by routine techniques with gimza solution (Sigma) to identify blood cells. The suspended cells were collected from the conditioned medium by centrifugation (2000 rpm for 5 min) and used for coculture.

Determination of human TGF-β3 concentration The concentration of human TGF-β3 in culture supernatant was determined by ELISA (Quantikine Immunoassay kits, R&D Systems, Minneapolis, MN, USA).

RESULTS

Effects of transplantation of porcine chondrocytes and human MSCs in the cartilage defect on cartilage tissue We harvested eight osteochondral discs from a condyle of one porcine femur using a mosaicplasty trephine and successfully created six partial defects on