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Xeno-free and shrinkage-free preparation of scaffold-free cartilage-like disc-shaped cell sheet using human bone marrow mesenchymal stem cells

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Aiming for the clinical application of cartilage regeneration, the xeno-free cultivation method to obtain a scaffold-free cartilage-like disc-shaped cell sheet using mesenchymal stem cells (MSCs) derived from human bone marrow without the shrinkage of the sheet was investigated. MSCs were inoculated into Cell Culture Insert (0.3 cm^2, pore size; 0.4 μm, pore density; 1.0 × $10^8/cm^2$) using serum-free chondrogenic differentiation medium containing TGF-β3, IGF-1 and dexamethasone or other modified media, and cultured at 37°C in 5% CO_2 for 3 weeks. Sheet thickness, cartilage specific genes expression, ECM accumulation were determined, and the sections of sheets were stained with alcian blue. A novel mixed medium consisting of a growth medium (10% FCS) with a serum-free chondrogenic differentiation medium could prevent the shrinkage of the sheet and produced a disc-shaped cell sheet. The depth of the sheet was approximately 0.7 mm and the gene expression levels were higher than those in cells in normal human cartilage. The use of human serum instead of FCS did not cause shrinkage and did not decrease the accumulation levels of sGAG and type 2 collagen in the sheet. The cultivation of MSCs grown with completely xeno-free materials using the mixed medium containing human serum in a cell culture insert showed a sheet depth of 1.0 mm and gene expression levels higher than those in normal cartilage. The scaffold-free and xeno-free cartilage-like cell sheet was successfully formed without shrinkage using human bone marrow MSCs and the chondrogenic differentiation medium containing human serum.

[**Key words:** Cartilage regeneration; Mesenchymal stem cells; Chondrogenic differentiation; Scaffold-free; Xeno-free culture]

Mesenchymal stem cells (MSCs) are an attractive candidate for cell-based cartilage repair because they can be harvested by a minimally invasive procedure and can differentiate into osteocytes, chondrocytes, and adipocytes. An artificial three-dimensional (3D) cell scaffold is usually utilized to form the 3D tissue of cartilage for implantation. A scaffold generally consists of synthetic polymers such as poly (D, L-lactide-co-glycolide) (1–3) and biological materials such as collagen and alginate (4–8). However, there are still several issues associated with the long-term safety of these materials. Synthetic polymers may have potential problems regarding their retention and degradation *in situ* (9). Biological materials potentially carry the risk of transmission of infectious agents and precipitation of immunological reactions (10). Taken together, to avoid these risks as well as unknown risks (11), such materials should ideally be excluded throughout the treatment procedure, and in this regard, an alternative scaffold-free 3D tissue system is required. There is a standard pellet cultivation method to obtain 3D chondrocyte tissues without using scaffolds, in which chondrocytes that aggregated after centrifugation are cultivated in a centrifuge tube (12). However, the pellets are globular, although the sheets of cartilage with a larger diameter are appropriate for transplantation for various sizes of cartilage defects.

Several methods of scaffold-free cultivation of MSCs to form a cartilage-like cell sheet have been developed so far. The cultivation of MSCs to prepare a cell sheet using a culture vessel, in which space is separated by a porous track-etched membrane (porosity 12%, poly-carbonate), was reported (13). The cell sheet on the membrane contains large amounts of the cartilage-like extracellular matrix (ECM) compared with that obtained by standard pellet culture, which might have resulted from an improved mass transfer across the membrane (13,14). However, the cell sheet often shrunk and could not maintain a constant size during the culture on the membrane (15), even after the optimization of membrane porosity (16). The shrinkage of the cell sheet should be avoided completely to obtain a reproducible cartilage therapeutic effect.

Previously, fetal calf serum (FCS) and other animal-derived materials such as trypsin were used for cell harvesting and cell growth in general. However, it is important in cultivation of cells for clinical transplantation to completely remove animal-derived materials, because these materials may be contaminated by agents causing diseases such as BSE. We have reported that bone marrow MSCs could grow in a xeno-free culture system with the combination of a xeno-free medium (MesenCult-XF medium, Stem Cell Technologies Co., Palo Alto, CA, USA), a recombinant protease (TrypLE Select, Invitrogen Co., Carlsbad, CA, USA), and a serum-free cryoprotectant (TC protector, DS Pharma Biomedical, Osaka, Japan) (17).

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TABLE 1. Sequences used in PCR.

Gene		Sequence
Aggrecan	F Primer	5′-AGTCCTCAAGCCTCCTGTACTCA-3′
	R Primer	5′-GCAGTTGATTCTGATTCACGTTTC-3′
	Probe	5′-ATGCTTCCATCCCAGCTTCTCCGG-3′
Col2A1	F Primer	5′-CGCTGTCCTTCGGTGTCAG-3′
	R Primer	5′-CCTTGATGTCTCCAGGTTCTCC-3′
	Probe	5′-CCAGGATGTCCGGCAACCAGGA-3′

The objective of this study was to develop an MSC culture system for the preparation of xeno-free and scaffold-free cartilage-like disk-shaped cell sheets without shrinkage.

MATERIALS AND METHODS

Cell isolation and cultivation of MSCs MSCs were isolated from bone marrow aspirates obtained by routine iliac crest aspiration from human donors (donor A: 19-year-old male, donor B: 19-year-old male). Both the donors gave their informed consent in this study, which was approved by our institutional committee on human research, as required by the study protocol. Briefly, bone marrow cells were seeded at a concentration of 6.0×10^5 nucleated cells (NCs)/cm^2 on a dish (55 cm^2; Corning, NY, USA) with the MSC growth medium (GM) containing Dulbecco's modified Eagle's medium-low glucose (DMEM-LG; Invitrogen) supplemented with 10% FCS (Invitrogen), 2500 U/l penicillin, and 2.5 mg/l streptomycin (Sigma–Aldrich, St. Louis, MO, USA). The cells were cultivated at 37°C in a humidified atmosphere containing 5% CO_2, during which the medium was changed with fresh medium on days 1, 2, and 9. When a heterogenous cell density was reached, the cells were detached using trypsin-EDTA (Sigma) and inoculated to the original dishes. When the culture reached near confluence, the cells were collected and further subcultured.

In the xeno-free system, the MesenCult-XF Attachment Substrate (Stem Cell Technologies) was diluted 28 times with PBS and the dish was coated with 1.75 ml of the diluted solution overnight before inoculation. MesenCult-XF (Stem Cell Technologies) containing the MesenCult-XF Supplement (Stem Cell) and L-glutamine (Stem Cell Technologies) was used as the growth medium. The bone marrow aspirate (donor C: 17-year-old male) was diluted with the growth medium, plated on the coated dish to a concentration of 6.0×10^5 NCs/cm^2 and cultured at 37°C in a humidified atmosphere containing 5% CO_2 for 13 days, during which time one-half of the medium was changed with a fresh one on day 10. On day 13, the cells were detached using TrypLESelect (Invitrogen) following the manufacturer's instructions, counted by the trypan-blue dye-exclusion method, suspended with the TC protector (DS Pharma Biomedical), and stocked in liquid nitrogen. The stocked cells were inoculated onto the coated dish (55 cm^2, Corning) at a cell density of 0.15×10^4 cells/cm^2 and 1/2 of the medium was changed with a fresh medium every 4–5 days thereafter. The cells were detached with TrypLESelect and subcultivated further when the culture reached near confluence.

Chondrogenic differentiation A chondrogenic differentiation medium (CD) consisting of DMEM-LG containing 3.5 g/l glucose (Wako, Osaka, Japan), 2500 U/l penicillin, 2.5 mg/l streptomycin (Sigma), 50 μg/ml ascorbic acid phosphate magnesium salt (Wako), 100 μg/ml pyruvic acid sodium salt (MP Biomedicals, Solon, OH, USA), 40 μg/ml proline (MP Biomedicals), 100 nM dexamethasone (MP Biomedicals), 1% ITS+ premix (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml bovine serum albumin, and 5.35 μg/ml linoleic acid; BD Biosciences, Franklin Lakes, NJ, USA), 10 ng/ml TGF-β3 (R&D Systems, Minneapolis, MN, USA), and 100 ng/ml IGF-1 (PeproTech, Rocky Hill, NJ, USA) was employed for the pellet and disc-shaped cell sheet cultures. For the disc-shaped cell sheet culture, cell suspensions (0.3 ml, 18.6×10^5 cells/well, passage 4) in CD, GM (DMEM-LG + 10% FCS), and mixed medium (MM, mixture of the same amounts of CD and GM) were added to empty wells of cell culture inserts (CCIs) (0.3 cm^2; thickness, 20–25 μm; polyethylene terephthalate; BD Biosciences) in a 24-well plate containing 1.0 ml of medium. In some case, the mixed medium contained human serum (HS, Off the Clot Type AB, PAA Laboratories, Dartmouth MA, USA) instead of FCS. All cells were incubated at 37°C in 5% CO_2 for 21 days. The medium was replaced with a fresh medium every 2 days.

Measurement of sheet size The sheet was removed from the well using a spatula. The pictures of the sheet, together with a scale bar, were taken using a digital camera (Canon). The diameter and thickness of the sheet were measured from the pictures and sheet volume was calculated.

Gene expression analysis The disc-shaped cell sheets were dissociated at 37°C for 40 min using 5 g/l collagenase (Wako), 5 g/l collagenase type II (Worthington Biochemical, Lakewood, NJ, USA), and 5 g/l trypsin (Sigma). Cell number was determined by the trypan-blue method after the dissociation. Total RNA was extracted from the cells using a GenElute Mammalian Total RNA Miniprep Kit (Sigma). DNase-treated RNA was used to produce the cDNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA) and a thermal cycler (Applied Biosystems 2720). Quantitative reverse transcription PCR (qRT-PCR) was performed with cDNA using AmpliTaq Gold PCR Master Mix (Applied Biosystems), primers, TaqMan probe, and the ABI PRISM 7700 system (Applied Biosystems). For all samples, aggrecan and type 2 collagen mRNA expression levels were normalized by β-actin mRNA expression level. The primers and probes for

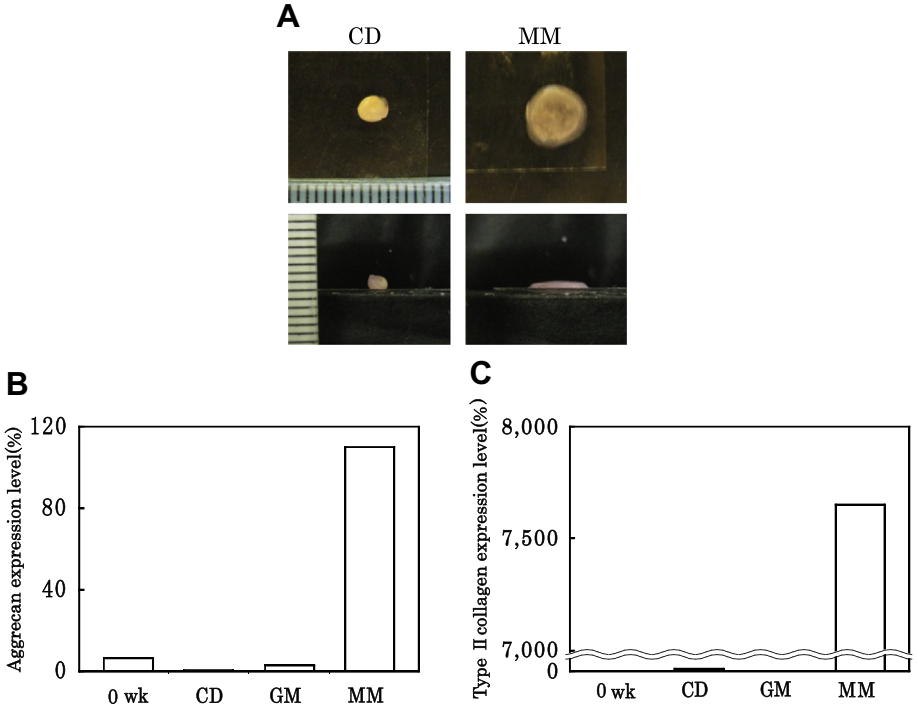

FIG. 1. Effect of medium composition on cell sheet preparation. Scaffold-free cultivations of MSCs were performed for 3 weeks using basic chondrogenic differentiation medium (CD), growth medium (GM), and mixed medium (MM). Top and side views of constructs with a scale of 1 mm (A). The relative gene expression levels of aggrecan (B) and type 2 collagen (C) of cells in the sheets were shown compared with those in chondrocytes in human articular cartilage.