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Chondrogenic Differentiation of Human Mesenchymal Stem Cells from Umbilical Cord Blood in Chemically Synthesized Thermoreversible Polymer

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Abstract

Scaffolds provide a template for cell distribution, growth, differentiation and extracellular matrix accumulation in a three-dimensional environment. Recent studies have demonstrated the potential of scaffolds for enhancing articular cartilage repair both *in vitro* and *in vivo* investigations. Mesenchymal stem cells derived from human umbilical cord blood (CBMSCs) have been characterized by their multipotency to differentiate into mesenchyme-lineage cell types, including chondrocytes, osteoblasts, and adipocytes. In this study, chondrogenesis of CBMSCs was performed in a chemically synthesized thermoreversible gelation polymer (TGP). CBMSCs were embedded in the TGP and supplemented with ascorbic acid and transforming growth factor-β3. After a 4-week induction, the results showed that CBMSCs formed into spheroid pellets and increased in size. The induced cells in the TGP expressed specific mRNA of collagen type II, aggrecan, and Sox9 for chondrocytes. Furthermore, CBMSCs embedded in TGP had higher ratio of glycosaminoglycan secretion to DNA content than the traditional induction method by aggregating pellet culture. These results demonstrated that chemically synthesized TGP provided a competent 3-dimentional culture environment for CBMSCs to differentiate into chondrocytes and may be applied clinically to induce chondrogenic differentiation of CBMSCs for cartilage repair in the future.

Key Words: chondrocyte, mesenchymal stem cell, thermoreversible gelation polymer, 3-dimentional culture

Introduction

Mesenchymal stem cells (MSCs) derived from human umbilical cord blood (CBMSCs) have been characterized by their capacity for self-renewal and multipotency for differentiation into mesenchyme-lineage cell types, such as chondrogenic, osteogenic, or adipogenic lineages depending on the culture conditions (10, 20). When MSCs were induced with ascorbic acid, transforming growth factor-β (TGF-β) family and other supplements, they could differentiate into chondrocytes (1, 23, 31). Therefore, CBMSCs and their induced chondrocytes have been regarded as practicable cell sources in cartilage tissue engineering to repair the defected sites.

Scaffolds provided a three-dimensional (3-D) template for cell distribution, growth, differentiation and extracellular matrix accumulation. Recent studies have demonstrated the potential of scaffolds for enhancing articular cartilage repair, based on *in vitro*

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and *in vivo* investigations. There were some materials for 3-D chondrogenesis of MSCs such as fibrin (15), alginate (4), collagen gel (14, 17, 32), Matrigel (18) and polymer of polyglycolic and polylactic acid (PLGA) (29). However, the uses of allogeneic or xenogeneic materials are associated with the risks of transmission of certain diseases such as HIV, hepatitis B and C viruses, and microbial infections, even xenografic contamination and immunologic rejection (14, 17, 18, 29, 32), and are not appropriate for clinical use. The uses of chemically synthesized scaffolds that are designed with the characteristics of biocompatibility, biodegradability, and the capability to form desired shapes and mechanical strength may solve the above shortages, and are emerging issues for tissue engineering (7).

Thermoreversible gelation polymer (TGP) is a chemically synthesized polymer that is composed of thermoresponsive poly (N-isopropylacrylamide-co-n-butyl methacrylate) and hydrophilic polyethylene glycol (poly(NIPAAm-co-BMA)/PEG), and has thermoreversible crosslinks between intermolecular poly(NIPAAm-co-BMA) blocks due to hydrophobic interaction (11, 21, 33). Cells can be seeded in the liquid form of TGP at the temperature lower than 20°C (this temperature is named as lower critical solution temperature), and cultured in the 3-D form at temperature higher than 20°C (27).

TGP has been applied in the culture of some cell lines (21) and osteogenesis of bone marrow MSCs (11). In this study, chondrogenesis of CBMSCs was performed in a synthesized TGP to show the capability of this novel scaffold to support the chondrogenesis of CBMSCs. CBMSCs were seeded in the TGP and supplemented with ascorbic acid and TGF-β3. To characterize the induced chondrocytes in TGP, we analyzed the specific mRNA expression of collagen type II, aggrecan, and Sox9 for chondrocytes, and compared the ratio of glycosaminoglycan (GAG) secretion to DNA content with aggregate culture of CBMSC pellet. The results demonstrated that chemically synthesized TGP provided an excellent 3-D culture environment for CBMSCs to differentiate into chondrocytes and would be beneficial to future applications in patients requiring chondrocyte transplantation.

Materials and Methods

CBMSC Establishment

CBMSCs were isolated from human umbilical cord blood after informed consent as previously described (5). Briefly, umbilical cord blood (UCB) was harvested in a standard 250-ml blood bag (Terumo, Shibuya-ku, Tokyo, Japan) and processed within 24 h. Buffy-coat cells were obtained from UCB by centrifugation. Mononuclear cells (MNCs) were collected after the buffy-coat cells were layered onto Ficoll-Paque solution (1.077 g/ml; Amersham Biosciences, Uppsala, Sweden) and centrifuged to deplete red blood cells, platelets, and plasma. MNCs (10^6 cells/cm^2) were seeded in a tissue culture flask with MSC proliferation medium that was composed of alpha-modified minimum essential medium (α-MEM, Hyclone, Logan, UT, USA) containing 20% fetal bovine serum (FBS, Hyclone), 4 ng/ml basic fibroblast growth factor (PeproTech EC Ltd, London, UK), 100 units/ml penicillin (Sigma, St. Louis, MO, USA), and 100 μg/ml streptomycin (Sigma), and cultured at 37°C in a humidified atmosphere with 5% CO_2. The nonadherent cells were removed by medium change, and the medium was changed twice a week. When well-developed colonies of fibroblast-like cells appeared, cells were characterized by immunophenotyping and differentiation potential for MSCs as previously described (5, 6), and the plastic-adherent cells with multipotent differentiation capacity *in vitro* were defined as CBMSCs for the following experiments (13).

In Vitro Chondrogenesis

Chemically synthesized TGP (Mebiol gel, Mebiol Inc., Tokyo, Japan) was liquefied by adding Dulbecco's modified Eagle's medium (DMEM, Hyclone) to lyophilized TGP in the 25T-flask, and incubated at 4°C. For chondrogenic differentiation, trypsinized CBMSCs were mixed with liquefied TGP at a cell density of 1×10^7 cells/ml and seeded 200 μl/well in the 24-well culture plate (pre-warmed in the 37°C) with chondrogenic medium. Chondrogenic medium was composed of DMEM supplemented with 0.1 mM ascorbic acid (Sigma), 10 ng/ml TGF-β3 (PeproTech), 1 mM sodium pyruvate (Sigma), 0.1 μM dexamethasone (Sigma) and 1 % ITS+ Premix (Becton Dickinson, San Jose, CA, USA) (20). For aggregate culture as a comparative study, 2×10^6 CBMSCs were centrifuged in a 15 ml polypropylene tube at 500 × g for 5 min, and resuspended in chondrogenic medium. Subsequently, the cells were recentrifuged to perform aggregated cell pellets (1). Both methods for chondrocyte induction were incubated at 37°C, 5% CO_2 for 4 weeks, and changed fresh chondrogenic medium twice per week. To harvest cells in the TGP, culture plate was kept at 4°C for 4 h to liquefy the TGP. Then the liquefied TGP was diluted with Dulbecco's phosphate buffered saline (D-PBS, Sigma), and centrifuged at 500 × g for 5 minutes. Supernatant was removed, and cell pellets were characterized by the following analyses.

DNA Assay and GAG Quantification

Cell pellets were treated with digestion buffer for 24 h at 60°C. The digestion buffer (pH 7.5) was

composed of D-PBS containing 125 μg/ml papain (Sigma), 5 mM L-cysteine (Sigma), 100 mM Na_2HPO_4 (Sigma), and 5 mM EDTA (Sigma). The aliquots of digested samples were subjected to acid hydrolysis (6 N HCl for 12 h at 100°C), and were immediately submitted to the Hoechst fluorescent DNA content assay and GAG secretion assay.

The DNA content was analyzed with Hoechst 33258 fluorometric detection method (12). A standard curve was generated using 1.25 to 25 μg of double-stranded calf thymus DNA (Sigma), dissolved and diluted in PBE buffer (pH 7.5) that was composed of D-PBS containing 100 mM Na_2HPO_4 and 5 mM EDTA. To determine the DNA content, 50 μl papain-digested samples in a 96-well plate were mixed with 200 μl TN buffer (pH 7.5) that was composed of D-PBS containing 5 μg/ml Hoechst 33258 dye (Sigma), 50 mM Tris (Sigma) and 150 mM NaCl (Sigma), and read by a Molecular Device Gemini II fluorescence plate reader at 360 nm excitation and 460 nm emission, with a 420 nm cutoff filter.

GAG secretion was detected by the colorimetric method (12). Fifty μl papain-digested samples in the 96-well plate were mixed with 200 μl dimethyl methylene blue (DMMB) solution (pH 3.0) that was composed of D-PBS containing 46 mM DMMB (Sigma), 40 mM NaCl, and 40 mM glycine (Sigma). Absorbance at 525 nm was read using an enzyme-linked immunosorbent assay (ELISA) plate reader (MRX Microplate Reader, DYNEX Technologies, Chantilly, VA, USA). A standard curve was generated using 0.125 to 1 mg of shark cartilage chondroitin sulfate C (Calbiochem, Lo Jolla, CA, USA) dissolved in PBE buffer.

Detection of mRNA Expression by RT-PCR

Cell aggregates were digested with 3 mg/ml collagenase (Invitrogen, Carlsbad, CA, USA), 1 mg/ml hyaluronidase (Sigma) and 0.25% trypsin (Sigma) for 3 h at 37°C (25). Total RNA was extracted by using TRIzol reagent (Invitrogen). RNA was converted to cDNA by the ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) following the manufacturer's instruction. Thirty cycles were used for cDNA amplification, and each cycle was consisted of 30 sec for denaturation at 94°C, 45 sec for annealing at 58°C, and 45 sec for polymerization at 68°C, and then 10 min for extension at 72°C in the final step. The reaction products were resolved by electrophoresis on a 1.5% agarose gel (Sigma) and visualized with ethidium bromide (Sigma). The gene-specific primer sets were following: Collagen type II (forward: TTCAGCTATGGAGATGACAATC, reverse: AGAGTCCTAGAGTGACTGAG), Aggrecan (forward: GCCTTGAGCAGTTCACCTTC, reverse: CTCTTCTACGGGGACAGCAG), Sox9 (forward: GAGCAGACGCACATCTC, reverse: CCTGGGATTGCCCCGA), and β-actin (forward: TGGCACCACACCTTCTACAATGAGC, reverse: CGCACTGTAATTCCTCTTCGACACG).

Statistical Analysis

The data analysis was performed with the Student's *t* test, and a value of $P < 0.05$ was considered significant.

Results

Morphology of In Vitro Chondrogenesis of CBMSCs in the TGP

CBMSCs were seeded into TGP in liquid form at 4°C in the 24-well culture plate, and induced with chondrogenesis medium. When TGP was incubated at 37°C, it formed an elastic hydrogel immediately because of the thermoreversible association of the copolymer chains between intermolecular poly(NIPAAm-co-BMA) blocks (Fig. 1., A-B). Chondrogenesis of CBMSCs was performed in the 3-D environment at 37°C. After a 4-week induction, CBMSCs were supported by TGP to form cell aggregates in the chondrogenic medium. The cell aggregates increased in size gradually with time increasing, and the surface of aggregates was smooth after a 4-week induction (Fig. 1., C-F). However, CBMSCs encapsulated in the TGP with MSC proliferation medium did not form any aggregates, and there were no significant differences in morphology and cell number between throughout 4-week culture (Fig. 1., G-H).

mRNA Expression of Induced Chondrocytes from CBMSCs in the TGP

Before the chondrogenic induction, CBMSCs did not expressed specific mRNA of collagen type II, aggrecan, and Sox9 for chondrocytes. When CBMSCs encapsulated in TGP and induced in chondrogenic medium, specific mRNA of collagen type II, aggrecan and Sox9 for chondrocytes began to exhibit (Fig. 2). Induced cells had the highest mRNA expressions of collagen type II and aggrecan at week 3 and week 2, respectively, and had the same level of Sox9 expression throughout the 4-week induction. As a control, CBMSCs encapsulated in the TGP with MSC proliferation medium did not have these three mRNA expressions for chondrogenesis.

GAG Secretion of Induced Chondrocytes from CBMSCs in the TGP

GAG secretion level was an important

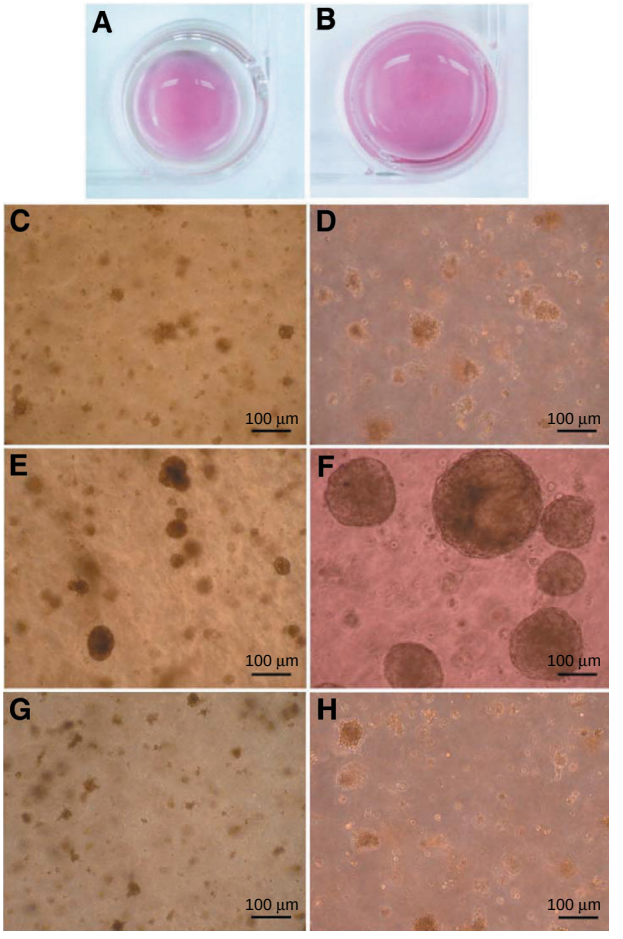

Fig. 1. Thermoreversible transformation of thermoreversible gelation polymer (TGP) and morphology of chondrogenic differentiation of cord blood mesenchymal stem cells (CBMSCs) in TGP (n = 4). TGP was in the liquid form (A, at 4°C) and 3-dimensional form (B, at 37°C) at the temperature higher and lower than lower critical solution temperature (20°C), respectively. Under microscope observation, CBMSCs formed into spheroidal pellets and increased in size in the TGP with chondrogenesis medium for a 1-week (C), a 2-week (D), a 3-week (E) and a 4-week (F) inductions. There were no significant differences in morphology and cell number between CBMSCs cultured in the Mebiol gel with MSC medium for 1-week (G) and 4-week (H) culture. Scale bar, 100 μm.

characteristic of functional chondrocytes. In this study, GAG secretion and DNA content of cultured cells were further determined. The cells induced with chondrogenic medium had similar total DNA content between the TGP culture (2.2 μg/ml at week 1 and 1.9 μg/ml at week 4) and aggregate culture of CBMSC pellets (2.2 μg/ml at week 1 and 1.8 μg/ml at week 4), but had a significantly lower level than the cells cultured with MSC proliferation medium in the TGP (3.4 μg/ml at week 1 and 3.3 μg/ml at week 4, Fig. 3A). After a 1-week chondrogenic induction, the cells had similar

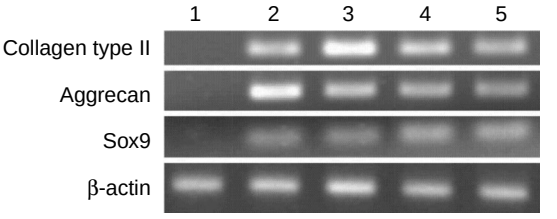

Fig. 2. RT-PCR analysis for chondrogenesis of CBMSCs (n = 3). Lane 1: CBMSCs before chondrogeneic induction. Lane 2, 3 and 4: chondrogenesis of CBMSCs in the TGP for 2, 3 and 4 weeks. Lane 5: chondrogenesis of CBMSCs in the 15 ml polypropylene tube by aggregate culture for 4 weeks.

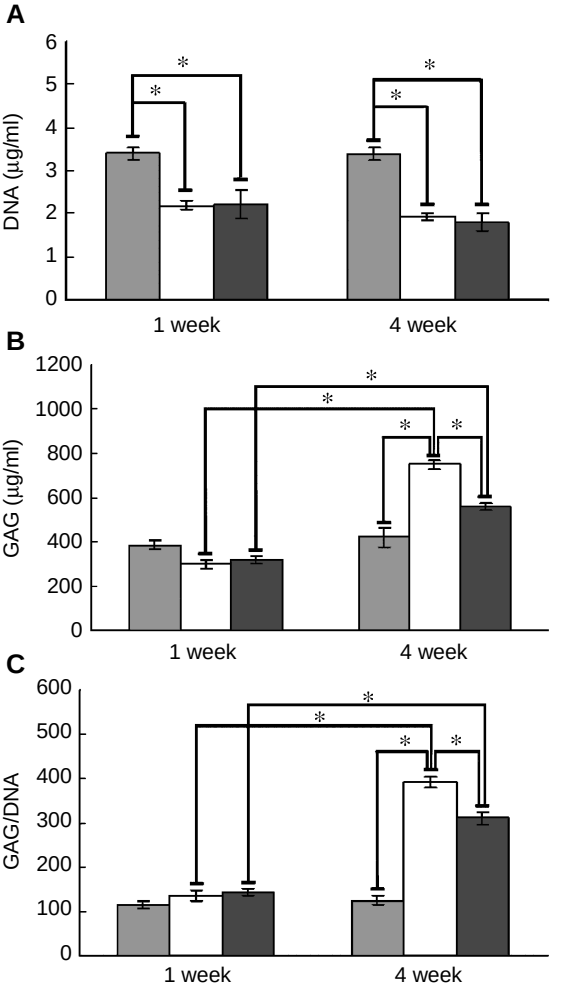

Fig. 3. The glycosaminoglycan (GAG) secretion and the DNA content of cord blood mesenchymal stem cells (CBMSCs) cultured with MSC proliferation medium (gray bar), and induced with chondrogenesis medium in the thermoreversible gelation polymer (TGP, white bar) and in the 15 ml polypropylene tube by aggregate culture of CBMSC pellets (black bar). (A) Total DNA content. (B) Total GAG secretion. (C) Total GAG secretion normalized to DNA content. *Represented a value of $P < 0.05$ analyzed by running Student's t test (n = 4).

level of total GAG secretion (300 μg/ml in the TGP and 320 μg/ml in aggregate culture) and GAG/DNA ratio (137 μg GAG/μg DNA in the TGP and 145 μg GAG/μg DNA in aggregate culture) to the cells cultured with MSC proliferation medium (388 μg/ml of total GAG secretion and 115 μg/μg of GAG/DNA ratio). However, the cells induced with chondrogenic medium in the TGP at week 4 had increased significantly in total GAG secretion (750 μg/ml) and GAG/DNA ratio (390 μg GAG/μg DNA), as compared with the aggregate culture (562 μg/ml of total GAG secretion and 310 μg/μg of GAG/DNA ratio), and the cells cultured with MSC proliferation medium (421 μg/ml of total GAG secretion and 124 μg/μg of GAG/DNA ratio) (Fig. 3B-C).

Discussion

Human UCB, collected from the postpartum placenta and cord, has been identified as a good source of mesenchymal stem cells (MSCs), and has the similar differentiation potential to MSCs from bone marrow (20). MSCs are defined by their capacities to be plastic adherent, surface markers of mesenchymal lineage and differentiation potentials, including chondrocytes, osteoblasts, and adipocytes (9). We have reported that CBMSCs have a superior ability in osteogenesis but lower adipogenesis than bone marrow MSCs (5). Therefore, CBMSCs and their induced lineage tissues have been regarded as practical cell sources in tissue engineering to repair the defected diseases.

As stated in many publications, investigators have already shown that human MSCs could grow and be induced into chondrocytes in monolayer on the tissue culture plates as 2-D culture (6, 20, 22). However 2-D culture method could not maintain the right morphology and long-term function of chondrocytes. 3-D culture methods such as aggregate culture of cell pellets or usage of scaffolds provide a template for chondrocyte distribution, growth, differentiation and extracellular matrix accumulation, and are thought to be an important issue for cartilage repair (1, 3, 30). However, the disadvantage of aggregate culture is low nutrition and metabolite transfer efficiency when the size of cell pellet is large (16, 25). Most scaffolds in tissue engineering are constructed of fibrin, alginate, collagen, Matrigel, and the uses of allogeneic or xenogeneic biomaterials, which are not appropriate for clinical use due to the risks of disease transmission, microbial infections, xenografic contamination and immunologic rejection (14, 17, 18, 32). Hishikawa *et al*. reported that TGP, a NIPAAm-based copolymer, could support and augment the osteogenesis of human MSCs (11). Yoshioka *et al*. demonstrated that this TGP has the potential as a scaffold material to support chondrocyte to generate tissue-engineered cartilage *in vitro* (33). These studies led us to hypothesize that TGP might be a suitable material to support chondrogenesis of CBMSCs.

After a 1-week induction of CBMSCs in the TGP with chondrogenic medium, the morphology of CBMSCs was formed into spheroid, and the size of the spheroids began to grow under microscope observation (Fig. 1). However, the increasing size of cell spheroids were not due to the proliferation of CBMSCs in TGP, which was demonstrated by the DNA content being consistent in chondrogenic medium (Fig. 3A). The morphological transformation and size increase were due to the abundant GAG accumulation in extracellular matrix, and represented that CBMSCs proceeded to differentiate into mature chondrocytes. CBMSCs cultured in the TGP with MSC proliferation medium did not show morphological change comparatively.

The specific phenotypes of mature chondrocytes were further characterized by the gene expression of collagen type II, aggrecan and transcription factor Sox9 (2, 35). Collagen type II and aggrecan were the main ingredients in the cartilage extracellular matrix, and Sox9 regulated a large number of cartilage matrix genes, such as Col2a1, Col9a1m Col11a2, and aggrecan (19, 24, 34). The gene expressions of Collagen type II, aggrecan and Sox9 of chondrocytes declined rapidly by 2-D culture method (8, 26). In our study, Collagen type II and aggrecan had the strongest expressions at week 3 and week 2 induction, respectively, and Sox9 had a stable expression throughout the 4-week induction. Importantly, collagen type II, aggrecan and Sox9 of induced chondrocytes expressed continuously for 4 weeks in the TGP, and these genes of induced chondrocytes in the TGP had similar expression level to those by aggregate culture at week 4 induction (Fig. 2).

Large amount of GAG secretion to construct extracellular matrix was an important characteristic of functional chondrocytes (12). In our study, CBMSC induced with chondrogenesis medium in the TGP had lower total DNA content and higher GAG secretion than those cultured with MSC proliferation medium in the TGP. It showed that CBMSCs differentiated, but not proliferated, into functional chondrocytes after 4-week induction. Furthermore, GAG/ DNA ratio of induced chondrocytes with chondrogenic medium in the TGP significantly increased after the 4-week induction, and was also significantly higher than that of the aggregate pellet culture. These results demonstrated that chemically synthesized TGP could provide a competent 3-D environment for CBMSCs to differentiate into chondrocytes.

In summary, chemically synthetic TGP has been used as 3-D environment to culture for various cells such as cancer cells, limbal stem cells, and CHO cells (11, 21, 27, 28), and have several advantages in

pathogen-free and animal source-free over other biomaterials for clinical application. We further showed that CBMSCs could differentiate into chondrocytes in the TGP according to their characteristics of morphology, gene expression and GAG accumulation. These results demonstrated that TGP could be used to study *in vitro* chondrogenesis of MSCs, and would be beneficial to the future applications in patients requiring chondrocyte transplantation.

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