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Chondrogenic differentiation of human mesenchymal stem cells cultured in a cobweb-like biodegradable scaffold

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Abstract

Human mesenchymal stem cells (MSCs) were cultured in vitro in a cobweb-like biodegradable polymer scaffold: a poly(DL-lactic-co-glycolic acid)-collagen hybrid mesh in serum-free DMEM containing TGF-β3 for 1–10 weeks. The cells adhered to the hybrid mesh, distributed evenly, and proliferated to fill the spaces in the scaffold. The ability of the cells to express gene encoding type I collagen decreased, whereas its ability to express type II collagen and aggrecan increased. Histological examination by HE staining indicated that the cells showed fibroblast morphology at the early stage and became round after culture for 4 weeks. The cartilaginous matrices were positively stained by safranin O and toluidine blue. Immunostaining with anti-type II collagen and anti-cartilage proteoglycan showed that type II collagen and cartilage proteoglycan were detected around the cells. In addition, a homogeneous distribution of cartilaginous extracellular matrices was detected around the cells. These results suggest the chondrogenic differentiation of the mesenchymal stem cells in the hybrid mesh. The PLGA-collagen hybrid mesh enabled the aggregation of mesenchymal stem cells and provided a promotive microenvironment for the chondrogenic differentiation of the MSCs.

Keywords: Mesenchymal stem cell; Chondrogenesis; Differentiation; Gene expression; Tissue engineering

Articular cartilage is avascular and consists of sparsely embedded chondrocytes in an organic matrix primarily consisting of type II collagen and proteoglycan. It possesses limited capacity for intrinsic repair or regeneration, and even minor lesions or injuries may lead to progressive damage and joint degeneration. Although many methods including subchondral drilling, osteochondral allografting, and periosteal or perichondral tissue grafting have been tried in attempts to repair defects in articular cartilage, these techniques cannot reproduce the reparative tissue characteristics of hyaline cartilage for optimal filling of the defects [1–5]. A cell-based tissue engineering technique has proved to be one of the most promising alternative therapies for articular cartilage defects [6–10]. This approach consists of an interactive triad of responsive cells, a supportive matrix, and bioactive molecules promoting differentiation and regeneration.

Mesenchymal stem cells (MSCs) are a prospective cell source for the engineering of cartilage tissue. They are relatively easy to obtain from a small aspirate of bone marrow and have multiple potentials to differentiate into different cell lineages such as osteoblasts, chondrocytes, adipose cells, ligament cells, and neural cells [11]. In addition, they are relatively easy to expand in culture under conditions in which they retain some of their potential to differentiate into multiple cell lineages [12].

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A three-dimensional microenvironment is necessary to promote the differentiation of MSCs into chondrocytes [13–15]. Various scaffolds such as porous sponges, mesh, fibers, and hydrogels have been prepared from biodegradable synthetic and natural polymers for the tissue engineering of cartilage [16–23]. However, these scaffolds have their respective problems. We have developed a new type of hybrid scaffold by forming collagen microsponges in the openings of a porous synthetic polymer skeleton [24,25]. The hybrid scaffolds exhibit the properties of high mechanical strength, ease of handling, and easy cell seeding and uniform cell distribution. The hybrid scaffolds facilitated the formation of cartilage tissue when dedifferentiated chondrocytes were cultured in them [26,27]. In the present study, one such hybrid scaffold—a cobweb-like hybrid mesh of poly(DL-lactic-co-glycolic acid) and collagen—was used for the three-dimensional culture of MSCs to provide a microenvironment for their chondrogenesis. Human mesenchymal stem cells were cultured in the hybrid mesh. The gene expression of type I collagen, type II collagen, and aggrecan was examined by RT-PCR; the morphological changes and cartilaginous ECM depositions were evaluated by histological and immunohistological staining.

Materials and methods

Scaffold preparation. A hybrid scaffold was prepared by forming collagen microsponges in the openings of a knitted mesh of PLGA. A Vicryl knitted mesh made of polyglactin 910 (a 90:10 co-polymer of glycolic acid and lactic acid) was immersed in an aqueous solution of collagen in acetic acid (porcine, type I, pH 3.2, 0.3 wt%, Nitta Gelatin, Osaka, Japan) and frozen at −80 °C for 12 h. The mesh was then freeze-dried under a vacuum of 0.2 Torr for 24 h and further cross-linked by treatment with glutaraldehyde vapor saturated with a 25% glutaraldehyde aqueous solution by placing the mesh and the solution in a closed box at 37 °C for 4 h. Subsequently, the mesh was treated with a 0.1 M glycine aqueous solution to block any unreacted aldehyde groups and then freeze-dried after complete washing with deionized water. The microstructure of the hybrid mesh was observed by scanning electron microscopy (SEM). The PLGA-collagen hybrid mesh was 200-μm thick. The hybrid mesh was cut into 1.0-cm diameter discs, which were then sterilized with ethylene oxide in preparation for cell culture.

Cell cultures. Human mesenchymal stem cells were available from Osiris (Worthington Biochemical, Lakewood, NJ) at passage 2. The cells were seeded in T-75 culture flasks using the proliferation medium from Osiris. The proliferation medium contains 440 mL of MSC basal medium and 50 mL of mesenchymal cell growth supplement, 10 mL of 200 mM L-glutamine, and 0.5 mL penicillin/streptomycin mixture. The cells were further subcultured once after reaching confluence and used at passage 4. The cells were collected by treatment with trypsin/EDTA solution and suspended in proliferation medium at a density of 2×10^6 cells/mL. The mesh discs were placed in the wells of a 24-well plate containing 1.0 mL proliferation medium and covered with glass rings having an internal diameter of 1.0 cm to protect cell leakage during cell seeding. The MSC suspension solution (100 μL/mesh) was dropped onto the PLGA-collagen hybrid mesh discs and cultured under an atmosphere of 5% CO_2 at 37 °C. After culture for 6 h, the discs were turned upside down and another 100 μL cell suspension was seeded on the other side of the hybrid mesh. The cells were cultured with the proliferation medium for 1 week. The medium was changed twice during the week. After culture for 6 h, 2 days, and 1 week, two discs were taken out for observation by scanning electron microscopy (SEM). For the SEM observation, the cells were fixed with 0.25% glutaraldehyde solution in PBS at room temperature for 1 h. After being rinsed three times with PBS and once with deionized water, the samples were freeze-dried. The discs were then coated with gold using a sputter coater and observed with the scanning electron microscope.

After culture for 1 week in the proliferation medium, the culture medium was changed to a chondrogenic induction medium. The chondrogenic induction medium consisted of serum-free DMEM containing 4500 mg/L glucose, 584 mg/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM nonessential amino acids, 0.4 mM proline, 50 mg/L ascorbic acid, 10^{-7} M dexamethasone, and 5 ng/mL TGF-β3 (Sigma–Aldrich, St. Louis, MO, USA). The TGF-β3 was thawed and supplemented immediately before use. The cells were cultured in the chondrogenic induction medium for 10 weeks. The medium was changed every 3 days. After culture in the chondrogenic induction medium for 1, 5, and 10 weeks, cells were harvested for histological examination and gene expression analysis.

Reverse transcription-polymerase chain reaction. The MSCs cultured in the hybrid mesh in the chondrogenic induction medium for 1, 5, and 10 weeks were washed with PBS and frozen in liquid nitrogen. The frozen discs were crushed into powder by an electric crusher. The powder from each disc was dissolved in 1 mL of Isogen reagent (Nippongene, Toyama, Japan). Total RNA was extracted with chloroform and precipitated with isopropanol. After washing with 70% ethanol, the RNA pellet was dried, dissolved in nuclease-free water, and the RNA concentration was determined spectrophotometrically at 260 nm. First strand cDNA was reverse transcribed from 2 μg of total cellular RNA using a commercially available kit for reverse transcription-polymerase chain reaction (RT-PCR) (Toyobo, Japan) according to the manufacturer's recommendations. PCR amplification was carried out in a reaction volume of 20 μl containing 20 pmol each of the sense and anti-sense primers, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 1 U rTaq polymerase (Toyobo, Japan). Human type I collagen, type II collagen, aggrecan, and 18S ribosomal RNA primers were used. The PCRs were done with an initial denaturation of 1 min at 95 °C, followed by a total of 25 cycles each consisting of 1 min at 95 °C, 1 min at 60 °C, 1 min at 72 °C, and a final extension of 10 min at 72 °C. The PCR products were separated electrophoretically using a 1.5% agarose gel in Tris–acetic acid/EDTA buffer, stained with ethidium bromide, and visualized under ultraviolet light. The PCR primers were as follows:

Type I collagen: (sense): 5′-GATGGATTCCAGTTCGAGTATG-3′; (anti-sense): 5′-GTTTGGGTTGCTTGTCTGTTTC-3′.
Type II collagen: (sense): 5′-CAGAAGACCTCACGCCTC-3′; (anti-sense): 5′-TAGTTTCCTGCCTCTGCCTTGAC-3′.
Aggrecan: (sense): 5′-CAGGTGAAGACTTTGTGGACATCC-3′; (anti-sense): 5′-CCTCCTCAAAGGTCAGCGAGTAGC-3′.
18S rRNA: (sense): 5′-GCCTGAGAAACGGCTACCAC-3′; (anti-sense): 5′-ACACTCAGCTAAGAGCATCG-3′.

Histological and immunohistological staining. The harvested discs were fixed in neutral buffered formalin, embedded in paraffin, and sectioned (10-μm thick). The cross-sections were stained with hematoxylin and eosin, safranin O/fast green, and toluidine blue. The type I collagen, type II collagen, and proteoglycan were immunohistologically stained using rabbit anti-human type I collagen antibody (Sanbio b.v., Uden, Netherlands), mouse anti-human type II collagen monoclonal antibody (Neomarkers, Fremont, CA), and mouse anti-human cartilage proteoglycan monoclonal antibody (Chemicon International, Temecula, CA) and DAKO LSAB Kit, Peroxidase (Dako, Carpinteria, CA) according to the instructions accompanying the kit. Briefly, the deparaffinized sections were incubated with proteinase K enzyme in

Tris-buffered saline (1:50 working dilution, pH 7.4, TBS) at room temperature for 5 min, and blocked with peroxidase blocking solution for 10 min and 10% goat serum solution for 30 min. The sections were then incubated with anti-type I collagen (1:400 working dilution), anti-type II collagen (1:200 working dilution), and anti-proteoglycan antibodies (1:1200 working dilution) for 30 min. Incubation with biotinylated anti-rabbit or mouse immunoglobulins for 10 min was followed by incubation with horseradish peroxide conjugated streptavidin for 10 min. The sections were then incubated with 3-amino-9-ethylcarbazole as the color substrate for 10 min to visualize the bound antibodies. The nuclei were counterstained with hematoxylin. All incubations were conducted at room temperature (RT).

Results and discussion

The PLGA-collagen hybrid mesh was prepared by forming microsponges of porcine type I collagen in the openings of a knitted mesh of PLGA. SEM observation showed the presence of the cobweb-like collagen microsponges in the openings of the synthetic PLGA mesh (Fig. 1A).

Passage 4 cultured human mesenchymal stem cells were seeded in the PLGA-collagen hybrid mesh and cultured in vitro in the proliferation medium for 1 week. Adhesion of MSCs in the polymer scaffold was observed by SEM (Figs. 1C–D). The MSCs adhered on both the cobweb-like collagen microsponges and the polymer fibers, and showed uniform distribution in the hybrid mesh. The cells proliferated and regenerated cartilaginous matrices filling the voids in the mesh.

After culture in the proliferation medium for 1 week, the MSCs were cultured in a chondrogenic induction medium for another 1–10 weeks. The gene expression of type I collagen, type II collagen, and aggrecan was examined by RT-PCR after the cells were cultured in the chondrogenic induction medium for 5 and 10 weeks. The expression of the genes encoding type II collagen and aggrecan was upregulated and that of type I collagen mRNA was downregulated (see Fig. 2).

Histological examination of the cells cultured in the hybrid mesh using hematoxylin and eosin stains indicated a uniform spatial cell distribution in the mesh (Fig. 3). The MSCs remained viable and continued to secrete extracellular matrix components to form homogeneously compact cartilage tissues. The MSCs showed a fibroblast morphology after culture for 1 week and a round morphology after culture for 5 and 10 weeks. The safranin O-positive stain indicated a low level of

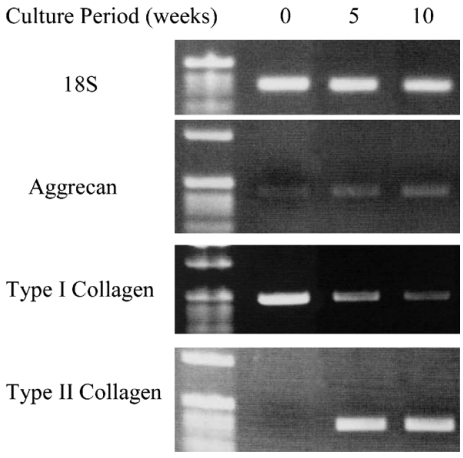

Fig. 2. RT-PCR results of gene encoding type I collagen, type II collagen, and aggrecan of human MSCs cultured in the chondrogenic induction medium in the hybrid mesh for 0, 5, and 10 weeks.

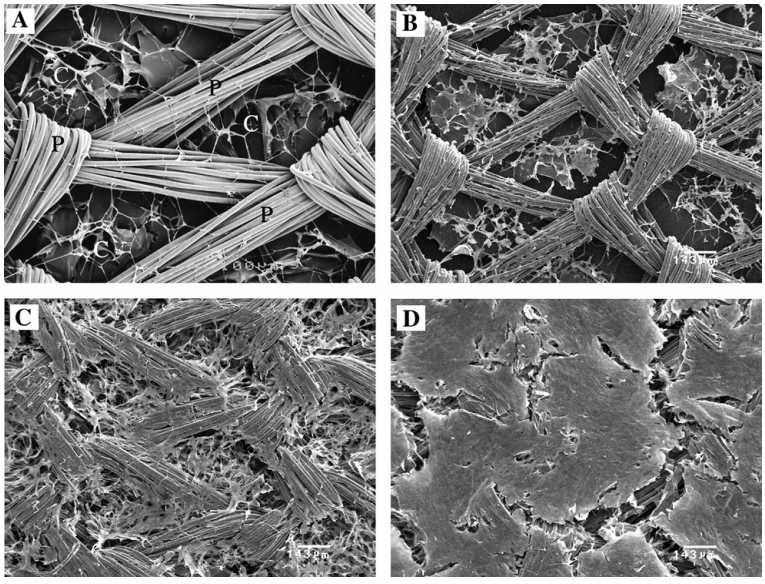

Fig. 1. SEM micrographs of PLGA-collagen hybrid mesh (A), mesenchymal stem cells cultured in the proliferation medium in the hybrid mesh for 6 h (B), 2 days (C), and 1 week (D). P indicates PLGA knitted mesh and C indicates microsponges of porcine type I collagen.

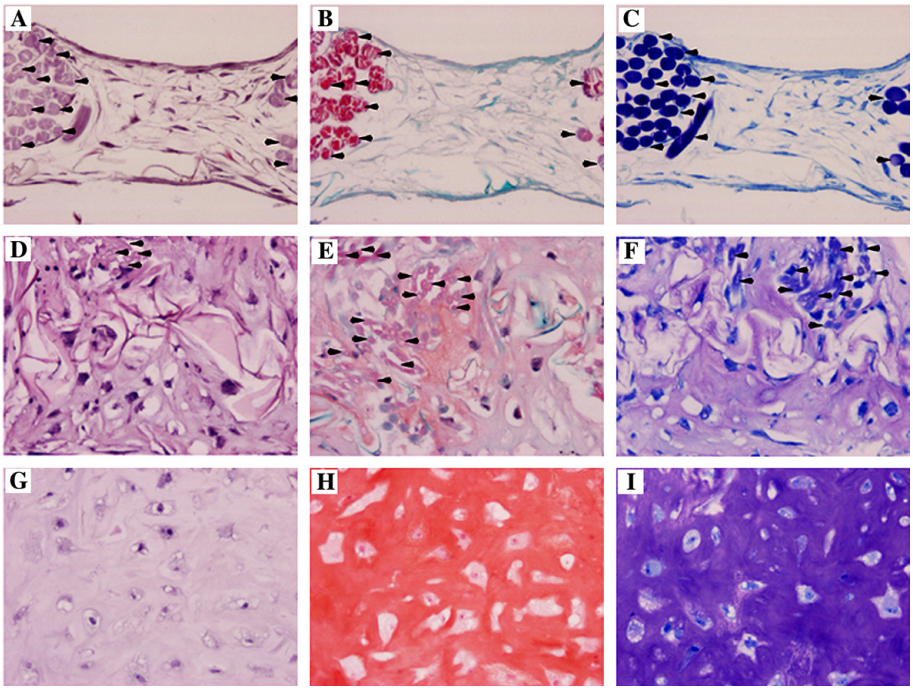

Fig. 3. Hematoxylin/eosin (A, D, and G), safranin O/fast green (B, E, and H), and toluidine blue (C, F, and I) staining of MSCs cultured in the chondrogenic induction medium in the hybrid mesh for 1 (A–C), 5 (D–F), and 10 (G–I) weeks. The round objects without nuclei are cross-sections of the remaining PLGA fibers (arrows). The magnification for (A–C) is 100 and for (D–I) is 200.

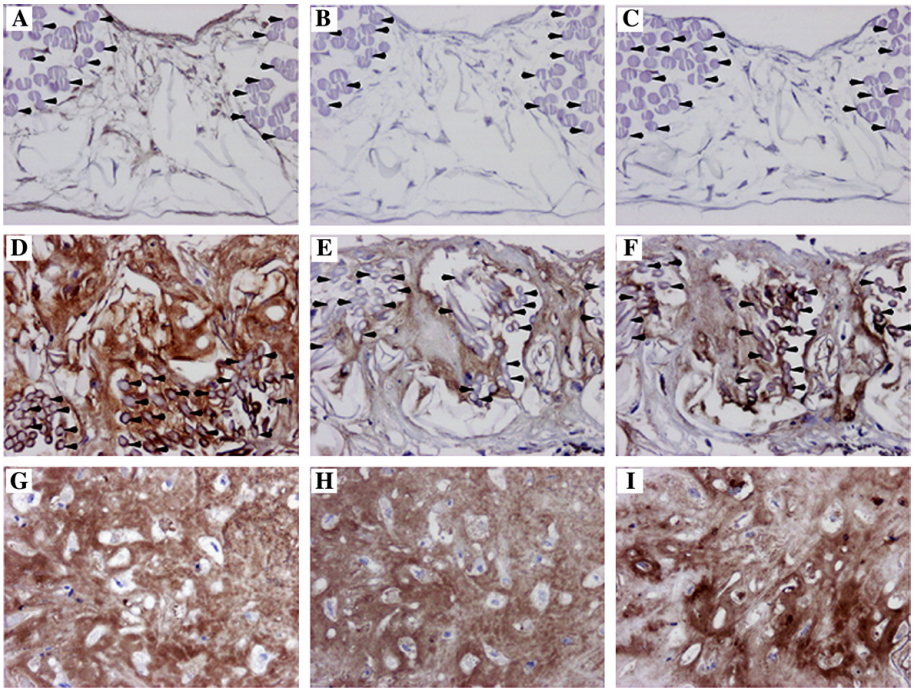

Fig. 4. Immunohistological staining of type I collagen (A, D, and G), type II collagen (B, E, and H), and proteoglycan (E, F, and I) of MSCs cultured in the chondrogenic induction medium in the hybrid mesh for 1 (A–C), 5 (D–F), and 10 (G–I) weeks. The round objects without nuclei are cross-sections of the remaining PLGA fibers (arrows). The magnification is 200.

glycosaminoglycans (GAG) during the first week, which were abundant and homogeneously distributed around the cells after 5 weeks. The matrices were further brightly stained with safranin O after 10 weeks culture. Toluidine blue staining demonstrated the typical metachromasia of cartilage.

Immunohistological staining with antibodies to type I collagen, type II collagen, and cartilage proteoglycan showed that the matrices were only stained for type I collagen and that the type II collagen and proteoglycan were not stained after culture for 1 week in the chondrogenic induction medium. After culture for 5 weeks, the type I collagen was brightly stained and the type II collagen and proteoglycan were weakly stained. After culture for 10 weeks, the staining levels of the type II collagen and proteoglycan increased and that of the type I collagen decreased (see Fig. 4). The immunohistological staining demonstrated a homogeneous extracellular staining for type I collagen, type II collagen, and proteoglycan. The histological and immunohistological results coincided with the results of gene expression, which suggests the chondrogenic differentiation of the MSCs in the hybrid mesh.

The polymer fibers of the hybrid mesh were histologically detectable after culture for 1 and 5 weeks. No evident pieces of polymeric fleece were detectable within the engineered specimens after culture for 10 weeks in the chondrogenic induction medium. The PLGA fibers gradually degraded and disappeared.

Mesenchymal stem cells have been used for both basic and clinical application studies of cartilage tissue engineering. Micromass pellet culture is an optimal model for the basic study of the chondrogenic differentiation capacity of MSCs [13–15]. Embedding in hydrogels has also been used frequently for cartilage tissue engineering with MSCs [17,28,29]. However, for a large defect, a three-dimensional scaffold is required to accommodate enough cells and to define the shape of the engineered tissue as desired. In the present study, a cobweb-like PLGA-collagen hybrid mesh was used for the three-dimensional culture of MSCs. The MSCs adhered in the hybrid mesh, proliferated, and produced extracellular matrices to fill the voids in the scaffold. The microsponges formed in the openings of the PLGA mesh provided abundant surface area for MSC attachment. The cells showed the morphology of spindle-like fibroblasts during the initial period and changed to a round morphology after a long culture period. The extracellular matrices also changed from being negatively stained by safranin O and toluidine blue to being positively stained. Immunohistological staining of type II collagen and proteoglycan demonstrated that the cartilaginous extracellular matrices such as type II collagen and cartilage proteoglycan were abundant and homogeneously distributed around the cells. Gene expression analysis agreed with the histological and immunohistological results. The MSCs cultured in the hybrid mesh showed a chondrogenic phenotype and were assumed to have differentiated into chondrocyte-like cells. Although type I collagen was detected by immunostaining, the gene expression level of the gene encoding type I collagen decreased with an increase of culture time. The type I collagen might be mostly synthesized during the early stage of culture. The gene expression results indicate that the cells switched gene expression from type I collagen to type II collagen, which is important for the regeneration of articular cartilage.

The effects of the hybrid mesh on the chondrogenic differentiation of MSCs might be caused by the specific structure of the hybrid mesh. The huge surface areas of the microsponges in the PLGA mesh openings facilitated cell adhesion and cell–cell contact. The cells in the open pores of the collagen microsponges formed aggregates and were connected to each other. The hybrid mesh provided an environment similar to a micromass pellet culture system and a network to put all the cells and matrices together to form a complete tissue of sufficient size. The combination of PLGA-collagen hybrid mesh with MSCs may provide us with a new strategy for cartilage tissue engineering.

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