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Characteristics of mesenchymal stem cells derived from Wharton's jelly of human umbilical cord and for fabrication of non-scaffold tissue-engineered cartilage

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Once cartilage is damaged, it has limited potential for self-repair. Autologous chondrocyte implantation is an effective treatment, but patients may suffer during cartilage harvesting and the donor-site morbidity may accelerate joint degeneration. Using autologous mesenchymal stem cells (MSCs) derived chondrocytes is another selection, while it also causes some injuring. The umbilical cord, an ecto-embryo tissue may be an ideal source of cells, because of its accessibility, abundant resources, painless procedures for harvesting, and lack of ethical issues. We isolated MSCs from Wharton's jelly of human umbilical cord (WMSCs), which expressed CD44, CD105 and CD271 but not CD34 and CD45 with flow cytometry analysis. RT-PCR showed not only positive expression of CD90, c-kit, Sca1, SH2 and SH3 but also positive expression of the chondrocyte markers Sox-9 and Col-2A1. WMSCs cultured in high-density in the presence of transforming growth factor β1 and dexamethasone showed cartilage extracellular matrix-secretion and integrated into a thin piece of cell-based membrane. The cell-based thin membrane cultured in rotary cell culture system formed a round, opaque, glistening non-scaffold cartilage-like tissue, larger and condenser than what was formed with conventional pellet culture. Glycosaminoglycan and type II collagen content significantly increased after 3-week culture. The human WMSCs express characteristics of pre-chondrocytes, low immunogenicity and are easy to be obtained with higher purity because there have no hematopoietic cells in Wharton's jelly, so it may be a new seed cells more suitable for constructing tissue-engineered cartilage.

[**Key words:** Umbilical cord Wharton's jelly; Mesenchymal stem cells; Cartilage; Tissue engineering; Non-scaffold; Characterization]

Once cartilage is damaged, little restoration occurs because the tissue has little self-healing capacity. Many attempts have been made to repair defects of cartilage due to trauma, osteochondritis (1–4). However, the treatment results were not satisfactory. Tissue engineering is a method that is prospective for the reconstruction of the damaged cartilage (5,6). The method includes scaffold and seed cells, while the autologous cells acquisition usually causes a further damage to the patient.

Due to the limitation of the autologous cells acquisition, researchers seek to obtain allogeneic seed cells for tissue engineering. While the allogeneic cells may cause immunological rejection, MSCs derived from embryo and the related tissues may have lower immunogenicity than adult cells (7–9). In recent years, MSCs also can be obtained from amniotic fluid, placenta, and umbilical cord blood (10–14). As well as derived from human umbilical cord blood, MSCs have been obtained from both the subendothelial layer of human umbilical vein (14,15) and Wharton's jelly (16). These latter cells showed a high proliferative rate (17) and were able to differentiate into adipocytes (18), osteoblasts (19,20), chondrocytes (21), cardiomyocytes (22), neurons, and glia (23). It may be a superior source for MSCs application without ethics limitation.

Wharton's jelly is abundant and containing significant amounts of hyaluronic acid and some sulfated glycosaminoglycans (GAGs) immobilized in an insoluble collagen fibril (24). We detected the cartilage-specific genes, Sox-9 and type II collagen, and found they are positive in Wharton's jelly stem cells (WMSCs). Due to the similar extracellular matrix and positive expression of cartilage-specific genes in WMSCs, we suppose that these cells may be superior to other stem cells, such as bone marrow stem cells or adipose stem cells, for construction of tissue-engineered cartilage.

During an earlier study, we found that Wharton's jelly MSCs cultured in chondrogenic medium secreted a large amount of GAGs and type II collagen (extracellular-matrix, ECM). This phenomenon was remarkable when a high-density of cells were cultured in chondrogenic medium. We highlighted the role of the ECM as a natural scaffold. So we fabricated non-scaffold tissue-engineered cartilage with MSCs derived from Wharton's jelly of human umbilical cord. Stimulated by gravity and shear force in the RCCS bioreactor, the cell pellet cultured in chondrogenic medium formed a larger and condenser bolus than conventional pellet culture. The aim of this study was to analyze the character of chondrogenic

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differentiation of human umbilical cord MSCs and whether these MSCs could be a new and better cell source for tissue engineering of cartilage.

MATERIALS AND METHODS

Reagents Culture medium and chemicals were purchased from the following companies: DMEM, dexamethasone, ascorbate-phosphate, proline, pyruvate, propidium iodide, fetal bovine serum (FBS), ITS + Primix and the alkaline phosphatase-conjugated antibody from Sigma Chemical (St. Louis, MO, USA); and 0.05% trypsin/0.02 mM EDTA solution from Gibco, Life Technologies (Grand Island, NY, USA); Trizol-LS, oligo-dT primers, and Superscript II from Invitrogen Life Technologies (Carlsbad, CA, USA); recombinant human TGF-b1 from Pepro Tech INC (Rocky Hill, NJ, USA); recombinant human basic fibroblast growth factor (bFGF) from Pepro Tech EC (London, UK),type-II collagen antibody was from Lab Vision (Fremont, CA, USA); antibodies of CD29, CD34, CD44, CD73, CD90, CD166, HLA-class I, and HLA-DR from BD Biosciences (PharMingen BD, CA, USA); antibody of CD105 from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and horseradish peroxidase-linked antibody, new fuchsine substrate system, Alcin blue solution, and toluidine blue solution from Dako (Kyoto, Japan).

Isolation and culture of Wharton's jelly MSCs Human umbilical cord samples (20 cm long, 20 g) were collected in sterile boxes containing D-Hanks' balanced salt solution. MSCs were isolated within 2 h of umbilical cords obtaining. Umbilical arteries and veins were removed, and then Wharton's jelly was peeled off from the remaining part of the umbilical cords. The Wharton's jelly was transferred to a sterile container and washed thoroughly for three times in serum-free Dulbecco's modified Eagle's medium (DMEM) containing penicillin 100 μg/ml, streptomycin 10 μg/ml, and amphotericin B 250 μg/ml. The jelly was then cut into pieces smaller than 0.5 cm^3. The minced Wharton's jelly was digested for 6–10 h in a sterilized bottle with 15 ml of culture medium containing collagenase of type I at 0.075% in 5% CO_2 37°C incubator with agitation. The cells were then washed three times with D-Hank's salt solution and centrifuged at 250× *g* for 10 min at room temperature, then (cells) were resuspended in culture medium DMEM supplemented with 10% FBS, antibiotics (penicillin, 100 μg/ml, and streptomycin, 10 μg/ml; Invitrogen Life Sciences) and glucose (4.5 g/l), and seeded at 5000–10,000 cells/cm^2 in 25- or 75-cm^2 culture flasks (Costar, Cambridge, MA, USA) with 5% CO_2 95% air in a 37°C incubator, and left undisturbed for 5–7 days. The MSCs were subcultured at approximately 80% confluence to prevent contact inhibition of growth and spontaneous differentiation (25). About 25 umbilical cords were collected for our tests and the obtaining of sampling human umbilical cords were approved by the ethics committee of general hospital of the People's Liberation Army (PLA). Written informed consent was obtained from all mothers before labor and delivery of infants.

Flow cytometry analysis The WMSCs from passage 2 or chondrogenic induction culture were harvested by use of a 0.05% trypsin/0.02 mM EDTA (pH 7.4) solution. For analysis, cells were stained by a combination of antibodies: PE-conjugated CD29, CD44, CD73, CD90, CD105, CD166, CD271 (27–30), HLA-class I; FITC-conjugated CD34, CD45, and HLA-class II; FITC-mouse IgG1; and PE-IgG1. After exposure to labeled antibodies, cells were washed with ice-cold PBS and resuspended in ice-cold PBS. The expression of the corresponding cell-surface antigen was assayed by FACS Calibur with CELL Quest software (Beckman Dickson, USA). The data were analyzed by use of Flow Jo software (Tree Star, Ashland, OR, USA).

Limiting dilution and colony-forming unit-fibroblast (CFU-F) assays According to the mean number of cells required to produce one colony, which was consequently determined to be 1 cfu-f per 333 cells, 8 incremental unit volumes 333 cells of Wharton's jelly cells from primary culture were seeded into individual wells on six-well plates. So WMSCs were diluted to $1^{\circ}\times 10^5$, 5×10^4, 2.5×10^4, 1×10^4, 5×10^3 and 2.5×10^3 and seeded onto six-well tissue culture plates, and fed every 2 days with DMEM Medium. The number of colonies, of >16 cells, were counted as CFU-F frequency in each well on day 10 of culture.

Immunophenotype change with cryopreservation and thaw of Wharton's jelly MSCs Serially passaged MSCs ($0.5–1\times 10^6$) were assayed for expression of MHC I and MHC II cell-surface antigens by flow cytometry. An amount of 1×10^6 MSCs were frozen by use of a freezing container (Coring Corp., USA) and stored in liquid nitrogen for 4 weeks in Cascade Biologics Synth-a-Freeze solution (Cascade Biologics). After 4 weeks of cryopreservation, the MSCs were thawed and analyzed (5×10^5 cells) by flow cytometry (see above), gating on the live cell population and observing them for expression of MHC I and MHC II cell-surface antigens.

Adipogenic, chondrogenic, osteogenic and Schwann cell differentiation of human WMSCs Cultured cells at passage 2 were incubated in adipogenic differentiation medium (DMEM containing 10% FBS, 50 μg/ml of ascorbate-1 phosphate, 10^{-7} M dexamethasone, and 50 μg/ml indomethacin) in chondrogenic differentiation medium (as cell pellets) [DMEM, insulin-transferrin-selenium (ITS) + premix (Gibco, Carlsbad, CA, USA), 10^{-8} M dexamethasone, 50 μg/ml ascorbate-2-phosphate, 40 μg/ml proline, 100 μg/ml pyruvate and 10 ng/ml transforming growth factor-β1 (Pepro Tech, Rocky Hill, NJ, USA)], in osteogenic differentiation medium (DMEM containing 10% FBS, 50 μg/ml ascorbate-2 phosphate, 10^{-8} M dexamethasone, and 10 mM β-glycerophosphate), in Schwann cell differentiation medium (DMEM, 10% FBS, 1 mM β-mercaptoethanol, 35 ng/ml All-trans retinoic acid, 5 μmol/L Forskolin, 10 ng/ml bFGF, 5 ng/ml PDGF, 200 ng/ml heregulin) or in DMEM supplemented with 10% FBS as a control. The medium was changed every 3 days, and the cells, after completion of differentiation had been established by morphology, were used for the histochemical staining and immunohistochemistry studies.

Pellet culture combined with rotary cell-culture system (RCCS) Culture-expanded MSCs were typically used at the end of second passage to prepare the cell aggregates. Briefly, 2×10^6 cells were cultured in culture disks with chondrogenic culture medium. Three days after chondrogenic induction, MSCs secreted extracellular matrix and formed a cell-based membrane. The cultures were rinsed with sterile D-Hank's salt solution to remove residual FBS present in the growth medium. The D-Hank's salt solution was then aspirated and 0.05% trypsin/0.02 mM EDTA (pH 7.4) solution was added; chondrogenic induction cells formed an integral membrane; bovine calf serum (Hyclone, Logan, UT, USA) was added to inhibit the trypsin. Then the cell-based membrane was transferred to a 15-ml polypropylene conical tube (Coring Corp.) and centrifuged for 5 min at 200× *g*. The supernatant was discarded, and 8 ml chondrogenic medium was added for centrifugation for 5 min at 200× *g* without shaking the polypropylene tube. The cell-based membrane pellet was cultured at 37°C with 5% CO2 in an incubator with chondrogenic medium. One week later, the cell-based membrane pellet contracted and turned into a sponge-like cell bolus. Next, the bolus was transferred to the RCCS with use of a pipette. The medium was replaced every 3–4 days for 14 days. After 3-week induction, the cartilage-like tissue was fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned for histological and immunohistochemical evaluation. Adjacent sections were stained with toluidine blue to evaluate proteoglycan content or anti-collagen type II antibody (Developmental Studies Hybridoma Bank, University of Iowa) to evaluate type-II collagen content. For immunohistochemistry, sections were deparaffinized in xylene in three 5-min washes, rehydrated in decreasing ethanol concentrations (in duplicate) for 2 min each, and washed with distilled water for 3 min. The sections were digested in a humidified chamber, with 1-mg/mL pronase (Sigma) in PBS solution for 15 min, and then blocked with 5% PBS–BSA for 30 min. The primary antibody, diluted at 1:200 in 1% PBS–BSA, was applied to the sections for 1 h. Horseradish peroxidase-conjugated goat anti-mouse IgG (MP Biomedicals, Irvine, CA, USA) was used as the secondary antibody, which was also diluted at 1:200 in 1% BSA in PBS and applied for 45 min, and the reactivity was detected by use of a diaminobenzidine tetrahydrochloride substrate. After each step in the protocol, the sections were washed with PBS for 5 min. The slides were then mounted with 5% *N*-propyl gallate in glycerol to slow photobleaching and photographed with use of a DP-70 digital camera (Olympus, BX51, DP 70 software) microscope.

Immunohistochemical assessment of human umbilical cord Human umbilical cords were immersed in 4% paraformaldehyde, a series of adjacent 5-μm-thick sections were cut in the coronal plane, the sections were stained with Hematoxylin and Eosin (H&E), toluidine blue and safranin O. For immunohistochemistry, an anti-type-II collagen monoclonal antibody was used. Sections were observed on light microscopy (Olympus).

RT-PCR To examine the cartilage-specific gene expression, total RNA was prepared from WMSCs after induction for 3 weeks. WMSCs (passage 2) not induced were used as a negative control. Total RNA was extracted by use of Trizol following the manufacturer's instructions. For examination of the chondrogenic-related gene expression, PCR amplification involved 30 cycles of 94°C for 30 s, 58°C for 45 s, and 72°C for 1 min. PCR products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide for visualization under UV light and photographic recording. PCR primers were as follows: Sox-9 (forward) 5′ CCC TTC AAC CTC CCA CAC TAC 3′, (reverse) 5′ TCCTCAAGGTCGAGTGAGCTG 3′ (272 bp); hCOL2A1 (forward) 5′ GCTCCCAGAACATCACCTACC 3′, (reverse) 5′ TGAACCTGCTATTG CCCTCT 3′ (135 bp); β-actin (forward) 5′ TCCTCCCTG GAG AAG AGC TA 3′, (reverse) 5′ TCA GGA GGA GCA ATG ATC TTG 3′ (302 bp).

To identify the MSC characters of WMSCs, we examined expression of the stem cell-related gene, including CD90, CD133, c-kit, Sca1, SH2, SH3, and lineage-related gene as cbfa1 and PPAR gamma. Primers are as following: CD90 (forward) (for) 5′ GCTCAGGGAGGAGGATAATGT 3′, (reverse) (rev) 5′ GGATGGGTGAACTGCTGGTAT 3′; CD 133 (for) 5′ CACTTACGGCACTCTTCACCT 3′, (rev) 5′ GGGATTTACCAGTCTGAGCCA 3′; c-kit (for) 5′ GGGAAAGAAGACAACGACACG 3′, (rev) 5′ ACAGACACAACAGGCACAGC 3′; Sca1 (for) 5′ GCAGCAAACCTCAGGGAAAC 3′, (rev) 5′ CTCAATGCCACAGTCCAGTTC 3′; SH2 (for) 5′ GCCTTTGATGAGTCAGGTAGC 3′, (rev) 5′ GGTCCCACTTTCCAGCAGAA 3′; SH3 (for) 5′ GTGCTTCTGGTCCTCAGTGTA 3′, (rev) 5′ AGTTCCACCTTCACCGTCAC 3′; cbfa1 (for) 5′ ACAGTAGATGGACCTCGGGA 3′, (rev) 5′ AGTGAGTGGTGGCGGACATA 3′; PPAR − γ (for) 5′ CAGGAGCAGAGCAAAGAGGT 3′, (rev) 5′ CACAGCAAACTCAAACTTGGG 3′. The expression level of these genes were analyzed with Gray-Scale software Autogel 106.

Detection of GAG content by biochemical assay For quantitation of GAG content, 1×10^6 WMSCs (passage 2) from both monolayer culture and monolayer three-week chondrogenic induction culture were collected. Each sample was mixed with a 4-M guanidine solution (Sigma) and placed on an orbital shaker at 4°C for 48 h. Digested samples were dialyzed by use of a 15-kDa cut-off cellulose membrane in a bath of distilled water for 12 h to remove acid. A 50-μL sample of digested tissue was mixed with 2.5 mL of Alcian blue 8GX (Sigma). The samples were analyzed with use of a spectrophotometer (DU640; Beckman Coulter, Fullerton, CA, USA). The