Enhancement of bone formation by genetically engineered human umbilical cord–derived mesenchymal stem cells expressing osterix

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Objectives. The aim of this study was to investigate if overexpression of osterix (Osx) in human umbilical cord–derived mesenchymal stem cells (UC-MSCs) would facilitate osteogenic differentiation in bone regeneration.

Study Design. UC-MSCs were isolated from UCs. A pEGFP-Osx plasmid was constructed and applied to transfect UC-MSCs. Cell proliferation, alkaline phosphatase (ALP) activity, and expression of bone-related genes were examined to evaluate the osteogenic potential of UC-MSCs. Bone regeneration in vivo was evaluated in nude mice using PLGA as a carrier.

Results. Reverse-transcription polymerase chain reaction showed that pEGFP-Osx transfection enhanced expression of bone matrix proteins. Overexpression of Osx in UC-MSCs enhanced ALP activity, while not inhibited their proliferation rate. The Osx-transduced group formed significantly more bone at 4 weeks.

Conclusions. Concerning their simple isolation and proliferation, it is believed that genetically engineered UC-MSCs could play important roles in the study and application of bone tissue engineering. (Oral Surg Oral Med Oral Pathol Oral Radiol 2013;116:e221-e229)

Therapeutic approaches for tissue-engineered repair of bone defects concentrate on combining autologous cells with appropriate extracellular matrix scaffolds and signal molecules.[1] The development of models that use various cell sources has led to tests of various methods of bone tissue engineering. Autogenous bone grafts are considered as the gold standard for bone replacement, mainly because they offer minimum immunologic rejection, complete histocompatibility, and provide the best osteoconductive, osteogenic, and osteoinductive properties.[2,3] Autologous bone grafting requires an invasive protocol, however, and is limited to the amount of available tissue, donor site morbidity, and difficulty in shaping the harvested autograft to effectively fit the defects.[4] Therefore, an alternative approach would be to apply a cell-based strategy, whereby umbilical cord–derived mesenchymal stem cells (UC-MSCs), for instance, could be grown and have potential for tissue-engineered cartilage and bone repair.[5]

UC-MSCs have been derived from the Wharton's jelly in UCs, which are a medical waste discarded after birth and can be attained without harm for the mother or infant.[6,7] In addition, UC-MSCs can be collected without an invasive procedure required for bone marrow–derived MSCs. Can and Karahuseyinoglu[6] reported that UC-MSCs have greater expansion capability and are more potent than bone marrow MSCs, showing that UC-MSCs may be a potential cell source for bone tissue engineering. Morever, UC-MSCs are a primitive MSC population that expresses certain embryonic stem cell (ESC) markers and exhibits high plasticity and developmental flexibility.[8] All these advantages make UC-MSCs a highly desirable stem cell source for tissue regeneration; however, despite its high promise, little has been published on osteogenic differentiation of gene-expressed UC-MSC delivery via bioactive scaffolds for bone tissue engineering.

In 2002, a new zinc-finger–containing transcription factor, osterix (Osx), was discovered and characterized.[9] There is growing evidence indicating that Osx plays an essential role in the differentiation of osteoblasts and bone formation. In Osx null mice, no bone formation occurs, and cells in the periosteum and the membranous skeletal elements cannot differentiate into osteoblasts. In a recently published in vivo study, Tu et al.[10] found that there was increased new bone formation in the wound sites where Osx was applied, as compared with controls. These results are the first demonstration that Osx may function during bone regeneration to control the differentiation of cells involved in the regenerative process.

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No reports have been available to focus on the functions of Osx in the osteogenic differentiation of UC-MSCs, however. Therefore, the aim of this study was to investigate if overexpression of Osx in UC-MSCs would facilitate osteogenic differentiation in bone regeneration in vitro and in vivo.

MATERIAL AND METHODS

Isolation and culture of UC-MSCs

Human UC-MSCs were isolated from umbilical cords from 2 patients who underwent full-term pregnancy (39-40 weeks) with signed consent forms, according to the methods described previously.[11] Briefly, human umbilical cord was rinsed several times with sterile phosphate-buffered saline (PBS) and cut into 2- to 4-cm lengths. Then, the fragments were incubated with 0.075% collagenase type II (Sigma, St. Louis, MO) for 30 minutes, followed by 0.125% trypsin (Sigma) for 30 minutes with gentle agitation at 37°C. The arteries and veins were removed under sterile conditions, and cell suspensions were collected using a 100-μm cell filter. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, MD) with 10% fetal bovine serum (Gibco BRL), 100 U/mL penicillin, and 100 U/mL streptomycin (Gibco BRL). The cells were cultured at 37°C with 5% CO_2 and 95% humidified air. After cells reached 90% confluent, trypsinization was performed with 0.25% trypsin (Gibco BRL, Gaithersburg, MD) in PBS for 10 minutes, and then the cells were subcultured until the third passage for the following analyses.

Flow cytometry analysis

After expansion, the cells of the third passage were analyzed by flow cytometry (FACsort; BD Biosciences Pharmingen, San Jose, CA). Briefly, cultured cells were trypsinized and resuspended at a concentration of 10^6 cells/mL in blocking buffer (0.5% bovine serum albumin, 0.01% Na azide, 1 × PBS). A total of 10^5 cells were treated with 10 μL of diluted primary antibody (CD34-fluorescein isothiocyanate [FITC], CD105-FITC, CD90-FITC, FITC) and analyzed on a FACSort (Becton Dickinson, San Jose, CA) as previously described.[12]

Plasmid transfection

The recombinant plasmid plasmid enhanced green fluorescent protein-osterix (pEGFP-Osx) was constructed by directed cloning technique by Shanghai Yuanxiang Biotech Co. Ltd. (Shanghai, China). For plasmid transfection, UC-MSCs were plated in 6-well plates at a density of 2.5×10^5 cells/well. Transfection of UC-MSCs was performed using Lipofectamine-2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The cells were observed under fluorescent microscopy (Leica, Tokyo, Japan) to determine the transfection efficiency. After 24 hours, the cells were passaged and subjected to G418 (500 μg/mL) selection for 2 weeks (pOsx-UC-MSCs). Control cells were mock transfected with "empty" vector (mock-UC-MSCs).

Cell apoptosis assay

Cellular apoptosis were detected by flow cytometry with Annexin-V-FITC/propidium iodide (PI) dual staining as described previously.[13] Briefly, cells were washed twice with cold PBS, then harvested into a tube. After adding 10 μL of annexin-V-FITC and 10 mL of PI to the tube and incubating at room temperature for 5 to 10 minutes in the dark, the samples were analyzed by flow cytometry. The emitted green fluorescence of annexin-V and red fluorescence of PI were detected by an flow cytometer (FACSCalibur, Becton Dickinson) with an excitation wavelength of 488 nm and an emission wavelength of 525 and 575 nm, respectively. For each sample, 10,000 events were recorded. The amount of early apoptosis, late apoptosis, and necrosis were determined as the percentage of annexin-V^+/PI^-, annexin-V^+/PI^+, and annexin-V^-/PI^- cells, respectively.

Alkaline phosphatase staining

Alkaline phosphatase (ALP) staining was also performed using the Gomori Calcium-Cobalt (Ca-Co) method. In brief, when the cells had reached confluence at day 14, after fixation with a solution of 95% alcohol for 10 minutes, the cells were incubated at 37°C for 4 to 6 hours. The cells were then stained with solutions of 2% cobalt nitrate and 1% ammonium sulfide in turn. After being air-dried, the slides were mounted and used for microscopy.[14]

Reverse-transcription polymerase chain reaction analysis for the expressions of bone-related genes

The osteogenic differentiation of pOsx-UC-MSCs, mock-UC-MSCs and UC-MSCs was evaluated by reverse-transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from cultured UC-MSCs using Trizol reagent (Tiangen Biotech, Shanghai, China). Specimens were lysed and homogenized with 800 μL of Trizol reagent, added to 200 μL of chloroform, and centrifuged at 12,000 rpm for 15 minutes. The RNA pellets were washed with 75% (vol/vol) ethanol, dried, and dissolved in RNase-free water. Quantity and quality of RNA were assessed by absorbance at 260 nm and agarose gel electrophoresis. Total RNA was reversely transcribed into first-strand cDNA by using the Takara RT-PCR Kit (TaKaRa Bio Inc., Shiga, Japan). Synthesized cDNA was amplified by PCR using human-specific primers shown in Table I. PCR was carried out for