Effect of cell culture using chitosan membranes on stemness marker genes in mesenchymal stem cells

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Received November 30, 2012; Accepted April 4, 2013

DOI: 10.3892/mmr.2013.1423

Abstract. Mesenchymal stem cell (MSC) therapy is a promising treatment for diseases of the nervous system. However, MSCs often lose their stemness and homing abilities when cultured in conventional two-dimensional (2D) systems. Consequently, it is important to explore novel culture methods for MSC-based therapies in clinical practice. To investigate the effect of a cell culture using chitosan membranes on MSCs, the morphology of MSCs cultured using chitosan membranes was observed and the expression of stemness marker genes was analyzed. We demonstrated that MSCs cultured using chitosan membranes form spheroids. Additionally, the expression of stemness marker genes, including Oct4, Sox2 and Nanog, increased significantly when MSCs were cultured using chitosan membranes compared with 2D culture systems. Finally, MSCs cultured using chitosan membranes were found to have an increased potential to differentiate into nerve cells and chrondrocytes. In conclusion, we demonstrated that MSCs cultured on chitosan membranes maintain their stemness and homing abilities. This finding may be further investigated for the development of novel cell-based therapies for diseases involving neuron-like cells and chondrogenesis.

Introduction

Stem cells may be used in the treatment of various diseases due to their differentiation activity and self-renewal potential. When subcultured over several generations, stem cells are unable to undergo differentiation or self-renewal, mainly due to the surrounding microenvironment, which is known to largely control the fate of stem cells. Recently, various biomaterials have been developed to maintain embryonic stem cell self-renewal. Mesenchymal stem cells (MSCs), one type of adult stem cell, are widely distributed in several tissues, including bone marrow, cord blood and adipose tissue. MSCs are able to differentiate into a variety of cell types, including adipocytes, osteocytes, chondrocytes, myocytes and hepatocytes of the mesodermal lineage and nerve and epithelial cells of the ectodermal lineage (1-6). Besides differentiation, MSCs also release numerous cytokines to support tissue regeneration and repair, including insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor-α (VEGF-α) and macrophage chemokines (7). Numerous studies have shown that MSCs stimulate the regeneration and repair of damaged tissues, particularly in acute ischemic diseases, including acute myocardial or cerebral infarction and various types of arthritis (1,3,5,6,8). Furthermore, previous studies have shown that MSCs have special immunological characteristics; therefore, MSCs may be transplanted without a rejection reaction (9,10). In addition, MSCs are involved in immune regulation, thus they have the potential to be used as a novel therapy to treat rejection following organ transplantation and autoimmune diseases. Consequently, MSCs may have a therapeutic value in the treatment of numerous serious diseases and a broad scope in future clinical applications.

The regeneration, repair and immune regulation abilities of MSCs are largely due to their multi-lineage differentiation potential and paracrine actions. Currently, it is difficult to maintain the stability of MSCs under *in vitro* culture conditions, since aging, spontaneous differentiation into osteocytes and a decreased proliferation ability have been reported in MSC cultures under normal conditions (11-13). The spontaneous differentiation of MSCs decreases their ability to differentiate into other important cells, such as nerve and cardiac muscle cells. The transplantation of these differentiated MSCs (which are not morphologically similar to osteocytes) to non-bone tissues has been shown to result in ectopic calcification and the impairment of tissue functions (14). The paracrine actions of MSCs cultured *in vitro* have also been found to decrease and their myocardial protection ability was affected (15). Finally, the aging and differentiation of MSCs cultured *in vitro* had significant effects on their therapeutic outcome and safety.

Chitosan is a chitin derivative that is widely found in nature. It is composed of D-glucosamine and N-acetyl-glucosamine, which are joined by β (1→4) glycosidic bonds. Due to its biocompatibility, non-immunogenicity and non-toxicity, chitosan is compatible and cross-linked with collagen. The

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Key words: chitosan, mesenchymal stem cell, stemness

Table I. Primers used for quantitative real-time PCR (qRT-PCR).

Gene	Primer sequence	Annealing temperature (˚C)
β-actin	Sense: AACTACCTTCAACTCCATC Antisense: TGATCTTGATCTTCATTGTG	55
Oct4	Sense: ACATCAAAGCTCTGCAGAAA Antisense: CTGAATACCTTCCCAAATAGAAC	60
Sox2	Sense: TGCGAGCGCTGCACAT Antisense: GCAGCGTGTACTTATCCTTCTTCA	58
Nanog	Sense: AATACCTCAGCCTCCAGCAGAT Antisense: TGCGTCACACCATTGCTATTCTT	60

different types of chitosan and its deacylation level have been found to affect cell adhesion and proliferation (16-19). Melanocytes and keratocytes cultured on chitosan-coated surfaces have been shown to form spheroids, which were maintained in three-dimensional (3D) culture systems (16,20). In the present study, we investigated the effect of cell culture using chitosan membranes on the stemness of MSCs and provided information with regard to the clinical application of MSC-based therapy.

Materials and methods

Ethics. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the First Affiliated Hospital of Xinxiang Medical University, Weihui, China.

MSC culture. The first generation of bone marrow-derived human mesenchymal stem cells (hMSCs) were provided by the Shengzhen Baiwang Biotechnology Corporation (Shengzhen, China). hMSCs (100 cells/cm^2) were thawed and recovered for 24 h, and then cultured in full medium (CCM) with 17% fetal bovine serum (FBS) for 7-8 days until 70% fusion. hMSCs were passaged under the same conditions and were used in the experiment before the third generation.

Preparation of chitosan membranes. Chitosan powder (molecular weight, 510 kDa; 77.8% deacetylation; Shengzheng Baiwang Biotechnology Corporation) was dissolved in 1% aqueous acetic acid solution to obtain a 1% chitosan solution. Next, 300 μl of the chitosan solution was coated on a glass coverslip and dried out at room temperature. Following neutralization with 0.5 N NaOH for 30 min, the glass coverslip was washed with PBS 5 times and exposed to ultraviolet radiation for 1 h. The prepared chitosan membranes were used in further experiments.

Generation and dissociation of spheroids. hMSCs were cultured at a low density (1,000 cells/well) in 6-well plates containing chitosan membranes. Cell growth was observed daily using an inverted microscope. To obtain spheroid-derived cells, spheroids were incubated with trypsin/EDTA for 5-30 min (depending on the size of the spheroid). During dissociation, the cells were pipetted every 2-3 min.

Proliferation and survival ratio of MSCs cultured using chitosan membranes. The cell numbers of MSCs cultured with or without chitosan membranes were analyzed using the fluorescent dye Hoechst 33528 on days 1, 3 and 10 of culture. Spheroids and cells were digested in papain solution (Sigma, San Francisco, CA, USA) and then incubated with 0.1 mg/ml Hoechst 33528 dye (Sigma). The concentration was measured by a fluorescence spectrophotometer (F2500; Hitachi, Tokyo, Japan) at room temperature using an excitation and emission wavelength of 365 and 458 nm, respectively. The cell number was calculated based on a standard curve and the cell activity was measured using a propidium iodide (PI) staining assay (Sigma Resources and Technologies, Inc., Santa Clara, CA, USA). PI solution (2 mg/ml) was added to the cell suspension and the percentage of unstained cells was defined as cell activity.

RNA extraction and quantitative real-time PCR (qRT-PCR) analysis. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The isolated RNA was reverse transcribed and amplified using the RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). cDNA was then used as a template for qRT-PCR to quantitatively analyze the gene expression levels with the DyNAmo Flash SYBR Green qPCR kit (Finnzymes, Espoo, Finland). β-actin was amplified and used as an internal standard to normalize the expression of tested genes. The primers of the genes investigated are listed in Table I.

MSC differentiation into nerve cells. MSCs were primarily cultured for 3 days to induce differentiation into nerve cells. Following the addition of 10 ng/ml epidermal growth factor (EGF; Gibco-BRL, Rockville, MD, USA), 20 ng/ml hepatocyte growth factor (HGF; R&D Systems, Minneapolis, MN, USA) and 20 ng/ml VEGF (R&D Systems) were added to the culture medium, and the cells were cultured for an additional 10 days.

Induction of chondrogenic differentiation. To induce chondrogenic differentiation, basic medium was replaced by chondrogenic differentiation medium following a primary culture for 3 days. Chondrogenic differentiation medium contained 10 ng/ml transforming growth factor β3 (TGFβ3)

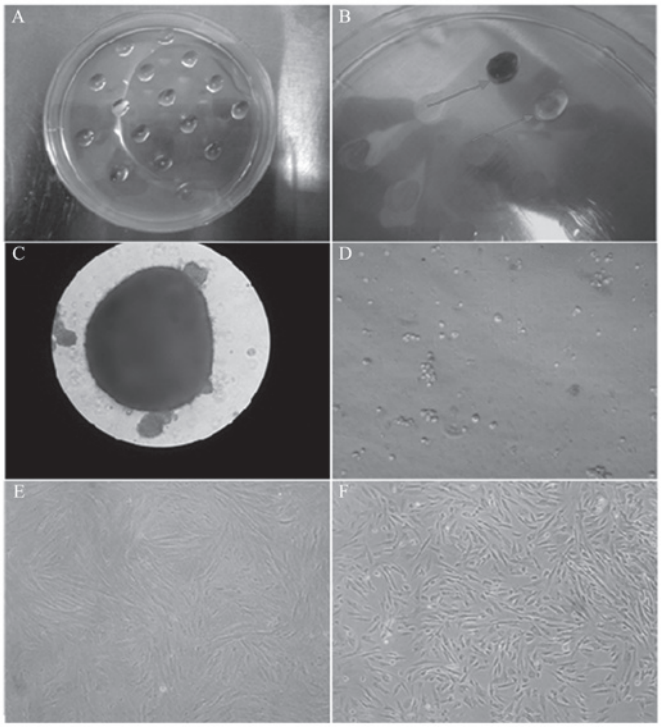

Figure 1. Effect of cell culture using chitosan membranes on MSC morphology. (A) Mode of colony formation following cell culture using chitosan membranes; (B) chitosan membrane culture was stained with trypan blue; (C) observation of spheroid formation following cell culture using chitosan membranes under a microscope; (D) the spheroid number was calculated following digestion with 0.25% trypsin and staining with trypan blue (x400); (E) P7 under general adherent culture (x400); (F) P7 under adherent culture following culture using chitosan membranes (x400). MSC, mesenchymal stem cell. P7, seventh passage cells.

(CytoLab/PeproTech, Rehovot, Israel), 0.1 mM dexamethasone, 50 mg/ml L-ascorbic acid 2-phosphate, 40 mg/ml L-proline, 1% insulin transferrin selenium (ITS-premix 100; Sigma), 1% penicillin and 1% streptomycin. The medium was replaced twice a week. For morphological observation, the cells were incubated in precooled acetone and washed with PBS. The cells were then stained with Mayer's hematoxylin solution and washed once with toluidine blue and water. Safranin O was then applied for 5 min to stain the cells. Subsequently, 95% alcohol, absolute alcohol and xylene were sequentially used to treat the cells once every 2 min. Finally, differentiation was observed under a microscope.

Statistical analysis. The data were analyzed using SPSS 12.0.1 software and are presented as the mean ± standard error (SE). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Morphology of MSCs cultured using chitosan membranes. MSCs cultured in a 2D culture system had a typical long polygonal morphology. However, spheroid formation was observed within 1-2 days when the cells were transferred to chitosan membranes. Cell size was reduced by 75% compared with cells cultured in a 2D system following trypsin digestion, similar to spheroids generated using the pendant drop method (Fig. 1).

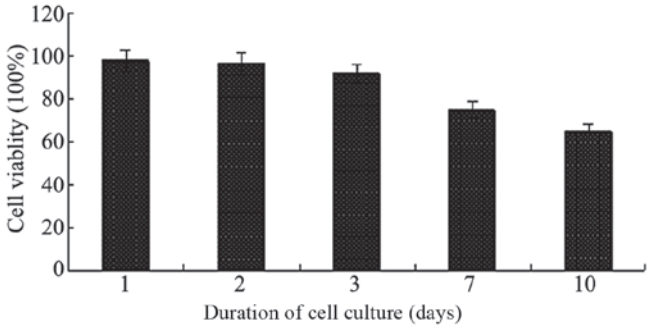

Figure 2. Survival percentage of MSCs following cell culture using chitosan membranes. The survival percentage was >90% during the first 3 days of culture and the cell survival ratio remained >65% after 10 days of culture. MSC, mesenchymal stem cell.

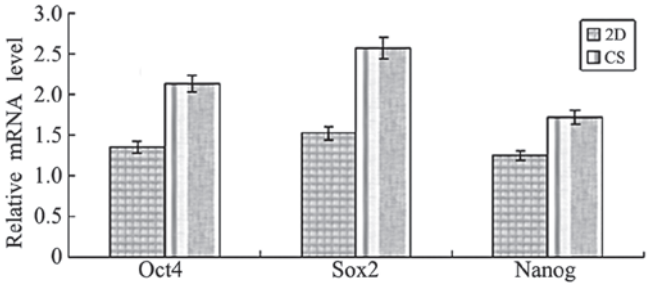

Figure 3. Gene expression levels of stemness marker genes Oct4, Sox2 and Nanog in MSCs following cell culture using chitosan membranes. Each experiment was repeated 3 times and the relative RNA level is presented as the mRNA ratio of each gene compared with GAPDH. CS, chitosan membrane cultures; MSC, mesenchymal stem cell; 2D, two-dimensional.

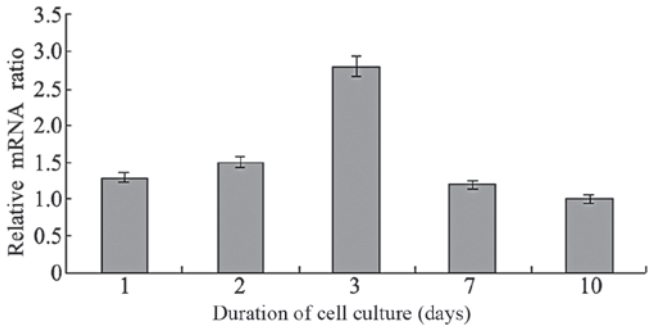

Figure 4. Relative Sox2 mRNA ratios of MSCs cultured using chitosan membranes compared with 2D culture on days 1, 2, 3, 7 and 10 of cell culture. The expression level of Sox2 was maintained when MSCs were cultured using chitosan membranes. MSC, mesenchymal stem cell; 2D, two-dimensional.

Spheroid cell viability. Since MSC spheroids have limited access to nutritional agents, cell viability was investigated. The 10,000 or 25,000 MSC spheroid was cultured for 3 days and 90% of harvested cells survived. The number of apoptotic cells slightly increased with the duration of culture (Fig. 2). Growth curves for cultured MSCs were plotted for the different culture materials used. MSC proliferation was found to be maintained after spheroid formation.

Effects of spheroid formation on the expression of stemness marker genes. To determine the regulatory roles of stemness

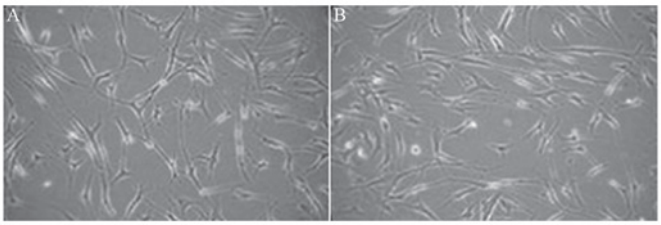

Figure 5. MSCs were cultured using chitosan membranes for 3 days and then transfered to a 2D culture system. The ability of the cells to differentiate into nerve cells in the two culture systems was compared. (A) MSCs cultured in a 2D culture system for 3 days (x400); (B) MSCs cultured using chitosan membranes for 3 days (x400). MSC, mesenchymal stem cell; 2D, two-dimensional.

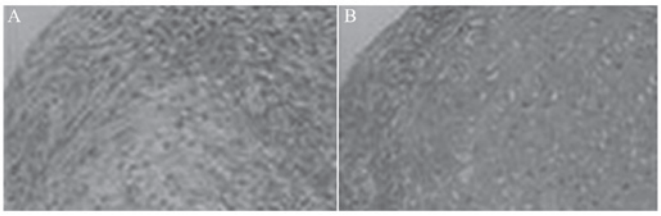

Figure 6. Ability of MSCs to differentiate into chondro metrocytes following treatment with TGFβ3. (A) MSCs cultured in a 2D culture system (x400); (B) MSCs cultured using chitosan membranes, followed by transfer to a 2D culture system (x400). MSC, mesenchymal stem cell; 2D, two-dimensional; TGFβ3, transforming growth factor β3.

marker genes in MSC culture, the expression levels of Oct4, Sox2 and Nanog mRNA with different culture materials were analyzed by RT-PCR (Figs. 3 and 4). A ratio >1 was considered to indicate upregulated gene expression, while <1 showed downregulation. The expression levels of these genes in MSCs cultured using chitosan membranes were upregulated within 1-3 days, and downregulated after 7 days. This indicated that spheroid formation by MSCs cultured using chitosan membranes may be able to maintain the expression of MSC stemness marker genes.

Effect of culture using chitosan membranes on MSC differentiation. Cells cultured in medium supplemented with growth factors were differentiated and transformed into cells with a long and thin morphology. The change in cell morphology was similar to that of nerve cells; for example, the cytoplasmic area around the nucleolus was smaller (Fig. 5). MSCs cultured using chitosan membranes had an increased differentiation efficiency ($P<0.001$). These results indicated that MSCs were able to differentiate into nerve cells in the presence of these growth factors, and that the MSCs cultured on chitosan membranes had an increased potential for this differentiation. When TGFβ3 was added to the medium of MSCs cultured using chitosan membranes, cells differentiated into cartilage after 2 weeks, and chitosan membranes were found to aid this type of differentiation (Fig. 6). These results indicated that spheroids formed by MSCs cultured using chitosan membranes were similar to MSC spheroids generated by the pendant drop method. This method maintained the stemness ability of MSCs and may aid the induction of differentiation under specific conditions.

Discussion

Stem cells are known for their self-renewal ability and may be induced to differentiate into specific cell types when cultured under particular conditions. They are also considered to be important candidates for tissue engineering. However, pluripotent stem cells lose their stemness ability when cultured *in vitro*. To maintain the self-renewal ability of undifferentiated embryonic stem cells (ESCs), the feeder layer, such as mouse embryonic fibroblasts (MEFs) or conditional medium, is required to provide a stable environment (21,22). Numerous natural or synthetic polymeric materials have been developed to replace the feeder layer in order to maintain the self-renewal ability of ESCs (23-27). Transcription factors Oct4, Sox2 and Nanog have been shown to be important in the maintenance of pluripotency in ESCs (28,29). These marker genes are also expressed in adult stem cells (30,31). In the present study, we investigated the effect of cell culture using chitosan membranes on the development and self-renewal ability of undifferentiated MSCs. MSCs were demonstrated to rapidly form 3D spheroids following culture using chitosan membranes, and the expression levels of marker genes (Oct4, Sox2 and Nanog) either remained stable or were upregulated.

3D multicellular states have been previously described in a number of cell types, including ESC-derived embryoid bodies and neurosphere-derived neurons (32). This information was useful for the development of ESCs. MSCs have been shown to form 3D spheroids when cultured using micropore membranes (33) or other types of surfaces (34,35). Human MSCs cultured using micropore membranes formed spheroids and exhibited increased anti-inflammatory properties in a previous study (35). Spheroid formation following 2D cell culture and the expression of differentiation-related genes have not previously been investigated. In the present study, the increased expression of stemness-related genes indicated an improved plasticity of MSCs. The accumulation of MSCs created a suitable environment for cell-cell interactions, and promoted the early differentiation of MSCs into nerve cells and chondrocytes (36). Various natural and synthetic 3D scaffolds have been demonstrated to be beneficial for the formation of nerve cells and cartilage (37,38). The spheroid formation of MSCs that occurred during culture using chitosan membranes not only enhanced cellular plasticity, but also improved nerve cell and cartilage formation. The disadvantage of 3D multicellular status may be the finite spreading of center. However, our results indicated that the cell survival ratio was >70%, even 7 days after spheroid formation, and that ≥90% cells had survived 3 days after spheroid formation.

In conclusion, 3D MSC spheroids were observed in 2D cultures within 1-2 days when using chitosan membranes. The expression levels of stemness marker genes remained stable in these spheroids; however, decreased expression levels of stemness marker genes suppressed spheroid formation. These results indicate that undifferentiated MSCs are able maintain their original state while forming 3D spheroids when cultured using chitosan membranes. Furthermore, spheroids exhibited improved nerve cell formation and chondrogenic differentiation abilities following induction. 3D spheroid formation on biomaterials may constitute a novel strategy to maintain the self-renewal ability of MSCs and increase the differentiation

potential of MSCs into cartilage, and has the potential to be applied in the treatment of diseases of the nervous system and cartilage tissue engineering. However, the underlying mechanism regulating spheroid formation requires further investigation.

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