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Unique characteristics of human mesenchymal stromal/progenitor cells pre-activated in 3-dimensional cultures under different conditions

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Abstract

Background aims. Human mesenchymal stromal cells (MSCs) are being used in clinical trials, but the best protocol to prepare the cells for administration to patients remains unclear. We previously demonstrated that MSCs could be pre-activated to express therapeutic factors by culturing the cells in 3 dimensions (3D). We compared the activation of MSCs in 3D in fetal bovine serum containing medium and in multiple xeno-free media formulations. *Methods.* MSC aggregation and sphere formation was studied with the use of hanging drop cultures with medium containing fetal bovine serum or with various commercially available stem cell media with or without human serum albumin (HSA). Activation of MSCs was studied with the use of gene expression and protein secretion measurements and with functional studies with the use of macrophages and cancer cells. *Results.* MSCs did not condense into tight spheroids and express a full complement of therapeutic genes in α-minimum essential medium or several commercial stem-cell media. However, we identified a chemically defined xeno-free media, which, when supplemented with HSA from blood or recombinant HSA, resulted in compact spheres with high cell viability, together with high expression of anti-inflammatory (prostaglandin E2, TSG-6 TNF-alpha induced gene/protein 6) and anti-cancer molecules (TRAIL TNF-related apoptosis-inducing ligand, interleukin-24). Furthermore, spheres cultured in this medium showed potent anti-inflammatory effects in a lipopolysaccharide-stimulated macrophage system and suppressed the growth of prostate cancer cells by promoting cell-cycle arrest and cell death. *Conclusions.* We demonstrated that cell activation in 3D depends critically on the culture medium. The conditions developed in the present study for 3D culture of MSCs should be useful in further research on MSCs and their potential therapeutic applications.

Key Words: *MSC, pre-activation, sphere, stem cell, xeno-free, 3D*

Introduction

Mesenchymal stromal/progenitor cells (MSCs) are being used in clinical trials for a large number of diseases. Currently, there are more than 100 registered clinical trials (www.clinicaltrials.gov) with MSCs or related cells. Also, more than half a dozen biotech companies are in Phase II and III trials in efforts to commercialize the cells [1]. The results from some of the trials are encouraging, but few have provided universally accepted data on efficacy [2]. One of the hurdles encountered has been questions as to how to best prepare the cells for administration to patients: Should the cells be extensively expanded in confluent cultures or expanded at low densities to a limited number of passages and population doublings so that the cells retain most of their stem cell like characteristics [3]? Should fetal bovine serum (FBS) be removed from medium formulations and replaced with chemically defined factors [4]? Should the cells be administered from freshly thawed frozen vials or as cells directly isolated from tissue culture [5]? Should the cells be preconditioned in culture to activate therapeutic genes before administration [6–9]?

MSCs are traditionally isolated and expanded as adherent monolayers on tissue culture plastic that is commonly used to culture mammalian cells and that has been treated with proprietary protocols to increase the oxygen content of the surfaces and thereby increase cell adherence and spreading [10,11]. There has been increasing interest, however, in culturing cells in non-adherent conditions in which most cells remain viable and form spheres/spheroids. One of the advantages of culturing cells in 3 dimensions (3D) as spheres is that it more closely reproduces the *in vivo* environment, including the delicate

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cell-to-cell and cell-to-matrix signaling networks [12,13]. A number of investigators have demonstrated that MSCs will form spheroids if incubated in hanging drops or other conditions that prevent their adhesion to planar surfaces [14–31]. Assembly into spheres improved many properties of the cells linked to their therapeutic potentials such as differentiating into hepatocyte-like cells [14], supporting migration and survival of endothelial cells [16], enhancing cardiac function [15,17], differentiating into insulin-producing cells [19], differentiating into chondrocytes [31], enhancing cartilage repair [25], supporting expansion of hematopoietic cells [26], anti-cancer effects [20] and suppressing inflammation [27,29,30]. However, the properties of the spheroid MSCs vary with the culture conditions such as cell concentration and the time in culture [30]. We previously demonstrated that if prepared with FBS-containing medium that was optimized for expansion of MSCs in monolayers, spheroid MSCs significantly decreased in size (to approximately one fourth of the volume of adherent MSCs) and fewer cells were entrapped in the lungs of mice after intravenous injection of the cells when compared with standard preparations of the cells [30]. Also, MSCs in spheroids significantly increased their production of prostaglandin E2 (PGE2), a potent inflammatory mediator; TNF-alpha induced protein 6 TSG-6, a protein that modulates the inflammatory responses; and stanniocalcin 1 STC-1, a calcium/phosphate-regulating protein that reduces reactive oxygen species when compared with adherent MSCs [27,29,30,32,33]. As the cells assembled into spheroids, there was increased activation of caspases that drove the activation of interleukin (IL)-1 signaling which, in turn, drove secretion of TSG-6 and STC-1 [27]. The activation of both IL-1 and contact-dependent Notch signaling was required for secretion of PGE2 [27]. Moreover, the cells were more effective in suppressing inflammation in a zymosan-induced model for peritonitis [30] and in promoting transition of lipopolysaccharide (LPS)-stimulated macrophages from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenoptype [29].

Because the culture medium components are important in determining the properties of MSCs and because the use of animal components in the medium to prepare cells results in lot-to-lot variations and limits the therapeutic uses of the cells, we tested a series of different media for culture of MSCs in hanging drops. In the process, we identified a chemically defined xeno-free medium that optimized sphere formation and pre-activation of MSCs to express and secrete several therapeutic molecules. Therefore, the procedure used here offers novel and effective methods for preparing pre-activated MSCs for research and clinical trials.

Methods

MSC culture

Human MSCs, isolated from 3 adult bone marrow aspirates and cultured as previously described [30], were obtained from the Center for the Preparation and Distribution of Adult Stem Cells (http://medicine.tamhsc.edu/irm/msc-distribution.html). Briefly, 1–4 mL of bone marrow aspirate was obtained from the iliac crest of normal adult donors. Nucleated cells, obtained by density gradient centrifugation (Ficoll-Paque; GE Healthcare Waukesha, WI), were resuspended in complete culture medium (CCM) consisting of α-minimum essential medium (MEM-α, Gibco Grand Island, NY) supplemented with 17% FBS (Atlanta Biologicals), 100 units/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco) and 2 mmol/L L-glutamine (Gibco), seeded in 175-cm^2 flasks (Nunc Rochester, NY), and subsequently cultured at 37°C in a humidified atmosphere with 5% CO_2. After 24 h, non-adherent cells were discarded. Adherent cells were incubated 4–11 d until approximately 70% confluent, harvested with 0.25% trypsin and 1 mmol/L ethylenediaminetetraacetic acid (EDTA, Gibco) for 5 min at 37°C and re-plated at 50 cells/cm^2 in an intercommunicating system of culture flasks (Nunc). The cells were incubated for 7–12 d until approximately 70% confluent, harvested with trypsin/EDTA and frozen as passage 1 cells in MEM-α containing 30% FBS and 5% dimethyl sulfoxide (Sigma St. Louis, MO). Frozen vials of each donor-derived passage 1 MSCs were thawed, suspended in CCM and plated on a 152-cm^2 culture dish (Corning Tewksbury, MA). After 24 h, cells were harvested with the use of trypsin/EDTA. The cells were plated at 100 cells/cm^2 and expanded for 7 d before freezing as passage 2 cells. For the experiments described here, a vial of passage 2 MSCs were recovered by plating in CCM on a 152-cm^2 culture dish for a 24-h period, re-seeded at 100–150 cells/cm^2 and incubated for 6–8 d in CCM. Culture medium was changed every 2–4 d and 1 d before harvest. Characteristics of the MSCs used in the study are described in Supplementary Table I.

Generation of spheroids and sphere-derived cells

To generate spheroids [30], MSCs were plated in hanging drops on an inverted culture dish lid in 35 μL of culture medium at 25,000 cells/drop. The lid was then rapidly re-inverted onto the culture dish that contained phosphate-buffered saline (PBS) to prevent evaporation of the drops. The hanging drop cultures were incubated for 3 d at 37°C in a humidified atmosphere with 5% CO_2. The MSCs in hanging drops were cultured in various media including CCM, α-MEM, MesenCult XF

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