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Combination of MSC spheroids wrapped within autologous composite sheet dually protects against immune rejection and enhances stem cell transplantation efficacy

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ABSTRACT

Mesenchymal stem cells (MSCs) are widely used in transplantation therapy due to their multilineage differentiation potential, abundance, and immuno-modulating ability. However, the risk of allograft rejection limits their application. Here, we proposed a novel method to facilitate MSC transplantation with enhanced applicability and efficacy. We cultured human adipose-derived MSCs in a 3D culture under *in vitro* expansion conditions and under conventional 2D adherent culture conditions. MSC spheroids promoted extracellular matrix molecules that stimulate MSC proliferation, and produced more angiogenic cytokines such as vascular endothelial growth factor, hepatocyte growth factor, and fibroblast growth factor than 2D-cultured MSCs. Further, MSC spheroids showed increased IDO expression, increased proportion of M2 macrophages, and decreased macrophage proliferation, compared to 2D-cultured MSCs. Next, we proposed the wrapping of autologous cell sheets from the recipient around *in-vitro*-grown MSC spheroids to prevent allogenic immune rejection during transplantation. Myoblasts from C57BL/6 mice were used to prepare a stem cell composite sheet containing human-derived MSC spheres. The transplantation of MSC spheroids increased the survival rate and decreased the inflammatory response of the immunocompetent C57BL/6 ischemic mice. Thus, combining 3D-cultured MSC spheroid technology with immune evasion stem cell composite sheet improved the outcome and strengthened the protection against allogenic immune rejection.

1. Introduction

Adult mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into various specialized cells, including osteoblasts, chondrocytes, adipocytes, and tenocytes (Caplan, 1991). Because of their excellent differentiation abilities, human MSCs have been widely utilized as a source for cell therapy and regenerative transplantation (Meirelles Lda et al., 2009). MSCs are derived from various tissues (Colter et al., 2000; Erices et al., 2000; Rodriguez-Lozano et al., 2011; Zuk et al., 2002) and extracellularly cultured; they are thus readily available as a source of cells with the potential to differentiate into various lineages (Pittenger et al., 1999; Prockop, 1997). However, there are some limitations to the applicability of MSC therapy that hinders the optimal outcome in the clinical setting (Mizuno, 2009; Yarak and Okamoto, 2010). Such limitations include the risk of severe hypoxia at the MSC graft site that prevents proper implantation (Adhami et al., 2006; Gimble et al., 2007), reduced functionality of MSCs from elderly donors, and reduced efficacy of transplantation in aged recipients (Capogrossi, 2004; Dimmeler and Leri, 2008; Fehrer et al., 2006; Zhang et al., 2005). To overcome these limitations, researchers have been focusing on developing strategies for increasing the survival rate of the transplanted cells. Solutions suggested so far include the transplantation of synthetic MSC scaffolds in combination with treatment with cellular growth and angiogenic factors (Banfi et al., 2012; Cao et al., 2003; Kim et al., 2011; Li et al., 2010), genetic modification of MSCs engineered for individualized adaptabilities (Keung et al., 2013), and three-dimensional (3D) culture of MSCs (Emmert et al., 2013; Spelke et al., 2011; Xie et al., 2016; Yeh et al., 2014). Among these, the 3D MSC culture method has been at the focus of particular interest.

The stem cell spheroids obtained from 3D culture are more adaptable to the application site in the human body than cells cultured with traditional two-dimensional (2D) culture. In particular, the physical

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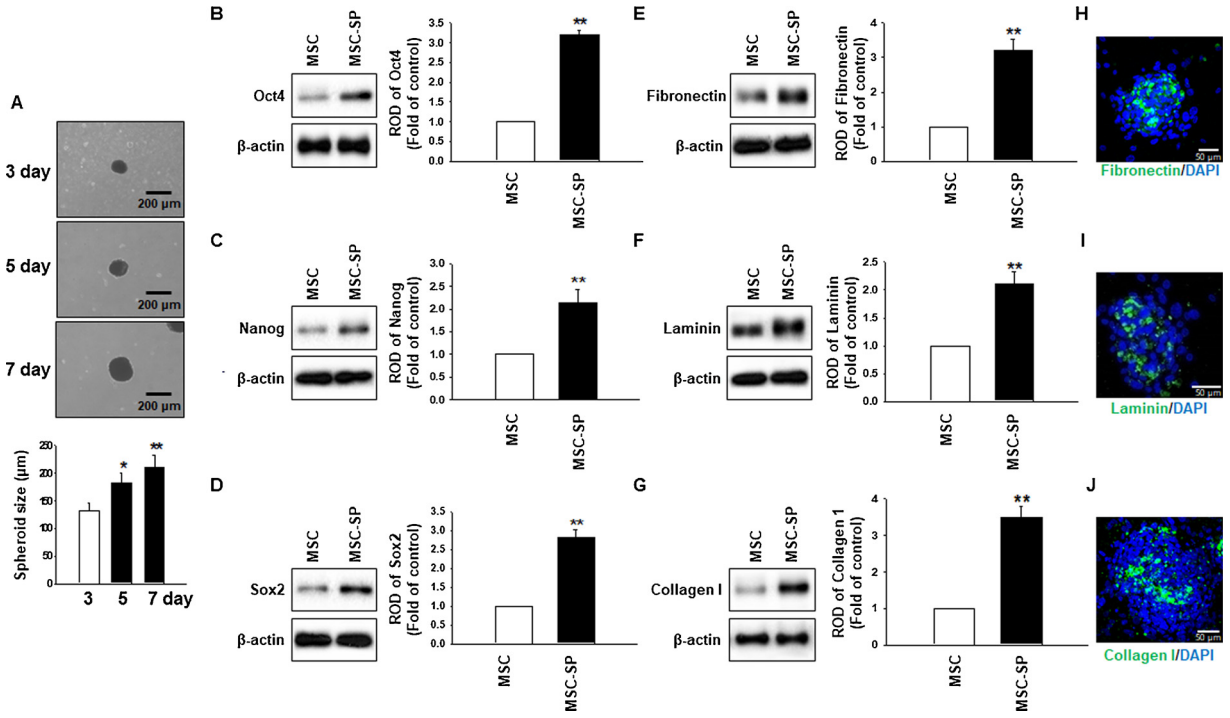

Fig. 1. Changes in stem cell function during MSC spheroid formation.
(A) Microscopic image showing the 7-day progression of ellipsoid formation from human adipose-derived MSCs. Sale bar = 200 μm. Values represent means ± SEM. * $p < 0.05$ and ** $p < 0.01$, vs. 3-day spheroid MSCs. (B–D) Western blot analysis for (B) Oct4, (C) Nanog, and (D) Sox2 in monolayer MSCs and MSC spheroids cultured for 7 days. (E–G) Western blot analysis for ECM components, including (E) fibronectin, (F) laminin, and (G) collagen-1 in monolayer MSCs and MSC spheroids cultured for 7 days. Relative expression of proteins normalized to that of β-actin. Values represent means ± SEM. ** $p < 0.01$, vs. monolayer MSCs. (H–J) Immunofluorescent staining (green) for ECM components, including (H) fibronectin, (I) laminin, and (*J*) collagen-1 in MSC spheroids cultured for 7 days. Sale bar = 50 μm.

shape of the MSC spheroids improves the efficiency of the therapy and applicability for transplantation, compared to the conventionally cultured cells. 3D-cultured MSCs also show increased expression of stem cell-associated genes, pluripotency, angiogenesis, secretion of anti-apoptotic and anti-inflammatory molecules, ECM secretion, and upregulation of hypoxic signals (Bhang et al., 2012; Lee et al., 2016; Park et al., 2014). The 3D culture of MSCs has also been reported to enhance anti-inflammatory properties by regulating the production of inflammatory cytokines by macrophages via increased PGE-2 and TSG-6 factors and IDO activity (Bartosh et al., 2010; Petrenko et al., 2017; Ylostalo et al., 2014). Thus, appropriate utilization of the 3D culture method could improve the function of stem cells and immunoregulation, enabling the production of optimized stem cells for transplantation. These observations suggest that spheroid-based cell therapy could be a promising technique for improving the applicability of the therapy.

Although it is clear from *in vivo* experiments that 3D culture of MSCs could improve therapeutic effects (Bhang et al., 2012; Lee et al., 2016; Park et al., 2014), allogenic immune rejection of Human embryonic stem cells (hESCs)-derived cells by the recipients of the transplant remains a significant limiting factor in the applicability of MSC therapy (Rong et al., 2014). Allogenic immune rejection, rejection of foreign materials by the body, significantly hinders the stable engraftment of the MSC transplants grown *in vivo*. Interestingly, previous studies suggest that autologous cells could increase angiogenesis and anti-apoptosis in the application site by secreting angiogenic factors such as vascular endothelial growth factor (Rehman et al., 2004). The utilization of autologous cells of the recipient would represent a potential method to overcome the allogenic immune rejection associated with MSC therapy.

In this study, we aimed to explore whether a combination of techniques, namely 3D culture of MSC spheroids and autologous composite cell sheets to protect the MSC spheroids from immune rejection, could produce a preferable therapeutic outcome *in vivo* in a hindlimb ischemic site. We hypothesize this combination of therapies would expand the MSC therapeutic options for patients.

2. Materials and methods

2.1. Cell cultures

Human adipose-derived MSCs that were negative for pathogens and mycoplasma were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The supplier certified that the MSCs expressed cell surface markers (CD73 and CD105, but not CD31) and possessed adipogenic and osteogenic differentiation potential when cultured in specific differentiation media. The MSCs were cultured in alpha-minimum essential medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco BRL), 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were placed in a humidified incubator at 37 °C at 5% CO_2.

2.2. MSC spheroid cultures

The MSCs were cultured in suspension using ultra-low attachment 6-well plates (Sigma, St. Louis, MO, USA) for spheroid generation. MSCs were cultured in growth media and incubated at 37 °C at 5% CO_2. Spheroids were formed at day 3, and then further cultured for the different periods (3, 5, or 7 days) to assess their bioactivity. MSC spheroids were identified and measured using a visual inspection microscope (Olympus, Tokyo, Japan). We dissociated the MSCs spheroid using Trypsin-EDTA solution to calculate the cells number in the MSC shperoid, and then measured the number of cells through trypan blue staining. MSCs spheroids with a diameter of 200 μm each contain an average of 25,000 cells.