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Regulation of the secretion of immunoregulatory factors of mesenchymal stem cells (MSCs) by collagen-based scaffolds during chondrogenesis

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ABSTRACT

In the latest decade, mesenchymal stem cells (MSCs) have wildly considered as a source of seeded cells in tissue engineering, not only because of its multi-differentiation potentials, but also due to its immunoregulation ability. The main immunoregulatory features of MSCs could be divided into low self-immunogenicity and secretion of soluble factors. In this study, we explored how scaffold structures modulated the secretion of soluble immunoregulatory factors in MSCs under an allogeneic cartilage tissue engineering background. MSCs were seeded in four different collagen-based scaffolds. Their proliferation, differentiation, and secretion of various soluble factors associated with the immunosuppressive effects were evaluated. In this study, qRT-PCR, ELISA and immunoregulation results showed a great variability of the factor secretion by MSCs seeded in scaffolds with different structures. Compared with two-dimensional (2D) monolayer culture condition, three-dimensional (3D) groups (hydrogels and sponge) could effectively promote the mRNA expression and the protein production of soluble immune-related factors. Also, the supernatants collected from 3D groups obviously showed inhibition on allogeneic lymphocyte activating. These results suggested that scaffold structures might modulate MSCs' secretion of soluble immunoregulatory factors, and our study might enlighten the scaffold designs for desired tissue regeneration to control the host immune rejection through immune-regulation reaction.

1. Introduction

Mesenchymal stem cells (MSCs) characterized as multipotent stromal cells have served as an excellent cell source for cell therapy, tissue engineering and organ transplantation, due to their capacity for long-term self-renewal, low immunogenicity, and multi-differentiation into mesenchymal lineages, including bone, cartilage, muscle, adipose and so on [1–4]. MSCs show low immunogenicity and are considered to be "immunologically privileged", for they express a relatively low level of cell-surface class I and II major histocompatibility complex (MHCs) and lack the surface expression of co-stimulatory molecules (CD80, CD86 and CD40), which are required for fully activating T cells [5–7]. More importantly, some active immunoregulatory factors secreted by MSCs regulate the immunological reactions of both the cells and the surrounding tissues.

Numerous studies have demonstrated that the paracrine effects involve in the immunoregulatory activity of MSCs through the secretion of soluble factors, including transforming growth factor-Beta1 (TGF-β1), prostaglandin-E2 (PGE2), hepatocyte growth factor (HGF), indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO), and interleukine-6 (IL-6) and so on [7,8]. NO is known as an inhibitor of CD4+ T cell proliferation under inflammatory and oxidative conditions [9]. Meanwhile, the expression of PGE2 can regulate the immune function through restraining the proliferation of CD4+ T cells, the production of B cells as well as the differentiation and maturation of dendritic cells (DCs) [6,10]. HGF can suppress both CD4+ and CD8+ T lymphocyte proliferation [6,11], and IL-6 interferes with the differentiation and maturation of DCs [6,9,10]. IDO can reduce the proliferation of CD4+ T-cells, inhibit the differentiation and maturation of DCs, and meanwhile stimulate Treg cell generation [7,10]. Thus, previous studies that revealed the immunoregulatory features of MSCs primarily focused on these soluble factors.

Tissue engineering is used to improve or regenerate the organ function with three essential elements, including scaffolds, cells and biofactors [12,13]. The multiple-differentiation and low immunogenicity ability ensure the key position of MSCs as seeded cells. In tissue engineering, the features of scaffolds are supposed to effectively modulate the biological behaviors of seeded cells, and thus dynamically control the properties and performance of engineered tissues [14,15]. Numerous investigations about scaffold modulating cell behaviors mainly focused on the differentiation of seeded cells and the deposition of extracellular matrix [16,17]. Our previous studies also found that scaffold structure could mediate the expression of cell surface marker molecules of seeded allogeneic MSCs, such as MHC-1 and -2, which are essential for stimulating immune responses [18,19]. However, the underlying cellular and molecular mechanisms on how the scaffold structure affects allogeneic MSC-mediated immunomodulation are poorly

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known. Our hypothesis is that scaffold properties can modulate the release of soluble immunoregulatory factors by seeded MSCs, and in turn improve the immunosuppressive ability of allogeneic MSCs-derived engineered tissue in a paracrine way. It is believed that a comprehensive understanding of how the scaffold properties modulate the secretion of these factors by MSCs can strongly support the application of MSC-based tissue-engineered constructs in a complicated environment, particularly in clinical long-standing local diseases, which often cause severe inflammatory or immune disorder.

In cartilage tissue engineering, scaffolds and seeded cells have been considered as crucial parts responsible for repairing. Based on previous studies, scaffolds with proper design (*e.g.* collagen-based hydrogel) could induce the chondrogenic differentiation of bone marrow-derived MSCs to form cartilage-like tissues [16,20]. The seeded cells (MSCs) could be readily encapsulated into tissue-engineered constructs with good rheological properties following the gelation of temperature-sensitive collagen solutions under a mild condition, in order to maintain their biological functionality and differentiation potential [20–22]. Furthermore, the study of immunoregulatory factors secreted by MSCs in this process could not be isolated from the chondrogenesis background. Thus, this study focused on investigating how the collagen-based scaffold structure regulated the immunological properties of allogeneic MSCs through modulating their secretion of immunoregulatory factors, and also exploring the scaffold-inducing chondrogenic differentiation as a background.

In light of these thoughts and hypothesis above, neonatal rat bone marrow-derived MSCs were cultured in three-dimensional (3D) collage-based scaffolds (including hydrogels and sponge), as well as two-dimensional (2D) tissue culture plate, respectively. Then, the chondrogenic differentiation and the secretion of several soluble factors by seeded MSCs were detected at the different time points, in order to systematically clarify the changes of immunological properties of allogeneic MSCs during scaffold-inducing chondrogenesis process. The findings of this study might improve the understanding of the relationship between scaffold properties and immunosuppressive capacity of allogeneic seeded MSCs, and provide a useful principle for optimizing scaffold design to create “immunological safe” allogeneic tissue-engineered composites with no or little host immune rejection for regeneration of cartilage and other tissues.

2. Material and methods

2.1. Isolation, culture and characterization of rat MSCs

In this study, all of the animals were used following the Guideline for the Care and Use of Laboratory Animals of Sichuan University and the standard of ISO 10993-2:2006. Long bones were removed from neonatal (three- to five-day) SD rats, and both sides were dissected in an aseptic condition. Bone marrow was flushed out of cavities and cultured with the improved alpha-MEM medium (α-MEM) containing 10% fetal bovine serum and 1% penicillin–streptomycin (Gibco, USA). Cell culture medium was changed every other day. After they reached an 80%–90% confluence in culture plate, MSCs were subcultured using trypsin (Gibco, USA).

To characterize the cellular phenotype, the expression profile of lineage-specific markers (*i.e.*, CD29, CD34, CD45 and CD90) was analyzed by flow cytometry. Briefly, cells of the third passage were harvested and resuspended to approximately 5×10^5 cells in 0.5 ml PBS containing 0.1% bovine serum albumin. Then, they were labeled with fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat antibody CD45 and CD90 (Abcam, USA), FITC-conjugated anti-mouse/rat antibody CD29 (Biolegend, USA) and Alexa Fluor 647-conjugated mouse anti-rat CD34 (Abcam, USA) for 1 h at 37 °C in darkness, respectively. After washing, cells were analyzed using a BECKMAN flow cytometry (Beckman Coulter, USA). About 25,000 events were collected from each run of flow cytometry.

2.2. Fabrication of cell-encapsulated collagen-based hydrogels and sponges

According to the methods reported in previous publications [16,17,23], type I collagen was extracted from calf skin by dealing with pepsin (Sigma-Aldrich, USA) in 0.5 M acetic acid (Kelong Chemical Industry, China) under a sterile condition, and then purified by sodium chloride fractionation and fibril reconstruction.

When reached a high confluence at the third passage, MSCs were collected with 0.25% trypsin-EDTA and counted with a hemocytometer. Trypan blue staining was used to confirm that cell mortality was < 5%. To fabricate cell-hydrogel constructs, MSCs (3×10^7 cells/ml) were thoroughly mixed with the neutralized collagen solution on ice with the final collagen concentration of 5, 10 and 15 mg/ml respectively. And then 150 μl homogenous suspension was injected into the customized moulds with 9.6 mm in diameter and 5 mm in height, and incubated at 37 °C for about 20 min to form hydrogel. Finally, the hydrogels were transferred into 6-well plates (Corning Coster, USA) with improved α-MEM culture medium supplied with 10% FBS and 1% PS. Meanwhile, collagen sponges with similar size were also synthesized by a freeze-dry process. Briefly, cell-free neutralized collagen solution was injected into 96-well plates (Corning Coster, USA) at 150 μl per well, pre-frozen at −80 °C for 4 h and then freeze-dried at −44 °C under 10–30 Pa under a sterile condition for 48 h. To form cell-sponge constructs, 100 μl culture medium with the similar final concentration of MSCs was added in each sponge, and incubated at 37 °C for 4 h to allow cell adhesion. And then cell-sponge constructs were also transferred into 6-well plates with fresh medium. MSCs seeded in both hydrogels and sponges were considered as three-dimensional (3D) culture groups, while, those seeded in tissue culture plate were considered as two-dimensional (2D) monolayer culture group. All groups were cultured in the incubator at a constant temperature of 37 °C and a constant CO_2 concentration of 5%. The culture medium was changed three times per week. The samples and culture supernatant were harvested at 7, 14 and 21 days, respectively.

2.3. Characterization of cell-scaffold constructs

As cell-induced matrix contraction plays an important role in cellular behaviors, the contractile characteristics of MSC-scaffold constructs were analyzed, including 5 mg/ml, 10 mg/ml and 15 mg/ml hydrogels as well as sponge, referred to as 5M, 10M, 15M and SPM, respectively. After seeding cells for 7, 14 and 21 days, cell-scaffold constructs were collected, and the scaffold contraction was measured *via* the method previously reported [24]. Briefly, scaffolds were placed on the top of a transparent metric ruler under a dark background, and optimal visibility of scaffold edges was obtained. As treating the scaffold as a round disc, the average of major and minor axes was taken as the mean diameter. The contraction ratio at a designated time was calculated as a ratio of the reduced diameter of scaffold to its initial diameter following the equation as below: contraction ratio = (DI − DR) / DI × 100%; where DI is the initial diameter of scaffold at day 0, and DR is the remaining diameter of contracted scaffold at a designated time.

2.4. Confocal laser scanning microscopy (CLSM)

Cell-scaffold constructs were harvested at 7, 14 and 21 days. For CLSM, some samples were treated with 1μg/ml fluorescein diacetate (FDA, Topbio Science, China) and 1 μg/ml propidium iodide (PI, Topbio Science, China) for 5 min, and then the fluorescence was visualized by a CLSM (TCS SP 5, Leica, Germany). According to the manufacturer's instruction, live cells are dyed green by reacting with FDA, while dead cells are dyed red by PI.