Identification of Myocardial Telocytes and Bone Marrow Mesenchymal Stem Cells in Mice

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Abstract
Objectives: The aim of this study was to compare the morphology, immune phenotype, and cytokine profiles between myocardial telocytes (TCs) and bone marrow mesenchymal stem cells (MSCs), and explore the difference between those two types of interstitial cells. Methods: TCs and MSCs were cultured in vitro and cell morphology was observed with a light microscope. The expression levels of CD34, c-kit, and vimentin were detected by immunofluorescence, RT-qPCR, and Western blotting in both TCs and MSCs. The related supernatant was collected and total of 49 cytokine profiles were detected by RayBio Mice Cytokine Antibody Array. Significantly different cytokines were further confirmed by ELISA. Results: TCs have small cellular body and very long prolongations and they were CD34+/c-kit+/vimentin+, whereas MSCs have no telopodes and they were CD34−/c-kit−/vimentin+. Cytokine profile analysis and ELISA showed that 19 of 49 cytokines were increased dramatically in the supernatant of TCs compared with those of MSCs. Moreover, 9 of 19 cytokines were increased 2-fold at least in the supernatant of TCs compared with those of MSCs. Of 49 cytokines, 30 exhibited no significant changes in the supernatant of TCs compared with those of MSCs. Conclusions: Using various technologies, we identified that myocardial TCs and MSCs are significantly different in terms of cell structure and cytokine profiles. Keywords: TCs, MSCs, morphology, phenotype, cytokine antibody array

Introduction
Telocytes (TCs) are a newly discovered interstitial cell type characterized by small and fusiform cellular body with telopodes. TCs are regarded as connecting cells involved in intercellular communication, either directly via homo-and heterocellular junctions, or by extracellular vesicle release at long distance. Recent studies have identified TCs in the interstitial space of many organs in mammals, and they considered to participate in the neo-angiogenesis, repair, and regeneration of specific tissues. So far, the biological functions of TCs remain unclear, although TCs have been characterized by genomic and proteomic approaches by comparison with several other cell types, including mesenchymal stem cells (MSCs).

Bone marrow MSCs are morphologically characterized by a small cell body with a few cell processes that are long and thin. The cell body contains a large and round nucleus with a prominent nucleolus, which is surrounded by finely dispersed chromatin particles, giving the nucleus a clear appearance. MSCs are considered to play a pivotal role in tissue regeneration. Upon stimulation by trauma and other factors, MSCs could participate in the repair of injured tissues under the direction of fibronectin or MSC growth factor. Zheng et al. showed that expression of genes and proteins are totally different in TCs and MSCs, although TCs and MSCs are both interstitial cells. Additionally, Cismasiu et al. also reported that TCs did not express miRNA, which could differentiate TCs from other stromal cells such as MSCs.
In the present study, we attempted to identify the difference of mouse myocardial TCs and MSCs from morphology, gene expression, and immune phenotype and cytokine profiles. Our results revealed that myocardial TCs and MSCs differ significantly in terms of cell structure, gene expression, protein expression, and cytokine profiles.

**Materials and Methods**

**Cell Culture**

The mice MSCs were purchased from Chinese Academy of Science, Kunming Cell Bank (Cat. no. KCB200537, HSF, Kunming, China) and were preserved at the Biomedical Research Center of Zhongshan Hospital (Fudan University, Shanghai, China). MSCs were cultured in 6-well plates at a density of 1×10^5/well and maintained at 37°C in a humidified atmosphere (5% CO_2 in air); the cells were cultured continuously in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, Carlsbad, CA, USA) (2 ml per well) without fetal bovine serum (FBS) for 48 h. Subsequently, the supernatant was collected and stored at –80°C for further cytokine antibody array measurement.

For culture of myocardial TCs, myocardial samples were isolated from BALB/c mice. The study was approved by the Ethics Committee of Fudan University. The methods used for isolation and culture of TCs were described previously^20,21. Briefly, myocardial tissues were cut and harvested under sterile conditions, and collected in sterile tubes containing DMEM. Next, the samples were rinsed with DMEM and minced into small fragments of around 1 mm^3 and digested with 1 mg/ml collagenase type II in PBS without Ca^2+ and Mg^2+, which were then incubated at 37°C for 4 h on an orbital shaker. The reaction of collagenase was terminated by 10% FBS (Gibco, Carlsbad, CA, USA). Dispersed cells were separated from the non-digested tissue by filtration through a 40-μm strainer (BD Falcon, NJ, USA) and collected by centrifugation at 2000 rpm for 5 min. The cells were seeded on 25 cm^2 plastic culture flasks in DMEM with 10% FBS and 1% penicillin-streptomycin (Sigma Chemical Co., St. Louis, MO, USA), at a density of 1×10^5 cells/cm^2, and maintained at 37°C in a humidified atmosphere (5% CO_2 in air) until becoming sub-confluent (usually 4 days after plating). Culture medium was changed every 48 h. At day 5, the adhered cells were collected and re-plated into a new 6-well culture plate at a density of 1×10^5/well. The cells were cultured continuously in DMEM (2 ml per well) without FBS for 48 h, and the supernatant was collected and stored at –80°C for cytokine antibody array measurement. For morphology study, the cells were observed and photographed under a light microscope through a 40× objective (Olympus 1X51, Tokyo, Japan).

**Immunofluorescence**

Cultured TCs and MSCs were washed with PBS and fixed by 4% paraformaldehyde (PFA) for 20 min. The cells were then rinsed with PBS and incubated with 0.5% Triton X-100 for 30 min at room temperature. Next, the cells were incubated in blocking buffer (5% normal goat serum) for 1 h at room temperature. Immunostaining was performed with goat anti-vimentin (ab11256, Abcam, Cambridge, MA, USA), mouse anti-CD34 (ab6330, Abcam), rabbit anti-c-kit (ab5506, Abcam). The primary antibodies were diluted in blocking buffer at a dilution of 1:100 for vimentin, 1:200 for CD34, and 1:100 for c-kit and incubated at 4°C overnight. The cells were washed with PBS and incubated with indicated Alexa Fluor secondary antibodies at a dilution of 1:500 for 2 h at room temperature. Finally, the cells were washed and counterstained with DAPI. Stained cells were observed and photographed by a fluorescence microscope through a 40× objective (Olympus 1X51, Japan).

**Total RNA Extraction and RT-qPCR**

Total RNA was extracted using TRIZol reagent (Invitrogen), and 1 μg total RNA was used to perform reverse transcription. Gene expression was measured by quantitative real-time PCR (qPCR) using SYBR Green reagent (Bio-Rad, Hercules, CA, USA) and normalized to GAPDH. Primers used in this study were: CD34 forward: 5’AGGGCTGG GTGAAGACCCCTA3’; reverse: 5’TGA ATGCGCGTT TCTGGAAGT3’; c-kit forward: 5’TCACTGAGTGTTG ATGGA AAA3’; reverse: 5’GGTGACCTTTTCAG GCACA3’; vimentin forward: 5’CGTCCA CACGCA CCTACAG3’; reverse: 5’GGGGGTAGAGGAATAG GCT3’. GAPDH forward: 5’TGGATTTGGACGCTTGG TC3’; reverse: 5’TTTGCACTGGTACGTGTGAT3’.

**Western Blot Analysis**

Western blot analysis was performed according to standard procedures. Total protein was isolated from TCs and MSCs. Protein concentrations were determined by BCA Protein Assay kit (Beyotime Biotechnology, Haimen, China). The proteins were separated by SDS polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with antibody against vimentin (ab11256, Abcam), CD34 (ab6330, Abcam), and c-kit (ab5506, Abcam). β-actin antibody (Abcam) was served as a loading control. Signals were detected by secondary antibodies labeled with HRP (horseradish peroxidase) and signal intensity was determined using Quantity One software (Bio-Rad, Richmond, CA, USA).

**Cytokine Profile Analysis**

The supernatants of cultured myocardial TCs and MSCs were analyzed with specially manufactured cytokine antibody array (Mouse Cytokine Antibody Array, Kangchen
Bio-tech, Shanghai, China). Briefly, cytokine array membranes were blocked in blocking buffer for 30 min, and then incubated with samples at room temperature for 2 h. The buffer was then gently removed and membranes were washed with wash buffer. Subsequently, the membranes were incubated with diluted biotin-conjugated antibodies at room temperature for 2 h. The membranes were washed again with wash buffer; horseradish peroxidase-conjugated streptavidin was then added and incubated for 2 h at room temperature. The membranes were then washed thoroughly and incubated with detection buffer in the dark. By comparing the signal intensities, relative expression levels of cytokines could be obtained. The intensities of signals were quantified by densitometry. Biotin-conjugated immunoglobulin G served as a positive control.

ELISA

According to the results of the cytokine antibody array analysis, several cytokines with significantly different expression between TCs and MSCs were selected and further detected with corresponding ELISA kits, strictly following the protocol recommended by the producers. The following ELISA kits were used: IL-1β (Sigma, St. Louis, MO, USA), IL-2 (Thermo Fisher, Waltham, MA), GM-CSF (R&D systems, Minneapolis, MN), TGF-β1 (R&D systems), and FGF-6 (R&D systems).

Data Analysis

The SPSS 17.0 software was used to process the data. All experiments were carried out in triplicate under identical conditions and data were represented as means ± standard error of the mean (SEM). To calculate a P value for comparisons between two samples, statistical analyses were performed using Student’s t-test. The threshold of significance was set at p < 0.05.

Results

Morphology of Cultured Myocardial TCs and MSCs

The morphology of mouse myocardial TCs and MSCs can be observed clearly with a light phase contrast microscope. As shown in Fig. 1A, myocardial TCs could be identified according to their characteristic morphologies. For instance, TCs had a small ovoid nucleus surrounded by a thin rim of cytoplasm. Furthermore, the morphology of TCs displayed cellular elongation and dichotomous branches—named telopodes—from the cell body. In contrast, MSCs looked spindle-shaped without extensive dichotomous branching, and the nucleus was larger than in TCs (Fig. 1B). Taken together, these results demonstrate that the morphology of TCs and MSCs is distinctly different.

Identification of TCs and MSCs by immunofluorescence, RT-qPCR, and Western Blotting

Next, we further investigated the difference between TCs and MSCs through detection of specific markers by various methods. Firstly, as shown in Fig. 2A and 2B, immunofluorescence data showed that TCs were positive for CD34, c-kit, and vimentin, whereas MSCs were negative for CD34 and c-kit and positive for vimentin. Secondly, the mRNA expression levels of CD34, c-kit, and vimentin were measured by RT-qPCR in both TCs and MSCs. As shown in Fig. 2C, the mRNA expression level of CD34 and c-kit was barely detectable, but the mRNA expression level of vimentin showed no significant difference between TCs and MSCs. Finally, the protein expression level of CD34, c-kit, and vimentin in TCs and MSCs was measured by Western blotting. As shown in Fig. 2D and 2E, CD34 and c-kit were strongly expressed in TCs, but were barely detectable in MSCs. The vimentin expression level was similar in both TCs and MSCs. Altogether, these results further confirm the difference between TCs and MSCs.
Cytokine Expression Profile in Supernatants of TCs and MSCs

Subsequently, we investigated cytokine expression in supernatants of TCs and MSCs using a cytokine antibody array. As shown in Fig. 3, a total of 49 cytokines were detected in the supernatants of TCs and MSCs. Of 49 cytokines tested, 30 were not significantly changed in supernatants of TCs compared with MSCs (Fig. 3A–3C). Strikingly, 19 of 49 cytokines were dramatically increased, and 9 of 19 cytokines were increased at least 2-fold in supernatants of TCs compared with MSCs (Fig. 3D–3E). These results reveal that the cytokine expression profile is different in TCs and MSCs.

Fig. 2. Identification of differences between TCs and MSCs by immunofluorescence, RT-qPCR, and Western blotting. (A, B) The expression of CD34, c-kit, and vimentin was detected by immunofluorescence in TCs and MSCs (n = 3). Scale bar = 200 μm. (C) mRNA expression of CD34, c-kit, and vimentin was measured by RT-qPCR in TCs and MSCs (n = 3). *p < 0.05. (D) The protein expression level of CD34, c-kit, and vimentin was evaluated by Western blotting in TCs and MSCs. Representative pictures are shown. (E) Quantitative analysis of changes in expression of CD34, c-kit, and vimentin in TCs and MSCs. (n = 3). *p < 0.05.
ELISA Assay of Cytokines Expressed Differentially in TCs and MSCs

In order to confirm the results of cytokine profiles identified using antibody arrays in myocardial TCs and MSCs, we detected six cytokines: GM-CSF, IL-1β, IL-2, TGF-β1, and FGF-6 with the corresponding ELISA kits and the results are shown in Fig. 4. The results of the ELISA assay indicated that GM-CSF, IL-1β, IL-2, TGF-β1, and FGF-6 were markedly elevated in TCs compared with MSCs (Fig. 4A–4F), and these results further confirmed the cytokine expression profile assay. Altogether, our data demonstrate that expression of secreted cytokines is different between TCs and MSCs.

Discussion

In the present study, we compared differences between mouse myocardial TCs and MSCs. Firstly, we confirmed previous descriptions of TCs and MSCs in terms of morphology and cellular structure. TCs have specifically moniliform feature and extremely long and thin cellular elongations, which were named telopodes. The shape of the TC body depends largely on the number of telopodes, and it can be piriform, spindle-like, or triangular depending on the number of telopodes. The features of MSCs are very different from those of TCs. Typical MSCs are moniliform, polygon, flat star and so on, in shape. MSCs have a bigger cellular body with cellular elongations, but there is no telopode structure on the cells. Therefore, all the morphologic characteristics identified in the present study are consistent with previous reports.

Secondly, we further confirmed several specific markers in TCs and MSCs using immunofluorescence, RT-qPCR, and Western blotting. Our results revealed that CD34, c-kit, and vimentin were positive in mouse myocardial TCs, whereas CD34 and c-kit were negative and vimentin was positive in MSCs. Because TCs and MSCs are interstitial cells, they were both positive for vimentin as well. Because TCs are identified around the blood vessels and separated from the endothelial cells of the capillaries by collagen bundles, fibrocytes, and pericytes, they are c-kit positive. Additionally, TCs have been identified as being closely related to stem cells in these tissues; thus, they are also CD34 positive. Altogether, these findings not only uncovered differences between TCs and MSCs, but also further confirmed previous studies that reported the features of TCs in vivo.

Thirdly, we detected 49 various types of cytokines in TCs and MSCs using a cytokine antibody array, including transforming growth factor, tumor necrosis factor, inflammatory cytokines, angiogenesis factors, growth factor, chemokine, and matrix metalloproteinases. The cytokine expression profiles identified in the current work for mouse myocardial...
TCs and MSCs were different from results of protein profiles obtained when comparing human lung or myocardial TCs and MSCs. These results indicate that the cytokine expression specificity for TCs is closely related to a specific tissue, and also indicates the existence of TCs subtypes. Here, we showed that the expression of 19 of 49 cytokines differed completely between myocardial TCs and MSCs. In particular, the expression of GM-CSF, IL-1β, IL-2, TGF-β1 and FGF-6 was significantly higher in TCs than in MSCs.

GM-CSF stimulates stem cells to produce granulocytes (neutrophils, eosinophils, and basophils) and monocytes, and plays a central role in anti-infectious immunity. In recent studies, it was shown that GM-CSF participates in tissue repair. TGF-β1 was first identified in human platelets as a protein with a potential role in tissue repair, and it also plays an important role in controlling the immune system, showing different activities on different types of cell, or cells at different developmental stages. FGF is a potent epithelial cell-specific growth factor involved in cell growth and tissue repair. These studies in mouse and rat demonstrate the roles of cytokines in the healing of wounds and in bone repair.

Taking into account that the myocardium is rich in connective tissue, the higher expression of TGF-β1 and FGF in myocardial TCs might indicate that TCs participate in the repair of heart tissues, which has also been hypothesized previously. Kostin et al. reported that myocardial TCs could be involved in tissue regeneration and the repair of injured heart tissues. There were 19 cytokines with a higher expression level in TCs than in MSCs, especially GM-CSF, IL-1β, IL-2, TGF-β1, and FGF-6. These cytokines are destined for the extracellular matrix, and play a role in enhancing the production of extracellular matrix components and wound healing. More recent findings also reported that rat myocardial TCs secrete some cytokines, including IL-2, IL-10, IL-13, and GRO-KC, and concluded that the TC secretome plays a modulatory role in stem cell proliferation and differentiation. Other findings suggest that subsets of TCs belong to the endothelial lineage while other subsets of cells with TC-like morphology might be actually stem/progenitor cells.

In conclusion, our results not only confirm that TCs and MSCs are different types of interstitial cells, but also reveal that TCs and MSCs have different morphology, immune phenotype, and cytokine secretion profiles. Further investigation will clarify the specific functions of TCs and MSCs in vivo.
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Ethical Approval
The study was approved by the Ethics Committee of Fudan University.

Statement of Human and Animal Right
The mice MSCs were purchased from Chinese Academy of Science, Kunming Cell Bank (Cat. no. KCB200537, HSF, Kunming, China) and were preserved at the Biomedical Research Center of Zhongshan Hospital (Fudan University, Shanghai, China).

Statement of Informed Consent
There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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