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Human diploid MRC-5 cells exhibit several critical properties of human umbilical cord-derived mesenchymal stem cells

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ABSTRACT

MRC-5 is the most common human diploid cell line used in production of viral vaccines; mesenchymal stem cells (MSCs) is a type of adult multipotent stem cells. Both cell types share the same fibroblast-like morphology and maintain a normal diploid karyotype over long *in vitro* expansion. However, other than these similarities, very little is known about MRC-5 in terms of biological properties possessed by MSCs. In this study, we compared MRC-5 with human umbilical cord-derived MSCs (hUC-MSCs), which serves as a representative of human MSCs, in expression of cell surface markers, abilities to differentiate into multiple cell lineages, inhibition of lymphocyte proliferation and promotion of Regulatory T lymphocytes (Treg), and IDO1 expression in response to inflammatory cytokines, all of which are critical properties of MSCs. It was revealed that MRC-5 was almost identical to hUC-MSCs in expression of both positive and negative surface markers of MSCs. Similar to hUC-MSCs, MRC-5 was also able to differentiate into osteocytes and chondrocytes, effectively inhibit mitogen-activated lymphocyte proliferation and promote Tregs, and express IDO1 in response to inflammatory cytokines IFN-γ and TNF-α. In addition, both MRC-5 and hUC-MSCs were non-tumorigenic with an extremely low telomerase activity. Moreover, both cells demonstrated a similar sensitivity to infection by EV71 and rubella viruses, which served as model viruses, in a virus infectivity assay. Therefore, this study suggests that MRC-5 is very likely a previously undefined MSC cell line, thus suggesting the feasibility of developing MSCs of at least umbilical cord origin as new cell substrates to be used in production of viral vaccines.

1. Introduction

MRC-5 is the most commonly used fibroblast-like human diploid cells (HDC) in the production of viral vaccines[1,2]. It was originally derived from human lung tissues of a 14-week-old male fetus and able to continuously grow for more than 40 passages while keeping normal diploid karyotype [3]. MRC-5 is sensitive to infection of a large variety of viruses, such as polio, herpes simplex, rubella, measles, hepatitis A and enterovirus 71 (EV 71) viruses [4–9], and has thus been used as a common cell substrate for production of viral vaccines. However, other than these, further in-depth biological understanding of MRC-5 has not yet been achieved.

Mesenchymal stem cells (MSCs) are fibroblast-like multipotent stromal cells existing in nearly all somatic tissues, such as bone marrow, adipose tissue and tendon of both adult and fetus, or in birth-related tissues, like umbilical cords and placenta [10,11]. MSC of various tissue origins possess abilities to differentiate into cell lineages, such as adipocytes, chondroblasts, and osteoblasts [12]. The International Society of Cellular Therapy (ISCT) has defined MSCs with three minimum criteria: (i) adherence to plastic; (ii) greater than 95% of cell population express CD73, CD90, CD105 positive surface markers and less than 2% express CD11b, CD14, CD34, CD45 and HLA-DR negative surface markers; (iii) the abilities to differentiate into osteoblasts, adipocytes and chondroblasts through each standard differentiation protocol [13].

MSCs also exhibit unique immunomodulatory functions. Within inflammatory environments, MSCs can be activated by proinflammatory cytokines to suppress proliferation of lymphocytes and polarize naïve T lymphocytes toward immunosuppressive Regulatory T cells (Treg) for promoting tissue homeostasis [11]. Thanks to their immunomodulatory functions, MSCs has been used in cell-based therapies for treating various diseases with uncontrolled inflammatory responses underlying the

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major pathogenesis [14–17]. In addition, it is believed that the immunomodulatory properties of human MSCs are mediated by biologically active molecules, especially indoleamine 2,3-dioxygenase 1 (IDO1)[18,19]. IDO1 catalyzes a rate-limiting step in degradation of tryptophan along the kynurenine pathway, the downstream metabolites of which contribute to the immunomodulatory activities of IDO1-expressing cells [20].

Similar to MRC-5, MSCs also exhibits a substantial *in vitro* growth activity and cytogenetic stability. Human MSCs can grow continuously over 25 passages without inducing senescence, while maintaining normal diploid karyotype [21]. Moreover, human MSCs are non-tumorigenic as they do not form tumors in both animals and humans [22]. Supportively, MSCs of different origins generally exhibit low activity of telomerase reverse transcriptase (hTERT) comparing with embryonic stem cells or malignant cells [23,24].

Given the similarities in cell morphology, growth activity and cytogenetic stability shared by MRC-5 and MSCs, it is of great interest to further determine whether MRC-5 may also possess other critical properties of MSCs, such as expression of surface markers, differentiation potentials, and immunomodulatory functions. It is also interesting to investigate whether human MSCs exhibit a similar sensitivity to viral infection as MRC-5. The comparative studies will provide critical inputs for developing new quality control technologies to evaluate the qualities of currently existing HDCs. More importantly, these studies can inspire developing MSCs as new cell substrates, which may meet the same requirements as the classical HDCs for vaccine production.

2. Materials and methods

2.1. Materials

(1) *Cells*: MRC-5 was purchased from ATCC (Rockville, MD) and cultured in MEM complete medium; hUC-MSCs was isolated from Wharton's Jelly of a human umbilical cord after normal labor and cultured in α-MEM complete medium. It was validated in our laboratory for their freedom from adventitious agents; Vero and RK-13 used for viral titration were from ATCC. (2) *Viruses*: EV71 virus, strain SH06, was from Sinovac Biotech Ltd; Rubella virus, strain RA27/3, was from ATCC. (3) *Antibodies*: The IDO1 antibody was from Cell Signaling (MA, USA) and β-actin antibody was from Sigma–Aldrich (St. Louis, MI). The FITC-conjugated CD4, APC-conjugated CD25, PE-conjugated CD127, and PE-conjugated CD166 antibodies were from BD (San Diego, CA, USA). (4) *Chemicals*: IFN-γ and TNF-α were from R&D system (Minneapolis, MN); Phytohemagglutinin (PHA) was from Sigma–Aldrich; Hybond^{TM} ECL was from GE Healthcare (Piscataway, NJ); STEMPRO Differentiation Kits were from Life Technologies (Grand Island, NY); BD Stemflow hMSC Analysis Kit for testing CD44, CD73, CD90 and CD105, and the negative cocktail was from BD Biosciences. 1-Methyl-L-Tryptophan (1-L-MT) [20], an IDO1 inhibitor, was from Sigma–Aldrich.

2.2. Detection of cell surface markers

The cell surface markers were examined using each dye-conjugated antibody included in the BD Stemflow hMSC Analysis Kit or PE-conjugated CD166 antibody. Briefly, the cells in approximately 90% confluence were trypsinized, washed and then re-suspended at 5×10^6 cells/ml PBS. Each 100 μl cell suspension was stained through incubation at room temperature for 30 min with the fluorescent dye-conjugated antibodies against positive marker CD73, CD90, CD105, CD44, CD166, or the negative marker cocktail containing equally mixed PE-conjugated antibodies against CD11b, CD19, CD34, CD45 and HLA-DR, or each isotype control antibody. After staining, the cells were washed with PBS, and analyzed using BD FACS Calibur flow cytometer. The expression of each marker or cocktail markers was determined by subtracting each fluorescent value from the value of each control antibody.

2.3. Cell differentiation

The abilities of hUC-MSCs or MRC-5 to differentiate into adipocytes, chondrocytes and osteocytes were analyzed by using STEMPRO Differentiation Kit. Briefly, the cells growing in approximately 60% confluence for testing adipogenesis and osteogenesis, or in small masses with each containing 8×10^4 cells for testing chondrogenesis, were incubated at 37 °C, 5% CO_2 in each well of a 12-well cell culture plate with each differentiation medium for 14–21 days. After incubation, the cells were fixed with 4% formaldehyde for 30 min, and then stained with 0.3% Oil Red O solution for 50 min, with 2% Alizarin Red S solution for 3 min, or with 1% Alcian Blue solution for 30 min for testing adipogenesis, osteogenesis or chondrogenesis, respectively. After thorough washing with water, the images from each staining were analyzed under a light microscope. In each differentiation assay, the cells growing in regular complete medium were used as the negative control.

2.4. Flow cytometry assays for detecting MSC-induced changes in lymphocyte proliferation and Tregs subpopulation

For testing lymphocyte proliferation, 1×10^6 peripheral blood mononuclear cells (PBMCs) freshly isolated from one healthy donor using Ficoll solution and suspended in 1 ml PBS were incubated with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) at room temperature for 5 min [25]. After thorough washing with PBS containing 5% FBS, the CFSE-labeled PBMCs were then co-cultured with MRC-5 or hUC-MSCs in 5:1 ratio for 7 days in RPMI1640 complete medium containing 10 ng/ml PHA at 37 °C, 5% CO_2 in a well of a 12-well cell culture plate. After incubation, all lymphocytes were collected and cell proliferation was determined by gradual reduction of CFSE fluorescence using a BD FACS Calibur flow cytometer. The PBMCs without labeling, PHA stimulation, or co-culturing were used as negative controls.

For testing Tregs, 1×10^6 fresh PBMCs were co-cultured with MRC-5 or hUC-MSCs in 5:1 ratio with or without 0.5 mM 1-L-MT in each well of a 12-well cell culture plate for 5 days in RPMI1640 complete medium containing 10% FBS. Then, the PBMCs in each well were collected and stained at room temperature for 30 min with FITC-conjugated CD4, APC-conjugated CD25 and PE-conjugated CD127 antibodies together. After staining, the cells were washed twice with PBS, and analyzed using BD FACS Calibur flow cytometer. The $CD4^+CD25^+CD127^-$ cells were identified as Treg subpopulation of lymphocytes.

2.5. Viral infectivity assay

EV71 and rubella viruses were chosen as model viruses in this assay. Equal number of each cell type growing in MEM medium with 2% FBS in full confluence in T25 cell culture flasks was inoculated with diluted EV71 virus of MOI 0.0025 or rubella virus of 1:30 dilution from viral seeds. The infection with EV71 continued at 37 °C, 5% CO_2 until the appearance of significant cytopathic effect (CPE). At the end of EV71 infection, virus suspension was prepared through a quick freezing-thawing method and used for viral titration and antigen measurement. For rubella virus, the supernatant of the virus-infected cells were collected at day 4, 8, 12, and 16, and used for viral titration. The cells from mock infection were used as negative control.

For viral titration, each serial dilution from EV71 or rubella viral suspension was inoculated in triplicate onto 1×10^4 Vero cells,