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Biological characters of human dermal fibroblasts derived from foreskin of male infertile patients

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ABSTRACT

Dermal fibroblasts play a vital role in maintaining skin function. They not only synthesize and secrete extracellular matrix molecules, but also produce a complex mixture of bioactive factors, which both contribute to immune regulation and wound healing. Fibroblasts isolated from skin tissue exhibit wide range of potentials, especially in regenerative medicine. The use of fibroblast cultures for medical purposes requires standardization of cell preparations. To achieve this, we isolated and characterized dermal fibroblasts from human foreskin with a standardized method. The obtained cells grew as typical morphology of fibroblasts, and expressed intermediate filament protein vimentin and nestin. Immunophenotypic analysis indicated that the isolated fibroblasts expressed mesenchymal surface markers CD73, CD90, CD44 and CD105, and were negative for haematopoietic markers CD45 and CD34. Growth kinetics analysis of the cells showed high proliferative properties. Furthermore, cryopreservation had no influence on cell morphology and growth properties. Here, we describe a standardized, repeatable method for isolation of fibroblasts from human foreskin tissues and identify their biological characters according to morphologic, immunohistologic and proliferative criteria, which would be meaningful for future clinical trials and regenerative medicine purposes.

1. Introduction

Fibroblasts are the principal cellular constituents of connective tissues, which appear in most tissues and organs of human body. Fibroblasts are often described morphologically as elongated, spindle shaped cells that are characterized by high proliferative and migration potential. Fibroblasts have multiple biological functions. They not only synthesize and secrete extracellular matrix (ECM) molecules, but also produce a complex mixture of bioactive factors (growth factors, cytokines, and chemokines), which both contribute to immune regulation and wound healing. In addition, fibroblasts interact with nearby tissues and cells through paracrine, autocrine, and other forms of communication (Sorrell and Caplan, 2009).

Fibroblasts isolated from skin tissue exhibit wide range of potentials, especially in regenerative medicine (Bussmann et al., 2016; Guerreiro et al., 2014; You and Han, 2014). In the last years, rapid development of regenerative medicine is primarily related to achievements of cell cultures for restoration of lost functions of the body. The use of cells including fibroblasts, epithelial cells and stem cells, as replacement therapy of damaged tissue and for stimulation of regenerative processes in the body, creates hope for structural and functional recovery in pathologies that are difficult to cure by other methods (Gennai et al., 2015; Novak et al., 2014; Yang and Jia, 2014). Moreover, human fibroblasts play a significant role in drug testing or toxicology screening (De Falco et al., 2013).

Human fibroblasts cultures in vitro may also provide a model for understanding of important process as wound healing, extracellu-

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lar protein synthesis, drug delivery, cell migration and proliferation in pathological or physiological conditions. This interest has arisen largely as a consequence of recent work demonstrating that human fibroblasts can be reprogrammed into induced pluripotent stem cells by defined transcription factors (Takahashi et al., 2007). Successful reprogramming of differentiated human fibroblasts into a pluripotent state allow the creation of patient- and disease-specific stem cells which has opened up new pathways to better understanding of many human diseases, and has created new therapeutic approaches (Chen et al., 2014; Goodridge, 2014; Qiu et al., 2013).

Fibroblasts could be isolated from different tissues with varied protocols. Compared with other sources, human skin tissue is easily accessible and with less ethical controversy and rejection problems. Therefore, human fibroblasts isolated from skin tissue are a convenient material for future clinical applications. Explant adherent method and tissue dissociation method are two basic methods for establishing primary fibroblast cultures from tissues. However, treating small pieces of tissue extensively with proteinases like trypsin-EDTA or collagenase may compromise cellular viability by an overly incubation with enzymes. Thus, the most commonly used method to establish primary fibroblast cultures is the explant adherent culture method (Balin et al., 2002).

Here, we present a standardized protocol to isolate and culture fibroblasts from foreskin of male infertile patients with explant adherent culture method. These fibroblasts could be maintained in Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum (FBS) without specific growth factors, easily trypsinized and passaged for more than 10 passages. We also identified their biological characters according to morphologic, immunohistologic and proliferative criteria. The obtained fibroblasts may be clinically useful for regenerative medicine and potentially effective for cell biological research.

2. Materials and methods

2.1. Ethical approvement and informed consent

Foreskin specimens were collected from the center of reproductive medicine of Tongji Medical College. Tissue specimens were obtained from male infertile patients under circumcision. Surgical procedures were carried out by trained and authorized urological surgeon. Written informed consents were obtained from all patients before surgery. The research has been carried out in accordance with the Declaration of Helsinki (2008) of the World Medical Association and the Institutional Review Board of Tongji Medical College approved the study.

2.2. Collection of human foreskin tissue and transport

Pieces of human foreskin of 1–2 cm^2 were derived from voluntary circumcisions of infertility patients aged 25–40 years old. The biopsy tissue was placed into a sterile tube filled with phosphate buffered saline (PBS) without $Ca^{2+}Mg^{2+}$ containing 1% penicillin/streptomycin (Invitrogen, USA), and transported to laboratory on ice for immediate processing or stored at 4 °C until the following day.

2.3. Establishment of fibroblasts cultures from foreskin tissue

The subsequent steps are performed in a tissue culture hood under aseptic conditions and using sterile instruments. Place the human foreskin tissue in a 100-mm Petri dish and rinse in 75% (v/v) ethanol for 5 min. Wash the tissue with PBS for three times in a clean Petri dish. Put the washed human foreskin tissue in another new 100-mm Petri dish. Carefully and completely remove the subcutaneous fat and capillaries. Wash in PBS repeatedly and transfer the tissue into a 50 ml falcon tube, add 4 ml of 0.05% trypsin/EDTA (Invitrogen, USA), and incubate overnight at 4 °C. After that, inactivate the trypsin by adding about 4 ml of freshly prepared fibroblasts culture medium (DMEM containing 10% FBS, 1% penicillin/streptomycin, 1% glutammine and 1% non essential amino acids, all Invitrogen, USA). Discard the supernatant and the epidermis is manually removed from the tissue piece.

Then, dissect the dermis tissues into small pieces (about 1 mm^3) by sterilized scissors and forceps. Transfer the chopped tissues to a 100-mm Petri dish with a distance of about 5 mm between every two pieces, and incubate at 37 °C, 5% CO_2 for 2–4 h to make the pieces adhere to the bottom of the dish. Then, add 2 ml of fibroblasts culture medium to cover the bottom and keep the pieces humidified. Continue to incubate at 37 °C, 5% CO_2.

The following day 3 ml of fibroblasts culture medium was slowly added, and subsequently the medium was changed every 3 days. The cultures were monitored daily by an optical microscope.

When dense outgrowths of fibroblasts appear, remove the tissue pieces from the dish carefully using sterile forceps, aspirate the medium and add fresh culture medium to keep the fibroblasts growing (passage 1). Continue to passage by trypsin/EDTA at a ratio of 1:3 every 5–7 days when the cells have reached 80% confluence.

2.4. Cryopreservation and recovery

After trypsinization and washing, the cells were centrifuged at 1000 rpm for 5 min. The supernatant was discarded. The cell pellet was re-suspended in a freezing medium containing 60% fibroblasts culture medium, 30% FBS and 10% dimethyl sulfoxide (DMSO) (Sigma, USA). The cell suspensions were kept in cryovials (Corning, USA) that were placed at 4 °C for 15 min followed by −20 °C for 2 h, −70 °C overnight, and then transferred to liquid nitrogen for long-term storage. To check the recovery rate and viability after freezing, the frozen fibroblasts were thawed in a 37 °C water bath and plated into 6-well culture dishes.

2.5. Immunofluorescence staining

Fibroblasts were fixed in 4% paraformaldehyde in PBS and permeabilized with 0.4% Triton X-100 (Sigma, USA) in PBS for 10 min. After blocking, the primary antibodies against vimentin (CST, USA) and nestin (RD, USA) were diluted at 1:100 and incubated overnight at 4 °C. A staining for DAPI was performed before staining with appropriate secondary antibodies conjugated to CY3 or FITC (Jackson ImmunoResearch, USA). Slides were examined under fluorescence microscope (Olympus, Japan). We counted the number of cells positive for nestin but negative for vimentin in five images of random fields, calculated its percentage in total cells, and took the average as the final result.

2.6. Analysis of immunophenotype by fluorescence-activated cell sorting (FACS)

The cells were harvested with 0.05% trypsin/EDTA, counted with a hematocytometer, washed, and resuspended in PBS before incubation with antibodies. Monoclonal antibodies against surface markers including CD34, CD44, CD45, CD90, CD73 and CD105 labeled with fluorochromes (eBioscience, USA) were used for staining, and isotypic antibodies conjugated with the corresponding fluorochrome served as the negative control. Incubation with the antibodies was carried out in 100 μl suspension containing 10^6 cells at 4 °C for 30 min in dark. The cells were washed twice with PBS. Analysis was performed on FACSAria sorter (BD Bioscience, USA).