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Research Report

The transdifferentiation potential of limbal fibroblast-like cells

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Abstract

We report the identification and isolation of limbal fibroblast-like cells from adult corneo-limbal tissue possessing self-renewing capacity and multilineage differentiation potential. The cells form cell aggregates or clusters, which express molecular markers, specific for ectoderm, mesoderm and endoderm lineages in vitro. Further, these cells mature into a myriad of cell types including neurons, corneal cells, osteoblasts, chondrocytes, adipocytes, cardiomyocytes, hepatocytes and pancreatic islet cells. Despite originating from a non-embryonic source, they express ESC and other stem cell markers important for maintaining an undifferentiated state. This multipotential capability, relatively easy isolation and high rate of ex vivo proliferation capacity make these cells a promising therapeutic tool.

Theme: Development and regeneration
Topic: Cell lineage and determination

Keywords: Eye; Limbal stem cell; Stromal cell; Isolation; Cell aggregate; Transdifferentiation

1. Introduction

Human pluripotent embryonic stem (ES) cells derived from the inner cell mass of blastocyst can be propagated indefinitely in an undifferentiated state [30,41]. These pluripotent stem cells can be differentiated into all cell lineages both in vivo and in vitro conditions [36,51]. However, the use of human ES cells has raised substantial ethical and technical issues [28]. One way to obviate such ethical issues is to generate pluripotent stem cells directly from other sources, of adult origin. ES-like pluripotent cells have been identified in many tissues such as bone marrow [42,48], brain [45], amniotic fluid [40], inner ear [25], skeletal muscle [1,64] and hair follicle [50], neonatal mouse testis [19] and brachial arch [9]. Similarly, recent studies have shown that tissue-specific stem cells can differentiate into lineages other than the tissue of origin including hematopoietic, neural, gastrointestinal, epidermal, hepatic and mesenchymal tissues [2,14,18,39,57,58]. This raises the possibility that either pluripotent cells or cells with the ability to transdifferentiate into multiple different lineages may exist in the limbal system as well.

Over the past several decades, human ocular stem cell research has been mainly focused on the tissue-specific differentiation that may be of clinical significance [10,16,21,22,24,32,54,60]. More recently, it has been reported that corneal cultures do have transdifferentiation capabilities [49,62], though the in vitro potential and isolation of specific fractions of limbal stromal cells have not been reported [11]. Isolation of pluripotent stem cells that forms a readily accessible source that is already clinically used has many potential advantages compared to some of the other sources of pluripotent cells and we have begun assessing the properties of specific fractions of such cells.

In this report, we describe a subpopulation of limbal stromal cells that possess a unique set of characteristics. We found that these fibroblastic appearing cells show an

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unambiguous expression of stem cell markers important for the maintenance of undifferentiated state. The derived cells after enrichment showed the ability to form cell aggregates or clusters, and exhibit a multi lineage transdifferentiation potential in vitro. The source of these cells may prove to be a promising candidate in autologous transplantation, for various degenerative diseases in the future.

2. Materials and methods

2.1. Ex vivo expansion of limbal cells on matrigel

After securing the approval of the Institutional Review Board and informed consent from the patients, 2–3 mm^2 of limbal biopsies were obtained [10] from patients undergoing cataract surgery with no ocular surface or intra-ocular disease, adhering to the "Tenets" of "Helsinki Declaration" in 1964. The limbal biopsy was incubated in Phosphate Buffer Saline (PBS) with penicillin–streptomycin and gentamycin for 2–5 min. The biopsy was explanted as such on matrigel [47] and cultured for 10–14 days, which was further checked for the expression of progenitor cells and committed cell markers as an initial experiment.

2.2. Derivation of limbal stromal cells

After the initial enzymatic treatment, the upper-pigmented epithelial segment was completely separated from the stromal zone [11] of the corneo-limbal biopsy with a sterile blade under stereomicroscope. Further, the isolated stromal tissue segment was separately plated on 1% matrigel-coated, 35 mm tissue culture plates (Nunc) and cultured in an expansion medium constituting of DMEM/F-12 (1:1) supplemented with 10% knockout serum (Invitrogen, USA), basic-FGF 4 ng/ml insulin 5 μg/ml, transferrin 5 μg/ml, sodium selenite 5 μg/ml (Sigma, USA) and human LIF 10 ng/ml (Chemicon, USA) for 12–15 days.

2.3. Isolation of SSEA-4^+ limbal fibroblast-like cells by MACS

After an initial passage, the limbal stromal cells were trypsinized (0.25% Trypsin-EDTA; Gibco-BRL, USA) and resuspended in PBS at a concentration of 5×10^4 cells/μl and incubated with 1 μl anti-SSEA-4 antibody (DSHB, USA) for 30 min at 4 °C. The cells were then washed twice with PBS to remove unbound antibody. After washing, the cell suspension was incubated with 15 μl of secondary antibody magnetic beads tagged with goat anti mouse IgG (1:4; Miltenyi Biotech, Germany) for 20 min at 4 °C followed by a wash with PBS. The cell suspension was then passed through magnetic column (Miltenyi Biotech, Germany). The positive fraction of SSEA-4 cells thus derived from limbal stromal cultures were then washed twice with PBS and plated on 1% matrigel-coated plates in the respective media mentioned earlier. These SSEA-4^+ cells were replated every 5–6 days at a dilution of 1:3 under similar culture conditions.

2.4. Clonogenic assay

The confluent SSEA-4^+ cells were dissociated with 0.05% trypsin-EDTA and viability determined. The cells were then diluted in the expansion medium and preconditioned medium in a ratio of 1:1 and plated in 96-well plate. Preconditioned media was obtained from the fully confluent SSEA-4^+ cells grown in expansion medium. The culture media was pooled, filtered and stored at −80 °C [64]. Each well of a 96-well plate was monitored under the microscope for the presence of a single cell. Wells with more than one clone were not considered. The clones with a high proliferative capacity were selected, propagated further, expanded and used for in vitro differentiation and characterization.

2.5. RNA isolation and RT-PCR

Total RNA was isolated by TRIzol method (Invitrogen, USA) and 1 μg of RNA treated with RNase-OUT ribonuclease inhibitor (Invitrogen, USA) was used for cDNA synthesis. Reverse-transcription was carried out using Superscript reverse-transcriptase II (Invitrogen, USA) and Oligo dT (Invitrogen, USA) to prime the reaction. 2 μl of cDNA was amplified by polymerase chain reaction using Abgene 2× PCR master mix (Abgene, USA) and appropriate primers (Table 1). The set of undifferentiated stem cell markers evaluated were Oct-4, Nanog, Rex-1, TDGF-1 and Sox-2 [25–29] and tissue-specific markers were NFH, keratin, cardiac-actin, AFP, albumin and AE-5 (Table 1). GAPDH was used as a housekeeping gene control.

2.6. Immunofluorescence analysis

The cells grown in 2-well chamber slides (Becton Dickinson, USA) were fixed in freshly prepared 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS. The non-specific binding sites were blocked with 1% bovine serum albumin in PBS. The cells were then incubated overnight at 4 °C with primary antibody. Undifferentiated stem cell markers like Oct-4, Tra1-60, Tra1-80, SSEA-4 and an exhaustive list of differentiated markers corresponding to three lineages like ectoderm, mesoderm and endoderm like nestin, NFH, TH, MAP-2, Oligodendrocyte (O4), GAD-65, glutamate, beta-MHC, albumin, PDX-1, stro-1, P-63, Cytokeratin 19 and AE-5 as shown in the Table 2 were evaluated. FITC/Texas Red coupled secondary antibody was applied at a dilution of 1:500 for 1.5 h at room temperature. Immunostained cells were mounted and visualized through inverted fluorescence microscope (Nikon Eclipse E600). Cells were also counterstained with DAPI (1 μg/ml; Molecular Probes).