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EPITHELIAL CELLS

Comparative study of isolation, expansion and characterization of epithelial cells

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Abstract

Background aims. The human epithelial cells (EPCs) have been identified as the essential element for the regeneration of skin construct for burns, wounds and various tissue engineer–based products. *Methods.* In this study, the isolation, expansion and characterization of EPCs from various sources such as juvenile foreskin (JSK), buccal mucosa (BM), penile skin (PS) and urothelium (UR) in serum-free and xeno-free EpiLife media were evaluated. *Results.* The growth kinetics study revealed that EPCs from JSK and BM had notably higher growth rates compared with the others. Overall, the EPCs from all sources retained basic morphological characteristics and the functional characteristics such as Pan Cytokeratin (AE1/AE3). In addition, the cryopreservation stability of EPCs was accessed for post-thaw viability and found to be greater than 80% at 1 year of storage, but demonstrated reduced cell recovery (51%) at the second year in fetal bovine serum–free cryopreservation media. *Conclusions.* Our result suggests that the EPCs from four cell sources can be grown in feeder-free, serum-free and xeno-free systems using commercially available EpiLife medium without losing epithelial cell characteristics even after passage 4. However, its suitability for clinical application must be accessed by preclinical and clinical studies.

Key Words: *cell therapy, epithelial cells, growth kinetics, epithelial cell source, tissue engineering*

Introduction

Epithelial cells (EPCs) hold considerable promise for therapeutic application in tissue engineering to repair, replace and regenerate damaged or aged cells and tissue. In most tissue-engineered products (TEPs), the EPCs or keratinocytes are the active cellular ingredients to form living skin substitutes, apart from other biomaterial excipients [1,2]. Transplantation of cultured epithelium to treat second- or third-degree burns on large areas was first successfully demonstrated in 1980. In addition, EPCs have been extensively studied in clinics to assess their safety and efficacy for damaged ocular surfaces [3,4], chronic non-healing wounds [5], venous leg ulcers [6–8], burns [9–11] and urethral stricture [12–14]. Several EPC-based products, such as Apligraf, OrCel [15], Epicel [16], EpiDex [16] and Bioseed [17], have been successfully commercialized. Such EPCs require clinical manufacturing and production, which starts with cell isolation, expansion, cryopreservation, current Good Manufacturing Practice compliance and adequate cell numbers without comprised quality or function.

The traditional isolation procedures and subculturing of cells use feeder and fetal bovine serum (FBS)-based methods and have the advantage of rapid proliferation with strong resistance to apoptosis [18]. However, residual animal source serum content and risk of bovine spongiform encephalopathy or transmissible spongiform encephalopathy are unavoidable, even after post-trypsinization washes of cells. The manufacturing process of some EPC-based TEPs (e.g., Apligraf) still include an FBS-based method, and although there has been significant positive clinical outcome, the risk of immunogenicity exists [2,19]. Therefore, the EPCs for clinical manufacturing require appropriate cell isolation and expansion procedures without using serum, porcine trypsin and the 3T3

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mouse cell line for feeder to ensure safety, consistency and reliability and xenogeneic-free media.

EPCs were first successfully isolated and cultured more than 40 years ago [20–22], and have subsequently been isolated from various tissues, including skin, penile skin, embryonic cells, oral, buccal mucosa, neonatal foreskin and amniotic membrane [21–28]. However, there is a no consensus on the preferred cell source, which would be determined by the achievable cell number at lower passage and the type of disease indication. Hence, in the present study, we optimized isolation enzyme concentration for clinical-scale expansion of EPCs for therapeutic applications. We compared the cell yield, morphological characteristics, growth kinetics and functional analysis of EPCs derived from four sources: juvenile foreskin (JSK), buccal mucosa (BM), penile skin (PS) and urothelium (UR). Generalized post-thaw viability (stability) of EPCs were accessed up to 2 years.

Methods

Biopsy collection

The Rangadore Hospital ethics committee approved this study. The patients undergoing circumcision (for JSK and PS) and urethroplasty with buccal mucosa for urethral strictures (for BM and UR) provided the samples for the study after providing informed consent. In the case of the minors, parents gave informed consent.

Biopsy samples were rinsed in povidone-iodine for 5–10 s and then with 10 mL of ofloxacin solution for 5 min, followed by rinsing with normal saline solution for 10 s. The biopsy was preserved in the transport medium containing 10 mL of 10× (50 IU/mL penicillin-streptomycin and 5 mg/mL amphotericin B) Anti-Anti-100× antibiotic-antimycotic (Life Technologies/Gibco)–enriched Hank's Balanced Salt Solution (Life Technologies/Gibco) solution. The transport medium containing the biopsy was shipped at 2–8°C for clinical manufacturing facility for further processing.

Hematoxylin and eosin staining

The histological characteristics of tissue biopsy of the four tissue sources were assessed by careful sectioning and then fixing in formalin solution for 1 day at room temperature. Tissue samples were dehydrated with a series of increasing ethanol concentrations of 30%, 50%, 70% and absolute alcohol. This was followed by immersing in xylene and embedding in paraffin. The paraffin cubes were prepared using microtome and then stained by Harris hematoxylin and eosin (H&E) Y stain. The stained slices were examined under a light microscope (Olympus INV) after mounting on the glass slides. The images were captured at 280× magnification.

Biopsy processing and isolation

The tissue samples from all four sources was processing aseptically in a biological safety cabinet in a class 10,000 clean room. These tissue samples were trimmed to remove the dermis and thus be as thin as possible. Tissue samples were washed thrice with 10× Anti-Anti antibiotics for 20 min each. The size of each biopsy tissue sample was recorded. The tissue samples were cut with a scalpel blade into multiple pieces of 0.5 × 0.5 mm, and then the pieces were transferred to a 5-mL solution containing 1 mg/mL of Dispase II enzyme (Life Technologies) for overnight incubation. The dispase-treated tissue samples were rinsed once with Dulbecco's phosphate-buffered saline (PBS), and the epithelial cell sheets were separated by gentle peeling with sterile forceps from the dermal layer. The epithelial sheets were treated with 100 units (1 mg/mL) of collagenase type IV (Life Technologies) and dispase at (1:1 ratio) for 2 h after mincing and then treated with TripLE Select 1× for 5 to 10 min at 37°C. The released cells were neutralized with 9 mL of complete EpiLife medium (EpM) and then pipetted for several times to a get single cell suspension. The re-suspended cell solution was centrifuged for 10 min at 1400 rpm after being passed through a 100-μm mesh for JSK, PS and UR and 40-μm mesh for BM. The viability of the isolated cells was determined by the trypan blue dye exclusion method after re-suspending the cells in culture medium.

P0 seeding and sub-culturing

Six-well plates were coated with a coating matrix kit (Life Technologies) as per the manufacturer's instruction; EpM was prepared using S7 supplements (Life Technologies) and EpiLife basal media (Life Technologies) for serum-free and xeno-free cultures as per manufacturer's instruction. The single cell suspension of the four sources was seeded in a pre-coated six-well plate at a seeding density of 50,000 cells/cm^2 at passage 0 (P0). The cells were incubated at 37°C in a humidified 5% CO_2 incubator. During the culture process, the media were exchanged with freshly prepared EpM every 48 h until completion of five media changes or reaching 80–90% confluence. The epithelial cells were lifted from the culture vessels by digestion for 3–5 min at 37°C with TryPLE Express 1× (Life Technologies). The morphological changes during cultivation were captured by phase contrast microscopy. Experiments with the four cell sources were performed in triplicates.

Sequential expansion and screening of EPCs

The P0 harvested EPCs from respective cell sources were sequentially sub-cultured at a plating density of 4000 to 12,000 per cm^2. Cells were screened every other

day and complete media change was done at approximately 50% confluency and harvested at approximately 80–90% confluency. The respective culture flasks were screened for morphological observations, and the images were captured using a phase contrast inverted microscope (Olympus INV) at 280× magnification.

Growth kinetics

Epithelial cells from four sources (three donors for each source) from P1 to P5 were used to study the growth kinetics. Population doubling time (PDT) of EPCs from four cell sources were calculated. Cumulative population doublings (CPD) were calculated by sequential sub-culturing from P1 to P and harvested at 80–90% confluency at each passage.

MTT assay

Cell proliferation was determined using a 3-(4, 5-dimethylthiazol-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay at P2. EPCs from the four sources were seeded at a density of 5×10^5 to 1.25×10^4 cells/well in 96-well plates. Proliferation was assessed 24 h post-seeding by thiazolyl blue tetrazolium bromide (Sigma-Aldrich) according to manufacturer's instructions. The absorbance of the samples was measured at 570 nm using a microplate reader. Experiments were performed in triplicate.

Immunocytochemistry and immunophenotyping

The immunocytochemistry (ICC) of cultured cells at P3 of the four tissue sources was assessed for identification of epithelial cell markers such as Pan Cytokeratin (AE1/AE3). Twenty thousand cells were seeded on a glass cover slip and left for 2 days to reach confluence. The smear was fixed in cold methanol, acetone for 10 min and washed in Dulbecco's phosphate-buffered saline with Tween-20 for 10 min. These were then subjected to immunostaining of AE1/AE3 markers (BioGenex) per manufacturer's instructions. Briefly, after avidin-biotin blocking solution, the smear was painted with primary antibody and kept at 4°C overnight, and biotinylated secondary antibodies were incubated for 30 min. Visualization was carried out by H&E staining after 3,3'-diaminobenzidine stain.

A quantitative expression profile of epithelial cell adhesion molecule (EpCAM), cytokeratin (Cyto; cytokeratin 4 + 5 + 6 + 8 + 10 + 13 + 18), Involucrin (Invo) and human leukocyte antigen D related (HLA-DR) of EPCs from the four cell sources was established by flow cytometry. The EPCs at P3 were used and repeated thrice. The harvested cells were washed and adjusted to a concentration of 1×10^6 cells/mL in PBS solution. Cells were painted with 0.1–10 µg/mL of respective primary antibody (Phycoerythrin [PE] or fluorescein isothiocyanate [FITC], Abcam) in polystyrene round bottom 12 × 75 mm² falcon tubes and incubated for 30 min at room temperature. As negative controls, isotypes were run in parallel by omitting the primary antibody at the same concentration as the test samples. The cells were analyzed on a flow cytometer (Beckman Coulter, FC500 series). Thirty thousand events were acquired per sample, and side and forwarded scatter gates were set to exclude dead cells and debris using unstained cells. Isotypes followed by testing samples were run and analyzed. The following markers were analyzed: Cyto-FITC, Invo-FITC, EPCAM-PE and HLA-DR-PE (Abcam).

Post-thaw viability

One million EPCs from four sources at P1 were cryopreserved in standard serum-free cryopreservation medium containing 10% dimethyl sulphoxide, and EpM and post-thaw viability was assessed by the dye exclusion method for 2 years of storage. One cryovial from each tissue source at P1 was revived at each time point (n = 5 at each time point). Each vial from the four tissue sources was accessed at a selected stability time point. The post-thaw viability was accessed at the following time points: within 3 months, 1 year and 2 years. Each sample was thawed after removing the cryovials from the cryo-freezer and immediately thawed in a 37°C water bath until ice crystals had just disappeared. The contents of each sample vial were transferred to a 15-mL centrifuge tube and diluted at a ratio of 1:9 within 1–3 min in EpM to a final volume of 10 mL. These were centrifuged to remove the dimethyl sulphoxide and suspended in complete medium, and viability was then assessed.

Statistical analysis

All values were expressed as mean ± SEM. Data were analyzed by paired Student's *t*-test using Graphpad Prism (version 5, Graphpad Software). Student's *t*-test (2-tailed) was performed to compare means between groups. $P < 0.05$ was considered significant.

Results

Biopsy processing

The biopsy samples were received at a manufacturing facility in transportation media at 2–8°C. The H&E staining of biopsy sections revealed that cells in the basal layer with rete ridges (which are epidermal projections into dermis) were found in all biopsy samples (Figure 1A–D). The demographic profile of donors is shown in the Table I. The average cell yield per square centimeters after isolation was significantly higher in JSK compared with the other cell sources (Figure 2A). Similarly, the viability of isolated cell suspension