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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 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

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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

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