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Using human epithelial amnion cells in human de-epidermized dermis for skin regeneration

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ABSTRACT

Background: Human amniotic epithelial cells (hAECs) is a desirable reserve of stem cells. Human de-epidermized dermis (DED) retains basic tissue structure and parts of the basement membrane (BM) components at the acellular dermal surface, and provides a potential tool for skin regeneration.
Objective: To evaluate the potential role of hAECs in skin regeneration, we used DED to perform organotypic culture of hAECs to develop organotypic skin.
Methods: HAECs were isolated and cultured. Biological characteristics of hAECs were determined by immunocytochemistry and flow cytometry. To prepare DED, the epidermis was removed and then repeated freeze-thaw cycles. HAECs and fibroblast were seeded onto DED to perform the submerged culture for 3 days and then to be maintained at the air-liquid interface for 14 days to form organotypic culture. To identify whether the obtained DED retain the BM structure and components, the histological characteristics of DED and the BM were detected by immunohistochemistry. To evaluate whether the organotypic skin has similar histological characteristics with normal human skin, the marks of epidermal proliferation and differentiation and basement membrane component were detected by immunohistochemistry. Moreover, cell ultrastructure, cell–cell contact and ultrastructure of BM were examined under the transmission electron microscopy.
Results: HAECs has stem-cell characteristics with strong pluripotent Oct-4 and embryonic marker SSEA-4 expression. DED has effectively cleansed the cell components and continuous distributions of laminin and collagen IV. The histological appearance of tissue-engineered skin in vitro has 4 to 9 continuous layers of stratified epithelium and is similar to normal human skin in morphology. Immunohistochemical studies revealed that proliferation and differentiation markers such as Ki67, CK19, CK14, CK10, filaggrin but not CK18 expressed similar pattern characteristics to normal human epidermis. In addition, Periodic acid-Schiff stain showed that a uniform red staining strip located at the epidermal-dermal junction. BM component proteins (type IV collagen and laminin) and cell adhesion protein (desmoglein) were detected by immunohistochemistry in organotypic skin. Ultrastructurally, desmosomes, hemidesmosomes and BM zone (BMZ) were observed in organotypic skin.
Conclusions: Our studies indicate that the hAECs is a promising stem cell source for tissue-engineered skin, and DED with hAECs is a potential application prospects in regenerative medicine.

1. Introduction

Bioengineered skin equivalents such as Apligraf VR and Dermagraft VR are recently used in wound treatment. The seed cells of these skin equivalents are mainly allogeneic keratinocytes [1], which is limited by the availability of suitable source of cells. Furthermore, graft rejection, the infection between donors and patients and the high cost of the procedure are also concerned clinically. In addition, the efficiency of skin substitute reconstruction with autologous keratinocytes is also a concern. Specifically, it takes for 2–3 weeks to grow confluent epithelial sheets*in vitro*, in which patients have to wait with high opportunity for infection [2] .To address this concern, dermal substitute with a population of human amniotic epithelial cells (hAECs) provide a better and novel option. Specifically, hAECs remain somewhat “plastic” on the choices of differentiation and maintain the capability of

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differentiation and contribute to cells form all three germ layers [3]. Furthermore, hAECs were confirmed to express growth factors (EGF, KGF, and HGF), anti-inflammatory cytokines and antibacterial, all of which are benefit for wound-healing [4–6]. From discarded placenta, abundant hAECs can be easily and quickly isolated [7]. It is therefore a noncontroversial source of cells for regenerative medicine. Importantly, unlike human embryonic stem cells, hAECs do not express telomerase and are not tumorigenic upon transplantation [3]. Moreover, hAECs develop on the 8th day of fertilization, which is long before gastrulation (days 15–17). Thus, hAECs are believed to retain some or all of the epiblast pluripotency [3]. Finally, hAECs have immunoprivileged characteristics and therefore released the risks of rejection upon transplantation [8]. All together, hAECs are definitely a promising source of cells for skin regeneration. Here, we used hAECs to develop stratified epithelium as a new epithelial cell resource for skin grafting.

The development of favorable dermal substitute has always been a main focus in skin tissue engineering research and the basis of composite skin equivalents [9]. The earliest dermal matrix used as a cultured tissue engineering skin incorporated fibroblasts into a gel of acid-extracted collagen overlayed with a suspension of keratinocytes [10]. Previous literature also reported the following other synthetic dermal substitutes include the polyglycolic acid mesh [11], collagen sponge [12], fibrin gels [13], hyaluronic acid-derived biomaterial [14] and a collagen/glycosaminoglycan mixture [15–18]. Although most dermal analogues can mimic the structural and functional characteristics of normal dermis, nearly all of them lack effective BM structures. Epidermal keratinocytes therefore gradually lose their proliferative ability on the dermal scaffold. To solve these concerns, more and more studies have focused on constructing dermal scaffolds with BM structures. BM is a very important structure in normal skin, located at the epidermal-dermal junction. Ultrastructurally, the BM zone (BMZ) is divided into hemidesmosomes, lamina lucida (LL), lamina densa (LD) and reticular lamina [19] and plays a vital role in maintaining the fine function of skin. There are abundant studies showing that by adding natural or artificial BM components onto the dermal substitutes, the *in vitro*proliferation rate of epidermal keratinocytes was significantly enhanced, and the function of the epidermal layer was also improved [20]. The DED is detached from the epidermis at the level of the LL, and thus it retains most of the components of the BM such as laminin, collagen IV and collagen VII except hemidesmosomes on the papillary dermal surface of the DED [21,22]. Some studies have demonstrated that this pre-existing BM in acellular dermal matrix was beneficial to the adhesion and proliferation and differentiation of keratinocytes and promoted new BM formation [23–25]. All together, the DED with an intact BM structure was able to enhance hAECs differentiation, stratification and cornification to display strong similarity to skin tissue in skin regeneration. Here, we used organotypic culture of hAECs in DED populated with fibroblasts for skin regeneration.

2. Materials and methods

2.1. Cell isolation and identification

2.1.1. Isolation and culture of hAECs

Human term placentas were obtained from uncomplicated, elective caesarean sections on healthy mothers of ages ranging from 25 to 35 years at Department of Obstetrics, Affiliated Hospital of Guiyang Medical College. All donors have given informed consents. Human amnion was mechanically peeled off the chorion of a placenta and washed several times with phosphate-buffered saline (PBS) supplemented with penicillin 100 UI/ml/streptomycin 100 μg/ml (Sigma, USA) to remove blood and cellular debris. It was then cut into 2 × 2 cm amnion fragments and processed as previously described [26]. Briefly, 20 pieces of human amnion fragments was incubated at 37 °C with 0.05% trypsin containing 0.02% ethylene diamine tetraacetic acid (EDTA) (Gibco-Invitrogen, USA) with constant agitation. The cells from the first 10-min digestion were discarded to exclude debris. The cells from the second and third 30-min digestion were collected and centrifuged and washed with PBS, and then cultured in hAECs media (L-DMEM (Gibco-Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA), 10 ng/mL epidermal growth factor (EGF)(Peprotech,USA), 1% Glutamax (Gibco-Invitrogen, USA), 1%penicillin-streptomycin solution (Sigma, USA) at 37 °C, and 5% CO_2, once the culture reached 80–90% confluence, hAECs subcultured to subsequent passages with a split ratio of 1:2.

2.1.2. Normalhuman fibroblasts and keratinocytes (KC) preparation

Human dermal fibroblasts and KC were isolated from discarded circumcised foreskin on healthy boys of ages ranging from 3 to 15 at Urology Surgery, Affiliated Hospital of Guiyang Medical College. All donors have given informed consents. The circumcised foreskin rinsed with PBS containing 1%penicillin-streptomycin solution (Sigma, USA). Cultures of human fibroblasts and KC were established as described previously [27,28]. The fibroblasts were cultured in DMEM (Gibco-Invitrogen, USA) supplemented with 10% FBS (Hyclone, USA), 1%penicillin-streptomycin solution (Sigma, USA). KC suspension were plated on 25 cm^2 cell culture flasks in Keratinocytes serum-free medium(K-SFM, Gibco-Invitrogen, USA) containing 2ng/ml EGF (Gibco-Invitrogen, USA), 30 μg/ml Bovine pituitary extract (BPE) (Gibco-Invitrogen, USA), 0.3 g/L glutamine (Sigma, USA), 1%penicillin-streptomycin solution (Sigma, USA). After the cells became confluent, they were used for passage or experiment.

2.1.3. Biological characteristics and verification of hAECs/fibroblasts

HAECs/fibroblasts respectively cultured on microscope cover glass (NEST, USA) were fixed in 4% paraformaldehyde for 15 min, and then incubated in 1%BSA for 30 min at 37 °C and 0.5% TritonX-100 15 min for blocking and permeablization. HAECs were then incubated at 4 °C overnight with a primary antibody including mouse anti-human pan-Cytokeratin Antibody (1:100, sc-8018, Santa Cruz, USA), mouse anti-human Oct-3/4(1:100, sc-365509, Santa Cruz, USA), mouse anti-human SSEA-4 (1:100, sc-21704, Santa Cruz, USA). Fibroblasts were incubated with anti-Vimentin (1:100, M0725, DAKO, Denmark). PBS was added as negative control in each experiment. After being rinsed with PBS, the samples were incubated with biotinylated secondary antibody (DAKO, Denmark) at 37 °C for 30 min followed by being visualized with 3,3-diaminobenzidine tetrahydrochloride (DAB,DAKO) and hematoxylin nuclear counterstaining. In immunofluorescence analysis, hAECs were incubated with FITC-conjugated secondary antibodies for 1 h at 37 °C. Cells were rinsed with PBS and the nucleus were counterstained with DAPI (4,6 diamidino-2-phenylindole, 5 ug/mL, Beyotime, China). Finally, the cells were examined under fluorescence microscope to capture fluorescent images. At the same time, normal human KC were used as the negative control group. The cell cycle of hAECs were examined by flow cytometry. HAECs concentration adjusted to 5×10^6 and re-suspended with PBS on ice, Propidium iodide (PI) staining was performed (50 μg/mL; BD Pharmingen) and then measured by flow cytometry, whereas the cell cycle of normal human KC were used as a control group. The distribution of two kinds of cell cycle phase was drawn as a histogram.