Video Article

Generation and Culturing of Primary Human Keratinocytes from Adult Skin

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Abstract

The main function of keratinocytes is to provide the structural integrity of the epidermis, thereby maintaining a mechanical barrier to the outside world. In addition, keratinocytes play an essential role in the initiation, maintenance, and regulation of epidermal immune responses by being part of the innate immune system responding to antigenic stimuli in a fast, nonspecific manner. Here, we describe a protocol for isolation of primary human keratinocytes from adult skin, and demonstrate that these cells respond to calcium-induced terminal differentiation, as measured by an increased expression of the differentiation marker involucrin. In addition, we show that the isolated keratinocytes are responsive to IL-1β-induced activation of intracellular signaling pathways as measured by the activation of the p38 MAPK pathway. Taken together, we describe a method for isolation and culturing of primary human keratinocytes from adult skin. Because the keratinocytes are the predominant cell type in the epidermis, this method is useful to study molecular mechanisms in cutaneous biology *in vitro*.

Video Link

The video component of this article can be found at https://www.jove.com/video/56863/

Introduction

The skin is the biggest organ of the human body and serves as a protective barrier against the external environment. The skin is composed of two main layers: the dermis and the epidermis, where the epidermis constitutes the outermost layer of the skin. The most abundant cell type in the epidermis is the keratinocytes comprising more than 95% of the cell mass[1,2]. The keratinocytes are maintained at various stages of differentiation in the epidermis and are organized into basal, spinous, granular, and cornified layers that correspond to specific stages of differentiation[3]. The primary function of keratinocytes is to provide the structural integrity of the epidermis, thereby producing an intact barrier to the outside world.

The keratinocytes also represent the first line of defense against pathogens in the skin, and therefore play an important role in the innate immune response[4,5]. Exposure of the keratinocytes to external stimuli leads to activation of intracellular signaling pathways and subsequently, production of a number of various inflammatory mediators including cytokines, chemokines, and antimicrobial peptides. These keratinocyte-derived proteins participate in the inflammatory response by recruiting and activating immune cells such as dendritic cells, neutrophils, and specific T cells[6,7]. Thus, because keratinocytes play a crucial part in numerous biological processes, the rationale behind the technique presented here was to generate an *in vitro* model to study skin biology. Primary keratinocyte cultures obtained from neonatal foreskin are often used to study skin biology[8,9]. However, with the technique described here, keratinocytes from both genders are obtained resulting in a higher biological diversity of the cells.

Here, we present a detailed protocol for the isolation and generation of primary human keratinocytes from adult skin, including maintenance and freezing of the keratinocytes. The overall goal of this method is to generate primary human keratinocytes that can be used as a model to study cutaneous biology *in vitro*.

Protocol

The collection of skin samples from healthy adult volunteers undergoing plastic surgery requires approval from the ethical committee in the host institutions. This protocol was approved by the Regional Ethical Committee of Region Midtjylland, Denmark (M-20110027). The method described here is derived from similar studies by Maciaq *et al.*[10] and Liu and Karasek[11].

1. Isolation of Keratinocytes from Human Skin

1. Start by making the following solutions: 50 mL solution of 0.25% trypsin and 0.1% glucose in DPBS. Mix and filter sterilize (0.2 µm) the solution. Prepare 10 mL of RPMI-1640 + 2% FBS solution, 50 mL of DPBS and keratinocyte serum-free medium (KSFM). Add KSFM supplements and 250 µL of gentamycin to 500 mL of KSFM. Pre-warm the solutions to 37 °C before use.

2. Collect skin from healthy adult volunteers undergoing plastic surgery. The skin samples used are approximately 10 cm x 15 cm, but can vary in size. Keep the skin cool under transportation by transporting the skin samples in a Styrofoam box containing a cooling element. If necessary, the skin sample can be stored at 4 °C overnight.
3. Remove fat from underneath the skin section using sterilized scissors, scalpels, and forceps.
4. Buckle out the skin section (approximately 10 cm x 15 cm) on a sterile cover on top of a plate using needles. First, clean the skin with a dry sterilized gauze pad. Then clean the skin using a sterilized gauze pad with 70% ethanol.
5. Using a foot planer, cut of the upper layer (epidermis) of the skin section and put it in a 9 cm Petri dish. Then, immediately add 25 mL of the DPBS/trypsin/glucose solution (prepared in step 1.1) to the Petri dish. Incubate for 30 min at 37 °C.
6. Using a pipette remove the DPBS/trypsin/glucose solution from the Petri dish and add 10 mL of RPMI-1640 + 2% FBS to inactivate the trypsin.
7. Using two forceps, now release the epidermal cells into the medium by gently scraping and agitating both the epidermal and the dermal compartment of the skin sections.
8. Filter the epidermal cell suspension through a metal filter (1 mm hole size), and collect in a 50 mL tube. Add the remaining skin sections to a 50 mL tube containing 10 mL of RPMI-1640 without FBS and vortex for 10 s. Filter the suspension through the metal filter into a 50 mL tube containing the epidermal cell suspension. Add DPBS to a total volume of 50 mL.
9. Centrifuge the cell suspension for 10 min at 450 x g at room temperature.
10. Remove the supernatant and resuspend the epidermal cell pellet in approximately 10 mL of 37 °C KSFM depending on the size of the cell pellet. Count the cells using the Trypan Blue staining method under microscope. The number of cells obtained from a 10 cm x 15 cm skin section is approximately 50 - 100 x 10^6 cells. To each 75 cm^2 culture flask, transfer 8 x 10^6 cells together with 12 mL of 37 °C KSFM. Gently shake the culture flasks to ensure uniform distribution of the cells.
11. Incubate the keratinocytes in a 37 °C incubator with 100% humidity and 5% CO_2. Change the medium after 2 days and three times weekly. Passage cells when the culture is 70 - 80% confluent, which takes approximately 2 weeks depending on the proliferation rate of the keratinocytes.

2. Passaging of Keratinocytes

1. Heat the appropriate amount of 0.05% Trypsin-EDTA solution to 37 °C in an incubator. Use 4.5 mL of 0.05% Trypsin-EDTA solution for a 75 cm^2 culture flask.
2. Remove medium from the cells, and add 4.5 mL/75 cm^2 culture flask pre-warmed 0.05% Trypsin-EDTA solution to the cells. Place the culture flask in the incubator (37 °C) and after approximately 5 min, check under the microscope if the cells have started loosening.
3. When approximately 50% of the cells have loosened, gently hit the culture flask against the hand to loosen the remaining cells. Then, to inactivate the trypsin, add 6 mL of RPMI-1640 + 2% FBS (37 °C) to the 75 cm^2 culture flask and transfer the cell suspension to 50 mL tubes.
4. **Rinse the culture flasks with 3 mL of RPMI 1640 + 2% FBS and add to the 50 mL tubes. Centrifuge cells for 10 min at 450 x g at room temperature.**
    1. Resuspend the pelleted cells in 10 mL of KSFM (37 °C). Count the cells and transfer 3 x 10^6 cells to a 150 cm^2 culture flask together with 20 mL of KSFM (37 °C). Gently shake the culture flask to ensure uniform distribution of the cells. Freeze the cells when the culture is 80 - 90% confluent, which takes approximately 4-6 days depending on the proliferation rate of the keratinocytes (see Section 3, freezing protocol below). Expand the keratinocytes to generate appropriate frozen stocks.

3. Freezing of Keratinocytes

1. Heat the appropriate amount of 0.05% Trypsin-EDTA solution to 37 °C in an incubator. Use 9 mL of 0.05% Trypsin-EDTA solution for a 150 cm^2 culture flask.
2. Remove medium from the cells, and add 9 mL/150 cm^2 culture flask pre-warmed 0.05% Trypsin-EDTA solution to the cells. Place the culture flask in the incubator (37 °C) and after approximately 5 min, check under the microscope if the cells have started loosening.
3. When approximately 50% of the cells have loosened, gently hit the culture flask against the hand in order to loosen the remaining cells. Then, to inactivate the trypsin, add 12 mL of RPMI-1640 + 2% FBS (37 °C) to the 150 cm^2 culture flask and aspirate the cell suspension to 50 mL tubes.
4. Rinse the culture flasks with 6 mL of RPMI 1640 + 2% FBS and add to the 50 mL tubes. Centrifuge the tubes for 10 min at 450 x g at 4 °C. Prepare ice-cold cell freezing medium (KSFM + 10% DMSO).
5. Resuspend the cell pellet in KSFM + 10% DMSO, count the cells, and freeze cells at a density of 6 x 10^6 cells/mL using standard slow-freezing cryopreservation methods[12].
6. Store the keratinocytes in liquid nitrogen (vapor phase) until ready to use.

4. Thawing and Culturing Frozen Keratinocytes

1. Rapidly thaw the appropriate frozen vials in a 37 °C water bath. Use 70% ethanol to clean the outside of the cryovial. Transfer the appropriate number of cells (approximately 15,000 cells/cm^2) to a culture flask and immediately add pre-warmed growth medium (KSFM) to the culture flask.
   Any size of culture flasks can be used depending on the setup of the experiment.
2. Use 5 mL culture medium for a 25 cm^2 culture flask. The number of cells added the culture flask might vary because the growth of the cells varies from donor to donor. Incubate cells in a 37 °C incubator with 100% humidity and 5% CO_2.
3. Change the medium after 2 days and three times weekly using fresh pre-warmed KSFM.
   Note: Our data have demonstrated that on average 95% of the cells isolated by the protocol described here are positive for the keratinocyte-marker cytokeratine-14[13].

Representative Results

Calcium-induced Terminal Differentiation

Human keratinocytes undergo terminal differentiation upon treatment with calcium[14,15,16]. Primary human keratinocytes were isolated and cultured as described in the above protocol. When approximately 50 - 60% confluent, the cells were stimulated with calcium (1.2 mM) or vehicle and pictures of the cells were taken on day 0, 1, and 2. **Figure 1** shows the morphological changes of the keratinocytes observed upon calcium stimulation.

The Expression of Involucrin is Increased Upon Calcium Stimulation

Cultured human keratinocytes were grown until approximately 50 - 60% confluent after which the cells were stimulated with calcium (1.2 mM) or a vehicle for 24 and 48 h. We demonstrated that in parallel with the increased differentiation of the cells as observed by the morphological changes of the keratinocytes, the mRNA expression of the differentiation marker involucrin increased significantly upon calcium stimulation. After 24 and 48 h of stimulation, the involucrin mRNA expression was increased approximately 5.5-fold and 3.5-fold, respectively, compared with vehicle (**Figure 2**).

IL-1β-induced Phosphorylation of p38 MAPK

To determine if the isolated human keratinocytes were responsive to cytokine-induced activation of intracellular signaling pathways, cultured keratinocytes were stimulated with IL-1β (10 ng/mL) for various time points. Within 5 min, IL-1β stimulation led to a rapid activation/phosphorylation of p38 MAPK, as determined by Western blotting. After 1 h, IL-1β-induced p38 MAPK phosphorylation had returned to basal level (**Figure 3**). Only the phosphorylated form of p38 MAPK was increased, as IL-1β stimulation had no effect on the total protein level of p38 MAPK (**Figure 3**).

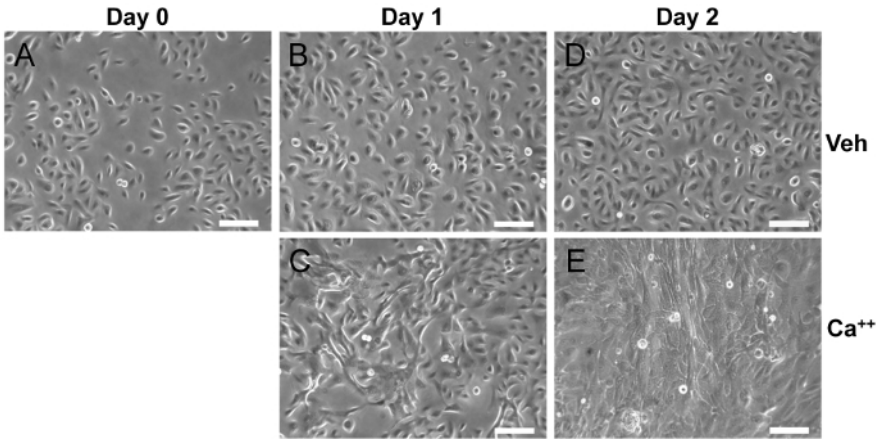

Figure 1: Representative images of calcium-induced differentiation of keratinocytes. Cultured primary human keratinocytes were stimulated with vehicle (dH_2O) or calcium (1.2 mM) for the indicated time points. (**A - E**) Phase contrast images of keratinocytes on day 0 (**A**), day 1 (**B** and **C**), and day 2 (**D** and **E**) after calcium stimulation. Scale bar = 100 µm. Please click here to view a larger version of this figure.

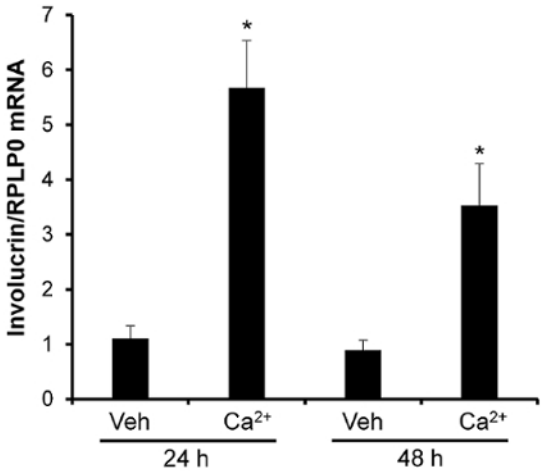

Figure 2: Increased mRNA expression of involucrin upon calcium stimulation. Calcium (1.2 mM) or vehicle (dH_2O) was added to cultured primary human keratinocytes for the indicated time points (n = 3). RNA was isolated and the expression of the differentiation marker involucrin analyzed by qPCR. RPLP0 (Ribosomal Protein Large P0) mRNA expression was used for normalization. Results represent mean ± S.D. from three different experiments. *p <0.05 compared with vehicle. Please click here to view a larger version of this figure.

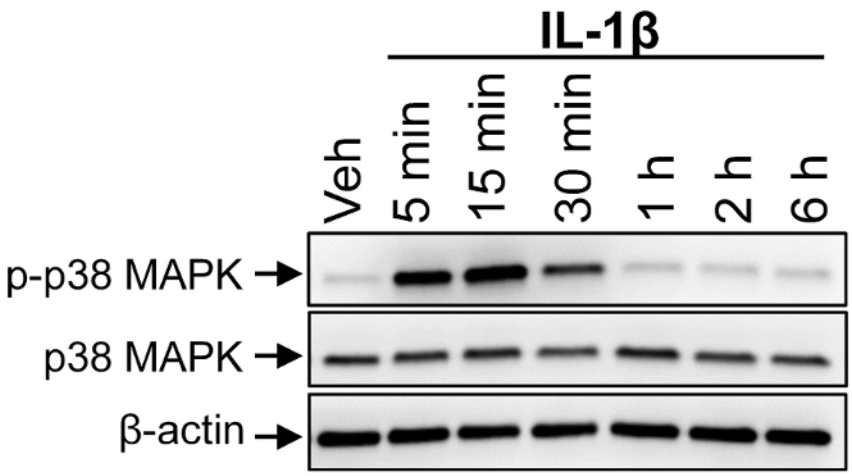

Figure 3: IL-1β-induced phosphorylation of p38 MAPK. Cultured primary human keratinocytes were stimulated with vehicle (PBS + 0.15% BSA) or IL-1β (10 ng/mL) for the indicated time points. Protein extracts were isolated and Western blotting analysis used to measure the phosphorylated level of p38 MAPK and total p38 MAPK. Equal loading was verified by incubation with an anti-β-actin antibody. Data from one representative experiment out of three are shown. Please click here to view a larger version of this figure.

Discussion

Here, we describe how to easily isolate primary human keratinocytes from adult skin, and how to culture them *in vitro*. This model can have a broad application for investigation of epidermal cell biology, and can be useful for researchers interested in studying cutaneous diseases.

Some of the advantages of the protocol described here is that in contrast to keratinocytes isolated from neonatal foreskin obtained from newborn males undergoing circumcision, primary human keratinocytes from adult patients are isolated from both men and women and can include any age ≥18 years. Thus, the biological diversity is much larger in these cells compared with keratinocytes from neonatal foreskin. Moreover, relatively large pieces of skin samples can be obtained from patients undergoing plastic surgery, such as breast reduction surgery or weight loss surgery.

As mentioned above the epidermis is organized in different layers corresponding to the specific differentiation stage of the keratinocytes. In order to study skin biology, the model described here is limited by the lack of a three-dimensional microenvironment. The different layers of the epidermis as well as the different cell types present in the skin are not mimicked in this model. To overcome this, human skin equivalent models can be used, which consist of a multilayered epithelium where keratinocytes differentiate upon exposure to an air-liquid interface, and thus, more closely mimicking the native epidermis[17,18,19]. Another model which can be obtained in order to study skin biology is the *ex vivo* skin model, in which skin biopsies are kept in cultures at an air-liquid interphase as previously described[20].

Disclosures

.The author has no conflict of interest.

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