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Isolation of small-sized human epidermal progenitor/stem cells by Gravity Assisted Cell Sorting (GACS)

Yasushi Fujimori [a], Kenji Izumi [b,*], Stephen E. Feinberg [c], Cynthia L. Marcelo [a]

[a] Division of Plastic and Reconstructive Surgery, Department of Surgery, University of Michigan, Ann Arbor, MI 48109-0654, USA
[b] Division of Oral Anatomy, Niigata University Post-Graduate School of Medical and Dental Sciences, 2-5274 Gakkocho-dori, Chuo-ku, Niigata 951-8514, Japan
[c] Department of Oral and Maxillofacial Surgery, University of Michigan, Ann Arbor, MI 48109-0654, USA

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ABSTRACT

Background: Small diameter characterizes epidermal progenitor/stem cells. We have developed Gravity Assisted Cell Sorting (GACS) to simply enrich small-sized epidermal progenitor/stem cells.
Objective: The cells sorted by GACS were characterized by fluorescence-activated cell sorting analysis, and cultured for up to 7 weeks. The cultured cells were then used for reconstruction of skin equivalent.
Methods: GACS was performed on primary cultures (primary cell) and passage 6–7 cultures (cultured cell) of keratinocytes. A keratinocyte suspension was sized into two groups: cells trapped by a 20 μm filter (trapped cells), and cells flowing through both a 20 and 11 μm filter (non-trapped cells).
Results: In the primary cell groups, viability of the trapped cells was 62.5 ± 7.2% compared to 77.0 ± 3.7% for the non-trapped cells. In the cultured cell groups, viability of the trapped cells was 64.3 ± 14.9%, compared to the non-trapped cells (93.1 ± 2.0%). Flow cytometric analysis showed better discrimination by cell size between trapped and non-trapped cells in culture than in the primary cell suspension. Non-trapped cells contained a larger number of cells with high levels of α6 integrin and low levels of CD71 (α6 integrin^{bri}CD71^{dim}), indicating an enriched progenitor/stem cell population. The difference in these markers between the non-trapped and trapped cells was seen in both the primary and cultured cell groups although this difference was more distinct in cultured cells. Culture of both groups showed that cultures originating from the trapped cells senesced after approximately 15 days while the non-trapped keratinocytes grew for up to 40 days. Manufacture of an epidermis/dermal device (artificial skin) showed that non-trapped cells formed a significantly thicker epithelial layer than the trapped cells, demonstrating the enhanced regenerative capability of the smaller diameter, α6 integrin^{bri}CD71^{dim} cells separated by GACS.
Conclusion: These results indicate that GACS is simple and useful technique to enrich for epidermal progenitor/stem cell populations, and is more efficient when used on cells in culture.

1. Introduction

The isolation of human epidermal progenitor/stem cells is clinically important for the treatment of burns and skin defects. Many researchers have tried to isolate human epidermal stem cells using cell markers, rapid adherence to collagen type IV, and other methods [1–7]. One of the important characteristics of epidermal stem cells is that these cells have a smaller diameter than other differentiating epidermal cells [2,5,7,8].

For separating different sized cells, a method applying density gradient centrifugation has been used [9,10]. In a simpler protocol, other studies have reported a method using nylon filters for separating cells by cell size from various tissues, for example, spermatozoa, leukocytes, islets and adult marrow-derived cells [11–15]. Another technique, cell isolation based on fluorescence-activated cell sorting (FACS) provides a reliable method for separating epidermal stem cells by size. However, FACS has many inherent disadvantages: (1) it is expensive to run; (2) it is time consuming; (3) there is a large potential for contamination; (4) cells can be traumatized [5].

We have developed Gravity Assisted Cell Sorting (GACS), a serial filtration system that enables us to isolate small diameter epidermal cells. In this study, adult human epidermal cells were harvested from skin and sorted by size using GACS. To evaluate the efficacy of GACS, sorted cells were characterized by FACS analysis to determine size distribution, viability and expression of two phenotypic markers (α6 integrin and CD71), and were cultured over the long term to determine proliferating capacity and life span

* Corresponding author. Tel.: +81 25 227 2816; fax: +81 25 223 6499.
E-mail address: izumik@dent.niigata-u.ac.jp (K. Izumi).

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of the cells. The cultured cells were used for reconstruction of skin equivalent in an organotypic culture system to examine the regenerative capability of post-GACS cells.

2. Materials and methods

2.1. Materials

Millipore Nylon net filters (11 and 20 μm) were purchased from Millipore (Bedford, MA). EpiLife medium and EpiLife defined growth supplement were purchased from Cascade Biologics (Portland, OR). Trypsin type IX, trypsin inhibitor type I-S, ethylenediaminetetraacetic acid, amphotericin B solution, propidium iodide, and 0.4% trypan blue solution were purchased from Sigma–Aldrich (St. Louis, MO). Newborn calf serum, gentamicin solution, and Dulbecco's phosphate buffered saline were purchased from Invitrogen (Grand Island, NY). Crystal violet was obtained from J.T. Baker Chemical (Phillipsburg, NJ). Methanol and bovine serum albumin were obtained from Fisher Scientific (Fair Lawn, NJ). Tissue culture flasks, centrifuge tubes, six-well plates, and 48 micro-well plates were obtained from Corning (Corning, NY). AlloDerm® was obtained from LifeCell (Branchburg, NJ). Type IV collagen was purchased from Fluka, through Sigma (St. Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated anti-integrin α6 monoclonal antibody (GoH3) as well as 7-amino actinomycin D and phycoerythrin (PE)-conjugated anti-CD71 (Ber-T9) monoclonal antibody were purchased from BD Biosciences (San Diego, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

2.2. Preparation of cell suspension

GACS was performed on primary cultures (primary cell group; $N = 17$) and on passage 6–7 (P6–7; $N = 8$) cultures of keratinocytes that were prepared by trypsinization of adult human breast skin (cultured cell group).

2.2.1. Primary cell group (Fig. 1)

An 8 cm × 10 cm piece of full thickness adult human breast skin was soaked in Ca^{2+} and Mg^{2+} free phosphate buffered saline with added gentamicin and amphotericin B (50 and 0.65 μg/mL, respectively) for 3–4 h at room temperature. The skin was then digested in a 0.125% trypsin solution in phosphate buffered saline overnight at room temperature. The upper cornified and differentiated cell layers were gently removed, and the basal layer cells were scraped from the dermis into culture medium plus 10% normal calf serum (added to stop trypsin activity). Debris and clumped cells were removed by filtration through a sterile 240 μm filter, and the resulting cell suspension was centrifuged at 600 rpm for 5 min at 24 °C. The supernatant was aspirated and the cell pellet was resuspended in EpiLife culture medium containing 2% newborn calf serum. To ensure complete removal of the debris, this process of filtering, centrifuging, and re-suspension was then repeated a second time. The viable cell count was determined using 0.4% trypan blue staining and a hemacytometer. A cell suspension containing 0.6–1.0 × 10^6 cells/mL was prepared.

2.2.2. Cultured cell group (Fig. 1)

To prepare keratinocyte cultures, the primary cell suspension containing 2% newborn calf serum EpiLife medium was plated into T-75 or T-150 plastic tissue culture flasks, at approximately 2.0 × 10^5 cells/cm^2. After 2 days of plating, the cell culture was fed with serum-free, low (0.06 mM) calcium EpiLife medium with defined growth supplement every 48 h until 80% confluence was reached. The cells were passaged by incubation at 37 °C with 0.025% trypsin + 0.01% ethylenediaminetetraacetic acid and plated at 2 × 10^6 cells per T150 flask. The keratinocytes were passaged until passage 6 (P6) or 7 (P7). The cultured cells were harvested and counted using the same method as the primary cell group, and a cell suspension 0.6–1.0 × 10^6 cells/mL was prepared.

2.3. Gravity Assisted Cell Sorting

Millipore Nylon net filters (pore size; 11 and 20 μm) were folded and stapled to form a funnel. They were soaked in 95% ethanol for a minimum of 2–3 h to avoid contamination, and then rinsed in phosphate buffered saline. Cell suspensions of 0.6–1.0 × 10^7 cells in 10 mL were pipetted at a rate of 2–3 drops per second into each filter funnel, where they passed through into a 50 mL centrifuge tube. Each keratinocyte suspension was sized into two groups: cells trapped by a 20 μm filter (trapped cells), and cells flowing through both a 20 and 11 μm filter (non-trapped cells). Once the cell suspension had been filtered, the stapled

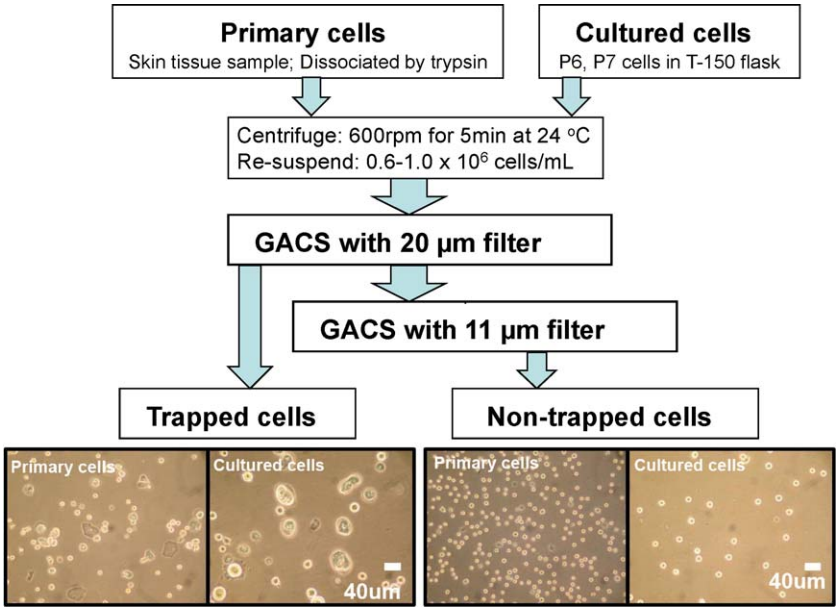

Fig. 1. Procedures for primary cell group and cultured cell group.