REQUIREMENT OF HYDROCORTISONE AND INSULIN FOR EXTENDED PROLIFERATION AND PASSAGE OF RAT KERATINOCYTES

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SUMMARY

A procedure for the preparation and cultivation of rat epidermal basal cells from full thickness skin resulted in greater than 99% viability and 90% plating efficiency. However, attempts to subculture monolayers of these epithelial cells grown in medium with serum as the only supplement were totally unsuccessful. When hydrocortisone and insulin were added to the medium, subcultivation of primary growth was obtained. It was demonstrated that hydrocortisone at concentrations as low as 0.1 μg/ml was necessary for at least the initial attachment of the cells to the substrate — an essential step in subcultivation. Increasing concentrations of insulin (0.1 to 50 μg/ml) caused the rate of proliferation and the cell density to increase, but insulin alone did not support subcultivation.

Key words: hormone; keratinocyte; hydrocortisone; insulin; passage.

INTRODUCTION

Two types of cells are the major components of the healthy mammalian skin, i.e., fibroblasts and keratinocytes. Fibroblasts, found in the dermis and responsible for the formation of collagen, can be cultured in vitro and subcultured in basal media with serum the only supplement (1). Keratinocytes, which form the epidermis, do not proliferate as readily in vitro in primary cultivation or passage when serum was the only supplement to basal medium. Only the basal cells harvested from the living epidermis engage in proliferative activity (2,3). Several systems have been developed to promote increased serial cultivation of keratinocytes. Improvements resulted when cells were grown on collagen (4,5) on feeder layers of irradiated fibroblasts (6), or in conditioned medium (7). Recent reports indicate that these systems may not be necessary (7–9) and that serial cultivation of keratinocytes can be achieved by supplementing basal media with various concentrations and combinations of hydrocortisone (HC) and insulin (1).

MATERIALS AND METHODS

Primary cultures. Fisher albino rats (Charles River, Portage, MI) were used to obtain viable cells from the epidermis. Epidermal-dermal separation was accomplished using previously reported procedures (3,13). Skin from 1 to 2 d old rats was cleansed with 70% alcohol after the animals were killed; the back skin was aseptically removed and placed, stratum corneum down, on a glass petri dish (100 × 15 mm). Loose connective tissue and blood vessels were scraped from the dermis. This scraping also caused the skin to stretch and adhere to the bottom of the dish. The tissues were then chilled to 4 °C and 15 to 20 ml of 1% crude trypsin (1:250) (Difco Laboratories, Detroit, MI), prechilled to 4 °C, was poured over the adhering skins. This combination of trypsinization and stretching of the skin at 4 °C not only caused the dermis and epidermis to separate at the basement membrane after 2.5 to 3 h but also loosened intracellular attachments between lower epidermis cells, which were then suspended
as aggregates by stirring the epidermis in basal medium containing fetal bovine serum (FBS) (Flow Laboratories, Rockville, MD) (13). The epidermal fragments, containing only differentiated keratinocytes and stratum corneum, were discarded. Also suspended into complete medium were single cells (basal cells and fibroblasts) and cellular debris, most of which were removed and discarded by the following method: the suspension was layered on 10% Ficoll (Pharmacia, Piscataway, NJ) and centrifuged for 5 min at 13 \times g. The single cells and cellular debris that remained in the supernatant fluid were discarded. The pellet containing aggregates of basal cells was resuspended in fresh medium and this washing procedure repeated seven times to remove most of the single cells and debris. Cell aggregate viability was determined by the dye exclusion test with 0.5% erythrosin B (14). The clarified suspension was pelleted at 53 \times g to obtain a packed volume and a 0.2% (vol/vol) suspension prepared in complete medium. This suspension contained \(5 \times 10^6\) cells/ml as determined by dissociating an aliquot of the cell aggregates with 0.02% EDTA and counting the single cells electronically. Complete medium contained 10% FBS and either 90% Eagle's minimum essential medium (MEM), 90% Waymouth 752/1 (WM), or 90% MEM:WM (1:1) (KC Biologicals, Lenexa, KS). Antibiotics, penicillin (100 U); streptomycin (100 \mu g/ml); and Fungizone (0.5 \mu g/ml) (GIBCO, Grand Island, NY) also were included in the medium. The cells were then seeded into plastic culture vessels, the medium changed 10 h after seeding, and the cells fed three times per week. Confluent monolayers were observed after 7 to 10 d at 35°C in a humidified 5% CO\(_2\) air atmosphere.

Subcultivation of primary cultures. Epithelial monolayers after varying times in culture were detached from the plastic substratum by replacing the nutrient medium with either of two solutions and incubating at 35°C: (a) 0.25% trypsin in Earle's balanced salt solution (EBSS), or (b) 0.02% trypsin and 0.01% EDTA in calcium and magnesium-free phosphate buffered saline (CMF-PBS). An equal volume of complete medium was added to the detached cells and the suspension centrifuged at 53 \times g for 10 min at 21°C. The cell pellets were resuspended in complete medium and seeded into new vessels using a 1:2 passage.

Supplements to nutrient medium. Hydrocortisone (HC) (Sigma, St. Louis, MO) was dissolved in absolute ethanol to a final concentration of 0.1 to 10 \mu g/ml. Insulin (I) (Sigma) was suspended in distilled water to concentrations of 1 to 50 \mu g/ml and the pH of the water adjusted to 4.0 with HCl to solubilize the hormone.

Histological examinations. Cultured basal cells were observed daily with an inverted phase contrast microscope. Permanent fixation was accomplished with a solution of butyl alcohol:acetic acid, 3:1 (vol/vol). After 10 min, the fixative was removed and the cells were stained with a hematoxylin and eosin (H & E). Stained cultures were quantitated by counting the nuclei contained in measured areas with ocular and stage micrometers, or automatically determining this number with a quantimet analytical system (15).

Ultrastructural examination. Growth medium was removed from the culture vessels and replaced with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) at room temperature. The cells were then cooled to 4°C by refrigeration for 10 min, fixed for 1 h at that temperature, and rinsed with phosphate buffer. The cultures were then dehydrated and embedded in Epon. Thin sections were cut on a Reichert Om-U2 ultramicrotome and stained first with 3% uranyl acetate then with lead citrate. Sections were examined with an AEI Cornith microscope.

RESULTS

Primary cultivation. A combination of stretching and trypsinization of full thickness skin for 2.5 to 3 h at 4°C resulted in the suspension of basal cells in large aggregates after brushing the epidermal cells into complete medium (12). Some of the larger aggregates were broken into smaller aggregates as a result of centrifugations in Ficoll. However, these centrifugations removed single cells and cell debris, leaving predominantly basal cell aggregates plus a few clumps of spinous cells. The viability of these suspensions of basal cell aggregates was routinely greater than 98% as indicated by the dye exclusion test. When these aggregates were seeded into plastic culture vessels and incubated at 35°C they attached, and growth centers were established around each aggregate within 12 to 18 h. Spinous cells attached initially but did not display any proliferative activity (3). These soon detached and were removed during the first feeding of the cultures (after 24 h of incubation).

Several experiments comparing the respective abilities of WM, MEM, and combinations of the