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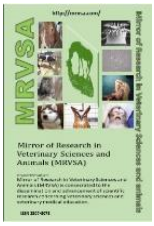

Culture technique of rabbit primary epidermal keratinocytes

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Abstract

The epidermis is the protective covering outer layer of the mammalian skin. The epidermal cells are stratified squamous epithelia which undergo continuous differentiation of loss and replacement of cells. Ninety per cent of epidermal cells consist of keratinocytes that are found in the basal layer of the stratified epithelium called epidermis. Keratinocytes are responsible for forming tight junctions with the nerves of the skin as well as in the process of wound healing. This article highlights the method of isolation and culture of rabbit primary epidermal keratinocytes *in vitro*. Approximately 2cm x 2cm oval shaped line was drawn on the dorsum of the rabbit to mark the surgical area. Then, the skin was carefully excised using a surgical blade and the target skin specimens harvested from the rabbits were placed in transport medium comprising of Dulbecco's Modified Eagle Medium (DMEM) and 1% of antibiotic-antimycotic solution. The specimens were transferred into a petri dish containing 70% ethanol and washed for 5 min followed by a wash in 1 x Dulbecco's Phosphate Buffered Saline (DBPS). Then, the skin specimens were placed in DMEM and minced into small pieces using a scalpel. The minced pieces were placed in a centrifuge tube containing 0.6% Dispase and 1% antibiotic-antimycotic solution overnight at 4°C in a horizontal orientation. The epidermis layer (whitish, semi-transparent) was separated from the dermis (pink, opaque, gooey) with the aid of curved forceps by fixing the dermis with one pair of forceps while detaching the epidermis with the second pair. The cells were cultured at a density of 4 x 10^4 cells/cm^2 in culture flask at 37°C and 5% CO_2. The cell morphology of the keratinocytes was analyzed using inverted microscope.

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Keywords: Culture, Rabbit, Primary, Epidermal, Keratinocytes

Introduction

The epidermis is the outer layer of the skin (William *et al.,* 2005) which functions as a protective covering of the mammalian body, with an average of about 0.2 mm thickness. The epidermal cells are stratified squamous epithelia which undergo continuous differentiation of loss and replacement of cells. Ninety-five per cent of the epidermal cells consist of epidermal keratinocytes which is proliferated and divided in the basal layer. After the cells are mature, it will move up to the upper layer to form the cornified cells. In the epidermis, the different stages of maturation of keratinocytes are divided into four levels (horny, granular, suprabasal and basal) cell layer. In the normal skin, the turn over time of the production of daughter epidermal cells is approximately 28 days. During the process of production of new cells, the cells undergo terminal differentiation which can change the characteristic of the cells. It is reflected as highly coordinated, sequential expression of morphological and biochemical markers of cell differentiation (Green, 1980; Green *et al.,* 1982). Many of the skin diseases whether it is benign or malignant are characterized to interfere with the normal regulation rate of proliferation and differentiation in the epidermis causing a severe disturbance in tissue homeostasis (Sybert *et al.,* 1985; Bernard et al, 1988; Rehfeld *et al.,* 1988; Turbitt *et al.,* 1990). The keratinocyte cell is a specialized epidermal cell that synthesizes keratin and it is the major cell type of the epidermis. Keratinocytes can be isolated from skin biopsies and are able to undergo expansion *in vitro* which can be used in patients with deep dermal burns as cultured epidermal autograft (CEA) (Dedovic *et al.,* 1998). Keratinocytes become activated and turn into hyper proliferative cells in the wound healing conditions which produce and secrete extracellular matrix components and signalling polypeptides. In the meantime, the production of specific keratin proteins will alter their cytoskeleton. Keratinocytes and other cutaneous cell types also contribute to the changes in growth factors, chemokines and cytokines. A culture system which enables growth of cells *in vitro* has become an important tool of study for normal and pathological cells (Jensen *et al.,* 1991). A significant trend of methods for culturing human epidermal keratinocytes has been successfully developed during the last 26 years. These achievements have made the analysis of the growth and differentiation pattern of epidermal keratinocytes in culture open to possible new strategies for therapeutic intervention. This research describes the protocol of isolating and culturing rabbit primary epidermal keratinocytes *in vitro*.

Materials and Methods

Ethical approval

This study was approved by the animal ethics committee of Universiti Sains Malaysia vide ref. USM/Animal Ethics Approval/2010/ (54) (175) dated 22 March 2010.

Skin harvesting

Three adult New Zealand White male rabbits, (*Oryctolagus Cuniculus*) were used in this study. The rabbits were administered a premedication of atropine (1mg/kg bwt i/m) and then anaesthetized using xylazine (5mg/kg bwt i/m) and ketamine (35mg/kg bwt i/m). The surgical site was shaved and povidine iodine was applied. The rabbit was observed for reflexes and when the rabbit was completely unconscious, it was moved to the animal operation theatre to perform the skin surgery. Approximately, 2cm x 2cm oval shaped line was drawn on the dorsum of the animal to mark the surgical area. Then, the skin was carefully excised using a surgical blade and the target skin specimens harvested from the rabbits were placed in transport medium comprising of Dulbecco's Modified Eagle Medium (DMEM) (Gibco®, USA) and 1% antibiotic-antimycotic solution (Invitrogen, USA). Then, the surgical wound was sutured and bandaged. The steps are shown in (Figure.1). The animal was monitored for reflexes throughout the surgical procedure.

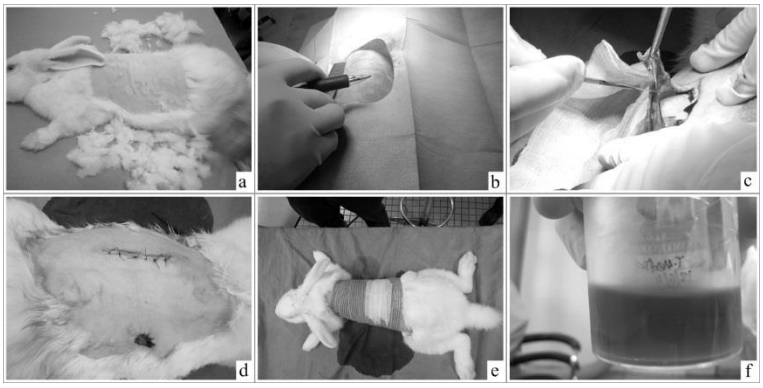

Fig 1. Skin harvesting in rabbit a) Shaved area on the dorsum of the rabbit b) 2cm x 2cm oval shaped area depicting the surgical area c) Skin excision using a surgical blade d) Sutured surgical wound e) Bandaged surgical wound and f) Transfer of target skin specimens harvested from the rabbits into transport medium comprising of DMEM and 1% antibiotic-antimycotic solution

Culture of keratinocytes

The specimens were transferred into a petri dish containing 70% ethanol and washed for 5 min followed by a wash in 1 x Dulbecco's Phosphate Buffered Saline (DBPS) (Gibco®, USA) for 5 min. Then, the skin specimens were placed in DMEM and minced into small pieces using a scalpel. The minced pieces were placed in a centrifuge tube containing 0.6% Dispase (Sigma-Aldrich, USA) and 1% antibiotic-antimycotic solution overnight at 4°C in a horizontal orientation. The next day, the specimen was transferred into a petri dish together with the dispase. Each of the specimens was transferred to a new petri dish containing CnT-57 (CELLnTEC, Switzerland) medium to wash away the excess dispase. While holding the skin submerged in CnT-57 medium, the epidermis layer (whitish, semi-transparent) was separated from the dermis (pink, opaque, gooey) with the aid of curved forceps by fixing the dermis with one pair of forceps while detaching the epidermis with the second pair. The separation of the rabbit dermis and epidermis was very difficult because the dermis was tightly bound to the epidermis by their thick and barely visible hair which serves to insulate the rabbit. The separated epidermis was placed in a 50 ml

centrifuge tube containing 10 ml of pre-warmed trypsin solution (TrypLE^{TM} Express, Invitrogen, USA) and incubated at 37°C for 10-15 min. Later, 10 ml of CnT-57 medium was added to this 50 ml centrifuge tube to deactivate the enzyme activity. The suspension was then carefully filtered using a 70 µM nylon cell strainer (BD Biosciences, USA) into a new 50 ml centrifuge tube. This was done to separate the cells and epidermis layers. The cell suspension was then centrifuged at 1700 rpm for 7 min. The supernatant was discarded and the cell pellet was re-suspended in CnT-57 medium. The cells were counted using a haemocytometer and the cell viability was estimated using 1% Trypan blue. The viable cells were cultured at a density of 4 x 10^4 cells/cm^2 in culture flasks (Nunc, Australia). The cells were cultured at 37°C and 5% CO_2. The cell morphology of the keratinocytes was analyzed using inverted microscope (Leica Microsystems, Germany).

Results and Discussion

The rabbit primary epidermal keratinocytes were cultured in the Cnt-57 progenitor cell targeted media. This medium favored only the growth of rabbit primary epidermal keratinocytes (Figure. 2). CnT-57 media is a low calcium (0.07 mM) formulation containing a low bovine pituitary extract (BPE) concentration of 6 µg/mL. A study by Kuo *et al.,* (2005) has shown that the addition of BPE aids attachment and migration and results in improved colony forming efficiency and maximum cell yield. BPE, derived from the pituitary gland (a small endocrine gland) that produces and secretes various hormones is important for the regulation of various bodily functions. It contains a full spectrum of putative mitogens and growth factors that have been used effectively in promoting robust growth in a wide range of cells *in vitro,* especially those of epithelial origin and stem cells. This medium has been used in humans (De Kock *et al.,* 2011) and mouse epidermal keratinocytes (Yazdi *et al.,* 2010). However, in a previous study by De Kock *et al.,* (2011), their differentiation strategy on human foreskin-derived precursor cells showed that the CnT-57 did not contribute to their cell differentiation with or without addition of CnT-02 and Epilife. Hence, it can be postulated that the BPE present in CnT-57 medium could be the factor promoting the growth of rabbit primary epidermal keratinocytes in addition to the components present therein. There are a limited number of reports suggesting the technique in isolation the rabbit primary epidermal keratinocytes. A study by Davison *et al.,* (1980) described the procedure for the isolation and cultivation of endothelium from the marginal vessels of the rabbit ear. The endothelial cells were isolated by slow perfusion with a trypsin solution and were cultured in minimal essential medium supplemented with 10% fresh rabbit serum for up to 6 months. They found that the fibroblast growth factor was not mitogenic for rabbit marginal vessel endothelium *in vitro.* In other study, Lapi *et al.,* (2008) found that for culturing rabbit bone marrow mesenchymal stem cells, DMEM showed significantly lower plating density compared to alpha minimal essential medium (α-MEM). Rutten et al, (1990) developed a method for rabbit skin organ culture in a two-compartment model. The skin discs were cultured on a Millicell-HA insert unit with a microporous membrane which allowed transport of culture medium via the dermis into the epidermis, whereas the epidermal side remains free of direct contact with culture medium. They concluded that at the end of the 7 days of culture period, the distance between single dermal collagen fibrils had increased as compared to non-cultured skin. In contrast, there are many

different methods reported for cultivating human epidermal keratinocytes which suggests two major categories (Fusenig, 1986; Watt, 1987); a simple system which retains some of the properties of the epidermis *in vivo* after serial passage of cells, while the second major categories are elaborating on reconstructing a true epidermal tissue in primary culture by approximating the *in vivo* epidermal environment. Between these two major categories of the culture systems, the system developed by Rheinwald and Green, (1975) has been the most successful system. The feeder layer of irradiated mouse 3T3 cells which was seeded by disaggregated keratinocytes enhances the plating efficiency and stimulates the keratinocytes growth (Green *et al.,* 1977; Rheinwald & Green, 1977; Watt and Green, 1981). In contrast, other studies have reported that irradiated human dermal fibroblasts can substitute for the 3T3 cells as a feeder layer (Limat *et al.,* 1989; Limat *et al.,* 1990). The Rheinwald-Green method is now used in almost every research because the method allows serial passage for many generations. It also gives a major impact on the study of many cellular and molecular aspects of proliferation and terminal differentiation of the keratinocytes (Fuchs and Green, 1980; Watt and Green, 1981). Furthermore, it allows large-scale production of epidermal cultures suitable for the covering of skin defects such as burn wounds (Green *et al.,* 1979; Gallico *et al.,* 1984). Growing keratinocytes in the absence of dermal cell products by altering the conventionally used tissue culture medium has now become possible. There were also few reports on chemically defined media which were serum-free (Tsao *et al.,* 1982; Boyce and Ham, 1983; Rikimaru *et al.,* 1990). These defined media which comes with the low calcium concentration gives rise to undifferentiated monolayer cultures of uniform small polygonal cells. Growth in defined media may be advantageous for some applications such as the study of growth regulatory substances (Wille *et al.,* 1984; Pittelkow *et al.,* 1986). The development of reliable methods for recreating and manipulating normal and pathological human epidermis in culture make human keratinocytes readily accessible for exploitation in basic and clinical research.

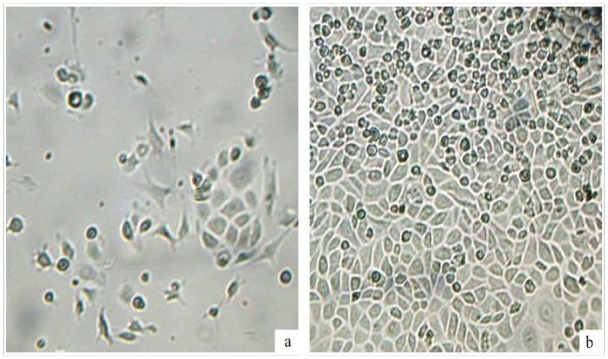

Fig 2. Rabbit primary epidermal keratinocytes a) Day 2 and b) Day 7 of the culture with magnification (1000x)

Moreover, the enzyme dispase has made the cultured epithelium easily detachable (Gallico *et al.,* 1984). Cultured keratinocytes thus also represent an attractive model system for somatic gene therapy and the successful transfer and expression of foreign DNA have been accomplished in these cells by a number of different techniques (Teumer *et al.,* 1990; Jiang *et al.,* 1991). Similar to the culture of human keratinocytes as reported

by the researchers described as above, more research is deemed necessary for exploiting the culture advantages of rabbit keratinocytes which could be employed in skin grafting and other therapeutic interventions.

In conclusion, Keratinocyte cultures have a great potential in cell differentiation and tissue development. In particular, we are fascinated by the possibilities of establishing the culture method of rabbit primary epidermal keratinocytes. Although some valuable results have already been achieved, this research is still in its infancy. New technical developments are clearly needed to make use of the full potential of the cultured rabbit primary epidermal keratinocytes.

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