Original Article

Ex vivo culture of oral keratinocytes using direct explant cell culture technique

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Abstract

Background: Culture of cells and tissues is a standard research method practiced in many laboratories. In most of the cases, these cultures are being used as substrates for cell products or as investigative tools for delving the mechanism of gene expression, cell proliferation and transformation. Primary monolayer cell culture has been beneficial to study the general biology of both oral and skin keratinocytes. There are two different techniques of primary cell cultures followed, which include direct explant and enzymatic techniques.

Aims: The aim of the study was to optimize the culture of keratinocytes obtained from patients with normal oral mucosa by direct explant technique.

Materials and Methods: Keratinocytes were isolated from 15 patients and were cultured *in vitro* and observed under an inverted microscope. The cultured cells were characterized by immunocytochemistry method using pan-cytokeratin.

Results: The total success rate of primary culture of the oral epithelial cells by direct explant technique was 88.6%. No contamination of microorganisms in the primary cell cultures was obtained.

Conclusion: Within the limited numbers of samples used in the current pilot study, we conclude that the direct explant technique appears to be a simple and successful technique for the isolation of oral mucosal keratinocytes if we follow the appropriate protocol.

Keywords: Cell culture, direct explant technique, immunocytochemistry, oral keratinocytes

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INTRODUCTION

Cell culture is one of the major tools used in cellular and molecular biology, which provides excellent model systems for studying the normal physiology and biochemistry of cells, the effects of drugs and toxic compounds on the cells, mutagenesis and carcinogenesis. Primary culture refers to the stage of the culture after the cells are being isolated from the tissue and have been proliferated under the appropriate conditions until they reach adequate confluence. It is used for drug screening and development and for large-scale manufacturing of biological compounds

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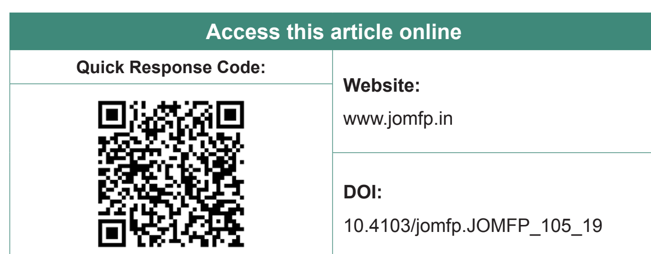

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such as vaccines, therapeutic proteins, tissue/organ replacement and stem cell therapy.[1,2]

The oral mucosa which is produced *ex vivo* has the ability to provide oral tissues that have similar characteristics of the original mucosa composed of an epithelial or dermal component. Most of the attention developed in recent years on kinetics of stem cell regeneration, mechanisms of differentiation and neoplasia has been focusing on the epithelial cells. Apart from this, cell culture of the oral and dermal keratinocytes has gained popularity in oral and maxillofacial surgery due to loss of epithelial structure found in thermal or chemical burns, trauma, penetrating gunshot injuries or ablative cancer surgery in the maxillofacial region.[3,4] Cells can be isolated from tissues for *ex vivo* culture by either purification from the blood (blood cells), released from soft tissue by enzymatic digestion (mononuclear cells) or explant culture.[5] Primary cells generally have limited lifespan in culture, known as the Hayflick limit, after which they stop dividing yet remain metabolically active, a phenomenon known as senescence.[6]

The first technique of culturing oral epithelial cells *in vitro* using 3T3 mouse fibroblasts as a feeder layer was proposed by Rheinwald and Green in 1975.[7] Although this method made it possible to grow keratinocytes with superior yield, these cultured sheets of oral mucosa with an irradiated feeder cell layer had the risk of introducing high mouse DNA content onto proliferating human cells.[8,9] This procedure was later modified by Kitano and Okada, who later introduced dispase to separate the epithelial sheet from the underlying dermis of the skin.[10] Boyce and Ham introduced a serum-free medium for the culture of primary keratinocyte, where the 3T3 feeder layer was no longer needed for the growth of keratinocytes, and it has benefits for use in clinical applications.[11]

Two techniques have been used to cultivate the oral keratinocytes from tissues for *ex vivo* cultures. Cells can be isolated from solid tissues by digesting the extracellular matrix using enzymes such as trypsin, collagenase or pronase in various concentrations, before agitating the tissue to release the cells into suspension.[12] The other method involves pieces of tissue placed in growth media, and the cells that grow out are available for culture. This method is known as explant culture and appeared to be more successful than the enzymatic technique in culturing human oral keratinocytes.[13,14] Carrel and Burrows in 1910 described a method for the extraction of epithelial cells called direct explant to culture human gingival and buccal sample.[9] In addition, Klingbeil *et al.* stated that the direct explant technique obtained the first keratinocytes yield faster than the enzymatic technique.[15] The aim of the present study was to re-evaluate the percentage of success in culturing oral mucosa keratinocytes by direct explant technique.

MATERIALS AND METHODS

Isolation of primary gingival keratinocytes

The study was approved by the ethics committee of our institution. Oral tissue samples were obtained from volunteers undergoing dental surgeries such as implant surgery, third molar extraction and crown lengthening procedures at the Oral and Maxillofacial Surgery Department. Primary cell cultures were performed from the keratinized gingival tissues of 10 healthy human subjects (4 males and 11 females aged ranging from 20 to 55 years). Patients were asked to rinse with 10% povidone-iodine solution before collecting the tissue sample. The tissue specimens were later carried to the cell culture laboratory in a 10 ml culture medium (Dulbecco's modified Eagle's medium [DMEM/F-12]: Gibco, New York, USA; pH 7.2) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.5% amphotericin B (Gibco, New York, USA) to prevent growth of microorganisms. All tissue culture was undertaken inside a laminar flow hood using aseptic technique. The tissue specimens were later washed and disinfected in a 10% povidone-iodine solution for 1 min followed by washing twice with phosphate-buffered saline (PBS) and later with culture medium. The tissue specimens were then cut into pieces, approximately 1 mm in size, and placed in the six-well culture plates using a sterile needle of a dental injector. Tissue pieces were left in the culture plate for 45 min to 1 h. The culture media were gently dropped on the tissue pieces placed inside the plates. After 18–20 h, the culture plate was flooded with 5 ml culture media. The culture plate was incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The culture plates were examined daily by an inverted microscope. The old culture medium was replaced with a fresh one twice a week. After the keratinocytes, which were squamous in shape, started to multiply around the tissue sample origin to a diameter of 2–5 mm [Figure 1a and b], the culture medium was changed to keratinocyte serum-free media (defined keratinocyte SFM from Gibco) containing basal media, keratinocyte growth supplement and bovine pituitary extract, 125 μg/ml gentamycin and 1 μg/ml amphotericin B (Gibco, USA) as described by Izumi *et al.* [3,16] with a calcium concentration of 0.06 mM. Thus, fibroblast overgrowth was prevented, and fibroblasts were eliminated [Figure 2]. The culture was fed every other day with the culture medium. After about 10 days, when the primary cell culture

reached 50%–60% confluence [Figure 3], oral mucosa keratinocytes were harvested with a solution of 0.025% trypsin-ethylene diamine tetra-acetic acid (EDTA) at 37°C. After 4–5 min, trypsin-EDTA activity was inhibited with an equal volume of 0.0125% trypsin inhibitor. Disaggregated cells were collected, counted, centrifuged, re-suspended and re-plated into a new different culture plate at a density of 10^5 cells/ml. The first passage was subcultured to a 24-well culture plate and then later seeded on to the coverslip (HiMedia 12–15 mm, surface coated) kept on another well for the next passages. After the cells got adhered on the coverslip and started proliferating, the coverslip was removed and placed on a slide followed by rinsing briefly with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature for immunocytochemistry analysis of pan-cytokeratin.

Immunocytochemistry

After fixing the cells with 4% paraformaldehyde on coverslip, the cells were washed twice with PBS solution. Cells were incubated with peroxidase block for 10 min followed by rinsing with deionized water and then with PBS. The antigen retrieval buffer (100 mM Tris, 5% urea, pH 9.5) was preheated to 95°C for 3 min. The coverslip with cells was placed in the antigen retrieval buffer in the staining jar and heat for 10 min at 95°C. Remove the solution and rinse the coverslip thrice with PBS. Application of power block was done for 5–10 min to minimize nonspecific binding. Prediluted cocktail primary antibody of pan-cytokeratin (AE1/AE3 from Biocare) was added for 1 h and rinsed with PBS. The tissue was later covered with a super-enhancer reagent for 20 min and rinsed with PBS. Polymer HRP reagent was added for 30 min and then rinsed with PBS. 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate solution was added for 10 min and rinsed well with deionized water. Counterstaining was done using Mayer's hematoxylin for 10 min, and the side was rinsed gently under tap water, immersed in ammonia water for 10 s and then rinsed with tap water.[17]

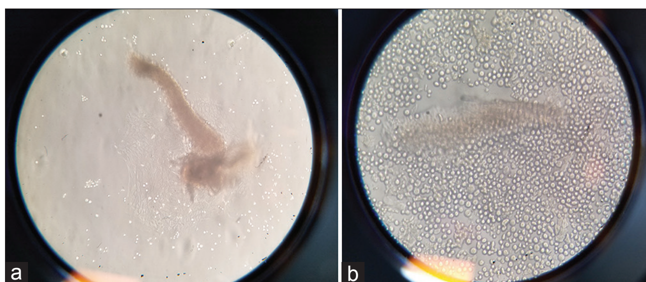

Figure 1: (a) Tissue has attached to the plates, and cells have started to disintegrate around the tissue. (b) Cells seen disintegrating from the tissue

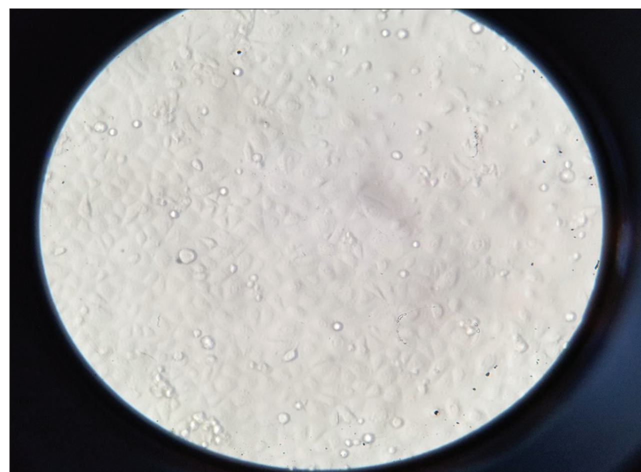

Figure 3: Monolayer of keratinocytes

RESULTS

The results showed that none of the primary culture of oral epithelial cells by direct explant technique failed, and the total success rate was 88.6%. Tissue samples of a size approximately 1 mm × 1 mm were found to adhere and grow faster than those tissues larger than 1 mm × 1 mm. The average days required for the epithelial cells to grow and migrate from the tissue was 5–7 days and to reach 50% confluency was 12–15 days. In this study, few wells

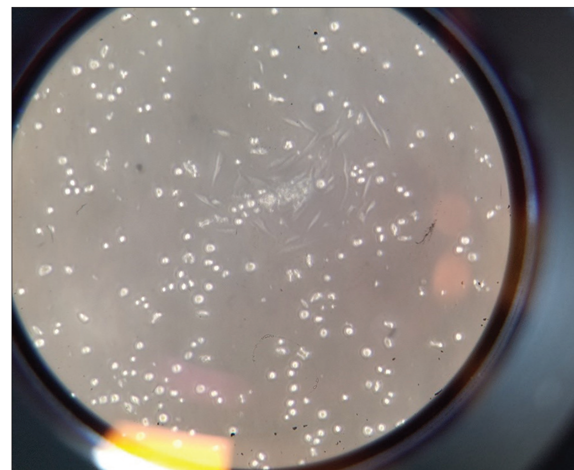

Figure 2: Mix population of polygonal- and spindle-shaped cells

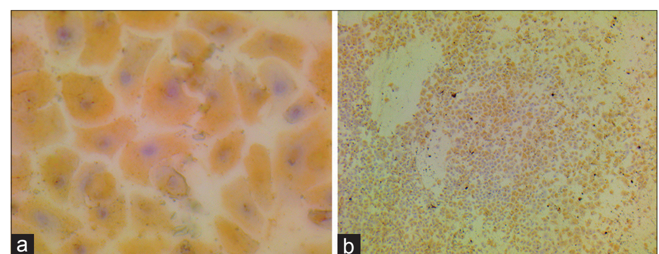

Figure 4: (a and b) Keratinocytes showing positivity for pan-cytokeratin antibody (a: 40X, b: 4X magnification)

with contamination of microorganisms in the primary cell cultures were observed. Morphology of the cells initially was round to polygonal and few cells with spindle shaped. Once the media were changed to keratinocyte growth media, cells appeared polygonal and formed a monolayer of sheet of cells. The structure of the oral keratinocytes *in vitro* was the same in primary and subcultures. The age and sex of the subjects did not influence much on the growth of cells during their culture.

Immunocytochemistry showed overall positivity of the cells which had taken up brown staining in the cytoplasm of keratinocytes [Figure 4a: 40X, 4b: 4X magnification].

DISCUSSION

The literature shows many published studies related to enzymatic and direct explant techniques for keratinocyte cultivation but still uncertainty remains about which one is ideal for obtaining the utmost number of clonogenic cells with good culture life span.[15] Freshney stated that when the tissue sample is limited, the direct explant technique should be opted for cell culture.[18] According to Breidahl *et al*., the direct explant method is more appropriate to grow more cells in number with good quality and viability.[19] Hence, we chose this method considering patient discomfort in extracting bigger tissue for isolation and growth of oral keratinocytes.

In our study, we could successfully isolate normal oral keratinocytes from the tissue sample. Kedjarune *et al*.[14] in their study compared both the direct explant technique and the enzymatic method in cultivation of human oral keratinocytes. They observed direct explant technique to be more successful for culturing human oral tissue keratinocytes than the enzymatic method and also found high rate of cell proliferation. They also noted that the direct explant method required only small pieces of samples when compared with the enzymatic method. However, more time was needed to subculture in the direct explant technique. However, these findings were in contrast with the results of the study performed by Klingbeil *et al*.,[15] who stated that the enzymatic method showed the best results in less time with good life span whereas the tissue size was larger to provide good seeding density. In their study, the average time required for cell growth by direct explant technique was 14.2 and 11.9 days for enzymatic method. In our study, it was 10–12 days for direct explant method where we used 12-well culture plates. This difference may be due to the use of bigger culture flask for culture of keratinocytes by Klingbeil *et al*. The success rate for the culture technique in our study was similar to Wanichpakorn and Kedjarune-Laggat's study, which was 88.9%, higher than Kedjarune *et al*.[14] and Reid *et al*. which was 82% and 80%, respectively.[1,4,20] The success rate of the culturing method was defined as the ability of the cells to grow from the original tissue sample, to become 60%–70% confluent and to survive at least until the second passage. The reason behind failure of the culture could be due to the placement of tissue in povidone-iodine for long time, tissue not getting adhered to the culture plate and microbial contamination. We also used rat tail collagen-coating solution from Sigma to enhance the adherence of keratinocytes. No difference was noted in the growth, adherence and subculture of keratinocytes between collagen-coated wells and noncollagen-coated wells.

The age and sex of the subjects who provided the tissue samples did not influence the success rate in the culture, which is consistent with the findings of Reid *et al*. and Kedjarune *et al*.[14,20] However, our results were in contrast with the study done by Lauer,[9] who found that keratinocyte growth and viability were influenced by age as tissue from younger subjects was cultured at the rate of 80% of cases when compared to older subjects with 65% success rates.

The disadvantage of the direct explant technique is that there is requirement of feeder layer when desired cell propagation is needed. Fibroblast overgrowth was also an issue in our study. The use of feeder layer and fibroblast growth was overcome by changing the culture medium from DMEM to keratinocyte growth media. Another issue we had to deal with was microbial contamination from the tissue samples associated with direct explant technique during removal of the tissue from the patient, transportation to the laboratory, tissue processing and media preparation.[5,13,20] Wanichpakorn and Kedjarune-Laggat[1] in their article stated that microbial contamination could be correlated with the size of the tissue sample being processed which can affect the success rate of cultivation. In our study, the tissue contaminated was overcome after placing it in a 10% povidone-iodine solution for 1–2 min and adding antibiotics and antimycotics to the media.

CONCLUSION

The direct explant technique for isolation and culturing of keratinocytes is a simple and easy method with minimum steps. It can be used in the research field which involves cell cultures and isolation from other tissues of the body. The age and sex of the subjects did not influence the growth and viability rate in culturing the oral keratinocytes. However, limitations were reported with contamination by oral flora

and fibroblast overgrowth. By taking appropriate measures, both the problems can be resolved. Further research could be helpful to study histological, immunocytochemical characteristics and cell behavior of the cells grown by the direct explant technique.

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Conflicts of interest

There are no conflicts of interest.

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