

A Pilot Study of the Primary Culture of the Oral Mucosa Keratinocytes by the Direct Explant Technique

Gurkan Rasit Bayar¹, Yavuz Sinan Aydintug², Aydin Gulses¹, Pinar Elci³, Meral Sarper³

¹ D.D.S., Ph.D. Oral and Maxillofacial Surgeon, Department of Oral and Maxillofacial Surgery, Gülhane Military Medical Academy, Ankara, Turkey. ² D.D.S., Ph.D. Professor, Oral and Maxillofacial Surgeon, Department of Oral and Maxillofacial Surgery, Gülhane Military Medical Academy, Ankara, Turkey. ³ Biologist (Laboratory Technician), Research and Development Centre, Laboratory of Cell Culture and Cancer Research, Gülhane Military Medical Academy, Ankara, Turkey.

Abstract

Introduction: A reliable source of cultured keratinocytes is essential as a component of oral mucosa substitutes to treat burns and wounds of the oral and maxillofacial region. Primary monolayer cell cultures have been also extremely helpful in the study of the basic biology and responses to stimuli, of both oral and skin keratinocytes, and many studies have used them. There are two techniques in primary culture, the enzymatic and direct explant techniques. The direct explant technique has been used for 30 years in the culture of human gingival and buccal tissues and has appeared to be a successful technique in culturing human oral keratinocytes. In addition, it has been suggested that the direct explant technique obtains the first keratinocytes yield faster than the enzymatic technique. **Aim:** The aim of this pilot study is to present our experience in *ex vivo* production of oral mucosa keratinocytes by using the explant technique. **Methods:** The explant technique was used to cultivate oral mucosa keratinocytes. **Results:** The total success rate of primary culture of the oral epithelial cells by direct explant technique was 88.9%. No contamination of microorganisms in primary cell cultures was obtained. **Conclusion:** Within the limited numbers of samples used in the current pilot study, it can be concluded that the explant technique piloted in this study appears to be a simple and successful technique in the primary culture of oral mucosa keratinocytes. A larger study is required to confirm this finding.

Key Words: Gingival Keratinocytes, Primary Cell Culture, Direct Explant Technique

Introduction

Thermal or chemical burns, trauma, penetrating gunshot injuries or ablative cancer surgery in the maxillofacial region can result in massive soft-tissue deficiencies. Thus reconstructive procedures in oral and maxillofacial surgery require skin and oral mucosa substitutes to compensate for soft-tissue loss and to enhance wound healing. Despite their disadvantages—such as the need for a second surgical procedure, limited graft amount and unsuitable texture of skin grafts—autogenous keratinised oral mucosa and split-thickness skin grafts are still being widely used for reconstruction of soft-tissue deficits in the circum-oral region.

In the last two decades, the ability to produce *ex vivo* oral mucosa and skin equivalents in one to two months from a punch biopsy composed of an epithelial or dermal component has started to assist surgeons from all fields and has also gained popularity in oral and maxillofacial surgery. An oral

mucosa equivalent, produced *ex vivo*, can provide an unlimited supply of oral tissues that will have similar characteristics to the original mucosa [1-4]. Additionally, cell culturing of human oral mucosa has many applications for oral biology research, including the study of differentiation processes, effects of drugs, and chromosomal analysis [5].

A mucosal equivalent must have two basic components: a superficial portion or epidermis that contains keratinocytes and a deeper portion or dermis [6]. Therefore, a reliable source of cultured keratinocytes is essential as a component of mucosa and/or skin substitutes.

Rheinwald and Green (1975) [7] proposed the first technique to fabricate cultured oral epithelial sheets by using a feeder layer composed of irradiated 3T3 mouse fibroblasts to grow keratinocytes *in vitro*. However, oral mucosal sheets cultured with an irradiated feeder cell layer are undesirable in elective surgery because of the undetermined risk of introducing

Corresponding author: Aydin Gulses, Gülhane Military Medical Academy Department of Oral and Maxillofacial Surgery, 06018 Etlik Ankara, Turkey; e-mail: aydingulses@gmail.com

high mouse DNA content onto proliferating human cells [8,9]. This process was modified by Kitano and Okada (1983), who introduced a milder protease known as dispase to separate the epidermal sheet from the underlying dermis of the skin. Boyce and Ham (1983) adopted a serum-free medium for primary keratinocyte culture [10]. With this technique, the 3T3 feeder layer is no longer needed and therefore it has benefits for use in clinical applications [11].

The dissociation methods of keratinocyte primary culture are well established. However, attempts to acquire reliable techniques to isolate high-quality progenitor keratinocytes and propagate them in culture are ongoing in many laboratories. To the best of the authors' knowledge, nowadays, there are basically two techniques in primary culture: the enzymatic and the direct explant technique.

Billingham and Reynolds (1952) described a technique for the separation of epithelial cells using an enzyme (trypsin) [12], thus called the enzymatic method, in order to obtain keratinocytes and at the same time prevent these cells from losing their viability and culture potential [13]. The enzymatic technique was developed by Daniels *et al.* (1996), who surveyed the success rate of human keratinocyte isolation with various concentrations including trypsin and dispase [14], the enzymatic condition, as well as the calcium concentration in the culture medium [12]. According to Lauer and Schimming (2001) [15], Carrel and Burrows described in 1910 a method for the extraction of epithelia cells called direct explant, which has been used since that time. The direct explant technique has been used for 30 years in the culturing of human gingival [16] and buccal tissues [17]. According to Lauer *et al.* (1991) [16], explant technique combined with autogenous serum can be used for culturing gingival autografts as well as for cultures with special tissues. Additionally, Klingbeil *et al.* (2009) [13] stated that the direct explant technique obtained the first keratinocytes yield faster than the enzymatic technique.

Aim

The aim of this study was to isolate and investigate the percentage of success in culturing oral mucosa keratinocytes by modifying the direct explant technique described by Wanichpakorn and Kedjarunelaggat (2010) [5].

Materials and Methods

This project was approved by the First Ethics

Committee of Clinical Researches of Ankara, under Licence Number 2010/01-214. Primary cell cultures were created by using human oral epithelial tissues from volunteers who were undergoing oral surgery (such as implant surgery, third molar extraction, and periodontal surgery) at the Oral and Maxillofacial Surgery Clinics in Gulhane Military Medical Academy. Oral epithelial tissues were obtained from the keratinised gingival tissues of nine healthy human subjects (five male, four female, with an age range of from 16 to 57 years).

The tissue specimens were carried to the cell culture laboratory in a 10 ml culture media (Dulbecco's Modified Eagle's Medium [DMEM]: Gibco BRL, New York, USA; pH 7.2) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin and 0.5% amphotericin B (Gibco BRL, New York, USA) to prevent growth of microorganisms. The tissue specimens were washed and disinfected in a 10% povidone iodine solution for one to two minutes and after that washed in culture media. The tissue specimens were then cut into pieces, approximately 11 mm in size, and placed in the culture plate (T-25 flask, Corning, New York, USA) using a sterile needle of the dental injector. Tissue pieces were left in the culture plate for 15-20 minutes and then the culture media was gently dropped on the tissue pieces. After waiting for three to four hours, 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% CO₂ (Incubator SANYO MCO 18-AIC, Osaka, Japan). 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 1*), the culture medium was changed to EpiLife (Cascade Biologics, Portland, OR, USA) supplemented with human keratinocyte growth factors (EDGS Cascade Biologics, Portland, OR, USA), 125 µg/ml gentamycin and 1 µg/ml amphotericin B (Fungizone, Sigma Chemical Co, St Louis, MO, USA) which was described by Izumi *et al.* (1999) [1], 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 EpiLife culture medium. After about 10 days, when the primary cell culture reached 70-80% confluence (*Figure 3*), oral mucosa keratinocytes were harvested with a solution of 0.025% trypsin-ethylenedi-

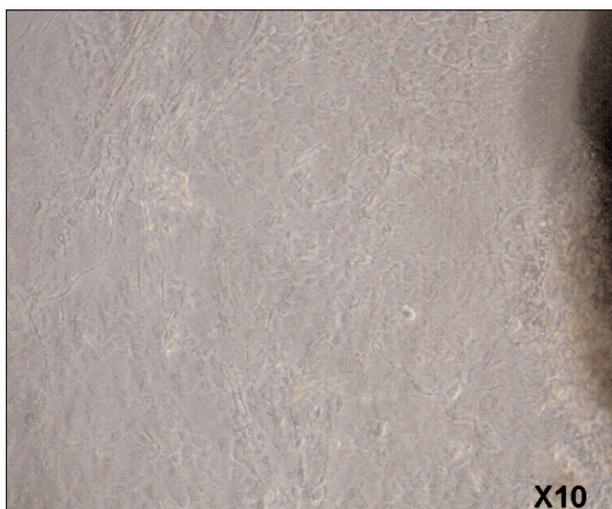


Figure 1. The morphology of the keratinocytes, which were squamous in shape, started to multiply around the tissue sample origin to a diameter of 2-5 mm.

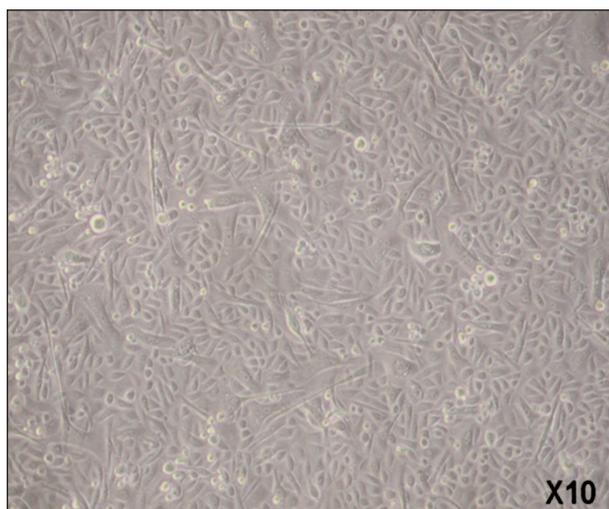


Figure 3. After around 10 days, when the primary cell culture reached to 70-80% confluence.

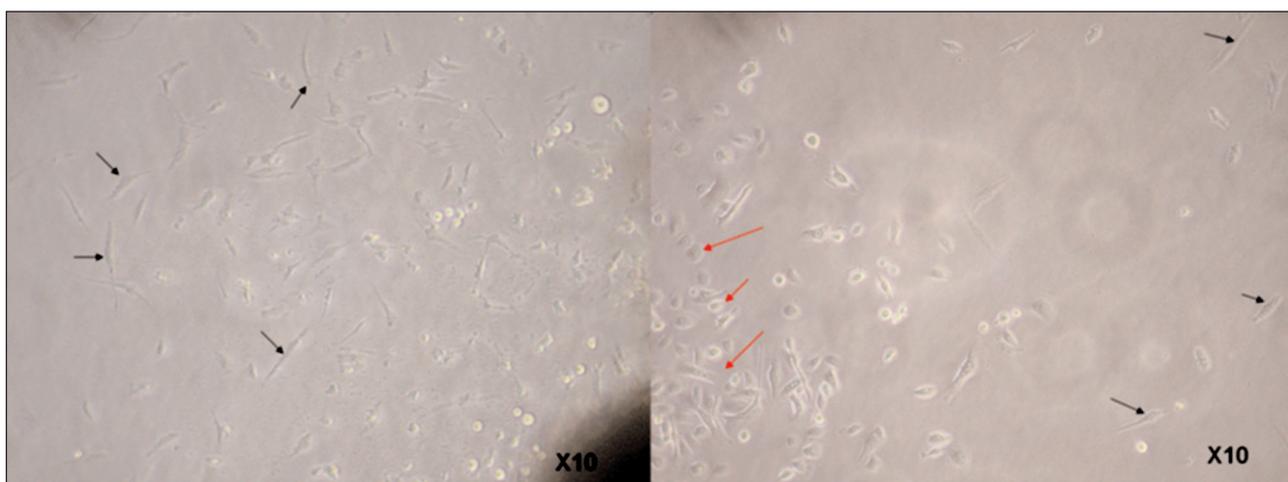


Figure 2. The elimination of the fibroblasts at second day after the changing of medium to EpiLife. Fibroblasts eliminated shown with black arrows. Keratinocytes attached shown with red arrows.

aminetetra-acetic acid (trypsin-EDTA, TE Cascade Biologics, Portland, OR, USA) at 37°C. After four to five minutes, 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 T-25 flask (Corning, New York, USA) at a density of 2.010^4 cells/cm². The first passage was subcultured to a T-25 flask and then moved to a T-75 flask (Corning, New York, USA) for the next passages. Primary cultured keratinocytes were used from the third through the fifth passages in a T-75 flask. In this study, the success rate of the culturing method was defined as the ability of the cells to grow from the original tissue sample, become 70-80% confluent, and to survive at least until the first passage.

Results

As previously described, the tissue samples were obtained from nine healthy human subjects (five males and four females) aged between 16-57 years (mean age 30.6 years \pm 12.8). The results showed that only one primary culture of oral epithelial cells by direct explant technique failed, and the total success rate was 88.9% (Table 1). In this study, no contamination of microorganisms in primary cell cultures was observed.

It was found that the tissue samples of a size approximately 11 mm were more successful for culturing the oral mucosa keratinocytes than those tissue samples larger than 11 mm. Oral mucosa epithelial cells developed from the original tissue after three to five days. The culture medium was

Table 1: The success and failure of the direct explant technique classified by sex and age

	Direct explant technique					
	Success			Fail		
	Female	Male	Total	Female	Male	Total
Oral mucosa keratinocyte cultivation	4	4	8 (88.9%)	0	1	1 (11.1%)
Age (mean±SD)	27.3±8.8			57		

Success: The ability of keratinocytes to grow out from the tissue sample origin, become 70-80% confluent and to survive at least until the first passage.

Fail: The cells cannot grow out from the tissue sample origin or survive at least until first passage.

changed to EpiLife, which is specific for culturing of the keratinocytes after nine to ten days [1]. In comparison, average time for the first development of keratinocytes was 20.8 days.

Discussion

Despite several published studies of enzymatic and direct explant techniques employed in keratinocyte cultivation, doubts still remain about which one is optimal for obtaining the greatest number of clonogenic cells, cell performance, and the best culture life-span. [13]

Klingbeil *et al.* (2009) [13] have stated that the average time needed to obtain the first keratinocyte was 11.9 days for the enzymatic method and 14.2 days for the direct explant method. Previous studies [5,13] have found that the average initial time for keratinocyte cultivation by the direct explant technique was 14.2 days. In the current study, it was 20.8 days, somewhat longer than in the previous studies. The use of a bigger culture plate (T-25 flask, 25 cm²) may have caused this difference. Nevertheless, the average initial time for keratinocyte cultivation in this study was not unreasonable compared to previous studies.

According to the results of the study performed by Klingbeil *et al.* (2009) [13], the operating procedure used in the direct explant technique process involves fewer steps compared with the enzymatic technique. The higher success rate of explant technique compared with the enzymatic may be interpreted with the number of the steps required. In this study, the success rate of mucosa keratinocyte culturing (88.9%) was similar to the findings of Wanichpakorn and Kedjarune-Laggat (2010) (88.9%) [5], higher than that reported by Kedjarune *et al.* (2001) [18], which was about 82%, and also higher than reported in the study of Reid *et al.* (1997) [19], which had

about 80% success, even though these three studies used the same direct explant technique.

According to the literature, the best possible concentration of trypsin-EDTA for detaching oral mucosa keratinocytes from a culture plate was 0.025% for four to five minutes. This was also suggested by Wanichpakorn and Kedjarune-Laggat's (2010) study [5]; these authors found that when using 0.05% trypsin-EDTA for harvesting of the keratinocytes, more than 30% of the cells would die in the first passage. Moreover, when the confluence of the cell number for keratinocytes subculture was 40-50%, it was found that each subculture growth rate slowed down. The same finding was also highlighted by Reid *et al.* (1997) [19].

The main disadvantage of the explant technique is that when cell propagation is desired, feeder-layer employment is needed and it also prevents the appearance of other undesired cells in the keratinocyte culture, such as fibroblasts. In this study, this problem was overcome by changing the culture medium that characteristically allows growing only keratinocytes in culture plate. *Figure 2* shows the elimination of the fibroblasts at the second day after changing the medium to EpiLife.

Bacterial contamination in the oral cavity from the tissue samples associated with direct explant technique is another problem that affects the success rate of the cultivation [5,13,18]. In addition, bacterial contamination was also reported to occur during medium preparation. Wanichpakorn and Kedjarune-Laggat (2010) [5] suggested that the contamination risk is correlated with the size of the tissue, because the tissue samples were very small and thin. The disinfection times for tissue samples can vary among different studies. In the current study, the tissue samples were not contaminated after placing in a 10% povidone iodine solution for one to two minutes.

Conclusions

The direct explant method used for this study provided successful results for primary culture of human oral keratinocyte. Within the limited number of samples cultured in the current study, it can be concluded that the explant technique has two advantages:

1. Technical handling involved in the direct explant method at the beginning of the process has fewer steps.
2. The direct explant method does not require a feeder-layer in order to obtain cells.

The use of direct explant cultivation protocol is adequate for obtaining oral keratinocytes in culture, and also shows the possibility of formation of a stratified epithelium. A larger study is needed to confirm these initial findings

References

1. Izumi K, Takacs G, Terashi H, Feinberg SE. *Ex vivo* development of a composite human oral mucosal equivalent. *Journal of Oral and Maxillofacial Surgery* 1999; **57**: 571-577.
2. Izumi K, Feinberg SE. Skin and oral mucosal substitutes. *Oral Maxillofacial Surgery Clinics of North America* 2002; **14**: 61-71.
3. Nakanishi Y, Izumi K, Yoshizawa M, Saito C, Kawano Y, Maeda T. The expression and production of vascular endothelial growth factor in oral mucosa equivalents. *International Journal of Oral and Maxillofacial Surgery* 2007; **36**: 928-933.
4. Bayar GR. *Ex vivo* produced oral mucosa equivalent preliminary report: a technical note. *Turkish Journal of Medical Sciences* 2011; **41**: 109-115
5. Wanichpakorn S, Kedjarune-Laggat U. Primary cell culture from human oral tissue: gingival keratinocytes, gingival fibroblasts and periodontal ligament fibroblasts. *Songklanakarinn Journal of Science and Technology* 2010; **32**: 327-331
6. Izumi K, Song J, Feinberg SE. Development of a tissue-engineered human oral mucosa: from the bench to the bed side. *Cells, Tissues, Organs* 2004; **176** : 134-152.
7. Rheinwald JG, Green H. Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma. *Cell* 1975; **6**: 317-330.
8. Raghoebar GM, Tomson AM, Scholma J, Blaauw EH, Witjes MJ, Vissink A. Use of cultured mucosal grafts to cover defects caused by vestibuloplasty: An *in vitro* study. *Journal of Oral and Maxillofacial Surgery* 1995; **53**: 872-878
9. Lauer G. Discussion: Use of cultured mucosal grafts to cover defects caused by vestibuloplasty: An *in vitro* study. *Journal of Oral and Maxillofacial Surgery* 1995; **53**: 878-879.
10. Kitano Y, Okada N. Separation of the epidermis sheet by dispase. *British Journal of Dermatology* 1983; **108**: 555-560.
11. Boyce ST, Ham RG. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *Journal of Investigative Dermatology* 1983; **81**(1 Suppl): 33-40.
12. Billingham RE, Reynolds J. Transplantation studies on sheets of pure epidermal epithelium and on epidermal cell suspensions. *British Journal of Plastic Surgery* 1952; **5**: 25-36.
13. Klingbeil MF, Herson MR, Cristo EB, dos Santos Pinto D Jr, Yoshito D, Mathor MB. Comparison of two cellular harvesting methods for primary human oral culture of keratinocytes. *Cell and Tissue Banking* 2009; **10**: 197-204.
14. Daniels JT, Kearney JN, Ingham E. Human keratinocyte isolation and cell culture: a survey of current practices in the UK. *Burns* 1996; **22**: 35-39.
15. Lauer G, Schimming R. Tissue-engineered mucosa graft for reconstruction of the intraoral lining after freeing of the tongue: a clinical and immunohistologic study. *Journal of Oral and Maxillofacial Surgery* 2001; **59**: 169-175.
16. Lauer G, Otten JE, Von Specht BU, Schilli W. Cultured gingival epithelium. A possible suitable material for pre-prosthetic surgery. *Journal of Craniomaxillofacial Surgery* 1991; **19**: 21-26.
17. Flaxman BA, Lutzner MA, Van Scott EJ. Cell maturation and tissues organization in epithelial out-growths from skin and buccal mucosa *in vitro*. *Journal of Investigative Dermatology* 1967; **67**: 8-14.
18. Kedjarune U, Pongprerachok S, Arpornmeaklong P, Ungkusonmongkhon K. Culturing primary human gingival epithelial cells: comparison of two isolation techniques. *Journal of Craniomaxillofacial Surgery* 2001; **29**: 224-231.
19. Reid CBA, Cloos J, Snow GB, Braakhuis BJM. A simple and reliable technique for culturing of human oral keratinocytes and fibroblasts. *Acta Oto-laryngologica* 1997; **117**: 628-633.

Acknowledgements

This project was funded by a grant from the Turkish Scientific and Technological Research Council (TUBITAK-Project no: 110S025).

Contribution of each author

GRB was research director, performed the surgical procedures.

YSA approved the study, checked the results and the paper.

AG wrote the paper, performed the surgical procedures.

PE performed the laboratory procedures.

MS performed the laboratory procedures.

Statement of conflict of interests

As far as the authors are aware, there was no conflict of interest.