The Primary Culture of Human Oral Mucosa Keratinocytes by Enzymatic Technique

Enzimatik Teknikle Gerçekleştirilen Primer Ağız Mukozası Keratinosit Hücre Kültürü

Gürkan Raşit BAYAR,[a]
Yavuz Sinan AYDINTUĞ,[a]
Aydın GÜLSES,[a]
Aydın ÖZKAN,[a]
Metin ŞENÇİMEN[a]

[a]Department of Oral and Maxillofacial Surgery, Gülhane Military Medical Academy, Ankara

Geliş Tarihi/*Received:* 05.05.2011
Kabul Tarihi/*Accepted:* 13.06.2011

This project has been granted by Turkish Scientific and Technological Research Council (TUBITAK- project no: 110S025)

Yazışma Adresi/*Correspondence:*
Gürkan Raşit BAYAR
Gülhane Military Medical Academy, Department of Oral and Maxillofacial Surgery, Ankara, TÜRKİYE/TURKEY
gurkanbayar@yahoo.com

ABSTRACT Objective: For reconstruction of oral mucosa defects, mucosal grafting is the ideal method, but limitation in the availability of graft material has made such reconstruction difficult. To solve this problem of autologous oral mucosa for reconstructive surgery, primary culture of oral mucosa keratinocytes has been developed. There are two techniques in primary culture of oral mucosa keratinocytes, which include the enzymatic and direct explant technique. The objective of this study was to present success rates and our experiences on the cultivation of oral mucosa keratinocytes using the enzymatic method. **Material and Methods:** Keratinocytes for primary cell culture were obtained from healthy oral mucosa specimens excised during operation of patients with no systemic disease who were referred for routine oral surgeries to the Oral and Maxillofacial Surgery Clinics. Tissue specimens were obtained from the oral mucosa of 12 different healthy human subjects (7 male, 5 female, aged between 9 and 59 years) oral mucosa. In this study, enzymatic technique was used for the primary cell culture of oral mucosa keratinocytes. **Results:** While a primary cell culture could be achieved in 8 tissue specimens, primary cell culture was not possible in 4 specimens, among which one developed fungal infection. **Conclusion:** The success rate was 66.6% by enzymatic technique in primary cell culture of oral mucosa keratinocytes. Enzymatic technique allows dissociation of keratinocytes and yields successful results in primary cell culture of oral mucosa keratinocytes.

Key Words: Primary cell culture; keratinocyte; enzymatic technique

ÖZET Amaç Ağız mukozası defektlerinin onarımı amacıyla gerçekleştirilen mukozal greft uygulamaları, ideal bir yöntem olmalarına rağmen, greft materyalinin elde edilmesi ile ilgili sınırlamalar, bu onarımı güçleştirmektedir. Bu sorunun çözümüne yönelik olarak oral mukoza keratinositlerinin primer kültürü yöntemi geliştirilmiş olup, bu yöntem, enzimatik ve direkt eksplant tekniği olmak üzere iki şekilde uygulanmaktadır. Bu çalışmanın amacı, enzimatik teknik kullanılarak ağız mukozası keratinositlerinin üretilmesi ile ilgili başarı oranlarını ve bu konudaki deneyimlerimizi sunmaktır. **Gereç ve Yöntemler:** Primer hücre kültürü için gereken keratinositler, Ağız, Diş, Çene Hastalıkları ve Cerrahisi Anabilim Dalı polikliniğine rutin ağız cerrahisi işlemleri için başvuran ve klinik olarak önemli bir sistemik hastalığı bulunmayan hastaların, operasyon gereği eksize edilmiş sağlıklı ağız mukozası doku örneklerinden elde edilmiştir. Doku örnekleri, yaşları 9 ile 59 arasında değişen 12 (7 erkek, 5 kadın) farklı sağlıklı bireyin ağız mukozalarından alınmıştır. Çalışmamızda, enzimatik primer ağız mukozası keratinosit hücre kültürü yöntemi uygulanmıştır. **Bulgular:** Doku örneklerinden 8'inde başarılı bir primer hücre (keratinosit) kültürü oluşturulabilirken, 4'ünde başarılı bir primer hücre kültürü elde edilememiş ve bunlardan birinde de mantar enfeksiyonu gelişmiştir. **Sonuç:** Çalışmamızın sonucunda, enzimatik teknikle gerçekleştirilen ağız mukozası primer hücre kültüründe %66,6 oranında başarı elde edilebilmiştir Enzimatik teknik, insan ağız mukozası keratinosit kültüründe başarılı sonuçların elde edilebildiği, kertinositlerin ayrıştırılmasına izin veren bir tekniktir.

Anahtar Kelimeler: Primer hücre kültürü; keratinosit; enzimatik teknik

Turkiye Klinikleri J Dental Sci 2011;17(3):237-42

Need for covering massive soft tissue deficiencies following traumas, ablative cancer surgeries, thermal or chemical burns, penetrating gun shot injuries or preprothetic surgeries on the maxillofacial region is among major problems faced by oral and maxillofacial surgeons. Reconstructive procedures in oral and maxillofacial surgery require autogenous skin and/or oral mucosa grafts for recompensing the soft tissue loss and enhancement of wound healing. Despite their disadvantages like the need for a second surgical procedure, limited graft amount and unsuitable texture of skin grafts, autogenous keratinized oral mucosa and split thickness skin grafts are still widely used for reconstruction of the soft tissue deficits of the circumoral region. Another solution for this problem may be to construct an oral mucosa substitute produced by the keratinocytes of the patient in required size.[1-5]

A mucosal equivalent necessitates basically the existence of two components: a superficial portion or epidermis that contains keratinocytes and the deeper portion or dermis. Therefore, a reliable source of cultured keratinocytes is essential as a component of mucosa and/or skin substitutes. Additionally, cell culturing of human oral mucosa has many applications for oral biology researches including the study of differentiation processes, effects of drugs, and chromosomal analysis.[6,7]

Rheinwald and Green[8] have 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 were undesirable in elective surgery because of the undetermined risk of introducing high mouse DNA content onto proliferating human cells.[9,10] This process was modified by Kitano and Okada[11] by introducing a milder protease known as dispase to separate the epidermal sheet from the underlying dermis of the skin. Boyce and Ham[12] adopted a serum-free medium for primary keratinocyte culture. This technique did not require the 3T3 feeder layer and therefore had benefits for use in clinical practice.

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 our knowledge, currently, there are basically two techniques in primary culture; the enzymatic and the direct explant technique.

In 1952, Billingham and Reynolds[13] described a technique for the separation of epithelial cells using an enzyme (trypsin), thus called the enzymatic method, in order to obtain keratinocytes and at the same time prevent these cells from loosing their viability and culture potential.[14] The enzymatic technique was further developed by Daniels et al.,[15] by surveying the success rate of human keratinocyte isolation with various concentrations including trypsin and dispase, the enzymatic condition, as well as the calcium concentration in the culture medium.

The aim of this study was to investigate the success rate of oral mucosa keratinocyte cultures by enzymatic technique described by Izumi et al.[1]

MATERIAL AND METHODS

This project was approved by the First Ethics Committee of Clinical Research of Ankara with the License Number 2010/01-214. Primary cell cultures were performed by using human oral epithelial tissues (4x4 mm or larger) obtained from volunteers who were undergoing dental surgeries such as implant surgery, third molar extraction, and gum surgery, at the Oral and Maxillofacial Surgery Clinics in the Gülhane Military Medical Academy (Figure 1). Oral epithelial tissues were obtained from 12 healthy human subjects (7 male, 5 female, aged between 9 and 59 years).

The basic "culture medium" (chemically defined culture medium, "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 amphothericin B (Sigma Chemicals Co., USA) was prepared as described by Izumi et al1 with a calcium concentration of 0.06 mM.

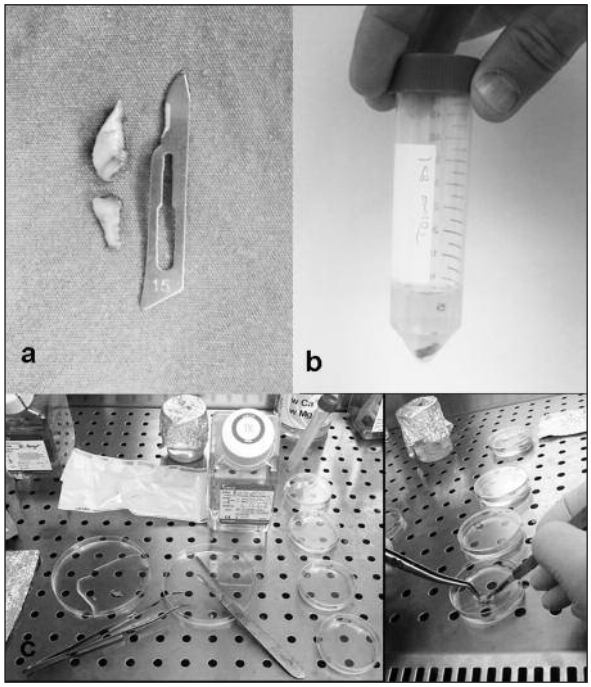

FIGURE 1: a; Human oral epithelial tissue samples, **b;** transportation of the tissue sample in a 50 ml falcon tube, c;d; preparation and cleaning of the tisssue sample for tyripsinization.

Tissue samples were transported to the cell culture laboratory in "washing solution" (Phosphate- Buffered Saline PBS, Ca++ and Mg++ free) which was supplemented with 125 µg/ml gentamycin, and 1 µg/ml amphotericin B (Sigma Chemicals Co., USA). Oral mucosal samples were placed in a new washing solution, were scraped clean to remove blood, and were trimmed of excess tissue (Figure 1). The mucosal tissues in the 0.04% trypsin solution (Sigma Chemicals Co., USA) were digested overnight at room temperature to allow the separation of the epithelium at the suprabasal layer. Trypsin was inactivated the next day by 0.0125% trypsin inhibitor ("DTI" Cascade Biologics, Portland, OR, USA). The epithelial layers were mechanically separated above the basal layer, and the interface areas were scraped to dissociate the basal cells from the submucosal layer. Collected cells were filtered with a nylon mesh filter (240 µm) and were counted with a hematocytometer (Figure 2). Following centrifugation of cells for 5 minutes at 1000 rpm at ambient temperature, they were resuspended with 5 ml culture medium and were plated at a concentration of 2.3×10^6 cells in a T-25 flask (Laboratory Science Co. Corning, NY, USA). They were incubated at 37ºC in 5% CO2 (Figure 3). The medium was changed two days after initial plating of the cells. The cultures were fed every other day with basic "culture medium" containing a low calcium concentration of 0.06 mM. After around 15 days, oral mucosa keratinocytes were harvested when they reached at 70-80% confluency and were replated onto different T-25 or T-75 flasks at a concentration of 2.0×10^4 cells/cm^2 (Figure 4). Replated keratinocytes in the new flasks were used for subsequent cell proliferation assays. In this study, the success rate of the culturing method was defined as the attaching capacity of the cells to the bottom of the flasks, become 70-80% confluent and to survive at least until the first passage.

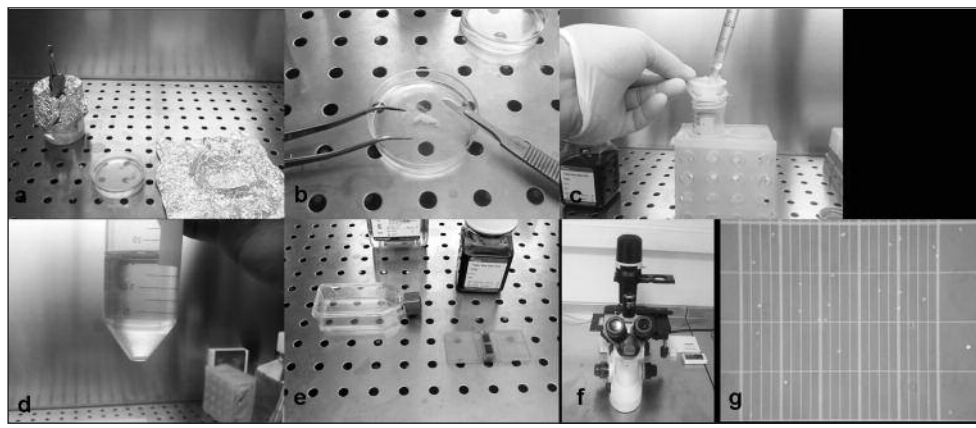

FIGURE 2: a;b; Tyripsinization and scraping of the tissue sample, **c;** filtration of the collected cells, **d;** appearance of the collected cells in a 50 ml falcon tube after centrifugation, and **e;f;g;** counting of the keratinocytes with a hematocytometer.

FIGURE 3: a; A T-25 flask plated oral mucosa keratinocytes, **b;** an incubator, and **c;** T-25 flasks plated oral mucosa keratinocytes on the shelf of the incubator.

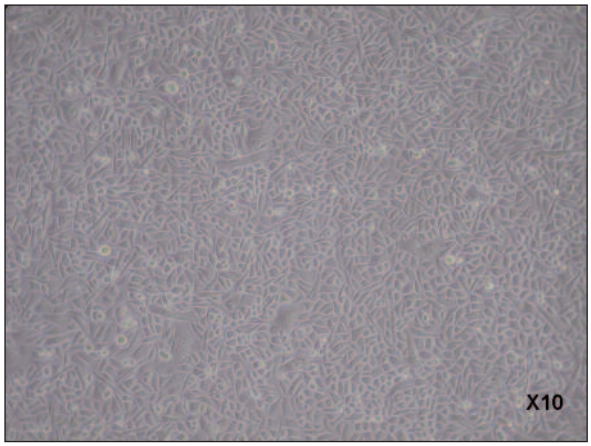

FIGURE 4: After around 15 days, when the primary cell culture reached to 70-80% confluence.

Descriptive statistical analyses were done for the tissue samples. SPSS 15.0 for Microsoft Excel (Chicago, USA) was used for data processing. Descriptive statistics were shown as mean ± standard deviation.

RESULTS

Tissue samples were obtained from 12 healthy human subjects (7 males and 5 females) aged between 9-59 years (mean age 36.1±15.3). Primary cultures of the oral epithelial cells by enzymatic technique failed in 4 samples and one developed a fungal infection. The overall success rate was 66.6%. Results were summarized in Table 1.

The ideal transportation period for the tissue samples to the cell culture laboratory proved to be between 1 and 3 hours. Less than 1 hour could increase the risk of infection and more than 3 hours could reduce the attaching capacity of the cells. The average time for keratinocytes in flasks to become 70-80% confluent in the first passage was 15.3 ± 1.6 days.

DISCUSSION

Although several published studies of enzymatic and direct explant techniques employed in keratinocyte cultivation, ther is still controversy around which one would be the best choice to obtain the greatest number of clonogenic cells, cell performance, and the best culture life span.[6,14]

According to Lauer and Schimming,[16] in 1910, Carrel and Burrows described a method for the extraction of epithelia cells, called direct explant, which has been used since then. The direct explant technique has also been used to culture human gingival and buccal tissues. According to Lauer et al.,[17] explant technique combined with autogenous serum can yield successful results for culturing gingival autografts. In addition, Klingbeil et al.[17] stated that although the direct explant technique was faster than the enzymatic technique in obtaining the first keratinocyte yield, the infection risk, which is a common problem in primary cell culture procedures, is more common in the direct explant technique than the enzymatic technique.

Klingbeil et al14 stated that the average time needed to obtain the first keratinocyte culture was

TABLE 1: The success and failure rates of the enzymatic technique classified by sex and age.

	Enzymatic Technique					
	Success			Fail		
	Female	Male	Total	Female	Male	Total
Oral Mucosa Keratinocyte Cultivation	2	6	8 (%66.6)	3	1	4 (%33.4)
Age (Mean±SD)		30.2 ± 14.4			48 ± 9.8	

Success: The attaching ability of the keratinocytes to the bottom of the flasks, to become 70-80% confluent and to survive at least until the first passage.

Fail: The failure of cells to attach to the bottom of the flasks or the infection of the culture.

11.9 days for the enzymatic technique and 14.2 days for the direct explant technique. The time in the current study was 15.3 ± 1.6 days for the enzymatic technique, which was longer than the time in the previous study. In this study, using a bigger culture plate (T-25 flask, 25 cm^2) than the one used in the previous study (culture dish, 4 cm) may account for this difference. Nevertheless, the average initial time for keratinocyte cultivation in this study was reasonable compared to the time previous study.

Higher succes rates have been reported for the direct explant technique compared to the enzymatic technique.18-20 In this study, the success rate of mucosa keratinocyte culture by the enzymatic technique (66.6%) was lower than that obtained by Kedjarune et al (88.9%), and Reid et al. (80%) by the direct explant technique. This may be attributed to the number of steps required in the direct explant technique.

Probably the main disadvantage of the explant technique is that for appropriate cell propagation, the culture medium should be changed; this prevents the appearance of other undesired cells like fibroblasts in the keratinocyte culture. In the enzymatic technique, changing of the culture medium was not required to allow the growth of only keratinocytes in the culture plate.

Bacterial contamination in the oral cavity from the tissue samples associated with direct explant technique is another problem, which affects the success rate of the cultivation. In addition, bacterial contamination was also reported to occur during medium preparation.6,14,20 Freshney20 suggested that the contamination risk was correlated with the size of the tissue and very small and thin tissue samples could be adequate for the direct explant technique. On the other hand, the success rate increases paralell to the size of the tissue sample in the enzymatic technique. Therefore, the infection risk may also increase in the enzymatic technique.

CONCLUSION

The enzymatic technique used for this study provided good results for primary culture of human oral mucosa keratinocytes. The enzymatic technique has two advantages:

1) In the enzymatic technique, changing of the culture medium was not needed to allow the growth of only keratinocytes in the culture plate.

2) According to previous reports, less time may be required in the enzymatic technique than the direct explant technique for the first cultivation of the keratinocytes.

The use of enzymatic cultivation protocol is adequate to obtain oral keratinocytes in culture, and allows the formation of a stratified epithelium.

Acknowledgement

This project has been granted by the Turkish Scientific and Technological Research Council (TUBITAK-Project no: 110S025).

REFERENCES

1. Izumi K, Takacs G, Terashi H, Feinberg SE. Ex vivo development of a composite human oral mucosal equivalent. J Oral Maxillofac Surg 1999;57(5):571-7.
2. Avcı G, Akan M, Yıldırım S, Aköz T. [Nerve repair and grafting (review of the literature)]. Turkiye Klinikleri J Med Sci 2002;22(4):428-37.
3. Izumi K, Feinberg SE. Skin and oral mucosal substitutes. Oral Maxillofac Surg Clin North Am 2002;14(1):61-71.
4. 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. Int J Oral Maxillofac Surg 2007;36(10):928-33.
5. Bayar GR. Ex vivo produced oral mucosa equivalent preliminary report: a technical note. Turk J Med Sci 2011;41(1):109-15.
6. Wanichpakorn S, Kedjarune-Laggat U. Primary cell culture from human oral tissue: gingival keratinocytes, gingival fibroblasts and periodontal ligament fibroblasts. Songklanakarin J Sci Technol 2010;32(4):327-31.
7. Izumi K, Song J, Feinberg SE. Development of a tissueengineered human oral mucosa: from the bench to the bed side. Cells Tissues Organs 2004;176(1-3):134-52.
8. Rheinwald JG, Green H. Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma. Cell 1975;6(3):317-30.

9. Raghoebar GM, Tomson AM, Scholma J, BlaauwEH, Witjes MJ, Vissink A. Use of cultured mucosal grafts to cover defects caused by vestibuloplasty: An in vitro study. J Oral Maxillofac Surg 1995;53(8):872-8.
10. Lauer G. Discussion: use of cultured mucosal grafts to cover defects caused by vestibuloplasty: An in vitro study. J Oral Maxillofac Surg 1995;53(8):878-9.
11. Kitano Y, Okada N. Separation of the epidermis sheet by dispase. Br J Dermatol 1983;108(5):555-60.
12. Boyce ST, Ham RG. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. J Invest Dermatol 1983;81(1 Suppl):33–40.
13. Billingham RE, Reynolds J. Transplantation studies on sheets of pure epidermal epithelium and on epidermal cell suspensions. Br J Plast Surg 1952;5(1):25-36.
14. 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 Tissue Bank 2009;10(3):197-204.
15. Daniels JT, Kearney JN, Ingham E. Human keratinocyte isolation and cell culture: a survey of current practices in the UK. Burns 1996;22(1):35-9.
16. Lauer G, Schimming R. Tissue-engineered mucosa graft for reconstruction of the intraoral lining after freeing of the tongue: a clinical and immunohistologic study. J Oral Maxillofac Surg 2001;59(2):169-75.
17. Lauer G, Otten JE, Von Specht BU, Schilli W. Cultured gingival epithelium. A possible suitable material for pre-prosthetic surgery. J Craniomaxillofac Surg 1991;19(1):21-6.
18. Kedjarune U, Pongprerachok S, Arpornmeaklong P, Ungkusonmongkhon K. Culturing primary human gingival epithelial cells: comparison of two isolation techniques. J Craniomaxillofac Surg 2001;29(4):224-31.
19. Reid CB, Cloos J, Snow GB, Braakhuis BJ. A simple and reliable technique for culturing of human oral keratinocytes and fibroblasts. Acta Otolaryngol 1997;117(4):628-33.
20. Freshney RI . Primary culture. Culture of Animal Cells: A Manual of Basic Techniques. 4th ed. New Jersey: Wiley and Sons; 2000. p.149-77.