

The Histological Characteristics of Cultured Oral Epithelium in Different Culture Conditions

Hassan Hashemi^{1 & 2}, Mojdeh Salehnia^{*3}, Morvarid Kamali¹ and Mandana Beigi Boroujeni⁴

¹Dept. of Ophthalmology, Farabi Eye Hospital, School of Medicine, Tehran University of Medical Sciences, Tehran;

²Noor Ophthalmology Research Center, Noor Eye Hospital, Tehran; ³Dept. of Anatomy, Faculty of Medical Sciences, Tarbiat Modares University, Tehran; ⁴Dept. of Anatomy, Faculty of Medical Sciences, Lorestan University of Medical Sciences, Khoramabad, Iran

Received 27 September 2008; revised 27 December 2008; accepted 5 January 2009

ABSTRACT

Background: This study was undertaken to establish the characterization of cultured oral mucosal epithelium and introducing them as an alternative source for reconstruction of ocular surface disease. **Methods:** Human oral epithelial cells were cultured on simple media (DMEM/HF12) as control and co-cultured on mitomycin C-treated 3T3 feeder layer, on the amniotic membrane (AM) without nitrocellulose and the mitotically inactivated 3T3 fibroblast, and on the sandwich layer of AM fastened on the nitrocellulose as insert and 3T3 fibroblast. After 3 weeks, the characteristics of the cells were assessed morphologically and also ultrastructurally using scanning electron microscopy and transmission electron microscopy and immuno-cytochemically. **Results:** The epithelial cells were cultured on AM spread on nitrocellulose insert and 3T3 feeder layer showed better growth than other groups and all groups of study were shown similar characteristics. The cultured oral epithelial shared the characteristics with corneal epithelium. **Conclusion:** Thus the oral epithelial could be an alternative source for transplantation. *Iran. Biomed. J. 13 (2): 109-115, 2009*

Keywords: Autologous, Co-culture, Corneal epithelium, Human oral epithelium

INTRODUCTION

Among different mucosal epithelia including oral, nasal, esophageal, tracheal and vaginal, the oral epithelium has been attracted as an available source for autologous cell transplantation [1-6]. The oral epithelial has also some advantages as candidate for usage in ocular surface disease such as the lower stage of differentiation and its short cell turnover time. It requires shorter culture period and can be maintained in culture for prolonged periods without keratinization [5]. Moreover, the oral mucosa is an available location for biopsy and its epithelial cells and cornea have shown the similar morphological and cytochemical characteristics [4]. However, there are some difficulties in developing a culture system for oral epithelial cells and a variety of factors may influence on the quality of the epithelial sheet, including source of cells,

preparation of the cells, types of substrates used, the use of feeder cells, and culture conditions.

Some investigations have been done on human and non human oral epithelial culture using denuded amniotic membrane (AM) as the substrate, 3T3 fibroblast layer to assist epithelial stratification and air lifting to facilitate the correct formation of epithelial sheet [1-8].

Fresh human AM secretes anti-inflammatory factor has anti-fibroblastic activity and anti-angiogenic property and it is the first carrier for corneal epithelial sheets [9, 10]. Nakamura *et al.* [3, 4] have developed a method for oral epithelial cell cultivation using human AM as carrier, and succeeded in transplantation of cultivated oral epithelial sheets.

Another supporting layer for proliferation and differentiation of epithelial cells is human fibroblasts or 3T3 mouse embryonic fibroblast. They were used

*Corresponding Author; Tel. (+98-21) 8288 3871; Fax: (+98-21) 8801 3030; E-mail: mogdeh@dr.com/salehnm@modares.ac.ir

for the first time in differentiation of mouse embryonic stem cells into skin epithelial cells [11].

Nishida *et al.* [7] grafted oral mucosal epithelial cell sheets cultured on temperature responsive cell-culture surfaces with 3T3 feeder cells. Inatomi *et al.* [1, 2] cultured the oral mucosal epithelium onto denuded AM spread on the bottom of culture inserts, and co-cultured with mitomycin-C inactivated 3T3 fibroblasts.

As it has been mentioned the transplantation of human oral epithelial sheets grown on various substrates can be useful for corneal reconstruction [1-8] but little is known about their histological and ultrastructural characteristics in the same culture system. However more studies need to improve the oral epithelial culture system.

This study was undertaken to compare and evaluate a cell culture system for the expansion of oral epithelial cells on AM carrier with or without nitrocellulose as insert and 3T3 fibroblast. In the present study, the growth, morphology and ultrastructural characteristics of cultured cells in different conditions were compared with light, scanning, and transmission electron microscopy (TEM) and immuno-cytochemistry for epithelium-specific keratin and stem cell marker "β1-integrin" [8, 12].

MATERIALS AND METHODS

Experimental design. The preparation of human samples (AM and oral epithelium biopsies) and the experimental studies were approved by the Institutional Review Board of Tehran University of Medical Sciences (Iran). To evaluate and compare the characteristics of human oral mucosal epithelium in different culture conditions, the following culture systems were considered (Fig. 1). Group A: The epithelial cells were co-cultured on the mitotically inactivated 3T3 fibroblast feeder layer. Group B: The epithelial cells were co-cultured on the AM without nitrocellulose and the mitotically inactivated 3T3 fibroblast. Group C: The epithelial cells were co-cultured on the sandwich layer of AM fastened on the nitrocellulose as insert and 3T3 fibroblast. Group D: The epithelial cells were cultured without any supporting feeder layer as control.

Amniotic membrane preparation. AM were prepared according to Bouchard and John method [10]. Briefly, AM was obtained in a sterile manner during cesarean section after written consent had

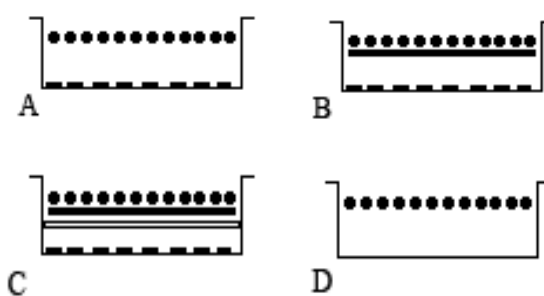


Fig. 1. The diagram of experimental design in all groups of study. ●●, epithelial cells; —, 3T3 fibroblast feeder layer; —, amniotic membrane; —, nitrocellulose insert.

been obtained from donors. We confirmed that maternal serum was negative for human immunodeficiency virus, hepatitis B and C viruses and cytomegalovirus. The AM was first washed free of blood clots with normal saline containing antibiotics (1% penicillin-streptomycin-amphotericin B) under sterile condition. The inner layer of AM was separated from the rest of the chorion by blunt dissection. Then, it was rinsed in sterile saline and later by 4%, 8% and 10% DMSO in PBS containing antibiotics for 5 min in each step, respectively. Some of the membrane was then fastened on nitrocellulose paper, with the epithelium/basement membrane surface up position and the remainder without the nitrocellulose inserts. The AM was then cut into 3 × 3 cm pieces. Each piece was placed in a sterile dishes containing 10% DMSO medium and frozen at -70°C. The membrane was defrosted immediately before use by warming the container at room temperature for 10 min, and rinsed three times in PBS.

Oral tissue sampling and isolation. Small biopsy specimens (approximately 2 mm³) of clinically normal oral tissues (buccal part) were obtained from 18-20-year-old men (n = 3) under local anesthesia. The biopsy specimens were washed three times with PBS containing antibiotics. The submucosal connective tissue was first carefully removed with scissors. The oral epithelium was then incubated at 37°C for 2 hours with 1.2 IU dispase II (Gibco, Paisley, UK) and treated with 0.25% trypsin-2.65 mM EDTA solution at room temperature for 20 minutes to scatter the epithelial cells. The single harvested cells were cultured in all groups of study as "Experimental Design".

The feeder layer preparation. 3T3 fibroblast cell line was prepared from Cell Bank of Pasture Institute of Iran (Tehran) and cultured on DMEM

(Gibco, Paisley, UK). Confluent 3T3 cells were incubated with 4 µg/ml of mitomycin C under 5% CO₂ at 37°C for 2 hours, then rinsed with PBS, trypsinized and placed in 96-well plastic dishes at a density of 2×10^5 cells/cm².

Oral mucosal epithelial cell culture and media.

After preparation of oral epithelial cells suspension, AM and mitomycin C-treated 3T3 fibroblasts, the epithelial cells were cultured with density of 1×10^5 /ml in 96-well dishes in all groups of study as described in "Experimental Design". The culture medium in all groups was the same according to the Nakamura *et al.* method [4] with some modifications. It was consisted of DMEM (Gibco, Paisley, UK) and Ham's F12 (Sigma-Aldrich, Hamburg, Germany) in 1:1 ratio and supplemented with 10% fetal bovine serum, 10 ng/ml epidermal growth factor (Sigma-Aldrich, Hamburg, Germany), 1% penicillin-streptomycin-amphotericin B (Sigma-Aldrich, Hamburg, Germany) and 5 µg/ml human recombinant insulin (Sigma-Aldrich, Hamburg, Germany). The medium was changed every day and culture was maintained with 5% carbon dioxide and 95% air at 37°C for 3 weeks. The morphology of cultured cells was assessed during culture period under inverted microscope.

Immuno-cytochemistry for Keratin and $\beta 1$ -integrin. Three weeks after culturing, the cytoplasmic slides of different groups of study were prepared for indirect immuno-cytochemical study. The samples were fixed with 4% paraformaldehyde in PBS and blocked with 10% normal goat serum (Sigma, Hamburg, Germany) in PBS. Cells were permeabilized with 0.3% triton X-100 (ICN, USA) and incubated with primary antibody against mouse anti-cytokeratin 3/12 (Santa Cruz Biotechnology Inc., USA) or mouse anti- $\beta 1$ -integrin (Santa Cruz Biotechnology Inc., USA) then were incubated with FITC-conjugated secondary antibody in dark room for 2 hours. After mounting with medium containing glycerol buffer, the slides were examined by fluorescent microscopy.

Transmission electron microscopy. The cultured oral epitheliums in different groups of study were examined by TEM. The specimens were fixed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS, pH 7.4) and post-fixed with 1% osmium tetroxide in the same buffer for 2 hours. After dehydration in an ascending series of ethanol, specimens were placed in propylene oxide and

embedded in Epon 812 (TAAB, Berkshire, UK). Semithin sections (0.5 µm) were stained with toluidine blue for light microscopy. Ultrathin sections (60-80 nm) were stained with uranyl acetate and lead citrate and then examined by TEM (Zeiss, Gottingen, Germany).

Scanning electron microscopy (SEM). For SEM analysis, after fixing and dehydrating samples in the same manner as described before for TEM study, they were dried, mounted and coated with gold particles (Bal-Tec, Switzerland) and examined using a scanning electron microscope (Philips XL30, Netherland).

RESULTS

In vitro growth and the morphology of cultured cells. Oral mucosal tissues were safely expanded without any fungal and bacterial contamination. By three-week culturing, mature oral mucosal epithelial sheets were generated in all groups of study and the superficial layer showed a typical epithelial morphology with polygonal appearance (Fig. 2). The growth of epithelial cells was different in experimental groups. It was higher in group C than other groups since they reached to the confluency near 18 days after culturing. Group D showed lower rate of growth than other groups and in this group, the cells reached to the confluency at the end of 3 weeks of culturing.

TEM ultrastructural observation. In group A, the cells formed a thin epithelial layer and the height of the cells was lower than the other groups (Fig. 3a). However, well-defined desmosomes were formed among the adjacent cells (Fig. 3b). Low magnification electron micrographs of the groups B and C confirmed that the sheets were comprised of well-differentiated stratified epithelium (Fig. 3c). They were consisted of cuboidal basal cells, several polygonal cells in intermediate layer, and flat apical cell surface layer. There were a lot of lateral interdigitations among cells in different layers (Fig. 3d). Several desmosomes were formed at lateral surface of the cells. The cytoplasm was filled with a lot of fibrous keratin protein which extended to the desmosomes (Fig. 3 d). The intercellular spaces between the cells were narrow. The ultrastructure of group D was similar to that of group A and the cells formed a thin epithelial layer.

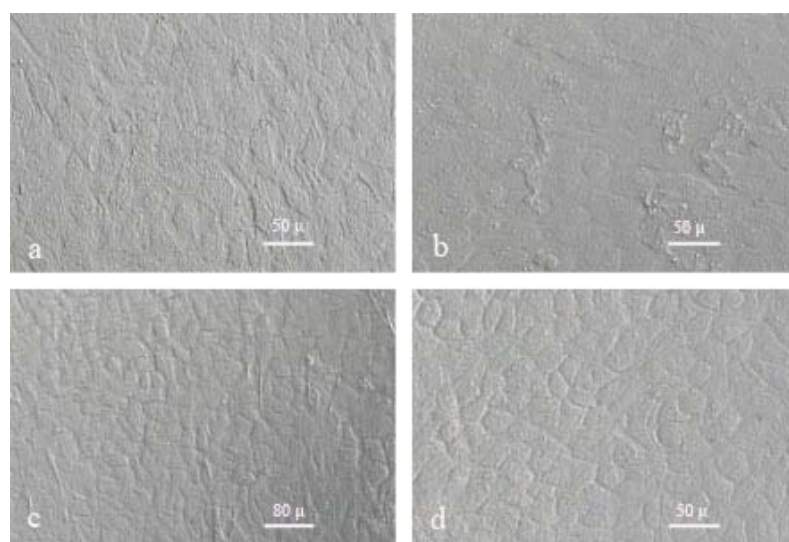


Fig. 2. The morphology of the cultured oral epithelial cells under inverted microscope. **(a)** The epithelial cells were cultured on the mitotically inactivated 3T3 fibroblast feeder layer (group A); **(b)** The epithelial cells were co-cultured on the amniotic membrane without nitrocellulose and the mitotically inactivated 3T3 fibroblast (group B); **(c)** The epithelial cells were co-cultured on the sandwich layer of AM fastened on the nitrocellulose as insert and 3T3 fibroblast (group C); **(d)** The epithelial cells were cultured without any supporting feeder layer as control (group D).

Scanning electron microscopy ultrastructural observation. SEM observations showed that the appearance of surface epithelial cells in groups A was flat squamous cells (Fig. 4 a-c) similar to the TEM. Their nuclei were projected to the surface. The surface of epithelial layer in groups B and C was similar and the apical surface of the cells showed a continuous layer of polygonal cells with prominent small microvilli (Fig. 4 d-f). The apical surface of cultured cells in group D was similar to that of group A.

Immuno-cytochemical observation. The immuno-cytochemistry of the cytoplasmic cell suspension showed the keratin 3/12 was expressed in all groups and the positive stained cells with FITC-conjugated secondary anti-cytokeratin antibody were detected in all groups of study (Fig. 5 a-c). Also the positive stained cells with FITC-conjugated secondary anti- β 1-integrin antibody were seen in all groups of study (Fig. 5 d-f).

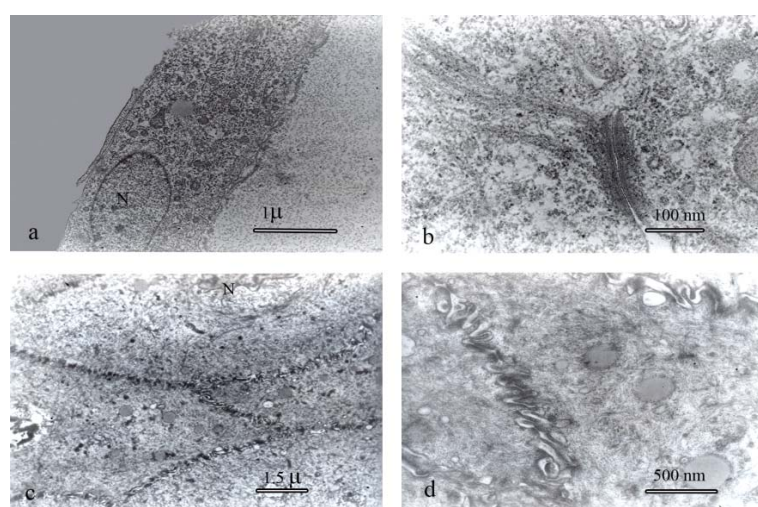


Fig. 3. TEM micrograph of oral epithelium grown on 3T3 fibroblast feeder layer. **(a)** A well-formed desmosomes between these cells; **(b)** The micrograph of stratified epithelial cells which is formed on the sandwich layer of AM fastened on the nitrocellulose as insert and 3T3 fibroblast and the epithelium was five to six layers thick **(c)** and exhibited many lateral interdigitations with well defined desmosomes **(d)**. N, nucleus.

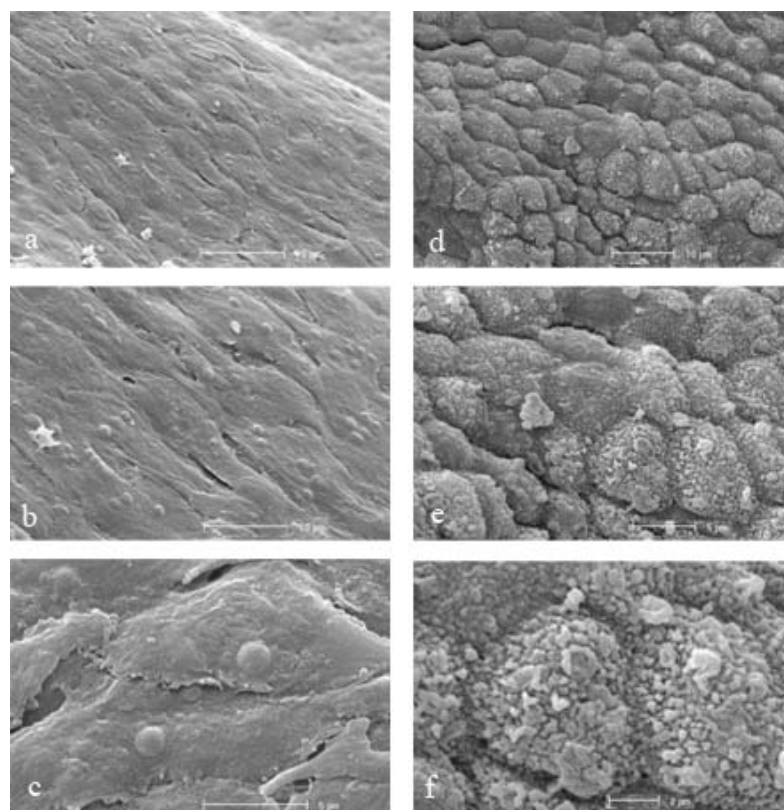


Fig. 4. Scanning electron micrographs of the cultured oral epithelium. The epithelial cells were cultured on the mitotically inactivated 3T3 fibroblast feeder layer showing the squamous flatted cells in different magnification. The nucleus projected from the surface of epithelial (a-c). The epithelial cells were cultured on the sandwich layer of AM fastened on the nitrocellulose as insert and 3T3 fibroblast. A lot of microvillus extended from the lateral and surface borders of the cells (d-f).

DISCUSSION

The main purpose of this study was to introduce the best culture condition for oral mucosal epithelial cells. We established a method for the culture of stratified oral epithelial on human AM with or without nitrocellulose as insert which was co-cultured with 3T3 fibroblast feeder layer. We modified this by using the nitrocellulose and deletion of the air lifting step. As our data show, the culture conditions in groups B and C were well supported the stratification of epithelia without any keratinization in the superficial layer. In this regards previous research emphasized that the air lifting is essential for stratification of epithelium [3]. In groups B and C, in the presence of both 3T3 feeder layer and AM, the growth and morphology of the cells were better than other groups so that they reached to confluency before 3 weeks of culturing. These results may be due to some factors which are produced by supporting layer of lethally irradiated 3T3 cells and AM. Also, in the presence of nitrocellulose inserts, nutrients can be passed via the

basal side of the cells through the insert membrane pores. This condition could be mimicked the *in vivo* state to support stratification of epithelial by a connective tissue sublayer.

A great advantage of our experimental system was the use of simple technique to obtain stratified oral epithelial sheet for transplantation in short cultured period. The SEM and TEM electron micrographs demonstrated that both cultured conditions in groups B and C produced a healthy and well-formed stratified cell layer (formation at least 5-6 layers). The similar features were showed *in vitro* culture corneal epithelial cells and *in vivo* normal corneal epithelium by other investigators [14, 15]. The human primary oral mucosal epithelial cells which were cultured on temperature-responsive culture inserts in the absence of 3T3 cell feeder layers showed the similar characteristics [16].

The results of the present study showed the primary cultured epithelial cells were positively immuno-stained by the antibody against cytokeratin 3/12 and similar observation was indicated by normal epithelial cells of oral mucosa *in vivo* [17].

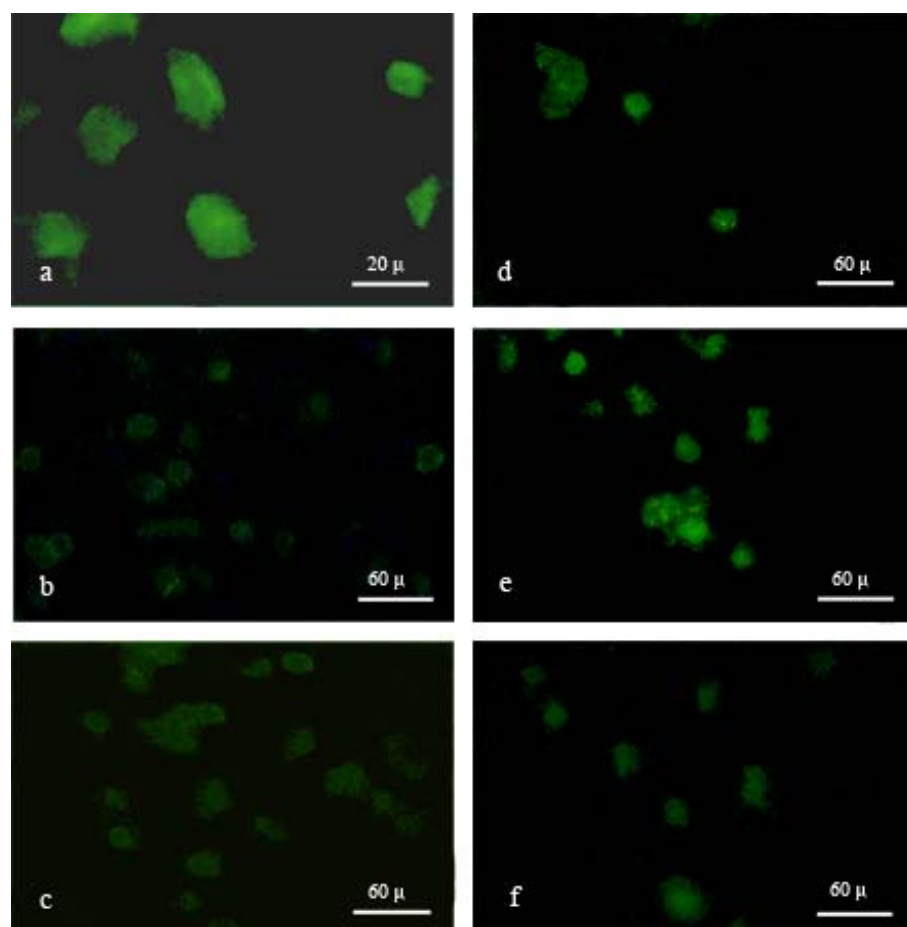


Fig. 5. The immuno-cytochemical staining for cytokeratin 3/12 in cytopspine slides of cultured cells. The cells in groups A, B and C were showed the expression of K3/K12, respectively (a-c). The β 1-integrin immuno-stained cells were cultured on the mitotically inactivated 3T3 fibroblast feeder layer (d), co-cultured on the AM without nitrocellulose and the mitotically inactivated 3T3 fibroblast (e) the sandwich layer of AM fastened on the nitrocellulose as insert and 3T3 fibroblast (f).

Therefore, it indicated that this characteristic is shared between oral and corneal epithelia, also it was considered as one of the *in vivo* corneal and conjunctival epithelia by some investigators [17-19]. However, it was showed that the keratin expression profiles in the tissue-engineered cell sheets before and after transplantation in comparison with those from native corneal and oral mucosal epithelia had some similarities [7]. These data support the occurrence of phenotypic modulation of oral mucosal epithelial cells during culturing and after transplantation [7].

The presence of the cells which were expressed the β 1-integrin suggested that they possessed the structural and regenerative characteristics of oral epithelium [20, 21]. Hayashida and associates [8] demonstrated p63 and β 1-integrin positively within the oral mucosa of rabbits, to imply the presence of stem cells in oral mucosal epithelium. The putative

oral epithelial stem cells may be converted into corneal epithelial-like cells under influence of the corneal micro-environment after transplantation with some similar characteristics [20]. Our results suggested the epithelial cells were cultured on AM spread on nitrocellulose insert and 3T3 feeder layer showed better growth. Also, the cultured oral epithelia were showed similar characteristics with corneal epithelium.

ACKNOWLEDGMENTS

This work was supported by a grant of Research Center of Noor and Farabi Eye Hospitals (Tehran, Iran). The authors thank Mr. Shahram Pour Bayranvand and Mrs. S. Ebrahimi for excellent technical support.

REFERENCES

- Inatomi, T., Nakamura, T., Koizumi, N., Sotozono, C., Yokoi, N. and Kinoshita, S. (2006) Midterm results on ocular surface reconstruction using cultivated autologous oral mucosal epithelial transplantation. *Am. J. Ophthalmol.* 141: 267-275.
- Inatomi, T., Nakamura, T., Kojyo, M., Koizumi, N., Sotozono, C. and Kinoshita, S. (2006) Ocular surface reconstruction with combination of cultivated autologous oral mucosal epithelial transplantation and penetrating keratoplasty. *Am. J. Ophthalmol.* 142: 757-764.
- Nakamura, T., Endo, K., Cooper, L.J., Fullwood, N.J., Tanifuji, N., Tsuzuki, M., Koizumi, N., Inatomi, T., Sano, Y. and Kinoshita, S. (2003) The successful culture and autologous transplantation of rabbit oral mucosal epithelial cells on amniotic membrane. *Invest. Ophthalmol. Vis. Sci.* 44: 106-116.
- Nakamura, T., Inatomi, T., Sotozono, C., Amemiya, T., Kanamura, N. AND Kinoshita, S. (2004) Transplantation of cultivated autologous oral mucosal epithelial cells in patients with severe ocular surface disorders. *Br. J. Ophthalmol.* 88: 1280-1284.
- Hata, K., Kagami, H., Ueda, M., Torii, S. and Matsuyama, M. (1995) The characteristics of cultured mucosal cell sheet as a material for grafting; comparison with cultured epidermal cell sheet. *Ann. Plast. Surg.* 34: 530-538.
- Henderson, H.W.A. and Collin, J.R.O. (2008) Mucous membrane grafting. *Dev. Ophthalmol.* 41: 230-242.
- Nishida, K., Yamato, M., Hayashida, Y., Watanabe, K., Yamamoto, K., Adachi, E., Nagai, S., Kikuchi, A., Maeda, N., Watanabe, H., Okano, T. and Tano, Y. (2004) Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. *N. Engl. J. Med.* 351: 1187-1196.
- Hayashida, Y., Nishida, K., Yamato, M., Watanabe, K., Maeda, N., Watanabe, H., Kikuchi, A., Okano, T. and Tano, Y. (2005) Ocular surface reconstruction using autologous rabbit oral mucosal epithelial sheets fabricated *ex vivo* on a temperature-responsive culture surface. *Invest. Ophthalmol. Vis. Sci.* 46: 1632-1639.
- Ang, L.P., Nakamura, T., Inatomi, T., Sotozono, C., Koizumi, N., Yokoi, N. and Kinoshita, S. (2006) Autologous serum-derived cultivated oral epithelial transplants for severe ocular surface disease. *Arch. Ophthalmol.* 124: 1543-1551.
- Bouchard, C.S. and John, T. (2004) Amniotic membrane transplantation in the management of severe ocular surface disease: indications and outcomes. *Ocul. Surf.* 2: 201-211.
- Koizumi, N., Inatomi, T., Suzuki, T., Sotozono, C. and Kinoshita, S. (2001) Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. *Ophthalmology* 108: 1569-1574.
- Sun, T.T. and Green, H. (1977) Cultured epithelial cells of cornea, conjunctiva and skin: absence of marked intrinsic divergence of their differentiated states. *Nature* 269: 489-493.
- Jones, P.H. and Watt, F.M. (1993) Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. *Cell* 73: 713-724.
- Koizumi, N., Cooper, L.J., Fullwood, N.J., Nakamura, T., Inoki, K., Tsuzuki, M. and Kinoshita, S. (2002) An evaluation of cultivated corneal limbal epithelial cells, using cell-suspension culture. *Invest. Ophthalmol. Vis. Sci.* 43: 2114-2121.
- Gipson, I.K. and Sugrue, S.P. (1994) Principles and practice of ophthalmology. In: Cell Biology of the Corneal Epithelium. (Albert, D.M., Jakobiec, F.A., Azar, D.T., Gragoudas, E.S., Power, S.M., Robinson, N.L. eds.), 2nd revised edition, Saunders (W.B.) Co. Ltd., Philadelphia, pp. 2-16.
- Murakami, D., Murakami, D., Yamato, M., Nishida, K., Ohki, T., Takagi, R., Yang, J., Namiki, H., Okano, T. (2006) Fabrication of transplantable human oral mucosal epithelial cell sheets using temperature-responsive culture inserts without feeder layer cells. *J. Artif. Organ.* 9: 185-191.
- Nakamura, T., Inatomi, T., Cooper, L.J., Rigby, H., Fullwood, N.J. and Kinoshita, S. (2007) Phenotypic investigation of human eyes with transplanted autologous cultivated oral mucosal epithelial sheets for severe ocular surface diseases. *Ophthalmology* 114: 1080-1088.
- Kawasaki, S., Tanioka, H., Yamasaki, K., Yokoi, N., Komuro, A. and Kinoshita, S. (2006) Clusters of corneal epithelial cells reside ectopically in human conjunctival epithelium. *Invest. Ophthalmol. Vis. Sci.* 47: 1359-1367.
- Tanioka, H., Kawasaki, S., Yamasaki, K., Ang, L.P., Koizumi, N., Nakamura, T., Yokoi, N., Komuro, A., Inatomi, T. and Kinoshita, S. (2006) Establishment of a cultivated human conjunctival epithelium as an alternative tissue source for autologous corneal epithelium transplantation. *Invest. Ophthalmol. Vis. Sci.* 47: 3820-3827.
- Gao, N., Wang, Z., Huang, B., Ge, J., Lu, R., Zhang, K., Fan, Z., Lu, L., Peng, Z. and Cui, G. (2007) Putative epidermal stem cell convert into corneal epithelium-like cell under corneal tissue *in vitro*. *Sci China C Life Sci.* 50: 101-110.
- Pearton, D.J., Yang, Y. and Dhouailly, D. (2005) Transdifferentiation of corneal epithelium into epidermis occurs by means of a multistep process triggered by dermal developmental signals. *Proc. Natl. Acad. Sci. USA* 102: 3714-3719.