Original Article

The Effects of Extracellular Matrices and Co-Culture Systems on Cultured Limbal Stem Cells

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Abstract

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Objective: To study the effects of different matrices and co-culture systems on cultured limbal stem cells (LSCs).

Materials and Methods: Limbal explants were co-cultured with limbal fibroblasts (LF) and/or mouse embryonic fibroblasts (MEF) on filter inserts coated with amniotic membrane (AM), matrigel (MAT) and collagen type I (COL).

Results: This study revealed that AM facilitated the cell migration and expansion significantly in comparison with other matrices. However, the gene expression profile of stemness markers of LSCs showed no significant differences among the experimental groups. The data indicated that at least in two-dimensional culture systems, the mentioned matrices have no significant effect on switching the expression of genes involved in differentiation process. In addition, the results of the two co-culture systems in case of different feeders, including MEF and LF were similar in growth rate and also preserving stemness quality of cultured limbal cells.

Conclusion: To exclude the pollution of transplantable cultivated cells with probable mouse viruses, LF with human origin is recommended as feeder. Hence, limbal explants grown on AM in co-culture with LF will promise a quick and safe model for preparing undifferentiated epithelial sheets suitable for transplantation.

Keywords: Limbal Stem Cells, Niche, Extracellular Matrix, Co-Culture Systems

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Introduction

The cornea is anterior, projecting transparent part of the eye's external tunica and the major site of refraction of light entering the eye. The most superficial layer of the cornea is a stratified, squamous non-keratinized epithelium, which is exposed to the environment on its outer surface, so undergoes a continuous process of self-renewal and regeneration (1). Ultimate cell sources for regeneration of corneal epithelium under normal and injured states are the cells reside at the corneo-sclera junction, named limbal stem cells (LSCs) and transient amplifying cells (TACs) (2-6). Multiple diseases or injuries destroy the LSCs, leading to a state known as limbal stem cell deficiency which carries the hallmark of conjunctivalization, in that, the corneal surface is covered by ingrowing conjunctival epithelium containing goblet cells and vessels. The result is severe corneal opacification followed by visual impairment (7, 8). The in vitro culture of remaining LSCs followed by transplantation provides a new modality for the treatment of limbal stem cell deficiency (9-11). Although this approach seems very promising, the behavior of stem cells changes when they are cultured in vitro. Many studies indicate that the functions of stem cells would be limited when detached from their in vivo niche (12-16). Stem cell niche includes soluble factors, cell-cell and

cell-extracellular matrix (cell-ECM) interactions (17-19). So to mimic the in vitro niche more close to those of in vivo, one approach is to utilize ECM in culture of stem cells (20). There is strong evidence that culture substrates variously affect cells. (21, 22). To find out which ECM is more suitable for limbal stem cell culture, we studied, for the first time, the effects of three common matrices of amniotic membrane (AM), matrigel (MAT) and collagen type I (COL) on cultured LSCs. Another method is using fibroblast feeder layers in co-culture with stem cells (23, 24). Mouse embryionic fibroblasts (MEF) has been proved to be effective in preserving stemness properties of embryonic stem cells (25). So we used MEF as one of experimental feeders. However, the dual growth of cells with MEF exposes the human LSCs to mouse retroviruses, which may prevent the future use of these cells in cell-based therapy. Therefore, we used limbal fibroblasts with human origins as a feeder layer and compared it with MEF.

Materials and Methods

Isolation and culture of MEF and LF as feeder layers

MEF as feeder cells derived from 12.5 days post-qoitus pregnant mice (NMRI), which were harvested according to Robertson's method (26). LF were obtained from limbal stroma taken from organ donors. The fibroblasts were routinely expanded in Dulbecco's Modified Egale's Medium (DMEM) (GIBCO; Invitrogen Corp., Carlsbad, California, USA) supplemented with 10% fetal bovin serum (FBS) (GIBCO). Fibroblasts obtained from passage 4-5 were used as feeder layer. Confluent cells was inactivated mitotically by 4µg/ml mitomycin C (Sigma-Aldrich, Saint Louis, Missuori, USA) for 2hr at 37 ºC in a 5% CO2 atmosphere then, rinsed three times in PBS (phosphate buffered saline), trypsinized with trypsin/EDTA 0.02% (GIBCO) for 2 minutes at 37 ºC and seeded into 6-well culture plates (TPP, Switzerland) at a density of 2×10^4 cells/cm^2.

Matrix Preparation

AM was prepared according to the method described previously with minor alterations (27). After the clinical confirmation of the absence of human HIV virus, hepatitis B and C viruses, human placenta was obtained shortly following cesarean surgery. Under sterile conditions, amniotic membrane was separated from the rest of placenta, cut into smaller sections, and stored in DMEM containing 50% glycerol (Sigma) at -70ºc. They were thawed gently before use, washed with PBS, then treated with trypsin-EDTA 0.02% for 30 minutes. The epithelial cells were removed by gentle scrapping using a cell scraper and were washed three times with PBS. Amniotic membrane pieces were placed with basement membrane side up on filter inserts (Millipore, Billerica, USA) fitted to 6-well tissue culture plate.

Matrigel (Sigma) and collagen I (INAMED Corp, Fremont, USA) were mixed with DMEM at the ratio of 1:5 and 8:1 respectively. Each filter insert was coated with 200µl of prepared matrices. Then, they were allowed at least 45 minutes for polymerization to occur.

Limbal epithelial cell isolation

Human eye globs (less than 72 hr post-mortem) were obtained from Iranian Eye Bank of Tehran. This study was approved by the Ethics Committee of Royan Institute, Tehran, Iran. Limbal epithelial cells were isolated from glob applying a method described by Grueterich (28), with minor alterations. In brief, limbal region was removed according to its anatomical position and then isolated tissues were rinsed with DMEM containing 50 µg/ml gentamicin (Sigma) and 1.25 µg/ml amphotericin B (GIBCO). Limbal stroma was removed after incubation with dispase II (1.2U/ml) (GIBCO) at 37 ºC for 5 minutes under an inverted microscope. They were cut into 1×2 mm^2 pieces then placed, epithelium side up, on the bottom of culture plate filter inserts coated previously with proposed matrices. Then, they were transferred into 6 well plates containing treated MEF and LF. The explants were submerged in the supplemental hormonal epithelial medium (SHEM), which contained Dulbecco's Modified Egale's Medium/AM's-F12

(DMEM/F12, 1:1) (Sigma) containing 5% FBS, 0.5% DMSO, 2ng/ml hEGF (Sigma), 5µg/ml insulin (Sigma), 5µg/ml apo-transferrin (Sigma), 5 ng/ml selenium (Sigma), 0.5 µg/ml hydrocortisone (Sigma), 50 µg/ml gentamicin, and 1.25 µg/ml amphotericin B. Cultures were incubated at 37°C under 5% carbon dioxide and 95% air humidity for 12±1 days. Explants were removed after 10 days.

Analysis of cell proliferation

The expansion of cells cultured on AM, MAT, and COL were followed under an inverted microscope. By 12±1 days, they were harvested with trypsin-EDTA, washed and resuspended in PBS. The cell number was determined by a hemocytometer count of viable cells following trypan blue-staining.

Transmission Electron Microscopy

The samples were fixed using 2.5% glutaraldehyde (TAAB, Berkshire, United Kingdom) in 0.1 M PBS (pH 7.4) for 2 h. After washing with PBS, samples were post-fixed with 1% osmium tetroxide (TAAB) for 1.5 h, washed again in PBS, dehydrated in an acetone series and then embedded in epoxy resin (Arald CY). After resin polymerization, sections of approximately 50 nm were cut and double stained with uranyl acetate (Merk, KGaA, Darmstadt, Germany) and lead citrate (Fluka). Electron micrographs were taken using a Zeiss EM 900 transmission electron microscope (Zeiss, Jena, Germany).

Immunostaining analysis

Immunostaining was done in order to detect P63, a transcriptional nuclear factor and also the ATP-binding cassette transporter G2 (ABCG2), a member of the multiple-drug resistance family of membrane transporters as markers for LSCs/TACs. Monoclonal antibodies anti-cytoplasmic cytokeratin K3 and anti-transmembrane protein Cx 43 were used to detect differentiated corneal cells. For this purpose, cultures were fixed in 2% paraformaldehide (Sigma) at room temperature for 10 minutes. Nonspecific staining was blocked with 10% goat serum for 20 minutes. Monoclonal antibodies were used to detect P63 (0.2mg/ml) (Chemicon, Temecula, California, USA), ABCG2 (500µg/ml) (R&D Systems, Inc., McKinley Place NE Minneapolis, USA), K3 (1mg/ml) (Chemicon) and Cx 43 (1:200) (Sigma) were applied and incubated for one hour at 37ºC. Samples were washed 3 times, for 5 minutes each, in PBS, and then incubated with anti-mouse IgG, FITC conjugate secondary antibody (1:200) (Sigma) for one hour. The nuclei were counterstained with Hoechst 33342 and/or propidium iodide (10µg/mL). Sections were examined and photographed with an epifluorescent microscope (Olympus, BX51) and a digital camera (Olympus, DP70).

Semi-quantitative RT-PCR

Semiquantitative RT-PCR was performed to assess the expression of a set of genes that may be markers of stemness and differentiation of proposed cells. RNA samples were digested with DNase I to remove contaminating genomic DNA. Nucleospin RNA II kit was used to extract total RNA from samples. Standard RT was performed using the RevertAid H Minus First Strand cDNA Synthesis Kit. Reaction mixtures for PCR included 2.5 µL cDNA, 1× PCR buffer (AMS, CinnaGen, Tehran, Iran), 200 µM dNTPs, 0.5 µM of each antisense and sense primer and 1 U Taq DNA polymerase. PCR amplification was carried out with specific primer pairs designed from published human gene sequences (Table 1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Table 1: Primer sequences used for semi-quantitative RT-PCR

Gene	DNA sequence
P63	Forward: CGG ACC TGA GTG ACC CCA T
	Reverse: TCC GTG ACG TCG TGA GGT
ABCG2	Forward: TGC GAG CTC TAG AAG AGG CTA
	Reverse: CCT CGT GGT TCT TCA TGT A
K19	Forward: TGC GAG CTC TAG AAG AGG CTA
	Reverse: CCT CGT GGT TCT TCA TGT A
K3	Forward: GAG CGG AGC AGG TGG CTT T
	Reverse: GGT CAG TCT CCA CTT TGA G
GAPDH	Forward: GAG GCG TAC AGG GAT AGC AC
	Reverse: GTG GGC ATG GGT CAG AAG

Semi-quantitative RT-PCR was stablished at intervals of 20, 25, 30, 35, and 40 cycle for each primer pairs and the products were analyzed using Bandmap software.

Statistical analysis

All experiments were done at least three times in six experimental groups. The differences within the two feeder groups and the three matrix groups were calculated using mann-withney and kruskal-wallis tests, respectively. $p<0.05$ was considered statistically significant. The data are presented as the mean±SEM.

Results

Phase-contrast Microscopy of Outgrowth

Cultures on AM started to grow on days 2±1, whereas those in MAT and COL groups began to grow on days 4±1. Some explants failed to start outgrowth. There was a significant difference ($p<0.05$) in the rate of growth start between all the groups. The most rate of success in growth start was observed in AM and the least in COL groups. There were no significant differences between the subgroups of feeder layers (Fig 1).

Phase-contrast microscopy showed that the morphology of expanded epithelial limbal cells on AM were small, compact, and uniform with round cell borders. Expansion area was continuous with semi-circular and regular margins. The outgrowth was about to cover the membrane within 12 days (Fig 2A, C, E).

However expanded cells in COL group showed larger size with polygonal borders. In contrast with AM group, uncontinuous cell expansions with irregular leading edges were observed in COL group. They usually did not reach confluence in 12 days (Fig 2B, D, F).

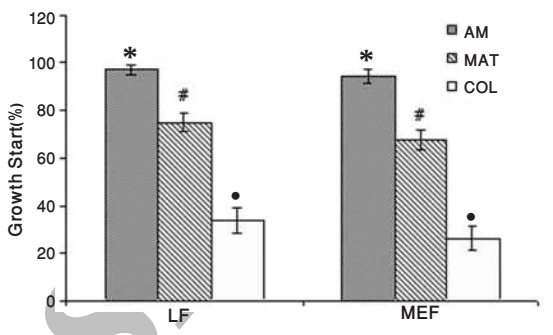

Fig 1, Table 2: Growth start (%). A significantly higher rate of growth start was noted in AM than other groups. (*p<0.05 vs MAT and COL), (#p<0.05 vs AM, COL), (•p<0.05 vs AM, MAT). AM=amniotic membrane, MAT= matrigel, COL= collagen, LF= limbal fibroblast, MEF= mouse embryonic fibroblasts.

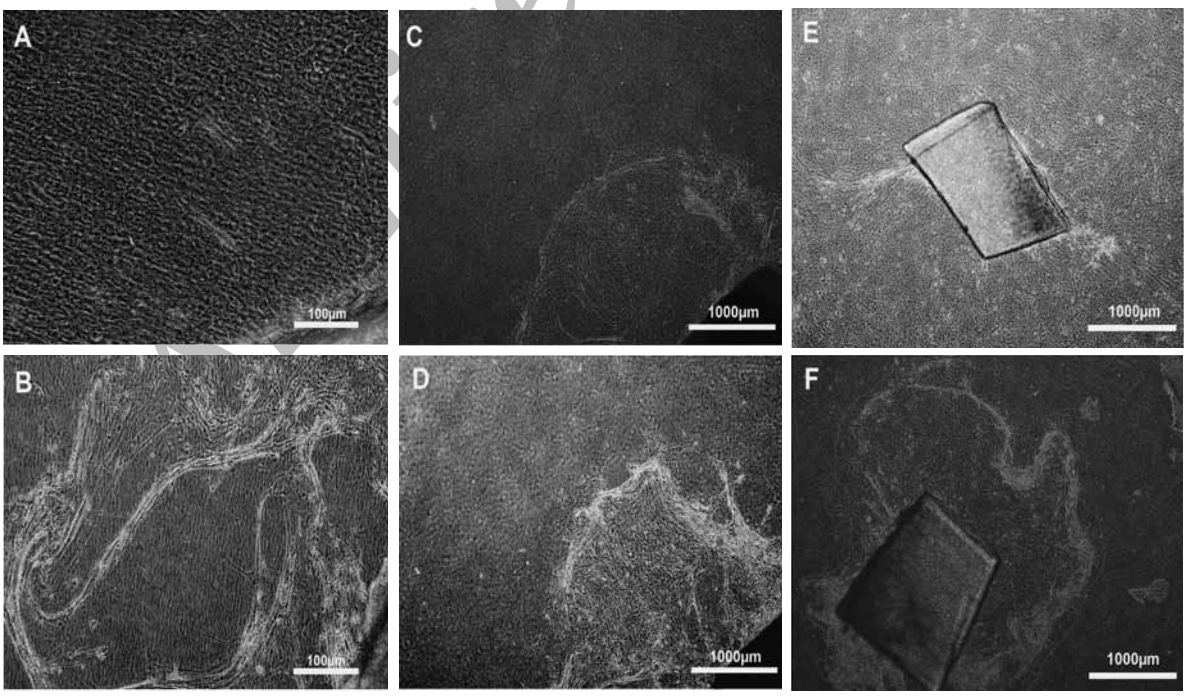

Fig 2: Phase-contrast microscopy of the outgrowth revealed a homogenous pattern of small, uniform and compact cells (A), continuously expanded (C) to reach confluence on AM matrix (E) and in COL group with larger size and less uniform (B), irregular leading edges (D) and less rate of outgrowth than the former (F).

Then, the cell expansions from limbal explants were harvested and stained with trypan blue. The living cells were counted using a hemocytometer. The most cell count was founded in AM and MAT ($p<0.05$). However the cell number in different feeder layers of the same matrices were similar (Fig 3).

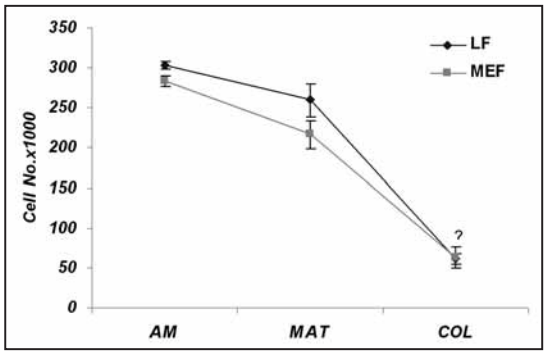

Fig 3: Comparison of cell number between different groups.
****p<0.05 vs AM, MAT. AM= Amniotic membrane, MAT= matrigel, COL= collagen, LF= limbal fibroblast, MEF= mouse embryonic fibroblast***

Ultrastructural examination

In photomicrographs of transmission electron microscope (TEM), we observed that the expanded epithelium was consisted of 4 or 5 cell layers in all groups. Cell expansions in AM group were elongated with round borders and high nucleus-to-cytoplasm (N/C) ratio while in COL, they exhibited polygonal borders with low N/C ratio (Fig 4A, B).

Cell expansions on MAT have an intermediate status. Precise observation of the basal domain of the cultured cells on AM revealed small patches of discontinuous electron-dense structures of the basal lamina with evidence of hemidesmosome-like attachments (focal adhesions) in some areas (Fig 4C, D). No organized basement membrane layer was observed, and correspondingly, no hemidesmosomes were formed in other matrix groups. Numerous ribosomes and extensive rough endoplasmic reticulum and Golgi stacks and mitochondria were observed in the cytoplasm of the cultured cells in all groups (Fig 4E).

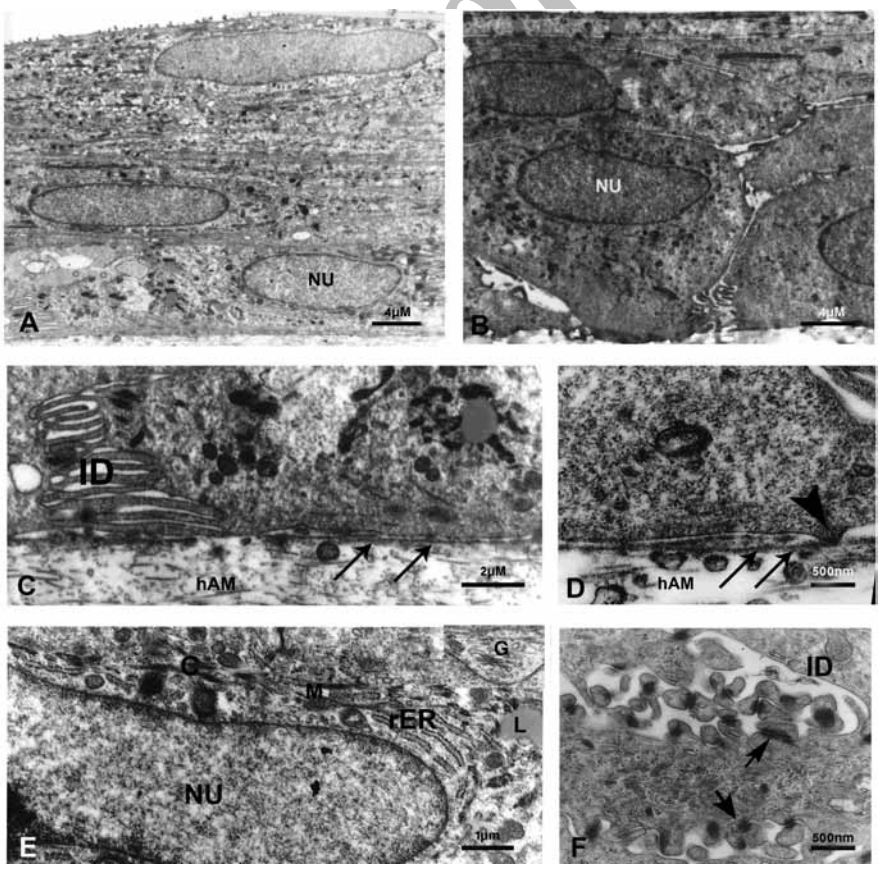

Fig 4: Ultrastructural futures of cultured limbal cells on amniotic membrane (A) and collagen (B) after 12 days: Both cultures produced an epithelial sheet with 5-6 layers. (C, D) The basal lamina: arrow (↑), hemidesmosome junction: heading (▼), Interdigitation junction: (ID). (E) Nucleus (NU), rough endoplasmic reticulum (rER), lipid (L), mitochondria (M), cytokeratin (C), Golgi (G). (F) desmosome junction: short arrow.

Vacuoles and degenerating nuclei were more common in cells cultured on COL Matrix. There was no prominent diversity in the distribution of cytokeratins and lipid droplets between different matrix groups. Desmosomes were prominent at the lateral surfaces of cultured cells, and some interdigitations were formed resembling that of the tissue (Fig 4F). There was no obvious difference between mentioned ultrastructures in corresponding LF and MEF co-culture systems in all groups.

Immunocytochemical and semi-quatitative RT-PCR

Immunostaining analysis was carried out to determine the expression of transcription factor P63, cytoplasmic cytokeratin K19, and transmembrane protein ABCG2, which were known to be expressed exclusively by LSCs/TACs in limbal region as well as cytokeratin K3 and transmembrane protein Connexin 43 (Cx43), known as the markers of differentiated corneal cells (5). ABCG2 and p63 were detected in all groups. The immunostaining, however, was negative for K3 and Cx 43 (Fig 5).

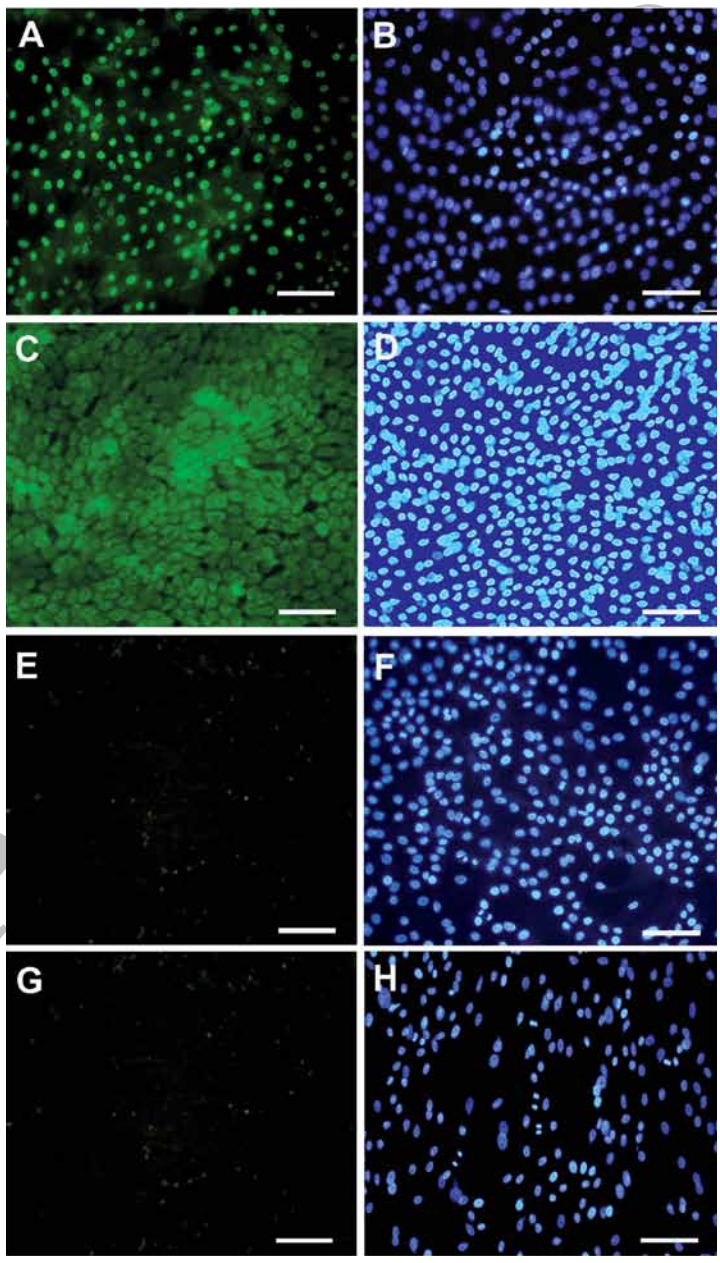

Fig 5: Immunofluorescence analysis of LSCs/TACs with (A) P63 in the nucleus and (C) ABCG2 in the cytoplasm and cell surface. The results were negative for K3 and Cx 43 (E and G). Hoechst 33342 was used as a counterstaining (B, D, F and H). Scale bars: 30µm.

The profiles of the gene expressions in different culture groups were compared using semi-quantitative RT-PCR. Primary culture of cells in different groups expressed P63, ABCG2 and K19 but K3 was not detectable in any group (Fig 6).

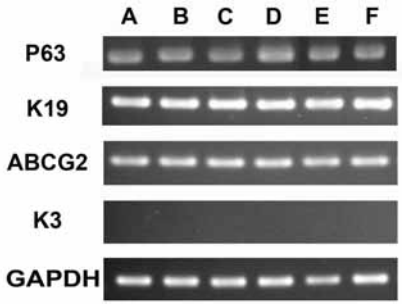

Fig 6: Representative semiquantitative RT-PCR profiles showing mRNA expression of P63, K19, ABCG2, K3, and GAPDH by six populations of LSCs/TACs cultured on AM/LF (A), AM/MEF (B), MAT/LF (C), MAT/MEF (D), COL/LF (E), COL/MEF (F). AM= Amniotic membrane, MAT= matrigel, COL= collagen, LF= limbal fibroblast, MEF= mouse embryonic fibroblast.

Semi-quantitative RT-PCR and immunocytochemical results showed no significant differences between the expression patterns of differentiation and stemness markers in the populations of cultured LSCs/TACs.

Discussion

A new modality for the treatment of corneal disorders is transplantation of LSCs cultured in vitro (12, 29). However, the problem of failure in the expansion of limbal biopsies has remained unsolved. Recently, it has been reported that stromal niche that includes ECM and also soluble factors secreted by feeder cells modulate the behavior and fate of the cultured stem cells (17, 18). Considering the important role of niche in the control of stem cell behavior, it seems that a proper niche might enhance the in vitro expansion of limbal biopsies, and make them appropriate for transplantation. Nevertheless, carefully conducted experimental manipulations of LSCs to examine the effect of different ECM and feeders on improving their expansion are mostly lacking. Thus, it prompted us to examine the effect of three different matrices and also two kinds of feeder cells on the growth and differentiation of cultured LSCs/TACs. The results of this study may provide the ground for the development of more appropriate microenvironment for culture of limbal biopsies suitable for transplantation.

In this study, we compared the effectiveness of inactive LF with MEF in the growth and differentiation state of cultured limbal cells. It has been assumed that soluble factors secreted by LF in the eye act as possible regulators of limbal basal epithelium (30). Hence, we selected LF with human origin as one of the feeder groups. On the other hand, the effect of MEF as feeder in maintaining stemness quality has been addressed for many years (25), however, the risk of cross-contamination of the cultured cells with infectious animal agents from the MEF remains problematic, making such cultured cells undesirable for clinical application. Our results revealed that inactive LF derived from adults are as effective as MEF in support and maintaining the stemness quality of cultured cells. Moreover, there were no significant differences in the growth state of cultured cells between the two groups. Thus, to exclude the cell contamination with probable mouse viruses, use of LF with human origin as a feeder for limbal biopsies could be a suitable substitution for clinical use.

To clarify the effects of different ECM on cultured limbal cells, three different matrices of AM, MAT, and COL were used as substrates in our culture model. We observed that AM compared to COL was significantly ($p<0.05$) more effective for enhancing the initiation of cell outgrowth and also ($p<0.05$) provided a significantly better condition for the expansion of cells. No significant differences in growth rate were observed between AM and MAT. Since MAT is too expensive and difficult to handle, preparation for coating and also transfer of sheets to eye for final transplantation, we concluded that AM might provide a more suitable substrate. Consistent with these findings, previous studies reported that AM contains purified soluble lumican glycoprotein that can facilitate the proliferation and migration of corneal epithelial cells during wound healing (31). Moreover, neural growth factor (NGF), which is a mitogen for cultured rabbit corneal and limbal epithelial cells, has been detected in AM too (20,32). When topically applied,

NGF facilitates epithelial healing and helps the recovery of the corneal sensitivity in patients suffering from neurotrophic ulcers (33-36). The proliferation and migration promoting factors of AM mentioned above are similar to the corneal stroma (37). These facts put more evidence for the preference of AM in case of improving cell expansion in our designed culture model. Recently, the effectiveness of AM as a patch in eye surgeries has been proved (38-42). The data provided in this study by cell culture on AM may be useful in understanding how AM enhances healing in eye surgeries. Here, we showed that COL acted as a poor matrix in enhancing LSCs/TACs growth. Our results provide more evidence for other studies indicating that integrins involved in signal transduction from COL, do not support cell survival signal transduction pathways in mouse mammary epithelial cells and mammary cell lines (43).

The results of ultrastructural studies showed some differences between morphology of cells expanded on AM compared with MAT and COL groups. Cell expansions on MAT and COL showed polygonal borders, while those cultured on AM were elongated with round borders, which were more similar to the morphology of undifferentiated limbal epithelial cells. Small patches of discontinuous electron-dence structure of basal lamina with rare hemidesmosomes in some areas was detecteded in AM group, while no organized basal lamina was observed in the other two groups. These results indicate that epithelial sheets cultured on AM have more similarity to those in vivo. Increased number of some organels like mitochondria and rER in AM and MAT groups may indicate more metabolic activity in those expanded on these matrices. Vacuoles were more commonly seen in cells cultured on COL group in comparison with AM and MAT. Taken together, it seems that cells may start a degenerative process on this matrix. However, mechanisms involved in this process remains to be elucidated.

Extracellular microenvironment can influence cellular responses from attachment and migration to differentiation and production of new tissue (44). Hence, we also examined the effect of mentioned matrices on differentiation state of the cultured limbal cells by immunocytochemistry and RT-PCR techniques. The results showed that there were no significant differences in the stemness state of cultured limbal cells among different matrices. This may indicate that mentioned matrices have no significant effect on differentiation pathways of these cells, at least in our two-dimensional design of culture systems. Regarding a significant difference among AM and MAT with COL in outgrowth rate and growth state of cultured cells, it seems that mentioned substrates may mostly influence attachment, migration, and survival of LSCs/TACs. There is also the possibility that the culture of these cells in a three-dimensional matrix or addition of inductive substances provide stronger tools for affecting differentiation pathways and switch of gene expression from stemness markers to the differentiated ones. The exact mechanisms involved in this regard needs further investigations.

Conclusion

According to the results of the study, we conclude that the cultivation of human limbal explants on AM as a matrix in co-culture with human LF on polarized condition similar to the model provided in this study will promise a quick, cheap and safe model for preparing undifferentiated epithelial sheets suitable for transplantation.

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