Influence of feeder layer on the expression of stem cell markers in cultured limbal corneal epithelial cells

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Received June 26, 2007

Background & objectives: The limbus is enriched with the stem cells of corneal epithelium. Auto- and allograft limbal transplantations are effective in restoring the corneal epithelium and inhibiting inflammation and neovascularization. Preserved human amniotic membrane (AM) is now widely used as a substrate for ocular surface reconstruction. The combination of limbal and AM transplantation has been shown to improve the surgical outcome in patients with total limbal stem cell deficiency (LSCD). The purpose of this study was to compare the expression of putative stem cell markers ATP binding cassette protein (ABCG2) and keratinocyte stem cell marker: p63 and differentiation markers. (connexin 43 and keratin 3 / keratin 12) on the limbal epithelial cells cultured over the denuded AM with and without the 3T3 murine fibroblast cells as feeder layer.

Methods: Human limbal tissues obtained from the cadaveric donor eyes were cultured over the denuded human amniotic membrane in the presence of mitomycin C treated 3T3 fibroblasts and the cultured cells studied for the expression of ABCG2 and p63 by immunohistochemistry and Western blot. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was done on the cultured cells at varying intervals of time for expression of ABCG2, p63, connexin43 (Cnx43), and keratin 3 (K3) and keratin 12 (K12).

Results: The growth rates were similar in both denuded AM and denuded AM + 3T3. The cells cultured over AM + 3T3 showed the expression of p63 and ABCG2 till 21 days of incubation by immunohistochemistry and Western blot. The expression of p63 and ABCG2 were retained till 21 days of incubation on the cells cultured over denuded AM + 3T3, whereas it was expressed only till day 8 on the cells cultured over the denuded membrane by semi quantitative RT-PCR. Cnx43 and K3/K12 were observed in both the conditions.

Interpretation & conclusions: The limbal epithelial cells cultured in the presence of mitomycin C treated 3T3 feeder layer were able to maintain the expression of putative stem cell markers. Further in vitro studies using feeder layer will enable us to understand the factors, which play a role in maintaining the limbal stem cell niche.

Key words ABCG2 - denuded AM - 3T3 feeder layer - p63 - semi quantitative RT-PCR

Ex vivo expansion of the limbal epithelium using amniotic membrane (AM) as a biological substrate is a well known technique for the management of ocular surface reconstruction in patients with partial or total limbal stem cell deficiency (LSCD). There are several approaches for the cultivation of the corneal limbal stem cells. The current practice employed for the expansion of the corneal limbal stem cells is use of AM with or without the feeder layer.
The use of feeder cells in cell culture was first reported by Puck et al. Feeder cells are especially effective for the support of growth of cells that are difficult to culture. Feeder cells also provide a suitable environment in the co-culture with a variety of cell types through different mechanisms, including cell to cell and cell to extracellular matrix (ECM) interaction, production of soluble growth factors and removal of toxicants from the culture medium.

Studies on the growth of the limbal stem cells showed that they have a greater growth potential in explant cultures and higher clonogenicity when co-cultured on irradiated murine 3T3 fibroblast as feeder layers and their proliferative potential is resistant to tumour promoting phorbol esters. Tseng and colleagues have reported that 3T3 culture system promoted the clonal growth of limbal progenitor cells. Both growth-promoting and anti-apoptotic activities were present in fibroblast-derived serum-free conditioned media. Several studies have shown favourable clinical outcomes, adopting different culturing techniques for ex vivo expansion, especially regarding the preparation of AM and the inclusion of 3T3 fibroblast feeder layers. Grueterich et al. have studied the modulation of connexin 43 (Cx43) and keratin 3/12 (K3/K12) expression on the cells cultured over the intact and denuded membrane with and without the presence of 3T3 feeder layer. However, not much information is available on the expression of the putative stem cell makers such as p63 and ATP binding cassette protein (ABCG2).

In this study, we compared the expression of the stem cell associated marker (p63 and ABCG2) and differentiation markers (connexin 43 and K3/K12) on the cells cultured over the denuded amniotic membrane with and without Mitomycin treated 3T3 feeder layer.

Material & Methods

Chemicals and reagents: Dulbecco’s modified Eagle’s medium (DMEM), F12, Hank’s balanced salt solution (HBSS), 4(2 hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) buffer, amphotericin B, and foetal bovine serum (FBS) were purchased from Hi-Media, Mumbai, India. The mouse monoclonal IgG antibody against p63 (1:100) (clone 4A4) was obtained from Santa Cruz, USA, and ABCG2 (1: 50) (clone BXP 21) was obtained from Chemicon, USA. The FITC-conjugated goat anti-mouse IgG and IgM antibodies from DAKO cytometry, USA. Hydrocortisone, epidermal growth factor, insulin-transferrin-sodium selenite media supplement were all from Sigma Chemicals Co, USA. ABC kit (DAKO, USA) was obtained from DAKO, USA. The tissue culture plastic plates and culture plate inserts were from Becton Dickinson (Lincoln Park, NJ). RNA extraction kit and cDNA conversion (sensiscript reverse transcriptase kit) were obtained from Qiagen, Germany, and the specific primer sequences were obtained from Sigma Chemicals Co., USA. Proprep protein extraction kit was procured from InTron Biotechnology, USA, and BrdU labeling kit from Roche, Germany.

Preparation of human amniotic membrane: Human AM was kindly provided by Vijaya Health Centre, Chennai, India, at the time of cesarean section, after proper informed consent. The AM was processed as described previously with little modifications and stored at -80°C for at least 3 months. AM was devitalized by freezing and thawing and washed three times with HBSS before being fastened onto a 12 well culture insert (BD Lincoln Park, NJ), to be placed in a 12 well plate, as previously described. Fifty per cent of the membranes used for limbal cultures were treated with 0.1 per cent sterile EDTA solution for 30 min and then gently scrubbed with an epithelial scrubber (BD Lincoln Park, NJ) to remove the amniotic epithelium without breaking the underlying basement membrane, as previously described. This method allowed removal of 90 to 100 per cent of the epithelium.

Preparation of 3T3 fibroblasts: Confluent murine 3T3 fibroblasts (National Centre for Cell Sciences, Pune) were incubated with 4 µg/ml mitomycin C (MMC) for 2 h at 37°C under 5 per cent CO₂, trypsinized and plated onto cell culture dishes at a density of 2.2 x 10⁴ cells/cm². These feeder cells were used 4 to 24 h after plating.

Limbal biopsy preparation: Corneal limbal biopsies of 2 mm³ were collected from the cadaveric donor eye obtained within 2 h of death, aged 8-85 yr (n=20, 10-denuded membrane and 10-denuded membrane + 3T3 feeder layer). The biopsy was collected in transport medium (DMEM containing 3% FCS and antibiotics) and was transported to the cell biology laboratory of Vision Research Foundation, Sankara Nethralaya, Chennai, for further processing. The biopsy tissue was gently washed three times using tissue culture growth medium. After careful removal of excessive sclera the tissue was cut into multiple small pieces using sterile sharp curved scissors/Bard-Parker blade.

Human limbal explants culture on the de-epithelialized amniotic membrane without the feeder layer: AM with the basement membrane facing upward was fastened
on the culture insert as described previously. On the centre of the EDTA treated AM an explant was placed and cultured in the medium containing equal volume of DMEM and F12 supplemented with 10 per cent FBS, 0.5 per cent DMSO, 50 µg/ml of gentamicin, 1.25 µg/ml of amphotericin B, 2 ng/ml of mouse epidermal growth factor (EGF), 5 µg/ml of insulin, 5 µg/ml of transferrin, 5 ng/ml of selenium, 0.5 mg/ml of hydrocortisone. Human limbal epithelial cells were thus maintained at 37°C under 95 per cent humidity and 5 per cent CO₂, and the medium was changed once in three days and cell growth was monitored daily for 3 wk with an inverted phase contrast microscope (Nikon, Tokyo, Japan). After 3 wk the cultures were embedded in optimal cutting temperature (OCT) compound and snap frozen for cryosections.

**Human limbal explants culture on the de-epithelialized amniotic membrane with feeder layer**: Ten out of the 20 cultures with the denuded AM were co-cultured on a 3T3 fibroblast feeder layer prepared as mentioned above on the tissue cultured plate. For the culture with the 3T3 the culture inserts with the AM and limbal explants were placed on top of the 3T3 layer. The medium was changed every 2 to 3 days. The cultures were incubated for about 3 to 4 wk until they reach the confluence. Once they reach confluence the cultures were proceeded for the immunostaining, RT-PCR and Western blot.

**Immunostaining**: When the cultures reach confluence the AM with the cells were removed and embedded in the OCT medium and frozen sections of 5 µg was taken and proceeded for immunostaining. After drying the slides were fixed in cold acetone for 20 min at -20°C. After rinsing with PBS three times for 15 min and preincubation with 5 per cent BSA in PBS to block the nonspecific staining, sections were incubated with primary antibody (against β-actin) and preincubation with 0.02 per cent trypsin in RNAse free vial and was extracted according to the manufacturer’s instructions. Samples were incubated for 15 min at -20°C, and then centrifuged at 12,500 g for 30 min at 4°C.

Protein concentration of the supernatant was determined by Lowery’s method. All samples were then diluted in 2X sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS; Gibco, Invitrogen, Carlsbad, CA), 20 per cent glyceral (Sigma, USA), 12 per cent 2-mercaptoethanol (Sigma) and boiled. Ten µg of each sample (5 µg for β-actin) were loaded on a 10 per cent SDS PAGE and proteins were run for about 1 h at 120 volts, and then transferred onto nitrocellulose membranes (Amersham CA, USA). Membranes were blocked with 5 per cent skim milk (Amersham, CA, USA) for 30 min at room temperature. Membranes were reacted with an anti-p63 (clone 4A4. Santacruz biotechnology dilution 1: 1000) and anti-ABCG2 (clone BXP21 Chemicon. dilution 1: 500) for overnight at 4°C. After three washes in TTBS, peroxidase conjugated anti-mouse IgG (Amersham CA, USA) was added for 2 h at room temperature. After intermittent washes the protein bands were detected using the detection reagents A and B. (Supersignal West Femto Maximum Sensitivity Substrate, Pierce, USA).

**RNA isolation and RT-PCR analysis**: Total RNA was isolated from tissue and cells at the end of 3 wk of incubation to see the expression of different markers by reverse transcriptase-PCR (RT-PCR). RNA was extracted from cells collected by treatment with 0.02 per cent trypsin in RNase free vial and was extracted using Qiagen RNAeasy mini Kit (Qiagen, Germany), the end of 8th, 14th and 21st days of incubation to determine the pattern of expression of different stem cell markers specific for corneal limbal stem cells, according to the manufacturer’s recommended protocol the total RNA extracted and was stored at -80°C until use. Using a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an internal control, the mRNA expression of different molecular markers was analyzed by semiquantitative RT-PCR as described previously.
RT-PCR was performed using sensiscript reverse transcriptase, which is recombinant heterodimeric enzyme. PCR amplification of the first-strand cDNA synthesis was performed using specific primer pairs, designed from published human gene sequences\(^{24}\) (Table) for different markers in Eppendorf PCR systems. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an internal control, the mRNA expression of different molecular markers was analyzed by semiquantitative RT-PCR. PCR products were fractionated by electrophoresis using 2 per cent agarose gel containing 0.5 per cent ethidium bromide with molecular marker Hinf I digest to confirm the size of the resultant product of the amplification curve. The semiquantitation was done with Quantity G software in Bio-rad gel documentation system, UK. The fidelity of the RT-PCR products was verified by comparing their size with the expected cDNA bands and by sequencing the PCR products.

**Results**

Cultivation of corneal limbal explants on denuded AM in the presence of the 3T3 feeder layer: A total of 20 limbal biopsies were obtained from Sankara Nethralaya eye bank during the 6 month period (December 2005-May 2006). The tissues from the donor aged 8-90 yr were collected within 2 h after death. Fig. 1 shows the growth of the cells on the denuded AM and with 3T3 feeder layer. The growth of cells from the explants were started by the end of day 3 and almost reached the confluence by the end of day 21.

Cultivation of corneal limbal explants on denuded AM in the absence of the 3T3 feeder layer: The outgrowth rate of the 10 cultures was photographed and measured each time the culture medium was changed until the cultures reached the confluence till 3-4 wk time (data not shown). The cells cultured over the denuded AM and denuded AM with 3T3 had similar growth rate and reached almost a confluent growth by the end of day 21.

\[^{24}\text{BALASUBRAMANIAN et al: EFFECT OF 3T3 ON LIMBAL EPITHELIAL CELL CULTIVATION}\]

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Annealing temp (°C)</th>
<th>PCR product size (bp)</th>
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<td>∆Np63</td>
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\[^{24}\text{Source: Taken with permission from Ref. 24}\]
of this marker was confirmed by Western blot. Western blot results showed the faint positive expression of both p63 and ABCG2 on the cells harvested at the end of 3 wk (Fig. 3).

**ABCG2 and p63 expression by immunohistochemistry and Western blot on the cultured corneal limbal explants on denuded AM in the absence of the 3T3 feeder layer:**
ABCG2 expression was completely absent on the cells cultured over the denuded membrane but the p63 expression was seen on the basal cells at the end of 3 wk (Fig. 2c and 2d). This was confirmed by the Western blot on the cells harvested at the end of 3 wk. There was complete absence of the ABCG2 but there was faint expression of the p63 protein (Fig. 3).

**Semiquantitative RT-PCR data on the expression of ΔNp63, ABCG2, connexin 43 and K3/K12 on the cells cultured over the denuded AM without 3T3 feeder layer:**
Faint expression of p63 and ABCG2 was seen only on the cells harvested at 8th day but it was completely absent on cells harvested till 21st day. Similarly the expression of connexin -43 and keratin 3 and 12 was comparatively more on the cells cultured over the denuded AM without 3T3 feeder layer (Fig. 4).

**Semiquantitative RT-PCR data on the expression of ΔNp63, ABCG2, connexin 43 and K3/K12 on the cells cultured over the denuded AM with the 3T3 feeder layer:**
The cultured cells showed the expression of p63 and ABCG2 till 21 days of incubation. There was a gradual decrease in the expression of these stem cells associated markers; it was able to retain the expression till 21 days of incubation. Similarly the expression of connexin43 and keratin 3 and 12 was seen but it was comparatively less than those cultured only on the denuded AM (Fig. 4).

**Discussion**
The limbal corneal epithelial cells cultured over denuded AM under the influence of feeder layer were able to retain the expression of the putative stem cell markers, ABCG2 and p63 in contrast to cells cultured over denuded AM minus feeder layer. Immunohistochemical expression of p63 and ABCG2 expression was observed at the end 21 days and the expression was confirmed by the Western blot. Western blot results show that the expression of p63 was seen both the culture conditions whereas the ABCG2 expression was seen only the cells cultured over the denuded AM with 3T3 feeder layer. RT-PCR results showed that expression of p63 and ABCG2 gradually decreased by the end of day 21 on the cells cultured in presence of 3T3, but were able to retain the expression of the p63 and ABCG2 but cells cultured on the denuded AM minus 3T3 were not able to retain the ABCG2 marker. Similarly, the expression of gap
junction Cnx43 and K3/K12 was observed in cells cultured over denuded AM, in the absence of feeder layer. There was a gradual increase in the expression of connexin 43 and keratins markers from day 8 to 21 on the cells cultured over the denuded AM. Our results are in concurrence with previous investigators. Liu et al. observed that the cells co-cultured with 3T3 feeder layer shows the expression of p63 and faint expression of K3 and K12. On the continuous passage the expression of p63 was lost. They also showed that the human limbal cells from the cadaveric donor eyes when co-cultured with the mitomycin treated 3T3 feeder layer were able to maintain the features of limbal epithelial cells. Low level expression of the keratin was reported on the cells cultured in the presence of 3T3. Experimental evidence showed that a more differentiated epithelial phenotype of limbal explant cultures on the denuded AM is downregulated by 3T3 fibroblast feeder layers. Thus, inclusion of 3T3 feeder layers appears to prevent epithelial differentiation when denuded AM is used for ex vivo expansion of limbal epithelial progenitor cells.

The reason for the maintenance of the stem cell markers in the cultured cells under the influence of feeder layer is not clearly understood, but could be multifactorial such as the possible release of diffusible factors or cytokines released by the 3T3 fibroblast system, presence of anti-apoptotic survival factor in 3T3 fibroblast conditioned medium, and the similarity of the 3T3 feeder layer in mimicking the numerous signal transduction pathways between the limbus and the limbal epithelial cells in maintaining the stemness of the cells.

3T3 feeder layer has also been used for the in vitro propagation of human ocular epithelial transplantation. Lindberg et al. have demonstrated the proliferative capacity of the cells isolated from the limbus when co-cultured with lethally irradiated mouse 3T3 fibroblasts. And the cells co-cultured were able to form a stratified epithelium confluent limbal cultures on transplantation onto the dermal bed of the nude mice, the cells adhered firmly and cells exhibited the limbal phenotype properties. Grueterich et al. have compared the growth of the limbal epithelial cells on the denuded AM with and without the 3T3 feeder layer. Xenotransplantation of the cultured cells with denuded +3T3 onto the nude mice demonstrated the limbal phenotype properties whereas cells with the denuded AM showed more of differentiated phenotype. These experimental evidences support that the differentiated epithelial phenotype of the limbal explant cultures on the denuded AM can be downregulated by the inclusion of 3T3 feeder layer.

Thus human limbal epithelial cells isolated from human cadaveric donor eyes when co-cultured with 3T3 feeder layer with denuded AM maintains the limbal epithelial cell properties. This culture system can be useful for the clinical application of the limbal stem cell culture and study of limbal stem cell mechanisms.

However, there are limitations in the use of feeder layer for culturing stem cells (either embryonic or mesenchymal) for human clinical applications. Recently,
human embryonic stem cell lines cultured on mouse feeder cells were reported to be contaminated by the xeno-carbohydrate N-glycolylneuraminic acid (Neu5Gc) and considered potentially unfit for human therapy. We do not know whether this holds true for adult limbal stem cells cultured under the influence of 3T3 feeder layer. In future, further in vitro studies using feeder layer will enable us to understand the factors, which play a role in maintaining the limbal stem cell niche.

Acknowledgment

The authors acknowledge the financial support received from the Indian Council of Medical Research, New Delhi.

References


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