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Cultivation of human corneal limbal stem cells in Mebiol gel® - A thermo-reversible gelation polymer

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***Background & objectives*: Cultivated limbal stem cell transplantation is being used as a current treatment modality for limbal stem cell deficiency. However, use of allogenic biological material as substrate is associated with risks of transmission of certain diseases and allograft rejection. Therefore development of non-toxic biodegradable synthetic polymers is important. We undertook this study to evaluate the use of a synthetic polymer Mebiol gel as a substrate for the growth of limbal phenotype cells and cornea phenotype cells from limbal explants.**

***Methods*: Human cadaveric limbal explants cells were cultivated on Mebiol gel. The proliferative capacity of cultivated cells was analyzed with thymidine incorporation studies. Immunostaining for presumed limbal stem cell association markers and cornea differentiation markers was performed and confirmed with reverse transcription (RT-PCR).**

***Results*: The limbal explants underwent proliferation *in vitro*. The cultivated cells expressed the presumed limbal stem cell association markers (ABCG2 and p63), the transient amplifying cell markers (connexin 43, integrin α9) and the cornea differentiation marker (K3). RT PCR confirmed the immunohistochemical data.**

***Interpretation & conclusion*: Our findings showed that the synthetic polymer Mebiol gel was able to support limbal explant proliferation. The cultured cells expressed presumed limbal stem cell association markers, transient amplifying cells and cornea phenotype markers. Mebiol Gel can be used as a scaffold for growing limbal explants.**

Key words Limbal explants - limbal stem cell transplation - Mebiol gel®

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Ocular surface diseases, such as Stevens-Johnson syndrome, chemical and thermal burns, and inflammatory lesions damage the corneal, limbal, and conjunctival epithelia, often resulting in significant visual morbidity. Cultivated limbal stem cell transplantation has been used in the treatment of limbal stem cell deficiency[1-3]. The substrates used for the development of ocular surface tissue-equivalents include human amniotic membrane (HAM), collagen gel and fibrin glue, of which HAM is currently the most commonly used substrate for ocular surface transplantation[1-9].

However, the use of allogeneic biological material is associated with the risks of transmission of certain diseases such as HIV, hepatitis B and C, and bacterial and fungal infections and allograft rejection. In addition, many of the substrates lack the mechanical properties that allow easy handling and suturing, as well as prolonged endurance after transplantation. As such, there is a need to develop new methods of ocular surface epithelial cell replacement. The use of biosynthetic materials as stromal substitutes to support epithelial cell growth would overcome some of the problems related to the use of allogeneic tissue and biological substrates. These could be custom fabricated to suit each condition and could provide a ready supply of material for clinical use and avoid the shortcomings, such as poor mechanical strength and risk of immunologic rejection. In this context developing synthetic polymers, which are non-toxic and biodegradable with adequate tensile strength is important.

Mebiol gel is a copolymer composed of thermoresponsive polymer block [poly (N-isopropylacrylamide-co-n-butyl methacrylate) (poly NIPAAm-co-BMA)] and the hydrophilic polymer block [polyethylene glycol (PEG)]. This polymer block is hydrophilic at temperatures below 20^0C and hydrophobic at temperatures above 20^0C forming cross-linking points and homogenous three-dimensional (3-D) network of Mebiol gel in water. Cells or tissues can be embedded in a liquid Mebiol gel solution at lower than 20^0C and cultured three-dimensionally in a hydrogel state at 37^0C. The sol-gel transition temperature can be controlled by altering chemical composition of thermo reversible gelation polymer (TGP)[10-12]. The present study was undertaken to investigate the growth characteristics and the phenotype of the limbal explants embedded in Mebiol gel.

Material & Methods

Chemicals and antibodies: Dulbecco's modified Eagle's medium (DMEM), penicillin, gentamicin, amphotericin B, 3 per cent foetal calf serum (FCS) trypsin, and EDTA were purchased from Hi-Media, Mumbai, India. Mouse monoclonal antibodies of P63 (4A4 isoform), ABCG2, Connexin 43, Integrin α9 and secondary antibody anti Mouse HRP conjugate for immunohistochemistry were procured from Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA. Secondary antibody mouse monoclonal FITC conjugate was procured form DAKOcytomation DAKO, USA. The Vision Research Foundation and the Institute Ethics Committees approved the study protocol.

Human limbal tissues: Human limbal biopsies (n=24) of approximately 2 mm^3 sizes were obtained from the superior and the inferior portion of the limbus from the human donor eyes using sterile Bard-Parker blade within 3-6 h of death from the C.U Shah eye bank of Sankara Nethralaya, Chennai, India between September 2004 and January 2005. Each human limbal biopsy was placed in one ml of tissue culture (TC) medium of DMEM containing 3 per cent FCS and antibiotics (penicillin, gentamicin and amphotericin B) and transported to cell biology laboratory immediately.

Preparation of Mebiol gel-tissue culture growth medium mixture: Mebiol gel provided in 10 ml amount as lyophilized and sterilized form in a flask was purchased from Mebiol Inc. (Tokyo, Japan,

through Nichi-In Bio Sciences Pvt. Ltd, Chennai, India). The gel in the flask was dissolved in 10 ml of 2X strength of TC growth medium of DMEM + Ham's F-12 containing 20 per cent FCS at *p*H 7.0 and placed in a refrigerator at 4°C-8°C overnight, yielding a viscous transparent Mebiol gel-TC growth medium mixture (gel-TC medium mixture) of uniform liquid without any air bubbles for use in the experiments.

Cultivation of corneal limbal tissue embedded within the Mebiol gel: Limbal biopsy was washed thrice using TC growth medium and the limbal epithelium was carefully dissected from the underlying stroma, cut into 0.5 to 1 mm pieces, and cultivated as explants within the Mebiol gel. A drop of the liquefied (4-8°C) gel-TC medium mixture was placed in the center of the 24-well TC plate and solidified at 37°C for about 20-30 min. The explant piece of tissue was placed on the surface of the solid gel and another cold liquefied drop of the gel tissue culture medium mixture was added to cover the gel with the tissue bit inside. Equal volume of DMEM + F12 was added and the tissue culture plates were incubated in 10 per cent CO_2 atmosphere at 37°C. The growth pattern of limbal explants within the gel was observed.

Morphology and viability of cultivated cells: Cultures were monitored under an inverted phase-contrast microscope (Nikon, Japan). The viability of cultivated cells was determined by staining with trypan blue (Hi-Media, Mumbai, India). The cells were harvested and washed twice with PBS and 0.5 per cent trypan blue solution in PBS was added to the cell pellet and incubated at room temperature. About 10 µl of the sample was loaded on the heamocytometer chamber and number of viable and non-viable cells counted.

Proliferative capacity of cultivated cells: The proliferative capacity of cultivated cells was evaluated by using the ^{3}H thymidine assay. Growth rate of human corneal limbal tissue (HCLT) cells within Mebiol gel was determined by the rate of uptake of ^{3}H thymidine by the growing cells[13] and modified according to Beckmann instruction manual along with control wells with and without gel. (Beckmann Instruction LS 6500 scintillation system operating manual, Beckmann Instruments Inc, USA).

The HCLT biopsy bits were placed over the 12-well tissue culture plate. One µCi of ^{3}H thymidine (BRIT, Mumbai, India) was incorporated into each well. Fifty µl of medium from each well was sampled every day for ten days and relative consumption percentage was measured and calculated using the disintegrations per minute of test and reference standard using LSS (Beckmann Instruments Inc, LS6500, CA, USA) scintillation counter. Reduction of ^{3}H thymidine indicated its incorporation by multiplying cells for DNA synthesis. The cultivated HCLTs along with the outgrowth of cells were harvested by liquefying Mebiol gel by placing the 12-well TC plates in the refrigerator for 2 h to release tissue and cells. Their viable count was done by trypan blue dye exclusion test[14].

Immunohistochemistry and immunofluorescence: The cultured cells from the limbal biopsy 1, 2 and 3 (Table II) were washed in cold PBS, *p*H 7.0, cytospinned (Cytospin 2, Shandon, UK) on the microscopic slides and stained for the various stem cell association markers using monoclonal antibodies against transcription factor p63 (clone 4A4), ABCG2 (ATP binding cassette super family G member 2) and differentiation markers Connexin 43 and Integrin α9 (Santa Cruz Biolgicals, Santa Cruz, CA, USA) by immunoperoxidase and immunofluorescent methods as described by Chen *et al*[15].

Immunoperoxidase staining was performed on cytospinned smear preparation. The smears were fixed in methanol and then dried and treated with primary antibody for 2 h at room temperature, further washed with Tris-HCl buffer, *p*H 8.0 and then incubated for one hour with horse radish peroxidase conjugated anti-mouse secondary antibody at 1:100 dilution. Substrate diamino benzidine (1:100) (DAKO cytomation corp, Glostrup, Denmark) was

added and incubated for 5 min washed and counterstained with Harris Haematoxylin for 30 seconds.

Immunofluorescence staining was performed on smears fixed in cold acetone. Staining was done with primary antibodies at 1:50 dilution with 1 h incubation and after washing with PBS-Tween 20 (PBST) smears were treated with anti mouse conjugated with FITC (DAKOcytomation corp, Glostrup, Denmark) at 1:5 dilutions for an hour and counterstained with 0.5 per cent Evans blue. The slides were mounted with glycerol and observed under the fluorescent microscope (Optiphot, Nikon, Japan).

Reverse transcription-polymerase chain reactions (RT-PCR): Total RNA was isolated from all the biopsy tissues and cells on days 2, 4, 6, 8 and 10 of incubation to determine the pattern of expression of different presumed corneal limbal stem cell association markers and cornea differentiation markers on the cultured cells by RT-PCR[15]. Cells were collected, treated with Tri-Reagent (Sigma Aldrich St Louis, USA) according to the manufacturer's recommended protocol and total RNA was extracted and stored at -80^{o}C until use. Reverse transcription was performed using sensiscript reverse transcripatase (Qiagen, Germany), which is a recombinant heterodimeric enzyme. PCR amplifications of the first-strand cDNAs were performed using specific primer pairs, designed from published human gene sequences along with House keeping gene, glyceraldehyde-3 phosphate dehydrogenase (GAPDH) as internal control, expression of the following markers *viz.*, ΔNp63, ABCG2, Integrin α9 Connexin 43, keratins K3 and K12 were performed on the cultured cells (Table I) in the Eppendorf PCR systems. PCR products were fractionated by electrophoresis using 2 per cent agarose gel containing 0.5 per cent ethidium bromide with molecular marker *Hinf I* Φ digest to confirm the size of the resultant product.

Table I. Primer sequence and reaction condition for the reverse transcription PCR

Gene	Primer sequences	Annealing temp. (^{0}C)	PCR product size (bp)
ΔNp63	FP: CAGACTCAATTTAGTGAG	54	440
	RP: AGCTCATGGTTGGGGCAC		
ABCG-2	FP: AGTTCCATGGCACTGGCCATA	62	379
	RP: TCAGGTAGGCAATTGTGAAGG		
Integrin α9	FP: TGGATCATCGCCATCAGTTTG	61	123
	RP: CCGGTCTTCTCAGCTTCGAT		
Connexin 43	FP: CCTTCTTGCTGATCCAGTGGTAC	66	154
	RP: ACCAAGGACACCACCAGCAT		
K3	FP: GGCAGAGATCGAGGGTCTC	64	145
	RP: GTCATCCTTCGCCTGCTGTAG		
K12	FP: ACATGAAGAAGAACCACGAGGATG	63	150
	RP: TCTGCTCAGCGATGGTTTCA		
GAPDH	FP: GCCAAGGTCATCCATGACAAC	63	498
	RP: GTCCACCACCCTGTTGCTGTA		

FP, forward primer; RP, reverse primer
Source: Ref. 15

Results

Morphology and viability of cultivated cells: The growth of cells from the limbal biopsy tissue embedded within the Mebiol gel commenced on day 4. The cells further multiplied with outgrowth migrating out of the margin of Mebiol gel forming monolayer outside the gel margin. (Fig. 1). Viable count, as estimated by trypan blue exclusion test, ranged from 95 to 98 per cent with approximate yield of 4 x 10^4 cells per mm^3 of liquefied gel.

Thymidine incorporation studies: There was rapid incorporation of thymidine in limbal explants embedded within the Mebiol gel. Rapid ^{3}H thymidine incorporation occurred from day 1 by cells in Mebiol gel and control cells did so only on the initial day. Fig. 2 shows the results of ^{3}H-thymidine incorporation studies performed on one of the limbal explants cultivated in Mebiol gel for 10 days.

Immunohistochemistry and immunofluorescence: The cultured limbal epithelial cells demonstrated a positive immunoreactivity for antibodies for presumed limbal stem cell associated markers: ABCG2 and p63 and cornea differentiation markers Connexin 43 and Integrin α9 by both immunoperoxidase and immunofluorescence methods (Table II).

Table II. Results of immunomarkers studies of each of the human corneal limbal explants cultivated within Mebiol gel

Sl.no.	Marker study				Day of harvesting
	p-63	ABCG-2	Connexin-43	Integrin α9	
1*	+	+	+	+	13th
2*	-	+	+	-	12th
3*#	+	+	-	-	10th
4#	Cultivated tissues & cells were lost during processing				10th
5#	+	+	+	+	10th
6	+	+	+	+	11th
7	+	+	+	+	12th
8	+	+	-	+	10th
9	+	+	-	-	11th
10	+	-	-	-	12th
11	+	+	-	-	11th
12	+	+	+	+	13th
13	+	+	+	-	13th
14	+	+	-	-	13th
15	+	+	-	-	10th
16	+	+	-	+	11th
17	+	+	-	-	11th
18	+	+	-	+	12th
19	+	+	+	+	11th
20	+	+	+	+	10th
21	+	+	+	+	10th
22	+	+	-	-	10th

*Immunofluorescence staining was performed ; #Tritiated thymidine uptake study was performed
+Positive expression of the marker-Negative for the marker

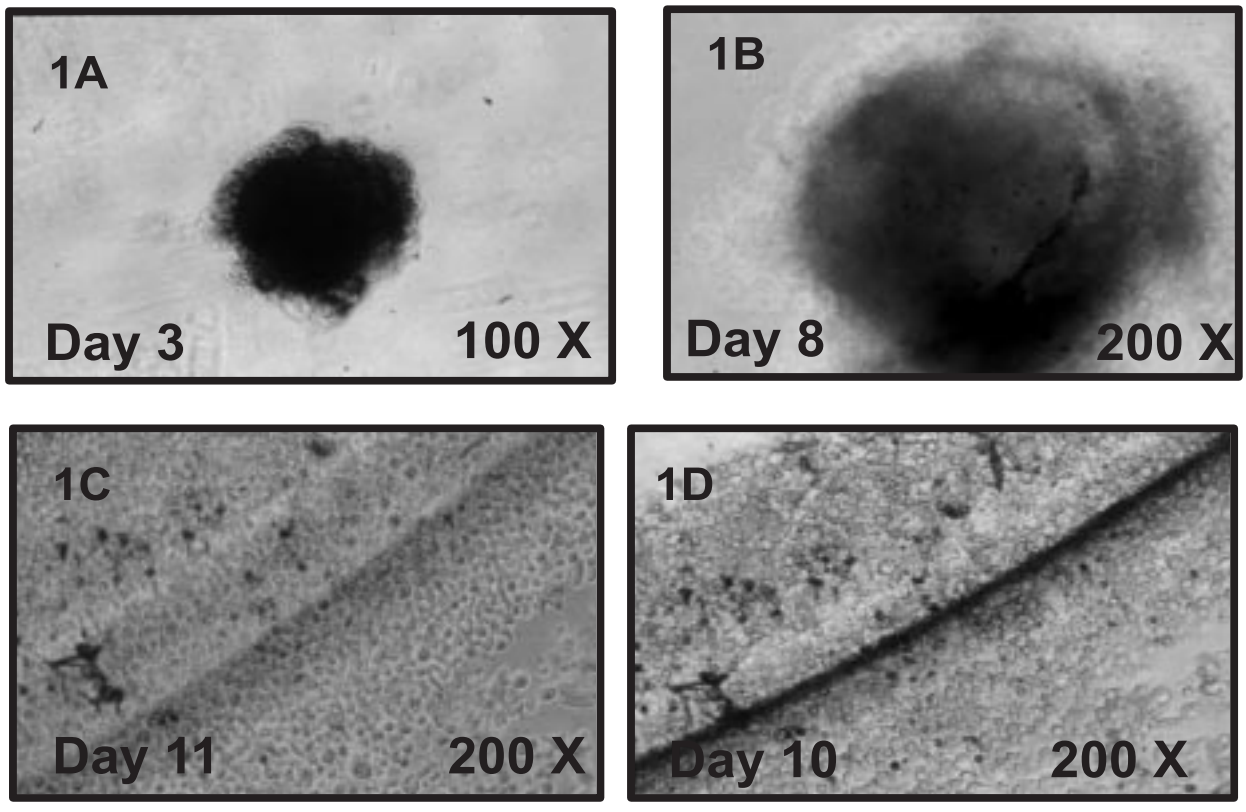

Fig. 1. A, B, C & **D** show the sequential growth and increase in size of human corneal limbal biopsy embedded within the Mebiol gel. **A** shows the growth as on day 3 with increase in size of the tissue and cells proliferation as seen in **1B**. The **1C** shows the migration of proliferating cells breaking through the margin of Mebiol gel on day 10. 1**D** shows the proliferating cells outside Mebiol gel margin forming a monolayer on day 11. Magnification 100X in 1A, 200 X in B,C D.

Expression of association and differentiation markers on the limbal explants cultured within the Mebiol gel: Results of RT-PCR on the RNA extracted from the HCLT cultivated cells harvested from the Mebiol gel on days 4, 8, 12, 16 and 20 of incubation showed expression of p63 and ABCG2 on all days (Fig. 3).

Expression of ABCG2 by immunophenotyping and RT-PCR on all days showed proliferating pluripotent stem cells in the cells cultivated within Mebiol gel.

In this study corneal limbal stem cells were p63 positive and Connexin 43 negative whereas transient amplifying cells were positive for both markers. Expression of this marker from day 8 on the cells cultivated within Mebiol gel indicated multiplication of transient amplifying cells (Fig. 3).

Integrin α9 expression was seen from the 12th day in our study (Fig. 3). Integrin α9 positivity in cells cultured in Mebiol gel demonstrated multiplying transient amplifying cells, further confirmed by results of RT-PCR experiments.

K3 and K12 are cornea specific keratins expressed by the mature corneal epithelium. RT-PCR results showed the expression of the K3 from the 12th day. Whereas K12 expression was absent (Fig. 3).

Discussion

The objective of our study was to observe whether the polymer Mebiol gel supported the growth of the cells from the limbal explants, whether the cultured cells were able to multiply, were viable and

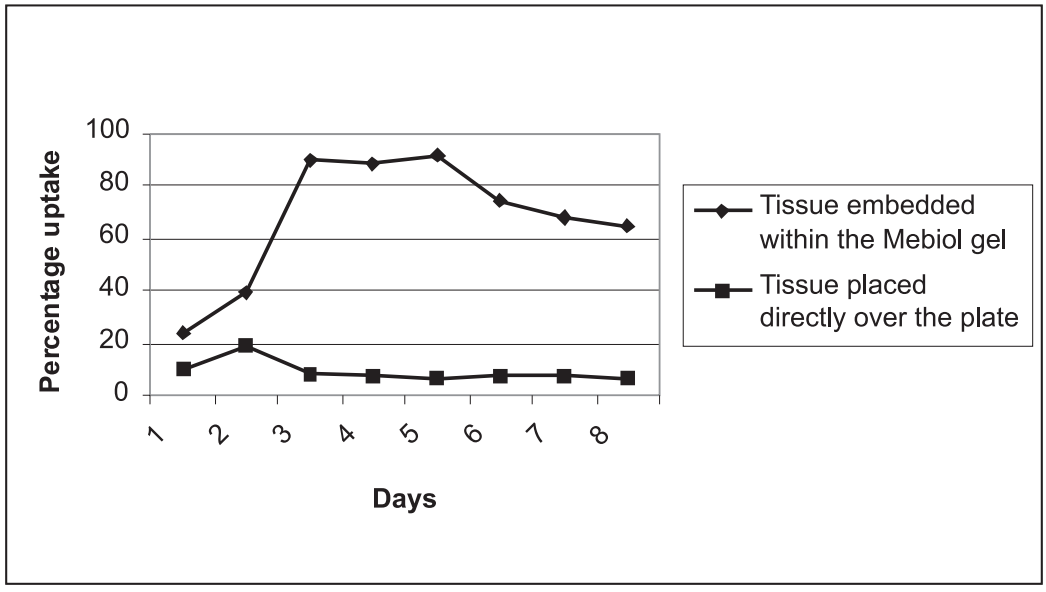

Fig. 2. Results of tritiated (^{3}H) thymidine incorporation studies carried out on the human corneal limbal biopsy embedded in Mebiol gel for 10 days. Cells within the Mebiol gel showed rapid multiplication from day 1 as demonstrated by ^{3}H thymidine incorporation several folds (as shown as ♦) more than control human corneal limbal tissue cells placed directly over the plate (as shown as ♦). Control limbal biopsies incorporated tritiated (^{3}H) thymidine at the initial day or two and did not do so afterwards indicating death of these cells.

most importantly, whether the cultured cells were able to retain the limbal phenotype and to show cornea-specific differentiation.

We observed that the Mebiol gel facilitate the growth of cells from the limbal explants. This was also supported by the ^{3}H-thymidine incorporation studies. The cultured cells were shown to be viable by the trypan blue exclusion test. The results from the immunochemistry and the RT-PCR studies established that the cultured cells were limbal phenotype and showed corneal differentiation.

The cells cultured in Mebiol gel, were positive for presumed limbal stem cell association markers ABCG2, and p63 cells from the day 4. ABCG2, found in a variety of stem cells, is expressed on the plasma membrane with a functional role in developmental stem cell biology. Ocular surface epithelia contain ABCG2 dependent side population cells exhibiting features associated with stem cells representing putative corneal epithelial stem cells[15,16]. Transcription factor p63 initially was considered as a new marker for limbal stem cells. p63 is expressed in committed progenitor cells of both corneal and conjunctival epithelium as well as in basal/parabasal cells in other squamous epithelial tissues[17]. Using the monoclonal antibody clone 4A4, which reacts with all p63 isoforms, our immunocytochemical investigation showed p63 staining on cells harvested on all the days. Since p63 was expressed on the cells harvested initially and on day 20, it suggests that p63 may represent both presumed limbal stem cells and corneal epithelial cells in a proliferative state, such as transient amplifying cells (TAC)[15,18].

The differentiation markers connexin 43, belonging to the family of 21 transmembrane proteins, is widely expressed in all layers of the corneal epithelium with the exception of the most superficial cells. This was not found on the expanded limbal epithelial cells on amniotic membrane[19]. Integrin β1, a marker for transient amplifying cells and K3 corneal phenotype markers were positive

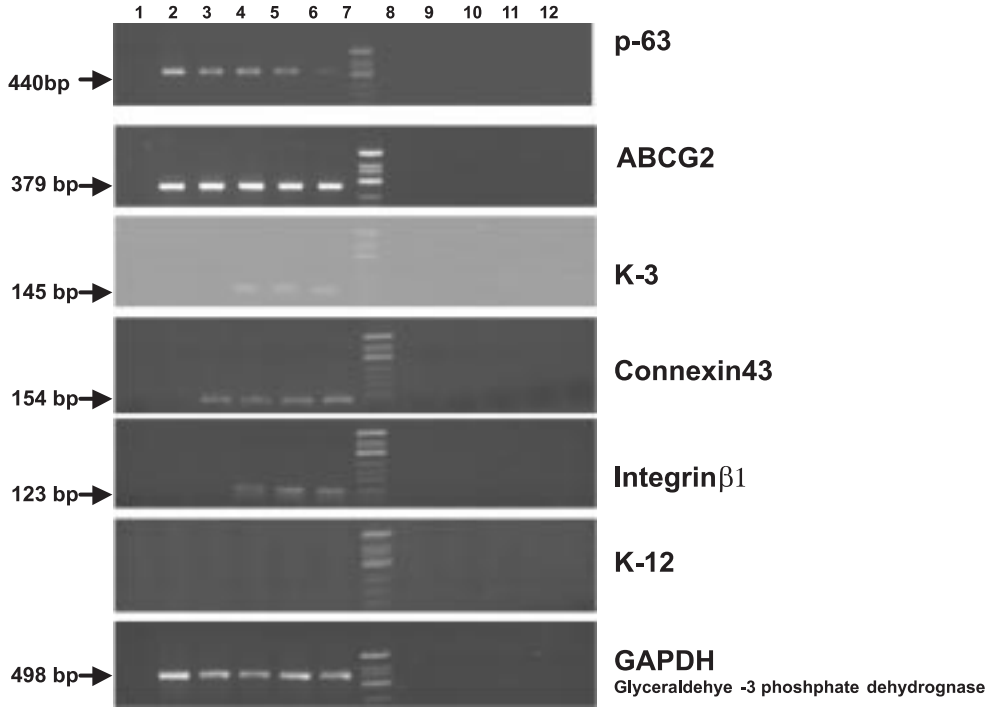

Fig. 3. Results of reverse transcription-polymerase chain reaction (RT-PCR) for m-RNA expression of ΔNp63, ABCG2, and Integrin β1 Connexin43, K3 and K12 markers on the cells grown within the Mebiol gel. Tissues along with cells cultivated were harvested on days 4, 8, 12, 16 and 20. **Lane 1** is the negative control and **lanes 2-6** is the m-RNA expression of the cells harvested on days 4, 8, 12, 16 and 20 respectively. **Lane 7** is the molecular weight marker ϕ X *Hinf* I Digest, **Lanes 8-12** are controls *viz*., DNA controls. p63 and ABCG2 expression was seen on all days. Connexin 43 expression was seen from day 8 and K3 and Integrin β1 expression from 12^{th}-20^{th} day. K12 expression was absent.

from day 12. K3 and K12 are specifically expressed in corneal epithelial cells and are regarded as markers of corneal epithelial differentiation. The appearance of the K3/K12 keratin pair during migration from the limbal to the corneal stroma has been interpreted as differentiation of SC into TAC[20].

Thus, our study demonstrated that Mebiol gel supported the growth of cultured cells from limbal explants. Since we were able to observe the expression of presumed limbal stem cell association markers in limbal explants cultured in Mebiol gel, there was no need for using 3T3 feeder layer when culturing cells for human clinical use.

Mebiol gel has been used as wound dressing, microcapsule for islets[21], electrophoretic gels for DNA separation[22], and three dimentional culture matrix for various cells[23]. Cancer cells rapidly grew in Mebiol gel while fibroblasts did not grow[24]. Our earlier investigations[24] on cultivation of standard animal cell cultures embedded in Mebiol gel indicated their rapid multiplication with formation of monolayers without signs of cytotoxicity.

In summary, we demonstrated the use of a synthetic polymer for the growth of corneal limbal epithelial cells. The Mebiol gel supported the proliferation of epithelial cells in culture and the cells were viable. The cells expressed presumed limbal stem cell association markers and cornea phenotype suggesting that Mebiol gel retains the stemness of the cultured cells. The use of biosynthetic, biocompatible membranes offers several advantages in terms of eliminating the risk of disease transmission, reducing the inconsistency in tissue composition associated with biological substrates, being able to be custom fabricated to suit specific requirements, and possibly providing a readily available alternative tissue source for clinical use.

The Mebiol gel is also far more transparent than HAM. This promising material may have the potential to be used in tissue engineering ocular surface equivalents in the future. These findings have important clinical implications and are an important step toward the development of a safe and effective bioengineered tissue equivalent for clinical use.

Conflict of interest

Authors have declared that they have no conflict of interest.

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