Effect of cord blood serum on ex vivo human limbal epithelial cell culture

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Abstract Limbal cell transplantation is an efficacious procedure for rehabilitation of visual acuity in patients with severe ocular surface disorders. Cultivation of limbal epithelial stem cell with fetal bovine serum for transplantation has been a promising treatment for reconstructing the ocular surface in severe limbal stem cell deficiency caused by Steven Johnson syndrome, chemical or thermal injury. This technique of “cell therapy” has been accepted worldwide but the cost of cultivating the cells for transplantation is high. The objective of this study was to investigate the effect of cord blood serum in place of fetal bovine serum on the growth of human limbal epithelial cell culture. Our group has experimented with human cord blood serum which was obtained free of cost from willing donors. The use of human cord blood serum in place of fetal bovine serum for ex vivo culture of limbal stem cell has helped us in reducing the cost of culture. Fresh human limbal tissues from donor cadavers were cultured on intact and denuded amniotic membrane. Cells were proliferated in vitro with cell culture media containing human cord blood serum. Reverse transcription-polymerase chain reaction and

immunofluorescence cytochemistry of cultured human limbal epithelial stem cell was done for characterization of the cells.

Keywords Limbal cell transplantation · Fetal bovine serum · Cord blood serum · Ocular surface disorders · Cell culture

Introduction

The intervening junction between the cornea and conjunctiva is rich in limbal epithelial stem cells producing epithelial cell for the corneal surface. Corneal epithelium exists in a state of dynamic equilibrium with the superficial cells being constantly shed into tear pool. The cells ultimately responsible for repopulating are termed as “stem cells,” which are cells with high capacity for self-renewal extending through the adult life [1].

In many of the ocular surface diseases such as Stevens-Johnson syndrome, chemical and thermal burns, ocular surface tumors, immunological conditions, radiation injury and inherited syndromes, limbal stem cells are damaged leading to corneal vascularization and scarring results in a marked decrease in vision [2]. Standard procedures like corneal transplantation do not restore the stem cell population and provide a stable ocular surface only as long as the donor epithelium survives. After the inevitable sloughing of the donor epithelium, conjunctivalisation will occur which increases the risk of allograft rejection [3].

In patients with ocular surface disorder with corneal stem cell deficiency, limbal stem cell transplantation is essential for corneal surface reconstruction. Tsubota et al. have shown that the transplantation of stem cell from limbal region has helped in restoring normal vision to many patients suffering from unilateral or bilateral ocular surface disorder [4].

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Over the past few years, different methods have been applied for limbal epithelial cell culture, which have resulted in the successful transplantation and reconstruction of damaged ocular surface [5].

In this study, we report the successful use of human cord blood serum in place of fetal bovine serum (FBS) for ex vivo culture of limbal epithelial cell required for clinical transplantation. Cord blood serum acts as a viable alternative to fetal bovine serum and is obtained free of cost from the willing donors with informed consent. The use of cord blood serum has helped in the development of a cost effective technique of limbal stem cell culture, which will probably benefit the poorer section of society. Intact and denuded human amniotic membrane (HAM) has been used as a substrate for the culture.

Materials and methods

Ethical approval regarding the protocol was obtained from the Institutional Review Board. Human cord blood is obtained from hospital after informed consent from donors and in accordance with the tenets of Declaration of Helsinki. At the time of procurement of cord blood, serum was tested for human immunodeficiency virus (HIV-1 and 2), hepatitis B surface antigen, hepatitis C antibody, and syphilis antibody. Cord blood is centrifuged at 5,000 rpm for 15 min. The upper supernatant of serum is separated and stored at -20°C after filtration through millipore filter paper.

Fresh human limbal tissues (less than 24 h postmortem) from donors aged 19–67 years were obtained from Eye bank for primary culture. Limbal epithelial cells were isolated from fresh limbal tissues as described by Zhang et al. [6]. In brief, the tissues were washed in ringer lactate solution containing 50 $\mu\text{g}/\text{ml}$ gentamicin and 1.25 $\mu\text{g}/\text{ml}$ amphotericin B. Limbal tissues were dissected out aseptically and were treated with trypsin (0.05 %) at 37°C for 90 min to loosen cell from tissue. The loosened tissue was then passed through fire polished Pasteur pipette (2 mm bore), for 10–15 passages. Cell was counted before plating. The isolated limbal epithelial single cell was then used for cell culture experiment.

Explant culture of limbal epithelial cell was performed by using an established method proposed by Noriko et al. [7]. In brief, each limbal rim was cut into 12 equal pieces (about $2\times 2\text{-mm}$ size each). Two pieces with their epithelium side up were directly placed into a well of 24-well culture plates and explants were then cultured in a medium, consisting of Dulbecco's Modified Eagles Medium (DMEM), 10 % cord blood serum, non-essential amino acid and human corneal growth supplement (Cascade Biologics) containing pituitary bovine extract, bovine insulin, hydrocortisone, bovine transferrin and mouse epidermal growth factor, 50 $\mu\text{g}/\text{ml}$

gentamicin, 1.25 $\mu\text{g}/\text{ml}$ amphotericin B, at 37°C under 5 % CO_2 and 95 % humidity. The medium was changed every alternate day and cell growth was observed under phase contrast microscope.

To compare the use of cord blood serum supplemented medium with that of the conventional FBS supplemented medium, we also cultivated the limbal epithelial cell in FBS-supplemented medium. We compared the morphological and immunohistochemical results of the limbal epithelial cell cultured in cord blood serum supplemented medium with the corresponding limbal epithelial cell grown in FBS-supplemented medium.

The standard protocol proposed by Kim et al. was used for the preparation of HAM [8]. In brief, placentas used in the study were obtained from cesarean section of seronegative donors with informed consent from donors and in accordance with the tenets of Declaration of Helsinki. They were then tested for the absence of HIV-1 and HIV-2, hepatitis B surface antigen, hepatitis C, and syphilis. Under sterile conditions the membranes were washed in ringer lactate containing antibiotics (amphotericin B and gentamycin) until blood was removed. Amnion was separated from chorion. HAM was cut into small pieces ($2''\times 2''$) with sterile scissors and forceps and adhered to nitrocellulose paper keeping the epithelium side up. HAM was stored in vials containing media (DMEM/glycerol 1:1) at -80°C before use. Prior to use, the HAM was thawed to 37°C for 30 min. Membranes were removed aseptically and kept in DMEM containing 10 % serum and allowed to be incubated under 5 % CO_2 , 95 % air at 37°C and inspected under microscope at different days for microbial contamination. Observations until 30 days did not show any signs of contamination.

Denuded HAMs (dHAMs) were also used for limbal epithelial cell culture. HAM was trypsinized with 0.05 % trypsin for 30 min at 37°C followed by scrapping of membrane with cell scraper. Limbal cells, either obtained from cadaver eyes as described previously or those obtained after prolonged primary culture, were seeded on the denuded membrane and maintained for 15 days and observed routinely under microscope for cell growth and proliferation.

Immunofluorescent staining of limbal epithelial cell culture was performed for characterization of the cell. Cells cultured on coverslip and dHAM were both stained to study their morphological pattern. After a few days, coverslip and denuded HAM with cultured limbal cell were fixed in ice-cold methanol for 10 min. Cells were then rehydrated in phosphate-buffered saline (pH=7.5) for 1 h at 37°C . Non-specific binding was prevented by blocking with normal goat serum (NGS) (1:10) for 45 min at 37°C . After blocking with NGS, primary monoclonal antibodies against integrin $\beta 1$, nuclear p63, and ATP-binding cassette sub-family G

Table 1 Human primer sequences used for semi-quantitative RT-PCR

Gene	Sense primer	Antisense primer	PCR product
Integrin β 1	CAGCCTGAGGCAGCCACTTCG	GGGGTCTTGCCACACCCACAG	443
δ Np63	TGGGCCGACGCTGCTTTGAGG	GTTGGCTCCCATGCCATCAGG	504
ABCG2	ACGCCATCCTGGGACCCACAG	TCCTGAGCAGGCCCGTGAAC	590
Nestin	CAGGACAGTGCTGAGCCTTCTGG	TCCCCTGAGGACCAGGA	380
Connexin43	CCTGGGTACAAGCTGGTTACTGG	ATCTCCAGGTCATCAGGCCGAGG	296
GAPDH	TGGCGCTGAGTACGTCGTGG	TAGCCCAGGATGCCCTTGAG	559

member 2 (ABCG2) were applied and incubated for 1 h at room temperature. Cells were stained with stem cell specific markers since no specific limbal cell marker has been identified till date. Fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Sigma), was applied for 45 min at 37 °C. Cells were observed under microscope in presence of mounting fluid.

RNA extraction from limbal epithelial cell culture was, at first, carried out by treating the cells with Trizol and passing through 1-ml syringe followed by chloroform treatment. This was followed by centrifugation at 12,000 $\times g$ for 15 min under cold conditions. The upper aqueous layer was transferred carefully to a fresh tube and isopropanol was added and kept at 10–15 °C overnight. Gel-like precipitate of RNA was obtained. Washing was done with 75 % ethanol to remove salts and pellet was vacuum dried. The dried pellet was dissolved in 0.1 % DEPC-treated water. The RNA was quantified by its absorption at 260 nm and stored at –80 °C before use. Using a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an internal control, the mRNA expression of different molecular markers was analyzed by semi-quantitative reverse transcriptase Polymerase chain reaction (RT-PCR). Briefly, first-strand complementary deoxyribonucleic acid (cDNAs) was synthesized from 5 μ g of total RNA with Moloney murine leukemia virus reverse transcriptase. PCR amplification was

performed with specific primer pairs designed from published human gene sequences (Table 1) for different markers [9].

During the standardization of this technique, cultures were tested for endotoxin and mycoplasma contamination. Endotoxin in cell culture was tested by *Limulus* amoebocyte lysate test. Mycoplasma was tested by Hoechst 33258 staining of coverslip culture containing cultured limbal epithelial cells. These testing were essential as contamination frequently spoil cell culture especially in tropical countries.

Results

Morphological appearance of ex vivo limbal epithelial cell culture with cord blood serum and fetal bovine serum were similar as observed under microscopy (Fig 1). Successful cultures from both primary and explants culture technique of limbal cell were established. In all cultures, the growth of cells was monitored under phase contrast microscopy. On an average, clusters of round cells were seen after 1–3 days of culture with cord blood serum-supplemented medium and FBS-supplemented medium. Cells further divided to form a monolayer by 5–7 days. These cells were expanded to confluency covering the entire membrane within a period of 2 weeks. It was observed that cell proliferation was better

Fig. 1 Limbal cell culture with cord blood serum and with fetal bovine serum: cells grown with cord blood serum (left figure) and fetal bovine serum are similar in morphology

Fig. 2 Immunofluorescence cytochemistry of limbal cells cultured on coverslips. Three different antibodies were applied for staining different regions of limbal cell, namely ABCG2 (1:25), integrin β 1 (1:200),

and p 63(1:25). ABCG2 is a cell body-specific marker, integrin β 1 is a cytoskeletal protein marker, while p63 stains the cell nucleus

when initially grown on petriplate for few days before transferring to HAM rather than cells grown directly on HAM. In the case of the former, confluency was reached within 10–12 days.

Immunofluorescent staining of human corneal cell culture on coverslip and dHAM showed that all the cells showed positive immunoreactivity to ABCG2 (1:25), integrin β 1 (1:200) and p63 (Figs. 2 and 3) thereby justifying purity of the preparation. ABCG2 is a cell body-specific marker; integrin β 1 is a cytoskeletal protein marker, while p63 stains the cell nucleus. It was observed that integrin β 1 antibody brightly stained the membranes of small cells, whereas weak or no staining was observed in the larger cells. The nuclear p63 antibody had stained the nucleus of limbal cell that was cultured on coverslip and dHAM. Cells stained positively against ABCG2 antibody although fluorescent-stained cells were less in number showing that in a population of cultured cell, number of stem cells is few and sparsely populated.

Semi-quantitative RT-PCR disclosed a differential expression pattern of stem cell associated and differentiation markers in these selected populations of limbal epithelial stem cell. Integrin β 1 mRNA was expressed at the highest

level. ABCG2 mRNA, a newly proposed stem cell marker, was expressed at a low rate explaining the fact that in a population of corneal epithelial cell, few numbers of limbal stem cells are present. Nuclear p63 mRNA was highly expressed by cultured cells. It is already reported that p63-expressing cells have high proliferative capacity. In contrast, the mRNAs encoding markers, connexin 43 and nestin, were not detected. No difference in the mRNA levels of GAPDH, a housekeeping gene used as an internal control, was observed among all four populations. Results demonstrated the presence of ABCG2, δ Np63 and integrin β 1 mRNAs, the three positive markers for limbal cells and absence of any detectable nestin and connexin mRNA, which were otherwise, detected in human blood cells (Fig. 4).

Discussion

The rise in ocular surface-related problems led to the first transplantation attempt in mice by Lindberg [10]. Subsequently, various groups started experimenting with various substrates such as collagen IV, fibronectin, laminin, silk, etc. for culturing of limbal epithelial cell on the surface

Fig. 3 Immunofluorescence cytochemistry of limbal cells cultured on denuded HAM. Limbal epithelial cell cultured on denuded HAM and stained with antibodies, namely ABCG2 (1:25), integrin β 1 (1:200), and p63(1:25)

Fig. 4 RT-PCR analysis for the presence of mRNAs in cultured limbal cells. Presence of five mRNAs were analyzed. (1) ABCG2, (2) δ Np63 and (3) intergrin β 1 are for positive limbal cell. Nestin and Connexin

43 are marker negative for limbal cell. (6) GAPDH, a housekeeping gene, was used as a positive control. A 100 bp DNA ladder is shown in the first left lane

of these substrates. HAM was one of the substrates used for culturing of limbal cell because of its wound healing potential by Talbot et al. [11].

Interestingly, the choice of technique for the culturing of these cells has varied among different investigators but the cost of culture still remained high. In this study, human cord blood serum was used in place of the traditionally used fetal bovine serum. This replacement had not only reduced the cost of culture but also has shown similar growth and proliferation rate as fetal bovine serum. Moreover, as obtained free of cost from human being, the need to use animal product could be minimized. The aim of the study was to establish a low-cost limbal cell culture technique, affordable for countries where ocular surface disorder is very common among people living below poverty line.

We have used HAM as a substrate for culturing of limbal cell because of its easy availability and also it has been reported to have high wound healing potential. Moreover, for transplantation of the cultured cell, HAM is considered to be a very good substrate [12].

After transplantation, HAM is easily degraded without any external force. Using HAM as a substrate has also benefited us in reducing the cost of limbal cell culture. A single specimen of placenta can provide us with as many as 100 small pieces (2"×2") of amniotic membrane required for limbal epithelial cell culture.

Generally, cholera toxin, sodium selenite, etc. are used for the culture of limbal cell. We have not used the abovementioned compounds in our cell culture media. Instead, we have used human cord blood serum which is easily available human byproduct, generally discarded as waste. Comparative analysis of the growth pattern of cell cultured in fetal bovine serum and cord blood serum have given similar results. The proliferative capacity of cells grown in cord blood serum is similar to those grown in fetal bovine serum. No differences in cell morphology were observed under microscope.

Animal serum is routinely added to culture media as a source of nutrients and other factors, despite its technical disadvantages and high cost. Technical disadvantages to using animal serum include the undefined nature of serum,

batch-to-batch variability in composition, and the risk of contamination. There are increasing concerns about fetal sensitivity to pain during blood collection.

Conclusion

This cost-effective technique of cell culture has helped us to perform sub-confluent limbal stem cell culture with human cord blood serum in place of fetal bovine serum. Transplantation of cultivated limbal stem cell with cord blood serum will benefit poor patients suffering from ocular surface disorder. However, development of cell line has not yet been done. The long-term objective is to develop a method for faster growth of limbal stem cell in vitro, which will benefit patients requiring transplantation. This work will probably add knowledge to the already existing technique of limbal epithelial cell culture and transplantation.

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