Transfusion and Apheresis Science xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Transfusion and Apheresis Science

journal homepage: www.elsevier.com/locate/transci

Comparison of three human platelet lysates used as supplements for *in vitro* expansion of corneal endothelium cells

Tsung-Jen Wang[a,b], Ming-Sheng Chen[c], Ming-Li Chou[c,1], Hsiu-Chen Lin[d,e], Jerard Seghatchian[f,*], Thierry Burnouf[c,g,**]

[a] Department of Ophthalmology, Taipei Medical University Hospital, Taipei, Taiwan
[b] Department of Ophthalmology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
[c] Graduate Institute of Biomedical Materials and Tissue Engineering, College of Biomedical Engineering, Taipei Medical University, Taipei, Taiwan
[d] Department of Pediatrics, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
[e] Department of Clinical Pathology, Taipei Medical University Hospital, Taipei, Taiwan
[f] International Consultancy in Blood Components Quality/Safety Improvement, Audit/Inspection and DDR Strategies, London, UK
[g] International PhD Program in Biomedical Engineering, College of Biomedical Engineering, Taipei Medical University, Taipei, Taiwan

ARTICLE INFO

Article history:
Available online xxx

Keywords:
Platelet lysate
Corneal endothelium
Expansion
Fetal bovine serum

ABSTRACT

Background: Human platelet lysates (HPLs) are emerging as the new gold standard supplement of growth media for *ex vivo* expansion of cells for transplant. However, variations do exist in the way how HPLs are prepared. In particular, uncertainties still exist regarding the type of HPL most suitable for corneal endothelium cells (CEC) expansion, especially as these cells have limited proliferative capacity.
Material and methods: Three distinct HPL preparations were produced, with or without calcium chloride/glass beads activation, and with or without heat treatment at 56 °C for 30 min. These HPLs were used to supplement basal D-MEM growth medium, each at a protein concentration equivalent to that of 10% fetal bovine serum (FBS; control). Impact on CEC (BCE C/D-1b cells) *in vitro* morphology, viability and capacity to express Zonula occludens-1 (ZO-1) tight junction marker was assessed by Western blotting.
Results: BCE C/D-1b cells grown in all HPL supplements exhibited four of essential characteristic properties: adhesion capacity, microscopic morphology and viability similar to that observed when using 10% FBS. In addition, Western blots analysis revealed an expression of the ZO-1 marker by BCE C/D-1b cells in all conditions of culture.
Conclusion: CECs can expand *ex vivo* in a basal medium supplemented with the three HPLs without noticeable difference compared to FBS supplement. These data support further studies to evaluate the potential to use HPLs as a clinical-grade xeno-free supplement of CEC for corneal transplant.

1. Introduction

The corneal endothelium (CE) is located in the inner part of the cornea, at the junction between the posterior surface of the Descemet's membrane and the aqueous humor [1]. CE is formed by a fragile monolayer of CE cells (CECs) that exert particularly crucial physiological roles in visual function by ensuring transparency, clarity, and hydration of the corneal stroma [1–4]. Alterations of the CE, as may occur as a result of injury or ocular pathologies, cause corneal hyper-hydration and opacification that lead to severe visual impairments and, eventually, blindness. Unfortunately, the CEC tissue is particularly devoid of self-repairing capacity *in situ*, thereby requiring the implementation of specific external therapeutic repair strategies. The gold-standard treatment for CE damage is corneal transplantation [5–7], but this therapeutic approach suffers from the serious shortage in donor corneas worldwide [8,9]. Difficulties in corneal tissue supply is stimulating the development of alternative regenerative medicine and tissue engineering procedures [10], some based on isolation and *ex vivo* expansion of CECs as material for transplants [11]. However, any cell therapy procedure intended for therapeutic use needs to meet the requirements for licensed clinical applications, in particular

* Corresponding author.
** Corresponding author at: Graduate Institute of Biomedical Materials and Tissue Engineering, College of Biomedical Engineering, Taipei Medical University, 250 Wu-Xing Street, Taipei 110, Taiwan.
E-mail addresses: jseghatchian@btopenworld.com (J. Seghatchian), thburnouf@gmail.com (T. Burnouf).
[1] Current address: INSERM, UMRS 938, Saint-Antoine, Laboratory Immune System, Neuroinflammation and Neurodegenerative Diseases, 184 Rue du Faubourg, Hôpital Saint-Antoine, F-75012 Paris, France.

http://dx.doi.org/10.1016/j.transci.2017.08.021
1473-0502/

with regards to the components of the growth medium for cell expansion. In that regard, some highly regulated countries are banning the use of fetal bovine serum (FBS) [12,13], a material exposing transplanted patients to immunological [14,15] and zoonotic infection [16] risks. Therapeutic-grade human platelet lysates (HPLs) are emerging as a robust substitute for FBS in clinically focused applications [17,18] following the pioneering work by Lataillade's group in France [19]. The technical possibility of replacing FBS by HPLs as a source of nutrients and growth factors for *in vitro* cell growth has been demonstrated without ambiguity for various types of human mesenchymal stem cells or other cells for transplant [17,20]. Use of HPL is also gaining interest in direct clinical use and tissue engineering, and was shown to exert anti-microbial [21] and anti-inflammatory [22] activities. Serum eye drops and HPL are also considered of clinical interest and without demonstrated toxic effects in treatment of epithelium ocular disease, including dry eye syndrome, as commented in this journal [23]. Recently, we proved that primary bovine CECs could be expanded in a basal growth medium solely supplemented with HPL [11], without the need for the additional supplements like growth hormones or recombinant growth factors [24–30]. The optimal production process of HPL to expand specific types of cells is, however, not yet defined [17]. To which extent variations in HPL preparation can affect the capacity to multiply CEC *in vitro* has not been explored. Here, we have evaluated the robustness of HPL supplementation by testing the capacity of 3 different types of HPLs to expand CECs *in vitro* using bovine BCE C/D-1b as a cellular model.

2. Materials and methods

2.1. Cell line and cell maintenance

The bovine BCE C/D-1b was provided by one of us (Prof. Tsung-Jen Wang) and obtained from a culture collection (BCE C/D-1b, Bioresource Collection and Research Center, Taiwan). The cells were maintained in Dulbecco's Modified Eagle's Medium (D-MEM, Gibco, Grand Island, NY, USA) containing 3.7 g/l of sodium bicarbonate ($NaHCO_3$) and 1% Penicillin Streptomycin, pH 7.2–7.4, originally supplemented with 10% FBS decomplemented at 56 °C for 30 min.

2.2. Platelet concentrates and HPL preparation

Four HPL batches were prepared from pooled therapeutic-grade expired non leucofiltered apheresis platelet concentrates collected by Taiwan Blood Center (Guandu, Taiwan). All processes were conducted under aseptic conditions. Three different methods to prepare HPL were evaluated. FTPL ("freeze-thawed platelet lysate") was obtained by subjecting the platelet concentrates to three freeze (−80 °C)–thaw (30 °C) cycles. GBPL ("glass beads platelet lysate") was prepared by serum-conversion of the thawed platelet lysate using 23 mM of calcium chloride in the presence of glass beads (0.5 g/mL) at 25 °C for 30 min, followed by removal of the glass beads/fibrin clot [11]. GBHPL ("glass beads heat-treated platelet lysate") was obtained by further subjecting GBPL to a heat-treatment at 56 °C for 30 min. All fractions were centrifuged at 6000 × *g* for 30 min at 25 °C to remove any cell debris or insoluble particles. Heparin (3 IU/ml, final concentration) was added to FTPL to prevent further coagulation of the growth medium during cell cultures. Samples were frozen in aliquots at −20 °C until use.

2.3. Protein content

The protein content of FBS, FTPL, GBPL and GBHPL was quantified by Coomassie blue method (Bradford) using BSA as a standard. 150 μl of standard and of diluted samples were added into 96-well plate, and then mixed with 150 μl Coomassie reagent. After 10–15 min, absorbance at 595 nm was measured by using spectrophotometer. The standard curve was made by plotting the blank-corrected O.D value. The protein concentration of each sample, as calculated from the standard curve, was used to normalize the protein content of the three HPL fractions to be equivalent to 10% FBS medium for BCE C/D-1b cultures.

2.4. Cell experiments

2.4.1. Cell culture

1 ml of frozen cells was thawed at 37 °C and mixed immediately with 9–10 ml of cultured medium into a culture dish. Fresh culture medium was added after 1 day. Cells were passaged every two to three days after washing with 10 ml normal PBS buffer twice. 2 ml of trypsin–EDTA (0.05%) was added, and cells were incubated at 37 °C in 5% CO_2 humidified atmosphere for 7 min. 8 ml of culture medium was added to stop the trypsin/EDTA reaction. The cell suspension was collected into a 15 ml-centrifuge tube at 200 × *g* for 5 min. Supernatant was removed, ca. 3 ml of culture medium was added, cells were resuspended by mild pipetting and 1 ml of cellular suspension added into a new culture dish together with 9 ml of fresh culture medium.

BCE C/D-1b cells were cultured in 6-well or 24-well dishes at 37 °C in a controlled atmosphere containing 5% CO_2. Cells were allowed to adhere for 1 day and then cultured for up to 7 days in the condition medium. 10% (v/v) FBS medium was used as control. Four conditions were evaluated for each cell: 10% FBS, and (a) FTPL (containing 3 unit/ml heparin), (b) GBPL, and (c) GBHPL at a final protein content equivalent to that of 10% FBS to avoid the impact of the total protein content. Medium was changed every 2 days.

2.4.2. Cell morphology

Cell morphology was observed by optical microscopy at 100× to observe the impact of each culture condition on cell morphology and compare to the FBS condition used as control.

2.4.3. Trypan blue exclusion tests

Following cultures, the cellular suspensions were collected. 10 μl cellular suspensions aliquots were mixed with 10 μl trypan blue, and then 10 μl of mixture was put on the slide to determine cell count by using an automatic cell counter.

2.4.4. Cell viability

BCE C/D-1b cells were seeded in 96-well plate at a density of 3×10^4 cells/well, then cultured with the basal medium supplemented with 10% (v/v) protein content FBS or the equivalent (based on total protein) in HPL. Cells were grown for up to 7 days. 100 μl of MTT working solution (0.5 mg/ml MTT in DMEM) and cells were incubated at 37 °C in 5% CO_2 humidified air for 3 h, and then the MTT working solution removed. 100 μl DMSO was added for dissolving the purple crystals. Absorbance was read at 540 nm with a spectrophotometer. Cell viability was expressed as a percent of the control calculated as follows: cell viability

$$(\%) = \left(\frac{\text{O.D. sample} - \text{O.D. blank}}{\text{O.D. control} - \text{O.D. blank}}\right) \times 100$$

2.5. Western blot for Zonula occludens-1 (ZO-1)

The Western blot analysis was performed as described before [11]. Cells were detached by scrapers and collected into Eppendorf. After centrifugation at 200 × *g* for 5 min to spin down and recover the cells, 50 μl of RIPA buffer containing protease inhibitors were then added to resuspend the cells. The suspension was strongly vortexed for 15 s seven times at 5-min intervals and centrifuged at 12,000 × *g* at 4 °C for 20 min. Protein extracts were

Please cite this article in press as: Wang T-J, et al. Comparison of three human platelet lysates used as supplements for *in vitro* expansion of corneal endothelium cells. Transfus Apheresis Sci (2017), http://dx.doi.org/10.1016/j.transci.2017.08.021