Tissue and Cell 46 (2014) 233–240

Contents lists available at ScienceDirect

Tissue and Cell

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

# The effects of human serum to the morphology, proliferation and gene expression level of the respiratory epithelium *in vitro*

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ARTICLE INFO

*Article history:*
Received 15 October 2012
Received in revised form 20 January 2014
Accepted 3 May 2014
Available online 9 May 2014

*Keywords:*
Human serum
Fetal bovine serum
Respiratory epithelium
Growth kinetics
Gene expression

ABSTRACT

The culture of human airway epithelial cells has played an important role in advancing our understanding of the metabolic and molecular mechanisms underlying normal function and disease pathology of airway epithelial cells. The present study focused on investigating the effects of human serum (HS) on the qualitative and quantitative properties of the human respiratory epithelium compared to the fetal bovine serum (FBS), as a supplement in culture. Respiratory epithelial (RE) cells derived from human nasal turbinate were co-cultured with fibroblasts, subsequently separated at 80–90% confluency by differential trypsinization. RE cells were then sub-cultured into 2 different plates containing 5% allogenic HS and FBS supplemented media respectively up to passage 1 ($P_1$). Cell morphology, growth rate, cell viability and population doubling time were assessed under light microscope, and levels of gene expression were measured via real time reverse transcriptase-polymerase chain reaction (qRT-PCR). RE cells appeared as polygonal shape and expanded when cultured in HS whereas RE cells in FBS were observed to be easily matured thus limit the RE cells expansion. Proliferation rate of RE cells in HS supplemented media ($7673.18 \pm 1207.15$) was 3 times higher compared to RE in FBS supplemented media ($2357.68 \pm 186.85$). Furthermore, RE cells cultured in HS-supplemented media required fewer days ($9.15 \pm 1.10$) to double in numbers compared to cells cultured in FBS-supplemented media ($13.66 \pm 0.81$). Both the differences were significant ($p < 0.05$). However, there were no significant differences in the viability of RE cells in both groups ($p = 0.105$). qRT-PCR showed comparable expressions of gene Cytokeratin-14 (CK-14), Cytokeratin-18 (CK-18) and Mucin-5 subtype B (MUC5B) in RE cells cultured in both groups ($p > 0.05$). In conclusion, HS is a comparatively better choice of media supplement in accelerating growth kinetics of RE cells *in vitro* thus producing a better quality of respiratory epithelium for future tracheal reconstruction.


## 1. Introduction

Respiratory epithelium is a ciliated pseudo-stratified columnar epithelium with numerous scattered goblet cells. It is derived from progenitor cells in the anterior foregut endoderm that contribute to formation of lung, thyroid, liver, and pancreas (Wells and Melton, 1999).

Pseudostratified respiratory mucosa consists of four different cell types which are ciliated cells, intermediate cell, basal cells and goblet cells. Ciliated cells occur from the trachea down to the last respiratory bronchiole, but their heights decreased with the reduction of the airway diameter. Basal cells lie on the basement membrane and have been considered the progenitors of columnar or goblet cells. Serous and mucous cells are the secretory cell types of the glands which are found in the wall of the large airways. The products of secretory cell types are essential for the mucociliary clearance and thus play an important part in defense. In many diseases, such as asthma or cystic fibrosis, the structure and function of these cells are altered in a characteristic way (Bals, 1997).

The airway epithelium is frequently injured because of permanent contact with the outer environment. Bacterial and viral infections, the inhalation of pollutants and toxic agents or mechanical stress can more or less severely alter the integrity of the

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http://dx.doi.org/10.1016/j.tice.2014.05.003
0040-8166/

epithelial barrier. The airway epithelium is a dynamic tissue normally undergoing slow, but constant renewal. On the basis of studies in experimental animals and limited studies in humans, the airway epithelium likely turns over every 30–50 days. If injured, unless the injury is too severe, extensive, or chronic, the airway epithelium responds vigorously to reestablish an epithelial sheet with normal structure and function, with resident cells as the source of the new cell population. Immediately after injury, the airway epithelium initiates a repair process in order to restore the barrier integrity (Coraux et al., 2005).

When stem-cell-based products involve more than minimal manipulation such as expansion or differentiation, the cells will probably be grown in *in vitro* culture. The standard culture medium contains bovine serum (Mannello and Tonti, 2007). Bovine serum is a product from bovine fetuses. However, there are several concerns when the tissue-engineered cell is generated for human use. There is possibility of contamination from bovine disease by prion, virus and zoonosis and immunological reaction due to the formation of antibodies to fetal calf serum proteins Patients can be sensitized with an Immunoglobulin E response against bovine serum albumin leading to anaphylactic reactions (Mackensen et al., 2000). A research done on mesenchymal stem cell that expanded in FBS showed that immunological reactions and anti-FBS antibodies have been observed and considered as having possibly affected the therapeutic outcome (Sundin et al., 2007).

Human supplements such as autologous or allogeneic human serum have been postulated as alternatives to FBS to provide nutrients, attachment factors, and especially growth factors (Bieback et al., 2009).

Moreover, it is easily accessible, readily available and considerably cheaper than FBS. Study has shown that human synovial MSCs expanded more in human serum than in FBS (Nimura et al., 2008). Human serum can support proliferation and differentiation of hMSC in vitro and can maintain their bone forming capacity in vivo (Aldahmash et al., 2011). The use of human serum in cell cultures of hMSC intended for cell-based therapy is preferable. However, studies employing human serum have reported conflicting results regarding their ability to maintain the biological characteristics of MSC with some studies demonstrating inhibitory effects on MSC biology (Kuznetsov et al., 2000).

Previous study has shown that human serum as a growth supplement is used in ex vivo expanded autologous limbal epithelial cells on amniotic membrane (Shahdadfar et al., 2011). In this study, patients with limbal stem cell deficiency (LSCD), transplantation of ex vivo expanded human limbal epithelial cells (HLECs) can restore the structural and functional integrity of the corneal surface.

Until present, there is no study on the human respiratory epithelium cultured with human serum. Thus our objectives were to compare the morphology of the human respiratory epithelial cells cultured in both serum, the growth kinetic of human respiratory epithelial cells including cell viability, proliferation rate and population doubling time; and the gene expression level of CK 14, CK 18 and MUC5B of human respiratory epithelium cells in human serum (HS) and fetal bovine serum (FBS) supplemented media.

## 2. Materials and methodology

This study was approved by the research ethic committee with approval number: UKM 1.5.3.5/244/SPP/FF-118-2012. All the human study subjects provided informed consent.

### 2.1. Human serum (HS) isolation for supplementation

Total of 200 ml of whole blood was withdrawn from 4 healthy donors (mean ± SEM age 23 ± 0.41 years) via venapuncture. Each 50 ml tube containing the serum from different individual was then centrifuged at 5000 rpm for 5 min. The supernatant was collected as the serum while the pallet comprises of blood cells and platelet was discarded. After centrifugation, the serum was sterility filtered through a 0.2-μm syringe filter into a 50 ml sterile tube and immediately stored at −20 °C prior to use.

### 2.2. Cell isolation

A consent form for obtaining nasal turbinate specimen from patient was prepared and the use of human nasal turbinate in this study was approved by the Research and Ethic Committee of Medical Faculty, Universiti Kebangsaan Malaysia. Samples were obtained from 6 consented patients (mean ± SEM age 20 ± 3.04 years) that underwent nasal turbinectomy at Seremban Specialist Hospital.

The specimen was thoroughly cleaned of mucus and blood three times using Dulbecco Phosphate-Buffered Saline (PBS) containing 100 μl penicillin and streptomycin. The soft tissue was then separated from the underlying bones and cut into 2 $mm^3$, subsequently digested in 0.3% Collagenase type 1 solution for 4–6 h in incubator shaker. After digestion, the cell suspensions containing fibroblasts and respiratory epithelial cells were centrifuged at 5000 rpm for 5 min. Then, the cell pellet was re-suspended and trypsinized using 10 ml Trypsin EDTA (Invitrogen) and incubated for 5 min at 37 °C to separate cell agglomerates into single cells.

### 2.3. Co-culture of respiratory epithelium

The co-culture system used defined keratinocyte serum-free medium (DKSFM) with growths supplement and F-12:Dulbecco's modified Eagle's medium (DMEM) at a ratio of 1:1 (FD) supplemented with 10% FBS solution with total volume of 2 ml medium per well in 6-well plate. The co-cultures were maintained in monolayer at 37 °C, 5% $CO_2$ in humidified atmosphere until they reached about 70–80% confluence.

### 2.4. Differential trypsinization of fibroblast from respiratory epithelium

Once the co-culture was 70–80% confluent, fibroblasts were removed via differential trypsinization. The 6-well plate was incubated with 2 ml 0.05% Trypsin EDTA in each well for 2 min at 37 °C for fibroblasts detachment. Trypsin inhibitor was added to inhibit the action of Trypsin EDTA on the remaining respiratory epithelial cells. The solution containing Trypsin EDTA, Trypsin Inhibitor and detached fibroblasts was then aspirated using pipette and discarded, leaving the respiratory epithelium cells attached on the well. Each well was then washed with 1 ml of DPBS and only DKSFM was added into each well to culture respiratory epithelium. The cell culture entered Passage 0 ($P_0$).

### 2.5. Re-seeding and sub-culture

Cells cultured at $P_0$ were allowed to proliferate and expand until reaching confluence of 70–80%. Cells were subsequently harvested via trypsinization. Cells were then detached from the plate by using 2 ml of 0.05% Trypsin EDTA into each well and incubated for 5 min. Same volume of Trypsin inhibitor was then added to neutralize the enzymatic action of Trypsin EDTA. Solution containing the detached cells was transferred into a 50 ml tube and centrifuged at 5000 rpm for 5 min. Supernatant was discarded. The remaining cell suspension were re-seeded into the new 6-well plate and sub-cultured into two different groups containing DKSFM supplemented with 5% human serum (HS) and 5% fetal bovine serum (FBS) respectively. Hence, cell cultures were incubated at 37 °C in humid air