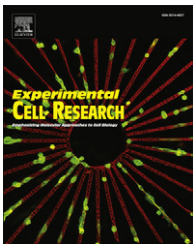
EXPERIMENTAL CELL RESEARCH 319 (2013) 1102–1110

Available online at www.sciencedirect.com

SciVerse ScienceDirect

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



Research Article

Generation and characterization of a spontaneously immortalized endothelial cell line from mice microcirculation

Rodrigo A. Loiola[a,*], *Tathiany C. Torres*[a], *Carla M. Aburaya*[a], *Maristella A. Landgraf*[a,b], *Richardt G. Landgraf*[a], *João Bosco Pesquero*[c], *Liliam Fernandes*[a]

[a]*Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Universidade Federal de São Paulo, Campus Diadema, Rua São Nicolau 210, Diadema, São Paulo 09913-030, Brazil*

[b]*Instituto de Ciências Biomédicas, Departamento de Imunologia, Universidade de São Paulo, Rua Professor Lineu Prestes 1730, São Paulo 05508-900, Brazil*

[c]*Departamento de Biofísica, Universidade Federal de São Paulo, Campus São Paulo, Rua Pedro de Toledo 669, São Paulo 04039-032, Brazil*

ARTICLE INFORMATION

Article Chronology:
Received 26 October 2012
Received in revised form
28 January 2013
Accepted 31 January 2013
Available online 14 February 2013

Keywords:
Microcirculation
Endothelial cell
Nitric oxide
Prostacyclin
Spontaneously immortalized cell line

ABSTRACT

Endothelial cells from microvasculature are directly involved in a large number of vascular diseases; however, culture of these cells is problematic, since most methodologies employ proteolytic enzymes or mechanical techniques, leading to cell damage and contamination of endothelial cultures with other cellular types. Besides, primary cultured cells have a short life span in vitro and undergo replicative senescence after 3–4 passages, limiting long-term studies. In the present work we report the generation of a spontaneously immortalized endothelial culture obtained from mice pulmonary capillaries. Firstly, primary (third passage) and immortalized (100th) cultures were established. Further, monoclonal populations were obtained by serial dilutions from immortalized cultures. Cells were analyzed according to: (1) morphological appearance, (2) expression of specific endothelial markers by fluorescent staining [von Willebrand Factor (vWF), endothelial nitric oxide synthase (eNOS), angiotensin converting enzyme (ACE) and Ulex europaeus (UEA-1)] and by flow cytometry (endoglin, VE-cadherin and VCAM-1), and (3) release of nitric oxide (NO), assessed by the specific fluorescent dye DAF-2 DA, and prostacyclin (PGI_2), quantified by enzyme immune assay. In both cultures cells grew in monolayers and presented cobblestone appearance at confluence. Positive staining for vWF, eNOS, ACE and UEA-1 was detected in cloned as well as in early-passage cultured cells. Similarly, cultures presented equal expressions of endoglin, VE-cadherin and VCAM-1. Values of NO and PGI_2 levels did not differ between cultures. From these results we confirm that the described spontaneously immortalized endothelial cell line is capable of unlimited growth and retains typical morphological and functional properties

Abbreviations: ACE, angiotensin converting enzyme; ACh, acetyl-choline; BK, bradykinin; DAF-2 DA, 4,5-diaminofluorescein diacetate; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified eagle's medium; DMSO, dimethylsulfoxide; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; PDT, population doubling time; PE, R-phycoerythrin; PGI_2, prostacyclin; PLA_2, phospholipase A_2; UEA-1, Ulex europaeus lectin agglutinin I; VCAM-1, vascular cell adhesion molecule; vWF, von Willebrand factor

*Corresponding author. Fax: +55 11 4043 6428.
E-mail address: rodrigoazl@gmail.com (R.A. Loiola).

0014-4827/$ - see front matter
http://dx.doi.org/10.1016/j.yexcr.2013.01.022

exhibited by primary cultured cells. Therefore, the endothelial cell line described in the present study can become a suitable tool in the field of endothelium research and can be useful for the investigation of production of endothelial mediators, angiogenesis and inflammation.

Introduction

Endothelial cells regulate the tonus of underlying vascular smooth muscle by releasing relaxing and contracting mediators [1,2]. Chemical or physical stimuli induce elevation of intracellular Ca^{2+}, followed by activation of endothelial nitric oxide (NO) synthase (eNOS) and phospholipase A_2 (PLA_2) [3]. eNOS catalyzes the conversion of the amino acid L-arginine to L-citrulline and NO [4], whereas PLA_2 induces production of arachidonic acid, the precursor of prostaglandin I_2 (PGI_2) [5]. Both NO and PGI_2 can diffuse throughout adjacent vascular smooth muscle cells, resulting in vascular relaxation. In addition, the combined release of NO and PGI_2 at the interface with the blood contributes to the protective role of endothelium against intraluminal platelet aggregation and thrombus formation [2].

It is widely accepted that a large number of important physiopathological events in which endothelial cells are involved (e.g., angiogenesis and inflammation) occur at the level of the microvasculature rather than in large vessels. Unfortunately, isolation and culture of microvascular endothelial cells is difficult and time-consuming, and most of the described techniques can induce cell damage, either by proteolytic enzyme effects or by mechanical trauma [6]. Another important observation is the fact that cultured microvascular cells undergo replicative senescence in vitro after a finite number of divisions [7]. To overcome this limitation, several approaches have been used to generate endothelial cell lines by inducing ectopic expression of viral oncogenes [8,9], overexpression of telomerase reverse transcriptase [10,11] or spontaneous immortalization [12–14].

Pulmonary microcirculation, comprised of capillaries that form a dense network in the alveolar wall, is essential for gas exchange and regulation of fluid and solute passage between the blood and interstitial compartments in the lung [15]. In culture, rat pulmonary microvascular endothelial cells exhibit a significantly lower hydraulic conductance [16] and form a more restrictive barrier to transport macromolecules [17] in comparison with endothelial cells from conductance vessels. In contrast to endothelial cells from large vessels, rat pulmonary microvascular endothelial cells maintain their proliferation independently of growth factors in order to restore cell confluence (and barrier function) following endothelial injury [18].

In the present study we report the generation and characterization of spontaneously immortalized cultures of endothelial cells obtained from mice pulmonary capillaries. Further, monoclonal populations were obtained from these immortalized cultures. Primary cultured (third passage) and immortalized/cloned cells (100th passage) were characterized according to morphological appearance, and specific endothelial markers were accessed by immunofluorescence and fluorescence-activated cell sorting (FACS). In parallel, functional studies evaluated and compared the functional ability of primary and cloned cultured cells to produce NO and PGI_2.

Methods

Animals

C57Bl/6 male mice, aged 10–14 weeks were obtained from the breeding stock of Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia (CEDEME—UNIFESP). Mice were kept in a temperature-controlled room on a 12 h light/day cycle, 60% humidity, standard mice chow and water ad libitum. All procedures were approved and performed in accordance with the guidelines of the Ethics Committee of the UNIFESP (protocol number 0928/05), conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised in 1996).

Endothelial cell isolation and culture

Cell cultures were established according to procedures previously related by Chen et al. (1995) [19]. Mice were anaesthetized (ketamine/xylazine association) and euthanized by cervical dislocation, lungs were excised, washed with phosphate buffered saline (PBS), cut into $1 \times 1 \times 1$ mm^3 pieces and placed in six-well (35 mm) dishes. Tissues were recovered with Dulbecco's modified eagle's medium (DMEM–low glucose) supplemented with fetal bovine serum (FBS, 20%) and gentamicin (40 mg/L), pH 7.4, and placed in a CO_2 incubator (Sheldon Mfg. Inc., USA) (37 °C). Lung explants were discarded after 60 h and medium was changed every 2–3 days. Cells were grown to confluence and further propagated in a 1:4 ratio using trypsin (0.1%). The cells were maintained in culture for 1 year, reaching a mean of 100 passages and 300 population doublings (PD).

Proliferation assay

At each passage, an aliquot of cell suspension was used to count trypan-blue-excluding cells in a hemocytometer and a known cell density was seeded in a tissue culture flask (25 cm^2). The number of PD occurring between passages was calculated according to the equation: $PD = \log^2 (C_H/C_S)$, where C_H is the number of viable cells at harvest and C_S is the number of cells seeded. The population doubling time (PDT) was determined using the time interval (hours) between cell seeding and harvest divided by the number of PD for that passage.

Cloning and growth curves

After 100 passages, endothelial cells (2×10^3) were dispersed by serial dilution in 96-well plates. In the following days, single colonies were transferred to a 12-well plate and cells were grown to confluence. The procedure was repeated twice to