Acta Astronautica 99 (2014) 16–23

Contents lists available at ScienceDirect

Acta Astronautica

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

Effects of simulated microgravity on cell cycle in human endothelial cells ☆

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ARTICLE INFO

Article history:
Received 20 March 2013
Received in revised form
15 December 2013
Accepted 30 January 2014
Available online 19 February 2014

Keywords:
Simulated microgravity
Cell cycle
Endothelial cells

ABSTRACT

The aim of the current study is to investigate effects of simulated microgravity on the cell cycle of endothelial cells. We analyze changes in the cell cycle after exposure of endothelial-like EA.hy 926 cells to simulated microgravity using a Desktop random positioning machine (RPM). Cell cycle profiles determined by flow cytometry show, that the percentage of the cells in the G0/G1 phase after 24 and 96 h of RPM-simulated microgravity is significantly increased as compared to the control group. However, no significant difference is observed after 120 h of RPM-simulated microgravity. In regard to S phase, the percentage of cells is significantly decreased after 24 and 96 h of RPM, respectively; whereas 120 h later, the number of S-phase cells is comparable to the control group. Thus, we show that simulated microgravity inhibits cell cycle progression of human EA.hy 926 cells from the G0/G1 phase to the S phase. We observe an effect of a hibernation-like state, when the growth of the cells in the RPM group slows down, but does not stop. Our results further show that simulated microgravity can affect adhesion of endothelial cells, and alpha-tubulin expression, as most cells begin to detach from the surface of OptiCell unit after 24 h, form aggregates after 48 h, and exhibit accumulation of alpha-tubulin around the nucleus after 48 h of exposure to simulated microgravity conditions. Our results demonstrate a chance in the cell cycle in a low gravitational field.
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1. Introduction

Investigation of weightlessness has shown that space flight can cause serious physiological and pathophysiological changes. Such changes include alterations in water-salt and mineral metabolism, muscle atrophy, as well as disruption of the hematological and immunological systems [1–3]. Many of these health problems are associated with effects of microgravity upon different hierarchical levels, including the cellular level [4,5]. Importantly, new insight regarding cell physiology under unusual extreme conditions may also contribute to better understanding of the pathophysiology of such diseases existing on Earth, for example osteoporosis, muscle atrophy, cardiovascular disease, and dysfunction of the immune system.

Impact of Microgravity on various cell types is an important topic for research, which during recent years has been aiming to investigate effects of microgravity on morphology, functions, and mechanisms of activity of different cells. In particular, most studies of cellular responses induced by

Abbreviations: RPM, Random positioning machine; PBS, phosphate-buffered saline; PI, propidium iodide; BSA, bovine serum albumin

☆ This paper was presented during the 63rd IAC in Naples.

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http://dx.doi.org/10.1016/j.actaastro.2014.01.032
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microgravity have been focusing on cells of bones, muscles, and of the immune system [6–8].

Many cellular events, metabolic pathways and signaling pathways have been determined in cells under microgravity conditions, including cell proliferation, differentiation, cell cycle, cell survival, and apoptosis [9,10].

The effects of microgravity on major cell functions were observed in various model systems during and after space flight. However, limited access to space flight necessitates the establishment of alternative methods for simulating microgravity conditions on Earth. Clinostats with one rotation axis running perpendicular to the direction of the gravity vector are called 1-D (seldom) or 2-D clinostats. In the 2D clinostat, which is widely used for experiments, a randomized gravity vector is obtained by rotation of cell cultures in a horizontal position with constant velocity. A 3D clinostat or two-axis clinostat can dispense the gravitational force over all directions. This apparatus is a type of rotating culture system, consisting of two frames, one positioned inside the other. However, both frames can also be operated with different speeds and different directions. In this case, the term "random positioning machine" (RPM) in combination with the description of the operational mode should be applied. These devices are characterized by randomly changing rotation speed and direction [11,12]. RPMs are valuable tools for simulating microgravity conditions in adherent mammalian cells.

The importance of cell shape and cytoskeletal changes controlling cell cycle progression by the extracellular matrix has been particularly emphasized in different adherent cell types such as hepatocytes [13], fibroblasts [14] and endothelial cells [15]. Endothelial cells are currently used as *in vitro* model systems for various pathological processes, and play a crucial role in different mechanisms of blood flow. They regulate coagulation, adhesion of cells, vascular smooth muscle cell growth, and form a barrier to transvascular diffusion of liquids [15,16].

Thus, investigation of cellular parameters such as cell proliferation, apoptosis, cell cycle and cell metabolism under simulated microgravity is important to understand the mechanisms of how microgravity is affecting cells *in vivo*.

In this study, we examined viability, cell cycle and several morphological properties of the human endothelial cells EA.hy 926 under simulated microgravity conditions using a Desktop RPM.

2. Materials and methods

2.1. Cell culture

The human endothelial cell line EA.hy 926 expressing CD105 and von Willebrand factor was kindly provided by Dr. Cora-Jean S. Edgell (University of North Carolina, USA) [17]. Cells were cultured in complete Dulbecco's modified Eagle medium containing 10% fetal calf serum, HAT (100 μM sodium hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine), 2 mM glutamine, and 50 mg/ml gentamicin (cell culture reagents were purchased from Invitrogen). Cultured cells were seeded in OptiCell units (Nunc OptiCell, Denmark), a cell culture system consisting of two gas-permeable polystyrene membranes enclosed by a cassette. Each OptiCell™ side contains a growth area of 50 cm², with membranes of 75 μm thickness. For experiments, cells were seeded in OptiCell units at a density of 3.0×10^4 cells/ml culture medium, according to the manufacturer's instructions and incubated at 37 °C in a humidified incubator supplemented with 5% CO_2. Cells were cultured under 1 g for 24 h before using a Desktop RPM for further analysis.

2.2. Random positioning machine (RPM)

Microgravity conditions were simulated using a Desktop RPM (Dutch Space, The Company Astrium EADS, Leiden, The Netherlands), a small "random positioning machine" which can be used as "microgravity simulator" by random rotation of cells around the Earth's gravity vector. The RPM consists of one frame rotating within a second rotating frame, each frame being driven by a separate motor.

To investigate effects of simulated microgravity, cells were divided into three groups, namely the simulated microgravity group (RPM group), the static control group, and the shaker group. The latter group was included in order to assess any effects of simulated microgravity on the mixing of medium.

For the RPM group, OptiCell units containing subconfluent cultures were fixed onto the Desktop RPM at the center of the platform, rotating at a speed of 60°/s and placed in a commercially available incubator at 37 °C supplemented with 5% CO_2. Rotation of the platform was regulated by computer software. Under the chosen experimental conditions (60°/s), a maximal residual acceleration of 10^{-3} g is achieved at the border of the OptiCell unit, which decreases towards the center [18].The static control group of OptiCell units was fixed onto the OptiCell rack in horizontal position and cultured in the same conditions (Fig. 1). For the shaker group, OptiCell units were placed on a shaker, rotating at 60 rev./min and placed in the same incubator. Half of the culture medium was replaced with fresh medium every day, according to the protocol for OptiCell.

To perform the analysis, cells were detached from OptiCell units with trypsin/EDTA solution, according to the manufacturer's instructions.

2.3. Cell viability analysis

Cell viability was assessed by trypan-blue vital dye exclusion (Invitrogen, USA). An aliquot of the suspension (~20 μl) from each sample was mixed with an equal amount of 0.4% (w/v) trypan blue dye and placed on the surface of the working slide for analysis on an automated cell counter Countess ™ (Invitrogen, USA). Each test sample of cells was counted in duplicate.

2.4. Cell cycle analysis

For cell cycle measurements, cells were detached from OptiCell units with trypsin/EDTA solution, washed in PBS (phosphate-buffered saline), gently fixed in ice-cold 70% ethanol, and kept for 24 h at 4 °C. The detection method was based on the use of the commercial kit

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