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Upregulation of erythropoietin receptor in UT-7/EPO cells inhibits simulated microgravity-induced cell apoptosis

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Abstract

Hematopoietic progenitor cell proliferation can be altered in either spaceflight or under simulated microgravity experiments on the ground, however, the underlying mechanism remains unknown. Our previous study showed that exposure of the human erythropoietin (EPO)-dependent leukemia cell line UT-7/EPO to conditions of simulated microgravity significantly inhibited the cellular proliferation rate and induced cell apoptosis. We postulated that the downregulation of the erythropoietin receptor (EPOR) expression in UT-7/EPO cells under simulated microgravity may be a possible reason for microgravity triggered apoptosis. In this paper, a human EPOR gene was transferred into UT-7/EPO cells and the resulting expression of EPOR on the surface of UT-7/EPO cells increased approximately 61% ($p < 0.05$) as selected by the antibiotic G418. It was also shown through cytometry assays and morphological observations that microgravity-induced apoptosis markedly decreased in these UT-7/EPO–EPOR cells. Thus, we concluded that upregulation of EPOR in UT-7/EPO cells could inhibit the simulated microgravity-induced cell apoptosis in this EPO dependent cell line.

Keywords: Simulated microgravity; Erythropoietin receptor; Apoptosis; Transfer gene

1. Introduction

Spaceflight anemia has been observed in several manned space missions since the 1960s, but the underlying mechanism is still unclear (Alfrey et al., 1996; Cogoli, 1981; De Santo et al., 2005; Udden et al., 1995a). There have been reports of decrease in responsiveness of the erythropoietic system and erythroid cells to erythropoietin (EPO) in both spaceflight (Allebban et al., 1996) and simulated experiments on the ground (Sytkowski and Davis, 2001).

Binding of EPO to the erythropoietin receptor (EPOR) is crucial for the survival, proliferation, and differentiation of erythroid progenitors (Stefan et al., 1999). BaF3 cells which stably expressed the transfected human EPOR cultured under modeled microgravity displayed the downregulation of EPOR cell surface expression (Xu et al., 2005), as well as IL-3, another member of the hematopoietin receptor superfamily (HRS).

Our previous research suggested that simulated microgravity downregulate the expression of EPOR in erythroid progenitors-like leukemia cell line UT-7/EPO, consequently inducing mitochondrial related apoptosis of UT-7/EPO cell through depression of the EPO–EPOR pathway (Zou et al., 2010). In this paper, it was found that simulated microgravity-induced apoptosis of UT-7/EPO cells was markedly decreased after EPOR was upregulated by transferring an EPOR gene into the cells. This presents a promising potential therapeutic treatment for spaceflight anemia, like directly active the down-stream cascade of EPO–EPOR pathway by some medicine, then the cell proliferation and differentiation could last out and do not depend on EPO–EPOR binding.

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2. Material and methods

2.1. Chemicals and reagents

Annexin V/FITC (fluoresce in isothiocyanate) was obtained from the Center for Human Disease Genomics, Peking University (Beijing, China). 4′, 6-diamidino-2-phenylindole, dihydrochloride (DAPI), propidium iodide (PI), penicillin, and streptomycin were obtained from Sigma Chemical Company (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from MDgenics Inc. (USA). Iscove's Modified Dulbecco's Medium (IMDM) was obtained from GIBCO^{TM} Invitrogen Corporation (USA). Recombinant Human Erythropoietin-β Injection (EPO, 2000 IU/300 μl) was obtained from Roche Registration Ltd. (UK). Monoclonal antibody of EPOR (MAB307) was purchased from R&D Systems (USA). FITC-conjugated goat anti-mouse monoclonal antibodies were purchased from Proteintech (USA). G418 was purchased from NALCO (USA). All other chemicals were of analytical grade.

2.2. Culture conditions

The UT-7/EPO cell line was maintained in liquid IMDM supplemented with 10% FBS and EPO (2 U/ml). Cell cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Simulated microgravity cultivation was accomplished using the NASA product Rotary Cell Culture System (RCCS, USA, Synthecon) at a constant rotation speed of 15 rpm. Control experiments were performed in the cell culture flask without rotation (Yi et al., 2009).

2.3. Gene transfer

Plasmid pCI-neo containing a human EPOR gene was kindly provided by A. Kirkeby (H. Lundbeck A/S, Denmark). DNA was transiently transferred into UT-7/EPO cells using lipofectamine 2000 (Invitrogen, CA, USA) according to manufacturer's instructions. G418 (100 g/ml) was used to select the positive cells.

2.4. Flow cytometry analysis of EPOR expression

UT-7/EPO cells were cultured to an initial density of 1×10^5 cells/ml in the RCCS, with cells cultured to the same density in a culture flask as a control. After culturing for 12 h and 24 h cells were washed intensively in PBS. EPOR antibody MAB307 was used at a 1:100 dilution in PBS containing 1.0% BSA, and 0.1% Tween-20 and incubated for 30 min at room temperature. After washing, cells were incubated for 30 min at room temperature with FITC-conjugated goat anti-mouse IgG antibody at a dilution of 1:100. The fluorescence intensity was analyzed using FACScan flow cytometry.

2.5. RT–PCR analysis of EPOR expression

Total RNA from UT-7/EPO cells was isolated using a standard TRIZOL method according to the manufacturer's protocol (Invitrogen Life Technologies, USA). The RNA quantity was identified from the absorbance at 260 nm. The isolated RNA had an A260/280 ratio of >1.8. A quality assay of the RNA by agarose gel electrophoresis showed no degradation. The RNA sample (4 μg) was added to 20 μl of the reaction mixture containing 1 μl oligo(dt)20, 1 μl SuperScript^{TM} III reverse transcriptase, 1 μl RNaseOUT, and 1 μl dNTP mix. Synthesis of cDNA was performed at 50 °C for 50 min, and the reverse transcription reaction was stopped by heating at 85 °C for 5 min. The cDNA was stored at −20 °C until further use. A total of 1 μl of cDNA was added to 5 μl of TaqPCR Colorless mixture containing 1 unit Taq polymerase, 1 μl of target gene primers. The primers used in the RT–PCR were F: 5′-CCTGCTCATCTGCTTTGG-3′ and R: 5′-AGGCT GTTCTCATAAGGG TTG-3′ for EPOR and F: 5′-GGG AAACTGTGGCGTGAT-3′ and R: 5′-GAGTGGGTGT CGCTGTTGA-3′ for GAPDH. PCR products were analyzed on a 2% agarose gel containing ethidium bromide.

2.6. Observation of cell nuclear damage

UT-7/EPO cells were cultured to an initial density of 1×10^5 cells/ml in the RCCS, with cells cultured to the same density in a culture flask as a control. After culturing for 12, 24, and 48 h, apoptotic nuclear morphology was visualized using the DAPI staining technique. Briefly, cells were harvested and washed three times in cold PBS then fixed with 3.7% paraformaldehyde for 10 min at room temperature, washed three times with PBS, and immersed in 0.1% Triton X-100 for 2 min. Paraformaldehyde fixed cells were stained using DAPI (10 μg/ml) under dark conditions for 10 min. After three PBS washes, cells were observed under a fluorescence microscope (Olympus IX71, Japan).

2.7. Cell apoptosis assays

UT-7/EPO cells were cultured to an initial density of 1×10^5 cells/ml in the RCCS, with cells cultured to the same density in a culture flask as a control. After culturing for 12, 24, 48, and 72 h, cell apoptosis was studied using Annexin V labeling and PI exclusion in combination with flow cytometry. Briefly, UT-7/EPO cells were collected, washed with PBS, and suspended in binding buffer containing 10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$. Cells at a concentration of 1×10^6 cells/ml were then stained with 10 μl Annexin V/FITC and 5 μl PI (50 μg/ml), incubated in the dark at room temperature for 15 min, and subjected to FACScan flow cytometry (Becton–Dickinson, USA).