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Alterations in Cell Cycle Dynamics in Human Endothelium Cell Culture Infected with Influenza Virus

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Abstract—The cell cycle of endothelium EAhy 926 cell culture infected with influenza virus has been studied. Cytometric analysis of cell culture synchronized by contact inhibition revealed the elongation of the S phase of the cell cycle in EAhy 926 cells under the influence of influenza virus. This result was shown in an EAhy 926 culture infected with influenza virus and treated with nocodazole. Comparison of a lung carcinoma A549 cell line in which influenza virus causes G_0/G_1 arrest and of an endothelial EAhy 926 cell line in which the same infection leads to S-phase elongation allows it to be suggested that different effects of influenza virus on cell cycle dynamics depend on the origin of infected cells.

Keywords: endothelial cell culture, cell cycle, influenza virus

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INTRODUCTION

The role of endotheliocytes in the functions of all organ systems can hardly be overestimated. Apart from the main function of maintaining homeostasis and regulation of the inflammation process in the organism, the endothelial cells are the basis of blood-tissue barrier, i.e., they regulate the diffusion, active compound transfer, and immune system cell migration from the blood flow to the surrounding tissues and back again; they also take part in the angiogenesis and endocrine processes (Petrischev and Vlasov, 2003; Lupinskaya, 2008).The barrier function of endothelium makes it a target for viral pathogens (Kota, 2012). It was shown previously by our group that influenza viruses are able to reproduce themselves in the endothelial cells both in vitro and in vivo and to cause their death by apoptosis or necrosis (Azarenok, 2014). According to recent data, the development of virus-induced apoptosis is preceded by changes in cell cycle dynamics, with it being arrested at a certain phase (Stewart et al., 1997; Fehr and Yu, 2013). Some DNA-containing viruses (adenoviruses, papilloma viruses) have been shown to induce the S phase, thus allowing the reproduction of large number of viral genomes (Cheng et al., 2013). The reproduction of the human immunodeficiency RNA virus is characterized by G_2/M arrest of infected cells (Stewart et al., 1997; Li et al., 2010). Other RNA-containing viruses (SARS, influenza virus) are able to block cells' G_0/G_1 exit (He et al., 2010).

The influence of the influenza virus on the cell cycle is being actively investigated at the present day. These studies are mainly performed on lung epithelium cell cultures (He et al., 2010; Wei et al., 2013). The cell cycle of an endothelial cell culture infected with influenza virus has yet to be investigated.

The aim of this study was to examine the dynamics of the cell cycle of a human endothelial cell culture infected with influenza virus.

MATERIALS AND METHODS

Cell culture. The study was carried out on human endothelialcell culture Eahy 926, generously provided by Dr. Edgell (University of North Carolina, United States). This cell line reproduces all main the phenotypic and functional characteristics of endothelium from large human vessels (Edgell et al., 1983; Azarenok et al., 2014). Cells were cultivated in DMEM/F-12 media with HEPES and L-glutamine containing 10% fetal bovine serum, HAT (hypoxanthine, aminopterin, thymidine), and 100 μg/mL gentamicin. Cells were passaged twice a week. The monolayer was disintegrated with 0.02% EDTA solution.

The synchronization of the EAhy 926 cell culture at the G_0/G_1 phases of cell cycle was assessed by contact inhibition in a dense cell culture; the cell proliferation was activated by the seeding of the cells at a low density about 4×10^5 cells per Petri dish (Jet Biofil, United States). Nocodazole treatment of EAhy 926 was per-

formed for 16 h with an end concentration of 0.05 μg/mL.

The cells were infected with the epidemic strain of human influenza virus A/Brisbane/10/2007 (H3N2) by virus absorption at 37°C and 5% CO_2 in maintaining media consisting of DMEM with 2 μg/mL tripsin. Uninfected EAhy 926 cells were used as control. The virus strain was provided by the Laboratory of Evolutionary Variability of Influenza Virus, Influenza Institution, Ministry of Health, Russia. The infectious activity of the virus was found from titration of virus containing material in a 1-day culture of EAhy 926 with a titration coefficient of 10 and calculated by the generally accepted method (Reed and Muench, 1938). A virus dose of $TCID_{50/cell}$ had been chosen in a previous investigation as it does not induce early apoptosis (Azarenok, 2014). Twelve hours after the start of proliferation, the EAhy 926 cell culture synchronized at the G_0/G_1 phase was incubated in a maintaining medium with virus for 1 h. The virus-containing media was then removed and the cells were cultivated in the maintaining media for 3 more days.

In the nocodazole experiments, 12 h after the start of proliferation, EAhy 926 cell culture was incubated in a maintaining medium with virus for 1 h. After that, the virus-containing medium was removed and the cells were cultivated in the maintaining medium for 8 h. The cell culture was then split into two halves, with nocodazole being added to one of them. Thereafter, all cells were cultivated in the maintaining medium for 16 h. Uninfected EAhy 926 cells with and without nocodazole were used as controls.

Cell cycle analysis. Once a day, control and infected cells were suspended in PBS; supplied with saponin (up to a concentration of 200 μg/mL), RNase A (250 μg/mL), and propidium iodide (50 μg/mL); and incubated for 1 h at room temperature in the dark; the distribution of the cells depending on the DNA amount was then found on an EpixXL flow cytometer (Becman Coulter, United States) equipped with a 488-nm argon laser. The analysis of the distribution of the cell population through the cell cycle phases was performed using WinMDI 2.8 and ModFitLT software (Verity Software House, Topsham, United States). Simultaneously, analysis of the growth of control and infected cells was carried out in a Goryaev's chamber. All experiments and controls were done in triplicate. Statistical analysis was carried out with Mann–Whitney's *U* test. The differences were assumed to be significant when $p < 0.05$ (Glotov, 1982).

Reagents. DMEM/F-12 media with HEPES and L-glutamine (Biolot, Russia), 10% fetal bovine serum (Sigma, United States), HAT (hypoxanthine, aminopterin, thymidine) (Sigma, United States), L-glutamine (Biolot, Russia), gentamicin (Biolot, Russia), EDTA (Biolot, Russia), nocodazole (Sigma, United States), tripsin (Sigma, United States), saponin (Fluka, United States), RNase A (Sigma, United States), and propidium iodide (Sigma, United States).

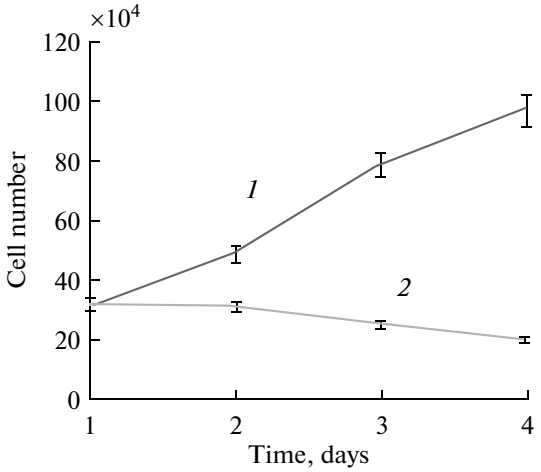

Fig. 1. EAhy 926 cell growth curves (*1*) in control and (*2*) after infection with influenza virus.

RESULTS AND DISCUSSION

Endothelial cells in vivo are mainly in the G_0 and G_1 phases of the cell cycle. The endotheliocytes enter the S phase, and then the G_2 and M phases in the case of reparative or oncogenic angiogenesis, as well as in the case of vascular pathology (Alimov, 1986; Lupinskaya et al., 2008).

The synchronization of the EAhy 926 cell culture at the G_0/G_1 phases of the cell cycle assessed by contact inhibition in the dense cell culture was aimed to mimic the normal status of endotheliocytes. Proliferation was induced by low-density seeding. The seeding dose had been chosen for cells to be in G_0/G_1 phases 12 h after the passage, and after 36 h to be actively proliferating.

The cell count in the Goryaev's chamber revealed the differences in the growth dynamics between control and infected EAhy 926 cells (Fig. 1). From 1 to 2 days after infection, the number of cells in the infected culture did not change, while, in the control culture, an increase in cell number was observed on day 2. Three days after the infection, the cell number in the infected cultures had decreased, indicating the death of some cells due to viral reproduction. In the control cultures, growth was still observed (Fig. 1).

Cytometric analysis of cell cycle dynamics of the EAhy 926 cells revealed the differences between the control and experimental cultures (Fig. 2). One day after infection, 78% of infected cells were in the G_0/G_1 phases, 16% in the S phase, and 6% in the G_2/M phases, while in control cultures 71% of the cells were in the G_0/G_1 phases, 13% in the S phase, and 16% in the G_2/M phase. On the second day after infection, the proportion of the infected cells in the S phase had increased up to 20%, thus exceeding the analogous population in the control culture (Fig. 2).

The number of the cells in the S phase in the experimental cultures had decreased slightly by the third day

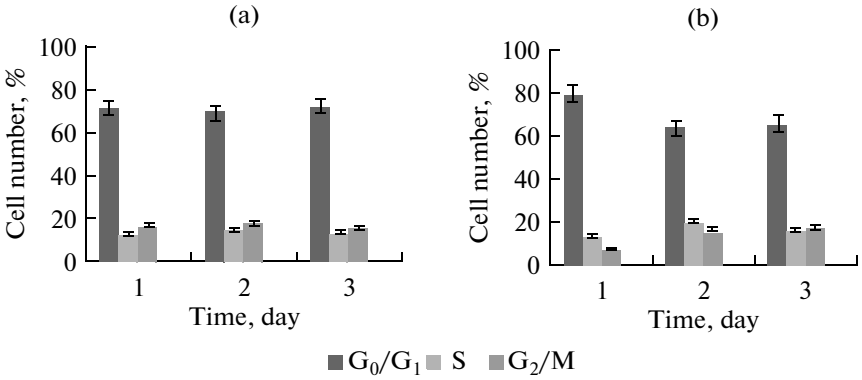

Fig. 2. Histogram of the distribution of the EAhy 926 culture over cell cycle phases (a) in control and (b) after infection with influenza virus.

after infection (Fig. 2). However, taking into consideration the total cell number decrease during viral reproduction (Fig. 1), these changes could be explained as results of different sensitivities of the cells at different cell cycle phases. Comparison of the cytometric data with the results of growth analysis allowed it to be suggested that the chosen virus dose slowed down the rate of the S phase in the cells of infected human endothelium culture. Nocodazole treatment of both control and infected cells (8 h after infection) was performed in order to prove this notion. The nocodazole synchronized the EAhy 926 cell culture in the G_2/M phase by disrupting the microtubules of the mitotic spindle. If the viral reproduction blocks or slows down the S phase of the infected cells, the following nocodasole treatment will weaken or cancel the G_2/M phase synchronization in the infected cultures. This effect was demonstrated in our experiments (Fig. 3).

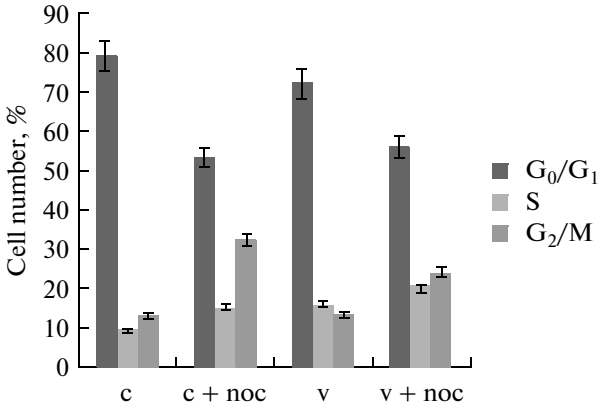

Fig. 3. Histogram of the distribution of the EAhy 926 culture over the cell cycle phases 24 h after infection with influenza virus combined with nocodazole treatment. c is control culture, c + noc is control culture treated with nocodazole, v is culture infected with the virus, and v + noc is infected culture treated with nocodazole. Means of three independent experiments are presented, $p < 0.05$.

One day after infection, in the EAhy 926 cell culture, accumulation of cells in the S phase occurred as shown in Fig. 3. Treatment with nocodazole of control cultures for 16 h led to an increase in the number of the cells in the G_2/M phases. The addition of nocodazole to the infected cultures caused only a slight increase in the proportion of the cells in the G_2/M phases. These results prove the idea that influenza virus causes deceleration of the S phase of the cell cycle.

These data revealed the response of the EAhy 926 endothelial cell line to the influenza virus reproduction, which to be a slowing down of the S phase. This effect is specific to the EAhy 926 cells when compared to other cell types, e.g., the lung carcinoma A549 cell line, in which influenza virus provokes G_0/G_1 arrest (He et al., 2010). The key factor in this G_0/G_1 arrest is the viral protein NS1 (Wei et al., 2013). It seems that the type of the viral effect on the cell cycle depends on the type of the cell culture, an idea that has been proven for the Ebola virus (Kota et al., 2012).

In our opinion, the results obtained provide a sufficient basis for further investigations of the influence of influenza virus itself, as well as its proteins, on the cell cycle dynamics in endothelium culture.

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