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ORIGINAL ARTICLE

Glial cells and fibroblasts cooperation and viability in simulated microgravity *in vitro*

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Abstract

OBJECTIVE: The main objective of the work was to clarify the question – how will cell cultures functional state change after microgravity simulation when the shift in full strength direction takes place?
METHODS: Proliferation processes and apoptosis intensity in cell lines of rat glioma and human fibroblasts were compared in changing the position of flasks with cell culture in relation to horizon. The detection of apoptosis and necrosis processes was carried out using flow cytofluorimety.
RESULTS: It was found that the change in full strength direction provides an inhibitory effect on tumor glial cells and fibroblasts' proliferative activity enhances along with inhibition of apoptosis processes.
CONCLUSIONS: Intensification of apoptotic processes in glioma cells and attenuation of cell death processes in normal cells – fibroblasts – are the result of cell cooperation disturbance.

Abbreviations:
AnV, annexin V; Apo-1/Fas, cluster of differentiation 95; C6, culture of rat glioma; CO2, carbon dioxide; DNA, deoxyribonucleic acid; F10, F-10 nutrient medium; FITC, fluorescein isothiocyanate; FLv, culture of human fibroblasts; PI, propidium iodide; rpm, rotations per minute; TNF, tumor necrosis factor.

Introduction

The shift of intercellular signaling pattern in brain and other organs and tissues of the organism under the change of body position (full strength direction shift) is verified (Cameron *et al* 2004; Buravkova *et al* 2005; Cazzaniga *et al* 2014), but poorly studied (Fortrat *et al* 2013). What really happened with cell-to-cell interactions in changed gravity? The answer to this question will help to approach the problem dysfunction during spaceflight (Buravkova *et al* 2005; Novoselova *et al* 2008; Navasiolava *et al* 2008). For example, how do separate cells or body cell populations react to changes in their position in space (shift in full strength direction)? Is the presence of neurohumoral regulation elements the condition for living cells response to the shift in full strength direction? Or maybe the change in body position in space directly reflects on separate cells or their populations.

The aim was set to answer these questions: to determine the characteristics of proliferative activity of living cells in changing their position in space. By the way, such condition is typical in microgravity, for example, on the space station. Passaged cul-

ture of C6 rat glioma was chosen as tumor object of investigation, and passage culture of human FLv-line fibroblasts – as non-tumor one. The state of cell cooperation in normal and pathologically changed cells in microgravity is poorly studied (Muid *et al* 2013; Herranz & Medina 2014). As an object of research chosen annexin. Annexin is a marker of apoptosis (Peng *et al* 2014). At the same time, annexin reflects the state of intercellular relationships (Nodin *et al* 2005; Wang *et al* 2014; Wu *et al* 2014). The function of annexin in cell cooperation is controversial. Some authors suppose annexin to be involved into the inhibition of glial cells proliferation (Wang *et al* 2014). The others argue in favor of proliferative role of annexin (Wu *et al* 2014). This experimental phenomenon should be studied more thoroughly.

Material and methods

Cell lines of C6 rat glioma and FLv-line human fibroblasts were obtained from the Russian cell culture collection of vertebrates (Cytology Institute of Russian Academy of Sciences, Saint-Petersburg).

C6 rat glioma cells and FLv human fibroblasts were cultivated (concentration 2×10^5 cells/ml) in 25 ml flasks in F10 medium with 10% fetal bovine serum and 10^{-4} g/ml gentamycin sulfates. Flasks were placed in CO_2 incubator at 5% CO_2 and 37°C.

Microgravity was modeled using UN-KTM2 clinostat, and the technology of flasks' position change relative to the horizon (Teacher's Guide 2013). Cell viability was monitored while using clinostat for 4 hours. The results of the experiments showed that the most pronounced changes in cells happen in 24 hours after the change of flasks' position relative to the horizon. So flasks' position was changed to ∠60° from the horizontal during experiments with C6 rat glioma cells and human fibroblasts cultures. Rotation was carried out in 40–48 hours after reaching 70% confluence. The change in full strength direction was made for 24 hours. When performing manipulations with flasks attention was paid to minimization of time needed to transfer cell cultures, careful implementation of procedures and the same stereotype of workflow.

The working surface, where microscope is attached to, was converted to horizontal position before starting the experiment for installation and fixation the flask with culture. Flasks stayed in this position for 1 hour for leveling the effects of transferring from CO_2 incubator to microscope. Then the slow slope of flasks to ∠60° relative to the horizon was carried out (Teacher's Guide 2013). Flasks stayed in this position for 24 hours. One flask stayed in horizontal position during the experiment (series 1) and the other one was tilted ∠60° relative to the horizon (series 2). The results of observations were compared. The monitoring of analogue events was carried over 24 hours using inverted microscope Opton ISM-405 with an increase in lens 16x and Leica DC 300F camcorder, and then the events were accumulated in digital form on the computer every 10 minutes. Photos were processed using Image G software.

Annexin V (AnV) – propidium iodide (PI) (Annexin V-FITC Apoptosis Detection Kit, No 556547, lot No 2195781, BD Pharmingen™, USA) system was used for detection apoptosis with the help of flow cytofluorometry. The distribution of dyes in cells allow establishing their characteristics and dividing them into living, necrotic and apoptotic.

Trypsinization was made for staining the cells with Annexin and Propidium iodide in flasks of 1 and 2 series. An equivalent amount of complete F10 culture medium was added into the flasks to mitigate the damaging effect of trypsin on cell membranes; flasks were also centrifuged for 5 minutes at 1500 rpm. Then the supernatant was removed and the cell pellet resuspended in 100 microliters of buffer included in Annexin V-FITC Apoptosis Detection Kit. The obtained cell suspension from each flask of 1 series was divided into 4 vials, and 2 series – into 3 ones. 5 microliters of both Annexin (FITC labeled) and Propidium iodide were added to each vial. In addition cells in one vial were stained with FITC and Propidium iodide – in second one. The cell suspension was incubated for 25 minutes in the dark with subsequent addition of 400 microliters of buffer into every vial. The determination of apoptotic cell number was made using flow cytofluorimeter BD FACS Canto II™ and BD Bioscience FACSDiva 7.0 Software (Becton & Dickinson, USA) by direct immunofluorescence.

The results of apoptosis and necrosis manifestation in cells were compared in flasks of 1 and 2 series. Results are expressed as mean ± s.e.m. Differences between means were evaluated by the Mann-Whitney test for unpaired observations.

Results and Discussion

The 24-hour monitoring was carried out to determine the features of proliferative activity of cultured cells. The research was made on tumor and non-tumor cell cultures. Passaged culture of rat glioma C6 was chosen as tumor cells, passaged culture of FLv line fibroblasts – as non-tumor cells. The calculation of the number of cells in the visual field was made considering the area the calculation took place in. The area of the field was 880 × 660 micrometers.

Figure 1 represents graphs that characterize the dynamics of the number of C6 glioma cells in a population with and without turning the flasks at ∠60°. Referring to the graph (Figure 1), the higher increase in cell mass is peculiar for the cells placed horizontally during the experiment (the increase was 86%) compared to the cells placed at ∠60° (the increase was 34%). It was found out that C6 glioma cell proliferative activity in ∠60° flasks rotation was reduced by 52% in eighteen hours of observation.

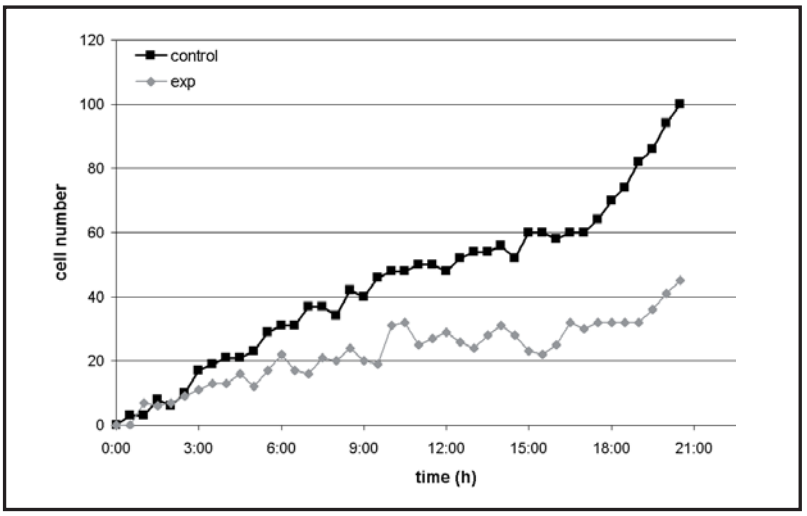
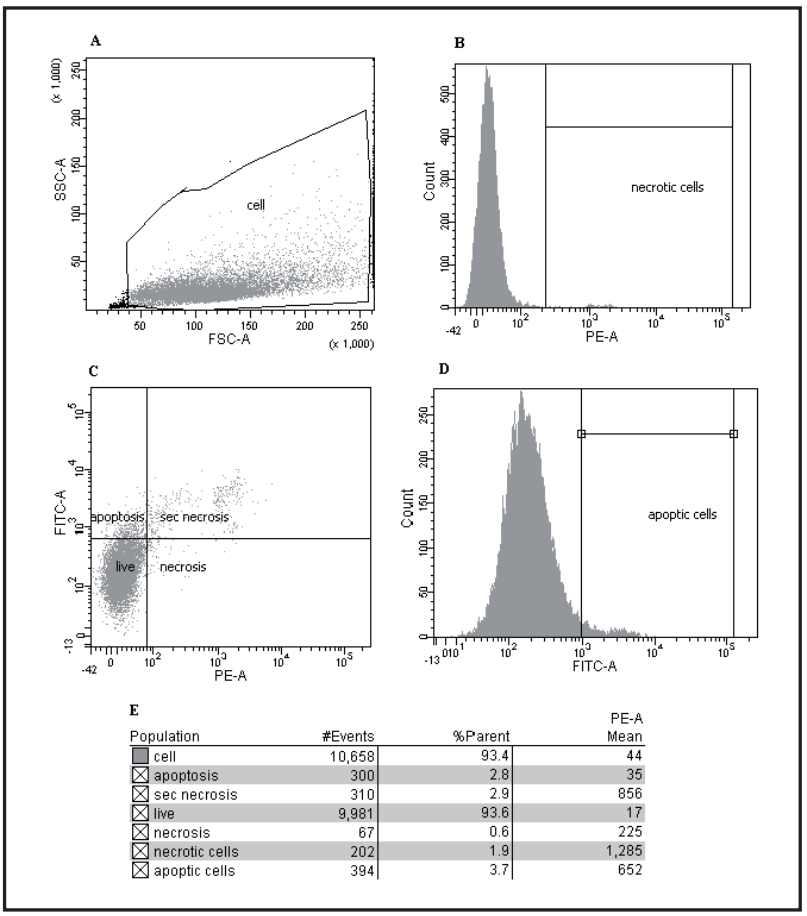


Fig. 1. Curves showing the changes in C6 glioma cells number in horizontal position and in ∠60° flasks rotation. Flasks were standing in horizontal position (control) and in ∠60° flasks rotation (exp).



E

Population	#Events	%Parent	PE-A Mean
cell	10,658	93.4	44
apoptosis	300	2.8	35
sec necrosis	310	2.9	856
live	9,981	93.6	17
necrosis	67	0.6	225
necrotic cells	202	1.9	1,285
apoptic cells	394	3.7	652

Fig. 2. The distribution of living, apoptotic and necrotic cells of C6 rat glioma (flow cytofluorimeter analysis). There were flasks standing in horizontal position during the whole experiment. (A) The manually circled area with cells separated from the debris and conglomerates, (B) propidium iodide fluorescence range in the cells at the stage of necrosis and late apoptosis, (C) the distribution of living, apoptotic and necrotic cells at different stages, (D) FITC labeled annexin fluorescence range in cells at early and late apoptosis, (E) numerical values of analyzed cells.

Figure 2 and 3 display the distribution of living, apoptotic and necrotic cells. The bulk of cell units comprise living cells. After changing the direction of the full strength their number was reduced, and the number of apoptotic cells in the later stages of apoptosis increased significantly (Figure 2E and 3E). Thus, it was found out that changing of direction of full strength reduces the viability of C6 glioma cells.

As shown in Figures 2E and 3E, change in the direction of full strength affects C6 glioma cells reaching the late stage of apoptotic and necrotic cells. Number of dying due to apoptosis and necrosis of cells was increased in comparison with a series of experiments where tumor cells were kept in horizontally placed flasks. Thus, a change in the direction of full strength is accompanied with the activation of apoptosis and necrosis in tumor cells.

So the change in the direction of the resultant force provides an inhibitory effect on C6 tumor glial cells. Therefore, the shift of this factor is reflected in the proliferative activity of pathologically transformed cells by changing of the direction of their development.

In what way will a change in the direction of full strength affect the functional state of non-malignized cells? To answer this question, one flask with FLv human fibroblasts was angled ∠60°. The other flask with fibroblasts was placed horizontally.

Figure 4 displays graphs that characterize the dynamics of human FLv fibroblasts in the population before and after the ∠60° flasks rotation. As seen from the graph (Figure 4), a higher cell mass increase was observed in cells when the object was located at 60º (the increase of 65%), in contrast to the horizontally placed cells (the increase of 16%).

Figure 5 and 6 display the distribution of living, apoptotic and necrotic cells. The overwhelming number of which are living cells, and their number increased after changing the direction of full strength (see Figure 5E and 6E). Attention should be paid to the fact that the change in the plumb line causes the reduction in the number of apoptotic cells (see Figure 6E). At the end, the events that take place in FLv fibroblasts culture after changing the direction

of full strength indicate the growth in number and viability of human fibroblasts (see Figure 6E).

As can be seen from Figures 5E and 6E, the change in direction of full strength primarily affects the cells that reached the late stage of apoptosis (their number was reduced in comparison with those cells that had not been affected by the changes in the plumb line). So after changing the direction of full strength proliferative activity of fibroblasts enhances and apoptosis in human fibroblasts is inhibited.

Currently, there are conflicting viewpoints on how the change in direction of full strength affects functional state of cells, including their cooperation and viability (Nodin *et al* 2005; Wang *et al* 2014; Wu *et al* 2014), which is characterized by a decrease or increase in the number of apoptotic and necrotic cells. By the way, the process of adaptation and settling into new qualities that contribute to "survival" in adverse situations may develop gradually and include apoptosis and necrosis at certain stages of ontogenesis. These processes hypothetically have evolved during phylogenesis, especially in the early period of the Paleozoic era, during the release of living creatures from water to land, when the direction of full strength significantly changed.

Necrosis is a destruction of cells and tissues structure in living organism which begins from cell membrane and leads to their death. Cell nucleus shrinks during necrosis because of activation lysosomal hydrolytic enzymes, chromatin concentrates (karyopyknosis) and then the nucleus splits into fragments (karyorrhexis) and dissolves (karyolysis). Denaturation and coagulation of proteins occurs in the cytoplasm of the cell following the disintegration of cytoplasm (plasmorrhexis) and its "melting" (plasmolysis) (Abdelhalim & Jarrar 2011). The main way of apoptosis initiation is realized through the system of membrane-associated tumor-necrotic proteins (tumor necrosis factor, TNF), Apo-1/Fas being one of them (Darmochwal-Kolarz *et al* 2012; A-Gonzalez & Hidalgo 2014). In recent years, along with the family of TNF proteins the great emphasis is paid to understanding the biological activity of annexin family pro-

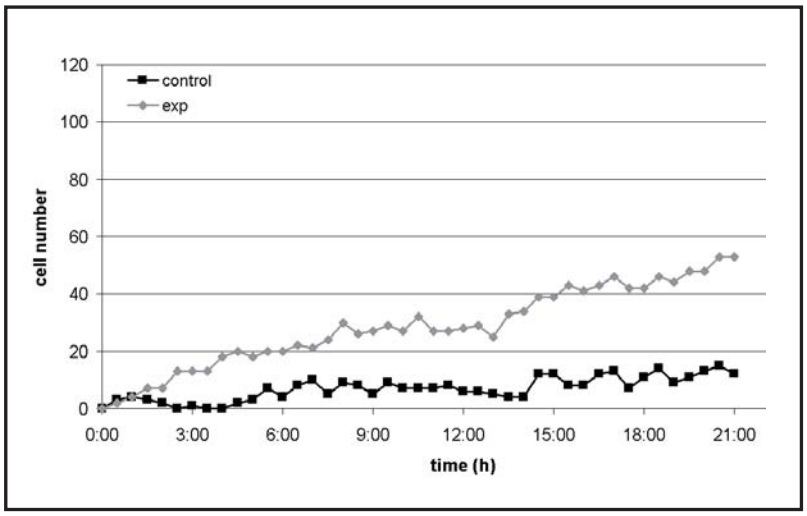
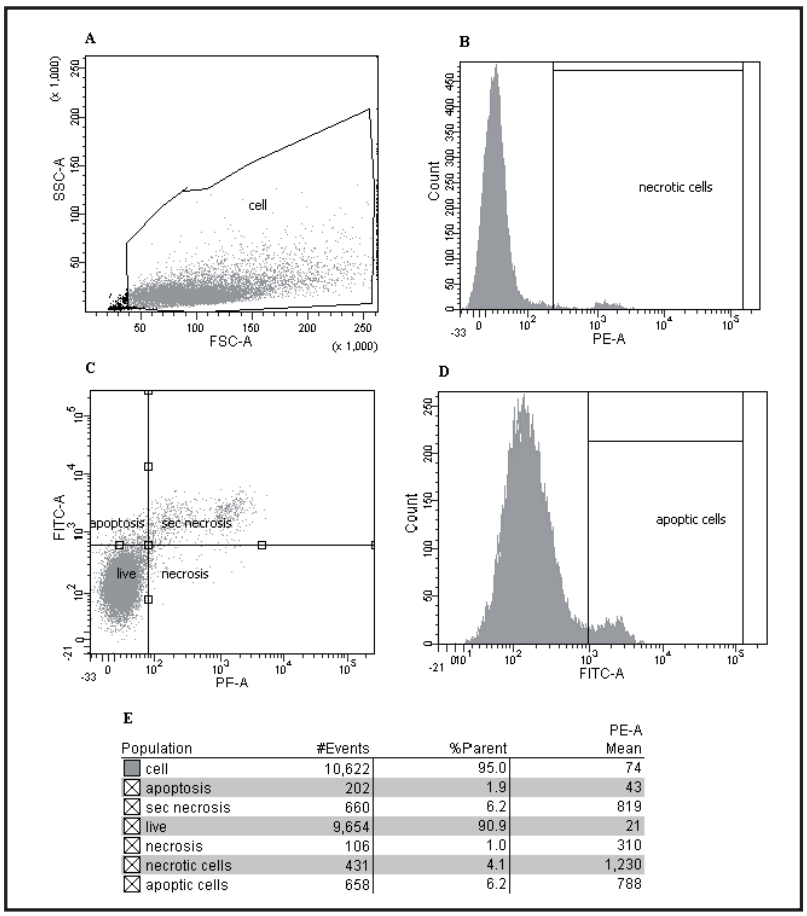


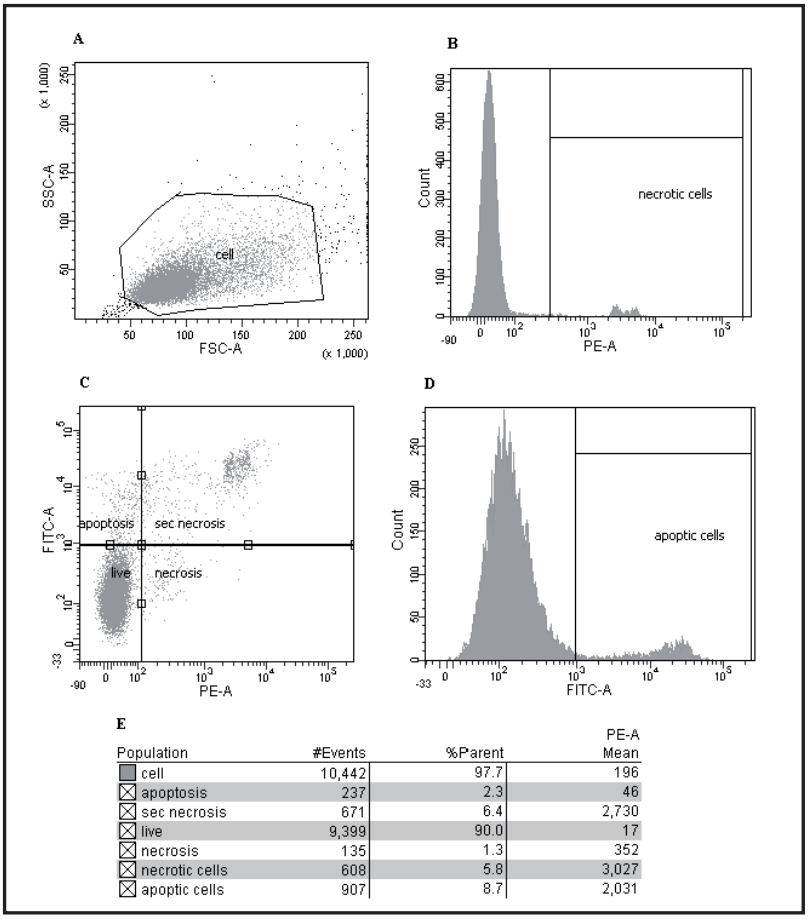
Fig. 4. Curves showing the changes in the number of human FLv-line fibroblasts. Flasks were standing in horizontal position (control) and in ∠60° flasks rotation (exp).



E

Population	#Events	%Parent	PE-A Mean
cell	10,622	95.0	74
apoptosis	202	1.9	43
sec necrosis	660	6.2	819
live	9,654	90.9	21
necrosis	106	1.0	310
necrotic cells	431	4.1	1,230
apoptic cells	658	6.2	788

Fig. 3. The distribution of living, apoptotic and necrotic cells of C6 rat glioma (flow cytofluorimeter analysis). There were flasks standing at ∠60° during the whole experiment. (A) The manually circled area with cells separated from the debris and conglomerates, (B) propidium iodide fluorescence range in the cells at the stage of necrosis and late apoptosis, (C) the distribution of living, apoptotic and necrotic cells at different stages, (D) FITC labeled annexin fluorescence range in cells at early and late apoptosis, (E) numerical values of analyzed cells.

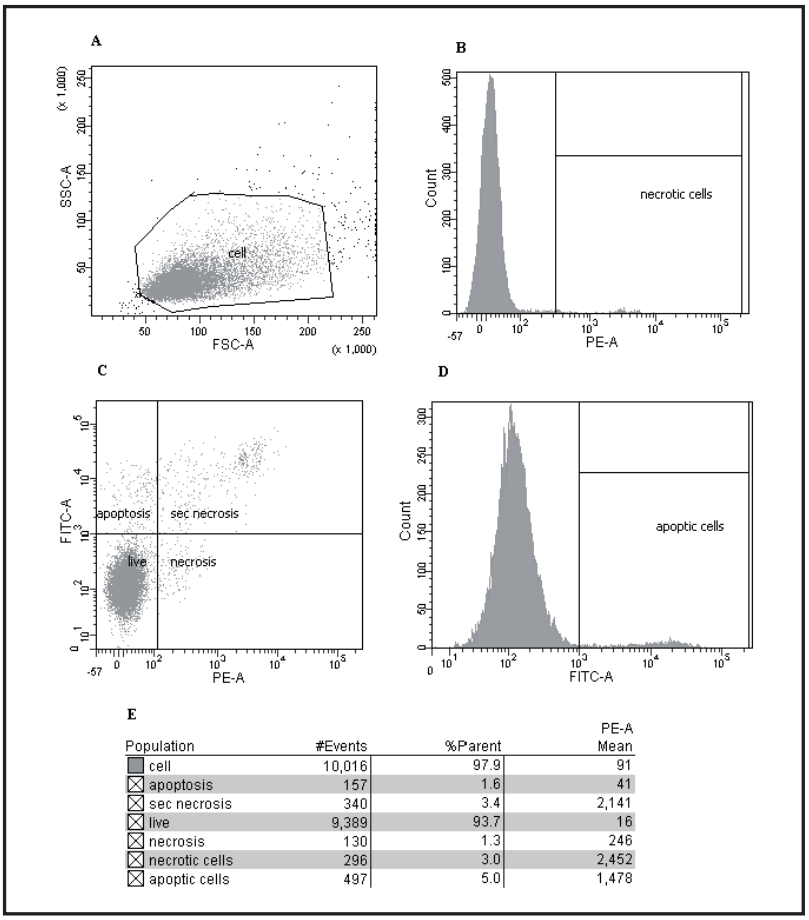


Population	#Events	%Parent	PE-A Mean
cell	10,442	97.7	196
apoptosis	237	2.3	46
sec necrosis	671	6.4	2,730
live	9,399	90.0	17
necrosis	135	1.3	352
necrotic cells	608	5.8	3,027
apoptic cells	907	8.7	2,031

Fig. 5. The distribution of living, apoptotic and necrotic cells of human fibroblasts (flow cytofluorimeter analysis). There were flasks standing in horizontal position during the whole experiment. (A) The manually circled area with cells separated from the debris and conglomerates, (B) propidium iodide fluorescence range in the cells at the stage of necrosis and late apoptosis, (C) the distribution of living, apoptotic and necrotic cells at different stages, (D) FITC labeled annexin fluorescence range in cells at early and late apoptosis, (E) numerical values of analyzed cells.

teins. The feature of annexin A5 is that it is expressed in apoptotic cells only, but not in healthy ones (Fu *et al* 2011; Wu *et al* 2014).

The data on the structural and functional shifts in cell cultures when changing their position in space were obtained in conducted experiments. Namely the shift of full strength direction, which is one of the factors of wildlife development under gravity, comes out the cause of transformations in living cells in space and the flask with culture position (Herranz & Medina 2014). The modeling of full strength direction shift in conducted experiments was accompanied with multidirectional changes in proliferative processes in fibroblasts (FLv) and C6 glioma cultures (Figures 1, 4). The processes of apoptosis and necrosis were initiated in different way in both cell cultures. The number of cells with signs of apoptosis and necrosis increased in tumor culture (C6 glioma) (Figures 2, 3). The number of cells with signs of apoptosis and necrosis decreased in fibroblasts culture (non-tumor) in comparison with the control (Figures 5, 6). There is information in scientific articles, devoted to the analysis of positive and negative effects of microgravity (Buravkova *et al* 2005; Fortrat *et*



Population	#Events	%Parent	PE-A Mean
cell	10,016	97.9	91
apoptosis	157	1.6	41
sec necrosis	340	3.4	2,141
live	9,389	93.7	16
necrosis	130	1.3	246
necrotic cells	296	3.0	2,452
apoptic cells	497	5.0	1,478

Fig. 6. The distribution of living, apoptotic and necrotic cells of human fibroblasts (flow cytofluorimeter analysis). There were flasks standing at ∠60° during the whole experiment. (A) The manually circled area with cells separated from the debris and conglomerates, (B) propidium iodide fluorescence range in the cells at the stage of necrosis and late apoptosis, (C) the distribution of living, apoptotic and necrotic cells at different stages, (D) FITC labeled annexin fluorescence range in cells at early and late apoptosis, (E) numerical values of analyzed cells.

al 2013), on the detailed mechanisms of these effects, including apoptosis and necrosis initiation (Wang *et al* 2014; Wu *et al* 2014), changes at the DNA level (García-Álvarez *et al* 2013).

Conclusions

The number of apoptotic and destroyed cells increases in glioma, and they are the sources of annexin V. Annexin V changes cell cooperation and the character of intercellular signaling after its fixation on cell membrane. Intensification of apoptotic processes in glioma cells and attenuation of cell death processes in normal cells – fibroblasts – are the result of cell cooperation disturbance. Thus, using *in vitro* models of full strength direction change the data were obtained allowing deepening the understanding of subtle mechanisms of whole organism various functions control (*in vivo*).

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