EXPERIMENT "REGENERATION" PERFORMED ABOARD THE RUSSIAN SPACECRAFT FOTON-M2 IN 2005

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ABSTRACT

The experiments on the newts performed earlier aboard Russian biosatellites showed that the rate of lens and tail regeneration in space was greater than on the ground. In parallel it was found that the number of cells in S-phase was greater in space-flown animals than in the ground controls. However, it was unclear whether cell proliferation stimulation was induced by micro- "g" per se. Molecular mechanisms underlying the change also remained obscure. These issues were addressed by the joint Russian-American experiment "Regeneration" flown on Foton-M2 in 2005. The method for in-flight delivering DNA precursor BrdU was developed. The experiment showed that during the flight the number of S-phase cells in the regenerating eyes and tails increased. These data together with those obtained earlier suggest that cell proliferation increases in response to the effects of both micro-"g" and 1 "g" after return to Earth. The expression of bFGF in regenerating tissues of "flown" newts and ground controls was examined using immuno-histochemistry. Obtained results suggest that this growth factor is a participant of the promotional effect of space flight upon cell proliferation in lens and tail regenerates.

Key words: microgravity, newt, tail, eye, regeneration, cell proliferation, BrdU, bFGF

1. INTRODUCTION

Study of space flight effects on repair processes in vertebrates is important because it can help predict organ/tissue recovery in mammals and humans during their exposure to the space environment and upon return to Earth. This is also of great scientific interest because it can help gain a better insight into the mechanisms underlying the effects of various factors, including microgravity, on organ/tissue regeneration. Lower vertebrates, particularly Urodelean amphibians, are known to have high regeneration capabilities. Moreover, they are capable of repairing simultaneously several injured organs/tissues. Due to this, regeneration processes in different organs/tissues of an animal can be investigated in one experiment. It should be noted here that tailed amphibians are very suitable for space experiments because they have high viability and can be flown in unsophisticated, inexpensive hardware. The Foton-M2 experiment flown in 2005 was performed using ribbed newts *Pleurodeles waltl* that were exposed to lensectomy and 1/3 tail amputation with the purpose of measuring lens and tail repair.

In 1985, we carried out the first experiment to study spaceflight effects on regeneration in Urodela. The 7-day flight experiment revealed specific changes in the repair parameters of adult newts [18]. In subsequent flights, we continued and expanded our newt studies. The data accumulated suggest that the space environment does not inhibit regeneration potentials of newts; moreover, it often stimulates organ/tissue recovery. In order to better understand these effects, we focused on the repair of eye lens and retina, forelimbs (muscles and bones), and tail [4, 7, 13, 19-20]. As compared to the ground controls, these organs/tissues regenerated at a faster and a better-synchronized rate. The accelerated recovery rate was shown to result from enhanced cell proliferation. Interestingly, it was not only regenerating cells but also the cells that were not involved in tissue/organ repair reached the S-phase within a shorter time. The stimulating space

flight effect proved to occur not only during but also after the exposure [8, 9, 18-21].

The major observations we made in space-flown experiments were well reproduced in ground clinostat studies that simulated microgravity effects [2-4, 6, 12-13]. It was therefore inferred that microgravity was the factor that played the key role in accelerating tissue/organ regeneration. However, we did not have any direct indications of an increase in the number of proliferating cells because fixations were made before launch and after recovery.

We also made attempts to better understand the factors responsible for the changes at the cellular level, i.e., those that shortened the time cells needed to reach the S-phase. We hypothesized that they can include: changed expression of growth factors and their receptors; synthesis of microgravity-specific stress-proteins induced by exposure to an altered gravity field; changes in circulatory and immune systems [13-15].

2. MATERIALS AND METHODS

In order to achieve the experimental goals in the Foton-M2 flight, we performed a large number of preflight tests the purpose of which was to optimize the delivery of bromo-deoxyuridine (BrdU), which is a thymidine analog and DNA synthesis precursor, to the flown newts. The first attempt was to use implanted Alzet minipumps filled with BrdU; then BrdU impregnated blankets were tried; finally, BrdU filled minipumps were inserted in the wet blankets covering the "Triton" container. In all preflight experiments BrdU incorporation in regenerating eyes and tails, as well as in the skin and internal organs of newts was measured. The measurements were done using BrdU immunoassays, which included immunochemical identification of the precursor using specific monoclonal antibodies and comparison with positive and negative controls. The precursor was detected not only in the cell nuclei of regenerating eyes and tails but also in internal organs. The preflight tests also helped to determine well-controlled and time-coordinated regimens of BrdU delivery. As a result of the preliminary tests, it was agreed to insert BrdU filled minipumps into the blankets covering the "Triton" container. The detailed description of the procedure can be found in the paper by Eduardo Almeida and coworkers from NASA Ames Research Center published in the *ISGP* Proceedings.

The Foton-M2 experiment was performed on adult *P. waltl newts* (11-12 cm long), which 10 days prior to launch underwent surgical removal of eye lens and 1/3 tail. The procedure was conducted on 15 flight, 5 basal and 15 synchronous control animals. The newts were anesthetized, operated and maintained in strict adherence to the Russian Academy of Sciences rules of animal care and use. On the launch day, the eyes and tails of basal controls were fixed to identify their regeneration stage. The Foton-M2 flight continued for 16 days. The synchronous control study began 48 hours after launch to simulate actual temperature variations in the capsule that were regularly downlinked. However, post-flight analysis of the temperature profile in the space capsule and in the synchronous control chamber demonstrated that the flight temperature was lower than expected (19 ºC vs. 26 °C) and that the synchronous chamber temperature during the last 5 days was higher than in flight (it occasionally rose to 22-23 °C).

After the successful flight, landing and animal return to the Institute of Developmental Biology, the regenerating eyes and tails of the flown newts and synchronous controls were fixed for further histology, immunochemistry and molecular biology examinations. Eye sections were prepared to study regeneration stages and cell mitotic activity in the lens, and tail sections were made to investigate tissue repair. Morphological parameters were measured by routine histology on serial sections. Immunohistochemical examination of the basic fibroblast growth factor (bFGF) expression was carried out using frozen eye and tail sections by means of indirect immunofluo-rescence with antibody against bFGF (Sigma). Images of regeneration morphology and immunospecific FITC-fluo-rescence were analyzed with the aid of an Olympus AH-3 microscope, digital camera, and Studio Lite and Viewfinder Lite software. BrdU incorporation was measured using a commercial BrdU kit (Zymed), which included specific anti-BrdU primary antibody, and

staining with secondary antibody. The study was limited to several eye and tail sections, which could not provide accurate quantification of the proliferation rate but allowed an adequate comparison of the pools of labeled cells in the flight and control animals.

3. RESULTS AND DISCUSSION

Preflight tests in which BrdU filled minipumps were inserted into the blanket covering the "Triton" container showed that the label reached not only the skin but also the internal organs of the animals. Moreover, the precursor was incorporated only in the DNA nuclei of synthesizing cells, and specific staining was characterized by a granular pattern that distinguished nuclear BrdU from nonspecifi-cally labeled cells (for example, red blood cells). The incorporation rate was particularly intensive in the skin and eye cornea. Distinct BrdU+ immunospecific staining and a greater number of BrdU+ cells were seen in every actively proliferating tissue (tail blastema, corneal epithelium, intestinal epithelium, etc.).

Tail regeneration. In basal controls (euthanized on launch day, 7 days after surgery), tail regenerates reached stage II (according to the Iten and Bryant [17] classification), which is characterized by complete epithelization of amputated area and by the beginning of cell differentiation and proliferation. Two reproducible immunochemical reactions proved that there was no BrdU incorporation prior to launch. This was in a good agreement with our expectations: BrdU delivery was delayed by the use of distilled water and mineral oil in the attachments to the minipumps and was designed to start upon insertion into orbit.

Comparison of tail regeneration in the flight newts and synchronous controls did not show any significant differences in the regenerate size or the regeneration stage: in both flight and control animals' regenerates were 0.4-0.5 mm long and reached stage Til. Examination of tail sections demonstrated BrdU incorporation in the proliferating cells of the skin epithelium, early blastema cells and nuclei of melanophores of both flight and control animals. The difference was that the number of BrdU+ cells in the flight specimens was about 1.5 times greater than in the controls; moreover, labeled cells occurred as clusters in the former case and as discrete cells in the latter (fig.l).

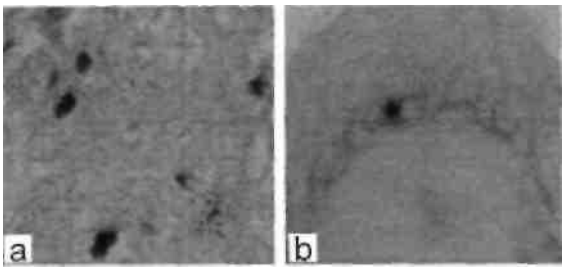

Fig. 1. The incorporation of BrdU inlo nuclei of lail regcncraie cells of space-flown (a) and synchronous control (b) animals.

It can therefore be concluded thai ihc experimental procedure and hardware could provide BrdU delivery to lail regenerating cells in flight and on the ground and that in space the cell proliferation activity was higher than on the ground.

In the previous Bion-11 flight [5] and clinostat experiments [4, 14] we detected an accelerated growth rate and an enhanced cell proliferation in the regenerating tissue and, in particular of tail, soon after recovery and during a lengthy follow-up period. Together with the Foton-M2 findings, these observations allow the conclusion that the stimulating space flight effect on cell proliferation and tissue regeneration results from gravitational changes the animal experienced at launch, in orbit, during landing, and readaptation to Earth gravity.

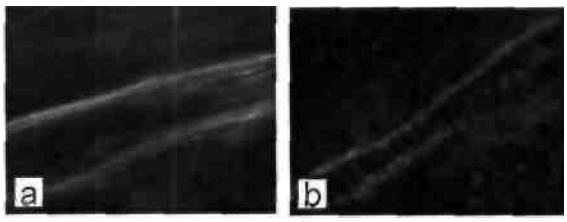

Fig. 2. bFGF expression in the cpendyma of spinal cord in regenerating tail in space-flown (a) and synchronous control (b) animals.

In amphibians, tail regeneration is controlled by various factors, including neuro-trophic factors, extracellular matrix components and growth factors [1, 10-11, 22, 24]. It is known that bFCiF and its receptors are involved in the regulation of cell differentiation and proliferation in the course of Urodela eye and tail development and regeneration [1, 16, 24]. Our study of the bFGF expression pattern using specific antibody demonstrated differences between the flight animals and synchronous controls. In the basal controls, the most intense bFGF expression was associated with dedifferentiated cells of the tail early blastema. Sixteen days after recovery highly intense immunospecific fluorescence was localized in the tissues (skin, muscles, spinal cord, vertebral column) adjacent to the amputation area i.e., in the area where cell dedifferentiation and growth continued.

Comparison of bFGF expression patterns in identical areas and at identical regeneration stages recorded using identical microscope settings revealed distinct differences between llighl and control animals. In the flight animals, immune response of bFGF-antigene determinant proved to be more stable and intense (fig.2). Interestingly, the difference was seen not only in the area of muscle-stump dedifferentiation but also in actively growing areas (spinal cord cpendyma and developing spinal column chondroblasts). The specific pattern of bFGF expression in the flight animals suggests that bFGF may be part of the mechanism(s) underlying enhanced tail cell proliferation and growth rates in the space environment.

Lens regeneration. Study on lens regeneration in the basal controls showed that 10 days alter surgery the operated areas reached regeneration stages II to IV, according to the T Yatnada classification [23]. At those stages iris cells. which were the regeneration source, underwent dedifferentiation and began to proliferate actively. This was indicated by specific mitotic patterns visible in the pigmented cells of the inner layer of dorsal iris. No labeled cells were delected by means of the BrdU test that confirmed, as was the case with amputated tails, that the label was not delivered on ihc ground. Posl-flighl examinations that look place 28 days after surgery showed that lens regeneration was noticeably advanced in both flight and control animals. Willi respect to the regeneration stage, there was no significant difference: the flown animals predominantly reached stages VIII-IX and the controls achieved stages IX-X. The number of mitoses per unit of lens regenerate thickness corresponded to the regeneration stage: 0.84 ± 0.19 and 0.98 ± 0.17 in the flight and control newis, respectively. The fact that the synchronous controls showed a slightly higher regeneration rate and greater mitotic activity can be explained either by individual variations or by the above-mentioned short-term temperature rise.

Our study of bFGF expression in basal controls demonstrated a low intensity reaction in the eye growth area (pars ciliaris - ora serraia) and a high intensity reaction in vascular membranes of the iris and retina. Comparison of bFGF expression in the flight and control newts, in whom lens regenerate was already formed, showed that bFGF positive immune reaction occurred in vascular membranes and *lie novo* in the lens epithelium. When comparing immunospecific fluorescence corrected for bFGF expression in epithelial cells of regenerating lenses, we noticed the differences observed in tail regenerates, viz., epithelial cells in the equatorial area of lenses developed a high level of expression in the flight animals and a lower level in the controls. These observations can be viewed as an indication of bFGF potential involvement in the regulation of lens regenerate development in space. An intensive expression of the cytokine detected in regenerating tails and eyes post-flight may be the factor or one of the factors causing a greater regeneration rate several days after flight that we previously reported.

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