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THE DYNAMICS OF PROLIFERATION AND DIFFERENTIATION OF OSTEOGENIC CELLS UNDER SUPPORTIVE UNLOADING

With the use of radioactive marker of DNA synthesis – ^{3}H-thymidine we have studied the dynamics, peculiarities of proliferation and differentiation of osteogenic cells under hind limb unloading of white rats («tail suspension» method at an angle 35°) during 28 days. The ^{3}H-thymidine was administered at a single dose at the end of the experiment, the biosamples were taken from femoral bones in 1, 48, 96 hr. Light and electron-microscopic radioautography with ^{3}H-thymidine (in 1 hour) have shown, that basic fraction of DNA synthesizing cells in the zones of adaptive remodelling of bone tissue is represented by little-differentiated perivascular cells (that include osteogenic cell precursors). A tendency for a decrease of a labelling index in the ^{3}H-thymidine osteogenic cells on metaphyseal bone trabeculae under hind limb unloading has been established. The dynamics of labelled cells during various time intervals after ^{3}H-thymidine injection testifies to a delay in the differentiation precursors in osteoblasts and their transformation to osteocytes in experiment animals. The obtained data have shown that a long-term supportive unloading leads to lowering the intensity of osteogenetic processes in long bones and reducing bone mass.

Introduction. The investigations carried out on the space stations, biosatellites and in modeled experiment, have shown weight unloading leads to the loss of bone mass (clinically known as osteopenia, some time osteoporosis), especially in the bones that receive supportive loading [1–3].

The peculiarities of proliferation, differentiation and the specific functional activity of osteogenic cells under hind limb unloading and microgravity conditions have been little-studied and the information about them in contradictory. According to some authors' view [4–6] the osteogenic precursors cells appeared to be the first target for the influence of the lowering of support load.

The experiments, conducted on «Cosmos 1987» with the use of morphometry of the osteogenic cells nuclei from rat periodontal ligaments of the upper jaw, showed a decrease of the population of early precursors and an increase (up to 42 %) in the number of preosteoblasts after 12,5 days of flight and 55 hours of post-flight stress. It is considered, that microgravity suppresses osteogenesis at the stage commitive precursor – preosteoblast [6]. The results of the experiment aboard «Bion-10» showed, that although the proliferation of the little-differentiated osteogenic cells (MN7) was not affected by microgravity, the differentiation of cells as judged by the synthesis of ALP and type I collagen was slowed down [7]. Some researchers [8, 9] associate reduction of the intensity of osteoplastic processes in support-load-bearing bones under hind limb unloading and microgravity with the reduction in the number of osteoblasts; the other researchers did not observe the reduction of osteoblasts' number [10]. Our previous historadiographic investigations with a use of ^{3}H-glycine have also showed a tendency of collagen biosynthesis reducing in osteogenic cells at hypokinesia [11, 12].

Therefore, the following questions are topical and need to be solved. Is the proliferation of osteogenic precursor cells suppressed and/or does their rate of differentiation slows down? What is the response of osteogenic cells to the reduction of supportive loading? Does dynamics of transformation osteoblasts in osteocytes change?

It is necessary to study the proliferative peculiarities of the osteogenic precursor cells, the dynamics of their differentiation into osteoblasts in the zones of osteogenesis under the lowering of supporting load with the use of the radionuclides

^{3}H-thymidine. ^{3}H-thymidine is the specific precursor for the DNA synthesis. Due to selective inclusion of ^{3}H-thymidine into DNA, only that cells are labelled, which synthesize DNA and are at the stage of synthesis (S-phase) of mitotic cycle.

Material and methods. The investigation was carried out on 36 white rats of a Wistar line (males, weight 170–180 gm) under the model hind limbs unloading. It was modeled according to the «tail suspending» method at an angle 35°. At the end of the experiment the animals were injected with ^{3}H-thymidine («Amerhsam», Austria). The radionuclide was administered to animals intraperitoneally at the dose of 0.5 μCu/gm body weight. In ^{3}H-thymidine experiments the rat femoral bones were taken in 1, 48, 96 hr after the radionuclide injection. The animals were anesthetized before the sacrifice. Six animals were used in each experiment and for each exposure time (control and experiment).

The biosamples were fixed in a 10 % neutral formaldehyde solution, decalcified in trillion B («Sigma», Germany), embedded in paraffin. The histological sections were covered with the «M» type photo emulsion (Plant of technical photo plates, Russia). The obtained histoautographs were stained with hematoxyline-tionin-eosin («Sigma», Germany).

In ^{3}H-thymidine historadioautographs the counts were made of nuclear labeling index (%) per 600–800 cells and cell labeling intensity per 50–100 cells. The counts were made at the microscope magnification 1250. The data were statistically processed with Microsoft Excel program. The method of electron-microscopic radioautography was used for the identification of DNA synthesizing cells in the osteogenesis zones. ^{3}H-thymidine was injected intraperitoneally to three rats (males, 170–180 gm of weight) at the dose 10 μCu/gm. The biosamples were taken at 1 hour after injection of radionuclide. Then they were fixed in 2.5 % gluteraldehide on the phosphate buffer, finally fixed in 1 % solution of osmic acid, and embedded in araldite. The ultra-thin sections were contrasted, covered with the «M» type photo emulsion and investigated under the electron microscope «TESLA-BS 500».

Results and discussion. It is known, that during early stages of the skeleton formation the little-differentiated mesenchymal cells serve as sources for osteoblasts. Later they acquire a perivascular localization to participate in the development of osteoblasts in the periosteal and enchondral osteogenesis and also in the formation of the bone marrow stroma cells. The population of mature osteoblasts is replenished due to the proliferation and differentiation of these precursor cells. These data were provided by studies employing ^{3}H-thymidine as a labelled DNA-precursor [13–16].

With a help of electron microscopic ^{3}H-thymidine radioauthography it was established that the little-differentiated perivascular forms make up the basic proliferating fraction in a population of the perivascular cells. Preosteoblasts proliferate less intensely. The overwhelming majority of functionally mature osteoblasts, osteoblasts undergoing transformation to osteocytes, as well as osteocytes are considered as irreversibly blocked forms relative to proliferation [14, 15, 17]. Morphologically the differentiation of precursor cells into osteoblasts is manifested in a successive development of the organelle systems involved in the biosynthesis of the organic substrates of the bone matrix, first and foremost of the rough endoplasmic reticulum and the Golgi complex [16].

In the experiments employing a single ^{3}H-thymidine administration the study was carried out the proliferation peculiarities and dynamics of cell populations in the metaphyseal zones of rat femoral bones under hind limb unloading compared with the control. In a definitive bone the growth and morphogenesis processes do not occur, here take place only the processes of physiological regeneration of bone structures and their adaptive remodelling (under the change of support-force loads). The latter processes happen also at hind limb unloading and are mostly pronounced in the metaphyseal zones.

The metaphyseal bone trabecules surfaces reveal DNA-synthesizing cells incorporating ^{3}H-thymidine at 1 hr after the isotope injection. Perivascular cells and preosteoblasts that leave G_0 phase and enter the mitotic cycle represent the population of labelled cells (Fig. 1). This is also confirmed by electron-microscopic radioautography (Fig. 2). The labelling index of these cells (counted in total in a microscopic field of vision) makes up 3.72 ± 0.18 % in the experiment and 4.31 ± 0.20 % in the control ($p < 0.05$) (Fig. 3). It should be noted that some bone trabecules' surfaces do not show ^{3}H-thymi-

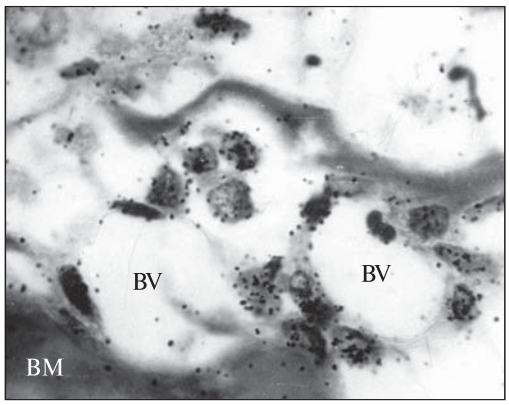

Fig. 1. ^{3}H-thymidine-labelled perivascular cells and preosteoblasts in the osteogenic zone 1 hr after the radionuclide injection. Hind limb unloading. A historadioautograph. Haematoxyline-tionin-eosin. Ob. 100, oc. 12,5. BV – blood vessel, BM – bone matrix ×1250

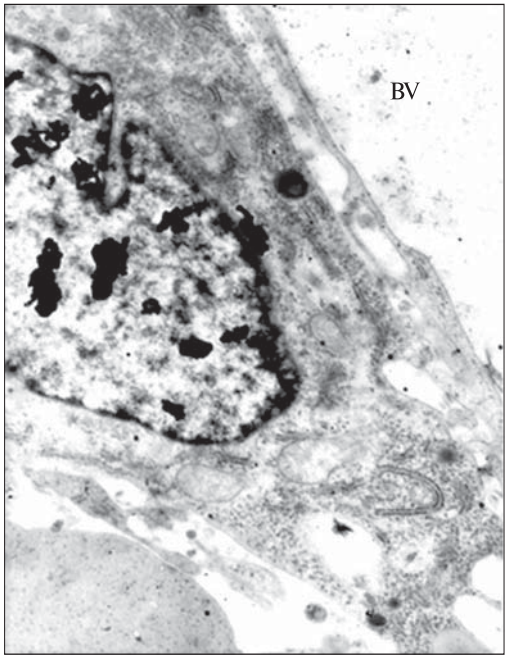

Fig. 2. A perivascular cell labelled with ^{3}H-thymidine. An electron radioautograph, exposure 1 hr. BV – blood vessel. ×12 000

dine labeled cells at 1 hr following the isotope injection. Thus, in hind limb unloading there appears a tendency to reduce in the number of proliferating osteogenic precursor cells in metaphyseal bone trabeculae. Osteoblasts and osteocytes do not incorporate the ^{3}H-thymidine label 1 hr following the injection. The nuclear labeling intensity of the precursor cells (preosteoblasts) 1 hr after injection is approximately similar both in the experiment and control (Fig. 4).

By 48 hr the average labelling intensity in a preosteoblastic population decreases almost 2-fold both in the experiment and control animals, whereas by 96 hr it shows a 3-fold reduction. We can conclude that by these time intervals the cells have undergone division 1 to 2 times. However the labelling index of cells decreases almost 1,6 times in the experiment i.e. a less degree than in the control (3,3 times).

This also confirms to some extent, the decrease of proliferate intensity in the precursor-cells under hind limb unloading. Insignificant part of initially little-labelled cells (4–8 silver grains) following division has been excluded from counts. By 48 hr the labeled osteoblasts and individual labelled osteocytes appear.

The labelling indices of preosteoblasts (3.54 ± ± 0.18 % in control and 3.29 ± 0.16 % in experiment) and osteoblasts (3.24 ± 0.16 % in control and 2.87 ± 0.14 % in experiment) show that in preosteoblastic population about a half of labelled cells leave the population due to the differentiation into osteoblasts. A half of preosteoblasts remain as a cell «reserve» at the surface of bone trabecules. By 48 hr both in control and experiment the bone trabecules show single labelled osteocytes. Their labelling indices make up 1,4 % in control and 1.2 % in experiment.

By 96 hr after the experiment the osteoblasts (labelled after 48 hr) were transformed into osteocytes. The labelling index of osteocytic nuclei including also formerly labelled osteocytes becomes equal to 3.6 ± 0.18 in control and 3.36 ± 0.16 in experiment. The labelling intensity of osteocytes in experiment and control by 96 hr approximates to that of osteoblasts after 48 hr. Insignificant number (≈2.0 %) of labelled osteoblasts in control and experiment observed before 96 hr were formed due to a repeated division of precursor cells. We can judge of this on the basis of similar values of the average labelling intensity of preosteoblasts and osteoblasts by 48 and 96 hr.

In the bone trabecules remodeling zones there are regions containing groups of ^{3}H-thymidine-labelled cells (at 1 hr exposition). These are mainly perivascular and fibroblast-like cells. 48 and

96 hr register these labelled cells in tissue formations (fibrous zones) at the superficial and deep zones of bone trabecules.

The analysis of the obtained results allows allous to suggest a tendency for a delay of growth processes in bone structures and physiological regeneration of osteogenic cells at the bone structures of the femoral bone metaphyses at hind limb unloading. It should be mentioned; in osteogenesis zones at hind limbs unloading, part of labelled by ^{3}H-thymidine stromal little-differentiated cells loss osteogenic gene type and after division differentiates into fibroblasts and adipocytes. It leads to appearance of fibrosis sections in remodeling zones of bones structures, also to increasing of adipocytes number on the bone surfaces [12, 18, 19]. In conditions of microgravity a loss of osteoblastic phenotype gradually takes place [20]. It was indicated clear influence of microgravity on internal nuclear structure. Rebuilding happens in the structure, location and contrast of nuclear parts. It is connected to expression shortening of autocrine genes and genes of the cell cycle, at parallel inhibition of anabolic reaction at microgravity [21]. MC3T3-E1 osteoblasts at cultivations at the conditions of modeled microgravity preserve the expression of RUNX2, osteocalcine and collagen type 1, but expression of alkaline phosphatase is decreased [20].

Some authors [22] suppose that the delay in the differentiation of osteoprecursor cells to osteoblasts under the gravity load decrease. According to electron-microscopic findings under hind limb unloading the population of perivascular cells and preosteoblasts show a reduction in the number of cells containing alkaline phosphates (one of the osteogenic differentiation markers) and an increase in the number of resting preosteoblasts [23].

An in vitro morphometric analysis of the little-differentiated cells (MN7) (Bion-10), showed a tendency for a cell differentiation delay [24]. An increase of the osteogenic cells' proliferation was observed (Bion-10) in cell cultures from monkey iliac bone spongy tissue using ^{3}H-thymidine marker [9]. Our findings correlate with results of studies of cultured mice osteoblasts (MC3T3-E1) exposed in a space system «Biopak», which demonstrated a delay of the osteoblastic differentiation from the very beginning of their cell cycle [25].

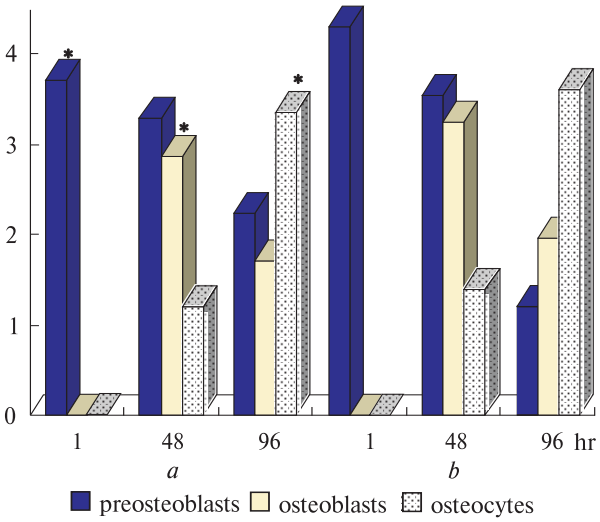

Fig. 3. The change of the ^{3}H-thymidine cells labelling index (%) in zone of the rat femoral bone metaphyses under hind limb unloading: *a* – experiment; *b* – control. * The difference is significant compared to control, $p < 0,05$

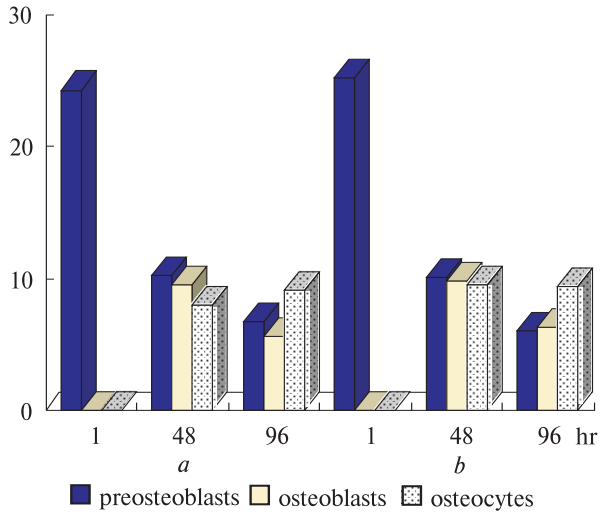

Fig. 4. The change of the intensity of ^{3}H-thymidine cells labelling (the number of silver grains) in zone of the rat femoral bone metaphyses under hind limb unloading: *a* – experiment; *b* – control

Conclusions. With the use of radioactive marker of specific biosynthesis – ^{3}H-thymidine we studied the dynamics and peculiarities of proliferation, differentiation of osteogenic cells and transformation osteoblasts in osteocytes under hind limb unloading of white rats («tail suspension» method) during 28 days. Light and electron-microscopic radioautography with ^{3}H-thymidine have shown,

that basic fraction of DNA synthesizing cells in the zones of adaptive remodelling of bone tissue is presented by little-differentiated perivascular cells (that include osteogenic cell precursors). A tendency for a reduction of a labelling index in the ^{3}H-thymidine-labelled osteogenic cells in metaphyseal bone trabeculae under hind limb unloading has been established. The dynamics of labelled cells during various time intervals after ^{3}H-thymidine injection testifies to a delay in the differentiation precursors in osteoblasts and their transformation to osteocytes in experiment animals. The obtai- ned data have shown that a long-term supportive unloading leads to lowering the intensity of osteogenetic processes in long bones and reducing bone mass.

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ДИНАМИКА ПРОЛИФЕРАЦИИ И ДИФФЕРЕНЦИРОВКИ ОСТЕОГЕННЫХ КЛЕТОК ПРИ СНЯТИИ ОПОРНОЙ НАГРУЗКИ

С использованием радиоактивного маркера синтеза ДНК – ^{3}Н-тимидина изучали динамику, особенности пролиферации и дифференцировки остеогенных клеток белых крыс при снятии опорной нагрузки с задних конечностей методом «вывешивания» за хвост под углом 35° в течение 28 дней. В конце эксперимента однократно вводили ^{3}Н-тимидин. Биообразцы отбирали из бедренной кости через 1, 48 и 96 ч. Методом световой и электронно-микроскопической радиоавтографии было показано, что основная фракция ДНК-синтезирующих клеток в зонах адаптивного ремоделирования костной ткани представлена малодифференцированными периваскулярными клетками, включающими в себя остеогенные клетки-предшественники. Установлена тенденция к уменьшению индекса мечения остеогенных клеток ^{3}Н-тимидином в костных трабекулах метафизов при снятии опорной нагрузки. Динамика меченых клеток в различные сроки после введения ^{3}Н-тимидина показывает замедление дифференцировки клеток-предшественников в остеобласты и процессов их трансформации в остеоциты у экспериментальных животных. Полученные данные свидетельствуют о том, что длительная опорная разгрузка приводит к снижению интенсивности остеогенетических процессов в длинных костях и редукции костной массы.

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ДИНАМІКА ПРОЛІФЕРАЦІЇ ТА ДИФЕРЕНЦІЮВАННЯ ОСТЕОГЕННИХ КЛІТИН ПРИ ЗНЯТТІ ОПОРНОГО НАВАНТАЖЕННЯ

З використанням радіоактивного маркера синтезу ДНК – ^{3}Н-тимідину вивчали динаміку проліферації та диференціювання остеогенних клітин білих щурів при знятті опорного навантаження із задніх кінцівок методом «вивішування» за хвіст під кутом 35° впродовж 28 днів. В кінці експерименту одноразово вводили ^{3}Н-тимідин. Біозразки відбирали із стегнової кістки через 1, 48 і 96 год. Методом світлової та електронно-мікроскопічної радіоавтографії було показано, що основна фракція ДНК-синтезуючих клітин у зонах адаптивного ремоделювання кісткової тканини представлена малодиференційованими периваскулярними клітинами, що включають остеогенні клітини-попередники. Встановлено тенденцію до зменшення індексу мічення остеогенних клітин ^{3}Н-тимідином в кісткових трабекулах метафізів при знятті опорного навантаження. Динаміка мічених клітин в різні терміни після введення ^{3}Н-тимідину демонструє уповільнення диференціювання клітин-попередників в остеобласти і процесів їхньої трансформації в остеоцити у експериментальних тварин. Отримані дані свідчать про те, що тривале опорне розвантаження призводить до зниження інтенсивності остеогенетичних процесів в довгих кістках і редукції кісткової маси.

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