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COMPLEX HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY OF MONOCYTOPOIESIS IN RATS WITH DETERMINATION OF PROLIFERATIVE ACTIVITY BY EXPRESSION KI67 AND CD68+ IN THE EXPERIMENT

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Ki67 is not only one of the markers of proliferation, but also an indirect determinant of the rate of cell division. CD68 is exploited as a valuable cytochemical marker to immunostain monocyte/macrophages in the histochemical analysis of inflamed tissues, tumor tissues, and other immunohistopathological applications. The purpose of the study was to determine the cell proliferation index of the monocytic line of red bone marrow using antibodies to CD68+ and Ki-67 in the control and intact groups of animals. There were established an equivalent degree of proliferative activity by progenitor cells with Ki-67 expression in the control and intact groups.

Key words: monocytopoiesis, Ki-67, CD68+, rat bone marrow.

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КОМПЛЕКСНЕ ГІСТОЛОГІЧНЕ ТА ІМУНОГІСТОХІМІЧНЕ ДОСЛІДЖЕННЯ МОНОЦИТОПОЕЗУ У ЩУРІВ З ВИЗНАЧЕННЯМ ПРОЛІФЕРАТИВНОЇ АКТИВНОСТІ ПО ЕКСПРЕСІЇ KI67 ТА CD68+ В ЕКСПЕРИМЕНТІ

Ki67 є не тільки одним із маркерів проліферації, але й непрямим визначником швидкості поділу клітин. CD68 використовується як цінний цитохімічний маркер для імунного фарбування моноцитів/макрофагів під час гістохімічного аналізу запалених тканин, тканин пухлини та інших імуногістопатологічних досліджень. Метою дослідження було визначення індексу проліферації клітин моноцитарної лінії червоного кісткового мозку за допомогою антитіл до CD68+ та Ki-67 у контрольній та інтактній групах тварин. Встановлено еквівалентний ступінь проліферативної активності клітин-попередників з експресією Ki-67 у контрольній та інтактній групах.

Ключові слова: моноцитопоез, Ki-67, CD68+, кістковий мозок щурів.

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In humans, the red bone marrow is the central and universal organ of hematopoiesis, starting from 4–5 months of embryonic development. Cytokines (interleukins, colony-stimulating factor, growth factors), hormones and other humoral factors, such as hematopoietins, which include erythropoietin, leukopoietin, thrombopoietin, monopoietin, are involved in the regulation of hematopoiesis. The importance of monocytopoiesis in the formation of transitional states is especially obvious: from the norm to pathology and in the transformation of pathological processes from one quality to another [16]. With all the variety of these processes, they include typical components associated with the development of tissue stress, which is characteristic not only of canonical, but also non-classical inflammation (parainflammation), which develops in response to low-intensity damage without the development of typical local signs of inflammation [7, 10, 11]. Ki67 is not only one of the markers of proliferation, but also an indirect determinant of the rate of cell division [6, 12]. Cyclins differ in expression at certain stages of the cell cycle and, accordingly, regulate mitotic cell division at different stages. CD68 is exploited as a valuable cytochemical marker to immunostain monocyte/macrophages in the histochemical analysis of inflamed tissues, tumor tissues, and other immunohistopathological applications. Cell-specific CD68 expression and differentiated expression levels are determined by the complex interplay between transcription factors, regulatory transcriptional elements, and epigenetic factors [5]. The cell receptor CD68+ is part of the macrophage receptor family that is associated with macrophages that have polarization for the M1 phenotype [2]. The growth of the cellular fraction of macrophages with CD68+ cannot always explain all changes in the activity of marker enzymes, since cells of non-macrophage origin can activate the transcription of the iNOS and Arg genes under certain conditions. It is considered that the rapid recirculation of CD68 from endosomes and lysosomes to the plasma membrane allows macrophages to crawl over selectin-carrying substrates or other cells [8, 9]. CD68 is also involved in the uptake of apoptotic and damaged cells by macrophages through interaction with phosphatidylserine [5]. Meanwhile, the homeostasis of monocytopoiesis, both in ensuring metabolic homeostasis and cell turnover, and in the

processes of immune and inflammatory reactivity in tissue damage of various origins, is insufficiently described.

The purpose of the study was to determine the cell proliferation index of the monocytic line of red bone marrow using antibodies to CD68+ and Ki-67 in the control and intact groups of animals.

Materials and methods. The study was performed on 20 adult male rats. Animals were randomly divided into 2 groups: control (10 animals) and intact (10 animals). Animals of the control group were injected with saline in dosage 0.3 ml in the thigh. Animals of the intact and control groups were kept in standard conditions of Poltava State Medical University vivarium. Experimental animals were euthanized in strict accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986) and the General Ethical Principles of Animal Experiments adopted by the First National Congress on Bioethics (Kyiv, 2001) [1].

To study the biopsy material, pieces of tissue from the red bone marrow of rats were fixed in 10 % buffered formalin, pH 7.4 for 24 h, and embedded in paraffin. Then, serial sections with a thickness of 5 μm were made, which were automatically stained with hematoxylin and eosin on a Microm HMS 740 Thermo scientific apparatus and embedded in Bio-Mount (Bio Optica Milano S.P.A., Italy).

Immunohistochemical examination of the biopsy material was performed in accordance with the standard protocol. Serial sections with a thickness of 5 μm were mounted on glasses coated with poly-L-lysine. Deparaffinization and high-temperature unmasking of antibodies were carried out using a PT-module for 20 min at a temperature of 98°C. Further procedures were performed automatically on an Autostainer 360 Thermo scientific using a QUANTO imaging system. The Autostainer protocol included 10 min H2O2, 10 min protein block, 30 min primary antibodies, 10 min secondary antibodies, 5 min DAB. Washing in TRIS-Buffer pH 6 with Tween 20. Tissue antigens were determined using mouse monoclonal antibodies to Ki-67 (MM1, "Diagnostic Biosystems", USA) and CD 68 "Chemicon" (USA).

The Ki-67 proliferation index in % was determined by the ratio of cells with immunoreactive nuclei to the total number of cells. The expression of the CD68 receptor was assessed on the outer membrane of the cells of the monocytic line of the red bone marrow in arbitrary units: 0 – no expression was observed; 1 – weak staining of cell membranes; 2 – moderate staining of cell membranes; 3 – intense staining of cell membranes.

Histological slices were examined using Olympus C 3040-ADU light microscope with digital microfilter with software adapted for these studies (Olympus DP – Soft, license No. VJ285302, VT310403, 1AV4U13B26802) and Biorex 3 (serial No. 5604).

Statistical processing of the survey results was performed using Microsoft Office Excel software and the extension of Real Statistics 2019 to it. The nonparametric Mann–Whitney test was used to determine the statistical significance of differences between groups. The difference was considered statistically significant at $p<0.05$.

Results of the study and their discussion. Our histological study of semi-thin sections of the rats red bone marrow of two experimental groups made it possible to establish that the stromal component was represented by a thin layer of compact mineralized substance, parallel to the oriented bone plates, in the depths of which there was a spongy substance. The weakly mineralized trabeculae were going in different directions, joining, forming arcs that formed interconnecting cavities of various volumes. The red bone marrow cells in different stages of differentiation were inside the cavities (lacunae).

The microenvironment for differentiating cells was formed by fibroblastic reticular cells of irregular shape with light cytoplasm. These cells connected to each other with their processes, forming a network. On the specimens of red bone marrow, adipocytes were visible like large cells of a round shape. The central part of the cells is light, unstained. The cytoplasm has the form of a narrow strip, the nucleus is displaced to the periphery.

Visual identification of cells of a monocytic lineage was a little difficulty, due to the large number of the erythroid cells.

The cells of the monocytic lineage are progenitor cells of monocyte and tissue macrophages. Most of them were large cells with a large nucleus, which occupied the main area of the cell. The nuclei are dark, dense. The cytoplasm is visible as a thin strip, basophilic, sometimes neutral. Figures of mitosis were observed in some cells. The above described cells in the population of the monocytic clone are visually unrecognizable. They become morphologically distinguishable from the monoblast stage (fig. 1).

Thus, during the morphological and morphometric assessment of the cells of the monocytic line, we did not find visible changes in the two experimental groups. Monoblast was one of the most morphologically recognizable cells of a monocytic lineage. The cell was rounded, with a small amount of cytoplasm. The nucleus was red-lilac in color, usually round, sometimes oval in shape. The nucleus

diameter averaged 14.06±0.28 μm. The cytoplasm was thin, blue, and did not contain inclusions. The nuclear-cytoplasmic ratio was 3.92:1.

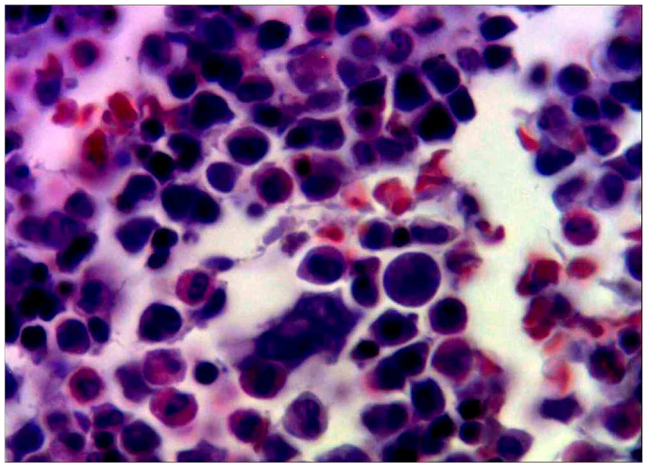
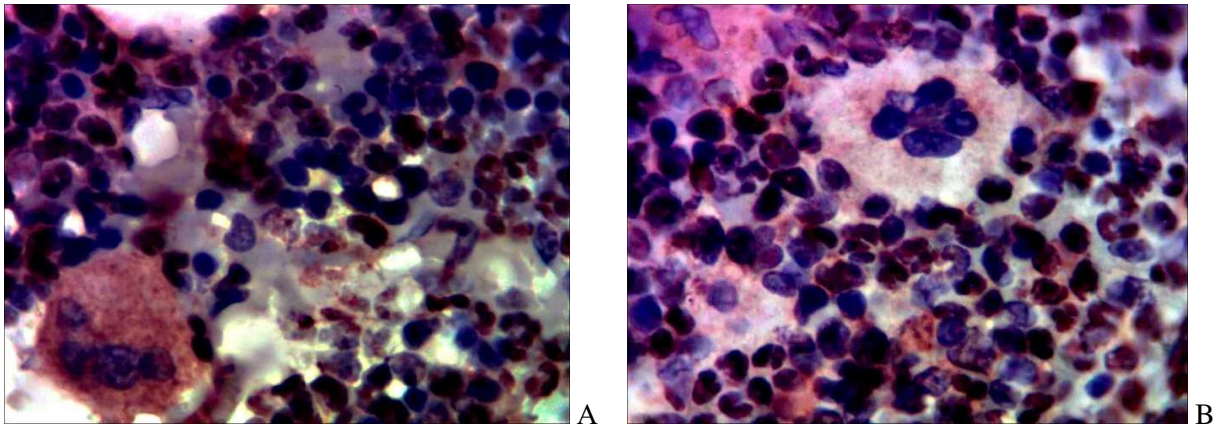


Fig.1. Red bone marrow of the control group. Monocytic line. H&E stain. Lens: 20: Ocular lens: 10.

A promonocyte was a round-shaped cell with an oval-shaped nucleus, the average diameter was 12.94±0.23 μm. The cytoplasm was blue-gray with insignificant granularity. Nuclear-cytoplasmic ratio 3.02:1

Monocyte was a round-shaped cell. Its nucleus had an irregular, bean-like shape with uneven edges, the average diameter was 16.38±0.19 μm. The cytoplasm of the monocyte was blue-grey, opaque, and contained small azurophilic granules. Nuclear-cytoplasmic ratio 1:1.

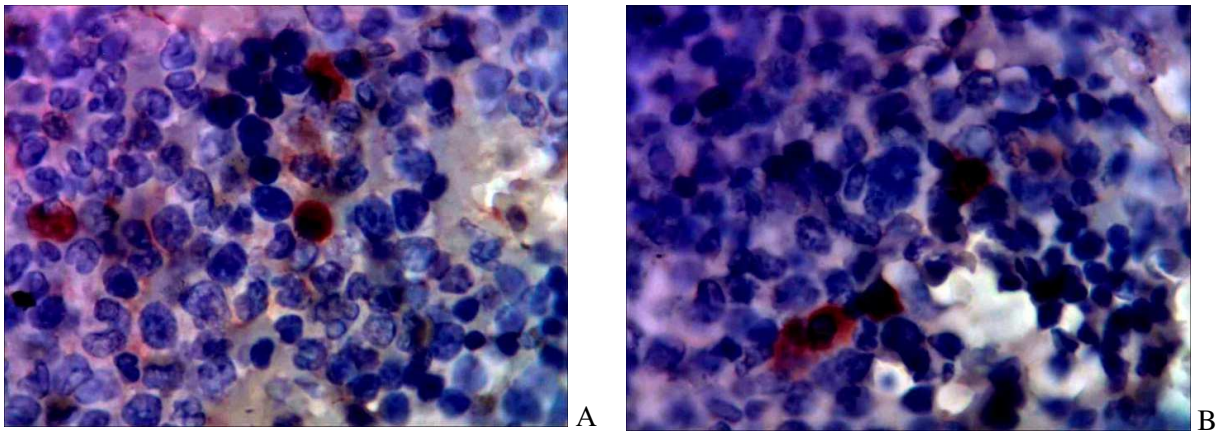
We have carried out a morphological and morphometric assessment of the immunohistochemical study of Ki-67 and CD68+ expression and confirmed counting of the mitotic index of the monocytic lineage of red bone marrow cells in all experimental groups. Hematologic specimens of all cases were reviewed and extensive immunohistochemical staining was performed. Monocytic clones, consisting mainly of precursor cells of monocytes, had a high expression of Ki-67 at the early stages of monocytopoiesis, and less pronounced in differentiating cells (monoblast and promonocyte with CD68+ immunostaining). On the contrary, CD68+ monocytes had an absolutely unexpressed degree of expression to Ki-67. We did not find the apoptotic mechanism of death of the cell elements of the monocytic lineage. (fig.2).



A B

Fig. 2. Expression of Ki-67 in cells of rat red bone marrow. A. Control group. B. Intact group.

Having carried out a quantitative calculation of the content of the monocytic lineage of red bone marrow in the population of CD68+ cells per percentage of expressed cells to nonexpressed, we found that their number was 88 % by 12 %, respectively 7.33:1, in the control group. And 89 % by 11 % in the intact group, respectively 8.09:1 (fig. 3).



A B

Fig.3. Expression of CD68+ in cells of rat red bone marrow. A. Control group. B. Intact group.

Consequently, we revealed a positive proliferative reaction in the cells of hematopoietic tissue at the early stages of monocytopoiesis with inhibition at later stages, which reflects the cyclical processes of cell proliferation and differentiation during the assembly of the macromolecular complex during each cell cycle.

Thus, our histological analysis of the stromal and hematopoietic components (monocytic lineage) of the red bone marrow in the control and intact groups of animals showed that the obtained results do not contradict the literature data [3, 10]. Among the cells of the microenvironment, we have identified fibroblastic reticular cells and adipocytes. These cells play an important role in the regulation of hematopoiesis [13, 14].

The study of progenitor cells of monocytes has a certain difficulty, since these cells do not contain specific cytoplasmic granules or lobules of the nucleus, which facilitate their morphological differentiation. Cells of the monocytic series of hematopoiesis become recognizable from the monoblast stage.

Maintaining the constancy of the quantitative and qualitative composition of each cellular link of the blood system under the conditions of constantly changing needs of the body for blood cells is based on the observance of the basic law of the kinetics of hematopoiesis: "The same number of cells is born and dies per unit time" [15]. Compliance with this law is ensured by complex mechanisms of regulation of hematopoiesis. Thus, our histological study of hematopoietic cells of the monocytic lineage showed phenotypic heterogeneity in the population of proliferating and differentiating cells. The results obtained are consistent with the literature data, in which the basic principles and regularities of the organization of the population of a monocytic clone of rats are set forth [4, 8, 10]. Also, in the cell population, we calculated immunohistochemically the coefficient of the proliferative activity of the monocytic lineage of the rat red bone marrow by expression to Ki67 and CD68. We will use these results in the following studies to determine the kinetics of monopoiesis and redistribution of the direction of hematopoiesis with the formation of M1 macrophages to perform their inherent functions.

Conclusions

1. We revealed an equivalent degree of proliferative activity by progenitor cells with Ki-67 expression in the control and intact groups.

2. Cell populations of the monocytic lineage in all experimental groups are predominantly in a state of active proliferation. In relation to maturing to mature forms, they are 7.33:1 and 8.09:1 with expression of Ki-67 and CD68+.

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