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AGE-DEPENDENT FEATURES OF THE BONE MARROW RESPONSE TO TOXICOGENIC LIVER FIBROSIS IN THE EXPERIMENT

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Summary. *Liver fibrosis has been shown to affect bone marrow cell morphotypes. This study investigated the response of bone marrow cells to fibrosis. Bone marrow cells from rats of different ages were the object of study. It was shown that the proliferative activity of bone marrow cells in animals with Cu-induced fibrosis was significantly lower than in animals with CCl_4-induced liver fibrosis. Bone marrow plays an important role in the development of liver fibrosis.*

Introduction. It is known that liver cirrhosis is the fourth leading cause of death among older people [1]. This terminal pathological stage is formed in the process of "pathological evolution" and can be represented by a sequence of the following stages: hepatitis, fibrosis and cirrhosis. There are studies showing that the early stages of fibrosis can be reversible [2-3]. At the same time, the problem of

treating liver fibrosis and preventing its progression to cirrhosis, despite intensive study of its mechanisms, remains one of the most acute problems of modern medicine. This may be due to a number of reasons: the stages of pathology development and the effectiveness of its treatment may be influenced by age, factors that induce its formation, the presence of comorbidities, etc. Finding answers to these questions is of great interest in solving this fundamental and important practical problem.

The study investigated the possible systemic effect of liver fibrosis on such an important immunocompetent organ as the bone marrow, since its function plays a key role in the formation of the immune response to fibrosis and this response may depend on the age of the animals. The inducer of this pathological process may play an important role in shaping the body's response to the development of fibrosis. Currently, tetrachloromethane is frequently used today to model toxicogenic fibrosis, which provides a fairly rapid transition from fibrosis to cirrhosis. Our laboratory has developed a new toxicogenic model of Cu-induced fibrosis [4-5].

Methods. The study involved male *Wistar* rats, both young (3 months old) and old (20 months old), which were divided into control groups and groups induced with Cu and CCL_4 fibrosis similarly for young and old animals. Each group consisted of 8 animals. Bone marrow cells (BMCs) were isolated from two rat femoral bones according to the method described in [6]. Cells were sedimented by centrifugation at 1,000g for 15 minutes at 4°C. They were gently suspended in DMEM medium containing 20% inactivated fetal calf serum and antibiotics (1% gentamicin and 1% streptomycin). The viability of the cells, as determined by the trypan blue test, was greater than 90%. The number of cells in the suspension was counted in a Goryaev chamber. The culture medium always had an initial cell concentration of 2 million cells per ml. Determination of morphological cell types was performed before the start of cultivation (characterization of baseline or initial level) and on the 2nd and 4th day of growth as described in [7]. Cell suspension was stained by Romanovsky-Giemsa staining, cell morphotypes were analyzed at 100x magnification on a microscope "Zeiss Primo Stari LED" (Germany). The obtained results were statistically processed using the Mann-Whitney method.

Results and their discussion. All cell types in the bone marrow can be represented as finally differentiated, i.e. morphologically identifiable: myelocytes, metamyelocytes, band and segmented neutrophils, lymphocytes, basophils and monocytes and morphologically unidentifiable cell types, which include stem cells and committed (finally undifferentiated) cells. In the bone marrow of young control animals, morphologically identifiable cell types accounted for about 60% and 40% were unidentifiable, i.e., not mature cell types (Fig. 1). At the same time, in old control animals this ratio was 80% identifiable and only 20% precursor cells (Fig. 1).

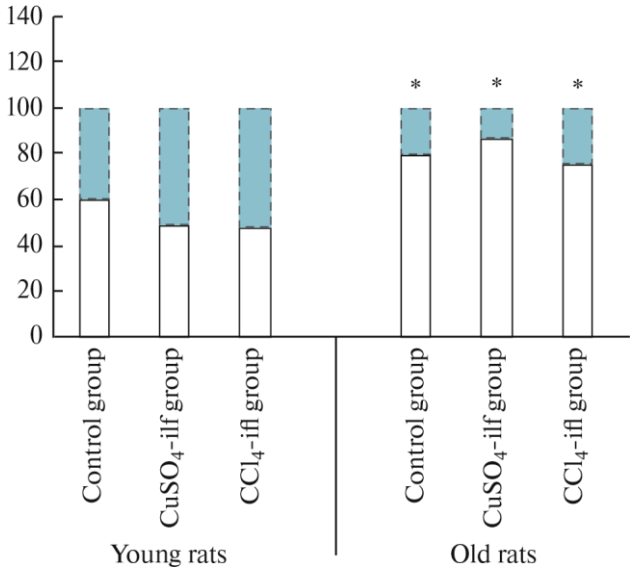

Fig. 1. **Number of morphologically identifiable cells and number of unidentifiable cells as a percentage of the total number of bone marrow cells obtained in young (A) and old (B), respectively, in the control group (1), in the group that received tetrachloromethane (2) and copper sulfate. Mean values obtained in 8 animals of each group and their standard errors are presented. The asterisk marks the variants for which P < 0.05 of old animals compared to young animals, and two asterisks control and experimental groups, according to the Mann-Whitney criterion.**

These results may indicate that differentiated (mature) cells, which are primarily immunocompetent cells, are "retained" in the bone marrow longer in old animals, or are transported into the bloodstream more slowly compared to young animals, or the pool of precursor cells is reduced in the bone marrow of old animals. Of course, this assumption requires further research on this important issue, but this indicator may be useful in assessing the functional characteristics of bone marrow in modeling liver fibrosis.

It was found that the ratios between identifiable and non-identifiable morphotypes of bone marrow cells of young and old animals changed differently depending on the inducer of fibrosis (copper sulfoxide or tetrachloromethane). Thus, against the background of liver fibrosis development, the number of morphologically identifiable cell types in the bone marrow of young animals decreased while the number of unidentifiable cells increased irrespective of the inducer type (Fig. 2). At the same time, in old animals, on the contrary, the number of morphologically identifiable cell types in the bone marrow decreased after the

administration of copper sulfate and remained at the control level in CCl_4-induced fibrosis (Fig.2).

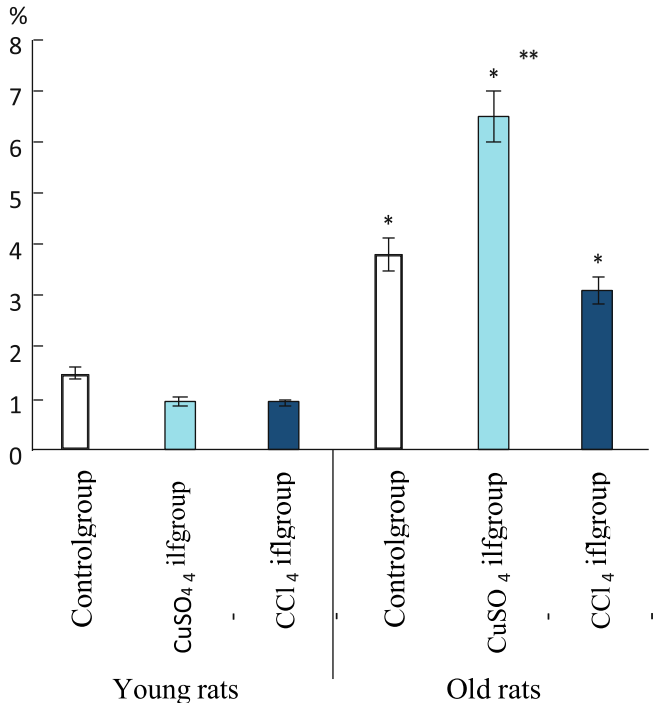

Fig. 2 **Ratios in percentages of identifiable to unidentifiable cell types in the bone marrow of young and old animals for the control group, the group with Cu-induced liver fibrosis and the group with CCl_4-induced fibrosis, respectively. Mean values and their standard errors obtained in 8 animals in each group are presented. An asterisk indicates differences between young and old animals for which P<0.05 and two asterisks between control and experimental groups for which P<0.05, according to the Mann-Whitney test**

These results suggest that the bone marrow of young and old control animals differs in the content of different cell types. If liver fibrosis was induced in young animals using different toxicogenic compounds, the ratios between identified and unidentified cell types in bone marrow did not change compared to their initial levels. In contrast, induction of liver fibrosis by different toxicogenic compounds in old animals resulted in different changes in the ratios of bone marrow cell types. These age-dependent differences may reflect both changes in the rate of transport of bone marrow cells into the bloodstream and differences in the rate of proliferation and direction of cell differentiation in the bone marrow of young and old animals.

When determining the number of band neutrophils, metamyelocytes, lymphocytes, segmented neutrophils, myelocytes, eosinophils, basophils and monocytes, we found that the bone marrow of the control group of old animals contained 160% more lymphocytes compared to the control group of young animals, and 35% fewer segmented neutrophils, respectively (Fig. 3a). The differences in the rest of the identifiable cell types between young and old animals in the control groups were insignificant. (Fig. 3 a).

As can be seen from the data presented in Fig. 3 b and c, both hepatotoxic compounds caused different changes in the number of immunocompetent cells in the bone marrow, and these quantitative changes were different in young and old animals with Cu-induced and CCl_4-induced fibrosis. For example, in old animals with Cu-induced liver fibrosis, the number of basophils in bone marrow decreased compared to their control levels, while in young animals at the same time, their number increased compared to their age-matched controls (Fig. 3b). It should be noted that the quantitative changes compared to the initial (control) values were more pronounced in animals with Cu-induced liver fibrosis in comparison with CCl_4-induced fibrosis, both for young and old animals (Fig. 3 b, c).

The available numerous data and the results obtained in the present work allow us to assert that the bone marrow takes an active part in the formation of adaptive response to fibrotic changes in the liver. Taking into account the fact that copper ions do not accumulate in the bone marrow, it can be assumed that changes in the functional activity of the bone marrow against the background of Cu-induced fibrosis are caused not by the direct action of these toxic compounds, but by changes in the characteristics of cell niches in the bone marrow of experimental animals. Changes in the characteristics of the redox system, formation of different DAMPs, which occur in animals with fibrosis, differ in their characteristics for different inducers, form in the organism of young and old animals specific low-molecular patterns that systemically regulate the formation of the adaptive response of the organism in response to the actions of hepatotoxic compounds.

Along with quantitative changes of cell types in the bone marrow of young and old animals, as well as animals with liver fibrosis, which were induced by different hepatotoxic compounds, may be accompanied by functional changes of immunocompetent cells, which are formed in these animals under extreme conditions. It is known that the same morphological cell types can perform different functions depending on the microenvironment and the "context" in which they are located at a given moment. The present study indirectly demonstrates this point. To experimentally verify this, we conducted a series of experiments to determine the proliferative ability of immunocompetent bone marrow cells obtained from control animals, animals with Cu-induced and CCl_4-induced fibrosis of different ages in an in vitro system. At the same time, we proceeded on the basis that proliferative activity

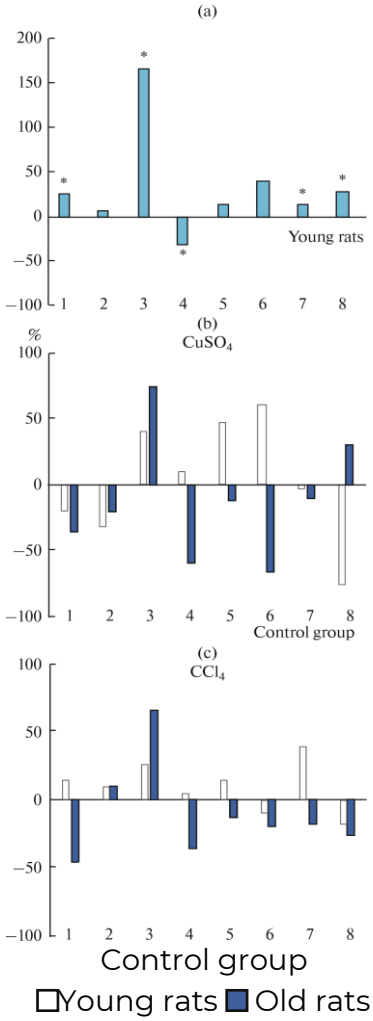

Fig. 3. **Differences in the number of band neutrophils (1), metamyelocytes (2), lymphocytes (3), segmented neutrophils (4), myelocytes (5), eosinophils (6), basophils (7) and monocytes (8) in old animals in percent compared to young animals, which are taken as zero level (a) and changes in the number of these cell types in young (□) and old animals (■) animals in percent against the background of Cu-induced fibrosis development (b) and, respectively, against the background of CCl_4 -induced fibrosis (c) in comparison with their control values, which are taken as zero level. Mean values and their standard errors are presented, and 8 animals were used in each variant. Asterisk indicates variants for which P<0.05 old versus young, by the Mann-Whitney criterion**

is one of the most important indicators of the functional activity of cells. Immunocompetent cells are of particular interest in this respect, as they have a relatively short lifespan.

When determining the proliferative activity, a suspension of bone marrow cells was prepared in such a way that the initial concentration of cells was the same in all studied variants and always amounted to 2 ml. cells per ml, and the cells were cultured simultaneously on the same nutrient medium and under the same conditions. It was found that during the first day of cultivation the number of bone marrow cells (BMC) obtained in control young animals increased by 60% of the initial number, at the same time in the same conditions the number of BMC obtained in old animals increased by 112% (Fig. 4a). Further, the number of cells in the culture obtained from young and old animals increased at the same rate and reached the stationary level in the primary culture by day 3 (Fig. 4a). The superiority of BMC in proliferation rate obtained in old control animals can be explained by the increased number of lymphocytes compared to young animals (Fig. 3a), which have a high proliferation rate compared to other types of bone marrow cells.

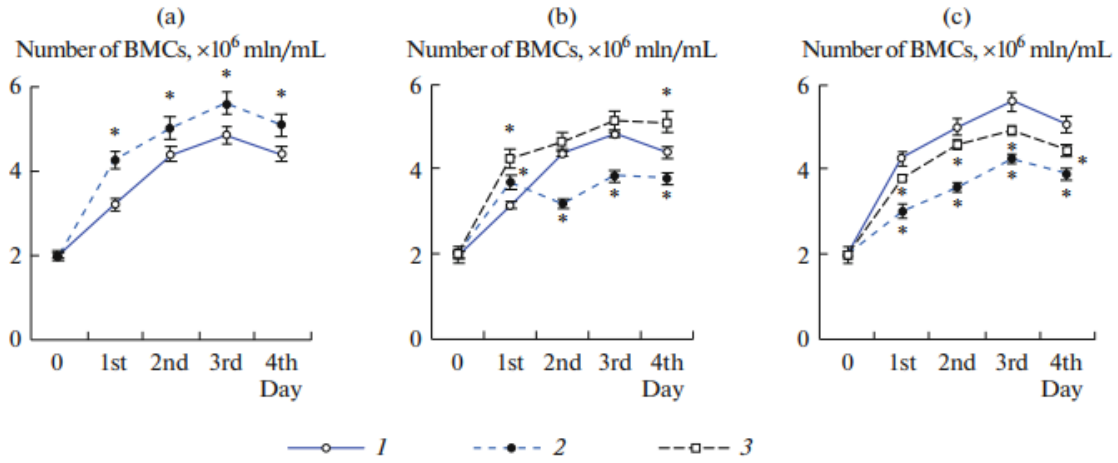

Fig. 4. **Change in the number of cells from 1 to 4 days of culturing on DMEM medium with 20% inactivated fetal calf serum with 1% gentamicin and streptomycin, which were obtained from bone marrow of young (___) and old animals (_ _) - (a) from young controls (1) with Cu-induced (2) and CCl_4-induced fibrosis (3) - (b) obtained from old controls (1) with Cu-induced (2) and CCl_4-induced fibrosis (3) -c. The mean values and standard errors for 8 animals in each experimental group are shown. The asterisk represents variants for which P<0.05 compared to the corresponding age-matched control, according to the Mann-Whitney test**

It cannot be excluded that the characteristics of the niches in which bone marrow cells differentiate in old animals differ from those in young animals, as a result of which the formed bone marrow cells may have insignificant structural and

functional differences in animals of different ages. These age-dependent features of bone marrow cells could be attributed to differences in the ionic composition of cells, which affects the rate of cell division. Calcium is known to have a wide variety of functions in the cell and in the organism as a whole, including serving as a secondary messenger and participating in the regulation of cell proliferation. It can be assumed that the "choice" of the function of polyfunctional molecules, which includes calcium, may be determined by the characteristic of the microenvironment of the molecule itself. There are works showing that an increase in intracellular calcium content leads to the activation of cell proliferation [8]. The content of calcium ions was determined in bone marrow cells obtained from young and old animals by the degree of fluorescence of Fluo-3 fluorescent probe specific for this ion. It turned out that the amount of calcium in bone marrow cells obtained from old animals was 109.9 + 4.2 (n=95) conventional units, while in young animals it was only 33.5 + 1.48 (n=68), i.e. 3 times less than in the cells of old animals.

The obtained results suggest that the higher proliferative activity of BMC in the culture of cells obtained from bone marrow of old animals can be associated with both a greater number of lymphocytes and the altered composition of cell components compared to young animals, at least in terms of calcium content. It should be noted that the determination of proliferative activity of cells in culture reflects their potential ability to proliferate and it does not follow from these data that in the body bone marrow cells of old animals realize this potential.

When analyzing the peculiarities of bone marrow cell proliferation in culture, it is important to note that if BMCs were isolated from young animals with Cu-induced liver fibrosis, their number increased slightly faster than the control level during the first day of cultivation. However, on the second day, their number decreased and did not change over time, and their number was significantly lower compared to the control at this time (Fig. 4b). If BMCs were obtained from young animals with CCl_4-induced liver fibrosis, their number increased even faster than in controls (Fig. 4b).

Consequently, bone marrow cells formed in the presence of different fibrosis inducers differ in their proliferative properties and possibly in other functional characteristics.

Other proliferative characteristics of BMCs were obtained for cells isolated from old animals with Cu-induced liver fibrosis and CCl_4-induced liver fibrosis (Fig. 4c). Thus, the dynamics of cell number increase in the process of cultivation was similar for all variants under study. The proliferation rate was the highest in control, insignificantly lower for BMC with CCl_4-induced liver fibrosis and significantly lower than control and CCl_4-induced liver fibrosis, if BMC was obtained from old animals with Cu-induced liver fibrosis (Fig. 4c).

Despite the fact that the number of leukocytes in the bone marrow of old animals with Cu-induced liver fibrosis and CCl_4-induced liver fibrosis remained

increased to the same extent, the proliferative activity for cells obtained from animals with Cu-induced liver fibrosis was significantly lower than that of cells from animals with CCl_4-induced liver fibrosis. The obtained data indicate that the change in the microenvironment for bone marrow cells, in this case after administration of copper sulfate and tetrachloromethane to animals, was accompanied not only by a change in the number of different types of cells in the bone marrow, but also that these types of cells formed different functional characteristics, at least in terms of proliferative activity in cell culture.

Therefore, the proliferative activity of bone marrow cells in animals with Cu-induced fibrosis was significantly lower than that in animals with CCl_4-induced liver fibrosis, and in addition, bone marrow cells changed their proliferative potential differently in response to the actions of different inducers in young and old animals.

Conclusions. Features of liver fibrosis development depend not only on the characteristics of the inducer of this pathological process, but also on the age of animals, which can "modify" the response to the actions of potogenetic factors and in this process an important role is played by the bone marrow as a central organ of immunogenesis. Further studies on the role of bone marrow, taking into account its age-specific features in the processes of liver fibrosis development, will allow us to develop a systematic approach to the diagnosis and treatment of liver diseases.

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