ISSN 2079-0570, Advances in Gerontology, 2017, Vol. 7, No. 4, pp. 336–344. © Pleiades Publishing, Ltd., 2017.
Original Russian Text © A.I. Bozhkov, S.L. Ohiienko, Yu.A. Kuznetsova, A.Yu. Bondar', V.P. Marchenko, M.S. Gumennaya, 2017, published in Uspekhi Gerontologii, 2017, Vol. 30, No. 3, pp. 457–467.

Donor Age Affects Behavior and Sensibility of Bone Marrow Cells to Copper Ions in Primary Culture

A. I. Bozhkov*, S. L. Ohiienko, Yu. A. Kuznetsova, A. Yu. Bondar', V. P. Marchenko, and M. S. Gumennaya

Research Institute of Biology, Karazin National University of Kharkiv, Kharkiv, 61022 Ukraine

**e-mail: bozhkov@univer.kharkov.ua*

Abstract—In the primary culture of bone marrow cells obtained from young (3-month-old) and old (20-month-old) rats, the change in the number of cells from day 0 to day 4, the pattern of cell morphotypes, and the lifespan of myelocytes, metamyelocytes, and stab and segment neutrophils was studied. It was shown that the number of bone marrow cells obtained from old animals (20-month-old) in the primary culture increased faster than the number of bone marrow cells in young animals (3-month-old). The presence of Copper-induced liver fibrosis in animals had a different effect on the rate of increase in the number of bone marrow cells obtained in young and old animals. The administration of 4 and 8 mmol of $CuSO_4 \cdot 5H_2O$ into the culture of bone marrow cells in young and old animals caused a dose-dependent inhibition of proliferative processes in the cells of young animals. When copper ions were administered into the culture of bone marrow cells obtained from old animals, the inhibition of proliferation was less pronounced than for young animals, and a concentration of 8 mmol of $CuSO_4 \cdot 5H_2O$ inhibited proliferation to a lesser extent than 4 mmol of $CuSO_4 \cdot 5H_2O$. The presence of liver fibrosis in animals accelerated the process of bone marrow cell death in the primary culture in young and old animals. However, this effect was more pronounced in young animals. It is suggested that bone marrow cells undergo such epigenetic changes, which change their functional properties, during ontogeny.

Keywords: bone marrow cells, aging, liver fibrosis, primary culture

DOI: 10.1134/S2079057017040026

INTRODUCTION

One of the most striking achievements of the biology of the 20th century, along with the decoding of the primary sequence of the genome, was the isolation, study, and development of stem cell cultivation methods [15, 18, 22]. The possibilities for the practical use of stem cells inspired not only medical workers but also gerontologists [4, 27].

There are new gerontological hypotheses, according to which stem cells can become the means to eliminate age-related changes, accelerate the regeneration of organs, and normalize the body's abilities to adapt to various degenerative changes [7, 19, 24].

The main arguments of the supporters of these ideas were based on two main positions: first, stem cells can be differentiated into any type of cells depending on the microenvironment or niche; second, the number of stem cells decreases with age, and age-related pathologies can be eliminated with the transplantation of stem cells to an aging organism.

Indeed, there are a sufficient number of studies in which it has been shown that the number of hematopoietic stem cells in the bone marrow decreases and leukemia develops more often in the aging organism than in young people [13, 29]. Along with this, there are data that do not confirm an obvious reduction in the number of stem cells in ontogeny [28, 30].

The results of fundamental studies on this problem have proved to be ambiguous and contradictory [25, 31]. The available data make it possible to conclude that the problem of aging is not reduced to quantitative or qualitative changes in stem cells; that the properties of stem cells are determined by their microenvironment, which can have an age-dependent nature; and that transplantation of stem cells does not solve the problem of age-dependent involution.

In this regard, it is important to establish patterns of interaction or interference of cells and environmental factors. Since cell–microenvironment or cell–organism dynamic systems are extremely complex, researchers resort to relatively simple in vitro models.

As it is known, the bone marrow, a system-forming organ, is the source for the obtainment and further study of stem cells [6, 23]. It provides hematopoiesis and synthesis of immunocompetent cells and is the "keeper" of the pool of mesenchymal stem cells, which in turn provide osteogenesis [16] and regeneration of the liver and other organs [26].

Bone marrow cells are represented by an extremely heterogeneous and rapidly changing cell population. Along with mesenchymal stem cells, these cells are erythroid, lymphoid, and myeloid cells of varying degrees of maturity. As it is known, blood cells have different lifespans. While progenitors of all blood cells and mesenchymal stem cells are immortal, differentiated blood cells have a short and greatly varying lifespan. Thus, while erythrocytes in the blood stream function for about 3 months, leukocytes function from several hours (neutrophils) and several weeks (lymphocytes), and memory cells function up to a decade [12, 21].

The program of cell death can be enabled under the influence of various exogenous and endogenous factors. In fact, the lifespan of cells is an integral indicator of the interference of various signals that determine the strategic choice between further functioning, proliferation, or apoptosis.

Consequently, the choice of cell behavior strategy depends on endogenous and exogenous factors. It can be assumed that, if a highly heterogeneous population of bone marrow cells is isolated, intact and pathological, in young and old animals and placed in the same conditions in vitro, then the different natures of their behavior under strictly controlled conditions will probably depend on the initial characteristics of these cells. If the pool of stem cells decreases in old animals, then the rate of the proliferation of bone marrow cells in the primary culture without the administration of, any inducers of proliferation, should be inferior to those in young ones. Reduction of the pool of stem cells in old animals can affect the quantitative and qualitative nature of cell differentiation in the bone marrow, that is, the degree of heterogeneity of the cell population and the time of cell cultivation in the primary culture.

In the study of stem cells, as a rule, they are isolated from a heterogeneous population in which they constitute no more than 0.01% in order to study their properties in culture. In this study, we determined the growth rate of the primary culture of bone marrow cells, the effect of copper ion concentrations on the growth rate, the pattern of the main morphotypes of bone marrow cells, and the lifespan of cells in the primary culture obtained in intact young and old animals and in rats with Copper-induced liver fibrosis.

MATERIALS AND METHODS

The experiments were carried out on mature male Wistar 3-month-old (young) and 20-month-old (old) rats. All procedures with animals were performed in compliance with bioethical rules and norms [10] and with an accounting for the circadian rhythms of the formation of biological responses. For this, animals received food at the same time of day and were kept in standard vivarium conditions, and all manipulations (administration of toxic drugs, isolation of bone marrow cells) were carried out at the same time of day before meals. Twelve hours before the isolation of bone marrow cells, the animals did not receive food. Bone marrow cells were always isolated from 8 to 10 a.m. local time; counting and morphological studies were carried out from 9 to 12 a.m. local time.

The experimental procedures were stopped by animal immersion in ether anesthesia and isolation of the bone marrow cells.

All animals were divided into four groups (Fig. 1). The first, the true control group, included bone marrow cells isolated from intact 3-month-old ($n = 8$) and 20-month-old ($n = 6$) animals. The second was comprised of bone marrow cells from 3-month-old ($n = 12$) and 20-month-old ($n = 6$) animals with Copper-induced liver fibrosis, which was modeled as described in [1]. The third included bone marrow cells from intact 3-month-old ($n = 6$) and 20-month-old ($n = 6$) animals whose bone marrow cell culture at the beginning of the incubation was administered with 4 and 8 mmol of $CuSO_4 \cdot 5H_2O$. The fourth consisted of bone marrow cells from 3-month-old ($n = 6$) and 20-month-old ($n = 6$) animals with Copper-induced liver fibrosis, and this culture was administered with 4 and 8 mmol of $CuSO_4 \cdot 5H_2O$ (see Fig. 1).

Three variants of culture for the in vitro system were formed from the obtained cells of all four variants. The first was administered with physiological solution (control culture), the second was administered with 4 mmol of $CuSO_4 \cdot 5H_2O$, and the third was administered with 8 mmol of $CuSO_4 \cdot 5H_2O$. After 1, 2, 3, and 4 days of cultivation, the number of cells, morphotypes, and the time of preservation of the main morphotypes in culture—the "life lifespan"—were determined.

Bone marrow cells were isolated from the two femoral bones of the rat by a method in the literature [2] and cultured in medium 199 with the addition of antibiotics (8% gentamicin and 8% streptomycin) and 20% inactivated fetal bovine serum. Cultivation was carried out at 37°C under standard conditions in an atmosphere of 5% CO_2 for 4 days. The culture of the cells was not changed (cumulative cultivation). The initial concentration of bone marrow cells in cultivation in all variants was always 2 million cells/mL.

The calculation and evaluation of the viability of bone marrow cells of animals was carried out as described in [8]. The morphotypes of bone marrow cells were determined immediately after the obtainment of a suspension of bone marrow cells, as well as on cultivation days 2 and 4 as described in the literature [20]. The cytological preparations were stained by Romanowsky-Giemsa and analyzed at 100× zoom with a ZeissPrimoStariLED microscope (Germany).

To determine the lifespan of myelocytes, metamyelocytes, and stab and segment neutrophils in the in

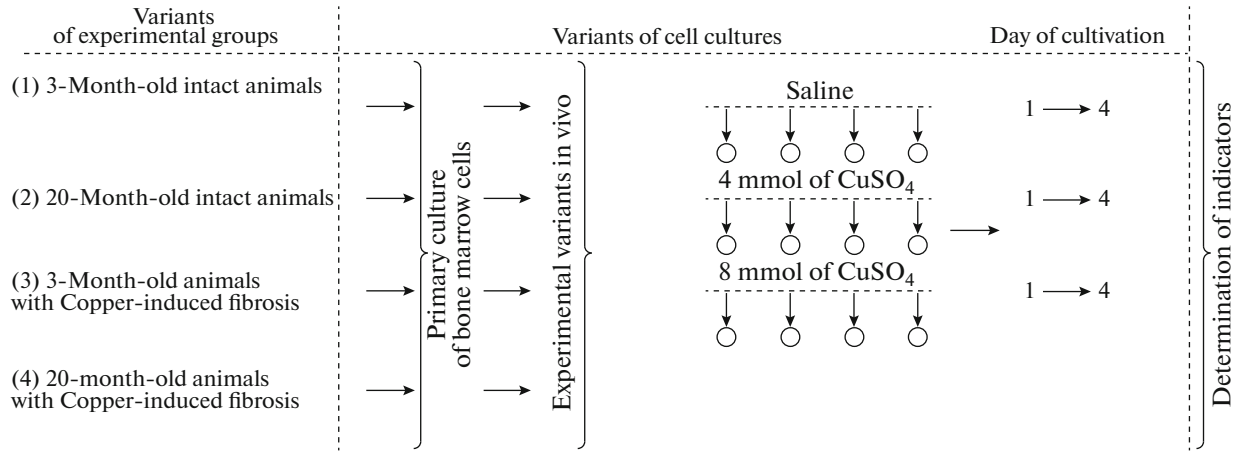

Fig. 1. Scheme of experimental procedures, which demonstrates that bone marrow cells were isolated from young and old animals, intact animals, and animals with Copper-induced fibrosis (variants of experimental groups).

vitro system, their numbers for 0, 48, and 96 h of culture were counted, with the counting of at least 500 cells of each of the morphotypes; the data were expressed in fractions of the total number of cells in the sample.

All experiments were repeated multiple times, with 6–12 animals used in each experimental group.

The characteristics of the obtained samples included the mean, standard deviation, standard error of the mean, and the sample size. The statistical significance of the differences between the two groups of data was estimated with the nonparametric Mann–Whitney test. The results were processed in Excel. Differences between the control and experimental data were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Growth Rate of Primary Cultures of Bone Marrow Cells of Young and Old Intact Animals and Rats with Copper-Induced Liver Fibrosis

The number of bone marrow cells in young rats increased by 44, 70, and 77%, respectively, on days 1, 2, and 3 as compared to the initial number, and a steady growth phase occurred on day 4 (Fig. 2a).

When bone marrow cells were obtained from old animals, their number in the culture increased on days 1, 2, and 3 twice as fast as in young animals, by 113, 140, and 171%, respectively, as compared to the initial quantity, while retaining the nature of the growth curve (see Fig. 2a).

Consequently, the rate of proliferation of bone marrow cells in the primary culture is more pronounced in old animals than in young animals. This indicates that there are more bone marrow cells (mesenchymal stem cells, primarily) capable of proliferation in old animals than in young animals. It is known that, in the presence of pathologies, in particular liver fibrosis, mesenchymal bone marrow stem cells migrate to the inflammatory focus and provide regenerative processes in the pathologically altered organ [5].

In the next series of experiments, liver fibrosis was induced in young and old animals [1], and the proliferative activity of bone marrow cells in these animals was determined in culture. It turned out that the number of bone marrow cells isolated in young animals with liver fibrosis also increased in the primary culture, as in the case of intact animals. Thus, on cultivation days 1, 2, and 3, their number increased by 30, 60, and 95% in comparison with the initial amount (see Fig. 2b).

At the same time, the number of bone marrow cells in the primary culture isolated from old animals with liver fibrosis increased to a lesser extent than in the case of intact old animals and differed little from this index in young animals with fibrosis (see Fig. 2b).

It is necessary to pay attention to the fact that the number of cells in culture obtained from old animals with fibrosis did not change linearly but rhythmically. Thus, their number increased by 65, 25, and 80%

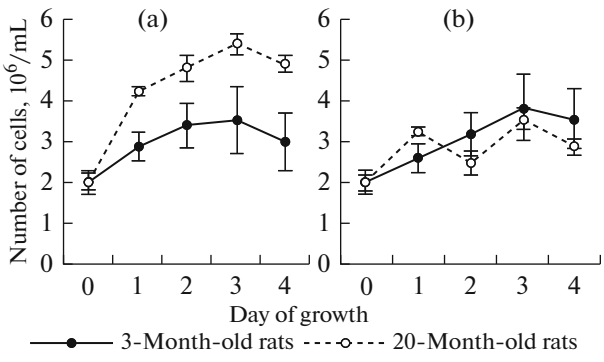

Fig. 2. Number of bone marrow cells in the primary culture isolated from intact 3-month and 20-month-old rats (a) and from 3-month and 20-month-old rats with Copper-induced liver fibrosis (b).

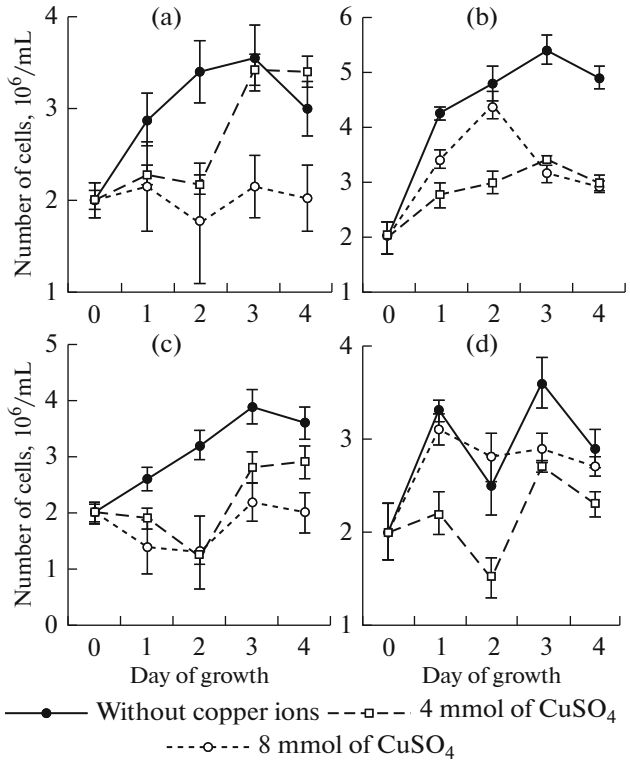

Fig. 3. Number of bone marrow cells in the primary culture isolated in intact 3-month (a) and 20-month-old rats (b) and 3-month (c) and 20-month-old rats (d) with Copper-induced liver fibrosis without administration of copper ions into culture, after adding 4 mmol of $CuSO_4$ and 8 mmol of $CuSO_4$ at the start of cultivation.

against 113, 140, and 171% for intact animals (see Figs. 2a and 2b). This type of change in the number of cells in culture indicates that the processes of cell degradation predominate over division processes in the primary culture on day 2.

Therefore, the proliferative activity in the primary culture of bone marrow cells obtained in young animals with liver fibrosis differed little from that obtained in the intact control group and significantly decreased in old animals with liver fibrosis as compared to old intact animals.

Since liver fibrosis was induced by successive administrations of copper sulfate to animals, it cannot be ruled out that copper ions can have both direct and indirect effects on the number and properties of bone marrow cells. Moreover, these changes may also have an age-dependent nature of the response to the administration of copper ions.

Effect of Exogenous Copper Ions on the Growth Rate of Bone Marrow Cell Culture of Young and Old Animals and Rats with Liver Fibrosis

The next series of experiments determined the effect of copper ions applied directly to the in vitro cell culture; the cells were isolated from intact animals in one case and from young and, respectively, old animals with Copper-induced fibrosis liver in another case (see Fig. 1).

Such an experimental approach should answer the following questions. Does the response of bone marrow cells in the culture to the effect of exogenous copper ions (toxicants) change depending on the initial state of the cells (intact animals and animals with fibrosis)? Is there is an age-dependent nature of the response to the exogenous effect of copper ions? Is it possible to use such an experimental approach to estimate the integral state of bone marrow cells? It turned out that, if bone marrow cells for further cultivation were obtained from intact 3-month-old animals and 4 mmol of exogenous copper sulfate were added to such a culture, then the number of cells did not change after 1 and 2 days as compared to the initial amount (2×10^6 cells/mL); that is, the culture did not grow (Fig. 3a).

However, on the third day, their number increased in comparison with the initial value by 70% and did not differ from the control culture, that is, without the administration of exogenous copper (see Fig. 2a). In the case when 8 mmol of $CuSO_4 \cdot 5H_2O$ was added to the culture, the number of cells from day 1 to day 4 remained constant and was about 2×10^6 cells/mL (see Fig. 3a).

The preservation of the number of bone marrow cells at an unchanged level from cultivation day 1 to day 4 can be explained by the fact that the number of dead cells corresponded to the number of newly appeared ones as a result of proliferation.

The viability of cells in culture was evaluated in a trypan blue test. It was found that, when the viability of cells was 96–98% in the initial suspension, it was 75 ± 1.2% by day 4. The administration of 8 mmol of $CuSO_4 \cdot 5H_2O$ into the bone marrow culture was accompanied by a slight (up to 10%) increase in nonviable cells.

In the case when bone marrow cells were isolated from intact 20-month-old animals and 4 mmol of $CuSO_4 \cdot 5H_2O$ were added to the culture, the number of bone marrow cells increased by 38, 50, and 71% as early as cultivation days 1 and 2 compared to their original quantity (see Fig. 3b).

After 8 mmol of $CuSO_4 \cdot 5H_2O$ was applied to the culture of cells obtained from 20-month-old animals, a paradoxical effect was observed in terms of the growth rate compared to the administration of 4 mmol of $CuSO_4 \cdot 5H_2O$ and compared with the same dose for bone marrow cells isolated from 3-month-old animals (see Figs. 3a and 3b).

Thus, after the administration of 8 mmol of $CuSO_4 \cdot 5H_2O$ into the culture, the number of bone marrow cells increased by 71% on day 1 and by 112% on day 3 and did not differ from the number of cells obtained from intact animals without adding copper ions (see

Fig. 3b); that is, copper ions in this case did not inhibit the growth of the culture, as it did in young animals. However, on day 3 and 4, the number of bone marrow cells decreased and did not differ from the number of cells after 4 mmol of $CuSO_4 \cdot 5H_2O$ was administered into the culture (see Fig. 3b); that is, there was a "lag" in the manifestation of the toxic effect of copper. Consequently, after the administration of copper ions, a dose-dependent response of the bone marrow cell culture is revealed, and it differed for the cell cultures obtained in young and old animals.

In the next series of experiments, the effect of exogenous copper was determined. It was administered into the cell cultures of young and old animals with Copper-induced liver fibrosis. It turned out that, when 4 mmol of $CuSO_4 \cdot 5H_2O$ was administered into the culture of bone marrow cells isolated from young animals with Copper-induced liver fibrosis, the number of cells in culture decreased by 5 and 40% on cultivation days 1 and 2 as compared to their the original quantity. After 2 days, the number of cells increased by 40% as compared to the initial quantity on day 3 and remained the same on day 4 (see Fig. 3c). When 8 mmol of $CuSO_4 \cdot 5H_2O$ was added to this cell culture, the number of cells decreased on day 1 by 30% and did not change further in comparison with the initial quantity (see Fig. 3c).

In the case when bone marrow cells were isolated from old animals with Copper-induced liver fibrosis and with the administration of 8 mmol of $CuSO_4 \cdot 5H_2O$, the number of cells was decreasing by day 2, increasing by day 3, and subsequently did not change significantly; that is, the share of the inhibition of the growth of the culture was expressed to a lesser extent than in young animals (see Fig. 3d). After the administration of 4 mmol of $CuSO_4 \cdot 5H_2O$ into the culture of bone marrow cells from old animals, a 55% increase in the number of cells from the initial value was observed on day 1, and it subsequently remained at the same level for up to 4 days; that is, 4 mmol had a smaller inhibitory effect than 8 mmol, in cells of intact animals as well (see Figs. 3b and 3d). Consequently, the age-dependent nature of the response of bone marrow cells to copper sulfate was maintained regardless of the state of the organism. At the same time, the effect of proliferation inhibition by endogenous copper in bone marrow cells isolated from animals with liver fibrosis was more pronounced than in the case of isolation of bone marrow cells in intact animals.

Pattern of Main Morphotypes of Bone Marrow Cells in Intact Animals and in Young and Old Rats with Copper-induced Liver Fibrosis

As it is known, the population of bone marrow cells is represented by extremely diverse types; that is, it is highly heterogeneous [5, 23]. Such heterogeneity in the morphotypes of bone marrow cells is due to several reasons: the functional feature of bone marrow cells (bone marrow is the site of differentiation of blood cells and immunocompetent cells); the difference in the rate of differentiation and transport of cells synthesized into the bloodstream; the functional "request" of the body for the type of immunocompetent cells and blood cells; and the difference in the responses of bone marrow cells to microenvironment factors.

In our experiments, eight basic morphotypes of bone marrow cells were morphologically clearly detected in young and old animals. The number of these morphotypes in the studied animals is shown in Fig. 4. It turned out that these eight morphotypes in young intact animals accounted for 58.6% of all bone marrow cells (see Fig. 4a).

It follows that the remaining 41.4% are morphologically unidentifiable cell types. At the same time, these eight morphotypes accounted for 79% in intact old animals; that is, unidentified cell types accounted for only 21% (see Fig. 4c).

In young animals with fibrosis, the number of detected morphotypes decreased by 13.4% as compared to intact animals, while it increased in old animals by 10% and amounted to 89% (see Fig. 4).

In old animals, not only the number of identifiable morphotypes was changed, but also the ration between the types of cells (see Figs. 4b and 4d). The greatest differences between young and old animals were found in the lymphocyte number: in old animals, it was 2.8 times greater than in young animals (see Fig. 4b).

Fibrosis induction was accompanied by changes in the pattern of morphotypes, and this was more pronounced for young animals than intact old animals. Thus, there was a decrease in their number of stab neutrophils and myelocytes (by 2.9 times) and eosinophils (by 11 times) in comparison with intact animals of the same age (see Figs. 4b and 4d).

At the same time, in old animals with liver fibrosis, the pattern of morphotypes changed little in comparison with intact animals: the number of stab neutrophils and lymphocytes increased insignificantly, while the number of metamyelocytes, segmented neutrophils, myelocytes, eosinophils, basophils, and monocytes did not change (see Figs. 4b and 4d). Consequently, young and old animals differed not only in the total number of identifiable morphotypes but also in the ratio of these cells (the pattern of distribution). The development of liver fibrosis altered the pattern of morphotypes in young animals and did not affect it in old animals.

As it is known, bone marrow cells vary greatly in life lifespan, from a few days to a decade [11, 17]. The next series of experiments determined the time of preservation of certain types of bone marrow cells in culture, conditionally their "lifespan."

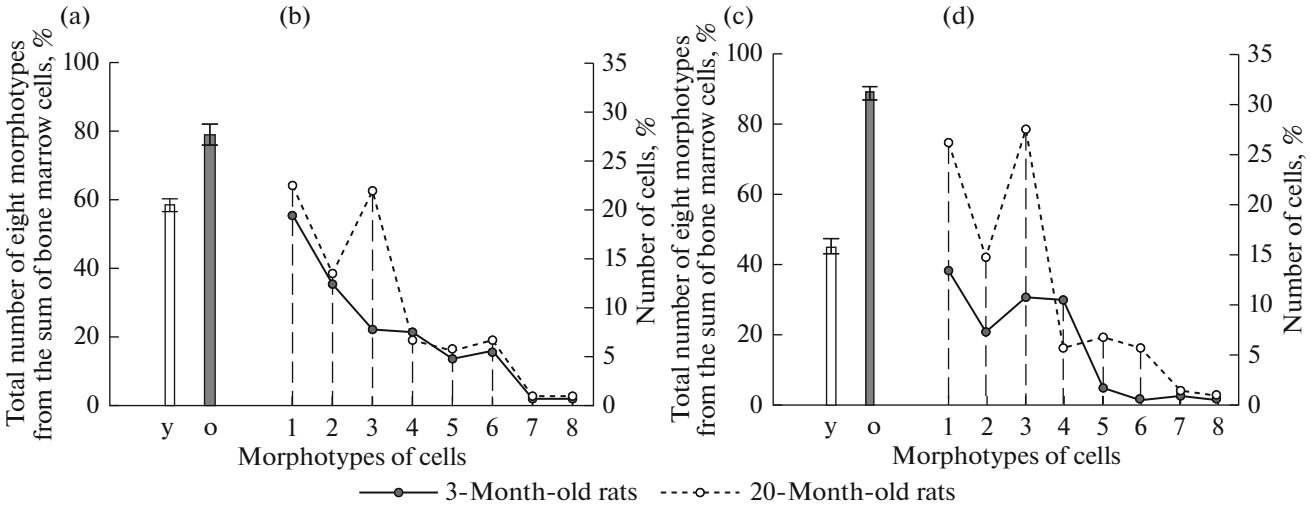

Fig. 4. Total number of bone marrow cells from the total number of those isolated from young (y) and old (o) rats; isolated from intact animals (a) and animals with liver fibrosis (c); the pattern of morphotypes of bone marrow cells, respectively: 1, stab neutrophils; 2, metamyelocytes; 3, lymphocytes; 4, segmented neutrophils; 5, myelocytes; 6, eosinophils; 7, basophils; 8, monocytes in young and old animals, for intact groups (b) and groups with fibrosis (d).

Lifespan of Bone Marrow Cells in Primary Culture from 0 to 96 h in Young and Old Intact Animals and in Rats with Copper-Induced Liver Fibrosis

As it is known, neutrophils are the most numerous group of leukocytes. Neutrophils mature in bone marrow cells from the stem cell and pass through a series of stages: promyelocyte → myelocyte → metamyelocyte → stab neutrophil [14].

The transfer of myelocytes to the primary culture with no differentiation factors can be accompanied by rapid degradation. We believe that the rate of their destruction can be different in young and old animals, in both intact animals and animals with Copper-induced liver fibrosis. These differences may be associated with epigenetic features, stages of differentiation and, as a consequence, different responses to a new microenvironmental system (in vitro).

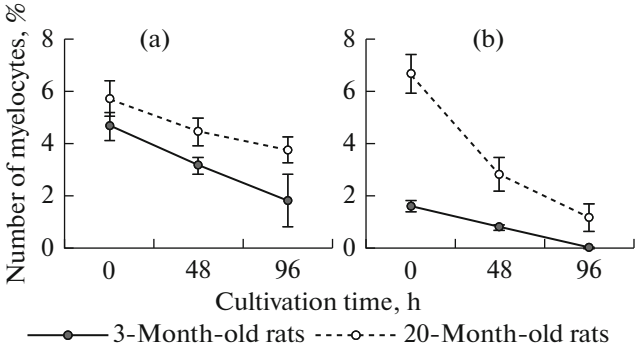

Fig. 5. Number of myelocytes in the primary culture obtained from intact 3-month-old and 20-month-old animals (a) and in 3-month-old and 20-month-old animals with Copper-induced liver fibrosis (b).

It turned out that the preservation, "lifespan," of different types of cells in the primary culture isolated from young and old animals varied. Thus, the number of myelocytes in the primary culture obtained from young intact animals decreased almost linearly; it reached 48% of the original amount after 48 h and 39% after 96 h (Fig. 5a). When the myelocytes were obtained from old animals, their number decreased at a lower rate. It was 78% after 48 h and 66% after 96 h; that is, their number was almost 2 times higher than in young animals at the same time (see Fig. 5a).

However, when myelocytes were obtained in young animals with liver fibrosis, the rate of their degradation was greater than for cells of intact animals. In this case, 96 h after transfer to culture, only 1.8% remained, and 17% of the initial amount remained in old ones (see Fig. 5b). Therefore, the presence of liver fibrosis in animals affected the lifespan of myelocytes in the primary culture.

The metamyelocyte number in the cultivation process decreased with a lower rate in comparison with myelocytes if they were obtained from intact animals (Fig. 6a). Thus, after 96 h, their number was 63% of the initial amount for young intact animals and 74% for old animals. The presence of liver fibrosis in experimental animals reduced the lifespan of metamyelocytes in the primary culture for young animals by 7.6 times compared to young intact ones and 4.3 times for cells of old animals 96 h after cultivation (see Figs. 6a and 6b).

The number of stab neutrophils in the primary culture of young and old intact animals decreased to the same extent (Fig. 7a). The presence of liver fibrosis in young animals differently influenced the preservation

of stab neutrophils in the culture of cells of young and old animals (see Fig. 7b).

The number of segmented neutrophils did not change during 96 h of cultivation when they were obtained from intact young and old animals (Fig. 8a). However, when they were obtained from young animals with liver fibrosis, their number decreased linearly; after 96 h, it was no more than 12% of the original. At the same time, the number of segmented neutrophils obtained in old animals with fibrosis did not change during cultivation (see Fig. 8b).

Consequently, the lifespan in the primary culture depends on the age of the animals. The presence of liver fibrosis accelerated the process of death of various cell morphotypes, with the exception of segmented neutrophils of old animals. The results of the work can be reduced to two main provisions:

—the proliferative potential of bone marrow cells of old animals was not inferior to or superior to that of young animals, at least in the in vitro system;

—the total pool of bone marrow cells of old animals differed from that of young animals by their cellular composition, the ability to respond to the action of exogenous copper ions in the primary culture, the lifespan in culture, and the formation of a response in liver fibrosis.

These results may indicate that bone marrow cells undergo epigenetic changes in ontogeny that affect not only the ratio of cell morphotypes but also the functional features within the identified morphotypes.

Considering this question, we are faced with the problem of identifying cell types and their functional features. As was already noted, bone marrow cells are represented by a highly heterogeneous, dynamically changing population. The morphometric method and/or characterization of surface markers are most commonly used for the identification of cell types [14]. The identification of cells by morphological features makes it possible to identify only those cell types that have expressed and long-lasting structural features, for example, lymphocytes, monocytes, neutrophils, etc.

However, this method does not make it possible to identify the wide variety of morphofunctional cell variants that exist in bone marrow cells, in particular multipotent mesenchymal stromal cells (MMSCs) and bone marrow cells located at different stages of differentiation.

Surface antigen markers are used to identify MMSCs. As a rule, different laboratories employ their own sets of such surface markers. Attempts to develop standard methods for determining the characteristics of MMSCs have not yet been successful. This can be explained by the lack of rigid specificity of surface markers for MMSCs. The task is even more difficult when it concerns not stem cells but other undifferentiated types of cells. We believe that these difficulties in identifying and defining MMSCs in old and young animals lead to ambiguity and contradictory answers

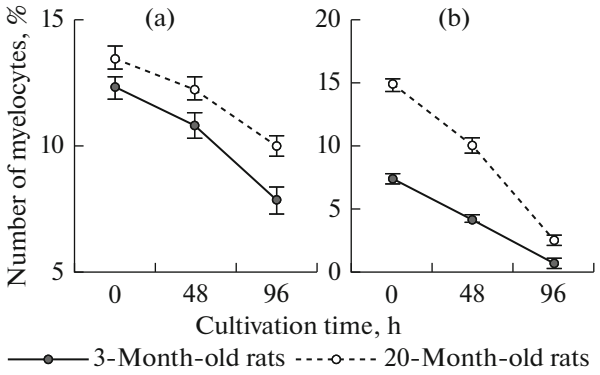

Fig. 6. Number of metamyelocytes in the primary culture obtained from intact 3-month-old and 20-month-old animals (a) and from 3-month-old and 20-month-old animals with Copper-induced liver fibrosis (b).

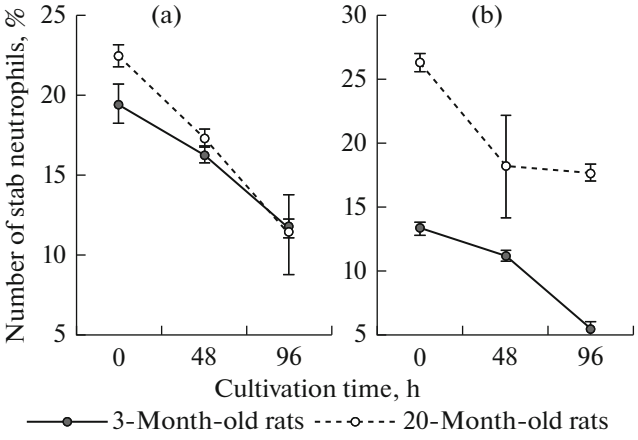

Fig. 7. Number of stab neutrophils in the primary culture obtained from intact 3-month-old and 20-month-old animals (a) and from 3-month-old and 20-month-old animals with Copper-induced liver fibrosis (b).

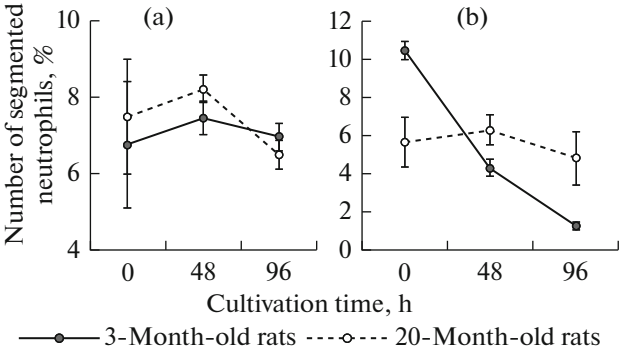

Fig. 8. Number of segmented neutrophils in the primary culture obtained from intact 3-month-old and 20-month-old animals (a) and from 3-month-old and 20-month-old animals with Copper-induced liver fibrosis (b).

to this question. In such a situation, it is advisable to evaluate not so much the structural composition of the bone marrow cell pool as the functional response of cells to various effects, which was realized in the present work.

As it is known, in the cultivation of various cell types (from bacteria to eukaryotes) under the regime of so-called accumulative cultivation, the culture passes through different stages: the lag stage, exponential growth stage, stationary stage, and death stage [3]. It is generally accepted that the rate of culture growth is always replaced by a stationary phase, during which two parallel processes (proliferation and degradation) "equalize," and the predominance of the death process over proliferation leads to the death of the culture [9].

Consequently, the culture growth curve reflects the interconnection of the processes of emergence of new cells and the reduction of the number of cells due to apoptosis, necrosis, or autophagy of cells in culture. From this, it follows that the greater rate of growth of the bone marrow cell culture of old intact animals can be due to both a greater number of dividing cells in the culture and a smaller number of dying cells in the culture.

Determination of the rate of "death" of myelocytes, metamyelocytes, and stab and segmented neutrophils in the primary culture of bone marrow cells obtained from intact young and old animals showed that they have a somewhat longer lifespan in old animals than cells from young animals in the in vitro system.

It is necessary to pay attention to the large differences in the lifespan of bone marrow cells of old and young animals with liver fibrosis. Thus, while only 1.8% of myelocytes and 8.2% of metamyelocytes were detected in young animals with liver fibrosis on the 96th h of cultivation, it was 17 and 17%, respectively, in old animals. Consequently, bone marrow cells obtained from animals with liver fibrosis were much more rapidly destroyed in the primary culture than cells obtained from intact animals. However, it occurred at different rates for different types of cells and animals of different ages. The rate of destruction of bone marrow cells in young animals was higher for both cells obtained in intact animals and for bone marrow cells obtained from animals with liver fibrosis.

Thus, while the number of myelocytes for young animals with fibrosis decreased 21 times faster over 96 h of cultivation than in the case of intact bone marrow cells in culture, it was only 3.8 times faster for the old animals. This difference was 7.6 times greater for metamyelocytes isolated from young animals with liver fibrosis and 4.3 times greater for those from old animals. Stab neutrophils in young animals with liver fibrosis were slowly destroyed, and their destruction in old animals did not differ from that in intact animals. In the case of segmented neutrophils, they were destroyed 7.2 times faster in young animals and 1.2 times faster in old animals only.

Consequently, the lifespan of bone marrow cells in the primary culture is affected by

—the age of the donor (in old animals, bone marrow cells persist longer in the primary culture);

—the presence of liver fibrosis (accelerates the process of death but to a greater extent in young animals);

—the differentiation stages by storage times are realized in the following order: segmented neutrophils → stab neutrophils and metamyelocytes → myelocytes for young and old animals.

At the same time, a lower rate of elimination of bone marrow cells in culture obtained from old animals does not make it possible to explain the twofold increase in the number of cells from growth day 1 to day 3 in comparison with those of young animals.

During ontogeny, it is not that the stem cell pool lessens but that epigenetic changes in bone marrow cells occur. These epigenetic changes are due to the fundamental properties of temporality of functioning, which is manifested in the accumulation of metabolic memory. Such cells acquire new characteristics and, in comparison with the cells of young animals, react to the same effects in a different way.

CONCLUSIONS

The ability of bone marrow cells to proliferate in primary culture in old animals was not inferior to or was superior to that of young animals.

The presence of liver fibrosis was accompanied by a decrease in the total number of identified cell types (stab neutrophils, metamyelocytes, lymphocytes, segmented neutrophils, myelocytes, eosinophils, basophils, and monocytes) in young animals and an increase in the number of unidentified cell types; in old animals, on the contrary, the number of identified increased and the number of unidentified cell types decreased.

Cultures of bone marrow cells of young and old animals responded differently to the administration of exogenous ions: they inhibited proliferation more strongly in young animals with manifestation of a direct-dose dependence. Bone marrow cells of old animals are less sensitive to copper ions and showed inverse dose dependence for both intact animals and animals with liver fibrosis.

The lifespan of bone marrow cells in the primary culture of old animals was greater than in young animals. The presence of fibrosis accelerated the rate of cell death in the primary culture in both young and old animals.

REFERENCES

1. Bozhkov, A.I., Nikitchenko, Yu.V., Klimova, E.M., Linkevych, O.S., Lebid, K.M., Al-Bahadli, A.M.M., and Alsardia, M.M.A., Young and old rats have different strategies of metabolic adaptation to Copper-

induced liver fibrosis, *Adv. Gerontol.*, 2017, vol. 7, no. 1, pp. 41–50.
2. Amend, S., Valkenburg, K., and Pienta, K., Murine hind limb long bone dissection and bone marrow isolation, *J. Visualized Exp.*, 2016, vol. 2016, pp. 12–22.
3. Bara, J., Richards, R., Alini, M., et al., Concise review: Bone marrow-derived mesenchymal stem cells change phenotype following in vitro culture: implications for basic research and the clinic, *Stem Cells*, 2014, vol. 32, pp. 1713–1723.
4. Beane, O., Fonseca, V., Cooper, L., et al., Impact of aging on the regenerative properties of bone marrow-, muscle-, and adipose-derived mesenchymal stem/stromal cells, *PLoS One*, 2014, vol. 12, p. e115963.
5. Berardis, S., Sattwika, P., Najimi, M., et al., Use of mesenchymal stem cells to treat liver fibrosis: Current situation and future prospects, *World J. Gastroenterol.*, 2015, vol. 21, pp. 742–758.
6. Bianco, P., Cao, X., Frenette, P., et al., The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine, *Nat. Med.*, 2013, vol. 19, pp. 35–42.
7. Blach-Olszewska, Z., Zaczynska, E., Gustaw-Rothenberg, K., et al., The innate immunity in Alzheimer disease—relevance to pathogenesis and therapy, *Curr. Pharm. Des.*, 2015, vol. 21, pp. 3582–3588.
8. Bozhkov, A., Kabachnyy, V., Kolot, N., et al., Hormesis effect and the influence of ultra-low glycosides doses on the bone marrow cells proliferative activity in culture, *J. Harmonized Res. Pharm.*, 2014, vol. 3, pp. 154–166.
9. Cooper, S., Distinguishing between linear and exponential cell growth during the division cycle: single-cell studies, cell-culture studies, and the object of cell-cycle research, *Theor. Biol. Med. Model.*, 2006, vol. 3, p. 10.
10. Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes, *Off. J. Eur. Union*, 1986, vol. 358, pp. 1–28.
11. Ding, L. and Morrison S. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches, *Nature*, 2013, vol. 495, pp. 231–235.
12. Franco, R., Measurement of red cell lifespan and aging, *Med. Hemother.*, 2012, vol. 39, pp. 302–307.
13. Gekas, C. and Graf, T., CD41 expression marks myeloid-biased adult hematopoietic stem cells and increases with age, *Blood*, 2013, vol. 121, pp. 4463–4472.
14. Glasser, L. and Fiederlein, R., Functional differentiation of normal human neutrophils, *Blood*, 1987, vol. 69, pp. 937–944.
15. Ideker, T., Galitski, T., and Hood, L., A new approach to decoding life: systems biology, *Ann. Rev. Genomics Hum. Genet.*, 2001, vol. 2, pp. 343–372.
16. Kim, J.M., Kim, J., Kim, Y., et al., Comparative secretome analysis of human bone marrow-derived mesenchymal stem cells during osteogenesis, *J. Cell. Physiol.*, 2013, vol. 228, no. 1, pp. 216–224.
17. Kolaczkowska, E. and Kubes, P., Neutrophil recruitment and function in health and inflammation, *Nat. Rev. Immunol.*, 2013, vol. 13, no. 3, pp. 159–175.
18. Krause, D., Fulzele, K., Catic, A., et al., Differential regulation of myeloid leukemias by the bone marrow microenvironment, *Nat. Med.*, 2013, vol. 19, pp. 1513–1517.
19. Krause, D., Theise, N., Collector, M., et al., Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell, *Cell*, 2001, vol. 105, no. 3, pp. 369–377.
20. Lewandowski, K., Kowalik, M., Pawlaczyk, R., et al., Microscopic examination of bone marrow aspirate in healthy adults—comparison of two techniques of slide preparation, *Int. J. Lab. Hematol.*, 2012, vol. 34, pp. 254–261.
21. McCracken, J. and Allen, R., Regulation of human neutrophil apoptosis and lifespan in health and disease, *J. Cell Death*, 2014, vol. 7, pp. 15–23.
22. Mezey, E., Chandross, K., Harta, G., et al., Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow, *Science,* 2000, vol. 290, no. 5497, pp. 1779–1782.
23. Morrison, S. and Scadden, D., The bone marrow niche for haematopoietic stem cells, *Nature*, 2014, vol. 505, pp. 327–334.
24. Orlic, D., Kajstura, J., Chimenti, S., et al., Bone marrow cells regenerate infarcted myocardium, *Nature,* 2000, vol. 410, pp. 701–705.
25. Pang, W., Price, E., Sahoo, D., et al., Human bone marrow hematopoietic stem cells are increased in frequency and myeloidbiased with age, *Proc. Natl. Acad. Sci. U.S.A.*, 2011, vol. 108, pp. 20012–20017.
26. Pellicoro, A., Ramachandran, P., Iredale, J., et al., Liver fibrosis and repair: immune regulation of wound healing in a solid organ, *Nat. Rev. Immunol.*, 2014, vol. 14, pp. 181–194.
27. Rando, T. and Wyss-Coray, T., Stem cells as vehicles for youthful regeneration of aged tissues, *J. Gerontol., Ser. A*, 2014, vol. 69, pp. 39–42.
28. Rossi, D., Bryder, D., Zahn, J., et al., Cell intrinsic alterations underlie hematopoietic stem cell aging, *Proc. Natl. Acad. Sci. U.S.A.*, 2005, vol. 102, no. 26, pp. 9194–9199.
29. Sharpless, N. and DePinho, R., How stem cells age and why this makes us grow old, *Nat. Rev. Mol. Cell Biol.*, 2007, vol. 8, pp. 703–713.
30. Sudo, K., Ema, H., Morita, Y., et al., Age-associated characteristics of murine hematopoietic stem cells, *J. Exp. Med.,* 2000, vol. 192, no. 9, pp. 1273–1280.
31. Zhou, S., Greenberger, J., Epperly, M., et al., Age-related intrinsic changes in human bone marrow-derived mesenchymal stem cells and their differentiation to osteoblasts, *Aging Cell*, 2008, vol. 7, pp. 335–343.

Translated by K. Lazarev