ИССЛЕДОВАНИЕ ФЕНОТИПИЧЕСКИХ И ЦИТОТОКСИЧЕСКИХ СВОЙСТВ ЭРИТРОИДНЫХ КЛЕТОК СЕЛЕЗЕНКИ ПРИ ГЕМОПОЭЗ-СТИМУЛИРУЮЩИХ ВОЗДЕЙСТВИЯХ

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Резюме. В последние годы исследования выявили большое разнообразие функций эритроидных клеток, в том числе в модуляции врожденного и адаптивного иммунного ответа. Анемический или гипоксический стресс стимулирует физиологический ответ в виде стрессового эритропоэза, направленного на увеличение доставки кислорода к тканям. При стрессовом эритропоэзе активируются клетки-предшественники и используются механизмы, которые отличаются от стационарного эритропоэза костного мозга. Для рассмотрения роли эритроидных клеток в регуляции гемопоэза были смоделированы гемопоэз-активирующие состояния: химически индуцированная гемолитическая анемия, острая кровопотеря, гипоксия. Серию экспериментов проводили на мышах-гибридах первого поколения CBA C57Bl6. Выделение эритроидных клеток проводили с помощью магнитной сепарации по маркеру CD71. Стадии дифференцировки эритроидных клеток определяли по сочетанию экспрессии маркеров TER-119, CD71 и параметров прямого светорассеяния в популяции как CD45-позитивных, так и CD45-негативных клеток селезенки. Для изучения иммунорегуляторной активности эритроидных клеток мы исследовали опосредованную цитотоксичность спленоцитов против опухолевых клеток линии мышиной меланомы B78 после культивирования с кондиционными средами селезенок после различных гемопоэз-стимулирующих воздействий. При различных гемопоэз-стимулирующих воздействиях происходит реорганизация количественного и качественного состава клеток селезенки в зависимости от компенсаторного механизма для восстановления гомеостаза. Анализ клеточного состава селезенки показал, что при гемопоэз-стимулирующих воздействиях происходит перераспределение популяций с маркером CD45: при гипоксии резко снижается количество CD45-негативных клеток и повышается количество CD45-позитивных клеток. Популяция базофильных эритробластов наименее подвержена количественному изменению при всех гемопоэз-стимулирующих воздействи-

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ях. При гипоксии наблюдается наиболее заметное изменение клеточного состава селезенки за счет повышенного накопления CD45-позитивных эритроидных клеток в селезенке. Медиаторы эритроидных клеток селезенки мышей после гипоксии не приводят к усилению цитотоксического проапоптотического действия спленоцитов на опухолевые клетки в отличие от эритроидных клеток нормальной селезенки, селезенки при анемии и кровопотере. Таким образом, именно тканевая гипоксия является процессом, который не только стимулирует эритропоэз, но и приводит к максимальному изменению супрессивных свойств окружающих клеток. Мы предполагаем, что реализация компенсаторных механизмов при исследованных гематопоэз-стимулирующих воздействиях направлена на активацию механизмов врожденного иммунитета и локальной иммуносупресии для предотвращения местного воспаления, накопления питательных веществ и привлечения клеточных элементов в очаг гемопоэза для восстановления гомеостатических функций.

Ключевые слова: эритробласты, селезенка, анемия, гипоксия, острая кровопотеря, терминальная дифференцировка

STUDY OF PHENOTYPIC AND CYTOTOXIC PROPERTIES OF ERYTHROID CELLS OF THE SPLEEN UNDER HEMATOPOIESIS-STIMULATING EFFECTS

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Abstract. In recent years, research has revealed a wide variety of erythroid cell functions, including modulation of innate and adaptive immune responses. Anemic or hypoxic stress stimulates a physiological response in the form of stress erythropoiesis, aimed at increasing oxygen delivery to tissues. Stress erythropoiesis activates progenitor cells and uses mechanisms that differ from stationary bone marrow erythropoiesis. To consider the role of erythroid cells in the regulation of hematopoiesis, hematopoiesis-activating states were modeled: chemically induced hemolytic anemia, acute blood loss, hypoxia. A series of experiments was carried out on first-generation hybrid mice CBA C57Bl6. Isolation of erythroid cells was performed using magnetic separation for the CD71 marker. The stages of differentiation of erythroid cells were determined by the combination of expression of TER-119 and CD71 markers and direct light scattering parameters in the population of both CD45-positive and CD45-negative spleen cells. To study the immunoregulatory activity of erythroid cells, we investigated the mediated cytotoxicity of splenocytes against tumor cells of the mouse melanoma B78 line after cultivation with conditioned spleen media after various hematopoiesis-stimulating effects. With various hemopoiesis-stimulating effects, the quantitative and qualitative composition of the spleen cells is reorganized depending on the compensatory mechanism for restoring homeostasis. An analysis of the cellular composition of the spleen showed that under hematopoiesis-stimulating effects, a redistribution of populations with the CD45 marker occurs: during hypoxia, the number of CD45-negative cells sharply decreases and the number of CD45-positive cells increases. The population of basophilic erythroblasts is the least susceptible to quantitative changes under all hematopoiesis-stimulating effects. During hypoxia, the most noticeable change in the cellular composition of the spleen is observed due to the increased accumulation of CD45-positive erythroid cells in the spleen. Mediators of erythroid cells of the spleen of mice after hypoxia do not lead to an increase in the cytotoxic proapoptotic effect of splenocytes on tumor cells, in contrast to the erythroid cells of the normal spleen, spleen with anemia and blood loss. Thus, it is tissue hypoxia that is the process that not only stimulates erythropoiesis, but also leads to the maximum change in the suppressive properties of surrounding cells. We assume that the implementation of compensatory mechanisms under the studied hematopoiesis-stimulating effects is aimed at activating the mechanisms of innate immunity and local immunosuppression to prevent local inflammation, accumulate nutrients, and attract cellular elements to the focus of hematopoiesis to restore homeostatic functions.

Keywords: erythroblasts, spleen, anemia, hypoxia, acute blood loss, terminal differentiation

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Introduction

In recent years, studies have revealed a wide variety of functions of erythroid cells, including modulation of the innate and adaptive immune response [8]. In research practice, the study of erythropoiesis for a long time took place separately from other lines of hematopoiesis, however, in the process of ontogenesis, myeloerythropoiesis occurs simultaneously with the development of lymphoid precursors, so their mutual influence during development is natural [10]. An important part of the functionality of immature erythroid cells is associated with their ability to produce cytokines or other immunomodulatory molecules with diverse, often opposite functions [11, 12, 13], which can be explained by the source of origin, influences, and the general heterogeneity of the erythroid cell population during terminal differentiation. Stationary bone marrow erythropoiesis maintains erythroid homeostasis throughout life. Anemic or hypoxic stress stimulates a physiological response in the form of stress erythropoiesis, aimed at increasing oxygen delivery to tissues. Stress erythropoiesis uses progenitor cells and signals that differ from stationary bone marrow erythropoiesis [1]. In this regard, the study of the phenotype of nucleated erythroid cells and their role in interaction with other cells, which manifest themselves during stimulation of hematopoiesis in pathological conditions, is relevant and significant today.

Materials and methods

In the work, we used mice hybrids of the first generation CBA C57Bl6 (3-5 months). The animals were kept in the NIIFKI vivarium under standard conditions with free access to food and drink. The study was carried out in accordance with the principles set out in the Declaration of Helsinki. Mice were removed from the experiment using cervical dislocation. To consider the role of erythroid cells in the regulation of hematopoiesis, hematopoiesis-activating states were modeled: chemically induced hemolytic anemia, acute blood loss, hypoxia. Similar intact mice were used as controls. Hemolytic anemia was induced by 3-fold (1.2 mg/mouse, 0.6 mg/mouse, 0.6 mg/mouse with 12-hour interval between injections) intraperitoneal administration of phenylhydrazine, which causes erythrocyte lysis. On the 4th day after the start of the experiment, the bone marrow and spleen were taken.

To simulate acute blood loss in mice under isoflurane anesthesia, blood was taken from the retroorbital sinus in a volume of ~ 0.5-0.7 mL, which corresponded to a loss of 12-14% of the circulating blood volume (average acute blood loss). On the 4th day after the start of the experiment, the bone marrow and spleen were taken. Hypoxic conditions were simulated in a pressure chamber (staying for 16 hours in a cage with bedding, food and water), where a pressure of ~ -46 kPa was created, which corresponded to an ascent to a height of 4200 m. At the end of exposure, mice were returned to standard vivarium conditions. Spleen sampling was carried out on the third day after the start of the experiment. Isolation of erythroid cells from a cell suspension purified on a ficoll-urografin density gradient ($\rho = 1.119$ g/cm^3) was performed using magnetic separation using monoclonal antibodies to CD71.

The stages of erythroid cell differentiation were determined by the combination of expression of TER-119 and CD71 markers and FSC forward light scatter parameters in the population of both CD45-positive and CD45-negative spleen cells. The analysis was performed on an Attune NxT Flow Cytometer (ThermoFisher Scientific, USA). To study the immunoregulatory activity of erythroid cells, we investigated the mediated cytotoxicity of splenocytes against tumor cells of the mouse melanoma B78 line after cultivation with conditioned spleen media after various hematopoiesis-stimulating effects. To do this, splenocytes were preliminarily cultured with a conditioned erythroblast medium for 24 hours, and then planted with B78 mouse melanoma cells for another 24 hours. The cytotoxic effect was determined by the expression of the apoptosis marker annexin.

Statistical analysis of the obtained data was performed using GraphPad Prism 8 software using ANOVA and Tukey's multiple comparison test. Data were presented as median and interquartile range – Me ($Q_{0.25}$-$Q_{0.75}$). Differences were considered statistically significant at $p < 0.05$.

Results and discussion

Analysis of the cellular composition of the spleen showed that during hypoxia, the number of CD45-negative cells sharply decreases, and the number of CD45-positive cells increases. In addition, there are significant differences in the cellular composition of the spleen between the states of anemia and hypoxia. For the total content of erythroid cells in the splenocyte population, the same trend is observed: a decrease in the number of CD45-negative erythroblasts and an increase in the number of CD45-positive erythroblast cells during hypoxia (Table 1).

Determining the stages of terminal differentiation of erythroblasts showed that among CD45-negative cells, polychromatophilic erythroblasts normally predominate, while their number decreases with anemia, blood loss and hypoxia, and the content of orthochromatophilic erythroblasts increases with the same effects. In the population of CD45-positive erythroblasts, the predominant stage of differentia-

TABLE 1. STRUCTURE OF THE SPLENOCYTE POPULATION IN MICE UNDER HEMATOPOIESIS-STIMULATING EFFECTS, Me ($Q_{0.25}$-$Q_{0.75}$)

	Intact mouse (n = 7)	Anemia (n = 7)	Acute blood loss (n = 7)	Hypoxia (n = 11)
CD45-negative splenocytes	83.73% (78.26-84.87)	75.09% (52.03-88.49)#	66.07% (24.14-68.32)	20.88% (16.25-66.79)*
CD45-positive splenocytes	16.27% (15.34-21.74)	25.47% (11.62-48.13)#	34.24% (31.93-71.86)*	78.66% (33.13-83.22)*
CD45-negative erythroblasts	42.54% (34.45-59.94)	41.29% (29.03-51.52)#	27.68% (13.86-41.33)	13.38% (12.01-43.63)*
CD45-positive erythroblasts	9.863% (8.314-13.240)	19.48% (5.661-32.670)#	17.58% (15.79-32.41)*	47.73% (20.78-58.55)*

Note. *, compared with the Intact mouse; #, compared with hypoxia. Differences were considered statistically significant at $p < 0.05$.

tion is basophilic erythroblasts both in intact mice and under hematopoiesis-stimulating effects. The content of polychromatophilic erythroblasts significantly increases during hypoxia compared to intact erythroblasts, and the content of orthochromatophilic erythroblasts in anemia is significantly higher than in hypoxia. In the total fraction of erythroblasts of the spleen, there is a decrease in the content of polychromatophilic erythroblasts and an increase in the content of otrochromatophilic erythroblasts under all types of exposure (Figure 1).

Since erythroblasts can play an important role in regulating the functions of other cells, we studied the effect of their conditioned media on the effector functions of splenocytes, namely, cytotoxic activity against melanoma B 78 tumor cells, i.e., cytotoxic effect on tumor cells (Figure 2).

In this work, it is shown that under various hematopoiesis-stimulating effects, the quantitative and qualitative composition of spleen cells is reorganized. Each of these effects requires a separate compensatory mechanism to restore homeostasis. With hemolytic anemia, it is necessary to restore the destroyed pool of erythrocytes, with hypoxia, it is necessary to increase the number of erythroid cells relative to the already existing baseline, and with acute blood loss, it is necessary to restore not only erythrocytes, but also lymphoid cells, as well as the circulating blood volume.

The population of CD45-positive cells traditionally includes T, B, NK cells, monocytes, macrophages, however, this molecule is also present on early-stage erythroblasts [4] and in foci of extramedullary erythropoiesis in pathological

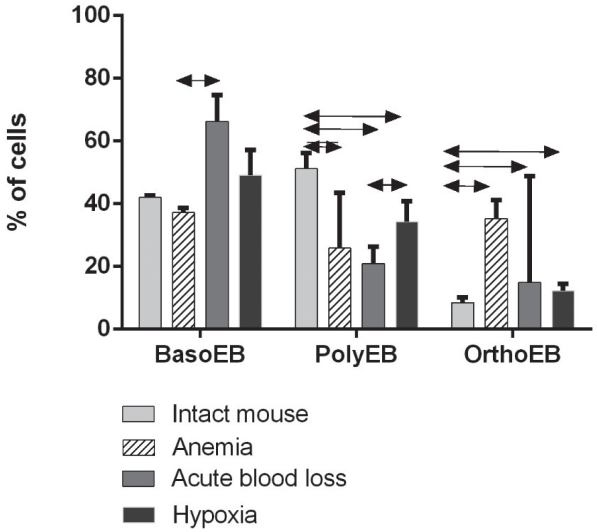

Figure 1. Determination of the stages of terminal differentiation of erythroid cells of the spleen under hematopoiesis-stimulating effects

Note. Data are presented as median and interquartile range – Me ($Q_{0.25}$-$Q_{0.75}$). Differences were considered statistically significant at $p < 0.05$.

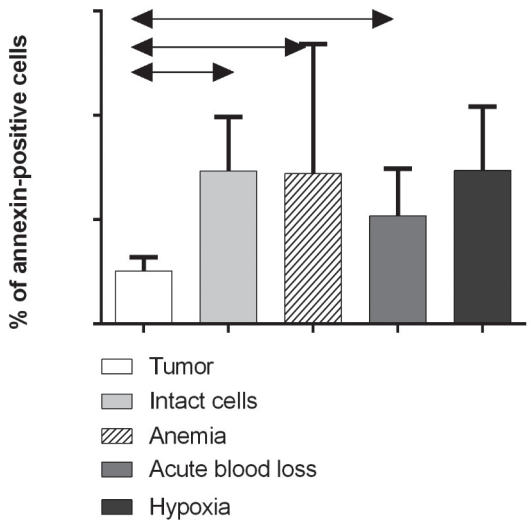

Figure 2. Cytotoxic effect of splenocytes treated with conditioned erythroblast media obtained under various hematopoiesis-stimulating effects against tumor line B 78 cells (n = 8)

Note. As for Figure 1.

conditions, for example, in the spleen of mice in transplantable tumor models [5]. During erythroid development, the CD45 marker arrests progenitors of $CD71^+TER119^+$ cells at undifferentiated stages, and its expression is a hallmark of early progenitors [7]. The population of basophilic erythroblasts is the least susceptible to quantitative changes under all hematopoiesis-stimulating effects. During hypoxia, the most noticeable change in the cellular composition of the spleen is observed, possibly due to the increased accumulation of CD45-positive erythroid cells in the spleen. $CD45^+$ erythroblasts are capable of greater suppressive activity compared to other cells [3]. In our work, we investigated the mediated effect of mediators that produce erythroid cells of the spleen on the cytotoxic activity of splenocytes. Mediators of erythroid cells of the spleen of mice after hypoxia do not lead to an increase in the cytotoxic proapoptotic effect of splenocytes on tumor cells, in contrast to the erythroid cells of the normal spleen, spleen with anemia and blood loss. It can be assumed that mediators secreted by erythroid cells under hypoxic conditions have the maximum suppressive effect on effector cells in the splenocyte population.

Tissue hypoxia is accompanied by the expression of the hypoxia factor HIF1a and the expression of the erythropoietin receptor, which protects cells from deep hypoxic damage [6]. Erythropoietin under conditions of stimulation of hematopoiesis leads to an increase in the production of erythrocytes, but an increase in the number of erythroid precursors and the priority of erythroid differentiation is compensated by a decrease in the number of precursors for other lines [14]. HIF induces a number of aspects of host immune function, from increasing the antibacterial capacity of phagocytes to stimulating T cell differentiation and cytotoxic activity [9]. Thus, it is tissue hypoxia that is the process that not only stimulates erythropoiesis, but also leads to the maximum change in the suppressive properties of surrounding cells.

Conclusion

We assume that the implementation of compensatory mechanisms under the studied hematopoiesis-stimulating effects is aimed at activating the mechanisms of innate immunity and local immunosuppression to prevent local inflammation, accumulate nutrients, and attract cellular elements to the focus of hematopoiesis to restore homeostatic functions.

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