PHARMACOLOGY AND TOXICOLOGY

Potentiation of Hemostimulating Effects of Erythropoietin with Pegylated Hyaluronate-Endo-β-N-Acetylhexosaminidase

A. M. Dygai, G. N. Zyuz'kov, V. V. Zhdanov, E. V. Udut, R. V. Gurto, L. A. Miroshnichenko, E. V. Simanina, T. Iu. Khrichkova, L. A. Stavrova, A. V. Chaikovsky, A. V. Artamonov*, A. A. Bekarev*, P. G. Madonov*, M. Iu. Minakova, and T. S. Markova

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Pegylated hyaluronate-endo-β-N-acetylhexosaminidase considerably potentiates the hemostimulating effects of erythropoietin due to intensification of proliferation and differentiation of erythroid precursors against the background of enhanced secretion of hemopoietins by nonadherent hemopoiesis-inducing environment cells and elevation of serum erythropoietin concentration. The use of the enzyme allows 10-fold reduction of the maximum effective erythropoietin dose.

Key Words: *hemopoiesis; erythropoietin; pegylated hyaluronate-endo-β-N-acetylhexosaminidase; hyaluronidase; precursors*

Recombinant forms of natural hemopoiesis regulator erythropoietin (EP) are often used in the therapy of pathologies associated with anemia [6,9,10]. However, practical use of EP is largely limited due to its toxicity and the risk of adverse side effects (hemostasis disturbances, hypertension, iron deficiency, etc.). Long-term EP treatment sometimes induces tolerance to this physiological regulator [6,9]. Since there are no alternative drugs comparable by their efficiency to EP for the treatment of anemia, the schemes of EP therapy should be optimized for reducing the risk of complications. A promising approach is modification of EP increasing its specific activity and allowing treatment with lower therapeutic doses. We have previously demonstrated the possibility of stimulating hemopoiesis with hyaluronidase pegylated using electron-beam synthesis nanotechnology [4] and the capacity of this substance to potentiate the hemostimulating effect of granulocytic CSF (G-CSF) [11].

Here we studied the blood system responses to combined administration of EP and pegylated highly purified testicular hyaluronidase, hyaluronate-endo-β-N-acetylhexosaminidase (Peg-HEAHA), and the mechanisms underlying these responses.

MATERIALS AND METHODS

The experiments were carried out on 2-month-old male and female CBA/CaLac mice (n=200, body weight 18-20 g, conventional mouse strain obtained from the nursery of Institute of Pharmacology, Tomsk Research Center, Siberian Division of Russian Academy of Medical Sciences).

Institute of Pharmacology, Siberian Division of the Russian Academy of Medical Sciences, Tomsk; *Scientific Future Management Company, Novosibirsk, Russia. ***Address for correspondence:*** zgn@pharm.tsu.ru. G. N. Zyuz'kov

Myelosuppression in experimental mice was modeled by single intraperitoneal injection of carboplatin in maximum tolerated dose (100 mg/kg, according to probit-analysis data). Starting from the next day after carboplatin injection, the experimental animals received subcutaneous injections of recormon (EP preparation, Hoffmann-La Roche) in doses of 5 or 0.5 U/mouse/day (therapeutic and 1/10 therapeutic doses [6]) for 5 days or EP in a dose of 0.5 U/mouse according to the same scheme in combination with Peg-HEAHA in a dose of 50 U/kg (Scientific Future Management Company) once a day for 2 days (intragastrically). The enzyme was immobilized on low-molecular-weight polyethylene glycol (molecular weight 1500 Da) using electron-beam synthesis nanotechnology [13]. Control mice received physiological saline according to the same schemes.

Total erythrocyte count, hemoglobin content, hematocrit, and mean corpuscular concentration of hemoglobin were evaluated on days 5, 7, 9, and 12 using a hematological analyzer ABACUS (Diatron) operated in a veterinary mode. The total number of reticulocytes and different leukocyte subpopoulations in the peripheral blood and parameters of bone marrow hemopoiesis were determined by routine hematological methods [7]. The number of erythroid precursors (CFU-E), their proliferative activity and intensity of differentiation, and erythropoietic activity (EPA) of the culture media conditioned by adherent and nonadherent elements of the hemopoiesis-inducing environment (HIM) [3] were evaluated by cell culture methods and the content of EP was assayed by ELISA. The data were processed statistically using Student's t test and nonparametric Mann–Whitney U test [1].

RESULTS

Carboplatin considerably decreased erythrocyte count (on days 5, 9, and 12), hematocrit (on days 5, 9, and 12), and total hemoglobin content (days 9 and 12) in the peripheral blood; the corpuscular concentration of hemoglobin increased (on days 5, 7, and 9). In parallel, the count of circulating reticulocytes increased (days 5, 7, 9, and 12), which represents a compensa-

TABLE 1. Dynamics of the Content of Some Erythrokaryocytes ($\times 10^6$/femur) in the Bone Marrow of CBA/CaLac Mice Receiving Physiological Saline (1), EP in Doses of 5 (2) or 0.5 U/mouse (3), or 0.5 U/mouse EP+50 U/kg Peg-HEAHA (4) against the Background of Single Carboplatin Administration in a Maximum Tolerated Dose ($X \pm m$)

Day of experiment	Group	Erythroblasts	Pronormoblasts	Basophilic normoblasts	Polychromatophilic normoblasts	Orthochromatic normoblasts
Before treatment		0	0.03±0.01	0.17±0.01	0.52±0.07	0.25±0.04
Day 5	1	0	0.04±0.01	0.06±0.01*	0.09±0.01*	0.04±0.01*
	2	0.01±0.01	0.05±0.02	0.13±0.05	0.20±0.04*+	0.18±0.02+
	3	0.02±0.01x	0.06±0.01	0.06±0.01*	0.08±0.02*	0.06±0.01*
	4	0	0.06±0.02	0.07±0.02*	0.16±0.02*+x	0.12±0.02+x
Day 7	1	0	0	0.02±0.01*	0.05±0.02*	0.01±0.01*
	2	0	0.02±0.01	0.04±0.01*	0.12±0.03*+	0.03±0.01*
	3	0	0.02±0.01	0.04±0.01*	0.10±0.01*+	0.05±0.02*+
	4	0	0.01±0.01	0.06±0.01*	0.14±0.02*	0.04±0.01*
Day 9	1	0	0.02±0.01	0.08±0.02	0.45±0.12	0.17±0.05
	2	0.01±0.01	0.07±0.02	0.23±0.04+	0.88±0.22+	0.49±0.07+
	3	0.01±0.01	0.03±0.01	0.26±0.08+	0.89±0.14+	0.33±0.08
	4	0.01±0.01	0.02±0.01	0.19±0.07	0.69±0.09	0.29±0.03
Day 12	1	0.01±0.01	0.10±0.02*	0.66±0.20+	0.16±0.02+	0.06±0.01
	2	0.02±0.01	0.02±0.01	0.23±0.03+	0.55±0.03+	0.14±0.02
	3	0.05±0.01*	0.05±0.02+	0.03±0.01	0.13±0.05	0.76±0.05*+x
	4	0.21±0.04*	0.20±0.05+	0.06±0.01+	0.01±0.01	0.32±0.02+x

Note. $p<0.05$ in comparison with: *intact animals (parameter before treatment), +group 1 (cytostatic control; administration of physiological saline against the background of myelosuppression), xgroup 3.

tory reaction of the body to the cytostatic treatment [2]. Administration of EP in both doses (5 and 0.5 U/mouse) was followed by a more pronounced increase in this parameter to 911 and 302% of baseline on days 9 and 12 after administration of 5 and 0.5 U/mouse, respectively. At the same time, administration of Peg-HEAHA together with 0.5 U EP induced a more pronounced increase in peripheral blood reticulocyte count. In these animals, this parameter practically attained the level observed in animals receiving EP in a dose of 5 U. The dynamics of erythrocyte count in the peripheral blood was similar, but less pronounced. Significant increase in the count of red blood cells in comparison with cytostatic control was observed on day 9 in mice receiving EP alone and on days 9 and 12 in mice receiving EP (0.5 U/mouse) with Peg-HEAHA.

These shifts in peripheral blood parameters reflect the processes of bone marrow hemopoiesis. Carboplatin treatment led to considerable suppression of both the granulomonocytic and erythroid hemopoietic lineages. The decrease in the content of morphologically discernible erythroid cell elements in the bone marrow was most pronounced. The counts of pronormoblasts, basophilic, polychromatophilic, and orthochromatic normoblasts were minimum on day 7 (0, 12, 10, and 4% of baseline values, respectively). EP considerably activated erythropoiesis. The content of practically all morphologically discernible erythroid cells in the bone marrow markedly increased (the increase in normoblast count was most pronounced on days 9 and 12 of the experiment). More drastic changes were observed in mice receiving EP in a dose of 5 U/mouse: the count of basophilic, polychromatophilic, and orthochromatic normoblasts attained 288, 196, and 288% of cytostatic control, respectively. EP in a dose of 0.5 U/mouse also produced an appreciable effect (Table 1). Additional administration of Peg-HEAHA in this case even more stimulated hemopoiesis. The efficiency of experimental therapy (combination of EP and Peg-HEAHA) was even superior to that of recormon (5 U/mouse). For instance, the content of polychromatophilic and oxyphilic normoblasts in the bone marrow on day 12 of the experiment was higher by 47 and 90%, respectively, than in the cytostatic control (Table 1). Additional administration of Peg-HEAHA also led to accelerated regeneration of the granulomonocytic compartment of the hemopoietic tissue. Peg-HEAHA treatment against the background of recormon therapy increased the count of imma-

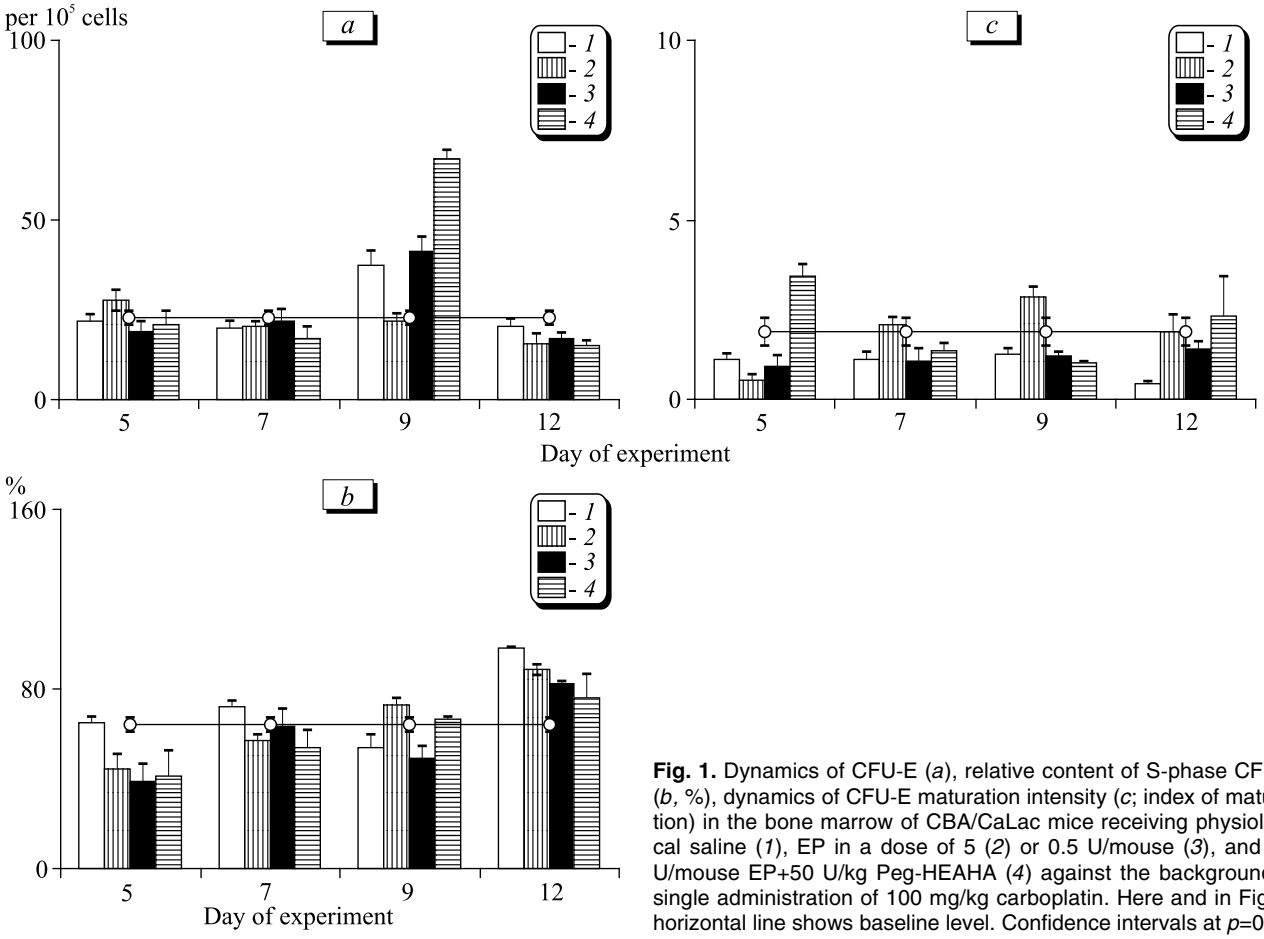

Fig. 1. Dynamics of CFU-E (*a*), relative content of S-phase CFU-E (*b*, %), dynamics of CFU-E maturation intensity (*c*; index of maturation) in the bone marrow of CBA/CaLac mice receiving physiological saline (*1*), EP in a dose of 5 (*2*) or 0.5 U/mouse (*3*), and 0.5 U/mouse EP+50 U/kg Peg-HEAHA (*4*) against the background of single administration of 100 mg/kg carboplatin. Here and in Fig. 2: horizontal line shows baseline level. Confidence intervals at p=0.05.

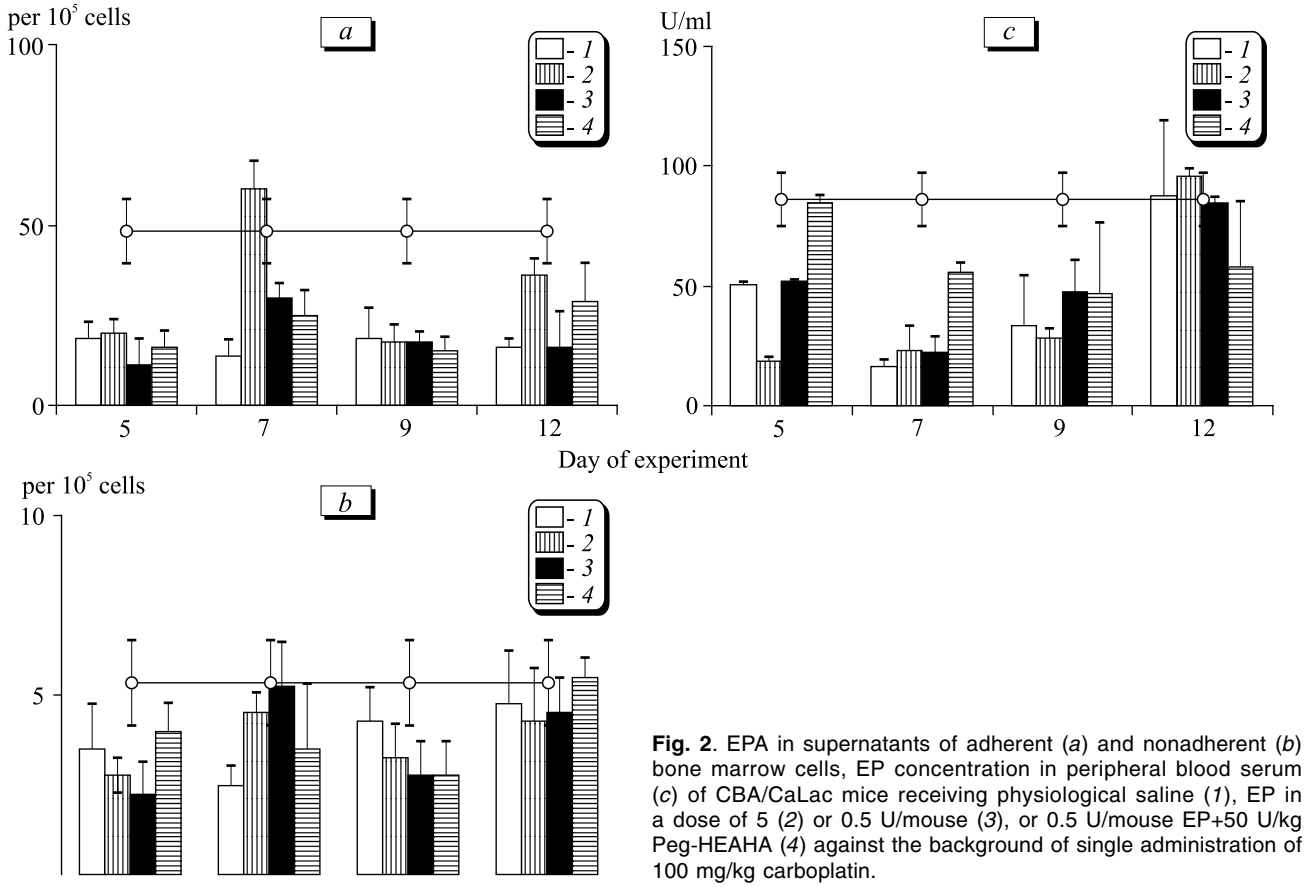

Fig. 2. EPA in supernatants of adherent (*a*) and nonadherent (*b*) bone marrow cells, EP concentration in peripheral blood serum (*c*) of CBA/CaLac mice receiving physiological saline (*1*), EP in a dose of 5 (*2*) or 0.5 U/mouse (*3*), or 0.5 U/mouse EP+50 U/kg Peg-HEAHA (*4*) against the background of single administration of 100 mg/kg carboplatin.

ture and mature neutrophilic granulocytes in the bone marrow at some time points. The development of this phenomenon was apparently a consequence of specific activity of the enzyme preparation [4,8].

Analysis of the bone marrow pool of hemopoietic precursors in animals with experimental cytostatic myelosuppression revealed increased content of erythroid precursors in the bone marrow on day 9 (to 160% of baseline) due to their enhanced proliferative activity (days 7 and 12) against the background of reduced intensity of maturation. Treatment with EP (5 U/mouse) led to more early increase in CFU-E count in the bone marrow (day 5) and acceleration of their division (days 9 and 12) and differentiation (days 7, 9, and 12). Changes in the latter parameter were so pronounced that the content of CFU-E in the hemopoietic tissue decreased on days 9 and 12 of the experiment. Administration of 0.5 U/mouse EP was not followed by marked intensification of CFU-E differentiation (except day 12); this together with stimulation of CFU-E proliferation led to their expected accumulation in the bone marrow on day 9. At the same time, additional treatment with Peg-HEAHA led to appreciable potentiation of EP effects on the pool of progenitor cells. On day 9 of the experiment, the count of erythroid precursors in animals receiving combined therapy surpassed that in mice receiving EP alone by 63%. Accumulation of CFU-E was observed against the background of marked enhancement of their proliferative activity and acceleration of maturation (day 5; Fig. 1).

An important role in determining the status of progenitor elements is played by HIM elements [2,6]. Their direct participation in the pathogenesis of anemia caused by carboplatin treatment has been demonstrated in the experiment. For instance, carboplatin treatment inhibited production of EPA by both adherent (days 5, 7, 9, and 12) and nonadherent (days 5 and 7) myelokaryocytes. At the same time, treatment with EP preparations increased EPA in the media conditioned by both bone marrow fractions isolated from mice on day 7 of the experiment. The reaction of adherent cells was more pronounced after treatment with EP in a dose of 5 U/mouse. Peg-HEAHA considerably increased (by 78%) the production of hemopoietically active factors by nonadherent HIM cells on day 5 (Fig. 2).

The basic mechanism of the myelosuppressive effect of carboplatin is suppression of endogenous EP, the main humoral hemopoietic factor regulating erythropoiesis [5], which was fully confirmed by the dynamics of serum EP content after cytostatic treatment: the decrease in this parameter to 19% of the baseline on day 7 with subsequent recovery only by

the end of the experiment (day 12; Fig. 2). Under these conditions, stimulation of the erythroid lineage cells by EP in the high dose (5 U/mouse) led to even deeper suppression of EP production on day 5. This was probably a result of feedback suppression of EP-producing cells in the kidneys by the excess of exogenous hormone-like substance [5]. Treatment with EP in a dose of 0.5 U/mouse did not affect EP production, while additional administration of Peg-HEAHA increased the hemopoietin concentration in the serum as a result of intrinsic pharmacological action of the enzyme [8].

These findings suggest that additional administration of Peg-HEAHA can considerably potentiate specific activity of EP preparations. The optimum pharmacological effects in this case can be attained even after treatment with 10-fold lower doses of EP. The observed modulating effect of the enzyme preparation is realized at the level of central, peripheral, and regulatory elements of the blood system; the potentiating effects of Peg-HEAHA are mediated via modification of hyaluronic acid in cell glycocalyx (including progenitor cells) increasing their susceptibility to EP [12].

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