NANOTECHNOLOGIES

Hemostimulating Effects of Erythropoietin Immobilized by the Nanotechnology Method of Electron-Beam Synthesis

A. M. Dygai, G. N. Zyuz'kov, V. V. Zhdanov, A. V. Artamonov*, A. A. Bekarev*, P. G. Madonov*, D. N. Kinsht*, E. V. Udut, L. A. Miroshnichenko, T. Yu. Khrichkova, E. V. Simanina, L. A. Stavrova, A. V. Chaikovskiy, T. S. Markova, and M. Yu. Minakova

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 151, No. 2, pp. 207-210, February, 2011
Original article submitted March 18, 2010

The hemostimulating effect of erythropoietin immobilized by the nanotechnology method of electron-beam synthesis was studied on the model of carboplatin-induced myelosuppression. Subcutaneous injection or oral administration of immobilized erythropoietin was followed by stimulation of erythropoiesis. The effect was most pronounced after parenteral treatment with this agent. The increase in proliferative activity and maturation of erythroid precursor cells serve as a factor determining acceleration of reparative processes in the hemopoietic tissue.

Key Words: *erythropoiesis; immobilized erythropoietin; nanotechnologies; cytostatic-induced myelosuppression*

Erythropoietin (EP) preparations are now extensively used for the therapy of anemia (specific disease or a pathological syndrome typical of various nosological forms. [1,8]. Protein nature of EP determines the only administration route (parenteral) and the risk of a variety of side effects and complications [6,10-12] related to immunogenicity of a linearly restricted growth factor for erythroid hemopoietic cells [2,3]. Protein toxicity can be reduced by immobilization on low-molecular-weight polyethylene glycol by using exposure to ionizing radiation. It should be emphasized that pharmaceutical products obtained by this method are protected from proteolytic enzymes. Hence, they can be used for oral administration [5-7].

Institute of Pharmacology, Siberian Division of the Russian Academy of Medical Sciences, Tomsk; *Scientific Future Management Company, Novosibirsk, Russia. ***Address for correspondence:*** mmu@pharm.tsu.ru. A. M. Dygai

The hemostimulating effect of EP immobilized by the nanotechnology method of electron-beam synthesis was studied on the model of carboplatin-induced myelosuppression.

MATERIALS AND METHODS

The experiments were performed on 2-month-old CBA/CaLac mice (n=156, body weight 18-20 g). The animals were obtained from the nursery of the experimental and biological clinic of laboratory animals (Institute of Pharmacology).

The mice received a single intraperitoneal injection of carboplatin (Teva) in the maximum permissible dose (MPD=100 mg/kg, Litchfield–Wilcoxon probit analysis). Immobilized EP (imEP) was developed by the Scientific Future Management Company in collaboration with the Institute of Pharmacology. imEP in

a daily dose of 5 U was administered subcutaneously or perorally on days 1-5 (starting from day 1 after carboplatin treatment). EP molecules were immobilized on low-molecular-weight polyethylene glycol by the nanotechnology method of electron-beam synthesis with directed flow of accelerated electrons [13]. Nonconjugated recombinant EP (rhEP, Rekormon, Hoffman la Roche) served as the reference preparation. rhEP in a dose of 5 U was injected subcutaneously for 5 days. Control animals received an equivalent volume of physiological saline (similar scheme of treatment).

Peripheral blood and bone marrow hemopoiesis were analyzed routinely using an ABACUS automatic blood analyzer (Diatron) on days 5, 7, 9, and 12 [4]. The content, proliferative activity, and differentiation of erythroid precursors (CFU-E) in the bone marrow were evaluated *in vitro* by cultural methods [4]. Serum EP concentration was measured by EIA with Biomerica kits.

The results were analyzed by methods of variation statistics (Student's *t* test and nonparametric Mann–Whitney *U* test).

RESULTS

Administration of carboplatin was followed by the development of anemia. We observed a decrease in the number of erythrocytes (days 5, 9, and 12), hematocrit (days 5, 9, and 12), and total hemoglobin (days 9 and 12) in the peripheral blood. It was accompanied by an increase in the corpuscular concentration of hemoglobin (days 5, 7, and 9) and number of peripheral blood reticulocytes (days 5, 7, 9, and 12). Changes in the peripheral blood reflect typical response of hemopoietic tissue. The content of morphologically distinguishable erythroid cells was shown to decrease in the hemopoietic tissue (Table 1). A significant increase in the amount of CFU-E in the bone marrow (day 9) was accompanied by an increase in proliferative activity (days 7 and 12; Fig. 1). These changes in the pool of parent cells probably result from an increase in functional activity of cells in the hemopoiesis-inducing microenvironment after cytostatic treatment [2]. EP concentration in the serum decreased sharply and was minimum on day 7 (19% of the baseline), but returned to normal by the end of measurements (day 12; Fig. 2). Our results are consistent with published data that abnormalities in the EP-secreting apparatus of the kidneys play an important role in the pathogenesis of carboplatin-induced myelosuppression [9].

EP products had a strong effect on the blood response. We observed an increase in the number of circulating reticulocytes, which reached maximum in rhEP-treated mice (911% of the baseline, day 9). Moreover, the content of morphologically discernible erythroid cells increased significantly in the bone marrow (Table 1). It should be emphasized that imEP was less effective than rhEP. For example, the number

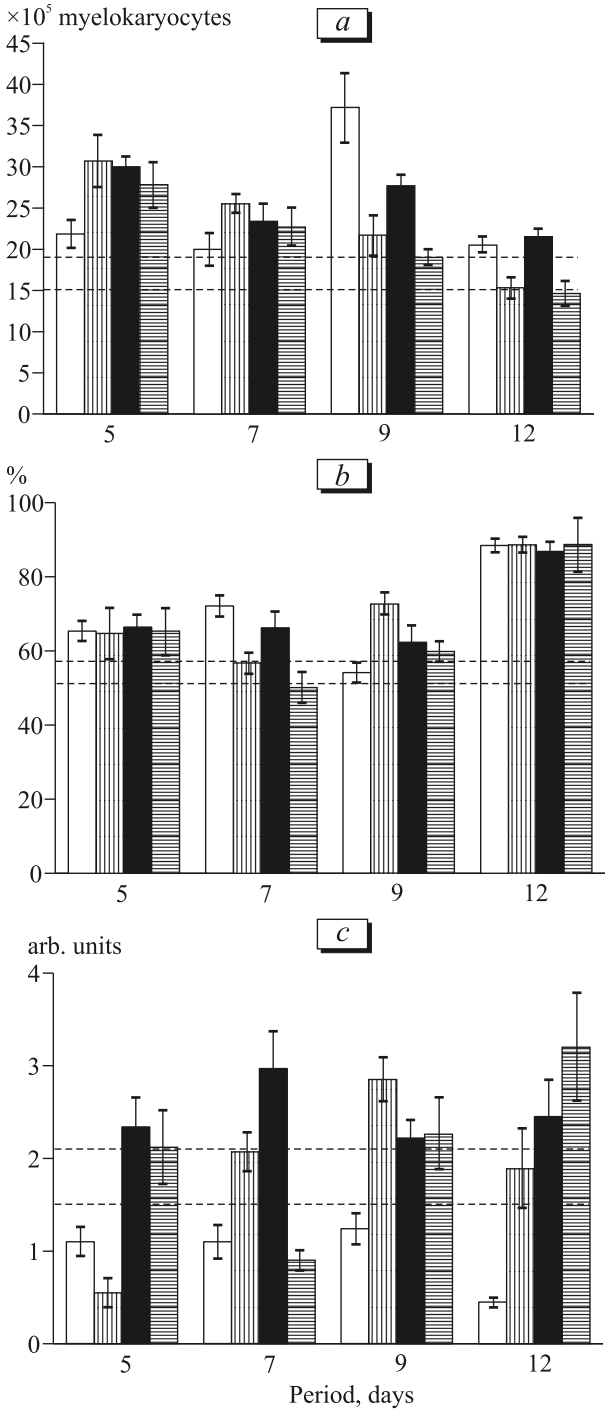

Fig. 1. Content of bone marrow CFU-E (*a*), ratio of CFU-E in S-phase of the cell cycle (*b*), and intensity of maturation (*c*) in CBA/CaLac mice with cytostatic-induced myelosuppression after administration of physiological saline (light bars), rhEP (vertical shading), and imEP (subcutaneously; dark bars; *per os*, horizontal shading). Here and in Fig. 2: space between dotted lines, confidence interval at $p \leq 0.05$ (intact mice).

TABLE 1. Number of Reticulocytes in the Peripheral Blood (‰) and Count of Erythroblasts, Pronormoblasts, and Basophilic, Polychromatophilic, and Orthochromic Normoblasts in the Bone Marrow ($\times 10^6$/femur) of CBA/CaLac Mice with Cytostatic-Induced Myelosuppression after Administration of Physiological Saline (1), rhEP (Subcutaneously, 2), and imEP (3, Subcutaneously; 4, *per os*; $X \pm m$)

Period, days		Peripheral blood reticulocytes	Bone marrow pronormoblasts	Bone marrow basophilic normoblasts	Bone marrow polychromatophilic normoblasts	Bone marrow orthochromic normoblasts
Baseline		3.88±0.30	0.03±0.01	0.17±0.01	0.52±0.07	0.25±0.04
5	1	7.33±0.42*	0.04±0.01	0.06±0.01*	0.09±0.01*	0.04±0.01*
	2	10.83±1.38*+	0.05±0.02	0.13±0.05	0.20±0.02*+	0.18±0.02+
	3	8.00±0.52*+	0.04±0.01	0.03±0.01*	0.10±0.01*o	0.05±0.02*o
	4	8.50±0.96*+	0.02±0.01	0.05±0.01*	0.14±0.01*+o	0.04±0.02*o
7	1	7.83±0.31*	0±0	0.02±0.01*	0.05±0.02*	0.01±0.01*
	2	13.00±1.75*+	0.02±0.01	0.04±0.01*	0.12±0.03*	0.03±0.01*
	3	11.83±0.65*+	0.02±0.01	0.04±0.01*	0.16±0.03*+	0.02±0.01*
	4	9.83±0.65*+o	0.01±0.01	0.05±0.01*	0.15±0.02*+	0.03±0.01*
9	1	7.67±0.49*	0.01±0.01	0.08±0.02*	0.45±0.07	0.17±0.05
	2	35.33±3.21*+	0.07±0.01*+	0.23±0.03+	0.88±0.07*+	0.49±0.06*+
	3	22.00±2.72*+o	0.05±0.01+	0.20±0.02+	0.69±0.02*+o	0.33±0.03+
	4	17.00±2.35*+o	0.04±0.01	0.17±0.02+	0.43±0.03o	0.31±0.03+
12	1	7.33±0.42*	0.02±0.01	0.05±0.01*	0.21±0.04*	0.10±0.02*
	2	30.50±1.69*+	0.05±0.01+	0.20±0.05+	0.66±0.02+	0.23±0.03+
	3	17.33±0.49*+o	0.05±0.01+	0.16±0.05	0.42±0.02+o	0.18±0.02+
	4	11.50±0.96*+o	0.04±0.01+	0.05±0.03	0.41±0.03+o	0.13±0.02

Note. $p<0.05$: *compared to the baseline; +compared to cytostatic control; ocompared to rhEP.

of bone marrow polychromatophilic cells on days 5, 9, and 12 after subcutaneous injection of imEP was lower than in the rhEP group (by 50, 22, and 36%, respectively). Even oral administration of imEP was followed by a statistically significant increase in the content of basophilic (day 9), polychromatophilic (day 7 and 12), and orthochromic erythroid cells (day 9) in the hemopoietic tissue. These phenomena provide support to the results of our previous studies. We showed that the protein structures conjugated to polyethylene glycol by the nanotechnology method of electron-beam synthesis can be absorbed in the gastrointestinal tract. Specific biological activity of conjugates remained unchanged under these conditions [5,7].

Cell culture study showed that administration of non-conjugated EP during experimental myelosuppression is followed by a more rapid increase in the number of bone marrow CFU-E (as compared to the control; days 5 and 7). We observed not only an increase in the rate of precursor cell division (day 9), but also acceleration of maturation (days 7, 9, and 12). These changes promoted a decrease in the content of CFU-E on days 9 and 12 (Fig. 1).

The use of imEP for correction of erythropoiesis was accompanied by an increase in the content of bone marrow CFU-E in experimental animals (days 5 and 7 after parenteral administration; and day 5 after oral administration). Despite significant increase in the rate of CFU-E maturation under both conditions, their amount in the hemopoietic tissue was elevated at various stages of the study (Fig. 2). imEP was more potent than the reference EP preparation in modulating differentiation of CFU-E at some stages of the study (days 5, 7, and 12 after subcutaneous injection; and days 5 and 12 after oral administration).

Our results are consistent with published data that EP products stimulate proliferation and differentiation of erythroid precursor cells [2,3].

However, stimulation of the erythroid hemopoietic stem with unmodified and pegylated EP was followed by greater suppression of EP production (as compared to the control group; Fig. 2). It probably results from negative feedback suppression of functional activity of EP-producing cells in the kidneys due to administration

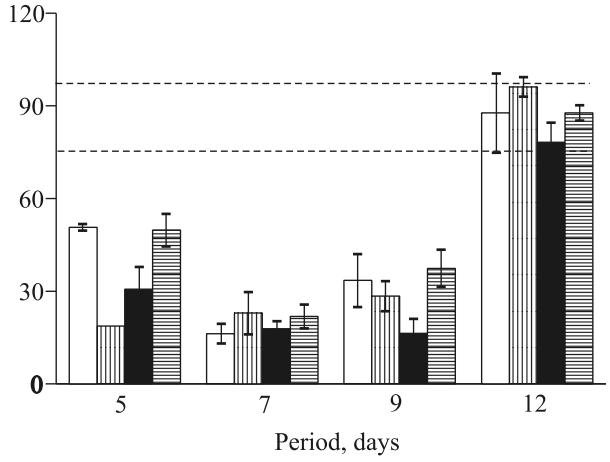

Fig. 2. Serum EP concentration in CBA/CaLac mice with cytostatic-induced myelosuppression after administration of physiological saline (light bars), rhEP (vertical shading), and imEP (subcutaneously; dark bars; *per os*, horizontal shading). Ordinate (logarithmic scale): parameter in mU/ml.

of considerable amounts of an exogenous hormone-like substance [9]. Production of this factor after treatment with imEP was suppressed most significantly in the later period compared to that observed in the rhEP group (from days 5 to days 9). These data attest to lower sensitivity of EP-recognizing cells in the kidneys to polyethylene glycol-conjugated EP.

We conclude that imEP has a strong stimulating effect on erythropoiesis. However, pegylation of EP is probably accompanied by a decrease in its biological activity. It should be emphasized that polyethylene glycol-conjugated protein substances exhibit extremely low toxicity. Therefore, repeated treatment with these agents induced no side effects or complications [5,13]. Moreover, imEP is highly effective after oral administration. Hence, the development of a new pharmaceutical product from this compound holds much promise for medical practice.

This work was supported by the Federal Target Program "Research and Scientific-Pedagogical Personnel of Innovative Russia for 2009-2013" (State contract No. 02.740.11.0781).

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