PHARMACOLOGY AND TOXICOLOGY

Role of Hemopoietic Precursors of Various Classes in the Effect of Granulocyte Colony-Stimulating Factor on Hemopoiesis during Cytostatic-Induced Myelosuppression

A. M. Dygai, E. G. Skurikhin, O. V. Pershina, T. V. Andreeva, E. S. Khmelevskaya, and M. Ju. Minakova

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 149, No. 4, pp. 400-404, April, 2010
Original article submitted June 11, 2009

Experiments were performed on the model of cyclophosphamide-induced myelosuppression. We showed that regeneration of the granulocytic hemopoietic stem is related to activation of multipotent, granulocyte-erythroid-macrophage-megakaryocyte, and granulocyte-macrophage precursors. The division and maturation of granulocyte colony-forming cells and significant decrease in the number of these cells in the bone were suppressed under these conditions. The granulocytopoiesis-stimulating effect of granulocyte CSF during myelosuppression was associated with an increase in functional activity of multipotent and granulocyte-erythroid-macrophage-megakaryocyte precursors (primarily of differentiation). In the period of regeneration, this effect was attributed to activity of granulocyte precursors.

Key Words: *multipotent hemopoietic precursor cell; precursor cells of the granulocyte-erythroid-macrophage-megakaryocyte, granulocyte-macrophage, and granulocyte types; granulocyte colony-stimulating factor; cyclophosphamide*

Granulocyte CSF (G-CSF) is one of the major hemopoietic growth factors playing a role in the maintenance of granulocytopoiesis in an equilibrium state under conditions of emergency activation [2]. G-CSF is a lineage-restricted regulator of neutrophil production. This agent is potent in stimulating the terminal stage of neutrophil development [3,6,7,9]. G-CSF plays a role of hemopoietin, which increases the survival and proliferation of immature hemopoietic cells [1,3] (*e.g.*, $CD34^+$ stem cells [5]). According to the modern notions, committing of early hemopoietic precursor cells occurs with no involvement of growth factors. At this stage of cell development, the membrane does not carry specific receptors [2]. Moreover, some cells (*e.g.*, bipotent hemopoietic precursors) carrying receptors for some growth factors are committed independently on the presence of cytokines. However, little is known about the relationship between division and maturation of hemopoietic stem cells and mature precursors under the influence of G-CSF.

Here we studied the effect of G-CSF on proliferation and differentiation of hemopoietic precursor cells of various classes under conditions of cytostatic treatment.

MATERIALS AND METHODS

Experiments were performed on 120 male CBA/CaLac mice (class I conventional strain) aging 2-2.5 months

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

and obtained from the nursery of the Institute of Pharmacology.

Cytostatic myelosuppression was induced by single intraperitoneal injection of an alkylating agent cyclophosphamide (one-third of the maximum permissible dose [MPD], 83 mg/kg). All mice of the treatment groups received subcutaneous injections of non-glycosylated human G-CSF Filgrastim (Neupogen, Hoffman-La Roche Ltd.) in a dose of 100 μg/kg for 5 days after administration of cyclophosphamide. Some mice received an equivalent volume of physiological saline (0.2 ml) under similar conditions (cytostatic control). The intact control group consisted of intact animals.

The number of neutrophilic leukocytes in the peripheral blood was measured on days 1-7 after cytostatic treatment. The animals were euthanized by craniocervical dislocation under ether anesthesia. The number of mature and immature neutrophilic granulocytes was evaluated in the bone marrow [4]. We estimated the number of granulocyte-erythroid-macrophage-megakaryocyte colonies (CFU-GEMM), which consisted of 4 types of hemopoietic cells (erythrokaryocytes, granulocytes, macrophages, and megakaryocytes). Granulocyte-macrophage colonies (CFU-GM) and granulocyte colonies (CFU-G) were also counted [4,8]. Proliferative activity of CFU-GM and CFU-G was studied by the method of cell suicide with hydroxyurea. The degree of differentiation was evaluated from the index of maturation (cluster/colony ratio in a well) [4]. G-CSF concentration in the culture was 2 ng/ml [4].

The concentration of bone marrow CFU with non-differentiated cells (CFU-N) was estimated by the method of limiting dilutions [4,8]. After culturing, CFU-N were collected, divided into individual nucleated cells (method of pipetting), and washed from methylcellulose. The cells were put in 35-mm Petri dishes with 2 ml medium, incubated for 12 days under standard conditions, and passaged (3 times). The number of colonies was evaluated after each passage. Cytomorphological study was performed. The effect of G-CSF on differentiation of CFU-N was studied. The aggregates were obtained after the 3rd passage and divided into individual cells. For the growth of CFU-GM and CFU-G, the concentration was brought to 10,000 cells per ml culture medium [4]. The samples (150 μl) were maintained in 96-well flat-bottom plates. The number of CFU-GM and CFU-G was evaluated after 7 days.

The results were analyzed by standard methods of variation statistics. The significance of differences was evaluated by parametric Student's t test or nonparametric Wilcoxon–Mann–Whitney U test. Exact Fisher test was used to analyze the rated data. The frequency of CFU-N was estimated by the standard linear method of Poisson distribution.

RESULTS

In series I, the regenerative capacity of hemopoietic tissue in animals was studied after treatment with cyclophosphamide in $^1/_3$ MPD. The cytostatic caused a significant decrease in the number of bone marrow neutrophilic granulocytes (days 1-3) and neutrophilic neutropenia in the peripheral blood (days 1-5; Table 1). However, the number of immature neutrophilic granulocytes in the blood increased significantly and reached the initial level on days 4-7. An increase in the number of mature granulocytes was observed in the later period (days 6-7).

Hemopoietic tissue regeneration during hypoplasia was shown to depend directly on the state of precursor cells for differentiation processes (erythroid burst-forming units and CFU, CFU-G) [2,3]. Administration of alkylating agent was followed by long-term inhibition of CFU-G growth (days 1-6) and division of granulocyte precursors (days 1-5; Fig. 1). The restoration of mitotically active CFU-G (up to the intact control level) and stimulation of their differentiation were observed when the number of mature neutrophils returned to normal (days 6-7). It seems unlikely that regeneration of hemopoietic tissue on day 4 after cytostatic treatment is related only to activity of CFU-G. We believe that immature hemopoietic precursors play a role in this process.

Activation of primitive hemopoietic precursors was observed in the period of myelosuppression. For example, the concentration of CFU-N in hemopoietic tissue was elevated on day 2 after cyclophosphamide administration (1 CFU per 14,600 nonadherent myelokaryocytes). This parameter in intact control animals was 1 CFU per 121,400 nucleated cells. The cells of colonies are characterized by long-term repopulation (more than 4 passages). The secondary and tertiary colonies practically did not differ from the primary culture in size (more than 1300 blast cells) and morphology. These colonies consisted of not only non-differentiated cells, but also of aggregates from erythroblasts, granulocytes, and mononuclear phagocytic cells. These CFU were probably formed from hemopoietic stem cells. The number of nucleated cells in CFU-N from the treatment group was 30-50% higher than in the intact control. The count of cell associations was shown to depend linearly on the number of nucleated nonadherent myelokaryocytes in a well (r=0.88, p<0.05). These data suggest that the cytostatic-mobilized hemopoietic cells have high proliferative activity. In the follow-up period (days 3-7) the number of CFU-N in this group practically did not differ from the control.

Cyclophosphamide treatment was followed by a sharp increase in the pool of CFU-GEMM (days 3-4;

TABLE 1. Effect of Granulocyte CSF on the Number of Cells of Granulocytic Hemopoietic Stem in the Bone Marrow ($\times 10^6$ cells per femur) and Peripheral Blood ($\times 10^9$ cells/liter) in CBA/CaLac Mice Receiving Cyclophosphamide in $^1/_3$ MPD (*M*±*m*)

Parameter			Intact control	Period, days						
				1	2	3	4	5	6	7
Bone marrow	immature neutrophilic granulocytes	PS	2.49±0.28	0.60±0.08*	0.46±0.03*	0.66±0.08*	2.84±0.25	1.86±0.41	3.20±0.31	2.51±0.14
		G-CSF		0.79±0.08*	0.85±0.08*+	1.39±0.24*+	2.13±0.15	1.95±0.01	2.77±0.28	2.02±0.35
	mature neutrophilic granulocytes	PS	6.35±0.49	2.23±0.26*	1.10±0.08*	0.77±0.09*	3.09±0.28*	3.09±0.45*	9.10±0.43*	8.640±0.052
		G-CSF		2.40±0.15*	1.26±0.16*	1.44±0.27*	4.84±0.42*+	7.11±0.52+	9.50±0.73*	8.10±0.97
Peripheral blood	stab neutrophils	PS	1.64±0.45	0.57±0.07*	0.33±0.04*	0.67±0.08*	2.36±0.20	3.44±0.33*	1.08±0.20	1.05±0.23
		G-CSF		0.37±0.05*	0.38±0.04*	0.44±0.10*	2.09±0.31	2.08±0.35+	0.55±0.07*	1.43±0.20
	segmented neutrophils	PS	5.05±0.28	2.60±0.29*	1.62±0.21*	0.76±0.13*	0.17±0.03*	1.37±0.25*	4.31±0.61	5.61±0.58
		G-CSF		3.20±0.37+	5.52±0.73+	0.23±0.07*	7.34±0.97*+	8.44±0.90*+	3.35±0.08	5.3±0.6

Note. PS, physiological saline. $p<0.05$: *compared to the intact control; +compared to the cytostatic control (PS).

Fig. 1). The number of cells in CFU-GEMM from animals of the treatment group was above 800. The count of nucleated cells in the intact control did not exceed 500. The number of precursor cells capable of forming CFU-GM in the culture of nonadherent myelokaryocytes was elevated on days 3, 5, and 7 (by 166, 733, and 167% higher than in the intact control, respectively). Experiments with with hydroxyurea showed that the rate of CFU-GM division increased during these periods. Activation of granulocyte-monocyte precursors was preceded by a 70% decrease in the rate of cell proliferation (by 70% compared to the intact control, $p<0.05$; day 1).

Our results indicate that cyclophosphamide-induced myelosuppression is accompanied by successive activation of multipotent, oligopotent, and bipotent hemopoietic precursors in the bone marrow.

In series II, we studied the response of hemopoietic precursors of various classes on G-CSF. The cytokine induced a more significant release of CFU-N on days 3 and 4 after treatment with alkylating agent (by 2 and 4 times, respectively, compared to the cytostatic control; $p<0.01$). The amount of CFU-N depended linearly on the concentration of nucleated cells in a well ($r=0.95$, $p<0.05$). Our findings illustrate high proliferative activity of this population. G-CSF had a stimulatory effect on the formation of CFU-G (days 3 and 6), but decreased the number of CFU-GM in the culture of non-differentiated cells from tertiary colonies (days 2 and 4; Fig. 2). Therefore, cyclophosphamide injection is primarily followed by the restoration of granulocyte precursors that have the greatest sensitivity to this cytostatic.

Parenteral administration of G-CSF had a strong stimulatory effect on the growth of CFU-GEMM (days 3-5; Fig. 1). The size of aggregates in these specimens was 40-70% greater than in the cytostatic control ($p<0.05$). Cytomorphological study showed that individual colonies consist of not only blast forms of granulocytes and promyelocytes (similarly to the cytostatic control, without preparation), but also of metamyelocytes and stab granulocytes. On days 2-5 after cytostatic treatment, the maturation index of CFU-G increased progressively in cytokine-receiving animals. This parameter was highest on day 5 (Fig. 1). Mitotic activity of granulocyte precursors returned to normal on days 3 and 5, but was below the intact control level by the 6thŒ ay.

After cyclophosphamide injection, G-CSF stimulated the differentiation of hemopoietic precursor cells. These changes contribute to the rapid increase in the number of neutrophils in the bone marrow and peripheral blood (Table 1). The effect of this cytokine on study cells was increased in the following order: CFU-N<CFU-GEMM<CFU-G.

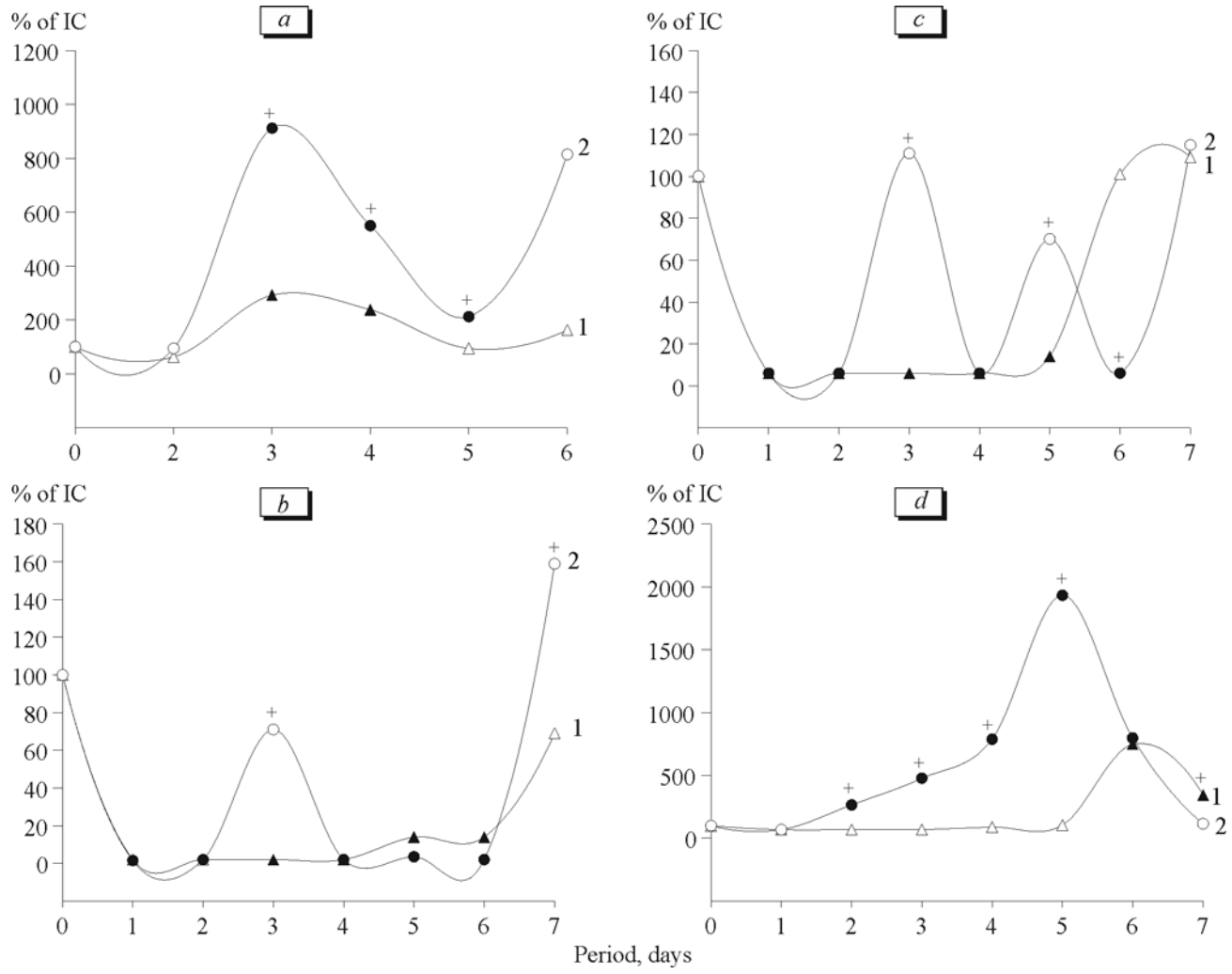

Fig. 1. Effect of course treatment with G-CSF on the number of CFU-GEMM (*a*) and CFU-G in the bone marrow (*b*), proliferative activity (*c*), and differentiation of granulocyte precursors (*d*) in cyclophosphamide-receiving CBA/CaLac mice. Here and in Fig. 2: physiological saline (cytostatic control, *1*); G-CSF (*2*). IC, intact control (period "0"). Filled symbol: significant differences of test parameter from IC ($p<0.05$). $^{+}p<0.05$ compared to *1*.

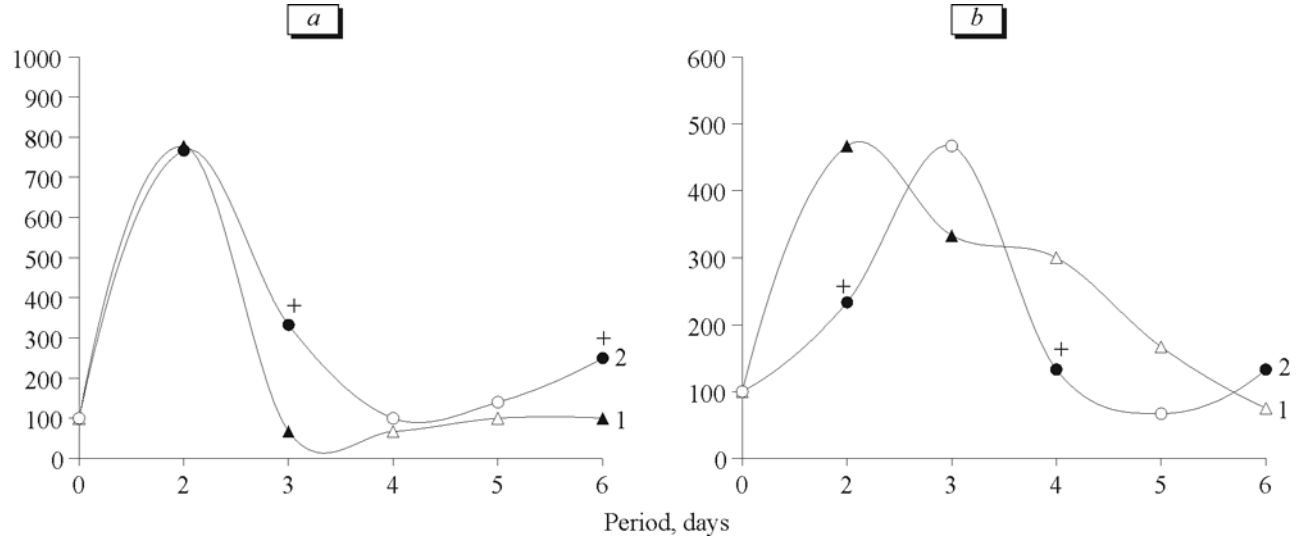

Fig. 2. Effect of course treatment with G-CSF on the growth of CFU-G (*a*) and CFU-GM (*b*) in tertiary cultures of multipotent hemopoietic precursors from cyclophosphamide-receiving CBA/CaLac mice. Ordinate: intensity of growth (% of IC).

We conclude that cyclophosphamide treatment is followed by the transition of primitive hemopoietic precursors in the bone marrow (CFU-N) from G_0 phase to the stage of proliferation. The mechanism of replenishment for the loss of blood neutrophils suggests not only the self-maintenance of multipotent precursors, but also the multistage differentiation of these cells into specialized blood cells (CFU-GEMM, CFU-GM, and CFU-G) and mature granulocytes. THE decrease in the duration and degree of neutropenia under the influence of G-CSF is provided by stimulation of maturation and, to a lesser extent, of the division of multipotent and oligopotent precursors (myelosuppression). Changes in granulocyte colony-forming cells were observed in the later period (regeneration). This phenomenon is probably related to high sensitivity of CFU-G to the cytostatic. When cyclophosphamide decreases significantly the number of mature precursors, the mitotic activity of these cells is increased in the presence of study cytokine. These data illustrate the high functional reserves of CFU-G. G-CSF provides activity of CFU-G and committing of multipotent hemopoietic precursors.

This work was supported by the grant of the President of Russian Federation (NSh-275.2008.7).

REFERENCES

1. M. A. Volkova, *Ter. Arkhiv*, No. 4, 80-84 (1998).
2. A. I. Vorob'ev, *Manual on Hematology*, Ed. A. I. Vorob'ev [in Russian], In 3 volumes, Vol. 1, Moscow (2002).
3. E. D. Gol'dberg, A. M. Dygai, V. V. Zhdanov, *et al*., *Pharmacological Regulation of the Blood System during Experimental Neuroses* [in Russian], Tomsk (2007).
4. E. D. Gol'dberg, A. M. Dygai, and V. P. Shakhov, *Tissue Culture Methods in Hematology* [in Russian], Tomsk (1992).
5. F. De Arriba, M. L. Lozano, F. Ortuño, *et al*., *Br. J. Haematol*., **96**, No. 2, 418-420 (1997).
6. G. D. Demetri and J. D. Griffin, *Blood*, **78**, No. 11, 2791-2808 (1991).
7. D. Metcalf, *Lancet*, **15**, 825-827 (1989).
8. G. J. Ventura, J. P. Hester, E. S. Buescher, *et al*., *Exp. Hematol*., **18**, No. 8, 878-882 (1990).
9. G. Williams, C. A. Smith, E. Spooncer, *et al*., *Nature*, **343**, 76-79 (1990).