Phenotype of Peripheral Blood Cells Mobilized by Granulocyte Colony-Stimulating Factor in Patients with Chronic Heart Failure

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Administration of granulocyte CSF preparation to patients with chronic heart failure produced a hemostimulating effect and increased the content of leukocytes and neutrophils in the peripheral blood. Granulocyte CSF induced mobilization of bone marrow progenitor cells into the peripheral blood. The content of hemopoietic $CD34^+$ progenitor cells, which attained 0.42% (0.25-0.64) by the end of mobilization, inversely correlated with the number of myocardial infarctions. Administration of granulocyte CSF not only led to mobilization of bone marrow hemopoietic cells, but also increased the pool of endothelial progenitor cells in the peripheral blood: the content of $CD34^+/CD133^+$ and $CD34^+/KDR^+$ attained 0.02% (0.013-0.075) and 0.1% (0.05-0.20), respectively. Peripheral blood is an available source of progenitor cells, while mononuclear cells after administration of granulocyte CSF can produce a reparative effect on ischemic myocardium.

Key Words: *progenitor cells; coronary heart disease; flow cytoemtry*

Coronary heart disease (CHD) is the leading cause of morbidity and mortality in developed countries. CHD and myocardial infarction (MI) usually result in chronic heart failure (CHF) with progressive myocardial remodeling. Despite evident progress in the therapy of heart failure, usual drug and surgical methods are not sufficiently effective in some patients. Due to limited proliferative potential of cardiomyocytes, the self-reparation capacities of the heart are far from optimal, which leads to impairment of myocardial function [17].

Heart transplantation is the golden standard in the therapy of progressive heart failure, but this approach is limited by shortage of donor organs, recipient's age, and other strict selection criteria. Cell cardiomyoplasty with autologous stem/progenitor cells is a new strategy in the therapy of cardiovascular pathologies [20].

BM is the major source of progenitor cells (PC) in the adult organism. PC are partially released from BM into circulation in various traumas, stress influences, and stimulations, thus providing reparation of damaged organs and tissues. Experimental and clinical studies demonstrated the safety and efficiency of using BM PC for the therapy of both acute MI [3,22] and chronic CHD [7,18].

BM SC/PC can also be isolated from the peripheral blood after mobilization with hemopoietic growth factors. In clinical practice, BM cells are mobilized by several successive injections of human recombinant granulocytic CSF (G-CSF) [12]. G-CSF is widely used for collection of peripheral blood PC in

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healthy donors for allogeneic transplantation [1,15]. Much recent attention is focused on the therapy with autologous cells, which are more preferable than allogeneic cells, because they induce no rejection reaction and do not require thorough testing for infectious and viral contamination.

Experimental studies on different animal species demonstrated the efficiency of G-CSF-mobilization in modeled MI, in particular, decrease in the infarction zone and improvement of heart function [10]. However, IM modeling cannot completely reproduce all processes accompanying CHD development in humans. Clinical studies in patients with cardiovascular pathology provide no definite conclusion on clinical efficiency of G-CSF administration in several successive injections [21], because cell therapy of CHD usually consists in combination of PC mobilization with local or systemic administration of isolated PC to the patient.

Effective mobilization leads to the release of PC from BM into circulation. Apart from hemopoietic precursors, the peripheral blood after PC mobilization is also a source of endothelial PC (EPC); the role of these cells in improvement of myocardial perfusion is now actively studied [2,5,6,12,16,23].

Here we studied the efficiency of mobilization of BM cells in patients with chronic myocardial ischemia and characterized the phenotype of isolated mononuclear fraction of blood cells for further intramyocardial transplantation.

MATERIALS AND METHODS

The study included 67 CHD patients (91% men) with CHF (NYHA functional class III-IV). The mean age of patients was 57.0±7.7 years, mean history of CHD was 7.2±5.4 years, the number of MI was 1.5±0.8. The patients were hospitalized at E. N. Meshalkin Research Institute of Circulatory Pathology (Novosibirsk). All patients signed informed consent for participation in the study. The experimental protocol was approved by local ethical committees and academic councils of both institutions.

Recombinant human G-CSF (TEVA Pharmaceutical Industries Ltd.) was injected subcutaneously in a dose of 3.3-5.0 μg/kg for 5 days. The blood was taken from the ulnar vein before mobilization (day 0) and after completion of G-CSF treatment course (day 6). The fraction of mononuclear cells was isolated by centrifugation on Ficoll-verografin density gradient (ρ=1.078).

Phenotyping of immunocompetent cells was performed on a FACSCantoII flow cytometer (Becton Dickinson) with FACSDiva software (Becton Dickinson) according to manufacturer's instructions. Monoclonal antibodies to CD3, CD4, CD8, CD14, CD19, CD34, CD45, CD133, and KDR labeled with FITC, phycoerythrin (PE), and allophycocyanin (APC) in amounts recommended by the manufacturers (Becton Dickinson, Sorbent) were used for surface labeling. Parameters of the peripheral blood were determined by standard hematological methods.

The data were processed by methods of descriptive nonparametric statistics using Statistica 6.0 software. For descriptive statistics, arithmetic means and errors of the means (M±m) were used; in case of non-normal data distribution, median and 25 and 75 quartiles were used. The significance of differences was evaluated using non-parametric Mann—Whitney test, the correlations between the studied parameters were analyzed by Spearman rank correlation test.

RESULTS

Evaluation of hemostimulating activity of G-CSF

Administration of G-CSF to healthy donors led to effective expansion of neutrophils and increased the count of leukocytes in the peripheral blood [1]. In our experiments, administration of G-CSF to patients with severe CHF also induced leukocytosis and granulocytosis. Leukocyte content in the peripheral blood increased by 4.9 times (from 6.39±1.20×10^9/liter to 31.25±6.00×10^9/liter) by day 6 (Table 1). The maximum rise was observed during the first day. The increase in leukocyte content was accompanied by the increase in both the relative and absolute count of neutrophils by 6.8 times (Fig. 1). The increase in neutrophil count directly correlated with the increase in leukocyte content (r_s=0.987 at p=0.004).

Biological effects of G-CSF are mediated by the receptor expressed not only on neutrophils. G-CSF in a dose of 10 μg/kg stimulates myeloid, lymphoid, erythroid, and megakaryocyte hemopoietic lineage cells [1]. We showed that administration of G-CSF in a dose of 3.3-5.0 μg/kg to patients with CHF led to a relative decrease in percentage of lymphocytes and monocytes; the absolute content of lymphocytes and monocytes increased by 1.9 and 2.9 times, respectively, but remained within the normal range. Flow cytometry revealed no significant increase in $CD3^+$ and $CD14^+$ cell populations, as well as T cell (CD4, CD8) and B cell (CD19) subpopulations (Table 2). The data are presented as the ratio of cell number after mobilization to that before G-CSF treatment. Administration of G-CSF in the specified dose did not change the content of eosinophils, basophils, platelets, erythrocytes, and hemoglobin and did not affect erythrocyte sedimentation rate. Taking into account the route of cell transplantation (directly in the myocardium), mobilization was performed with medium doses of

TABLE 1. Dynamics of Hematological Parameters of Peripheral Blood (*M*±*m*; *n*=28)

Parameter	Before G-CSF injection (day 0)	After G-CSF injection (day 5)
Leukocyte count, $\times 10^9$/liter	6.39±1.20	31.25±6.00 (p=0.0015)
Neutrophils, %	52.96±4.50	77.92±4.60 (p=0.0015)
Neutrophil count, $\times 10^9$/liter	3.53±0.78	24.18±4.50 (p=0.0015)
Lymphocytes, %	35.27±7.80	10.18±3.10
Lymphocyte count, $\times 10^9$/liter	2.23±0.38	4.29±1.32 (p=0.0025)
Monocytes, %	8.45±2.30	5.73±2.50
Monocyte count, $\times 10^9$/liter	0.61±0.20	1.77±0.84 (p=0.0094)
Platelet count, $\times 10^9$/liter	209.0±58.1	210.73±32.70
Erythrocyte count, $\times 10^{12}$/liter	4.24±0.70	4.17±0.80
ESR	14.36±9.00	11.82±6.70

Note. *p*: significance of differences from the parameter before mobilization (Mann–Whitney *U* test).

G-CSF (3.3-5.0 μg/kg) not stimulating megakaryocyte and erythroid lineage cells. These findings agree with published data [1.9].

In the delayed period (6 months after G-CSF administration), the content of leukocytes and neutrophils was 6.31±1.70×10^9/liter and 3.3±1.7×10^9/liter, respectively, which corresponded to the values before mobilization.

Effect of G-CSF on PC. The content and functional activity of PC decrease with age; the efficiency of G-CSF mobilization in healthy donors also depends on their age [6]. The patients included in the study not only belonged to the elder age group, but also had long history of CHD, from 1 to 5 IM, and progressive heart failure. Moreover, some drugs (β-blockers, acetylsalicylic acid, nitrates) can considerably limit mobilization and modulate the properties of PC [3,6,16].

For evaluation of the effect of PC mobilization into circulation, hemopoietic cell marker CD34 was used. Mononuclear fraction cells were counted with restriction of events with panleukocytic marker CD45 expression. In patients with CHF, the content of circulating $CD43^+$ cells is normally (without simulation) 0.01% (median). On day 6 of G-CSF treatment, the content of $CD34^+$ cells increased to 0.42% (Table 3). The content of $CD34^+$ cells increased by on average 20.7±16.8 times. It should be noted that in patients (11%) with initially high level of $CD34^+$ cells (>0.2%) this increase was less pronounced (by 2.4±1.0 times). These findings agree with published data on the content of $CD34^+$ cells and increase in these cells in patients with cardiovascular pathology [13]. In healthy individuals, the content of $CD34^+$ cells after mobilization attained higher values (up to 1%) [9]. Analysis of the content of $CD34^+$ cells before and after G-CSF stimulation revealed no significant correlations with the age of patients and duration of CHD history (Table 4). At the same time, a significant negative correlation was observed between the content of $CD34^+$ cells after G-CSF mobilization and a history of MI (from 1 to 5). Thus, the maximum number of progenitor $CD34^+$ cells after administration of G-CSF was revealed in patients with single MI, which suggests that the best effect of mobilization can be expected in patients with chronic myocardial ischemia.

Administration of G-CSF leads to not only mobilization of hemopoietic PC from BM, but also to an increase in EPC pool in the peripheral blood; these cells are identified by the expression of CD34 marker, early hemopoietic cell marker CD133, and vascular endothelial growth factor receptor $VEGFR_2$ (KDR) [4,5,8,23]. In patients with CHF on day 6 after G-CSF mobilization, we observed an increase in the number of not only $CD34^+$ cells, but also $CD34^+$ cells co-expressing CD133 marker (Table 4). Although no sig-

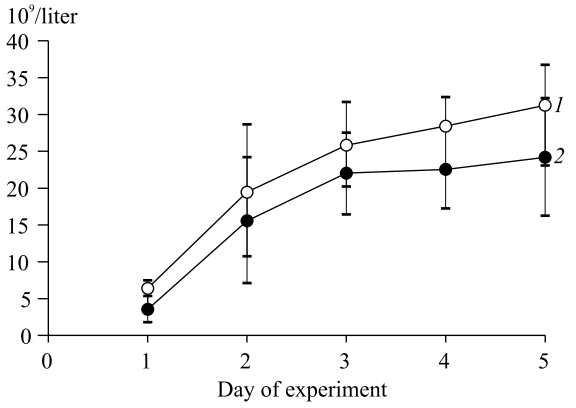

Fig. 1. Dynamics of leukocyte (*1*) and neutrophils (*2*) during G-CSF mobilization.

TABLE 2. Changes in the Percentage of Cells Carrying CD Markers on Day 6 (*M*±*m*; *n*=13)

CD marker	Multiplication of cell percentage
CD3	1.1±0.2
CD4	0.98±0.20
CD8	1.2±0.5
CD19	1.2±0.5
CD14	1.2±0.2

nificant increase of $CD34^+/CD133^+$ cells was revealed (p=0.06), the relative content of this cell population among mononuclear cells after mobilization was high and attained 0.02%, whereas before mobilization these cells were practically undetectable. At the same time, the population of $CD133^+CD34^-$ cells detected before G-CSF stimulation increased by 5 times after mobilization (to 0.1%). This population of $CD133^+CD34^-$ cells, similarly to population of $CD133^+CD34^+$ cells promote vascular regeneration [8]. It is known that the amount of circulating EPC decreases with age and in cardiovascular diseases [11]. We revealed no significant relationship between patient's age, CHD severity, and the content of endothelial precursors. The third marker typical of EPC is $VEGF_2$ receptor or KDR [6,19]. After administration of G-CSF, the content of $CD34^+KDR^+$ endothelial precursors increased and attained 0.1%. However, both $CD34^+$ and $CD34^-$ cells can serve as EPC. We showed that the population of $CD34^-$ cells expressing $VEGF_2$ receptor (KDR^+ cells) increased from 0.25 to 2%. High expression of $VEGF_2$ receptor (KDR) can suggest that after mobilization the mononuclear fraction contains either another population of endothelial precursors ($CD14^+CD34^-$ EPC) or mature endothelial cells [23].

Peripheral blood EPC after G-CSF mobilization represent a heterogeneous population. EPC can produce reparative effects on the ischemic myocardium by promoting neovascularization due to synthesis of growth factors potentiating local angiogenesis, by direct integration into the vascular wall (vasculogenesis), additional mobilization of PC from BM, and differentiation into cardiomyocytes [14,23]. Due to this, peripheral blood is a promising source of PC for the treatment of patients with CHF and further studies of the mechanisms of myocardium reparation are required.

Thus, G-CSF effectively mobilizes PC from BM into peripheral blood in patients with CHF. G-CSF probably produces no direct effect on hemopoietic SC and the effect of $CD34^+$ cell mobilization is mediated by cells carrying receptors to G-CSF (*e.g.* neutrophilic granulocytes and probably monocytes).

In these cells, G-CSF initiates the synthesis of second messengers (*e.g.* other cytokines (IL-8, IL-3, G-CSF, SDF-1) and/or collagenases (matrix metalloproteinases 9 and 2) disrupting binding of $CD34^+$ cells with stromal elements of BM [16].

Apart from mobilization of hemopoietic PC, G-CSF increases the pool of EPC. It is known that the content of EPC is reduced in CHD patients. It was

TABLE 3. Content of CD34, CD34CD133, CD34 KDR Cells during Mobilization of G-CSF*

Markers	Day observation	
	0	6
$CD34^+$	0.01 (0.01-0.067) (n=38)	0.42 (0.25-0.64) p=0.0001 (n=38)
$CD34^+/CD133^+$	0.001 (0-0.075) (n=8)	0.02 (0.013-0.075) p=0.06 (n=22)
$CD133^+/CD34^-$	0.06 (0.06-0.1) (n=8)	0.1 (0.05-0.5) p=0.11 (n=22)
$CD34^+/KDR^+$	0.00 (0-0) (n=8)	0.10 (0.05-0.2) p=0.108 (n=8)
$KDR^+/CD34^-$	0.25 (0.1-0.95) (n=8)	2.0 (0.9-2.2) p=0.06 (n=8)

Note. Expression of markers (% of positive cells). p: significance of differences from the parameter before mobilization (Mann–Whitney U test). The data are presented as median and quartiles (25-75).

TABLE 4. Correlation of the Content of $CD34^+$ and $CD34^+CD133^+$ Cells with Patient's Age, CHD History, and Number of MI

Parameter	Number of $CD34^+$ cells on day 0 (n=38)		Number of $CD34^+$ cells on day 6 (n=38)		Number of $CD34^+CD133^+$ cells on day 6 (n=22)	
	r_s	p	r_s	p	r_s	p
Age	0.046	0.85	0.396	0.115	0.354	0.2
CHD history	-0.352	0.16	0.207	0.425	-0.046	0.17
Number of MI	0.228	0.37	-0.467	0.05	0.198	0.44

Note. p: significance of differences between the parameters.

found that increased content of circulating EPC is associated with lower risk of cardiovascular complications, which accentuates the vasoprotective effect of EPC. Injection of cultured EPC into systemic circulation or increase of EPC pool *in vivo* caused by statins, estrogen, G-CSF, or physical exercise improved reendothelialization, suppressed neointima formation, and prevented restenosing [22].

Hence, the cardioprotective effect of intramyocardial injection of total mononuclear fraction cells after G-CSF mobilization in patients with CHF can be mediated by stimulation of neovasculogenesis [4].

It should be noted that the use of peripheral blood as the source of hemopoietic PC and EPC in patients with CHF has obvious advantages over other tissue sources.

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