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Differentiation and subpopulation composition of VEGFR2$^+$ cells in the blood and bone marrow in ischemic cardiomyopathy

Chumakova S.P.[1], Urazova O.I.[1, 2], Shipulin V.M.[3], Denisenko O.A.[1, 4], Kononova T.E.[1], Nevskaya K.V.[1], Andreev S.L.[3]

[1] Siberian State Medical University
2, Moscow Trakt, Tomsk, 634050, Russian Federation

[2] Tomsk State University of Control Systems and Radioelectronics (TUSUR)
40, Lenina Av., Tomsk, 634050, Russian Federation

[2] Cardiology Research Institute, Tomsk National Research Medical Center (NRMC), Russian Academy of Sciences
111a, Kievskaya Str., Tomsk, 634012, Russian Federation

[4] Tomsk Regional Blood Center
45, Vershinina Str., Tomsk, 634045, Russian Federation

ABSTRACT

Aim. To identify disturbances of differentiation and subpopulation composition of VEGFR2+ cells in the blood and bone marrow associated with the features of the cytokine profile in the blood and bone marrow in patients with coronary artery disease (CAD) with and without ischemic cardiomyopathy (ICM).

Materials and methods. The study included 74 patients with CAD with and without ICM (30 and 44 people, respectively) and 18 healthy donors. In all patients with CAD, peripheral blood sampling was performed immediately before coronary artery bypass grafting, and bone marrow samples were taken during the surgery via a sternal incision. In the healthy donors, only peripheral blood sampling was performed. In the bone marrow and blood samples, the number of VEGFR2$^+$ cells (CD14$^+$VEGFR2$^+$ cells) and their immunophenotypes CD14^{++}CD16$^-$VEGFR2$^+$, CD14^{++}CD16$^+$VEGFR2$^+$, CD14$^+$CD16^{++}VEGFR2$^+$, and CD14$^+$CD16$^-$VEGFR2$^+$ was determined by flow cytometry. Using enzyme-linked immunosorbent assay, the levels of VEGF-A, TNFα, M-CSF, and IL-13, as well as the content of MCP-1 (only in the blood) and the M-CSF / IL-13 ratio (only in the bone marrow) were determined.

Results. The content of CD14$^+$VEGFR2$^+$ cells in the blood of CAD patients with and without ICM was higher than normal values due to the greater number of CD14^{++}CD16$^-$VEGFR2$^+$, CD14^{++}CD16$^+$VEGFR2$^+$, and CD14$^+$CD16^{++}VEGFR2$^+$. In the bone marrow of the patients with ICM, the content of CD14^{++}CD16$^-$VEGFR2$^+$, CD14$^+$CD16^{++}VEGFR2$^+$, and CD14$^+$CD16$^-$VEGFR2$^+$ was lower than in patients with CAD without ICM, and the number of CD14^{++}CD16$^+$VEGFR2$^+$ cells corresponded to that in the controls. Regardless of the presence of ICM in CAD, a high concentration of TNFα and normal levels of VEGF-A and IL-13 were observed in the blood. In CAD without ICM, an excess of MCP-1 and deficiency of M-CSF were revealed in the blood. In the bone marrow, the levels of VEGF-A, TNFα, M-CSF, and IL-13 were comparable between the groups of patients against the background of a decrease in the M-CSF / IL-13 ratio in the patients with ICM.

Conclusion. Unlike CAD without cardiomyopathy, in ICM, no excess of VEGFR2$^+$ cells and MCP-1 in the blood is observed, which hinders active migration of CD14$^+$CD16^{++}VEGFR2$^+$ cells from the myeloid tissue, and a decrease in the M-CSF / IL-13 ratio in the bone marrow disrupts differentiation of other forms of VEGFR2$^+$ cells, preventing vascular repair.

Keywords: endothelial progenitor cells, monocytes, bone marrow, cytokines, vascular repair, ischemic cardiomyopathy, coronary artery disease

✉ *Chumakova Svetlana P.,* chumakova_s@mail.ru

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Conformity with the principles of ethics. All study participants signed an informed consent. The study was approved by the local Ethics Committee at Siberian State Medical University (Protocol No. 5046 of 28.11.2016).

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Дифференциация и субпопуляционный состав VEGFR2+ моноцитов крови и костного мозга при ишемической кардиомиопатии

Чумакова С.П.[1], Уразова О.И.[1, 2], Шипулин В.М.[3], Денисенко О.А.[1, 4], Кононова Т.Е.[1], Невская К.В.[1], Андреев С.Л.[3]

[1] Сибирский государственный медицинский университет (СибГМУ)
Россия, 634050, г. Томск, Московский тракт, 2

[2] Томский государственный университет систем управления и радиоэлектроники (ТУСУР)
Россия, 634050, г. Томск, пр. Ленина, 40

[3] Научно-исследовательский институт (НИИ) кардиологии, Томский национальный исследовательский медицинский центр (НИМЦ) Российской академии наук
Россия, 634012, Томск, ул. Киевская, 111а

[4] Томский региональный центр крови
Россия, 634045, г. Томск, ул. Вершинина, 45

РЕЗЮМЕ

Цель: установить нарушения дифференцировки и субпопуляционного состава VEGFR2$^+$ моноцитов в крови и костном мозге во взаимосвязи с особенностями цитокинового профиля крови и костного мозга у больных ишемической болезнью сердца (ИБС), страдающих и не страдающих ишемической кардиомиопатией (ИКМП).

Материалы и методы. В исследование вошли 74 больных ИБС, страдающих и не страдающих ИКМП (30 и 44 человека соответственно), и 18 здоровых доноров. У всех больных ИБС забор периферической крови производился непосредственно перед операцией коронарного шунтирования, а костного мозга – из разреза грудины во время операции. У здоровых доноров забирали только периферическую кровь. В костном мозге и крови методом проточной цитофлуориметрии определяли численность VEGFR2$^+$ моноцитов (CD14$^+$VEGFR2$^+$ клеток) и их иммунофенотипов CD14^{++}CD16$^-$VEGFR2$^+$, CD14^{++}CD16$^+$VEGFR2$^+$, CD14$^+$CD16^{++}VEGFR2$^+$, CD14$^+$CD16$^-$VEGFR2$^+$, методом иммуноферментного анализа регистрировали концентрацию VEGF-A, TNFα, M-CSF, IL-13, а также содержание MCP-1 (только в крови) и соотношение M-CSF/IL-13 (только в костном мозге).

Результаты. Содержание CD14$^+$VEGFR2$^+$ клеток в крови у больных ИБС без кардиомиопатии и с ИКМП было выше нормы из-за большей численности CD14^{++}CD16$^-$VEGFR2$^+$, CD14^{++}CD16$^+$VEGFR2$^+$ и CD14$^+$CD16^{++}VEGFR2$^+$ форм. В костном мозге у больных ИКМП содержание CD14^{++}CD16$^-$VEGFR2$^+$, CD14$^+$CD16^{++}VEGFR2$^+$ и CD14$^+$CD16$^-$VEGFR2$^+$ форм было ниже, чем у больных ИБС без кардиомиопатии, а количество CD14^{++}CD16$^+$VEGFR2$^+$ клеток соответствовало их числу в группе сравнения. Вне зависимости от наличия ИКМП при ИБС в крови отмечалась высокая концентрация TNFα, нормальный уровень VEGF-A и IL-13; при ИБС без кардиомиопатии – избыток MCP-1 и дефицит M-CSF в крови. В костном мозге концентрация VEGF-A, TNFα, M-CSF, IL-13 была сопоставимой между группами больных на фоне снижения M-CSF/IL-13 у пациентов с ИКМП.

Заключение. В отличие от ИБС без кардиомиопатии при ИКМП не формируется избыток VEGFR2$^+$ моноцитов и MCP-1 в крови, что затрудняет активную миграцию CD14$^+$CD16^{++}VEGFR2$^+$ клеток из миелоидной ткани, а снижение M-CSF/IL-13 в костном мозге нарушает дифференцировку остальных форм VEGFR2$^+$ моноцитов, препятствуя репарации сосудов.

Ключевые слова: моноциты, прогениторные эндотелиальные клетки, репарация сосудов, костный мозг, цитокины, ишемическая кардиомиопатия**,** ишемическая болезнь сердца

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Ischemic cardiomyopathy (ICM) is a complicated form of chronic coronary artery disease (CAD) and one of the most common causes of death from cardiovascular diseases worldwide [1]. ICM is characterized by dilatation of heart chambers, hypertrophy (mainly of the left ventricle), and a decrease in the heart pumping function with the formation of chronic heart failure [2]. It is believed that ICM development is based on impaired myocardial contractile function due to coronary microvascular dysfunction, which is a trigger for widespread myocardial ischemia and hibernation, necrosis, and apoptosis of cardiomyocytes, followed by fibrosis and ventricular remodeling [1, 2]. Endothelium plays an important role in the physiology and pathology of the cardiovascular system, modulating vascular tone, hemocoagulation, fluid and solute exchange, as well as inflammation and angiogenesis [3–5].

At the same time, endothelial dysfunction is considered mainly as an imbalance of vasoconstrictor and vasodilator stimuli, and due attention is not paid to endothelial regeneration in the literature. Meanwhile, impaired endothelial repair may be an important pathogenetic factor of ICM, which substantiates the study of the mechanisms of differentiation and migration of endothelial progenitor cells (EPC) in this pathology.

EPCs are mainly present in the bone marrow, maturing from hematopoietic stem cells, but they can also be isolated from peripheral blood and blood vessel walls [6, 7]. Endothelial cells, including EPCs, express the type 2 vascular endothelial growth factor receptor (VEGFR2) at all stages of differentiation. VEGF (VEGFR2) is associated with tyrosine kinase and is the main activator of angiogenesis, because phosphorylation of its Y1175 domain activates proliferation, and phosphorylation of Y951 and Y1214 domains activates cell migration [8]. It has been established that blood mononuclear cells *in vitro* can acquire endothelial markers and endotheliocyte morphology under the influence of proangiogenic stimuli [7], which indicates their mixed phenotype and suggests the relevance of studying monocytic VEGFR2$^+$ cells (CD14$^+$VEGFR2$^+$ cells) belonging to early EPCs, with weak expression of CD34 [9, 10]. The literature describes several subpopulations of CD14$^+$VEGFR2$^+$ cells with different phenotypes (classical CD14^{++}CD16$^-$, intermediate CD14^{++}CD16$^+$, non-classical CD14$^+$CD16^{++}, and transitional CD14$^+$CD16$^-$) in healthy donors [11], however, there is no information about changes in the ratio of CD14$^+$VEGFR2$^+$ cell subpopulations in cardiovascular pathology.

Normally, EPCs are a very small population of blood cells, but in case of damage or hypoxia, they are mobilized from the bone marrow under the action of cytokines and are attracted to the damaged area [12]. In a mouse model of angiogenesis, S.K. Chauhan et al. (2015) identified mononuclear cells expressing protein tyrosine kinase 7 and VEGFR2 (as descendants of a macrophage and monocyte subpopulation

located in the perivascular region of *de novo* forming vessels), as well as expressing pericyte markers and secreting angiopoietin-1 and other proangiogenic factors [13].

The process of EPC mobilization is activated by several cytokines: vascular endothelial growth factor (VEGF), hypoxia-inducible factor (HIF)-1, interleukin (IL)-6 [14, 15], monocyte chemotactic protein (MCP)-1 [15, 16], tumor necrosis factor α (TNFα) [6], and macrophage colony-stimulating factor (M-CSF) [17]. At the same time, it is possible that an increase in the number of EPCs in the blood may also be associated with the intensification of their differentiation in myeloid tissue. M-CSF, IL-6, and TNFα can be involved in EPC maturation as proinflammatory cytokines stimulating maturation of monocytic cells [18], while VEGF can contribute to this process as a cytokine interacting with specific receptors on the EPC membrane [10, 13]. In addition, anti-inflammatory cytokines can have a negative effect on myelopoiesis [19], such as IL-13 produced by regulatory T cells (Treg), which induces secretion of IL-10 by macrophages.

Information about the effect of cytokines on EPC differentiation and the content of monocyte progenitor cells of $CD14^+VEGFR2^+$ endotheliocytes in the bone marrow or blood of patients with ICM is not present in the literature. Changes of the subpopulation belonging of EPC to various immunophenotypes of monocytes in CAD, complicated and uncomplicated by ICM, have not been described. Meanwhile, identifying deviations of the above-mentioned parameters characteristic of ICM and understanding the role of cytokines in the differentiation and migration of EPCs in CAD could be the key to successful treatment of ICM.

The aim of the study was to identify the disturbances of differentiation and subpopulation composition of $VEGFR2^+$ monocytes in the blood and bone marrow associated with the features of blood and bone marrow cytokine profile in CAD patients with and without ICM.

MATERIALS AND METHODS

A single-stage, controlled (case-control), single-center, observational study was carried out involving 74 CAD patients with NYHA functional class II–IV angina pectoris and class I–III circulatory insufficiency, with ICM (27 men and 3 women, age 61.0 [56.0; 64.0] years) and without ICM (36 men and 8 women, age 64.0 [59.5; 68.0] years), with a history of myocardial infarction. The control group consisted of 18 healthy donors matched by sex and age with the cohorts of patients.

The diagnostic criteria for ICM were: left ventricular ejection fraction ≤ 40%, a history of acute myocardial infarction or revascularization, ≥ 75% stenosis of the left main or proximal part of the left anterior descending artery, or ≥ 75% stenosis of two or more epicardial vessels [20]. The patients did not have significant differences in the functional class of angina pectoris and heart failure, the incidence of hypertension (stage III), gastrointestinal tract, and lung diseases. However, ICM patients had a higher frequency of chronic cerebrovascular accidents (90 vs 59.09%, $p = 0.023$), and CAD patients without cardiomyopathy – of type 2 diabetes mellitus (31.82 vs 6.67%, $p = 0.046$). The exclusion criteria were the presence of autoimmune diseases, severe allergic reaction, cancer, hypoplastic, B12- or folate deficiency anemia, leukemia and other hematological diseases and syndromes, chronic infections (viral hepatitis, syphilis, HIV infection), treatment with iron-containing drugs, erythropoietin or immunosuppressive therapy before surgery, the presence of acute infectious diseases less than 3 weeks before surgery, as well as patient's refusal to enroll in the study.

The patients underwent coronary artery bypass grafting in combination with the left ventricular reconstruction using cardiopulmonary bypass at the Cardiovascular Surgery Department of the Cardiology Research Institute of Tomsk National Research Medical Center. At the preoperative stage, CAD patients in both study groups received similar drug treatment: antianginal therapy with long-acting nitrates, beta1-blockers, Ca^{2+}-channel blockers, hemostasis correction with platelet antiaggregants, and lipid metabolism correction with statins. Premedication and induction of general anesthesia in patients in both study groups was carried out in a similar way using sedatives and anesthetics, narcotic analgesics and muscle relaxants (diazepam, ketamine, fentanyl, promedol, pipecuronium) in comparable doses.

In all CAD patients with and without ICM, 5 ml of peripheral blood was collected from the cubital vein immediately before the surgery, with subsequent stabilization with heparin (25 U / ml). During the surgery, after gaining access to the heart via median sternotomy and before establishing cardiopulmonary bypass, red bone marrow was taken via a sternal incision in the amount of 2 ml in a test tube with the addition of heparin (25 U / ml).

The relative count of $CD14^+VEGFR2^+$ cells in the total population of monocytes (taking cells positive for CD14 as 100%), $CD14^+VEGFR2^+$ cell

distribution among monocyte subpopulations (classical $CD14^{++}CD16^{-}$, intermediate $CD14^{++}CD16^{+}$, non-classical $CD14^{+}CD16^{++}$, transitional $CD14^{+}CD16^{-}$ monocytes), and the proportion of $CD14^{++}CD16^{-}VEGFR2^{+}$, $CD14^{++}CD16^{+}VEGFR2^{+}$, $CD14^{+}CD16^{++}VEGFR2^{+}$, and $CD14^{+}CD16^{-}VEGFR2^{+}$ cells in the total number of $CD14^{+}VEGFR2^{+}$ cells (taking the number of cells positive for CD14 and VEGFR2 as 100%) were determined in the blood and bone marrow samples of patients of both study groups and in the blood of healthy donors by laser flow cytometry at the Central Research Laboratory of Siberian State Medical University.

Monoclonal antibodies CD14-FITC, CD16-PE, VEGFR2 (KDR, CD309)-Alexa Fluor 647 (BD Biosciences, USA) were used to identify subpopulations of the studied blood cells. The absolute count of the above cell subpopulations was assessed based on their proportion and the absolute count of monocytes in the bone marrow, determining the total number of myelokaryocytes with standard hematological methods using the Goryaev's chamber grid and the proportion of monocytic cells (monocytes and promonocytes) by the microscopic examination of bone marrow smears and with account of the absolute count of monocytes in the blood, which was recorded from the clinical patients' records.

Blood plasma and myeloplasma (bone marrow supernatant) of the patients were obtained by centrifugation of the corresponding biomaterial for 15 min at 2,000 g, preserved, and stored at –80 °C. The concentrations of TNFα, MCP-1, IL-13, M-CSF, and vascular endothelial growth factor A (VEGF-A) were determined using commercial enzyme immunoassay kits TNF alpha IFA-BEST and MSR-1-IFA-BEST (Vector-BEST, Novosibirsk), Human IL-13 Platinum ELISA kit (eBioscience, Austria), RayBio Human M-CSF ELISA Kit (RayBiotech, USA), and Human VEGF-A ELISA Kit (Cloud-Clone-Corp., USA) at the Pathophysiology Division of Siberian State Medical University.

The median and the interquartile range $Me\ [Q_1; Q_3]$ were calculated for statistical description of the study results. Nonparametric methods of statistical analysis were used due to the small number of samples and non-normality of data distribution. The Mann – Whitney U-test with the Benjamini–Hochberg correction for multiple comparison was used in order to test the null hypothesis when comparing independent variables. The Spearman's rank correlation coefficient was calculated to analyze the associations. The results were considered statistically significant at a p level of less than 0.05. A statistical analysis of the data was performed using Statistica 10.0 software package.

RESULTS

According to the results of the study, the total count of monocytes of all subpopulations in the blood of CAD patients, regardless of the presence of ICM, corresponded to the normal values (Table 1). At the same time, the number of $CD14^{+}VEGFR2^{+}$ cells was increased only in the CAD patients without ICM and significantly differed from the physiological values of this parameter in the ICM patients (Table 1).

Table 1

The content of monocytes, cytokines, and $CD14^{+}VEGFR2^{+}$ cells in the blood in CAD patients with and without ICM, $Me\ [Q_1; Q_3]$

Parameters	Healthy donors	CAD patients without ICM	CAD patients with ICM
Content of monocytes, $\times 10^9$ / l	0.58 [0.40; 0.66]	0.57 [0.50; 0.76] $p_c = 0.616$	0.67 [0.57; 0.73] $p_c = 0.189$ $p = 0.464$
Content of $CD14^{+}VEGFR2^{+}$cells, %	1.91 [0.75; 3.92]	4.98 [3.86; 10.12] $p_c = 0.006$	2.13 [0.95; 2.66] $p_c = 0.665$ $p = 0.002$
Content of $CD14^{+}VEGFR2^{+}$cells, $\times 10^6$ / l	15.25 [5.07; 22.70]	26.01 [24.24; 34.31] *pc = 0.049*	13.16 [8.51; 15.97] $p_c = 0.685$ $p = 0.024$
VEGF-A, pg / ml	6.50 [1.75; 13.25]	7.00 [4.50; 15.25] $p_c = 0.721$	7.00 [6.00; 13.00] $p_c = 0.415$ $p = 0.811$
TNFα, pg / ml	0.64 [0.04; 0.83]	1.16 [0.90; 1.82] $p_c = 0.012$	2.08 [1.04; 3.60] $p_c = 0.009$ $p = 0.247$
MCP-1, pg / ml	175.0 [145.0; 185.0]	225.0 [182.0; 280.0] $p_c = 0.027$	205.0 [170.0; 260.0] $p_c = 0.104$ $p = 0.660$

Table 1 (continued)

Parameters	Healthy donors	CAD patients without ICM	CAD patients with ICM
IL-13, (pg / ml)	0.50 [0.40; 0.75]	0.60 [0.41; 0.82] $p_c = 0.683$	0.82 [0.40; 0.95] $p_c = 0.420$
M-CSF, (pg / ml)	2.50 [1.60; 4.40]	0.40 [0.12; 2.37] $p_c = 0.046$	2.00 [1.21; 3.24] $p_c = 0.177$ $p = 0.097$

Note: the level of statistical significance of differences in the parameters compared with the content of cytokines and (or) cells in healthy donors – p_c, in patients with CAD – p (here and in Table 2).

The subpopulation composition of $CD14^+VEGFR2^+$ cells, depending on the intensity of CD14 and CD16 expression on peripheral blood monocytes in the ICM patients, fully corresponded to that in healthy donors (Fig. 1). At the same time, the CAD patients without ICM had an increased content of non-classical $CD14^+CD16^{++}VEGFR2^+$ monocytes relative to the norm and a clear trend ($p = 0.072$) toward an increase in the number of classical $CD14^{++}CD16^-VEGFR2^+$ cells in the blood. The content of $VEGFR2^+$ monocytes of all immunophenotypes was higher in this category of patients than in ICM patients (except for equal values for transitional $CD14^+CD16^-VEGFR2^+$ cells, Fig. 1).

In the bone marrow, the total content of monocytic cells and the relative and absolute count of $CD14^+VEGFR2^+$ monocytes were comparable between the groups of CAD patients, however, there were characteristic features of the subpopulation composition of these cells (Table 2, Fig. 2). Thus, the number of classical $CD14^{++}CD16^-VEGFR2^+$, non-classical $CD14^+CD16^{++}VEGFR2^+$, and transitional $CD14^+CD16^-VEGFR2^+$ cells in the bone marrow of CAD patients without ICM exceeded their content in ICM patient. It is worth noting that the number of intermediate $CD14^{++}CD16^+VEGFR2^+$ monocytes in the bone marrow in CAD patients in both groups was comparable, while in the blood it was different: in ICM patients it was 2.4 times less than in CAD patients without ICM (Fig. 2).

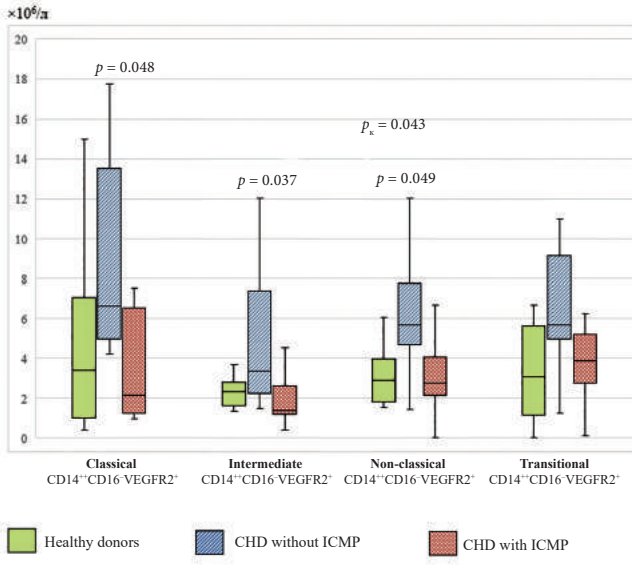

Fig. 1. Subpopulation composition of CD14+VEGFR2+ blood monocytes in patients with CAD with or without ICM: p_c – the level of statistical significance of differences in the parameters compared with the content of cells in healthy donors, p – in patients with CAD without ICM

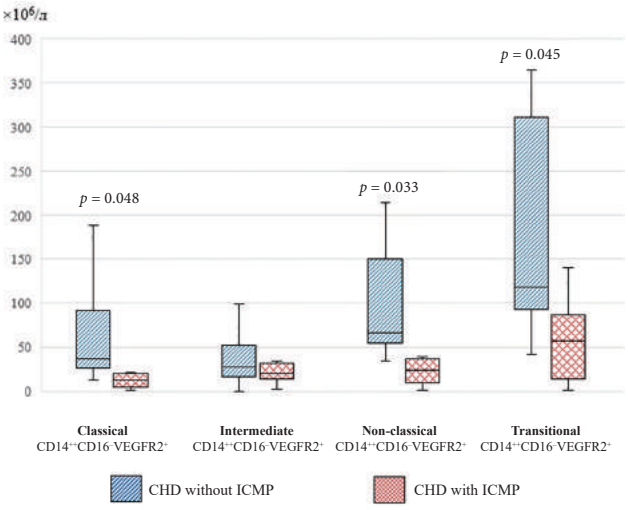

Fig. 2. Subpopulation composition of CD14+VEGFR2+ bone marrow monocytes in the patients with CAD with or without ICM: p – level of statistical significance of differences in the parameters compared with the content of cells in the patients with CAD without ICM

Table 2

The content of monocytes, cytokines, and $CD14^+VEGFR2^+$ cells in the bone marrow in CAD patients with and without ICM, *Me* [Q_1; Q_3]

Parameters	CAD patients without ICM	CAD patients with ICM
Content of monocytes, $\times 10^9$ / l	1.05 [0.84; 1.57]	0.89 [0.71; 1.40] $p = 0.525$
Content of $CD14^+VEGFR2^+$cells, %	27.85 [22.57; 42.88]	28.30 [11.98; 36.21] $p = 0.637$
Content of $CD14^+VEGFR2^+$cells, $\times 10^6$ / l	343.90 [208.75; 638.10]	223.90 [38.20; 546.10] $p = 0.325$
VEGF-A, pg / ml	18.50 [12.10; 25.30]	21.00 [16.00; 28.50] $p = 0.344$
TNFα, pg / ml	10.80 [9.90; 21.84]	18.06 [14.15; 19.40] $p = 0.517$
IL-13, pg / ml	1.00 [0.80; 1.23]	1.22 [0.80; 2.41] $p = 0.874$
M-CSF, pg / ml	7.16 [3.45; 16.33]	3.22 [1.20; 8.04] $p = 0.792$
M-CSF / IL-13	9.00 [2.13; 22.09]	1.02 [0.41; 2.00] $p = 0.047$

A correlation analysis of the parameters of $VEGFR2^+$ composition in the blood revealed two positive correlations in the CAD patients without ICM: between the absolute count of $CD14^+VEGFR2^+$ monocytes and the number of classical $CD14^{++}CD16^-VEGFR2^+$ cells ($r_s = 0.86$; $p < 0.01$) and between the number of transitional $CD14^+CD16^-VEGFR2^+$ monocytes and the number of non-classical $CD14^+CD16^{++}VEGFR2^+$ cells ($r_s = 0.93$; $p < 0.01$). We also found three positive correlations in ICM patients: between the absolute count of $CD14^+VEGFR2^+$ monocytes and the number of classical $CD14^{++}CD16^-VEGFR2^+$ ($r_s = 0.74$; $p < 0.01$), non-classical $CD14^+CD16^{++}VEGFR2^+$ ($r_s = 0.79$; $p < 0.01$), and transitional $CD14^+CD16^-VEGFR2^+$ monocytes ($r_s = 0.81$; $p < 0.01$). A similar correlation pattern was revealed in the bone marrow of ICM patients with a positive correlation between the number of classical $CD14^{++}CD16^-VEGFR2^+$ monocytes and the number of non-classical $CD14^+CD16^{++}VEGFR2^+$ cells ($r_s = 0.90$; $p < 0.05$). In the bone marrow of the CAD patients without ICM, a directly proportional relationship was identified between the absolute count of $CD14^+VEGFR2^+$ monocytes and their non-classical subpopulation ($r_s = 0.71$; $p < 0.05$).

Regardless of the presence of ICM, the concentration of VEGF-A and IL-13 in the blood of the CAD patients varied within physiological values, and the content of TNFα exceeded the normal values. At the same time, the CAD patients without ICM had an excess of MCP-1 and a deficiency of M-CSF compared with the ICM patients, in whom the content of these cytokines remained similar to that of healthy donors (Table 1). The concentrations of VEGF-A, IL-13, TNFα, and M-CSF in the CAD patients with and without ICM were comparable, characterized by a trend toward higher TNFα values and lower M-CSF values in the bone marrow in the ICM patients. At the same time, reciprocal changes in the M-CSF and IL-13 levels in the bone marrow were noted in many patients during the visual analysis of the data. It prompted the calculation of the M-CSF / IL-13 ratio, which turned out to be 9 times higher in the CAD patients without ICM than in the ICM patients (Table 2).

DISCUSSION

According to the obtained data, in the CAD patients without ICM, there is an almost twofold increase in the content of $CD14^+VEGFR2^+$ monocytes in the blood, both relative to the normal values and ICM patients (Table 1). This phenomenon in CAD patients without ICM can be considered as a compensatory reaction of the body in the conditions of atherogenesis. Accumulation of macrophages loaded with lipids in atheroma is accompanied by NADPH oxidase activation in them, followed by generation of reactive oxygen species, as well as by secretion of matrix metalloproteinases types 2 and 9 by macrophages, damaging the elements of the extracellular matrix and the basement membrane of blood vessels, which leads to destruction of the vascular endothelium [21].

At the same time, endothelial NO synthase (eNOS) activation due to vascular damage, ischemia, and hypoxia induces the release of proangiogenic factors

(HIF-1α, VEGF, etc.) from the endothelium and tissue cells, as well as EPC migration and proliferation [22]. Since $CD14^+VEGFR2^+$ monocytes are early EPCs that can stimulate vascular cell differentiation in a paracrine fashion and prevent their apoptosis by secreting VEGF, angiopoietin-1, and other proangiogenic factors [10, 13], the physiological level of $CD14^+VEGFR2^+$ monocytes in the blood of ICM patients (Table 1) with a verified atherosclerotic process can be considered as the absence of a compensatory reaction of the body aimed at repairing vessels in case of damage.

The analysis of the subpopulation composition of $CD14^+VEGFR2^+$ blood monocytes demonstrates that their transitional $CD14^+CD16^-VEGFR2^+$ immunophenotype is not involved in the increase in the total $CD14^+VEGFR2^+$ count in the blood of the CAD patients without ICM (Fig. 1). This is explained by the fact that transitional $CD14^+CD16^-$ cells are immature forms of monocytes concentrated mainly in the bone marrow, while their content in the blood is about 6% [23], so $CD14^+CD16^-VEGFR2^+$ cells do not enter the bloodstream in large numbers. However, despite the small number of this monocyte population in the blood, transitional $CD14^+CD16^-VEGFR2^+$ monocytes account for 25–35% of all $CD14^+VEGFR2^+$ cells in healthy donors and in CAD patients (Fig. 1). This may indicate a significant proangiogenic role of transitional $CD14^+CD16^-VEGFR2^+$ cells as younger representatives of monocytic cells in the blood, which are less subject to modulation of their own numbers in the bloodstream.

Mature forms of $CD14^+VEGFR2^+$ monocytes are most susceptible to quantitative changes in the blood. Thus, according to the data obtained, the increase in their total blood content in the CAD patients relative to those in the ICM patients occurs due to almost 3-fold accumulation of classical $CD14^{++}CD16^-VEGFR2^+$ and intermediate $CD14^{++}CD16^+VEGFR2^+$ monocytes with a 2-fold increase in the number of non-classical $CD14^+CD16^{++}VEGFR2^+$ cells. However, the number of the latter exceeds that even in healthy donors (Fig. 1). Therefore, the content of non-classical $CD14^+CD16^{++}VEGFR2^+$ cells in the blood is a parameter that varies the least and most accurately reflects the enhancement of vascular repair in cardiovascular pathology.

We have previously shown that a distinctive feature of ICM patients is the deficiency of non-classical monocytes in the blood (regardless of the VEGFR2 expression on their membrane), which are able to eliminate immune complexes and dead cells from the vascular intima surface and protect it from damage [24]. Due to the fact that intermediate monocytes are characterized by the highest expression of VEGFR2 and VEGFR1 and the highest proportion of $VEGFR2^+$ cells compared with classical and non-classical cells (8.25, 5.00 and 2.80% of the corresponding population of monocytes) [11], and, probably, it is the intermediate forms that have high proangiogenic activity, the trend toward a decrease in this cell subpopulation in the blood in ICM patients relative to the normal values is of great interest (Table 1). Therefore, it can be assumed that in ICM, there is no compensatory activation of vascular repair in atherosclerosis manifested by $CD14^+VEGFR2^+$ cell accumulation in the blood, endothelium clearance from immune complexes decreases with a non-classical monocyte deficiency, and there is a trend toward a lack of physiological regeneration in the vascular endothelium with the participation of intermediate $CD14^{++}CD16^+VEGFR2^+$ monocytes.

The reason for the absence of an increase in the number of $CD14^+VEGFR2^+$ cells in the blood in ICM is probably the peculiarities of the blood cytokine profile. With an equivalent surplus of TNFα in the cohorts of patients and physiological VEGF-A and IL-13 levels in the blood in patients of both groups, there was an excess of MCP-1 and a deficiency of M-CSF in the blood of the CAD patients without ICM, while the concentration of these cytokines in the ICM patients corresponded to the normal values (Table 1). Therefore, an increase in the content of $CD14^+VEGFR2^+$ monocytes in the blood in the CAD patients with ICM is associated with the MCP-1 accumulation and does not depend on the M-CSF plasma concentration. It is known that under physiological conditions, differentiation of monocytes and macrophages is determined by the levels of M-CSF, which is constitutively synthesized by stromal progenitor cells, fibroblasts, and macrophages. However, under conditions of inflammation, the colony-stimulating factor of granulocytes and macrophages (GM-CSF) becomes more important in the regulation of hematopoiesis and monocytopoiesis [25]. In addition, in the presence of M-CSF or GM-CSF, monocyte precursors in the bone marrow actively proliferate and differentiate, after which mature monocytes become refractory to these growth stimuli [26], and they need the M-CSF receptor, first of all, for regulation of macrophage differentiation [27]. Therefore, it is probably not M-CSF, but MCP-1, a pro-inflammatory cytokine and the most powerful chemoattractant for monocytes and macrophages [21,

24], that is involved in the migration of $CD14^+VEGFR2^+$ cells from the myeloid tissue into the blood in CAD patients without ICM.

A comparable number of $CD14^+VEGFR2^+$ cells in the bone marrow of CAD patients with and without ICM (Table 2) with differences in their count in the blood (Table 1) allows to tentatively conclude that only the migration of $CD14^+VEGFR2^+$ monocytes into the bloodstream is impaired in ICM without changes in their differentiation in the myeloid tissue. Meanwhile, when analyzing the subpopulation composition of $CD14^+VEGFR2^+$ cells, it becomes obvious that this phenomenon is observed only in intermediate $CD14^{++}CD16^+VEGFR2^+$ monocytes, the number of which in the blood turned out to be comparable between the groups of patients against the background of their increased values in the blood of CAD patients without ICM (Fig. 1, 2).

As discussed above, the high MCP-1 plasma concentration in these patients (Table 1) allows $CD14^+VEGFR2^+$ cells to actively migrate from the bone marrow into the blood, which is not seen in ICM patients. However, the bone marrow contains a large number of hematopoietic and stromal stem cells and progenitor cells, which are in a microenvironment abundant in factors at different stages of differentiation and become different subsets of cells [12]. The content of classical $CD14^{++}CD16^-VEGFR2^+$ and non-classical $CD14^+CD16^{++}VEGFR2^+$ monocytes in the myeloid tissue in the CAD patients without ICM was almost 3 times higher than in the ICM patients, and the number of intermediate $CD14^{++}CD16^+VEGFR2^+$ monocytes was 2 times higher (Fig. 2). Consequently, differentiation of these three subpopulations of $CD14^+VEGFR2^+$ monocytes is impaired in ICM, which explains the trend toward a decrease in the total number of $CD14^+VEGFR2^+$ cells in the bone marrow in the ICM patients.

The comparative analysis of the VEGF-A, TNFα, IL-13, and M-CSF concentrations in the myeloid tissue in the CAD patients did not reveal any differences between the groups of patients, which does not allow to select a specific cytokine responsible for impaired differentiation of $CD14^+VEGFR2^+$ cells in the bone marrow in ICM. At the same time, a clear trend toward elevated M-CSF values in the CAD patients without ICM with reciprocal (in many patients) IL-13 changes in the bone marrow made it possible to calculate the ratio of these cytokines and reveal a 9-fold decrease in the M-CSF / IL- 13 ratio in the ICM patients compared with CAD patients without ICM. This is important because these cytokines have different effects on monocytopoiesis.

Stimulation of the receptor for M-CSF (CSF-1R), the expression of which increases by 10 times in "the colony-forming unit of macrophages – monoblast – promonocyte – monocyte – macrophage" cell series, induces an early reaction of monocytic cells in the form of increased protein synthesis and cytoskeleton actin network rearrangement, later – macrophages differentiation and proliferation with trophic and growth-stimulating properties. During myelopoiesis, a proliferative response to M-CSF is possible precisely in myeloblasts due to increased activity of ζ-type protein kinase C, which stimulates the Erk1 / 2 pathway. This enzyme is absent in promonocytes, and CSF-1R stimulation only leads to their differentiation with the participation of the PI3K / Akt pathway, which also ensures the survival of monocytes / macrophages [28, 29].

Along with this, Treg-derived IL-13, acting on monocytes, has a differentiation potential (induces maturation of monocytes into alternative macrophages and secretion of immunosuppressive IL-10 by them) [19], but its proliferative and anti-apoptotic effects on monocyte cells have not been described. In monocytes, IL-13 activates STAT 1, 3, 5, 6 via Jak2 and Tyk2 kinases, inhibits synthesis of proinflammatory cytokines, including IL-1β, IL-6, IL-8, and TNFα, and downregulates the surface expression of Fc receptor for IgG (i.e. CD16 molecules) [30]. The latter probably explains the reduced content of non-classical $CD14^+CD16^{++}VEGFR2^+$ monocytes in the bone marrow in the ICM patients compared with the CAD patients without ICM (Table 2). The decrease in the number of classical $CD14^{++}CD16^-VEGFR2^+$ and transitional $CD14^+CD16^-VEGFR2^+$ cells that do not express CD16 molecules in the ICM patients (Table 2) is rather associated with both insufficient proliferation and reduced survival of monocytic cells in the bone marrow at low M-CSF / IL-13 ratio. The high M-CSF / IL-13 ratio in the CAD patients without ICM indicates the predominance of proliferative, anti-apoptotic, and differentiation M-CSF signals in monocyte progenitors over the IL-13-mediated stimulus that blocks maturation of the most differentiated $CD16^+$ forms of $VEGFR2^+$ monocytes. In ICM, these signals in monocytic cells are balanced (the M-CSF / IL-13 ratio is close to 1, Table 2).

The results of the correlation analysis for the parameters of the $VEGFR2^+$ subpopulation composition demonstrate that in the CAD patients, the content of $CD14^+VEGFR2^+$ cells in the bone marrow is

determined by the number of non-classical $CD14^{+}CD16^{++}VEGFR2^{+}$ cells and in the blood – by the number of classical $CD14^{++}CD16^{-}VEGFR2^{+}$ monocytes as the most numerous subpopulation of blood monocytes in these patients. In the blood of the ICM patients, the population of transitional $CD14^{+}CD16^{-}VEGFR2^{+}$ monocytes is the most numerous, determining, along with classical $CD14^{++}CD16^{-}VEGFR2^{+}$ and non-classical $CD14^{+}CD16^{++}VEGFR2^{+}$ cells, the total number of $CD14^{+}VEGFR2^{+}$ cells both in the blood and the bone marrow. At the same time, the ratio between $CD14^{+}VEGFR2^{+}$ cell subpopulations in the ICM patients is equivalent in both tissues (Fig. 1, 2), and in the CAD patients without ICM, the dominant populations are transitional CD14+CD16-VEGFR2+ monocytes in the bone marrow and classical CD14++CD16-VEGFR2+ monocytes in the blood.

Taken together, this means that in ICM, there is no further differentiation of $CD14^{+}VEGFR2^{+}$ cells and their subpopulation composition in the blood corresponds to that in the bone marrow, where non-classical $CD14^{+}CD16^{++}VEGFR2^{+}$ monocytes differentiate from classical $CD14^{++}CD16^{-}VEGFR2^{+}$ cells (according to the identified correlations, see above). In CAD without ICM, the formation of non-classical $CD14^{+}CD16^{++}VEGFR2^{+}$ cells occurs not only in the bone marrow, but probably continues in the blood from transitional $CD14^{+}CD16^{-}VEGFR2^{+}$ monocytes (according to the correlation). Apparently, the formation of classical $CD14^{++}CD16^{-}VEGFR2^{+}$ and transitional $CD14^{+}CD16^{-}VEGFR2^{+}$ cells occurs constitutively to a greater extent, while the non-classical $CD14^{+}CD16^{++}VEGFR2^{+}$ generation is inducible. It is worth noting that the content of intermediate $CD14^{++}CD16^{+}VEGFR2^{+}$ monocytes did not correlate either with the numbers of their other subpopulations or with the total number of $CD14^{+}VEGFR2^{+}$ cells. It can be explained by a combination of differentiation of these monocytes with their parallel homing into the vascular wall as a subpopulation of $CD14^{+}VEGFR2^{+}$ cells, which have the maximum $VEGFR2^{+}$ expression on monocytes [11]. It was shown that EPCs are involved in the angiogenesis directly by incorporation into the vascular wall in the area of vascular growth and indirectly by secretion of proangiogenic factors [6].

CONCLUSION

ICM development is apparently associated with insufficient vascular repair due to the absence of the compensatory response of the body manifested through an increase in the number of $CD14^{+}VEGFR2^{+}$ cells during the atherogenesis. The content of $CD14^{+}VEGFR2^{+}$ cells in the blood during ICM is reduced compared with their number in the CAD patients without ICM due to the lower number of classical $CD14^{++}CD16^{-}VEGFR2^{+}$, intermediate $CD14^{++}CD16^{+}VEGFR2^{+}$, and non-classical $CD14^{+}CD16^{++}VEGFR2^{+}$ monocytes. At the same time, in the bone marrow of the ICM patients, due to the low M-CSF / IL-13 ratio, the differentiation of their precursors (transitional $CD14^{+}CD16^{-}VEGFR2^{+}$ cells) and, as a result, classical $CD14^{++}CD16^{-}VEGFR2^{+}$ and non-classical $CD14^{+}CD16^{++}VEGFR2^{+}$ cells is reduced. The formation of the latter in ICM is not activated from transitional $CD14^{+}CD16^{-}VEGFR2^{+}$ cells either in the bone marrow or in the blood, which, on the contrary, is typical of CAD patients without ICM.

The maturation of intermediate $CD14^{++}CD16^{+}VEGFR2^{+}$ cells in the myeloid tissue is not affected, and their lower content in the blood is due to the absence of active migration of these cells from the bone marrow at the physiological concentration of MCP-1 in the blood, which is increased in CAD patients without ICM. At the same time, the VEGF-A and TNFα concentrations in the blood and bone marrow probably do not affect the number of $CD14^{+}VEGFR2^{+}$ cells and their subpopulations. Consequently, they differentiate and migrate from the myeloid tissue, like monocytic cells during inflammation, and the hypoxia mediator VEGF-A controls these processes to a lesser extent (due to the absence of an increase in its concentration in the blood and bone marrow). Knowledge about such patterns of EPC generation and migration will help to induce this process in ICM patients and develop a new treatment method for this severe heart disease in the future.

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Authors contribution

Chumakova S.P. – research design, review of literature, statistical processing of research results and their interpretation, drafting and design of the manuscript. Urazova O.I. – research design, coordination of laboratory research, interpretation of the results, editing of the manuscript. Shipulin V.M. – consulting on research planning and interpretation of the clinical aspects of the results. Denisenko O.A. – preparation of biomaterial samples, review of literature. Kononova T.E. – carrying out of the enzyme immunoassay, preparation of images. Nevskaya K.V. – carrying out of flow cytometry. Andreev S.L. – collection of patients' data, provision of clinical material, intraoperative bone marrow sampling.

Authors information

Chumakova Svetlana P. – Dr. Sci. (Med.), Professor, Pathophysiology Division, Siberian State Medical University, Tomsk, chumakova_S@mail.ru, https://orcid.org/0000-0003-3468-6154

Urazova Olga I. – Dr. Sci. (Med.), Professor, Corresponding Member of RAS, Head of the Pathophysiology Division, Siberian State Medical University; Professor, Department of Complex Information Security of Computer Systems, Tomsk State University of Control Systems and Radioelectronics, Tomsk, urazova72@yandex.ru, https://orcid.org/0000-0002-9457-8879

Shipulin Vladimir M. – Dr. Sci. (Med.), Professor, Honored Scientist of Russia, Scientific Head of the Cardiovascular Surgery Department, Cardiology Research Institute, Tomsk NRMC; Professor, Division of Hospital Surgery with a Course in Cardiovascular Surgery, Siberian State Medical University, Tomsk, shipulin@cardio-tomsk.ru, https://orcid.org/0000-0003-1956-0692

Denisenko Olga A. – Doctor of Clinical Laboratory Diagnostics, Tomsk Regional Blood Center, Tomsk, eolga-muraveinik@yandex.ru, http://orcid.org/0000-0003-4524-8491

Kononova Tatyana E. – Cand. Sci. (Med.), Associate Professor, Pathophysiology Division, Siberian State Medical University, Tomsk, kononova.te@gmail.com, https://orcid.org/0000-0001-8457-9440

Nevskaya Ksenia V. – Cand. Sci. (Med.), Junior Researcher, Central Research Laboratory, Siberian State Medical University, Tomsk, nevskayaksenia@gmail.com, https://orcid.org/0000-0003-1659-8812

Andreev Sergey L. – Cand. Sci. (Med.), Cardiovascular Surgeon, Senior Researcher, Department of Cardiovascular Surgery, Cardiology Research Institute, Tomsk NRMC, Tomsk, anselen@rambler.ru, https://orcid.org/0000-0003-4049-8715

(✉) **Chumakova Svetlana P.,** chumakova_s@mail.ru

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