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Effect of M-CSF on the expression of endothelial progenitor cell markers in blood mononuclear cell culture in coronary heart disease

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ABSTRACT

Aim. To evaluate the nature of changes in the expression of markers of endothelial progenitor cells (VEGFR2, CD34, CD14) and endothelial cells (CD146) in association with the expression of the leukocyte common antigen CD45 in the culture of blood mononuclear cells in the presence of M-CSF in patients with coronary heart disease (CHD) and healthy donors.

Materials and methods. The study included 12 patients with CHD with class III–V angina pectoris and 10 healthy donors, from whom 30 ml of venous blood was taken on an empty stomach in the morning and stabilized with heparin. Blood mononuclear cells were isolated by Ficoll density gradient centrifugation (1.077 g / cm^3) and subject to immunomagnetic separation using CD14-MicroBeads and CD34-MicroBead Kit (Miltenyi Biotec B.V. & Co. KG, Germany). The resulting $CD14^+$ and $CD34^+$ culture of mononuclear cells was incubated for 6 days in a complete nutrient medium with and without M-CSF 50 ng / ml (Cloud-Clone Corp., USA) with complete replacement of the medium and repeated application of M-CSF on day 3. After 6 days, the proportions of $CD45^+$, $CD14^+$, $CD34^+$, $VEGFR2^+$, and $CD146^+$ cells in the culture were assessed by flow cytometry using CD14-FITC, CD34-PE, VEGFR2-Alexa Fluor 647; CD45-FITC and CD146-PerCP antibodies (BD Biosciences, USA).

Results. It was shown that in healthy donors, the proportion of $CD146^+$ cells in the co-culture of blood mononuclear cells with M-CSF exceeded their number in the sample without it, with comparable expression rates of CD45, CD14, and VEGFR2 markers between the control and stimulated cultures. In CHD patients, the number of CD146+ and $VEGFR2^+$ cells did not change when M-CSF was added to the mononuclear cell culture; however, the proportion of $CD14^+$ cells increased and the proportion of $CD45^+$ cells decreased compared to the control sample. The number of $CD34^+$ cells was comparable both between control and stimulated samples, and between the groups of examined individuals. At the same time, in patients with CHD, an increased proportion of $VEGFR2^+$ cells was found in the control and stimulated samples compared to healthy individuals, while an increased proportion of $CD14^+$ cells was detected only in the stimulated culture.

Conclusion. The development of CHD disrupts the response of blood mononuclear cells to the effect of M-CSF, increasing the number of $CD14^+$ and reducing the proportion of $CD45^+$ cells in the culture in the absence of stimulating effects on the expression of endothelial cell marker CD146. At the same time, M-CSF does not affect the expression of CD34 and VEGFR2 in endothelial progenitor cells both in patients with CHD and in healthy individuals.

Keywords: endothelial progenitor cells, monocytes, M-CSF, vascular repair, endothelial cells, coronary heart disease

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Conformity with the principles of ethics. All individuals signed an informed consent to participate in the study. The study was approved by the local Ethics Committee at Siberian State Medical University (Protocol No. 9299 of 28.11.2022).

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Влияние M-CSF на экспрессию маркеров прогениторных эндотелиальных клеток в культуре мононуклеаров крови при ишемической болезни сердца

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РЕЗЮМЕ

Цель: оценить характер изменений экспрессии маркеров эндотелиальных прогениторных клеток (VEGFR2, CD34, CD14) и эндотелиоцитов (CD146) в ассоциации с экспрессией панлейкоцитарного маркера CD45 в культуре мононуклеаров крови в присутствии M-CSF у больных ишемической болезнью сердца (ИБС) и здоровых доноров.

Материалы и методы. В исследование вошли 12 больных ИБС со стенокардией напряжения III–V функционального класса и 10 здоровых доноров, у которых утром натощак забирали венозную кровь в количестве 30 мл и стабилизировали гепарином. Мононуклеары крови выделяли на градиенте фиколла 1,077 г/см^3 и подвергали иммуномагнитной сепарации с применением антител CD14-MicroBeads и CD34-MicroBead Kit (Miltenyi Biotec B.V. & Co. KG, Германия). Полученную смешанную по CD14 и CD34 культуру мононуклеаров инкубировали 6 сут в полной питательной среде с добавлением M-CSF 50 нг/мл (Cloud-Clone Corp., США) и без него с полной заменой среды и повторным внесением M-CSF на 3-и сут. Через 6 сут оценивали долю позитивных по CD45, CD14, CD34, VEGFR2, CD146 клеток в культуре с помощью антител CD14-FITC, CD34-PE, VEGFR2-Alexa Fluor 647; CD45-FITC и CD146-PerCP (BD Biosciens, США) методом проточной цитофлуориметрии.

Результаты. Показано, что у здоровых доноров доля CD146$^+$ клеток в смешанной культуре мононуклеаров крови при добавлении M-CSF превышает их количество в пробе без его внесения при сопоставимых показателях экспрессии маркеров CD45, CD14 и VEGFR2 между контрольной и стимулированной культурами. У больных ИБС численность CD146$^+$ и VEGFR2$^+$ клеток не изменялась при добавлении M-CSF в культуру мононуклеаров, однако доля CD14$^+$ клеток возрастала, а CD45$^+$ клеток снижалась относительно контрольной пробы. Количество CD34$^+$ клеток было сопоставимым как между контрольной и стимулированной пробами, так и между группами обследованных лиц. При этом у больных ИБС установлено превышение доли VEGFR2$^+$ клеток относительно здоровых доноров в контрольной и стимулированной M-CSF пробах, а для CD14$^+$ мононуклеаров – только в стимулированной культуре мононуклеаров.

Заключение. Формирование ИБС нарушает реакцию мононуклеаров крови на действие M-CSF, увеличивая число CD14$^+$ и уменьшая долю CD45$^+$ клеток в культуре при отсутствии стимулирующего влияния на

экспрессию маркера CD146 эндотелиальных клеток. При этом M-CSF не влияет на экспрессию маркеров CD34 и VEGFR2 ЭПК как у больных ИБС, так и у здоровых лиц.

Ключевые слова: моноциты, прогениторные эндотелиальные клетки, M-CSF, репарация сосудов, эндотелиоциты, ишемическая болезнь сердца

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

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INTRODUCTION

For many decades, cardiovascular diseases have been the main cause of death in many countries of the world [1, 2]. Studies of the pathogenesis of vascular pathology are often limited to studying vasomotor endothelial vascular dysfunction [3, 4], however, its angiogenic form which includes impairment of angiogenesis and reparative processes in the vessels is insufficiently studied [5].

In atherosclerosis, which underlies coronary heart disease (CHD), monocytes can have both a negative and a protective effect. On the one hand, plaque macrophages, maintaining chronic inflammation, prolong vascular alteration with the help of matrix metalloproteinases (MMPs) [1, 6, 7] and promote vascularization in atheroma, which increases the risk of plaque hemorrhage with subsequent plaque destabilization [3, 8]. On the other hand, monocytes containing a population of endothelial progenitor cells (EPCs) [9] can participate in the angiogenesis induction, which is necessary for the formation of collateral blood flow and repair of damaged vessels, which has a protective and adaptive value in CHD.

Angiogenesis is realized by early and late EPCs, which have monocytic VEGFR2$^+$CD34$^+$CD14$^+$ and non-monocytic VEGFR2$^+$CD34$^+$CD14$^-$ immunophenotypes, respectively [2, 9]. Early EPCs stimulate the mature endothelial cells survival in a paracrine way, can acquire their markers, but have limited proliferative activity; late EPCs have a high proliferative activity and differentiate into endotheliocytes [2, 9]. It is known that circulating EPCs can be isolated from peripheral blood mononuclear cells. Moreover, when monocytes are cultured in the presence of vascular endothelial growth factor (VEGF), they transform into an intermediate cell phenotype and further differentiate in endotheliocytes, losing the CD45 pan-leukocyte marker [10]. The ability of other growth factors to stimulate angiogenesis has also been demonstrated. Thus, the cultivation of bone marrow cells with granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF) increased the expression of endothelial markers CD31 and CD146 [11], and the cultivation of EPCs isolated from blood monocytes with granulocyte – macrophage colony-stimulating factor (GM-CSF) increased their proliferative activity [12]. Considering the above, it can be assumed that blood monocytes (CD14$^+$) enriched with hematopoietic stem (CD34$^+$) cells [2] can modulate their phenotype under the influence of various stimuli. Since early EPCs are monocytic cells, it is possible that the cultivation of a mixed culture of monocytes (CD14$^+$) and hematopoietic stem cells (CD34$^+$) in the presence of M-CSF can affect the expression of markers characteristic of EPCs.

The aim of the study was to evaluate the nature of changes in the expression of markers of endothelial progenitor cells (VEGFR2, CD34, CD14) and endotheliocytes (CD146) in association with the expression of the leukocyte common antigen CD45 in CD14$^+$ and CD34$^+$ blood mononuclear cell culture in the presence of M-CSF in patients with CHD and healthy individuals.

MATERIALS AND METHODS

A single-stage, case-control, single-center, observational study was conducted from December 2022 to May 2023. The study included 12 patients with CHD (10 men and 2 women, mean age 62.0 [56.5; 64.0] years) with class II–IV angina pectoris and mainly NYHA class II–III heart failure, who had a history of myocardial infarction and were admitted to the Cardiology Research Institute of Tomsk National Research Medical Center to undergo coronary bypass grafting. Patients received standard anti-anginal therapy with long-acting nitrates, β1-blockers, calcium channel blockers, angiotensin-converting enzyme inhibitors, as well as antiplatelet therapy with acetylsalicylic acid preparations or P2Y12 receptor blockers and lipid-lowering therapy with statins. The comparison group consisted of 10 apparently healthy donors (7 men and 3 women, mean age 57.5 [48.0; 65.5] years), who did not have any cardiovascular diseases and complaints of a corresponding nature.

The exclusion criteria were as follows: age over 70 years, autoimmune diseases, an allergic process in the acute phase, a tumor process, viral hepatitis, syphilis, HIV infection, anemia, treatment with iron-containing drugs, erythropoietin or immunosuppressive therapy, acute infections within less than 3 weeks before the study, as well as the patient's refusal to participate in the study.

The studies were carried out in accordance with the ethical principles set out in the Declaration of Helsinki (1975) and approved by the local Ethics Committee at Siberian State Medical University (Protocol No. 9299 of 28.11.2022).

The material of the study was blood from the cubital vein in a volume of 30 ml, taken in the morning on an empty stomach before exercise, diagnostic and therapeutic procedures, which was stabilized with heparin (25 IU / ml). Blood mononuclear cells were isolated by Ficoll density gradient centrifugation (1.077 g /cm^3) (PanEco LLC, Moscow). After double washing of mononuclear cells with 0.5% PBS (PBS, pH=7.2), immunomagnetic separation was performed using CD14 MicroBeads and CD34 MicroBead Kit antibodies (Miltenyi Biotec B.V. & Co. KG, Germany), MS separation columns (Miltenyi Biotec B.V. & Co. KG, Germany), and a MiniMACS magnet (Miltenyi Biotec B.V. & Co. KG, Germany) according to the manufacturer's instructions. The purity of isolation, i.e. the proportion of CD14+ and CD34+ cells in the culture, was 80–85% and 5–7%, respectively. Cell viability was determined in a test with 0.1% trypan blue (PanEco LLC, Moscow). If it was at least 96%, cells were added to 2 wells of a 24-well plate, 106 cells each. The samples were incubated for 6 days with 5% CO_2 in a complete nutrient medium (nutrient medium RPMI-1640 (PanEco LLC, Moscow) supplemented with fetal calf serum, L-glutamine, penicillin – streptomycin) with the addition of 50 ng / ml of recombinant human M-CSF (Cloud-Clone Corp., USA) to one of the wells. After 3 days of incubation, the medium was partially replaced and the stimulant was re-introduced at the same dose. The sample with recombinant M-CSF was considered stimulated, and the sample without M-CSF was considered control. After 6 days, the cells were removed from the plate surface by incubating them with 500 μl of 0.05% trypsin – EDTA solution (PanEco LLC, Moscow) per well for 5 min at 37 °C. After washing the cells with 500 μl of 0.5% PBS, the pellet was resuspended, and the cells were used for flow cytofluorometry.

To determine the expression of CD45, CD14, CD34, VEGFR2 (KDR; CD309), and CD146 molecules in the co-culture culture of blood mononuclear cells, flow cytometry was performed using monoclonal antibodies with two combinations of labels: CD14-FITC, CD34-PE, VEGFR2(KDR; CD309)-Alexa Fluor 647 and CD45-FITC, CD146-PerCP, VEGFR2(KDR; CD309)-Alexa Fluor 647, according to the manufacturer's instructions (BD Biosciences, USA). Dead cells were excluded from the analysis using DAPI staining (Wuhan Servicebio Technology Co., Ltd, China). The fluorescence intensity was measured on the CytoFLEX flow cytometer (Beckman Coulter International S.A., USA), and the data obtained were analyzed using the CytExpert 2.3 software (Beckman Coulter International S.A., USA). The positivity boundaries of the label luminescence were established using fluorescence minus one (FMO) controls. We estimated the proportion of cells positive for each marker as a proportion (%) of the total number of cases, excluding the area of small objects (FSC less than 100×10^4).

Statistical data was analyzed using the Statistica 10.0 program. In the statistical description of the results, the median and interquartile range were calculated (*Me* [*Q*1; *Q*3]). The Mann – Whitney test (for independent samples) and the Wilcoxon test (for dependent samples) were used to perform comparative analysis of sample data. The results of the statistical analysis were considered statistically significant at $p < 0.05$.

RESULTS

The comparative analysis of control and M-CSF-stimulated samples of $CD14^+$ and $CD34^+$ blood mononuclear cell co-cultures in healthy donors revealed a statistically significant increase in the proportion of $CD146^+$ cells only in the sample with M-CSF compared to the sample without M-CSF with comparable expression parameters for CD45, CD14, and VEGFR2 markers (Table). On the contrary, in patients with CHD, the number of $CD146^+$ cells did not change when M-CSF was added to the culture, as did the number of $VEGFR2^+$ cells, but the proportion of $CD14^+$ mononuclears increased significantly, and $CD45^+$ decreased compared to the control sample. In the meantime, CD34 expression did not differ between the control and stimulated samples, or between the groups of examined individuals.

The analysis of the expression parameters of the studied markers between the groups of examined patients revealed an excess of the relative number of $VEGFR2^+$ cells in CHD patients compared to healthy donors in both control and stimulated M-CSF samples, and for $CD14^+$ mononuclear cells – only in the stimulated sample (Table).

Table

Expression of markers of endothelial progenitor cells and endotheliocytes, as well as CD45 molecules in the $CD14^+$ and $CD34^+$ blood mononuclear cell co-culture stimulated and not stimulated with M-CSF in patients with CHD and healthy donors, *Me* [Q_1; Q_3]

The proportion of cells expressing the marker	Healthy donors		CHD patients	
	Control sample	Sample with M-CSF	Control sample	Sample with M-CSF
CD45, %	56.18 [40.55; 64.20]	30.93 [23.40; 47.12] $p_c = 0.288$	30.79 [19.66; 46.60] $p = 0.135$	17.68 [11.62; 23.90] $p = 0.217$ $p_c = 0.049$
CD14, %	25.82 [18.34; 31.10]	19.17 [12.32; 26.80] $p_c = 0.627$	26.43 [15.93; 30.12] $p = 0.916$	40.36 [25.14; 51.69] p = 0.046 $p_c = 0.031$
CD34, %	22.71 [18.14; 27.58]	19.18 [15.07; 23.76] $p_c = 0.743$	15.84 [10.86; 24.11] $p = 0.566$	17.32 [14.24; 22.31] $p = 0.920$ $p_c = 0.855$
VEGFR2, %	4.38 [1.75; 9.25]	5.62 [2.51; 11.43] $p_c = 0.315$	21.16 [13.05; 28.56] $p = 0.002$	25.47 [13.80; 32.16] $p = 0.013$ $p_c = 0.407$
CD146, %	1.79 [0.94; 2.70]	2.82 [1.63; 5.40] $p_c = 0.023$	1.44 [0.90; 3.82] $p = 0.821$	2.36 [1.59; 4.27] *p = 0.763* $p_c = 0.194$

Note: p_c – the level of statistical significance of the differences in the parameters compared with the cell content in the control sample, p – in healthy donors.

DISCUSSION

The study of the M-CSF effect on the marker expression inherent in early EPCs (VEGFR2, CD34, CD14) and more differentiated endotheliocytes (CD146) established the differences in the effects of this cytokine on the culture of blood mononuclear cells mixed in $CD14^+$ and $CD34^+$ in CHD patients and healthy donors (Table). It was shown that the physiological response of these cells to M-CSF consists in increased CD146 expression, which does not occur in patients with CHD.

The CD146 molecule is expressed on endotheliocytes and pericytes, promoting the formation of cell – cell interactions between them, increasing endothelial adhesiveness and endothelial cell survival, and also contributing to pericyte recruitment, EPC homing, vessel architectonics, and their stabilization [13, 14]. Its soluble form sCD146 was shown to enhance the angiogenic properties of EPCs, and sCD146 injection improved neovascularization in a murine ischemia model, which was mediated by VEGFR1, VEGFR2, angiomotin, and the shCD146 isoform [13]. Therefore, an increase in CD146 expression in the blood mononuclear culture in healthy individuals can be considered as a positive effect of M-CSF, which could have a protective effect on vessels of the ischemic myocardium affected by atherosclerosis

in patients with CHD, but it is not realized in CHD (Table).

Using a model of chronic obstructive pulmonary disease, it was shown that CD146 deficiency in pulmonary endothelial cells was associated with its increased permeability and tissue infiltration by monocytes, and the addition of sCD146 increased monocyte transmigration *in vitro* [13]. Accordingly, impaired CD146 expression under the effect of M-CSF in patients with CHD can not only inhibit angiogenesis, but also enhance monocyte migration into the vessel wall and tissues, contributing to inflammation and fibrosis. CD146 deficiency is associated with suppression and enhancement of the non-canonical and canonical Wnt pathways, which leads to a profibrotic state [15].

Since CD146 is more present on mature endotheliocytes and is often used as a marker of desquamated endothelial cells [14, 16, 17] and to a lesser extent – on EPC [9, 14], in healthy individuals, a mature phenotype of endotheliocytes is probably more likely to form under the M-CSF influence in blood mononuclear cell culture. At the same time, the expression of VEGFR2, which is inherent in EPCs and endotheliocytes, did not change in blood mononuclear cell culture in the presence of M-CSF in both healthy individuals and patients with CHD (Table).

Binding of VEGFR2 to its ligands VEGF-A and VEGF-C stimulates the expression of adhesion molecules, vascular permeability, cell survival (through activation of the PI3K/AKT pathway), cell attachment and migration (through activation of p38MAPK and focal adhesion kinase FAK), and proliferative response (through activation of mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK)) [18]. Therefore, it can be assumed that early $VEGFR2^+CD34^+CD14^+$ and late $VEGFR2^+CD34^+CD14^-$ EPCs present in the mononuclear cell culture (because they were separated by $CD34^+$) were transformed under the M-CSF influence in healthy donors into a more mature phenotype with CD146 expression, but without increasing the proliferative potential of cells through VEGFR2 signaling. This transformation obviously did not occur in CHD. Since the expression of VEGFR2 and CD34 in the blood mononuclear cell culture did not increase in the M-CSF presence in both healthy individuals and CHD patients (Table), it cannot be argued that M-CSF promotes EPC differentiation or proliferation. However, the proportion of $VEGFR2^+$ cells in the co-culture of blood mononuclear cells in CHD patients was higher than in healthy individuals, both with M-CSF and without it (Table). This fact is obviously associated with the initially greater $VEGFR2^+CD34^+$ cell separation in patients due to the high $VEGFR2^+$ cell content in the blood of CHD patients, which we discussed earlier [17].

A significantly higher percentage of $CD34^+$ cells in both groups of examined individuals after mononuclear cell culture (Table) is worth noting compared to that obtained using immunomagnetic separation (see Materials and Methods section). It has been shown that blood mononuclear cells in the culture can transform not only into endothelial cells and macrophages, but also into fibrocytes expressing CD34 [19]. We observed the spindle cell formation in culture (the results are not presented, they are being statistically processed), the shape of which is inherent in both fibrocytes [19] and terminal endothelial cells [2, 10]. However, without studying specific fibrocyte markers, such as the presence of intracellular collagen and CD34 in the absence of CD33, CD35, and CD93 expression [19], it is impossible to discuss the M-CSF effect on fibrocyte differentiation.

The unchanged proportion of $CD34^+$ mononuclear cells in the culture under the M-CSF effect in the examined individuals of both groups (Table) can also be associated with the induction of multi-directional processes by this cytokine: stimulation of its formation from monocytes ($CD14^+$) that do not express $CD34^+$ into fibrocytes carrying it, and with the transition of $CD34^+$ EPCs into mature endothelial cell forms that lose $CD34^+$. Thus, $CD34^+$ cells with high expression of endothelial cell markers, surrounded by spindle cells, were found in the blood monocyte culture *in vitro*.

Over time, two types of colony-forming units (CFU) were isolated in the monocyte culture: CFU-Hill cells and endothelial colony-forming cells. The former are phagocytic and express CD14, CD45, CD115, but do not have proliferative and vasculogenic activity, while the latter do not have CD14, CD45, CD115 and express markers of endothelial cells and form capillary-like structures *in vitro* and vessels *in vivo* [2]. Exposure to M-CSF caused $CD14^+$ cell accumulation (Table) in CHD patients compared to healthy individuals, which indicates increased CFU-Hill cell formation. At the same time, the decrease in the expression of the leukocyte common antigen CD45 on the cultured blood mononuclear cells in CHD patients with M-CSF can probably be explained by its stimulating effect on the transformation of monocytes into various cells not carrying CD45 (endothelial [2])

and weakly expressing it (fibrocytes and macrophages [19]).

CONCLUSION

In CHD, the nature of the response of $CD14^+$ and $CD34^+$ blood mononuclear cells to the M-CSF action changes, which is manifested by an increase in the CD14 marker expression and inhibition of CD45 molecule expression. At the same time, the physiological response of these cells to M-CSF stimulation in the form of increased endothelial marker CD146 expression is lost. Meanwhile, M-CSF does not change the EPC marker (VEGFR2, CD34) expression on $CD14^+$ and $CD34^+$ blood mononuclear cells both in healthy individuals and in CHD patients. The obtained knowledge creates the concept of the effectiveness of cytokine and cell therapy using M-CSF for the angiogenesis induction in CHD patients and the mechanisms of its possible therapeutic effect. These data can become the grounds for developing a new method for treating this disease. Provided that the recombinant M-CSF side effects are excluded, further research is required *in vivo*.

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Chumakova S.P. – conception and design, literature review, statistical processing of the research results and their interpretation, drafting and formatting of the manuscript. Urazova O.I. – conception and design, supply and equipment support for laboratory research, interpretation of the data, editing of the manuscript. Shipulin V.M. – consulting on research planning and interpretation of the clinical aspects and results obtained in cardiac patients. Gladkovskaya M.V. – sample preparation and carrying out of cell culture research. Andreev S.L. – interaction with cardiac patients, provision of clinical material. Nevskaya K.V. – carrying out of flow cytometry. Zima A.P. – supply and equipment support for laboratory research, interpretation of the data. Nikulina E.L. – statistical processing of the research results.

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