DOI: 10.31793/1680-1466.2018.23-4.384

Mechanisms of regeneration of the endothelium at diabetes mellitus

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Abstract. Vascular endothelium is the main organ, suffering from diabetes mellitus (DM) and cardiovascular diseases (CVD). In this review, the role of endothelial progenitor cells (EPCs) in the regeneration of the endothelium and in the formation of new blood vessels is considered. Mechanisms of migration and mobilization of EPCs from the bone marrow to the damage zone are described. The analyzed data show that CVD and DM cause the decrease in the number and the disturbance of the function of EPCs. The data on the heterogeneity of the population of EPCs are presented in article. The various combinations of surface markers for identification of these cells are assessed. At the same time, protocols for the identification of EPCs have not been developed, which confirms the relevance of the search for the phenotype of EPCs, which would be adopted as the standard.

Keywords: endothelial progenitor cells, angiogenesis, endothelial regeneration, endothelial dysfunction, diabetes mellitus.

This work is a fragment of research and development work 'Investigation of endothelial dysfunction in diabetes mellitus and the possibility and effectiveness of the use of a new method for treating patients with diabetes mellitus'. № of state registration 0118U001852.

Diabetes mellitus (DM) is a serious problem of the XXI century, and despite a wide range of modern medicines, type 1 (T1DM) and type 2 (T2DM) diabetes mellitus is inevitably progressing. The American Heart Association has classified T2DM as a cardiovascular disease (CVD) due to the high risk of vascular complications. The basis of micro- and macroangiopathies in DM is endothelial dysfunction (ED), which is manifested by an imbalance between the production of vasodilators and vasoconstrictors, thrombogenic and atrombogenic factors. Moreover, ED represents imbalance between the degree of damage and the ability to restore [1-3].

Previously it was thought that vasculogenesis occurs only during embryonic development. Currently, vascular repair and postnatal angiogenesis are associated with endothelial progenitor cells (EPCs), which are a unique population of cells that participate in the formation of blood vessels due to angiogenesis and vasculogenesis [4].

EPCs were isolated in 1997 by Asahara et al. [5] from human peripheral blood based on expression

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on the hematopoietic stem cells’ surface of the myeloid marker CD34 (cluster of differentiation 34) and marker of kinase insert domain receptor (KDR). These cells have been identified as immature bone marrow (BM) cells that have the ability to differentiate into mature endotheliocytes, therefore they are called «endothelial progenitor cells». The opening of EPCs has initiated a new era in studies of angiogenesis [5].

It is worth to note, that BM is the main, but not the only organ, that produces progenitor cells, possessing the ability to differentiate into mature endothelial cells (ECs). According to some reports, up to 70% of EPCs circulating in the blood are not cells of BM-origin. In particular, both circulating and localized in peripheral tissues, progenitor cells of other populations and monocytic-macrophage line cells can differentiate into endotheliocytes and stimulate angiogenesis [6].

In response to angiogenic growth factors, EPCs migrate from the BM niche into the blood, circulate and transform in the tissues into local adhesive EPCs. The participation of EPCs in neovascularization is not only due to their ability to differentiate into ECs and the replacement of dysfunctional ECs, but also due to the ability to secrete various regulatory growth factors and cytokines that stimulate vasculo- and angiogenesis. It was shown that EPCs account for up to 26% of all ECs in neovascularization [7].

When the peripheral tissues are under conditions of inflammation, damage or ischemia, EPCs exit from the BM in the bloodstream and migrate to the zone of injury. The entry of EPCs into the damaged area is a complex coordinated multi-step process that includes several consistent stages: mobilization, chemotaxis, adhesion, migration through the vessel wall and cell differentiation involving growth factors, chemokines and adhesion molecules. The process of attracting and migrating EPCs in the body is controlled by cells that are actually located in the area of the damage [1, 8].

The large group of substances that stimulate or inhibit angiogenesis is known **(Tabl.)**. [9].

Table. Angiogenic stimulators and inhibitors

PROANGIOGENIC FACTORS	ANTI-ANGIOGENIC FACTORS
Acidic & basic fibroblast growth factors (aFGF, bFGF)	2-Methoxyestradiol
Adenosine	Angiopoietin 2
Angiogenin	Angiostatin
Angiopoetin 1	Angiotensin II (AT-II)
Angiotensin I (AT-I)	Antithrombin III (AT III)
Angiotropin	Arrestin
Brain-derived neurotrophic factor (BDNF)	Canstatin
Cathepsin	Cartilage-derived inhibitor (CDI)
Cyclooxygenase 2 (COX-2)	Caveolin 1,2
Developmental endothelial locus 1 (Del-1)	Chondromodulin
Ephrin	Cortisol
Epidermal growth factor (EGF)	Endorepellin
Estrogen	Endostatin
Follistatin	Fibronectin
Glial cell derived neurotrophic factor (GDNF)	Fibronectin-binding integrins
Granulocyte-colony stimulating factor (G-CSF)	Heparin hexasaccharide
Granulocyte-macrophage colonystimulating factor (GM-CSF)	Human chorionic gonadothropin (hCG)
Growth-regulated oncogene β (GRO-β)	Interferon inducible protein (IP-10)
Heparin-binding growth factor 8 (HBGF-8)	Interferon α, β, g
Hepatocyte growth factor (HGF)	Interleukins (IL-4, IL-10, IL-12, IL-18, IL-18)
Histamine	Kallilrein-3
Hypoxia-inducible factor 1-α (HIF-1α)	Laminin
Insulin-like growth factor 1 (IGF-1)	Maspin
Interleukins (IL-1, IL-2, IL-6, IL-8, IL-18)	Pigment epithelial derived factor (PEDF)
Leptin	Placental ribonuclease inhibitor (PRI)
Leukotriene C_4	Plasminogen activator inhibitor 1,2 (PAI-1, PAI-2)
Matrix metalloproteinases (MMPs)	Prolactin
Monocyte chemoattractant protein-1 (MCP-1)	Proliferin-related protiei (PRP)
Nerve growth factor (NGF)	Prothrombin kringle-2
Nicotinamide	Restin
Nitric oxide synthase (NOS)	Retinoids
Placental growth factor (PLGF)	Soluble receptors for proangiogenic factors
Platelet factor IV	Transforming growth factor α, β
Platelet-derived growth factor (PDGF)	Thrombospondin 1,2
Progranulin	Tissue inhibitors of matrix metalloproteinases (TIMPs)
Proliferin	Tumor necrosis factor α (TNF-α), high doses
Prostaglandins E_1, E_2	Troponin I
Semaphorins	Tumstatin
Stromal-cell-derived factor 1 (SDF-1)	Vasculostatin
Tissue and urokinase plasminogen activator (tPA, uPA)	Vasostatin
Transforming growth factor α, β (TGF-α, β)	Vasohibin 1
Tumor necrosis factor α (TNF-α), low doses	
Vascular endothelial growth factors (VEGFs)	

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One of the most important inductors of angiogenesis is the group of vascular endothelial growth factors (VEGFs), angiogenic cytokines released under the influence of hypoxia by activated platelets and leukocytes. The angiogenic signal of VEGFs, mediated by tyrosine kinase receptors (VEGFR1 and VEGFR2), promotes proliferation, differentiation and EPCs chemotaxis in the area of injury or ischemia. VEGFs also induce the expression of stromal-cell-derived factor-1 (SDF-1). Additionally, VEGFs do not induce the proliferation of other vascular cells, such as pericytes, vascular smooth muscle cells (VSMCs) and fibroblasts, although VEGFs enhance the migration of VSMCs. Among VSMCs, the isoform VEGF165 is the most efficient regulator of angiogenesis in physiological and pathological states [10].

SDF-1 is produced by BM stromal cells and acts as a powerful chemoattractant for EPCs. SDF-1, binding to the CXCR-4 receptor on EPCs, stimulates targeted migration of cells to the ischemic zone [11]. Under physiological conditions, a low level of SDF-1 is determined in the blood, BM and other tissues, where SDF-1 is produced. This level creates a gradient that holds EPCs [11, 12]. In pathological conditions, this gradient in the BM is reversed by hypoxia-inducible factor 1-α (HIF-1α), which stimulates SDF-1 and VEGFs in damaged tissues [13].

Nitric oxide (NO), estrogens, high-density lipoproteins and erythropoietin via the PI3K/Akt signaling pathway (phosphatidylinositide3-kinase) by activation of the endothelial NO-synthase (eNOS) also contribute to the mobilization of EPCs [13, 14].

Activated M2-type macrophages, through the production of granulocyte-colony stimulating factor (G-CSF), also participate in the mobilization of EPCs from the BM in the bloodstream [15]. G-CSF induced mobilization of EPCs is associated with increased level of neutrophils in the blood circulation, which can lead to the release of VEGFs [16]. G-CSF stimulates the mobilization of hematopoietic progenitor cells (HPCs) through the release of elastase and cathepsin G by neutrophils. These proteases disconnect EPCs, causing proteolytic cleavage of the vascular cell adhesion molecule 1 (VCAM-1 or CD106), which is expressed on the mesenchymal stem cells [17].

The synthesis of NO and the local activity of matrix metalloproteinases (MMPs), in particular matrix metalloproteinase-9 (MMP-9), influence on the attraction of EPCs from BM. MMP-9, which is regulated by SDF-1 and VEGFs, promotes the release of EPCs from adhesive interaction with stromal cells. This leads to the release of EPCs into the peripheral blood, causing the release of a soluble Kit ligand, which in turn, binds to tyrosine kinase (c-Kit or CD117) receptors, which are expressed on the surface of EPCs [9, 18].

After exiting the BM, EPCs migrate to the zone of injury and act in one of three directions: integration (restoration of the damaged site), the formation of new vessels or paracrine (the allocation of angiogenic factors). Binding of EPCs with the damaged endothelium and transmigration of cells through the endothelial monolayer is realized due to molecules of cell adhesion and selectins. P/E-selectin, intercellular adhesion molecule-1 (ICAM-1 or CD54), the platelet endothelial cell adhesion molecule (PECAM-1 or CD31), and the integrins α4, β1, β2, β3 and β5 are the most studied adhesion factors [19]. Moreover, EPCs are able to adhere not only to the endothelium, but also to platelets through interaction with P-selectin and GPIIb integrin [20-22].

The surfaces of endothelial cells in undamaged and noninflamed vessels are nonadhesive to circulating cells, including platelets. When vascular damage is severe, platelets are activated by exposed extracellular matrix proteins, and adhere to the bared vascular wall. Activation of platelets leads to the microthrombi formation and to the expression of SDF-1, which directs EPCs to the damaged endothelium.

After the processes of mobilization, migration, adhesion and invasion, EPCs begin to differentiate into mature ECs. Undifferentiated EPCs and mature ECs exert mutual influence on the functional state of each other, stimulating proliferation and migration through paracrine mechanisms. The paracrine effects of pro-angiogenic biologically active factors that are secreted by undifferentiated and mature ECs are comparable to the stimulating effect of angiogenic cytokines [8].

Reduction of the quantitative content of EPCs, a change in their functional capacity and mobilization potential have been revealed at many diseases, in which ED is considered as one of the most important pathogenetic mechanisms.

The participation of EPCs in angiogenesis has been confirmed by numerous studies at various diseases such as myocardial ischemia [23, 24], limb ischemia [25], DM [1, 26], ischemic stroke [27, 28],

atherosclerosis [29], wounds [30] and others. At the same time, a decrease in proliferation, migration of EPCs to the lesion zone and changes in secretory activity are considered as a possible mechanism for the development of coronary heart disease and chronic heart failure. All these studies emphasize the diagnostic value of EPCs as a marker of CVD and DM [31].

In a cohort of patients with DM, a direct association between the amount of circulating EPCs and the intensity of oxidative stress was found [26]. At DM, a decrease in the concentration of EPCs is determined, which have a reduced ability to adhesion, proliferation and the formation of tubular-like structures [32]. Hyperglycemia and elevated levels of glycosylated hemoglobin are inversely correlated with the level of EPCs. The degree of inhibition of EPCs is associated with the severity of diabetic angiopathy [32].

An inevitable feature of T2DM is insulin resistance (IR). IR via the suppression of the PI3K/eNOS pathway has a significant effect on the development of ED and the progression of atherosclerosis. There is a decrease in the bioavailability of NO and the mobilization of EPCs from BM as a result of the inactivation of the PI3K/eNOS pathway, which is also associated with reduced MMP-9 activity.

In the conditions of hyperglycemia, the toxic effect of glucose suppresses proliferation and enhances the apoptosis of EPCs by stimulating expression of different genes such as $p21^{Waf1}$ and $p16^{Ink4a}$ [34].

Stimulation of $p16^{Ink\text{-}4a}$ and $p21^{Waf\text{-}1}$ pathways leads to blocking of the cell division cycle. P38 mitogen-activated protein kinase (MAPK) is also related to hyperglycemia-induced suppression of EPCs [35].

Akt is known to be involved in signaling pathways that mediate the metabolic effects of insulin. Akt activity in the tissues of patients with DM increases and the constitutive activation of Akt in ECs promotes senescence-like arrest of cell growth via a p53/p21-dependent pathway, impaires angiogenesis and increases inflammation. It has shown that insulin also increases p53 activity and expression of p21 and that it promotes cellular senescence in an Akt-dependent manner [34].

The number of EPCs correlates with factors of cardiovascular risk, such as age, male gender, arterial hypertension, dyslipidemia, smoking, obesity, hyperuricemia, hypodynamia and depends on the total number of risk factors [37, 38]. An inverse correlation between the amount of EPCs and the functional state of the endothelium was also found. In addition, in individuals with high cardiovascular risk, the senescence of EPCs was faster than in those without risk factors [37, 38].

Methods of identification of EPCs

The number of circulating EPCs is small and amounts to 1-5% of the total population of BM cells and less than 0,0001-0,01% of peripheral mononuclear cells circulating in the blood. There are 2 approaches to isolate them from peripheral blood, which are currently used: culture and colony formation analysis and selection of subpopulations based on surface markers. In clinical practice, the gold standard for the determination of EPCs is the flow cytometry method [39].

Identification of EPCs is a difficult task due to lack of standard protocol for the identification of EPCs, because the proposed combinations of markers are not completely specific for EPCs [39]. As a result, there are wide ranges of interlaboratory variations in the quantitative evaluation of EPCs [40].

EPCs express surface markers that are specific both for immature HPCs and mature cells of the endothelium. Markers of hematopoietic stem cells (CD34, CD133) and endothelial cell lines, such as endothelial growth factor receptor-2 (VEGFR-2), also known as KDR or CD309, are most often used to identify these cells. EPCs also express other endothelial markers, such as eNOS, tyrosine-protein kinase receptor (Tie-2), (c-kit or CD117) and E-selectin (CD62E) [40].

Other antigens, such as von Willebrand factor, PECAM-1 and vascular endothelial cadherin (VE-cadherin or CD144) are used to determine EPCs. It should be noted that these antigens are actually markers of mature ECs [42].

Circulating EPCs are not the result of differentiation of a one line of multipotent cells of bone marrow-derived cells (BM-EPCs), including those localized in peripheral tissues. They also include circulating progenitor cells of other populations, as well as cells of the monocyte-macrophage line, which are capable to differentiate into endotheliocytes. This is confirmed by the fact that $CD14^{+}$-myeloid subpopulations express both hematopoietic and endothelial markers and are able to differentiate in EPCs [43].

In dependence on the origin, EPCs express different surface markers. For example, CD31, CD144, CD146 and KDR are positive in EPCs, derived

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from umbilical cord blood, and are negative or weak in BM-EPCs. At the same time, BM-EPCs and the EPCs, obtained from umbilical cord blood, express the CD105, CD73 and CD34 markers [44].

CD34 is the main transmembrane protein, widely represented on the membranes of circulating EPCs of the hemopoietic and mesenchymal lineage cells. $CD34^+$-progenitor cells are thought to differentiate in the direction of the two main lines: peripheral blood cells and ECs [45]. CD133, also known as prominin or AC133, is a transmembrane protein and is expressed on the surface of HPCs and usually does not occur in mature endotheliocytes and monocytes [46].

Currently, the combined expression of $CD34^+KDR^+$, $CD34^+CD133^+$, $CD133^+KDR^+$, $CD14^+CD34^{low}$, $CD34^+CD45^-$, $CD14^+$/ Tie-2/ KDR^+, is widely used to identify EPCs [47].

It is believed that the $CD133^+KDR^+$ phenotype is associated with immature circulating EPCs, whereas the $CD34^+KDR^+$ complex can also be identified in immature endotheliocytes. Some authors suggest that the determination of CD133 antigen increases the specificity of EPCs [48]. Nevertheless, the number of $CD34^+KDR^+CD133^+$-cells in the peripheral blood is much lower. Therefore, the quantitative determination of this phenotype becomes less reliable [49].

The phenotype of EPCs described as $CD34^+KDR^+$ is the most common, has the highest sensitivity and specificity and it is characterized by low expression of the total leukocyte antigen CD45 [50].

It is considered that circulating EPCs with the phenotype $CD34^+$ $CD45^-$ have the greatest ability to differentiate into mature ECs and are most actively mobilized at endothelial damage. Concentration of the $CD34^+CD45^-$ and $CD14^+Tie2^+KDR^+$ phenotypes of EPCs in the peripheral blood is closely associated with the severity of atherosclerotic lesion and ED at various diseases of the CVS, including DM [51-53].

In the general population, the level of circulating $CD34^+/CD45^-$-EPCs positively correlates with obesity, left ventricular hypertrophy and the number of other traditional cardiovascular risk factors, and depends on the amount of cardiovascular risk factors [54]. It is supposed that the level of $CD34^+CD45^-$-cells may indirectly reflect the prevalence of atherosclerosis and correlated with the number of potentially endangered atheroma [55].

In 1996, the ISHAGE (International Society for Hematotherapy and Graft Engineering) protocol was developed to identify stem and progenitor cells, which was successfully implemented in multicenter trials [56]. Schmidt-Lucke and el. [36] adapted the ISHAGE protocol by including a fraction of CD45 cells in the analysis, suggesting that it could contain «true» circulating EPCs [57]. The quantification was performed after a consistent gating strategy [56], and the $CD34^+$-cells were further subdivided into 3 subpopulations: $CD45^-$, $D45^{dim}$ and $CD45^{bright}$.

The data obtained in this protocol revealed the diagnostic value of the phenotype $CD34^+CD45^{dim}KDR^+$. An inverse correlation was found between the number $CD34^+CD45^{dim}KDR^+$ and ischemic heart disease, as well as the number of cardiovascular risk factors, even after correcting the activity of the disease and the number of affected coronary arteries [36].

It is established that in response to ischemia in patients with acute coronary syndrome and unstable angina the concentration of $CD34^+CD45^-$-EPCs increase [36]. Additionally, the concentration of $CD34^+CD45^-$-cells may increase in patients with the obliterating atherosclerosis of arteries of the lower extremities [38, 58].

It is determined that $CD34^+CD45^-$-cells of non-hemopoietic origin are phenotypically indistinguishable from BM-EPCs and functionally differ only in ability to form a colony during cultivation [59]. This creates difficulties in identifying the origin of EPCs in the case of verification of CD34 antigen expression in CD45-negative mononuclear cells. More complex antigenic phenotypes may be more specific for EPCs, however, the lower reproducibility limits their use in clinical practice.

Consequently, more complex antigenic combinations, in spite of providing additional information about the cells, do not increase the efficiency of cell determination as clinical biomarkers [60]. Nevertheless, the functional ability of EPCs to stimulate the neoangiogenesis is not an attribute of their origin and is not directly related to their phenotype [61].

Conclusion

Endothelial progenitor cells are the main pool of cells involved in vascular regeneration and postnatal neovascularization, have the ability to synthesize

vascular growth factors and various cytokines that stimulate vasculogenesis and angiogenesis. Numerous studies have shown the importance of EPCs as an independent predictor of cardiovascular events in patients with high cardiovascular risk. However, there is no standard method for the identification of EPCs and the generally accepted combination of EPCs surface antigens. At the same time, the phenotypes of EPCs as $CD34^{+}CD45^{-}$ $CD34^{+}CD45^{-}KDR^{+}$ possess the highest potential and can be considered as a diagnostic marker of EPCs.

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(Надійшла до редакції 09.11.2018 р.)

Механизмы регенерации эндотелия при сахарном диабете

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Резюме. Эндотелий сосудов — основной орган-мишень, страдающий при сахарном диабете (СД) и сердечно-сосудистых заболеваниях (ССЗ). В обзоре рассмотрена роль эндотелиальных прогениторных клеток (ЭПК) в регенерации эндотелия и в образовании новых кровеносных сосудов. Описаны механизмы миграции и мобилизации ЭПК из костного мозга в зону повреждения. Приведены данные о снижении количества и нарушении функции ЭПК при ССЗ и СД. В статье представлены данные о гетерогенности популяции ЭПК, о различных комбинациях поверхностных маркеров, которые используются для идентификации данных клеток. Вместе с тем на данный момент не разработаны протоколы для идентификации ЭПК, что подтверждает актуальность поиска фенотипа ЭПК, который был бы принят за стандарт.

Ключевые слова: эндотелиальные прогениторные клетки, ангиогенез, регенерация эндотелия, эндотелиальная дисфункция, сахарный диабет.

Механізми регенерації ендотелію за цукрового діабету

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Резюме. Ендотелій судин — основний орган-мішень, що потерпає за цукрового діабету (ЦД) і серцево-судинних захворювань (ССЗ). В огляді розглянуто роль ендотеліальних прогеніторних клітин (ЕПК) у регенерації ендотелію та утворенні нових кровоносних судин. Описано механізми міграції та мобілізації ЕПК із кісткового мозку в зону пошкодження. Наведено дані про зниження кількості та порушення функції ЕПК за ССЗ і ЦД, а також про гетерогенність популяції ЕПК, про різні комбінації поверхневих маркерів, які використовуються для ідентифікації даних клітин. Наразі не існує протоколу для ідентифікації ЕПК, що підтверджує актуальність пошуку фенотипу ЕПК, який може буде прийнятий за стандарт.

Ключові слова: ендотеліальні прогеніторні клітини, ангіогенез, регенерація ендотелію, ендотеліальна дисфункція, цукровий діабет.