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Изучение количества циркулирующих прогениторных клеток эндотелия у больных сахарным диабетом 1 типа

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По современным представлениям, циркулирующие прогениторные клетки (ЦПК) играют важную роль в восстановлении поврежденного сосудистого эндотелия.
Цель. *Целью исследования было изучить влияние сахарного диабета 1 типа (СД1) на количество различных линий ЦПК.*
Материалы и методы*. Количество различных линий ЦПК определяли методом проточной цитофлюориметрии у 45 больных СД1 (средний возраст 26,6±6,5 лет) с различной длительностью заболевания и 14 здоровых добровольцев.*
Результаты. *Количество ЦПК в периферической крови больных СД1 было достоверно снижено в сравнении со здоровыми добровольцами и имело достоверную обратную корреляционную зависимость с уровнем гликированного гемоглобина (HbA_{1c}).*
Заключение. *В периферической крови больных СД1 количество ЦПК зависело от степени компенсации диабета. Так, количество ЦПК суммарной фракции Lin^-CD34^+ в группе больных СД1 оказалось статистически значимо снижено на 42% (p=006), клеток $Lin^-CD34^+c\text{-}Kit^-$ – на 42% (p=0,007); клеток $Lin^-CD34^+c\text{-}Kit^+$ – на 41% (p=0,022). Чем выше был уровень HbA_{1c}, тем меньше было количество ЦПК в периферической крови у больных СД1.*
Ключевые слова: *циркулирующие прогениторные клетки; сахарный диабет 1 типа; дисфункция эндотелия*

Examination number of circulating progenitor endothelial cells in patients with type 1 diabetes mellitus

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Aim. *Circulating progenitor endothelial cells (CPCs) play an important role in the regeneration of damaged vascular endothelium. We aimed to study the effect of type 1 diabetes mellitus (DM) on the number of different lines of CPCs.*
Methods. *The number of different lines of CPCs was evaluated by flow cytometry in 45 patients with type 1 DM (mean age: 26,6±6,5 years) of different duration and 14 healthy volunteers.*
Results: *The number of CPCs in the peripheral blood flow of patients with type 1 DM was significanlty reduced compared with that in healthy volunteers and had a reliable inverse correlation with glycated haemoglobin (HbA_{1c}).*
Conclusions: *The number of CPCs in the peripheral blood flow of patients with type 1 DM depended on the level of DM compensation. Of the different lines of CPCs, Lin^-CD34^+ cells were significantly reduced by 42% (p=0,006), $Lin^-CD34^+c\text{-}Kit^-$ cells by 42% (p=0,007) and $Lin^-CD34^+c\text{-}Kit^+$ cells by 41% (p=0,022). Thus, the number of CPCs in the peripheral blood of patients with type 1 DM is reduced with higher levels of HbA_{1c}.*
Key words: *circulating progenitor cells; diabetes mellitus 1 type; disfunction of endothelium*

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Diabetes mellitus (DM) is one of the most common, high-cost and disabling chronic disease. Despite a large number of clinical studies that have convincingly proven the relationship between DM and cardiovascular complications (United Kingdom Prospective Diabetes Study, Diabetes Epidemiology: Collaborative Analysis of Diagnostic Criteria in Europe, Atherosclerosis Risk in Communities Study, Action in Diabetes and Vascular Disease-Preterax and

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Diamicron MR Controlled Evaluation), the cellular mechanisms undermining the development of angiopathy in patients with DM remain unclear.

Endothelial dysfunction (ED) is known to cause vascular dysfunction in patients with DM, and at the cellular level, ED is related to impairment and accelerated apoptosis of endotheliocytes. A number of cytokines and growth factors are thought to cause mobilization and release of circulating endothelial progenitor cells (EPCs) from the bone marrow. Once they have reached the bloodstream, EPCs then migrate to sites of blood vessel damage and tissue ischaemia to help with angiogenesis. EPCs were first isolated from among circulating mononuclear cells as cells characterized by low expression or complete absence of the leukocyte common antigen cluster of differentiation (CD)45 and by the presence of the progenitor-specific CD34 antigen [1].

Currently, only the CD34 antigen has been recognized as an important marker of pluripotent hemopoietic cells [2]. Another marker, c-Kit, has been shown to regulate proliferation, differentiation and migration of hemopoietic cells, and has therefore long been considered a hemopoietic marker of bone marrow stem cells. In the bone marrow, approximately 1%–5% of all cells express c-Kit, whereas among CD34+ bone marrow cells, approximately 70% express c-Kit. The progenitor erythrocyte, megakaryocyte, granulocyte and lymphoid cells account for the maximum c-Kit expression, and although peripheral blood cells mostly do not carry c-Kit, it is expressed in the cells of a small population of circulating CD34+ bone marrow cells. However, there are limited quantitative data on the number of circulating c-Kit+ progenitor cells in patients with type 1 DM because researchers have tended to focus on the total proportion of CD34+ cells.

Another important factor is the contradicting assumptions regarding the antigenic phenotype of EPCs. However, these differences can be accounted for by significant overlaps in the surface markers between endothelial and hemopoietic progenitor cells, differences in study protocols and differences in the analysis details used in the quantification of EPC populations using flow cytometry. Therefore, later we will use the term CPCs, circulating progenitor cells.

Most previous clinical studies that have sought to quantify CPC numbers were conducted using patients with cardiovascular disease (CVD) and concomitant impairments of carbohydrate metaboli sm, including type 2 DM [3, 4]. Patients with type 1 DM, as opposed to those with type 2 DM, are younger and tend to have few CVD risk factors that could otherwise affect the CPC level. Studying the mechanisms of angiopathy development in type 1 DM therefore seems to be more reasonable.

Objective

The objective of the study was to quantify the amount of CPCs (i.e. the CPC count) in patients with type 1 DM, depending on the glycaemic control (i.e. the quality of carbohydrate metabolism control) and duration of the disease. To achieve the objective, the following three tasks were set:

1. Determination of the amount of progenitor cells of the following circulating populations in the bloodstreams of both patients with type 1 DM and healthy volunteers: Lin^-CD34^+, $Lin^-c\text{-}Kit^+$, $Lin^-CD34^+c\text{-}Kit^-$, $Lin^-CD34^-c\text{-}Kit^+$ and $Lin^-CD34^+c\text{-}Kit^-$.
2. Comparison of the amount of CPCs in the above populations against the glucose level in the blood plasma of fasting patients as well as the level of glycated haemoglobin (HbA1c) and the duration of type 1 DM
3. Comparison of the level of CPC apoptosis between patients with type 1 DM and healthy volunteers.

Materials and methods

The study involved 45 patients with type 1 DM and 14 healthy volunteers of comparable age and gender. We excluded participants with the following diagnoses: type 2 DM, ketoacidosis, acute or chronic inflammatory conditions, cancer or systemic disease, severe anaemia or haematological disease, alcoholism, heart defects, myocardial infarction, stroke, surgical intervention (including low-invasive surgical treatment) performed less than 6 months prior to the study, refractory arterial hypertension (AP > 180/100 mmHg against the background of hypotensive therapy administered), obesity, diabetic foot syndrome, diabetic macroangiopathy, terminal kidney insufficiency and dialysis. All the study participants gave their informed consent and the study protocol was approved by the local ethics committee of the Endocrinology Research Center of the Ministry of Health of the Russian Federation at the meeting held on 19 January 2009 (Protocol # 1).

The clinical characteristics of the study groups are shown in Table 1. The average age of patients with type 1 DM was 26.6 ± 6.5 years, the average duration of DM was 13.3 ± 7.5 years and the average HbA1c level was 8.4% ± 2.0%. At baseline, 11 patients (24%) had microalbuminuria and one patient had proteinuria. In accordance with the international classification of chronic kidney disease (CKD), seven patients were diagnosed with grade 1 CKD, four with grade 2 CKD and one with grade 3 CKD. Diabetic retinopathy was present in 10 patients (22%), of which seven had undergone laser treatment. Furthermore, 12 participants (27%) had chronic diastolic heart insufficiency and 19 (42%) had hypertension treated with angiotensin converting enzyme inhibitors. At the time of hospitalization, all patients were undergoing basal–bolus insulin therapy with human insulin analogues.

All participants underwent standard clinical and laboratory tests, which included a complete blood count, a blood biochemistry test and an HbA1c test. The HbA1c was assessed by the liquid chromatography method using the Sapphire 400 (Niigata Mechatronics, Japan) analyzer according to the manufacturer's guidelines. We also performed electrocardiography, echocardiography and exercise tolerance tests to exclude coronary disease. The stage of CKD was determined by the glomerular filtration rate, using the Modification of Diet in Renal Disease study formula [5].

The number of CPCs and percentage of apoptotic CPCs were determined by flow cytometry on a MoFlo™ cell sorter (DakoCytomation, USA). Mononuclear leukocytes were isolated by centrifugation in Ficoll solution (PanEco, Rus-

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Table 1

The clinical characteristic of the study groups

Parameters	Healthy volunteers	Patients with type 1 DM
Total, n = 59	14	45
Age, years	26.4 ± 3.3	26.6 ± 6.5
Male gender, n (%)	5 (36)	20 (44)
Duration of type 1 DM, years	-	13.3 ± 7.5
Fasting plasma glucose, mmol/l	4.5 ± 0.6	7.5 ± 2.4
HbA1c, %	4.7 ± 0.7	8.4 ± 2.0
Arterial hypertension, n (%)	-	19 (42)
GFR (MDRD ml/min/1.73 m^2	98.0 ± 7.9	105.9 ± 24.2
TC, mmol/l	4.3 ± 0.7	4.8 ± 1.4
LDL, mmol/l	2.0 ± 0.5	2.6 ± 1.2
HDL, mmol/l	1.9 ± 0.5	1.7 ± 0.6
TG, mmol/l	1.0 ± 0.4	1.1 ± 0.8
Haemoglobin, g/l	133.0 ± 7.8	139.7 ± 13.5
Leukocytes, 109 /l	6.9 ± 0.7	6.7 ± 1.6
Exercise, n (%)	7 (50)	24 (53)

Legend: Data are shown as M ± m (δ). DM, diabetes mellitus; GFR, glomerular filtration rate, calculated using the Modification of Diet in Renal Disease study equation; TC, total cholesterol; LDL (cholesterol), low-density lipoprotein cholesterol; HDL (cholesterol), high-density lipoprotein cholesterol; TG, triglycerides.

sia). Then, 2–5 × 10^6 cells were incubated for an hour with fluorescence-labelled antibodies to Lin-FITC (BD Biosciences, USA), CD34-APC (DAKO, Denmark) and c-Kit-PE (DAKO). To assess the degree of apoptosis, 1 × 10^6 cells were dyed in a similar way but without c-Kit-PE; after washing, the cells were additionally dyed for 15 min with Annexin V-PE (AnV-PE) (BD Biosciences) and 7-AAD (BD Biosciences) in buffer solution for AnV (BD Biosciences). After dying, the cells were washed, resuspended in a 500-ml phosphate-salt buffer solution and analysed. A zone of cells not carrying linear leukocyte markers was identified (Lin$^-$, Fig. 1A), and among those cells, three populations were identified (Lin$^-$CD34$^+$c-Kit$^-$, Lin$^-$CD34$^+$c-Kit$^+$ and Lin$^-$CD34$^-$c-Kit$^+$; Fig. 1B), and the percentage of cells was calculated for each subpopulation in relation to the total number of mononuclear leukocytes. The percentage of Lin$^-$CD34$^+$ and Lin$^-$c-Kit$^+$ was calculated as the sum of these two subpopulations. The percentage of apoptotic cells was determined for the population of Lin$^-$CD34$^+$ leukocytes (Fig. 1C). The apoptotic cells were identified as cells dyed with AnV-PE and not containing 7-AAD (i.e. AnV$^-$7AAD$^-$; Fig. 1D)

Statistical data analysis

We registered, edited and analysed the data using a Microsoft Office® package and the Statistical Analysis System.

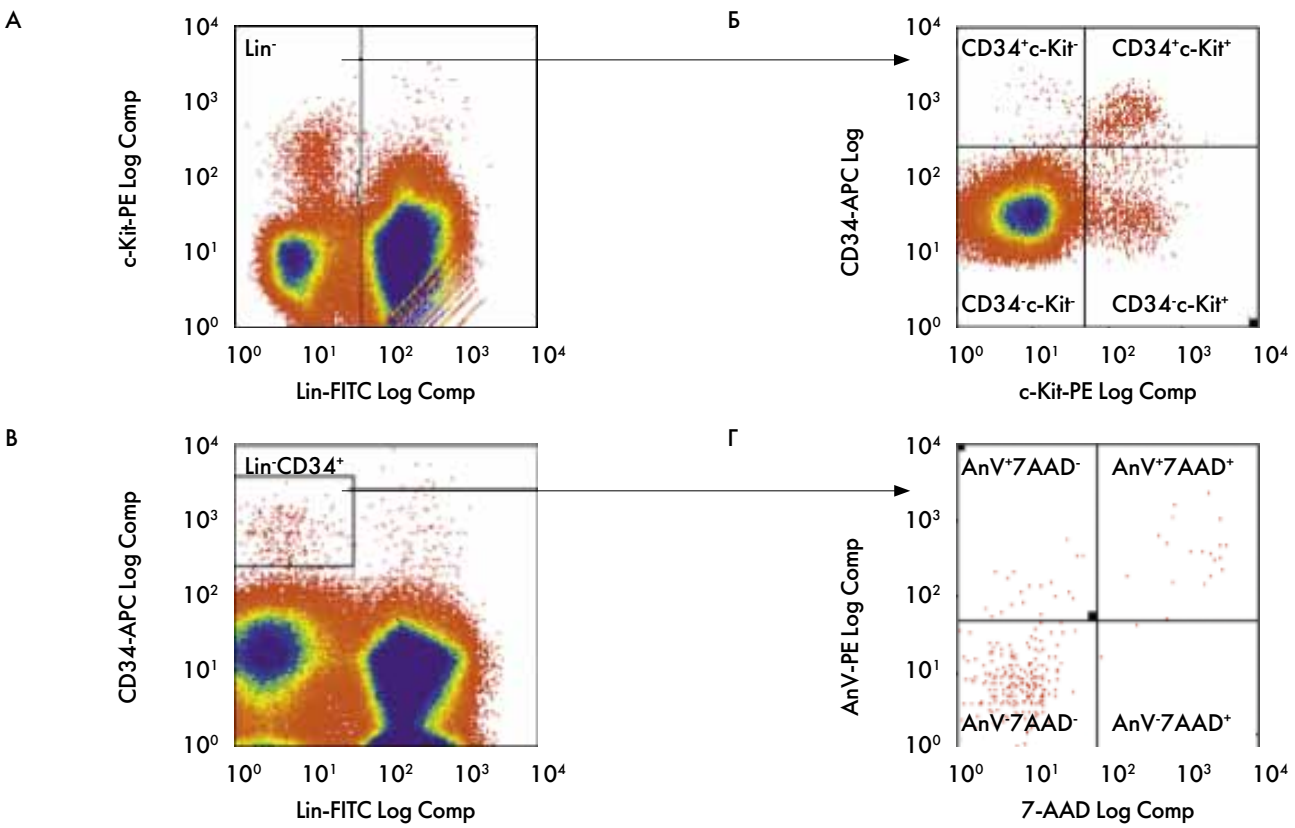

Figure 1.Analysis of cell subpopulations by flow cytometry.
A. Zones of leukocytes not carrying linear leukocyte markers are shown (i.e. Lin$^-$)
B. The analysis of Lin–cells by expression of the markers c-Kit (X-axis) and CD34 (Y-axis) is shown. The zones are involved in expressing one or more of the markers (i.e. Lin$^-$CD34$^+$c-Kit$^-$, Lin$^-$CD34$^+$c-Kit$^+$ or Lin$^-$CD34$^-$c-Kit$^+$);
C. The zone of Lin$^-$CD34$^+$ cells is shown;
D. The analysis of Lin$^-$CD34$^+$ cells in accordance with expression of the dead cell marker (7-AAD, X-axis) and apoptotic cells (AnV, X-axis). The zone of apoptotic cells is marked as AnV$^-$7AAD$^-$.

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Table 2

The amount of CPCs in the peripheral blood of study participants

CPC populations (cells/ml/leukocytes)	Healthy volunteers	Patients with type 1 DM	Lower in the patients' group, %	Confidence of differences, p-value
Lin^-CD34^+	74.1 ± 58.5	43.1 ± 23.7	↓42%	0.006
$Lin^-c\text{-}Kit^+$	203.2 ± 96.9	165.2 ± 80.5	↓19%	0.178
$Lin^-CD34^+c\text{-}Kit^-$	34.3 ± 30.4	19.8 ± 10.4	↓42%	0.007
$Lin^-CD34^-c\text{-}Kit^+$	163.4 ± 11.0	141.8 ± 78.4	-	0.460
$Lin^-CD34^+c\text{-}Kit^+$	39.8 ± 35.1	23.3 ± 16.3	↓41%	0.022

Legend: Data are presented as M ± m (δ). CPC, circulating progenitor cells; DM, diabetes mellitus

Descriptive data were calculated and presented as averages, frequency ratios, standard deviations and standard errors, using the procedures PROC SUMMARY, PROC UNIVARIATE and PROC FREQ. Extended variance-covariance analysis was conducted with the PROC GLM procedure to evaluate the impact of age, glucose level, HbA1c level, duration of DM, gender (binary) and the availability of type 1 DM signs (categories) on the number of Lin^-CD34^+, $Lin^-c\text{-}Kit^+$, $Lin^-CD34^+c\text{-}Kit^-$, $Lin^-CD34^+c\text{-}Kit^+$ and $Lin^-CD34^-c\text{-}Kit^+$ cells and the percentage of apoptotic cells. Standard significance tests were used, as appropriate: χ^2 test, student's t-tests (two-sample and paired tests) and Fisher's test for analysis of variance. Spearman partial correlations were calculated between variable blocks for gender, age and body mass index. To reveal independent inter-relations among the parameters, multivariate regression equations and multiple linear regression analyses were used. The null hypothesis was rejected when $p < 0.05$.

Results and discussion

The CPC count in patients with type 1 DM

The amount of CPCs in the study groups is shown in Table 2. Compared with the control group, patients with type 1 DM were characterized by reduced CPC counts. The CPC count as a proportion of all Lin^-CD34^+ cells among patients with type 1 DM was statistically reduced by 42%; meanwhile, the reduction was by 19% for $Lin^-c\text{-}Kit^+$ cells, 42% for $Linin^+CD34^+c\text{-}Kit^-$ cells and 41% for $Lin^-CD34^+c\text{-}Kit^-$ cells. No reliable differences were revealed for the amount of $Lin^-CD34^-c\text{-}Kit^+$ cells.

Similar data were obtained in the study by Palombo et al. (2011), in which compared with the control group, patients with type 1 DM (HbA1c = 7.7% ± 1.1%) demonstrated reduction in the population of CPC $CD34^+$ by 34.5% [6]. The authors also revealed a reciprocal correlation between the CPC count and intima-media thickness ($r = -0.31$, $p < 0.05$), attributing this to a more intense attraction of the progenitor cells to the atherosclerotic zones.

In the study by Głowin´ska-Olszewska et al. (2013), which involved 52 patients with type 1 DM (HbA1c = 8.5% ± 1.4%), the authors did not reveal any differences in the amount of CPCs among all $CD34^+$ cells compared with that in healthy controls. However, they did reveal a reduction in the CPC population of $CD34^+CD144^+$ ($VE\text{-}cadherin^-$) cells in patients with type 1 DM compared with that in healthy controls, and they demonstrated a relationship between this population and flow-dependent vasodilation ($r = -0.31$, $p = 0.03$) [7].

Among the possible mechanisms of CPC reduction, the following mechanisms are discussed: impaired mobilization of progenitor cells from bone marrow [8], increased 'consumption' of these cells in the ischaemic zones, endothelial damage, impairment of functional characteristics of CPC due to hyperglycaemia and accelerated CPC apoptosis [2]. In addition, mobilization of CPCs from the bone marrow, which is attributed to the specific type of bone marrow neuropathy [9], is also relevant. Diabetes is also supposed to lead to bone marrow angiopathy that has specific morphological features similar to other typical diabetes-induced microvascular complications, including thickening of the basal membrane, capillary thinning and increased apoptosis of endotheliocytes and progenitor cells of blood vessels [10].

Relationship between glycaemic control and the CPC count in patients with type 1 DM

Hyperglycaemia is known to be the main factor to damage blood vessel walls in patients with DM. During correlation analysis we did not reveal any interaction between the CPC count and fasting glucose levels. However, we found a statistically relevant negative correlation between the CPC count and HbA1c level that was present for the total proportion of Lin^-CD34^+ ($F = 7.4$, $p = 0.009$), $Lin^-CD34^+c\text{-}Kit^-$ ($F = 6.6$, $p = 0.01$) and $Lin^-CD34^+c\text{-}Kit^+$ cells ($F = 5.2$, $p = 0.02$) (Fig. 2, 3, 4).

The results obtained agree with those of other authors who reported that the relationship between the CPC count and poor glycaemic control in patients with DM is established [3, 4]. The negative relationship between the CPC count and HbA1c level in this study and the existing literature confirm the high significance of glycaemic control as an important factor for preventing micro- and macro-vascular complications in patients with either type 1 or type 2 DM [6, 11].

Association between the duration of type 1 DM and the CPC count

Some authors have argued that the duration of DM is associated with a reduction in CPC counts, with most authors agreeing that this reduction is caused by prolonged oxidative stress associated with chronic hyperglycaemia. Several other mechanisms have also been discussed, including impairment of the local production of nitrogen oxide (NO), excessive generation of endothelium-dependent superoxide, inactivation of synthesized NO molecules and stimulation of the oxidation of low-density lipoproteins as well as damage to endotheliocyte membranes caused by peroxynitrite and hydroxyl radicals [12].

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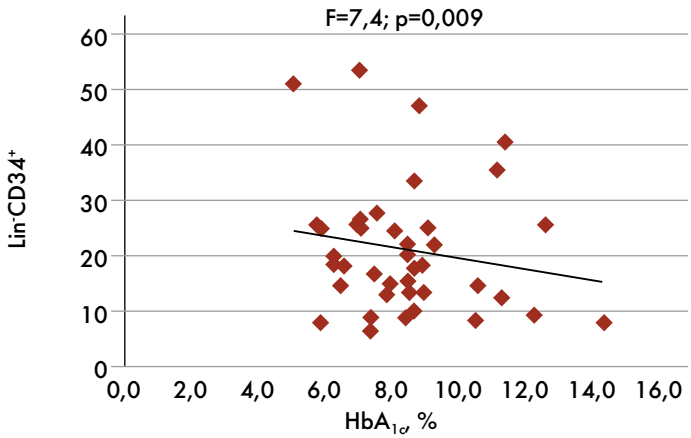

Figure 2. Relationship between the relative amount of Lin⁻CD34+ cells and the HbA1c level, n = 45

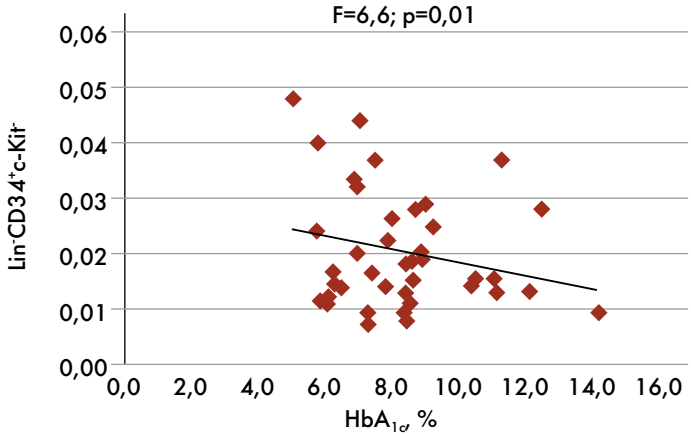

Figure 3. Relationship between the relative amount of Lin⁻CD34+c-Kit⁻ cells and the HbA1c level, n = 45

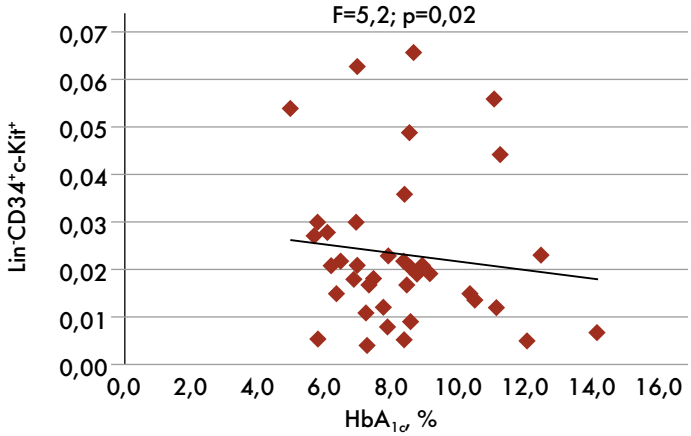

Figure 4. Relationship between the relative amount of Lin⁻CD34+c-Kit+ cells and the level of HbA1c, n = 45

Notably, our study did not reveal any statistically relevant differences when comparing patients by duration of DM (0–5 years, 6–10 years and ≥10 years).

CPC apoptosis

Apoptotic bodies of endotheliocytes have shown to exert dose-dependent stimulation on EPCs. This has led to the supposition that apoptosis products are physiological attractants for CPCs, stimulating their proliferation and mobilization from the bone marrow into the blood. Studies on the interaction between the carbohydrate metabolism impairments in patients with DM and the level of CPC apoptosis are few and have produced ambiguous results. In the study by Loomans et al. (2004), the authors found no differences in the level of apoptosis of cultivated CPCs between healthy controls and patients with type 1 DM [13]. In some studies, in addition to a reduction in the CPC count ($CD34^-/CD133^+/KDR^+$) in patients with type 2 DM compared with that of patients without type 2 DM, an increase in the amount of circulating early apoptotic progenitor cells ($CD34^+/7AAD^-/AnnexinV^+$) has been demonstrated. In our study, we did not find any statistically relevant differences in the level of apoptosis among the Lin^-CD34^+ CPC cells in any group; in addition, we did not find any relationship between the level of CPC apoptosis and the parameters of the carbohydrate metabolism.

Conclusions

1. The CPC count in the peripheral blood of patients with type 1 DM is reliably lower than that in healthy volunteers.
2. The CPC count has a reliable reciprocal correlation with the HbA1c level, indicating that hyperglycaemia plays a significant role in the formation of defects in EPCs required for vessel repair.
3. Duration of type 1 DM does not affect the CPC count.
4. The CPC apoptosis levels did not differ between patients with type 1 DM and healthy controls.

Thus, reduction in the circulating levels of EPCs in patients with type 1 DM, which was revealed in our study, is related to the quality of metabolic control rather than to the disease duration. This insight allows us to assume that there is a glucose-related impairment of the mechanisms of endothelial cell repair in blood vessels. This process undoubtedly exacerbates the vascular complications of DM, together with the other known mechanisms of glucose toxicity in relation to blood vessel walls.

Information on funding and on the conflict of interests

The authors declare the absence of potential conflicts of interests related to the publication of this paper. The study was conducted under an endocrinology platform-based research programme approved by a commission of the Russian government.

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