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Circulating endothelial progenitor cells and angiogenic factors in diabetes complicated diabetic foot and without foot complications

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ABSTRACT

Introduction: Data about angiogenic factors in diabetic foot syndrome (DFS) are insufficient. Therefore, in the present study we focus on circulating endothelial progenitor cells (EPCs) and two major angiogenic factors: vascular endothelial growth factor (VEGF-A) and fibroblast growth factor (FGF-2) in patients with DFS.
Materials and methods: We included 75 subjects: 45 patients with type 2 diabetes and 30 controls. The study group was divided into 2 subgroups: 23 patients with diabetic foot and 22 patients without diabetic complications. The concentration of VEGF-A, soluble VEGF receptor 2 (sVEGF-R2) and FGF-2 were measured in plasma samples. The number of circulating EPCs was determined in peripheral venous blood. The number of endothelial progenitor cells was measured with FACSCalibur flow cytometer using monoclonal antibodies directed against antigens specific for EPCs.
Results: In our study we observed significant higher levels of VEGF-A and FGF-2 and lower sVEGF-R2 concentration in patients with T2DM compared to healthy subjects. The conducted analysis showed decreased levels of VEGF-A and elevated levels of FGF-2 in patients with DM complicated DFS compared to diabetic patients without DFS. Increased circulating EPCs number was reported in patients with DFS, and the difference was almost statistically significant.
Conclusions: The high concentration of VEGF-A and FGF-2, and a positive correlation between them indicate their participation in the process of angiogenesis in T2DM. Decreased sVEGF-R2 may result from inactivation of VEGF-A during complexes formation.

1. Introduction

Diabetes is one of the major socioeconomic problems, and it is considered a global epidemic of the XXI century. According to the International Diabetes Federation in 2012 more than 371 million people suffer from diabetes, and it is postulated that in 2030 the number of patients with diabetes rises to 552 million (IDF Diabetes Atlas, 2012; Waniczek et al., 2013). Long lasting (prolonged) hyperglycemia leads to development of diabetic complications, like retinopathy, nephropathy or diabetic foot syndrome (DFS). It is considered that DFS is a significant cause of morbidity and mortality and is not typical for late diabetic complication, but also appears in patients with newly diagnosed diabetes. Normal wound healing is very important for patients with DFS because of development of diabetic foot ulcer. Impaired tissue regeneration, resulting from insufficient angiogenesis, contributes to non-healing ulcers. Diabetic non-healing ulcers account for more than 60% of all non-traumatic lower limb amputations (Jaiswal, Gambhir, Agrawal, & Harish, 2010).

The pathogenesis of each diabetic complication is undoubtedly multifactorial. Nevertheless, impaired angiogenesis is one potential component that might be common for many diabetic complications. Angiogenesis is a multi-stage process involving the endothelium, growth factors and their inhibitors, cytokines, endothelial progenitor cells (EPCs) and enzymes (Jiang & Brey, 2011; Kajdaniuk, Marek, Borgiel-Marek, & Kos-Kudła, 2011; Kota et al., 2012). The process of proper vascular network formation includes four phases: initiation, progression, differentiation and stabilization with maturation. Every phase is controlled by balance between stimulatory and inhibitory factors (Kota et al., 2012).

VEGF-A and FGF-2 (basic fibroblast growth factor) are two crucial angiogenic factors. First of them (VEGF-A) activates angiogenesis and is responsible for endothelial cells survival, migration and proliferation (Kajdaniuk et al., 2011). VEGF transmits signal through receptors:

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Flt-1 (VEGF-R1), Flk-1 (VEGF-R2) and Flt-4 (VEGF-R3) that are localized on cell surface. Further receptors are soluble forms of receptor: sVEGF-R1 and sVEGF-R2. Soluble receptors bind VEGF and reduce its biological activity. Soluble receptors for VEGF are known as angiogenesis inhibitor (Kajdaniuk et al., 2011; Shibuya, 2006).

Purified FGF-2 stimulates proteases synthesis (metalloproteases and urokinase-type plasminogen activator) that contributes to basement membrane degradation. In that way, cells can migrate into the new blood vessel. FGF-2 plays a critical role in the extracellular matrix (ECM) components synthesis that leads to the maturation of new blood vessels (Mizia-Malarz, Sobol, & Woś, 2008; Presta, Andres, Leali, Dell'Era, & Ronca, 2009; Qazi, Maddula, & Ambati, 2009). Thus, VEGF-A predominate in early angiogenic steps and FGF-2 regulates the later stages of angiogenesis. Co-operation between these factors is needed for property, well-functioning vessels development.

The discovery of endothelial progenitor cells has brought new insight into angiogenesis process. These cells exist in the bone marrow (BM) and are heterogenic group of cells. Highly immature EPCs (pre-EPCs) express early hematopoietic markers as CD117 and CD133. Early EPCs (CD133, CD34 and VEGF-R2 positive) may facilitate angiogenesis by secreting pro-angiogenic factors (mainly VEGF). The late EPCs express endothelial markers such as CD31, VEGF-R2, but they are CD133 negative (Lombardo et al., 2012; Qazi et al., 2009; van Ark et al., 2012). EPCs also express receptors for FGF. In order to various stimuli (e. g. ischemia), EPCs are recruited to the peripheral circulation (Lombardo et al., 2012). Recently experimental study has shown that EPCs applied to diabetic wounds increased local VEGF and FGF expression that improved vascularization and process of wound healing (Asai, Takenaka, Ii, et al., 2013).

From the angiogenesis point of view, diabetes is a paradoxical disease. An excessive angiogenesis is observed in retinopathy or nephropathy, while in diabetic foot syndrome angiogenic response is impaired. Data about angiogenic factors in DFS are insufficient. Therefore, in the present study we focus on circulating EPCs and two major angiogenic factors: VEGF-A and FGF-2 in patients with DFS.

2. Materials and methods

This study was conducted according to the tenets of the Declaration of Helsinki and was approved by the Bioethics Commission of the Collegium Medicum in Bydgoszcz Nicolaus Copernicus University in Toruń (no. KB/367/2009). Patients and volunteers were informed about the purpose of the study and the procedures involved. They all gave written informed consent.

We included 75 subjects and divided them into two groups: 45 patients with type 2 diabetes and 30 controls. The study group consisted of 22 women and 23 men. The study group was divided into 2 subgroups: 23 patients with diabetic foot and 22 patients without diabetic complications. The control group consisted of 30 healthy volunteers selected with regard to the age and gender (Table 1).

All patients were being treated with appropriate therapy in the Diabetic Foot Clinic, University Hospital and the Department of Vascular Surgery and Angiology, University Hospital of A. Jurasz, Bydgoszcz. The criteria for inclusion in the study were glycated hemoglobin value above 7% providing poor diabetes control and the presence of vascular complication with diagnosed diabetic foot syndrome. Classification according to Wagner was done to determine the deep foot lesions during the first visit. T2DM was diagnosed using blood glucose cut-off values as defined by WHO. The patients were treated with insulin, metformin and other appropriate therapy. Hypertension was defined as a systolic pressure $\geq$ 140 mm Hg or diastolic pressure $\geq$ 90 mm Hg. Patients with hypertension-received angiotensin-converting enzyme (ACE) alone or associated with other anti-hypertensive agents. The patients who had an operation within the last month, had the end-stage renal failure, chronic liver disease or any other severe medical conditions requiring active treatment were excluded from this study.

Table 1
Clinical characteristics of the patients.

Characteristic	Patients with DF	Patients without DF
Gender (M/F)	16/7	7/15
Age (mean)	67	63
DM duration (years)	12.4	5.3
DFS duration (months)	17	–
BMI		
Normal	7	12
Overweight	10	8
Obesity	6	2
Hypertension	18	16
Smoking	7	6
Retinopathy	12	–
Nephropathy	7	–
Neuropathy	12	–

DF – diabetic foot, DM – diabetes mellitus.

The comparative analysis of the examined group (only patients with DFS) included the influence of factors as: gender, smoking (n = 7), retinopathy (n = 12), nephropathy (n = 7), neuropathy (n = 12) and hypertension (n = 18) on measured parameters.

2.1. Immunoassays methods

The material for the research of angiogenic factors was blood samples drawn after 12 hours of fasting. Plasma was obtained from the whole blood collected into tubes containing ethylenediaminetetraacetic (EDTA) and centrifuged at 3000 rpm for 15 minutes. All the samples were stored at −80 °C until the analysis, but no longer than for 6 months. VEGF concentration was determined in the plasma samples considering the known release of it by platelets. The concentration of VEGF-R2 and FGF-2 was also measured in plasma samples. The growth factors and receptors were measured using a human Elisa kit: Quantikine Human Immunoassay produced by R&D Systems, Minneapolis, MN, USA.

2.2. Quantification of circulating EPCs by flow cytometry

To determine the number of circulating EPCs, 3 ml of peripheral venous blood was collected in EDTA tubes and processed within 5 h after collection. The number of endothelial progenitor cells was measured with FACSCalibur flow cytometer (Becton Dickinson, San Diego, USA) using monoclonal antibodies directed against antigens specific for endothelial progenitor cells (EPCs). Acquired data were analyzed by using CellQuest software (Becton Dickinson). The selection of antigens allowed to assess the population of early and late circulating EPCs.

The following monoclonal antibodies were used in this study: fluorescein isothiocyanate (FITC)-conjugated anti-CD31, PerCP-Cy5.5-conjugated anti-CD45, as well as APC-conjugated anti-CD34 antibody (all BD Biosciences, Pharmingen, San Diego, CA, USA), phycoerythrin (PE)-conjugated anti-CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany). Endothelial progenitor cells (EPCs) were defined as negative for the hematopoietic marker CD 45 and positive for the endothelial progenitor marker CD 133 and positive for the endothelial cell markers CD 31 and CD 34. At least 100,000 events were collected before analysis. TruCount tubes (BD Biosciences, San Jose, CA, USA) containing a calibrated number of fluorescent beads and `lyse-no-wash` procedures were used in the present study.

To evaluate immunophenotyping of circulating endothelial progenitor cells, 50 μl of whole blood (taken at the EDTA-K2) were incubated in TruCOUNT tube (Becton Dickinson) in the dark for 15 minutes with antibodies: anti-CD45, anti-CD31, anti-CD34,