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Endothelial progenitor cells in relation to endothelin-1 and endothelin receptor blockade: A randomized, controlled trial

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ABSTRACT

Aims: Endothelial progenitor cells (EPC) represent an endogenous repair mechanism involving rendothelialization and neoangiogenesis. Patients with both diabetes and vascular disease have low numbers of circulating EPC. The endothelium-derived peptide, endothelin-1 (ET-1), is increased in patients with type 2 diabetes and vascular complications and has been suggested to contribute to endothelial dysfunction. Therefore, we investigated the relation between EPC and plasma ET-1 and the effect of dual ET-1 receptor antagonist treatment.
Methods: In this double blind study patients with type 2 diabetes mellitus and microalbuminuria were randomized to treatment with the dual ETA/ETB receptor antagonist bosentan treatment (125 mg bid; n = 17) or placebo (n = 19) for four weeks. Different EPC subpopulations were enumerated by flow cytometry using triple staining (CD34, CD133, KDR) at baseline at the end of treatment. Viability was assessed by 7AAD and Annexin-V-staining.
Results: Baseline ET-1 levels correlated significantly with C-reactive protein levels. Patients with ET-1 levels above the median value had higher levels of $CD34^+CD133^+$ and $CD34^+KDR^+$ EPC. There was no difference in $CD34^+$ and $CD34^+CD133^+KDR^+$ cells, markers of EPC apoptosis or circulating markers of endothelial damage between patients with ET-1 levels below or above the median. Four week treatment with bosentan did not change EPC levels.
Conclusion: Among patients with type 2 diabetes and vascular disease, high plasma levels of ET-1 are associated with higher number of EPC. The recruitment of EPC does not seem to be regulated via ET-1 receptor activation since treatment with a dual ET-1 receptor blocker did not affect circulating EPC numbers.

1. Introduction

Endothelial progenitor cells (EPC) home to sites of ischemia and vascular injury as an endogenous repair mechanism of damaged endothelium. They are thought to contribute to reendothelialization, neovascularization, and normalization of endothelial function and restoration of blood flow at the site of injury [1]. In consequence, a reduced number and function of EPC have been linked to endothelial dysfunction [2] as well as increased risk for atherosclerosis and cardiovascular morbidity and mortality [3].

Cardiovascular risk factors have been linked to the number and function of circulating EPC [4]. Accumulating evidence indicates that also diabetes mellitus is associated with impaired number and function of EPC. The number of EPC is reduced in patients with type 2 diabetes mellitus (T2D) and the number of EPC was found to be negatively correlated with disease severity scores [5].

Different EPC subpopulations have been identified: a more primitive $CD34^+/CD133^+/KDR^+$ subpopulation and a more mature $CD34^+/KDR^+$ subpopulation. High numbers of the latter predict better cardiovascular outcome [6]. On the other hand, higher numbers of EPC have been used as marker of occult vascular damage in subjects at risk to develop cardiovascular disease [7,8]. In addition, staining of progenitor cells with markers for apoptosis provides additional important pathophysiological information. Apoptotic progenitor cells are elevated in patients with acute coronary syndrome, heart failure and diabetes [9,10].

A growing body of evidence supports the notion that EPC can be mobilized from bone marrow to the circulation by ischemia, drug treatment and a variety of cytokines [11]. Stimulating factors include granulocyte-macrophage colony-stimulating factor (GCSF) and vascular endothelial growth factor (VEGF). Preliminary data indicate that also endothelin-1 (ET-1) seems to be involved in the regulation of EPC recruitment [12]. ET-1 is a potent vasoconstrictor peptide initially detected in endothelial cells [13]. It stimulates a number of biological actions including vasoconstriction, pro-inflammatory actions, mitogenic and proliferative effects and formation of free radicals. These effects are mediated via the two ET-1 receptors named ET_A and ET_B receptor [13]. Plasma levels of ET-1 are elevated in patients with type 2 diabetes [13] contributing to endothelial dysfunction in patients with type 2 diabetes [14]. Four week treatment with the ET receptor antagonist bosentan improved peripheral endothelial function in patients with type 2 diabetes [14]. The aim of the present study was therefore to explore the relation between ET-1

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levels and EPC in patients with type 2 diabetes and vascular complications, and to investigate the effect of dual ET_A/ET_B receptor blockade on circulating EPC.

2. Materials and methods

2.1. Study subjects

Patients with type 2 diabetes of at least two year duration and known vascular dysfunction detected by microalbuminuria were recruited at Karolinska University hospital from autumn 2007 to spring 2010 by members of the study group. Patients were classified as having diabetes mellitus if fasting blood glucose exceeded 7.0 mmol/l (on at least on two occasions) or blood glucose concentration was >11.0 mmol/l 2 h after an oral glucose loading (75 g). Albuminuria was defined as urine albumin concentration>20 μg/l or >30 μg/12 h and a ratio of albumin/creatinine>3.0 mg/mmol. Patients were excluded if they had a myocardial infarction or unstable angina within the last three months, decompensated heart failure, childbearing potential, impaired hepatic function (2 times upper normal limit of serum aminotransferases), ongoing treatment with glibenclamide, cyklosporine or warfarin or concomitant disease that may have interfered with the possibility for the patients to comply with or complete the study protocol.

2.2. Study design

The study was approved by the local ethical committee of the Karolinska Institute and the Swedish Medical Product Agency and complies with the Declaration of Helsinki. Thirty-six patients were enrolled into the study after giving their written informed consent. A detailed description of the main study assessing the effect of the dual ET_A/ET_B receptor antagonist bosentan treatment on peripheral endothelial function has been described elsewhere (ClinicalTrial.gov NCT01357109) [14].

The study was randomized, double-blind and placebo-controlled with two parallel groups. Following baseline measurements and blood sampling, patients were randomized to treatment with bosentan or placebo. Bosentan treatment was started at a dose of 62.5 mg bid for 2 weeks, and in the absence of side effects, the dose was increased to 125 mg bid for 2 weeks. Additional blood samples were collected at the end of the 4 week treatment period. Treatment compliance was checked through pill count. A computer-generated randomization list was delivered by the producer of the drug/placebo (Actelion, Allschwil, Switzerland). The randomization code was kept in sealed envelopes and was not opened until completion of data collection and analyses. The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology.

2.3. Circulating EPC number

A venous blood sample (10 ml) was obtained in EDTA tubes for enumeration of EPC. Different subpopulations were identified by using the hematopoietic progenitor cell marker CD34, the immature hematopoietic progenitor cell marker CD133, and the endothelial cell receptor VEGFR2 (vascular endothelial growth factor receptor-2, also known as kinase domain receptor, KDR), as described previously [8,10,15]. Briefly, peripheral blood mononuclear cells (PBMC) were incubated with fluoresceinisothiocyanate (FITC)-conjugated anti-human CD34 monoclonal antibody (mAb) (BD Biosciences), phycoerythrin (PE)-conjugated anti-human CD133 mAb (Miltenyi Biotec) and allophycocyanin-conjugated monoclonal anti-VEGFR2 (R&D Systems) for 60 min at +4 °C. Control isotype immunoglobulin antibodies were obtained from Becton Dickinson. The cytometer (CyAn, Dako, Denmark) acquired at least 5×10^6 events. The frequency of peripheral blood cells positive for these reagents was determined by a two-dimensional side-scatter fluorescence dot-plot analysis of the samples, after appropriate gating. Data were processed using the Summit software program (Version 4.3, Dako).

2.4. Apoptotic progenitor cells

Apoptosis in progenitor cells was assessed using ApoScreen Annexin V Apoptosis detection kit (Annexin V-PE, 7-AAD solution, and Annexin V binding buffer; SouthernBiotech, Birmingham, AL), as described previously [9,10]. Using gradient centrifugation (Lymphoprep, Medinor, Sweden) peripheral blood mononuclear cells were isolated from 5 ml of freshly drawn heparinized blood. Cells were incubated with the CD34mAb for 60 min. Thereafter, apoptosis staining was done with Annexin-V binding buffer and V-PE labeled Annexin-V-mAb and 7AAD according to the manufacturer's instructions. By binding to DNA 7AAD stains dead cells while Annexin-V binds to phosphatidylserine usually present on the inner leaflet of the cell membrane. Phosphatidylserine is exposed to the external cellular environment, which occurs when membrane integrity is affected in early phase of apoptosis [16].

2.5. Circulating markers of endothelial damage

Circulating levels of Von Willebrand factor (vWF) and inter-cellular adhesion molecule 1 (ICAM-1) were determined using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions.

2.6. Peripheral endothelial function testing

The investigations were performed in the morning after a 20 min rest with the patient in the supine position. The patients were asked to refrain from caffeine-containing drinks or tobacco consumption and intake of drugs was withheld for the 12 preceding hours and until after the examinations. Non-invasive determination of digital endothelial function was measured with a pulse amplitude tonometry (PAT) device placed on the tip of each index finger (Endo-PAT2000, Itamar Medical, Caesarea, Israel). The PAT device comprises a pneumatic plethysmograph that applies a uniform pressure to the surface of the distal finger, allowing measurement of pulse volume changes. The inflation pressure of the digital device was electronically set to 10 mm Hg below diastolic blood pressure or 70 mm Hg (whichever was lower). The PAT signal was recorded at baseline and following 5 min arterial occlusion using an inflatable cuff while the contralateral arm served as a control. The blood pressure cuff was inflated 60 mm Hg higher than the systolic pressure or at least 200 mm Hg for 5 min. The post-occlusive hyperemia stimulates endothelium-dependent vasodilatation causing an increase in digital pulse amplitude. Pulse amplitude was recorded electronically in both fingers and analyzed by a computerized, automated algorithm (Itamar Medical). The change from the baseline measurement is expressed as the reactive hyperemia index (RHI) which in part reflects vasodilator function of the digital microcirculation [14].

2.7. Laboratory investigations

Hb1Ac (Mono S), blood lipids and creatinine were accessed with standard methods according to local laboratory routines. ET-1 was measured using ELISA (R&D systems, Minneapolis, USA). High-sensitive C-reactive protein (CRP) was analyzed using turbidimetry (Beckman Coulter, Fullerton CA, USA).

2.8. Statistical analyses

All data are expressed as mean ± standard deviation (SD). Continuous variables were tested for normal distribution with the Kolmogorov–Smirnov test. Non-normally distributed continuous variables groups were compared by the Mann–Whitney *U* test, while others were analyzed by *t* test (2 sided, including Levene's test for equality of variances). Bivariate correlation (Spearman's rank correlation coefficient) was used to correlate circulating EPC counts with risk factors or drug treatment. Comparisons between before and after treatment were compared using a paired test. Statistical significance was assumed if a null hypothesis could be rejected at $p<0.05$. All statistical analyses were performed with IBM-SPSS, version 19.0 (IBM Inc.).

3. Results

3.1. Baseline ET-1 and EPC

The baseline characteristics of the 36 subjects are summarized in Table 1. Baseline plasma ET-1 levels were 1.61 ± 0.83 pg/ml. According to the median (1.29 pg/ml), the cohort was divided into two groups in order to compare the association between ET-1 levels on EPC and markers of endothelial damage. The groups with high and low ET-1 levels were well matched for age, gender and medication. Patients with high ET-1 levels had increased body mass index (BMI), larger waist circumference and had higher systolic blood pressure than those with low ET-1 levels. Other important risk factors for cardiovascular disease including lipid levels and smoking habits were similar in the two groups. Medication was similar in the two groups except for a higher proportion of patients treated with angiotensin receptor blockers (ARB) among those with high ET-1 levels.

Patients with higher ET-1 levels had higher levels of $CD34^+CD133^+$ ($p=0.038$) and $CD34^+KDR^+$ EPC ($p=0.032$), whereas there was no difference in $CD34^+$ ($p=0.077$) and $CD34^+CD133^+KDR^+$ ($p=0.324$) cells between patients with high and low ET-1 levels (Fig. 1). There was no difference in the relative number of cells with markers of apoptosis between patients with high and low levels of ET-1 (22 ± 9% vs. 24 ± 15%, p = ns). The number of $CD34^+KDR^+$ EPC at baseline correlated significantly with ET-1 levels ($R=0.36$, $p=0.034$) whereas $CD34^+CD133^+$ cells did not ($p=0.08$). Furthermore, there was no difference in circulating levels of ICAM-1 (224.1 ± 48.0 ng/ml vs. 254.6 ± 77.1 ng/ml) or vWF (1.38 ± 0.62 kIE/l vs. 1.38 ± 0.76 kIE/l) between patients with high and low ET-1 levels.

Patients with high ET-1 levels had higher CRP levels (3.2 ± 2.2 mg/l versus 1.4 ± 1.2 mg/l; $p=0.008$). Furthermore ET-1 and