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ScienceDirect
Clinical Biochemistry 41 (2008) 82–87
CLINICAL BIOCHEMISTRY

Expression profile of total VEGF, VEGF splice variants and VEGF receptors in the myocardium and arterial vasculature of diabetic and non-diabetic patients with coronary artery disease

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Received 24 July 2007; received in revised form 30 August 2007; accepted 11 September 2007
Available online 21 September 2007

Abstract

Background: Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis and is implicated in the development of diabetic microvascular and macrovascular disease.

Methods: The expression of total VEGF, VEGF splice variants ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{165}$, $VEGF_{183}$ and $VEGF_{189}$), VEGFR-1 and VEGFR-2, was investigated in biopsies from the right atrium and left internal mammary artery (LIMA) of 32 non-diabetic and 20 diabetic patients undergoing coronary artery bypass grafting.

Results: Diabetes was independently negatively correlated to total VEGF mRNA expression in atrium. Total VEGF, $VEGF_{121}$ and $VEGF_{165}$ mRNA levels were upregulated in the LIMA of diabetics vs. non-diabetics. The expression of VEGF receptors in atrium and LIMA was similar between these groups. $VEGF_{121}$ and $VEGF_{165}$ were the major variants expressed, followed by $VEGF_{189}$ and $VEGF_{183}$, while $VEGF_{148}$ and $VEGF_{145}$ were detected in small amounts. The expression profile of VEGF splice variants displayed significant heterogeneity between the examined tissues.

Conclusions: This is the first study to quantify VEGF splice variants expression in cardiac and vascular tissue. Our results could help elucidate the role of VEGF splice variants in diabetic complications.

Keywords: VEGF; VEGF splice variants; VEGF receptors; Right atrium; Left internal mammary artery; Diabetes; Real-time RT-PCR

Introduction

Vascular endothelial growth factor (VEGF) is a key regulator of vascular development in physiological and pathological conditions, with crucial roles in developmental blood vessel formation and hypoxia-induced tissue angiogenesis [1]. The versatility of VEGF as a patterning molecule is likely linked to its expression as several isoforms with a different affinity for heparan sulfate proteoglycans in the extracellular matrix, resulting in the different distribution of these isoforms in the environment of VEGF-secreting cells. Furthermore, VEGF associates with various signalling receptor complexes: VEGF binds to receptors VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1), while there are also $VEGF_{165}$ isoform specific receptors, neurophilin-1 and neurophilin-2 [2,3].

Various splice variants resulting from alternative splicing of a single VEGF gene have been reported (subscripts denote the number of amino acids after signal sequence cleavage): $VEGF_{206}$, $VEGF_{189}$, $VEGF_{183}$, $VEGF_{165}$, $VEGF_{148}$, $VEGF_{145}$ and $VEGF_{121}$ [1,4–7]. Most VEGF-producing cells express $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ [1]. $VEGF_{183}$ also has a wide tissue distribution and may have avoided earlier detection due to confusion with $VEGF_{189}$ [8]. $VEGF_{145}$ is one of the main VEGF isoforms expressed by several cell lines derived from carcinomas of the female reproductive system [9] and was also detected in ovarian and breast cancer tissue [10]. $VEGF_{148}$ was identified in human glomeruli [7] and detected in breast and colorectal

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doi:10.1016/j.clinbiochem.2007.09.005

cancer cell lines [11]; however, there is currently no data concerning its biological role. $VEGF_{206}$ is a very rare isoform found mainly in human fetal liver library [12].

Much of the morbidity and mortality associated with diabetes is attributed to a series of microvascular and macrovascular complications [13]. It is now becoming evident that diabetes results in the increased expression of angiogenic growth factors, mainly VEGF, in numerous tissues (retina, glomeruli) as a response to both hyperglycaemia and tissue ischemia, eventually leading to complications such as diabetic retinopathy or diabetic nephropathy [14,15]. On the contrary, diabetes leads to the development of cardiovascular pathologies and in these cases the majority of complications result from a reduction in blood flow to various tissues. The development of collateral vessels can reduce the degree of ischemic damage and it is believed that VEGF may play a significant role in this adaptive process [16,17]. Impairments in VEGF expression and action have been reported in diabetes [18], but whether diabetes-related heart disease is associated with altered VEGF expression is still unclear since data are contradictory [19–24]. Despite the accumulation of data on the expression of VEGF and VEGFRs in a variety of cardiac and vascular beds, little is known about VEGF expression in the left internal mammary artery (LIMA), which is often used as a conduit during coronary artery bypass grafting (CABG) in patients with coronary artery disease. LIMA conduits have shown very high patency rates improving the long-term outcome after CABG [25].

In the present study, we applied our recently developed real-time RT-PCR assay for the quantification of total VEGF, six VEGF splice variants ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{165}$, $VEGF_{183}$ and $VEGF_{189}$), VEGFR-1 and VEGFR-2 [11,26] in biopsies of atrium and LIMA originating from diabetic and non-diabetic patients undergoing CABG. Quantification of the expression of the VEGF system as well as the expression profile of VEGF splice variants in cardiac and vascular tissue could provide valuable information regarding the biological role of VEGF splice variants in diabetic complications.

Methods

Selection of patients

Fifty-two consecutive patients, 20 diabetics and 32 non-diabetics, undergoing CABG for stable angina pectoris, were enrolled in the study. The patients' characteristics are presented in Table 1. Patients were excluded from the study on the basis of: acute myocardial infarction, unstable angina, heart failure, left ventricular ejection fraction <45% (estimated by left ventricular angiography), systolic pulmonary artery pressure >50 mmHg (estimated by echocardiographic studies), plasma creatinine >1.8 mg/dL, additional cardiac disease and severe non-cardiac disease. During the operation, a small part of the LIMA from the distal end and a small part of the right atrium was received from all patients. The specimens were immediately frozen in liquid nitrogen and stored at −80 °C until analyzed. Research was carried out in accordance with the Declaration of Helsinki. The protocol was approved by the Hospital Ethics Committee and informed consent was obtained from all patients.

Table 1
Clinical features of the patients studied

	Diabetics ($n=20$)	Non-diabetics ($n=32$)
Age, years	66 (59, 70)	66 (59, 74)
Sex, M/F	18/2	27/5
Body weight, kg	85 (69, 90)	83 (74, 88)
Left ventricular ejection fraction, %	55 (45, 58)	55 (48, 60)
Blood cholesterol, mg/dL	200 (191, 218)	206 (194, 224)
LDL-cholesterol, mg/dL	129 (118, 153)	139 (122,153)
HDL-cholesterol, mg/dL	39 (35, 42)	38 (34, 41)
Blood glucose before operation, mg/dL	156 (144,173)	105 (99, 111)*
HbA1c, %	7.2 (6.6, 7.9)	5.7 (5.1, 5.9)*
Systemic hypertension	13	21
History of old myocardial infarction	10	15

All continuous variables are summarized as medians and the interquartile range (25th and 75th percentiles). *$p<0.05$ diabetics vs. non-diabetics.

RNA extraction and cDNA synthesis

Total cellular RNA was isolated using the Qiagen RNeasy Mini Reagent Set (Qiagen, Germany) according to the manufacturer's recommendations. The concentration and purity of the RNA were determined by spectrophotometric analysis at 260 and 280 nm and the isolated RNA was stored at −80 °C until analyzed. Reverse transcription of RNA was performed with the SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen, USA) according to the manufacturer's instructions, using 1 μg of total RNA as template.

Real-time RT-PCR

Quantification of total VEGF, VEGF splice variants and VEGF receptors was performed with the LightCycler Instrument (Roche Diagnostics, Germany) as previously described [11,26]. For the normalization of our results, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used. Primers and probe from a recent publication were used [27]; however, the experimental procedure and cycling protocol applied were the same as those used for VEGF quantification [11,26].

Statistics

Data for each continuous variable were examined with the Shapiro–Wilk's W test to determine whether assumptions of normality were valid. Since the data were non-normally distributed, non-parametric tests were used. The expression of the investigated genes in different patient groups was compared using the Mann–Whitney test, whereas the Wilcoxon rank-sum test for matched pairs was used to estimate differences in gene expression levels between pairs of LIMA and atrium. Unadjusted associations between the investigated genes and independent variables were tested using Spearman's rank R. Adjusted associations were tested using multiple linear regression analysis. Variables that reached levels of significance ≤ 0.20 during