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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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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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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

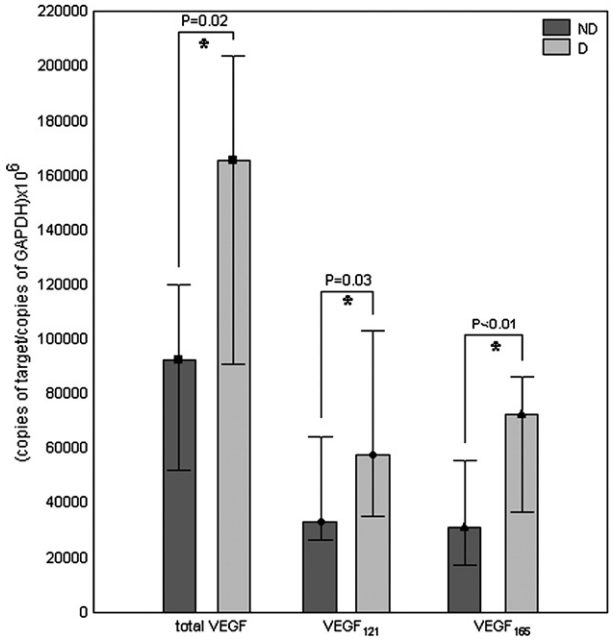

Fig. 1. Total VEGF, $VEGF_{121}$ and $VEGF_{165}$ mRNA levels in the left internal mammary artery of diabetic ($n=20$) and non-diabetic patients ($n=32$). Data are given as medians and the interquartile range (25th and 75th percentiles).

univariate analysis were included in the multivariate analysis. Descriptive data for continuous variables are reported as means ± SD unless stated otherwise. Two-sided p values below 0.05 were considered to be statistically significant. Data were analyzed with the Statistica software (version 7.0, StatSoft Inc., USA).

Results

The expression of total VEGF, VEGF splice variants and VEGFRs was investigated in biopsies of atrial myocardium and LIMA. All specimens investigated expressed total VEGF and both VEGFRs. Among the subtypes of VEGF, $VEGF_{121}$ and $VEGF_{165}$ were dominant in all the specimens investigated, followed by $VEGF_{189}$ and $VEGF_{183}$. $VEGF_{148}$ and $VEGF_{145}$ were detected in small amounts, in 81.3% and 75.0% of the atrial myocardium samples and in 36.7% and 55.1% of the LIMA samples, respectively. The expression levels of all genes investigated were normalized to the GAPDH housekeeping gene and are given as copy number of the gene of interest/copy number of the GAPDH gene $\times 10^6$ (Supplementary Table 1).

Expression of total VEGF, VEGF splice variants and VEGFRs in the right atrium

Atrial mRNA expression of total VEGF and $VEGF_{165}$ tended to be lower in diabetics, even though p values were not statistically significant ($p=0.17$ and 0.15 respectively). Patients with a history of old myocardial infarction (MI) had a tendency to overexpress total VEGF and $VEGF_{121}$ compared to patients without a history of old MI, although without reaching statistical significance ($p=0.12$ and 0.08, respectively). The presence or absence of hypertension did not differentiate mRNA levels of VEGF and VEGF splice variants in atrium. The expression of VEGFRs was similar between diabetic and non-diabetic patients, as well as between patients with or without a history of old MI. Hypertensive patients tended to overexpress VEGFR-2 but this also did not reach statistical significance ($p=0.08$), while no difference was observed for VEGFR-1.

We investigated the possible effect of diabetes, hypertension or the history of old MI on the relative expression of VEGF splice variants, i.e. the ratio of the copy numbers of a specific VEGF splice variant to the sum of all variants. While diabetes or the history of old MI did not change the relative expression of VEGF splice variants in atrium, samples originating from hypertensive patients displayed higher $VEGF_{183}$ expression ratios compared to samples originating from normotensive patients.

Analysis of unadjusted associations showed that total VEGF mRNA expression had a weak positive association with the history of old MI ($R=0.23$, $p=0.12$), a borderline negative association with diabetes ($R=-0.20$, $p=0.17$) and a significant negative association with cholesterol blood levels ($R=-0.41$, $p=0.003$). Strong negative correlations were found between the expression of $VEGF_{121}$ ($R=-0.33$, $p=0.02$), $VEGF_{148}$ ($R=-0.49$, $p=0.005$), $VEGF_{165}$ ($R=-0.45$, $p=0.004$), $VEGF_{183}$ ($R=-0.42$, $p=0.002$) or $VEGF_{189}$ ($R=-0.41$, $p<0.004$) and cholesterol values. We also observed a weak negative correlation between VEGFR-1 mRNA levels and cholesterol values ($R=-0.24$, $p=0.09$) and a weak positive correlation between VEGFR-2 mRNA levels and systemic hypertension ($R=-0.26$, $p=0.08$). Using multivariate analysis we found that, after adjusting for other possible explanatory variables, only the presence of diabetes (regression beta = −0.31, standard error beta = 0.13, $p=0.02$) and cholesterol blood levels (regression beta = −0.40, standard error beta = 0.13, $p=0.004$) were independent predictors of total VEGF mRNA expression, whereas no independent predictors of VEGFR-1 or VEGFR-2 mRNA were found.

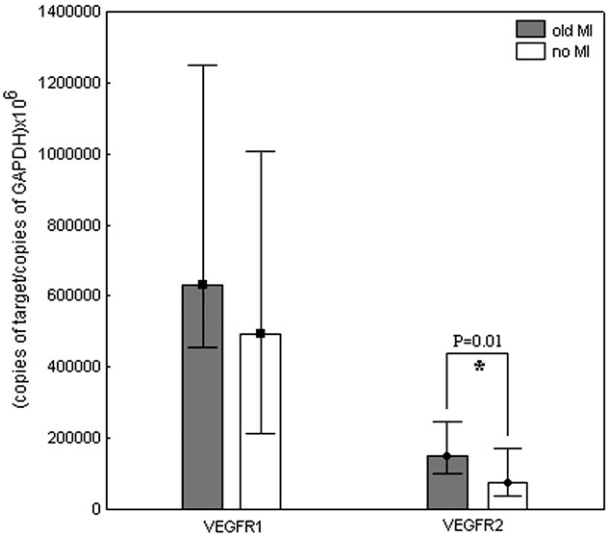

Fig. 2. VEGFR-1 and VEGFR-2 mRNA levels in the left internal mammary artery of patients with ($n=25$) and without ($n=27$) a history of old myocardial infarction (MI). Data are given as medians and the interquartile range (25th and 75th percentiles).

Left mammary artery mRNA expression of VEGF, VEGF splice variants and VEGFRs

Total VEGF, $VEGF_{121}$ and $VEGF_{165}$ expression in LIMA specimens was upregulated in diabetic compared to non-diabetic patients (Fig. 1), while the presence of hypertension or the history of old MI did not change total VEGF and VEGF splice variants expression in LIMA samples. The expression of VEGFR-1 was similar regardless of the presence of diabetes, old MI or hypertension. However, VEGFR-2 copy numbers were higher in patients with a history of MI compared to those without ($p=0.01$) (Fig. 2).

The relative expression of $VEGF_{165}$ as part of the total VEGF message was upregulated in LIMA specimens originating from diabetic patients compared to non-diabetic individuals, whereas the opposite was true for $VEGF_{189}$. Hypertension or the history of old MI did not differentiate VEGF splice variants expression pattern in internal mammary artery.

Analysis of unadjusted associations showed that total VEGF mRNA expression had a positive association with diabetes ($R=0.34$, $p=0.02$) and a significant negative association with cholesterol blood levels ($R=-0.37$, $p=0.009$). Also, strong negative correlations were found between the expression of $VEGF_{121}$ ($R=-0.40$, $p=0.004$) or $VEGF_{165}$ ($R=-0.33$, $p=0.02$) and cholesterol values. We did not find any statistically significant correlations between VEGFRs and independent variables. Using multivariate analysis we found that cholesterol blood levels (Regression beta $=-0.32$, Standard error beta $=0.14$, $p=0.02$) was an independent predictor of total VEGF mRNA expression in LIMA.

Expression of total VEGF, VEGF splice variants and VEGFRs in pairs of right atrium and LIMA

Next, we compared the expression profile of VEGF splice variants in paired samples of right atrium and LIMA. In LIMA specimens, $VEGF_{121}$ constituted on average $46.5\pm11.9\%$ of the total VEGF message, whereas in atrium the percentage was significantly lower, $19.2\pm7.7\%$ ($p<0.01$). The relative expression of $VEGF_{165}$, $VEGF_{183}$ and $VEGF_{189}$ was significantly higher in atrium ($59.1\pm7.7\%$, $3.3\pm1.6\%$ and $18.3\pm5.5\%$, respectively) than in matched LIMA samples ($40.1\pm10.9\%$, $1.6\pm1.1\%$ and $11.5\pm4.4\%$, respectively, $p<0.01$ in all cases) (Fig. 3). The expression profile of VEGF splice variants in tissue pairs did not differ depending on whether they originated from diabetic or non-diabetic individuals.

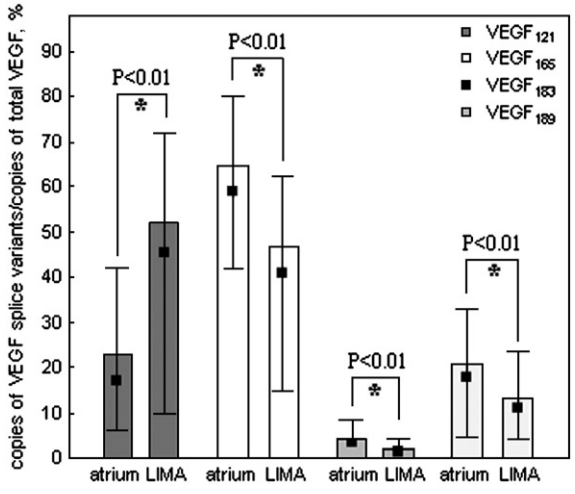

Fig. 3. Expression profile of VEGF splice variants (ratio of each variant to total VEGF) in atrial myocardium and left internal mammary specimens derived from patients undergoing coronary artery bypass grafting. Data are given as medians and the interquartile range (25th and 75th percentiles).

Discussion

Our data provide a comprehensive analysis of the expression of total VEGF, VEGF splice variants and VEGFRs in the atrium and LIMA of diabetic and non-diabetic patients. To our knowledge, this is the first quantitative study of the expression of six VEGF splice variants in cardiac and vascular tissue including the less abundant variants $VEGF_{145}$, $VEGF_{148}$ and $VEGF_{183}$. All VEGF splice variants were expressed in the examined specimens, with $VEGF_{121}$ and $VEGF_{165}$ being the most dominant. Our samples were obtained from a population with multiple cardiovascular risk factors, since it is very difficult to find patients with only one isolated risk factor undergoing bypass surgery. Thus, in order to be able to assess the effect of systemic hypertension, diabetes or history of MI on the expression of VEGF or VEGFRs we carried out a regression analysis.

Diabetes was negatively correlated with myocardial total VEGF expression but the expression of VEGFRs was similar between diabetic and non-diabetic patients. Several studies have examined the expression of VEGF and VEGFRs in the diabetic myocardium; however, their results are contradictory. Some studies report a downregulation of total VEGF, VEGFR-1 and VEGFR-2 mRNA and protein levels in the myocardium of streptozotocin-induced diabetic rats [20,24], which is in agreement to the findings in diabetic ventricular myocardium [20]. However, long-term experimental diabetic rats displayed increased myocardial VEGF mRNA expression and unaffected expression of VEGFR-1 and VEGFR-2 [21]. Similar results were found when examining biopsies of the left ventricle originating from type 2 diabetic and non-diabetic patients with chronic coronary artery disease [22].

The discrepancies between the results of these different studies could be ascribed to: the patient's quality of glucose control, the medications the patients were taking and whether those medications had been stopped before the study, the average duration of diabetes in the different populations studied, inter-individual variability or other unrecognized factors, gender issues, the different sampling sites in the human myocardium (to our knowledge, no studies have addressed the difference in the expression of VEGF or VEGFRs in different sites of the human myocardium, so it is possible that these genes are not homogenously expressed in the adult human heart), and the housekeeping gene used for the normalization of the results. In our study, GAPDH was used as a housekeeping gene because it is reported to be stably expressed in the human myocardium of patients with various cardiac conditions [27].

Diabetic patients displayed significantly higher mRNA levels of total VEGF, $VEGF_{121}$ and $VEGF_{165}$ in LIMA specimens, but no concomitant increase in VEGFR-1 or VEGFR-2 was found. Recently, it was reported that although the expression of VEGFR-1 and VEGFR-2 in LIMA is not significantly different between diabetics and non-diabetics, both gene transcription and protein expression of $VEGF_{165}$ are decreased in the diabetic vessels [28]. The discrepancies between our results and those reported in this study [28] can be attributed to some of the factors listed above as well as to differences between the patients' populations studied.

CABG is among the most common operations performed in the world and the choice of conduit plays a critical role in the prognosis of surgical revascularization. Arterial grafts of diabetic patients appear to maintain their biological integrity even in the presence of poorly compensated glucose metabolism, whereas vein conduits present functional and structural abnormalities, the extent of which is inversely correlated with the efficacy of glucose control [25]. Our finding of enhanced expression of total VEGF in the LIMA of diabetic patients provides a possible explanation for the long lasting and excellent patency rate of arterial grafts. This enhanced expression of total VEGF in the group of diabetic subjects is attributed to the shorter, diffusible variants $VEGF_{121}$ and $VEGF_{165}$ and it is possible that these variants can reach the diabetic myocardium, induce an angiogenic response and compensate for the slightly reduced myocardial VEGF expression observed in this group of patients.

Our results are in keeping with the angiogenic paradox observed in diabetics: In some microvascular tissues pathologic neovascularization and increased vascular permeability, which is attributed to increased growth factor expression, are observed [14,15]. This angiogenic response resulting from impairments in VEGF expression seems to be reduced in patients with diabetes associated macrovascular disease [13,17,19]. Impaired collateral blood vessel formation is one possible explanation for diabetic cardiovascular complications. VEGF seems to play a significant role in this adaptive process [17], but whether diabetes does in fact impair collateral development is under debate [29,30]. However, diabetes was shown to impair angiogenesis in a murine model of unilateral limb ischemia. This impairment was caused by reduced expression of VEGF which was successfully addressed by intramuscular gene transfer [19].

Through alternative splicing of a single VEGF gene various splice variants are produced. VEGF isoforms are not functionally equivalent, since previous studies have shown that the presence of all isoforms is required for normal vascular development [31]. Relative levels of $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ varied among different organs and their expression pattern changed during organ development [32]. Accordingly, we report that the expression profile of VEGF splice variants varied between cardiac and vascular tissue and did not depend on the diabetic status of patients. $VEGF_{165}$ was the major variant expressed in human myocardium followed by $VEGF_{121}$ and $VEGF_{189}$ which were expressed in similar amounts, while in LIMA the major variant was $VEGF_{121}$, followed by $VEGF_{165}$ and $VEGF_{189}$.

In conclusion, a different expression profile of total VEGF, VEGF splice variants and VEGFRs was found in atrium and LIMA of diabetic and non-diabetic patients with coronary artery disease. In atrium, diabetes was negatively correlated to VEGF mRNA expression, whereas in LIMA total VEGF, $VEGF_{121}$ and $VEGF_{165}$ expression were upregulated in diabetic compared to non-diabetic patients. VEGFRs expression was similar between these two groups in both tissues. Significant heterogeneity in the VEGF splice variants expression profile was observed between cardiac and vascular tissue. Our findings could lead to a better understanding of the biological role of the VEGF system in diabetic microvascular and macrovascular complications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clinbiochem.2007.09.005.

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