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Associations of vascular endothelial growth factor (VEGF) with adhesion and inflammation molecules in a healthy population

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ABSTRACT

Vascular endothelial growth factor (VEGF) is implicated in numerous pathologies through complex relationships with cellular adhesion molecules (CAMs) and inflammation markers. These have not been assessed in non-pathological conditions. Our aim was the evaluation of associations between VEGF and CAM/inflammation molecules in a healthy population, and of possible genomic interplays in order to better apprehend the underlying mechanisms leading to the pathology. We examined the associations between VEGF and ICAM-1, VCAM-1, E-, L-, P-selectins, TNF-α, CRP and IL-6 plasma levels in 403 healthy individuals. Gene expression of CAM/inflammation molecules and VEGF isoforms (121, 145, 165, and 189) were quantified in peripheral blood mononuclear cells (PBMCs). The effect of four genetic variants (explaining ~50% of the heritability of circulating VEGF levels) and of their interactions on plasma and mRNA levels of CAM/inflammation molecules was examined. VEGF was associated with ICAM-1 and E-selectin in plasma. In PBMCs, $VEGF_{145}$ mRNA was associated with ICAM-1, L-selectin and TNF-α expression. Interactions of the genetic variants were shown to affect ICAM-1, E-selectin, IL-6 and TNF-α plasma levels, while rs4416670 was associated with L-selectin expression. These findings propose a biological connection between VEGF and CAM/inflammation markers. Common genetic and transcriptional mechanisms may link these molecules and control their effect in healthy conditions.

1. Introduction

Vascular endothelial growth factor (VEGF) is a multifunctional cytokine that shows distinguished functions in angiogenesis, lymphangiogenesis, vascular permeability, and hematopoiesis [1]. VEGF has been linked with a number of vascular pathologies including cardiovascular diseases (ischemic heart disease, heart failure, stroke). VEGF is a highly conserved, disulfide-bonded dimeric glycoprotein of 34–45 kDa and it is produced by several cell types including fibroblasts, neutrophils, endothelial cells and peripheral blood mononuclear cells (PBMCs) and macrophages [2]. Six isoforms of human VEGF, which range from 121 to 206 amino acid residues, have been identified. $VEGF_{145}$, and $VEGF_{206}$ have a key role during the angiogenesis of the human ovarian as well as in bone and wound healing [1,3,4]. $VEGF_{183}$ also has a broad tissue expression and may have not been easily detected earlier due to confusion with $VEGF_{189}$ [5].

Elevated circulating VEGF levels have been also observed in several types of cancer and in various other disorders, for example: ischemic heart disease, diabetes, reproductive, immune-inflammatory disorders [6,7].

The expression of the VEGF isoforms regulate the gene expression of cellular adhesion molecules (CAMs) and inflammation markers. Cellular adhesion molecules (CAMs) are cell membrane receptors that mediate several interactions known to play a key role in a variety of physiological and pathological conditions related to traffic and interactions between cells, cell-matrix contact and in determining the specificity of cell–cell binding [8]. Their participation in the development of the atherogenic plaque is well described [9]. Furthermore, atherosclerosis is characterized by chronic inflammation and inflammation markers have been associated with cardiovascular pathologies [10].

Indeed, complex relationships have been observed between VEGF, CAM and inflammation markers in many diseases and especially in cardiovascular-related phenotypes. For instance, some studies reported the involvement of E-, P- and L-selectin, vascular cell adhesion molecule 1 (VCAM), intercellular adhesion molecule 1 (ICAM-1) and interleukin 6 (IL-6) in angiogenesis [11]. Also, hypoglycemia has been shown to increase circulating levels of

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VEGF, IL-6, E-selectin and ICAM-1 in healthy individuals and in patients with type 1 diabetes [12]. Moreover, it is noteworthy that several reports linked angiogenesis and inflammation, highlighting a key role of TNF-α (tumor necrosis factor-α) and CRP (C-reactive protein) [13–15]. Other important inflammation marker is interleukin 6 (IL-6). This anti- and pro-inflammatory cytokine is a primary determinant of the hepatic production of CRP [16]. Recent studies indicated the simultaneous increasing of IL-6 and VEGF circulating levels in some pathological conditions such as ovarian hyperstimulation syndrome induced ascites, diabetes, visceral obesity, cancer and hypertension [17–21]. Furthermore, IL-6 seems to induce VEGF expression in diabetic nephropathy and rheumatoid arthritis [22,23]. Serum levels of soluble adhesion molecules have also been correlated with serum levels of some acute phase proteins such as CRP in individuals with breast cancer [24]. In addition, in patients with hepatic cancer, the levels of soluble P-selectin have been associated with plasma levels of $VEGF_{165}$ [25].

The assessment of these relationships in different pathologies is progressing; however, due to the complexity of the pathophysiological mechanisms implicated in a disease, the origin of these biological connexions is difficult to be interpreted. Therefore, their investigation in the healthy state, where there is no implication of evident pathology, could offer important information concerning the physiological connexions between these molecules before the development of a specific disease. This could also be the key for the better understanding of the VEGF biological roles. However, to our knowledge, there is no published study of the link between VEGF and both CAM and inflammation markers in a healthy population.

Therefore, the aim of this study was to examine the associations between VEGF and ICAM-1, VCAM-1, E-, L- and P-selectins, TNF-α, IL-6 and CRP in plasma of supposed healthy individuals. Furthermore, we seek to investigate whether there is a relationship in expression profile of these molecules in peripheral blood mononuclear cells (PBMCs). Finally, we thought to assess possible genetic links between these molecules. We have recently identified four single nucleotide polymorphisms (SNPs) (rs6921438, rs4416670, rs6993770 and rs10738760) explaining ~50% of VEGF circulating levels heritability [7]. Thus, we aimed to determine possible associations of these SNPs with plasma levels of CAM/inflammation markers, as well as their effect on gene expression in PBMCs.

2. Materials and methods

2.1. Study population

The STANISLAS Family Study (SFS) is a 10-year longitudinal survey involving 1006 volunteer families from Vandoeuvre-lès-Nancy, France between 1993 and 1995 [26]. Individuals with chronic disorders (cardiovascular diseases or cancer) or having a personal history of cardiovascular disease were not included, as the aim of the study was the assessment of genetic susceptibility factors on the variability of intermediate phenotypes in physiological conditions without the influence of any long term medication and disease. The study protocol was approved by the Local Ethics Committee of Nancy and all subjects gave written informed consent for their participation in the study. Four hundred and three unrelated adults collected during the second examination of the SFS were involved in the present study for which all data were available.

2.2. Laboratory measurements

Blood samples were collected after overnight fasting. Sodium EDTA-plasma was separated by centrifugation at 2000*g* for 15 min at 4 °C and stored at −196 °C in liquid nitrogen until analysis. Circulating plasma levels quantification was performed by Randox Ltd. (Crumlin, UK) using a biochip array analyzer (Evidence®) [27].

Plasma IL-6, TNF-α, ICAM-1, E-, L- and P-selectins were determined by a commercially available enzyme-linked immunosorbent assay (ELISA) (R&D Systems, UK) according to the manufacturer's instruction. Concentrations of plasma CRP were determined using the BN II nephelometer (Germany).

Before the quantification of CAM and inflammation molecules, we tested the effect of the procedures of storage and sample collection on their concentrations. Fresh and frozen state had no significant effect on the concentration of the different molecules except from VCAM-1, which was less stable. Thus it was not included in the analyses of the present study.

2.3. Gene expression analysis

PBMCs were isolated by centrifugation on a density gradient of Ficoll [28] (Ficoll-Paque™ PLUS; Amersham BioSciences). Total RNAs were extracted from PBMCs with a MagNaPure automate, using the MagNA Pure LC RNA HP isolation kit and RNA HP Blood External lysis protocol (Roche Diagnostics, France) in short period of time after cell isolation. Reverse transcription of total RNAs were performed using 200 units of M-MuLV Reverse Transcriptase with 0.25 μg of oligos (dT) (Promega, France) according to a previously described protocol [29]. Quantification of the transcripts coding for the VEGF isoforms (VEGF121 [antisense: 5′-CTCggCTTgTCACATTTTTC-3′ and probe: 5′-TgCAgACCAAAgAAAgATAgCAAgACA], VEGF145 [antisense: 5′-CTTgTCACATACgCTCCAggAC-3′ and probe: 5′AAACgAAAg CgCAAgAAATCCCggTA-3′], VEGF165 [antisense: 5′-GCTTTCTCCgCTC TgAgCA-3′ and probe: 5′-AgCAAgACAAgAAAATCCCTgTgggCC-3′], VEGF189 [antisense: 5′-CCACAgggAACgCTCCAggAC-3′ and probe: 5′-AgCAAgACAAgAAAAAAATCAgTTCgAggAAA-3′]), ICAM-1, E-, L-, P-selectins, TNF-α, IL-6 and the beta 2 microglobulin (β2M) control gene were performed using TaqMan® and LightCycler technologies (LC TaqMan Master Kit, Roche Diagnostics, France). It should be mentioned that a common VEGF forward primer (5′-gAgCTTCCTACAgCA CAACAAA-3′) was designed based on the fact that all VEGF isoforms share exons 1–5. All experiments were performed in duplicate. RT-PCR optimization and specificity of Real Time-PCR products were conducted using SYBR® Green technology (LC FastStart DNA Master^{PLUS} SYBR Green I kit, Roche Diagnostic, France), melting curves analysis and agarose gel electrophoresis of the PCR amplicons, as previously described [29]. $VEGF_{183}$, $VEGF_{206}$, E-selectin and CRP expression in PBMCs were not quantified due to their insufficient expression in this cell type. In total, available mRNA data existed for a subsample of 65 subjects. All mRNA levels were normalized to the mRNA levels of β2M gene. Total VEGF mRNA was calculated as the sum of the ratio of the four isoforms present in PBMCs.

2.4. Genotyping

SNPs rs6921438, rs4416670, rs6993770, and rs10738760 were genotyped by Genoscreen (http://genoscreen.fr), using a Sequenom iPLEX Gold assay–Medium Throughput Genotyping Technology [30]. The significant results of this study were compared to previous findings in the literature (previous GWAS) by imputation analyses using Plink. SNPs with a correlation coefficient ⩾80% were considered in linkage disequilibrium (LD).

2.5. Statistical analysis

Continuous variables are presented as mean value ± standard deviation and categorical variables are given in percentages. Hardy–Weinberg equilibrium was tested using the chi-square test.