

Peripheral monocytes from diabetic patients with coronary artery disease display increased bFGF and VEGF mRNA expression

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Abstract

Background: Macrophages can produce vascular endothelial growth factor (VEGF) in response to hypoxia, transforming growth factor β 1 (TGF- β 1), angiotensin II, basic fibroblast growth factor (bFGF), and interleukin-1. These factors have been found in the serum of coronary artery disease (CAD) patients as well as in atherosclerotic lesions. The aim of the present study was to test the hypothesis that the expression of VEGF, TGF- β 1 and bFGF in peripheral monocytes and lymphocytes is related to CAD.

Methods: Human Mononuclear cells and lymphocytes from peripheral blood were isolated from 53 donors undergoing angiography. Seventeen were found to be healthy and 36 were diagnosed with CAD. The respective mRNAs were extracted and quantified.

Results: The statistical analysis revealed a significant increase of the basal level expression for macrophage VEGF and bFGF in the CAD SA (stable angina) patient group compared to the noCAD (control) ($p = 0.041$ and $p = 0.022$ respectively) and CAD UA (unstable angina) ($p = 0.024$ and $p = 0.005$ respectively) groups, which was highly dependent on the diabetic status of the population. Furthermore, we demonstrated with an in vitro cell culture model that the levels of VEGF and bFGF in monocytes of healthy donors are not affected by short term exposure to increased glucose levels (usually observed in the diabetic patients) and/or statin.

Conclusion: Our findings display a statistically significant association of the increased VEGF and bFGF levels in peripheral monocytes, with stable angina and diabetes in coronary artery disease. The results give new insight to CAD and the impaired collateral vessel formation in diabetics.

Background

Association of cardiovascular disease with several risk factors such as smoking, hypertension, hypercholesterolaemia, diabetes and family history is well established [1].

However, the critical molecular and cellular interactions that lead to the development, growth and rupture of atherosclerotic lesions remain to be identified. Monocyte derived macrophages are believed to play a pivotal role in

the initiation and progression of atheroma formation. The differentiation state of macrophages is directly related to macrophage metabolism of lipoproteins and cholesterol and consequently foam cell formation [2]. Progression of atherosclerosis relates to accumulation of macrophages, alteration of EC (endothelial cell) function, phenotypic modulation of SMCs (smooth muscle cell), and neovascularization of the plaque tissue [3,4]

A secondary to atherosclerosis event, also involving macrophages, is the process of collateral vessel development (arteriogenesis) which appears under hypoxic stress [5]. Arteriogenesis is beneficial to patients with CAD (coronary artery disease) where stenosis or occlusion causes repetitive or chronic regional myocardial ischemia [6]. Following the occlusion of a large artery, the stress of ischemia causes nearby arterioles to become activated. Their endothelial surface upregulates expression of adhesion molecules [7] and triggers the initial monocyte invasion into the arterial wall of the growing collateral arteriole. The proposed model [8] states that the infiltrating monocytes/macrophages produce VEGF (vascular endothelial growth factor), bFGF (basic fibroblast growth factor) and other factors which act on both the endothelium and the smooth muscle cells thereby inducing more VEGF production from these cells. Additional migration of monocytes is facilitated by the secretion of several growth factors including VEGF, bFGF and MCP-1 (monocyte chemoattractant protein 1). Arteriogenesis has been shown to vary between individuals [9] and it was defective in diabetic individuals where the migratory response of monocytes to VEGF was attenuated [10].

Modified low density lipoproteins, proinflammatory cytokines and chemotactic factors are known to affect the differentiation and migration status of macrophages and thus play a role in the macrophage mediated atherogenesis in the vascular wall [3] and collateral vessel formation [8]. Macrophages can produce VEGF in response to hypoxia, TGF- β 1, angiotensin II, bFGF, and interleukin-1 [11,12]. These factors have also been found to be expressed in atherosclerotic lesions.

CAD patients receive standard medication which among other compounds includes statins. HMG-CoA reductase inhibition by statins as established drugs for the treatment of hypercholesterolaemia [13] has been associated with beneficial effects on the progression and regression of atherosclerosis in humans and in animal models [14,15]. Statins have been reported to influence the surface phenotype of peripheral lymphocytes [16].

Atherogenesis is believed to be a system-wide inflammatory response. As such, all peripheral lymphocytes and monocytes could be affected. Although VEGF, bFGF and

TGF- β 1 are involved in the mechanism of atherogenesis and collateral vessel development, a detailed analysis of the steady state mRNA levels of these factors in isolated peripheral monocyte and lymphocyte populations of patients with CAD has not been previously performed. The aim of the present study was to test the hypothesis that the expression levels of VEGF, TGF- β 1 and bFGF in peripheral monocytes and lymphocytes is related to CAD. We also tested the effect of clinical parameters related to CAD (diabetes, type of myocardial ischemic episodes) on the above relationship. The strategy involved exploring the expression levels of VEGF, TGF- β 1 and bFGF in purified peripheral monocytes and lymphocytes from patients with CAD and normal donors. We observed a significant increase of VEGF and bFGF but not TGF- β 1 in CAD patients with stable angina, which is even more pronounced in the diabetics. Furthermore we demonstrated with an *in vitro* cell culture model that the short term effect of glucose and/or statins, which are usually observed in the diabetic CAD patients, did not increase the levels of VEGF and bFGF on monocytes from healthy donors.

Methods

Patients

Patients were recruited randomly from those undergoing diagnostic catheterization at the University Hospital (Heraklion, Crete, Greece) over a 4 month period. A total of 53 patients were included in the study. A group of 36 patients, that were submitted to angiography due to angina pectoris, showed ≥ 1 vessel coronary stenosis of $\geq 70\%$ by visual analysis and thus were considered as CAD group. A total of 17 individuals, that were submitted to angiography due to valvular disease or nontypical chest pain syndrome with a negative or non diagnostic exercise ECG or asymptomatic episodes of unsustained ventricular tachycardia, were found to have no stenosis and were considered to be the control group (noCAD). Due to poor RNA yields we excluded from the analysis 4 macrophage (1 CAD UA, 2 noCAD and 1 CAD SA) and 12 lymphocyte samples (5 CAD UA, 3 noCAD and 4 CAD SA).

The patients, and controls had no evidence of peripheral artery or cerebrovascular disease; all had normal echoduplex of cervical arteries, the aorta and lower limb arteries and/or resting and post-exercise ankle/ brachial pressure index > 0.85 . Criteria for exclusion were 1) age < 18.2) clinical or laboratory signs of acute or chronic inflammatory disease and 3) presence of overt neoplastic disease.

The definitions of the clinical parameters used in the study are presented below. **A.** Hypertension: systolic blood pressure ≥ 160 mm Hg and or diastolic > 90 mm Hg, **B.** Hypercholesterolaemia: LDL ≥ 160 mg/dl for patient without any artery risk factors for coronary artery

disease or LDL \geq 130 mg/dl for patient with one known risk factor for coronary artery disease or LDL \geq 100 mg/dl for patient with known coronary artery disease, C. Diabetes: Two separate values of fasting blood glucose \geq 126 mg/dl or two random values of blood glucose \geq 200 mg/dl. D. Levels of acid uric \geq 7 mg/dl. E. Unstable angina: crescendo angina of superimposed on a preexisting pattern of relatively stable exertion-related angina pectoris or angina pectoris at rest as well as with minimal exertion or angina pectoris of new onset which is brought on by minimal exertion. F. Stable angina: stable angina on effort without any clinical changes within two months. The present study was approved by the institutional ethics committee and the patients gave written informed consent to participate in the study. The investigation conforms to the principles outlined in the Declaration of Helsinki.

Blood collection, lymphocyte separation and cell culture

Twenty millilitres of blood was collected from the femoral venous catheter placed for the catheterization before angiography was begun. The blood was immediately placed in four 5-ml polypropylene heparinized tubes and kept on ice until it was used for monocyte isolation. In all instances, the blood was used within 1 hour of removal from the patients.

The 20 ml of heparinized blood was gently layered over 30 ml of Histopaque-1077 (Sigma) in a 50-ml polypropylene centrifuge tube. Tubes were centrifuged at 1800 rpm for 30 minutes at room temperature. The middle phase (buffy coat) containing the monocytes was isolated and placed in a fresh 50-ml polypropylene centrifuge tube. The cell yield was on average 10^6 cells/ml of blood. The isolated mononuclear cells were washed twice with sterile phosphate buffered saline (PBS). The cell pellets were resuspended in RPMI1640 medium (Sigma). The cells were plated on 25 cm² tissue culture flasks (Costar) and incubated in a 5% CO₂ incubator (Forma) at 37°C for 1 hour to allow for monocyte attachment. The non adherent cells were removed by washing twice with PBS. Purity assessment of the monocyte preparation was performed with anti CD14 FACS analysis (FACSCalibur, Beckton Dickinson) and showed consistently more than 95% purity. The lymphocyte fraction mentioned in the study represented the non adherent fraction of the ficoll purified peripheral leucocyte preparation. The lymphocyte fraction contained a very low level of granulocytes (<1%) and macrophages (<1%). The viability of our populations after the purification procedure was more than 99% as determined by trypan blue exclusion.

Analysis of the effect of glucose and Fluvastatin on the kinetics of mRNA expression was performed as previously described [17]. Due to the need of a large cell number for

the kinetic analysis we utilized buffy coats from healthy donors. Briefly Ficoll purified cells were incubated in 6-well plates (Costar) at a concentration of 2×10^6 cells/ml, in 2 ml RPMI1640 medium, for 1 hour, in a CO₂ incubator. Non adherent cells were removed by washing twice with PBS and new RPMI1640 medium was added containing 5% human serum. Fluvastatin (1 μ M) or glucose (6 mg/ml) was added according to the study design. At an appropriate time period (30 min, 1 h, 2 h, 4 h, 24 h, 48 h) cells were harvested and mRNA was extracted and purified.

Extraction and quantification of mRNA

Total RNA was isolated directly from the tissue culture dishes containing the adherent monocytes using the Trizol reagent (Life Technologies Ltd., U.K.). Briefly, 1 ml of reagent was added to each dish with vigorous pipetting and transferred to a 1.5 ml Eppendorf tube. Chloroform (200 μ l) was added, and the tube was vortexed and centrifuged at 14 000 rpm for 15 minutes. The RNA was precipitated with an equal volume of isopropanol and washed with 75% ethanol. The RNA was air-dried and resuspended in water treated with diethyl pyrocarbonate. The RNA preparation was treated with DNase I to remove residual traces of DNA, purified with the phenol-Chloroform method and precipitated with ethanol. The non-adherent/lymph fraction was harvested, centrifuged and RNA extraction was performed with the Trizol reagent according to the manufacturer's instructions. RNA concentration and purity was determined on a UV spectrophotometer (Hitachi Instruments Inc., U.S.A.) by the 260 nm absorbance and 260 nm to 280 nm absorbance ratio respectively. 1% agarose gel electrophoresis and ethidium bromide staining were used to examine RNA integrity.

Semi-quantitative RT-PCR

Each quantification set included two PCR reactions (the target and the β 2-microglobulin (β 2M) reference). Each PCR reaction (target and β 2M) was optimized individually for primer, Mg and Taq polymerase concentration using as a template a representative pool of all samples to be measured. Then the reactions were combined into a single tube in order to eliminate tube to tube variations. A new optimization was performed to ensure that there was no cross inhibition between the two PCR reactions. Subsequently another optimization was performed modulating the relative concentration of the two sets of primers to ensure that the two reactions reached the logarithmic phase of expansion in the same PCR cycles (reaction synchronization). Finally we determined the cycle in which the reaction reached the middle of the logarithmic expansion phase. The set of conditions that were established regarding primer, Mg, Taq polymerase concentration and cycle number, was applied specifically to the set of samples that were used for the standardization (sample pool)

and the corresponding target. The total standardization procedure was repeated for each quantification reaction (bFGF, VEGF, TGF- β 1).

Reverse transcription reactions for the preparation of first strand cDNA from 1 μ g of total RNA, were performed for 1 h at 52°C, using 15 U Thermoscript reverse transcriptase, 40 U RNaseOut, 50 ng of random hexamers and 1.0 mM of each dNTP in a total volume of 20 μ l of 1x First Strand cDNA Synthesis Buffer containing 5 mM dithiothreitol (DTT), ensued by incubation for 20 min at 37°C with 2 U of E. coli RNaseH to avoid RNA contamination of cDNA, according to the manufacturer's protocol (Life Technologies Ltd., U.K.).

PCR assays were carried out in a PTC-200 programmable thermal controller (MJ Research Inc., U.S.A.); 1 μ l of cDNA was amplified in a total volume of 10 μ l containing. The general PCR protocol included 1x PCR reaction buffer, 2.5 mM MgCl₂, 0.4 mM dNTPs, and 0.6 U Platinum Taq DNA polymerase (Life Technologies Ltd., U.K.), with 30 pmol of each primer set. Cycling parameters were as follows: 3 min for initial denaturation at 94°C; 30 sec at 94°C, 30 sec at 58°C for primer annealing, 40 sec at 72°C for primer extension, these steps were repeated for 35 cycles; final extension step at 72°C for 10 min. β 2-microglobulin was used as an internal control in all PCR reactions.

PCR products were analysed by 8% polyacrylamide gel electrophoresis (29:1 ratio acrylamide/bis-acrylamide) and silver stained. Gels were scanned on an Agfa Snap-Scan 1212 u (Agfa-Gevaert N.V., Belgium). The integrated density of the bands was used as quantitative parameter and was calculated by digital image analysis (Scion image). The ratio of the integrated density of each gene divided by that of β 2-microglobulin was used to quantify the results.

The oligonucleotide primers used in the study were: β 2-microglobulin;Forward (F):
TCCAACATCAACATCTTGGT,

β 2-microglobulin;Reverse (R):
TCCCCAAATTCTAAGCAGA,

TGF- β 1;F: ATGAACTCATTTCAGTCACCATAGC,

TGF- β 1;R: CTATCCCCACTAAAGCAGG,

VEGF;F: ACGATCGATACAGAAACCACG,

VEGF;R: CTCTGCGCAGAGTCTCCTCT,

bFGF;F: GCCACATCTAATCTCATTTCACA,

bFGF;R: CTGGGTAACAGCAGATGCAA.

Statistical analysis

Data are reported as mean \pm SEM. Initial statistical comparison of the mRNA expression results between the CAD and the noCAD (control) groups was performed utilizing the Mann Whitney test [18]. Subsequent analysis between noCAD, CAD SA (stable angina) and CAD UA (unstable angina) groups for statistically significant differences in enumerative data was performed with the use of the X^2 test. Analysis between groups for continuous variables such as age, number of diseased vessels and % of lymphocytes was performed with one-way ANOVA. The expression of VEGF, bFGF and TGF- β 1 mRNA was compared between the groups noCAD, CAD SA (stable angina) and CAD UA (unstable angina) by ANCOVA with age, number of diseased vessels, family history of heart disease, diabetes, smoking, hypertension, and hypercholesterolaemia as covariates. Bonferroni post hoc comparisons were performed to compare the adjusted levels of VEGF, bFGF and TGF- β 1 between the 3 groups.

Results

Quantitation of the mRNA for VEGF, bFGF and TGF- β 1 in peripheral monocytes and lymphocytes

A representative semi-quantitative RT-PCR assay demonstrating a range of differences in the production of VEGF, bFGF and TGF- β 1 mRNA in 5 different donors is shown in Figure 1. The quantification and statistical evaluation of the mRNA expression results (Mann Whitney test) for the CAD and the noCAD (control) groups showed no significant difference for any of the above factors. Stepwise regression analysis of the expression of VEGF with CAD-related clinical parameters (angina, age, number of diseased vessels, family history of heart disease, diabetes, smoking, hypertension, and hypercholesterolaemia) revealed a highly significant association to the angina parameter ($R = 0.5$, $p = 0.003$).

In order to investigate further the relation of the mRNA expression of VEGF, bFGF and TGF- β 1 with the angina parameter we divided our patients in the following groups: noCAD(control), CAD SA (CAD with stable angina), CAD UA (CAD with unstable angina) and proceeded with the statistical evaluation alone or in cooperation with other clinical parameters. The profile of the donors in the noCAD, CAD SA and CAD UA groups with regard to sex, levels of uric acid, hypertension, cigarette smoking, diabetes, family history, hypercholesterolaemia and number of diseased vessels is given in Table 1. Lymphocyte counts for the donors are shown in Table 2. There was no statistically significant difference between the 3 patient groups in any of these variables.

Table 1: The characteristics of patients

Covariate	Control	CAD SA ¹	CAD UA ²	X ²	F ³	p
n	17	19	17			
Male sex, %	59	89.5	70.6	4.45		0.100
Uric acid, % (7 mg/dl)	11.7	26.3	11.7	1.83		0.400
Hypertension, % (S.B.P. ⁴ ≥ 160 mm Mg, D.B.P. ⁵ > 90 mm Mg)	53	79	53	2.95		0.229
Cigarette smoking, %	41	53	53	1.09		0.578
Diabetes, % (≥ 126 mg/dl ⁶ ≥ 200 mg/dl ⁷)	23.5	52.6	41	3.2		0.201
Family history, %	35	37	53	1.36		0.507
Hypercholesterolaemia, % (LDL ≥ 160 mg/dl ⁸ . LDL ≥ 130 mg/dl ⁹ . LDL ≥ 100 mg/dl ¹⁰)	47	68	76	3.43		0.179
No. of diseased vessels (mean)	0	2.1 ± 0.87	1.9 ± 0.82	0.332	0.568	

¹:Coronary Artery disease, Stable Angina. ²:Coronary Artery disease, Unstable Angina. ³:ANOVA analysis of the number of diseased vessels from CAD SA and CAD UA patient groups. ⁴:Systolic Blood Pressure. ⁵:Diastolic Blood Pressure. ⁶:two separate values of fasting blood glucose. ⁷:two random values of blood glucose. ⁸:for patient without any artery risk factors for Coronary Artery Disease. ⁹:for patient with one known risk factor for Coronary Artery Disease. ¹⁰:for patient with known Coronary Artery Disease.

Table 2: Haematological parameters

	Control	CAD SA ¹	CAD UA ²	F ³	P
n	10	9	12		
Neutrophiles (mean)	0,617	0,689	0,559	1,066	0,354
Lymphocytes (mean)	0,293	0,226	0,334	0,845	0,436
Monocytes (mean)	0,053	0,062	0,078	0,608	0,551

¹ Coronary artery disease Stable Angina. ² Coronary artery disease Unstable Angina. ³ Analysis ANOVA

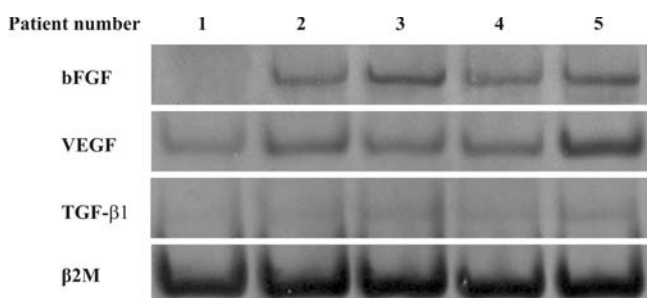


Figure 1
Acrylamide gel electrophoresis of RT-PCR products. RT-PCR products from 5 random patients demonstrating the interindividual differences in mRNA expression of VEGF, bFGF, and TGF-β1 in peripheral monocytes/macrophages. The RT-PCR and the mRNA semi-quantification for each factor were performed as described in the Methods section.

The statistical analysis of the mRNA quantification results revealed a significant difference in the basal level expression between the three patient groups for macrophage VEGF and bFGF. No statistically significant differences were detected for macrophage TGF-β1 and lymphocyte VEGF, bFGF and TGF-β1.

A summary of the results is shown in Table 3. In peripheral macrophages for donors with no coronary arterial disease (noCAD) the mean VEGF/β2M ratio was 32.13±8.38, for CAD patients with stable angina the mean VEGF/β2M ratio was 152.07±50.63 and for CAD patients with unstable angina the mean VEGF/β2M ratio was 23.86±6.7. The respective mean values of bFGF/β2M ratio in the above patient groups were: noCAD 16.73±8, CAD SA 85.98±25.26 and CAD UA 5.3±1.63. In the lymphocyte cell fraction the respective mean values of VEGF/β2M ratio in the above patient groups were: noCAD 4.31±3.72, CAD SA 22.66±14.16 and CAD UA 1.62±1.62.

Table 3: Summary of the results on VEGF and bFGF expression in monocytes (Mf) and lymphocytes (Ly)

	No CAD	CADSA	CADUA
VEGF/ β 2M Mf	32.13 \pm 8.38	152.07 \pm 50.63	23.86 \pm 6.7
bFGF/ β 2M Mf	16.73 \pm 8	85.98 \pm 25.26	5.3 \pm 1.63
VEGF/ β 2M Ly	4.31 \pm 3.72	22.66 \pm 14.16	1.62 \pm 1.62

Data are presented as mean \pm SEM (standard error of the mean). The values represent the VEGF/ β 2-microglobulin and bFGF/ β 2-microglobulin ratios which were calculated by the optical integrated density of each gene divided by that β 2-microglobulin.

The effect of glucose and statins in the expression of VEGF and bFGF

We selected 2 independent healthy male donors, age of 27 and 23 years, with no prior history of atherosclerosis or diabetes. These samples were processed in an identical fashion to those from patients from the catheterization laboratory. The intra assay variability of the mRNA measurements of our *in vitro* short term culture system was assessed by measuring the VEGF and bFGF mRNA in triplicate cultures each. For donor 1 the mean VEGF/ β 2M ratio was 97.5 \pm 1.53 and bFGF/ β 2M ratio 54.62 \pm 5.19. For donor 2 the mean VEGF/ β 2M ratio was 45.47 \pm 4.55 and bFGF/ β 2M ratio 18.87 \pm 2.37.

Furthermore we analyzed in the above model the effect of high glucose levels found in diabetic individuals and statins found in the medication of all our patients on the mRNA levels of VEGF and bFGF during a 48 hour period. We isolated a blood sample from healthy donor 1 in an identical fashion to those from patients from the catheterization laboratory and set cultures with appropriate concentrations of glucose, statin and glucose+statin. After the 1 hour attachment period the cultures were harvested at specific time intervals (30 min, 1 h, 2, 4, 24 h, 48 h) and the mRNA of VEGF and bFGF was quantified. The results show a gradual time dependent decrease of both mRNA levels (Figure 2) which was not affected by glucose or statin. The experiment was repeated with a different donor and the results were similar. Although the initial values of bFGF and VEGF differed slightly, consistent with the donor variation, the trend for decline of expression was the same. These results should be evaluated in the context of our *in vitro* system since *in vivo* gene expression is not always the same.

Statistical evaluation of the results between different patient groups

The mRNA quantification revealed a significant difference in the basal level expression between the three groups for macrophage VEGF and bFGF (Figure 3A,3C). No statistically significant differences were detected for macrophage TGF- β 1 and lymphocyte VEGF, bFGF and TGF- β 1.

The difference in the macrophage VEGF expression between noCAD control patients and CAD SA patients was statistically significant ($p = 0.041$), as was the difference between patients with CAD SA and CAD UA ($p = 0.024$) (Figure 3A). There was no statistical difference between the macrophage VEGF expression of noCAD and CAD UA patients ($p = 0.445$) (Figure 3A). Similarly, for macrophage bFGF, the difference in expression between patients with CAD SA and CAD UA was highly statistically significant ($p = 0.005$) as was the difference between patients with CAD SA and noCAD ($p = 0.022$) (Figure 3C). There was no statistical difference between noCAD and CAD UA patients ($p = 0.160$) (Figure 3C). No statistically significant differences were detected for macrophage TGF- β 1 and lymphocyte VEGF (Figure 3E), bFGF and TGF- β 1 in any of the patient groups.

The mRNA expression data from the 3 groups were subjected to ANCOVA using the variables age, sex, prior myocardial infarction, hypertension, family history, hypercholesterolaemia, cigarette smoking, diabetes, and number of diseased vessels as covariates. The simple analysis of variance gave a significant difference between the three groups for macrophage VEGF ($p = 0.011$) (Figure 3B) and macrophage bFGF ($p = 0.002$) (Figure 3D). Diabetes was the only covariate found to influence greatly the variance improving considerably the significance of our model both for VEGF ($p = 0.001$) (Figure 3B) and bFGF ($p = 0.000$) (Figure 3D). Bonferroni post hoc comparison between the groups noCAD and CAD SA and between the groups CAD SA and CAD UA revealed a statistically significant difference in VEGF expression ($p = 0.021$ and $p = 0.011$ respectively). No significant difference was found between the groups with noCAD and CAD UA. Bonferroni post hoc comparison in bFGF expression between the groups CAD SA – CAD UA and noCAD – CAD SA revealed a statistically significant difference ($p = 0.003$) and ($p = 0.016$) respectively. No significant difference of bFGF was found between the groups noCAD – CAD UA ($p = 0.843$). Pearson two tailed correlation of VEGF and bFGF expression was found to be highly significant ($p < 0.001$).

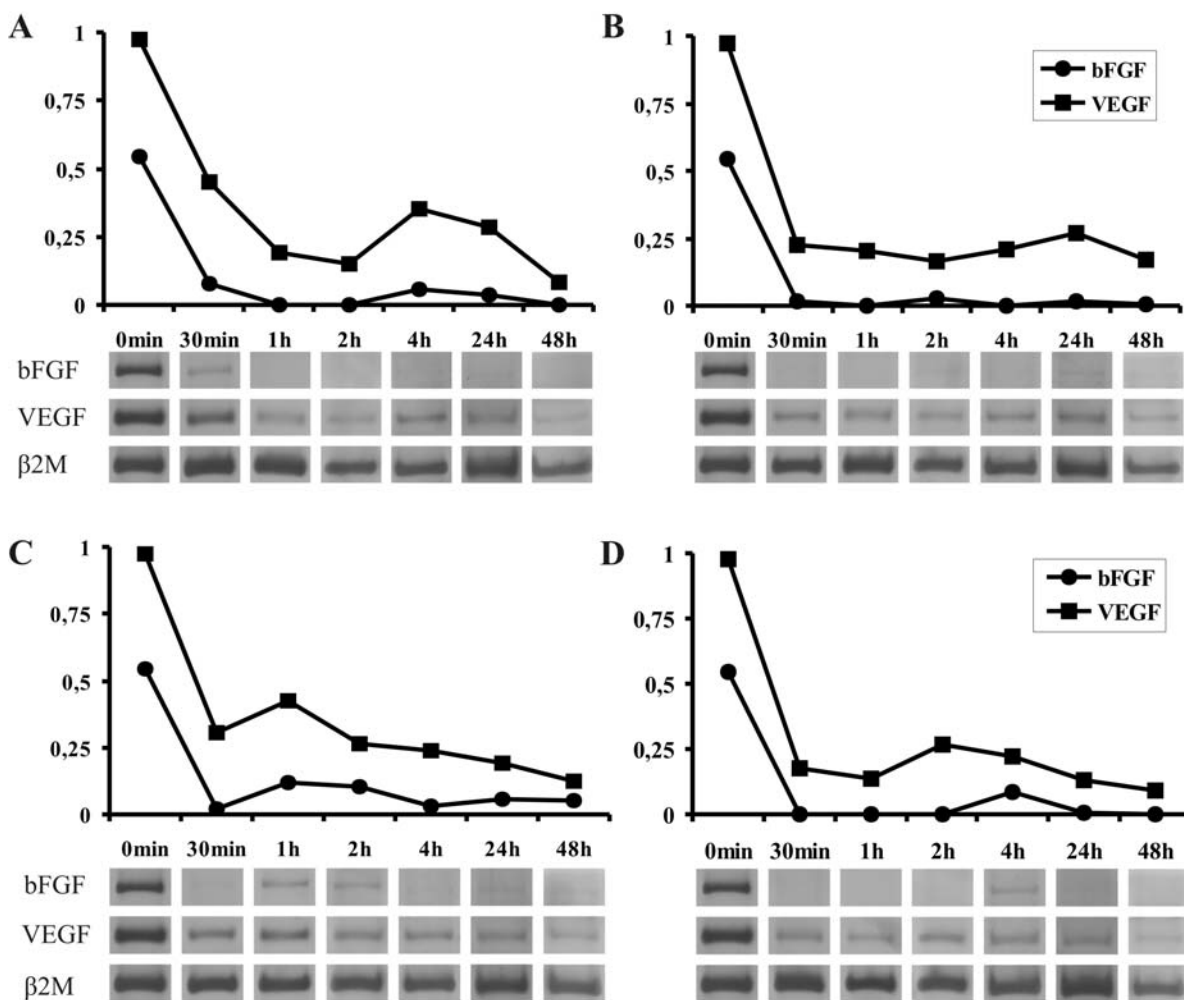


Figure 2
Analysis of the effect of glucose and fluvastatin on the kinetics of mRNA expression of VEGF (---) and bFGF (---) in normal monocytes/macrophages during a 48 hour in vitro treatment period. A, no treatment. B, treatment with glucose (6 mg/ml). C, treatment with fluvastatin (1 μM). D, treatment with glucose and Fluvastatin. The y-axis represents the VEGF/β2-microglobulin and bFGF/β2-microglobulin ratio of the integrated density respectively.

Discussion

The present study provides evidence on the relationship of the expression of VEGF, bFGF and TGF-β1 in the peripheral monocyte and lymphocyte cell populations from patients with coronary artery disease. The mRNA quantification revealed a significant increase of the basal level expression for macrophage VEGF and bFGF in the CAD SA patient group compared to the noCAD and CAD UA groups, which was highly dependent on the diabetic status of the population. Our findings contribute to the understanding of the role of VEGF, bFGF and TGF-β1 in cardiovascular disease.

A recent study on the hypoxic induction of VEGF reported a low level of VEGF expression from monocytes of atherosclerotic donors after 24 hours of culture [9]. Aiming to follow the kinetics of the mRNA expression of VEGF and bFGF during a 48 hour culture period we set up a control experiment with monocytes isolated from a healthy donor. We observed that the expression of both genes declined in a time dependent fashion reaching the lowest levels at 24 to 48 hours. The result explained the consistently low values reported previously [9]. Our measurements of the mRNA profile were performed immediately after cell isolation in order to ensure the representative mirroring of the *in vivo* condition. All samples were

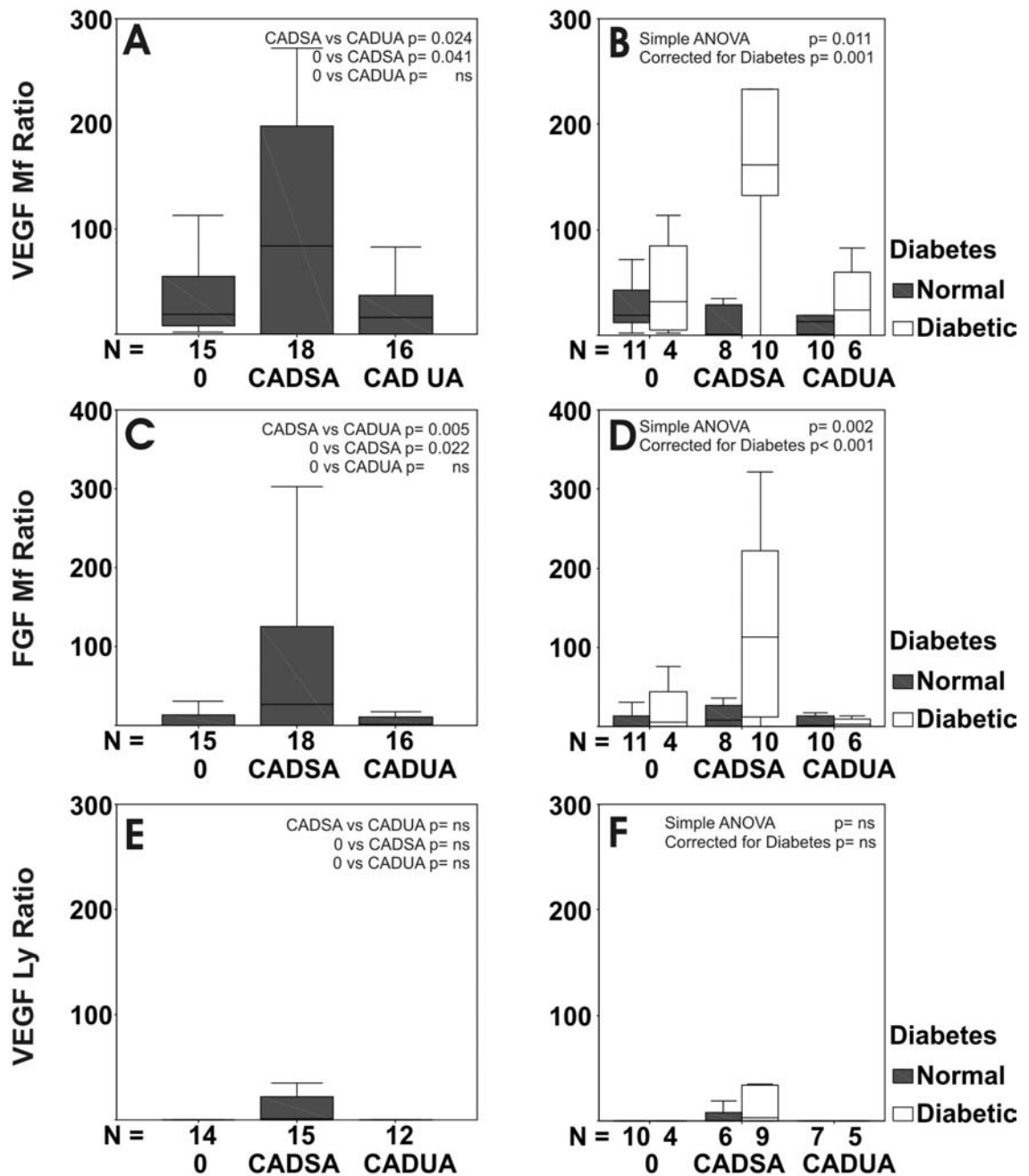


Figure 3

Boxplots of the expression of VEGF and bFGF. Distribution boxplot of the expression of VEGF in monocytes/macrophages (A, B), bFGF in monocytes /macrophages (C, D) and VEGF in lymphocytes (E, F). The boxplots A, C and E demonstrate the expression in the control, CAD SA and CAD UA group. The boxplots B, D and F demonstrate the expression in the above groups classified for diabetes. The y-axis values represent the VEGF/ β 2-microglobulin and bFGF/ β 2-microglobulin ratios accordingly. The ratios were calculated by the optical integrated density of each gene divided by that of β 2-microglobulin. The optical integrated density was calculated by digital image analysis (Scion image) of acrylamide gel electrophoresis of the RT-PCR products. **Mf:** macrophages, **Ly:** non-adherent lymphocyte fraction, **ns:** non-significant.

treated exactly the same way to ensure that the effect of cell handling and isolation, if any, was uniform.

All the patients in our study received standard medication which among other compounds included statins. Statins have been reported to influence the surface phenotype of peripheral lymphocytes [16]. To determine the effect of fluvastatin on the mRNA expression of bFGF and VEGF in monocytes of a healthy donor we performed kinetic analysis. We observed no significant effect on VEGF or bFGF during the 48 hour *in vitro* fluvastatin treatment. Glucose has been reported to be able to influence macrophage differentiation state in general and specifically CD36 through direct effect on the mRNA level [19]. Since our major finding is related to diabetic individuals usually exhibiting high glucose levels, we evaluated the short term effect of glucose on monocytes of a healthy donor similarly to the above kinetic analysis. We observed no significant deviation from the untreated mRNA levels for VEGF and bFGF during a 48 hour period after treatment with glucose alone or in combination with fluvastatin.

VEGF and bFGF have been isolated in human cardiac tissue where increased levels were observed among patients with acute myocardial infarction [20] and unstable angina pectoris [21]. Presence of VEGF and bFGF has been demonstrated in atherosclerotic lesions produced mainly by macrophages [22,23]. Furthermore it was reported that VEGF can induce migration and activation of monocytes through its receptor flt-1 [24], up regulation of adhesion molecules on endothelial cells [25] and secretion of monocyte chemoattractant protein 1 (MCP-1) [26].

Detection of VEGF in the serum in atherosclerotic patients requires the existence of an appropriate source producing it in high amount. Our results demonstrate that in CAD the VEGF, bFGF and TGF- β 1 are not systematically produced by peripheral lymphocytes or monocytes. Expression of the VEGF and bFGF gene was restricted to the monocyte population in the CAD patient group with stable angina. Corroborating with our results a previous study demonstrated that patients with stable angina pectoris had higher amounts of serum VEGF compared to old myocardial infarction and control patients [27]. In patients suffering from stable effort induced angina pectoris, repeated myocardial ischemic stimuli can be expected to continuously sustain collateral growth and remodeling and display increased amounts of activated macrophages in the periphery primed to produce angiogenic factors. The high level of serum VEGF and bFGF reported in other stages of atherosclerosis should be accredited to other potential sites of production like the atherosclerotic lesion itself. The serum VEGF concentrations in the early stage of myocardial infarction was reported to be high reaching maximum levels between

day 7–14 post MI. [28,29]. Elevated VEGF production in the serum has also been detected in diabetic retinopathy [30], hyperlipidemia [31] and hypertension [32] asymptomatic for atherosclerosis. Diabetes is a negative prognostic indicator for patients with coronary arterial disease. Our study revealed a striking restriction of VEGF and bFGF mRNA expression in the diabetic sub-group of the atherosclerosis patient group with stable angina pectoris while the non diabetic sub-group displayed levels similar to the normal group. In agreement with our observations a recent study reported that diabetic individuals with atherosclerosis display relatively increased levels of serum VEGF [33] but they did not test stable angina pectoris as a covariate. A factor related to the diabetic condition (unbalanced glucose levels, defective insulin regulation, etc) in cooperation with the myocardial ischemia could be responsible for the high levels of VEGF and bFGF observed in the diabetic patients (CAD SA). In agreement a recent study demonstrated *in vivo* a direct effect of the insulin induced modulation of the level of glucose on the serum levels of VEGF [34]. The fact that the CAD UA groups showed low levels of expression is interesting and could be attributed to the migration of monocyte producers from the periphery to the sites of ongoing tissue damage. The numerous biochemical and metabolic pathways postulated to have a causal role in the pathogenesis of diabetic vascular disease can be summarized to the following hypothesis: either the effect of increased reductive or oxidative stress to the cell, or activation of numerous protein kinase pathways, particularly protein kinase C and mitogen-activated protein kinases, induces growth factor expression among which the most important is VEGF. Our contribution to the proposed model is that the induction of VEGF in the periphery could require preconditioning of the monocytes which is performed during the chronic myocardial ischemic condition observed in the CAD SA group.

Collateral vessel formation termed arteriogenesis usually follows severe atherosclerosis. Arteriogenesis is severely reduced in patients with diabetes mellitus [35]. Recent studies showed that arteriogenesis requires active migration of peripheral monocytes to the site of ischemia and interaction with epithelium of the arteriole [8]. In addition it is known that the migration of monocytes is mediated through the action of VEGF and its receptor Flt-1 [36,37]. Trying to identify the molecular abnormality Waltenberger et al. demonstrated that in diabetics monocytes have a functional defect: they do not respond to migratory signals from VEGF although their flt-1 receptor level is normal and the intracellular pathway is functional [10]. Our results demonstrate that monocytes from diabetic CAD SA patients express very high amounts of VEGF mRNA. We propose that the migratory defect could be caused by the increased autocrine VEGF production of the

peripheral monocytes. In fact Waltenberger et al [10] included a control experiment demonstrating that monocytes migrate only when they encounter a gradient of VEGF concentration. Although additional experiments are necessary to evaluate the monocyte migratory responses in our patients, it is tempting to speculate that in diabetic patients of the CAD SA group, the VEGF gradient is disturbed causing defective collateral vessel formation. A direct consequence of this model is that any thought of intravenous administration of VEGF for a therapeutic intervention should be carefully examined since infusion of VEGF could derange monocyte migratory responses and lead to impaired collateral vessel development.

Conclusions

We observed a significant increase of the basal level expression for macrophage VEGF and bFGF in the CAD SA (stable angina) patient group compared to the noCAD (control) and CAD UA (unstable angina) groups. The increase was highly dependent on the diabetic status of the population. Our findings contribute to the understanding of the increased risk of atherosclerosis as well as to the impaired collateral vessel formation which is well documented for the diabetic CAD patients.

Competing interests

None declared.

Authors' contributions

DP carried out the experimental work, drafted the paper, evaluated the results, AZ conceived the study, participated in its design, evaluated the results, performed the statistical analysis, drafted the paper, EK performed the cell culture experiments, GEK evaluated the clinical aspects of the study, NEI helped in the gathering of clinical samples and data, DAS conceived the study, and participated in its design and coordination.

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