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Original Article

HYPERHOMOCYSTEINEMIA MODULATES VEGF PLASMA LEVELS AND ITS EXPRESSION IN LEUCOCYTES IN HEALTHY SUBJECTS AND IN PATIENTS WITH PERIPHERAL ARTERIAL DISEASE AND DIABETES MELLITUS

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Aim: *Both homocysteine and vascular endothelial growth factor (VEGF) are implicated in development and progression of atherothrombotic vascular disease. We sought to determine whether there is a relationship between homocysteine and VEGF in healthy individuals and in patients with peripheral arterial disease (PAD) or diabetes mellitus (DM).*
Methods: *Measurements of plasma homocysteine and VEGF and expression of VEGF in leucocytes were performed before and after intervention. Twelve healthy individuals were evaluated before and 4 h after methionine loading, whereas 10 patients with PAD and 15 patients with DM were evaluated before and 6 weeks after oral administration of B vitamins and folate.*
Results: *Basal homocysteine was elevated in patients with PAD and DM (21.5±.9 and 19.9±1 µmol/l, respectively). Methionine resulted in significant elevation, while B vitamins resulted in significant reduction of homocysteine and VEGF and there was a significant correlation between homocysteine and VEGF changes ($r = 0.73$, $p < 0.01$). Moreover, VEGF mRNA expression in leucocytes was upregulated after methionine loading and was downregulated after B vitamins and folate treatment.*
Conclusion: *These findings demonstrate that B vitamins and folate can successfully lower plasma homocysteine and VEGF expression in leucocytes in patients with PAD and DM.*

Keywords: *Peripheral arterial disease, diabetes mellitus, homocysteine, vascular endothelial growth factor.*

INTRODUCTION

Elevated plasma homocysteine has been implicated in the development and progression of atherothrombotic vascular diseases, including peripheral arterial disease (PAD), coronary heart disease, cerebrovascular disease, and venous thromboembolism.[1-6] Pathophysiological explanations for the adverse effects of homocysteine included impairment of the functions of endothelial and vascular smooth muscle cells and extracellular matrix as well as increased coagulation and impaired fibrinolysis.[2]

Homocysteine is a thiol-containing aminoacid that results from demethylation of methionine and is oxidized in plasma to the disulfide homocytine and to the mixed sulfide homocysteine-cysteine. These three compounds occur naturally in plasma in both free and protein bound forms. Normal levels of fasting plasma homocysteine, free and protein bound, are considered to be between 5 and 15 µmol/L. Mild, moderate, and severe hyperhomocystinemia refer to concentrations between 16 and 30, between 31 and 100, and greater than 100 µmol/L, respectively.[3,6,7]

Vascular endothelial growth factor (VEGF) is a peptide growth factor that promotes angiogenesis in normal physiologic conditions such as wound repair and in pathologic conditions including atherosclerosis, diabetic retinopathy, tumor growth and metastasis formation.[8-12] VEGF has been implicated in the progression of atherosclerosis.[13,14,15] In atherosclerosis, angiogenesis within the adventitia of arterial walls is seen in the development of plaques, and extends into the media and

intima as the lesions progress. Furthermore, the expression of VEGF has been positively correlated with the number of intimal blood vessels found within atherosclerotic plaques. VEGF has been demonstrated to be increased in patients with PAD, coronary artery disease and in patients with uncontrolled type 2 DM.[8,9,16,17,18,19] VEGF is remarkably expressed in endothelial cells, smooth muscle cells within human coronary atherosclerotic lesions and in activated macrophages (15). Additionally, elevated levels as well as a positive correlation was found between VEGF and tissue factor in patients with peripheral arterial disease.[8] Because conditions leading to neoangiogenesis such as tissue ischemia in PAD or hyperglycemia in DM are frequently associated with accumulation and activation of leucocytes, VEGF expression and release from leucocytes may play an important role in these conditions.[20]

We hypothesize that homocysteine affects the levels of VEGF and its expression in leucocytes both under physiologic condition and in pathologic states such as PAD and DM. To test this hypothesis, we measured plasma homocysteine and VEGF and expression of VEGF in leucocytes in healthy volunteers before and after methionine-induced acute hyperhomocysteinemia and in patients with PAD and DM before and after reduction of homocysteine with B vitamins and folate.

PATIENTS AND METHODS

Patient Selection: Patients with PAD comprised of ten consecutive nondiabetic patients with stable symptomatic lower extremity arterial disease (intermittent claudication) and with resting ankle brachial pressure index (ABI) less than 0.9 (1). Patients with DM consisted of 15 patients with type 2 DM and without evidence of PVD. Control subjects were 12 healthy nonsmoker adult volunteers without hypertension, PAD, DM, hyperlipidemia, or coronary atherosclerosis recruited from the Faculty staff. Informed consent was obtained from all subjects in accordance with protocols fully approved by the Faculty of Medicine Review Board.

Study Design:

Methionine loading intervention: Twelve healthy subjects (8 men) received 100 mg/kg L-methionine (Sigma) mixed with orange juice four hours before blood sampling.[21] Plasma homocysteine and VEGF levels and expression of VEGF in leucocytes were determined before and after methionine loading.

B vitamins and folate intervention: Ten patients with PAD (10 men) and 15 patients with DM (10 men) received vitamin B1 125 mg/d, B6 125 mg/d, B12 125 µg/d, and folic acid 0.5mg/d (Tri B, Nile Co., Egypt) for six weeks. Plasma homocysteine and VEGF levels and expression of VEGF in leucocytes were measured before and after B vitamins and folate administration.[1,22]

Sampling technique: Venous blood was drawn and plasma was separated by centrifugation at 6000 rpm. Plasma was divided into separate aliquots and stored at -70 °C until use. Blood cells were used to separate leucocytes for determination of VEGF expression. Plasma total cholesterol, HDL cholesterol, triglyceride, urea, total bilirubin, and glucose levels were analyzed by enzymatic colorimetric methods using commercially available kits.

Measurements of plasma homocysteine: The total plasma homocysteine was measured using Diazyme Homocysteine Microplate Test Kit (La Jolla, CA, USA) which applies EIA-like colorimetric assay and has a wide detection range (2-60 µmol/L).

Measurement of plasma VEGF: Quantitative VEGF measurements were performed with the use of kit from CytImmune Sciences; ACCUCYTE (Rockville, MD., USA), according to recommendations of the manufacturer. Plasma rather than serum was used to measure total VEGF165 so as not to include VEGF released from platelets and leucocytes during blood clotting.[23]

*RT-PCR analysis:*RT-PCR was used to assess VEGF mRNA expression in leucocytes as previously described.[23] Briefly, leucocytes were prepared from blood after separation of plasma by adding 5ml of RBCs lysis solution. Total RNA was extracted from leucocytes and was reverse-transcribed as described.[20] PCR amplification was performed in a DNA thermal cycler (Landgraf, Hannover, Germany). Forward and reverse primers used for the amplification of human VEGF165 mRNA were as follows (Biosynthesis Incorporated, USA): 5′-AGG GCA GAA TCA TCA CGA AG-3′ and 5′-CGC TCC GTC GAA CTC AAT TT-3′. The PCR products were analyzed by electrophoresis on 1.2% agarose gel according to the manufacturer's recommendations and stained with ethidium bromide.

Statistical Analysis: The results are expressed as means ± SE. Comparisons between pre- and post-intervention plasma concentrations of homocysteine and VEGF were performed using two-tailed paired sample t-test, while comparisons of baseline total cholesterol, urea and total bilirubin among groups were performed using one-way ANOVA. The relationship between changes in plasma homocysteine and VEGF was assessed using Pearson product-moment correlation coefficient. Statistical analysis was performed with the software Statistical Package for Social Sciences, SPSS version 13 (SPSS, Chicago, IL, USA). A p value of less than 0.05 was considered statistically significant.

RESULTS

Demographics and biochemical profiles: As expected, the baseline characteristics of the control (healthy) group

differed significantly from the PAD and DM groups. Table 1 summarizes the baseline demographics and risk factor profiles of subjects in the three groups. The mean age of patients with PAD and DM were significantly higher than the mean age of control group ($p < 0.05$). All subjects in the control group were normotensive, with normal biochemical profile. Baseline cholesterol concentrations were significantly elevated above the upper limit of normal in PAD group compared with control and DM groups ($p < 0.05$). Baseline postprandial blood glucose determinations showed euglycemic state in the control and PAD groups compared with mild hyperglycemic state in DM group ($p < 0.01$). Baseline blood urea and total bilirubin were within normal values in all groups.

Plasma homocysteine levels (Fig. 1). The mean basal homocysteine plasma levels in the PVD and DM group were above the upper limit of normal (15 µmol/L) and were significantly elevated compared with the mean basal levels of the control group (21.5±0.5, 19.9±1.0, 11.2±1.0 µmol/L, respectively) ($p < 0.05$). The mean plasma homocysteine concentrations in the control group (11.2±1 µmol/L; range, 5.5 to 19 µmol/L) were significantly increased after methionine loading test (16.1±1.2 µmol/L; range, 10.2 to 24.2 µmol/L) ($p < 0.001$). In patients with PVD and DM, the basal hyperhomocysteinemia significantly decreased after vitamin administration (21.5±0.9 to 17±1.1 µmol/L; 19.9±1.0 to 14.6±0.6 µmol/L, respectively) (both $p < 0.001$).

Plasma VEGF levels (Fig. 2). The mean plasma VEGF concentrations in the control group (30.5±3.1 pg/ml; range 18.2-48.5 pg/ml) were significantly increased 4 h after methionine loading test (39.6±3.8 pg/ml; range 20.8-58 pg/ml) ($p < 0.01$). In patients with PVD and DM, the basal VEGF concentrations (51.6 ±5.2 pg/ml and 52.8 ±4.5 pg/ml, respectively) were significantly decreased after B vitamins and folic acid administration (36.2±4.3 pg/ml and 31.5±3.3 pg/ml, respectively) ($p < 0.01$, $p < 0.001$, respectively).

Relationship between plasma homocysteine and VEGF levels (Fig. 3). The changes in plasma levels of homocysteine and those of VEGF as a result of intervention demonstrated a significant positive correlation ($r = 0.73$, $p < 0.01$).

Expression of VEGF in leucocytes. VEGF mRNA expression in leucocytes was increased in the control group after methionine loading test (Fig. 4), however, the level of expression decreases after folic acid and B vitamins administration in the PVD and DM groups (Fig. 5).

Table 1. Baseline subjects characteristics

Baseline characteristics	Control (n=12)	PAD (n=10)	DM (n=15)
Age, years	34.3±1.5	56.8±3.0	55±2.2
Cholesterol (<200mg/dl)	178±8	228±12	190±9
Bilirubin (<1.0mg/dl)	0.8±.02	0.7±.01	0.8±.01
Urea (<45mg/dl)	27±1.3	34±3.1	42±3.8
Glucose (<140mg/dl)	98±4.5	122±8.2	157±9.4

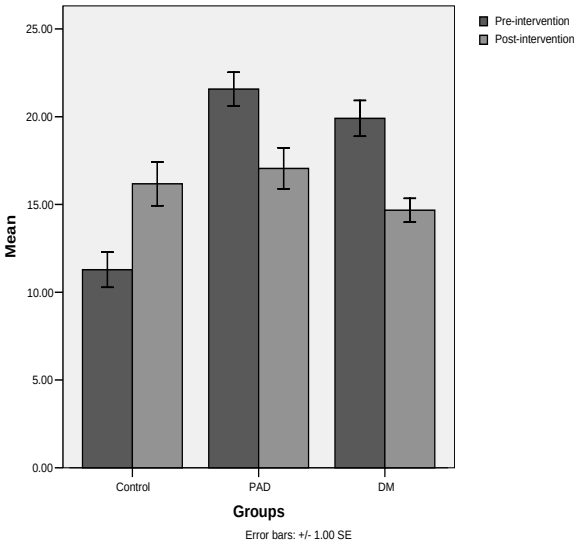

Fig 1. Mean plasma homocysteine levels (µmol/L) significantly increased ($p < 0.001$) after methionine loading in control group and significantly decreased ($p < 0.001$) after B vitamins and folate supplementation in PAD and DM groups.

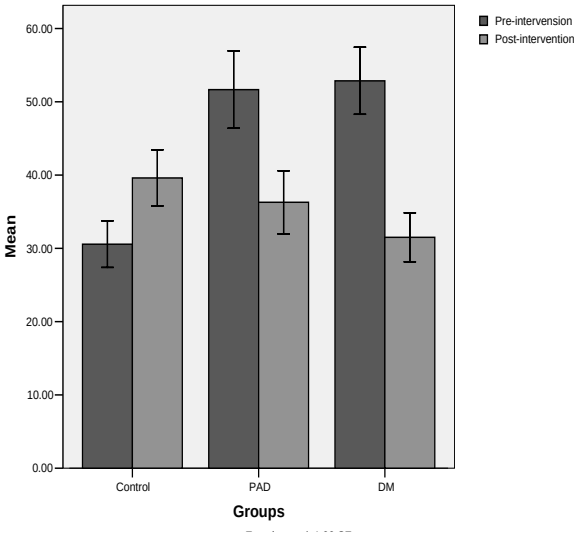

Fig 2. Mean plasma VEGF levels (pg/ml) significantly increased ($p < 0.01$) after methionine loading in control group and significantly decreased after B vitamins and folate supplementation in PAD and DM groups ($p < 0.01$ and $p < 0.001$, respectively).

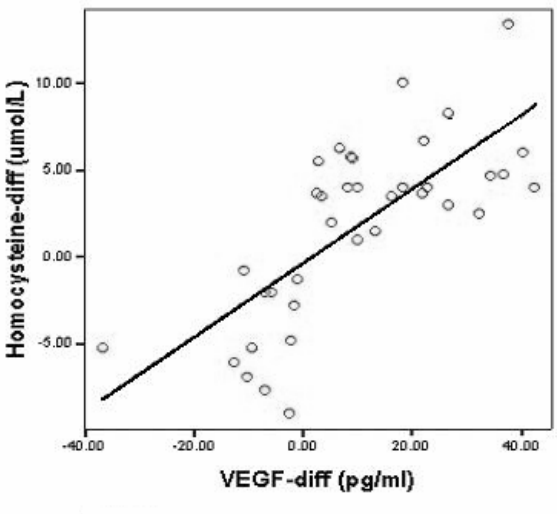

Fig 3. Scatter plot illustrating significant positive correlation between changes in homocysteine and VEGF ($r = 0.73$, $p < 0.01$).

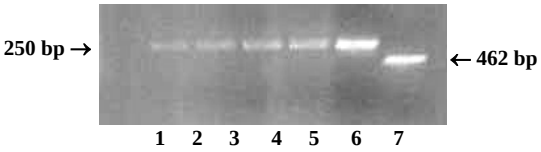

Fig 4. Agarose gel electrophoresis of amplification products generated by ThermalCycler RT-PCR (25 cycles) for VEGF165, allowing semiquantitative measurement of VEGF165 expression. Lane 1, negative control. Lanes 2-4 low expression in pre-methionine samples. Lanes 5 & 6 increased expression in post-methionine samples. Lane 7 positive control.

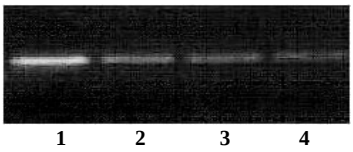

Fig 5. Agarose gel electrophoresis of PCR products at 25 cycles. Lane 1 shows VEGF expression in leucocytes from a patient with DM before vitamin treatment. Lane 2 shows decreased intensity of band of the same patient in lane 1 after treatment indicating decreased VEGF expression. Lane 3 shows VEGF expression in leucocytes from a patient with PAD before treatment. Lane 4 shows a decrease in intensity of band after treatment of the same patient in lane 3 indicating decreased VEGF expression.

DISCUSSION

Numerous studies have confirmed that elevated plasma homocysteine is an independent risk factor for the presence and progression of atherosclerotic vascular disease including coronary, cerebral and peripheral arterial atherosclerosis and for arterial and venous thromboembolism. Moreover, the risk is continuous across the concentration distribution of homocysteine.[1,3,5,6,7,24,25,26] Homocysteine concentrations exceeding the upper limit of normal (15 μmol/L) are common, and are found in almost 30% of patients with vascular disease.[27] Additional risk factors (smoking, arterial hypertension, diabetes, and hyperlipidemia) may additively or, by interacting with homocysteine, synergistically increase overall risk. Studies have shown that there is a dynamic and inverse relationship between plasma homocysteine and vascular endothelial function. Homocysteine exerts it effects by the generation of oxygen-derived free radicals, which in turn promote oxidation of low-density-lipoprotein (LDL), and deactivation of nitric oxide. Deactivation of nitric oxide, the major endothelium derived vasodilator, may lead to vasoconstriction, platelet aggregation, and monocyte adhesion, all of which promote atherosclerosis.[27,28]

Taylor et al in a prospective blinded study of the relationship between plasma homocysteine and progression of symptomatic peripheral arterial disease reported a mean plasma homocysteine level for 351 patients with PAD of 14.09 μmol/L.[1] Darius et al found that fasting homocysteine plasma levels in 6880 unselected primary care patients differed significantly between patients with and without PAD: 15.2 μmol/L versus 13.9 μmol/L.[25]

The basal plasma homocysteine levels in healthy volunteers in our study (11.2±1.0μmol/L) is slightly higher than those reported in the literature (5-10μmol/L), however, both the basal and the post-methionine increase are below the upper limit of normal.[29] This slightly elevated basal homocysteine is due perhaps to worse nutritional status in our subjects. This is supported by the finding of Chambers et al that elevated homocysteine in Indian Asians was due to reduced levels of vitamins B12 and folate.[27] This applies also to the finding of higher (19.9±1.0μmol/L) basal homocysteine levels in our patients with uncomplicated type 2 DM compared with those reported in the literature (~10μmol/L).[30,31] Also, our patients with PAD have basal homocysteine (21.5±0.9 μmol/L) levels that are higher than the upper limit of normal, and higher than patients with PAD reported in studies of Taylor et al and Darius et al.[1,25]

Plasma homocysteine concentrations can be reduced by 25-30% through oral supplementation with B vitamins (B6 and B12) and folate.[3,6,22] Recent clinical studies have shown that B vitamins and folate supplementation is associated with an improvement in endothelium dependent dilatation, and in serum markers of endothelial injury. Vitamin treatment had no effect on ankle-brachial pressure index, or on carotid and peripheral arterial outcome variables, although the current testing may not be sufficiently sensitive to detect modest changes in subclinical atherosclerosis.[27]

In our study the administration of folic acid, vitamin B1, vitamin B6, and vitamin B12 for six weeks in patients with PAD and DM resulted in significant reduction in homocysteine levels, which was more pronounced in the diabetic group (21% and 27% reductions, respectively). This reduction, however, did not normalize homocysteine levels in both groups probably due to the small dose (0.5mg/d) of folic acid administered. The American Heart Association recommendation is to treat mild to moderate hyperhomocysteinemia with a dose similar to that used in our study. If repeat analysis after 1 month showed that the treatment is ineffective, the dose can be increased to 1mg/d of folic acid, 25 mg/d of vitamin B6, and 0.5 mg/d of vitamin B12.[6]

VEGF has been implicated in the progression and instability of intimal plaques in atherosclerosis.[13,14,15] VEGF expression and secretion by human endothelial and vascular smooth muscle cells as well as from circulating blood cells has been described.[12,20] Because conditions leading to neoangiogenesis such as tissue ischemia in PAD or hyperglycemia in DM are frequently associated with accumulation and activation of leucocytes, VEGF expression and release from leucocytes may play an important role in these conditions.[20] Koehne et al demonstrated that, in contrast to human vascular smooth muscle cells, VEGF release or VEGF mRNA expression in polymorphnuclear leucocytes was not stimulated under hypoxic conditions, hypo- or hyperthermia, and acidosis. The authors suggested that these entirely different patterns of VEGF secretion reflect different and possibly complementary roles of resident cells and circulating blood cells in the initiation of the effects that are triggered by VEGF in tissue ischemia and inflammation.[20]

In our study, the levels of plasma VEGF in the control group 4 h after methionine loading were increased 1.3 folds relative to baseline levels. The level and time-course of VEGF increase corresponds with the previously demonstrated 1.3-fold increased secretion of VEGF observed with in vitro homocysteine-treated cultured cells.[9] Moreover, in this in vitro study, the expression of VEGF in the treated cells occurred within 1h and reached maximum at 2 to 5h depending on the dose of homocysteine treatment. These investigators demonstrated that the increase in VEGF mRNA levels produced by homocysteine was caused by direct activation of VEGF transcription rather than by a post-transcriptional mechanism.[9] Another study by Maeda et al showed that

VEGF mRNA was upregulated by homocysteine in a dose- and time-dependent manner in macrophages with the increase in VEGF secretion.[32] Therefore, our study is the first in vivo study to confirm that the increase in VEGF is due to elevation of homocysteine plasma levels. Another line of evidence comes from the finding of a positive correlation between the changes in homocysteine whether induced by methionine loading or vitamin treatment and the corresponding changes in VEGF.

In conclusion, the present study demonstrated the following findings. 1) All groups exhibited higher plasma homocysteine compared with levels reported in the literature. 2) Elevated plasma homocysteine levels following methionine loading induced the elevation of plasma VEGF levels and leucocytes expression of VEGF mRNA in healthy individuals. 3) The basal plasma concentrations of homocysteine and VEGF in patients with PAD and DM are elevated above the upper limit of normal. 4) A positive association between plasma homocysteine and VEGF plasma levels and it expression in leucocytes in all groups studied. 5) Administration of B vitamins and folate to patients with PAD and type 2 DM resulted in reduction of the elevated plasma levels of homocysteine and VEGF. However, a higher dose of folate than that used in this study is needed to normalize the elevated homocysteine levels. This will lead in reduction in progression of atherosclerosis and in vascular complications of DM.

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