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The Effect of Hypercholesterolemia on Serum Vascular Endothelial Growth Factor and Nitrite Concentrations in Early Stage of Atherosclerosis in Rabbits

Shaghayegh Haghjooyjavanmard[1], Mehdi Nematbakhsh[1] and Masoud Soleimani[2]
[1]Department of Physiology, Applied Physiology Research Center,
Isfahan University of Medical Sciences, Isfahan, Iran
[2]Department of Haematology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran

Abstract: Vascular Endothelial Growth Factor (VEGF) and Nitric Oxide (NO) play an important role for maintaining endothelial integrity. The purpose is to investigate the VEGF alteration during early atherosclerosis lesion formation in an animal model of hypercholesterolemia. We also measured nitrite to observe the relationship between VEGF and endothelial NO production. 20 white male rabbits randomly assigned in 2 groups (1% high-cholesterol diet, HC group, n = 14, or standard diet, control, n = 6) for 4 weeks. The serum levels of VEGF and nitrite (NO metabolite) were determined. Fatty streaks were measured in rabbit's aortas. The results indicated that the serum level of VEGF concentration was significantly higher in hypercholesterolemic rabbits and negatively correlated with fatty streak lesions ($r = -0.89$, $p < 0.05$). The serum level of nitrite was significantly higher in HC group than the control ($p < 0.05$). There was a significant negative correlation between serum level of nitrite and VEGF ($r = -0.55$, $p < 0.05$). It is concluded that, the increased VEGF in early atherosclerosis may be regarded as a safeguarding response to endothelial injury, which is responsible for maintaining endothelial integrity.

Key words: Atherosclerosis, vascular endothelial growth factor (VEGF), nitric oxide

Introduction

Atherosclerosis is the single most important cause of cardiovascular disease - a predominant health problem worldwide (Libby, 2002). The integrity and functional activity of the endothelium play a critical role in atherogenesis (Werner and Nickenig, 2006). Cardiovascular risk factors induce endothelial injury and thus trigger a cascade of proinflammatory events, resulting in infiltration of monocytic cells and smooth muscle cell proliferation, which lead to the formation of atherosclerotic lesions (Libby, 2002). VEGF, a 45 kDa heparin-binding growth factor elicits an array of biological effects on endothelial cells in vivo and in vitro including survival, proliferation and migration NO production and increases vascular permeability (Shibuya, 2001; Zachary, 2003). Recently raised plasma VEGF levels has been described in hypercholesterolemia and atherosclerosis in several studies (Blann et al., 2001; Couffinhal et al., 1997; Giurgea et al., 2006; Trape et al., 2006). These studies have obtained different conclusions as to whether VEGF can either inhibit or promote atherosclerotic lesion formation (Blann et al., 2001; Couffinhal et al., 1997; Giurgea et al., 2006; Hiltunen et al., 2000; Howell et al., 2005; Inoue et al., 1998; Kuzuya et al., 2001; Laitinen et al., 1997; Rutanen et al., 2003). Up regulation of VEGF in the vessel wall could account for the increased vascular permeability and could be part of a vascular inflammatory process (Couffinhal et al., 1997; Giurgea et al., 2006; Trape et al., 2006; Inoue et al., 1998). Although

VEGF up regulation as a known endothelial survival factor could constitute a vascular homeostatic mechanism for compensating endothelial injury (Hiltunen et al., 2000; Howell et al., 2005; Laitinen et al., 1997; Leppanen et al., 2005). Thus, the role of VEGF in atherosclerosis is controversial. Accordingly in this study we investigated the association of serum level of VEGF with early atherosclerosis lesion formation in a rabbit model of hypercholesterolemia. We also measured serum concentration of nitrite to observe the relationship between VEGF and endothelial NO production.

Materials and Methods

Animals and experimental design: This study was reviewed and approved by the Ethics Committee of Isfahan University of Medical Sciences. 20 white male rabbits were obtained from the Pasteur Institute of Iran. After 1-week acclimation period and an overnight fasting blood samples were taken as pre-experiment sampling. Collected blood samples were centrifuged (10,000g), and the resulting serum was stored at -70°C until measurements. The animals were then randomly assigned in 2 groups. The rabbits were fed rabbit chow supplemented with 1% cholesterol. (Hypercholesterolemic diet) (HC group, n=14) or standard diet (control group, n=6) for 4 weeks.
By the end of 4 weeks, the blood samples were taken and the animals euthanized by an overdose of sodium pentobarbital and exsanguinated. The serum was stored again.

Lipids measurement: Total cholesterol level was measured by standard enzymatic kit according to manufacture's instruction (Pars Azmoon Co, Iran)

Serum nitrite measurement: The serum level of nitrite (stable NO metabolite) was measured using a colorimetric assay kit (R and D Systems, Minneapolis, USA) that involves the Griess reaction according to manufactures instruction. Briefly, serums were added into wells (96-well enzymatic assay plate). A sulfanilamide solution was added to all experimental samples and after incubation, N-1-naphtylethy-lenediamine dihydrochloride solution was added. Then, absorbance was measured by a microreader in 520 nm wavelength. The samples NO concentration was determined by comparison to nitrite standard reference curve. The detection limit was 0.25 µM nitrite.

Serum VEGF measurement: Serum VEGF concentration was measured using enzyme-linked immunosorbent assay using available reagents and recombinant standards (R and D Systems, Minneapolis, MN) according to manufactures instruction. Briefly, 50 µl of standard or serum was added to the wells of the microplate, which was precoated with monoclonal antibody for VEGF and incubated for 2 h at room temperature. After any unbound substances had been washed away, an enzyme-linked polyclonal antibody against VEGF was added to the wells and incubated for 2 h. After a wash, 100 µl substrate solution was added to the wells and incubated for 30min. A 100µl stop solution was then added for color development. The optical density was determined at 450nm using a microplate reader. The VEGF assay has a minimum sensitivity of 3.0 pg/ml.

Pathological investigation: The abdominal aorta was subjected to pathological investigation to verify fatty dot or fatty streak lesions formation. Entire aorta, from the aortic arch to the external iliac arteries, was dissected out and cleaned of excess adventitial tissue. The aorta was fixed in buffered 10% formalin for 24 h and then embedded in paraffin. The paraffin - embedded specimen was stained with haematoxylin and eosin, sectioned at 5 µm (20 sections in succession) and examined by light microscopy to measure fatty streaks by two pathologists in a double-blinded manner.
Fatty streaks formation was determined by intima thickness and media thickness measurement in all sections. The data were averaged and were used to obtain the IMT ratio (intima thickness / media thickness).

Statistical analysis: The data are reported as the mean±SEM. A statistical software package, SPSS (version 13), was used to perform statistical analysis.

The data were tested for normality and homogeneity of variance. Otherwise, paired Student's t-test was used to assess the significance of any change within groups, while an unpaired Student's t-test (equal or unequal variance assumed accordingly) was used to assess the significance of any change between groups. The Spearman's rank correlation test was used to evaluate relationships between variables. Statistical significance was accepted at $p < 0.05$.

Results
Serum lipids: The cholesterol-rich diet induced a significant increase of total cholesterol in HC group (2.82±0.3 vs.55.10±4.4 mmol/L P < 0.05) while cholesterol level in the control group remained unchanged through the study (3.11±0.5 vs. 2.90±0.8 mmol/L, P>0.05). After 4 weeks of cholesterol-rich diet, animals of the HC group had significantly higher cholesterol level than control group ($p < 0.05$)

The effect of hypercholesterolemia on serum level of VEGF: The cholesterol-rich diet induced VEGF increment in HC group (30.96±6.6 vs. 239.91±78.2 pg/ml, P>0.05) (Fig. 1), while VEGF levels in the control group remained unchanged through the study (36.62±20.0 vs. 46.17±24.8 pg/ml, P>0.05) (Fig.1). After 4 weeks of cholesterol-rich diet, animals of the HC group had significantly higher VEGF level than control group ($p < 0.05$) (Fig. 1).

The effect of hypercholesterolemia on serum level of nitrite: There were no significant changes in serum level of nitrite of HC and control groups through the experiment (9.99±0.7 vs. 8.93±1.09 µmole/l, p>0.05). By the end of study, the serum level of nitrite was significantly higher in HC group than the control groups (12.66±0.7 vs 9.21±0.6 µmole/l , p<0.05) (Fig. 2). There was a significant negative correlation between serum level of nitrite and VEGF concentration in HC group (r = -0.55, p<0.05).

Fatty streaks formation: By the end of study, there were no fatty streaks lesions in control group aortas whether IMT ratio was 0.33±0.1 in HC group (p=0.07). There was a significant negative correlation between fatty streaks and VEGF concentration in HC group (r=-0.89, p<0.05).
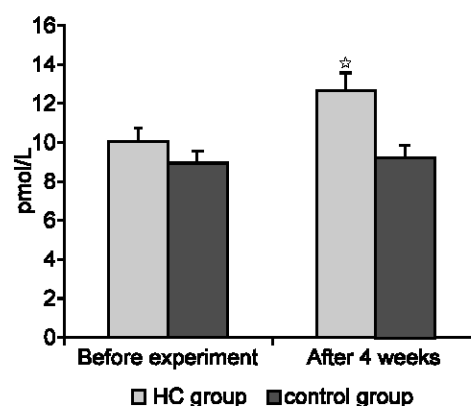
Discussion
The role of VEGF as a specific endothelial cells mitogen and survival factor responding for maintaining and or restoring endothelial integrity following arterial injury has remained less appreciated. This study was designed to investigate the VEGF alteration in an animal model using hypercholesterolemia as an endothelial cell stressor during early atherosclerosis lesion formation.
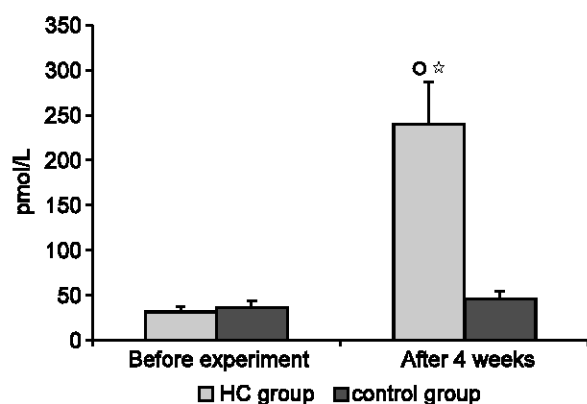
O Significant difference from before experiment
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Fig. 1: Serum VEGF concentration (pico mol/l) before and after experiment. Serum VEGF concentration significantly increased in hypercholesterolemic (HC) group (p<0.05) while remained unchanged in control group. Serum VEGF concentration in HC group was significantly higher than control group (p<0.05)

As the results of our study showed, VEGF significantly increased in hypercholesterolemic animals. This is in agreement with other studies who found higher VEGF concentration in patient with hypercholesterolemia and atherosclerosis than normal subject (Blann *et al.*, 2001; Giurgea *et al.*, 2006; Trape *et al.*, 2006).
The contribution of VEGF to atherogenesis has been challenged and still remained unclear. Although the progressive expression of VEGF-A in activated endothelial cells, macrophages and differentiated smooth muscle cells in atherosclerotic lesions might be suggesting a proinflammatory role of VEGF in atherogenesis but it has been shown in several studies that periadventitial and intra-arterial gene transfer of VEGF - A, - C and -D has inhibited neointimal growth (Hiltunen *et al.*, 2000; Leppanen *et al.*, 2004; Rutanen *et al.*, 2005; Yla-Herttuala *et al.*, 2007). In addition systemic adenoviral gene transfer of VEGF - A, - B, - C, or - D, as well as recombinant VEGF-A administration, did not alter plaque area or macrophage influx in LDLR/ApoB48 double knockout mice (Leppanen *et al.*, 2005). Furthermore, VEGF-A polymorphism causing higher VEGF-A expression recently was found to be associated with a lower risk of coronary artery disease in an epidemiological study (Howell *et al.*, 2005). VEGF up regulation has been also reported after arterial injury (Tsurumi *et al.*, 1997). As the result of our study showed there was a negative correlation between VEGF concentration and fatty streaks formation. Because of all the above mentioned materials. It is tempting to

☆ Significant difference from other group

Fig. 2: Serum NO concentration (µmol/l) before and after experiment. Serum NO concentration in hypercholesterolemic (HC) group was significantly higher than control group (p<0.05).

speculate that this over expression could be an endothelial protecting response in early atherosclerosis. Another open question in this field is the mechanism by which hypercholesterolemia may induce VEGF up regulation in the vessel wall, among the possible pathophysiological stimuli are hypoxia, oxidative stress, inflammation and low NO bioavailability (Rodriguez *et al.*, 2005). As our results showed there was a negative correlation between NO and VEGF in hypercholesterolemic rabbits. This is interesting in light of Tsurumi and Murohara study who found NO attenuate VEGF expression in a concentration dependent manner by inhibition of binding of the transcription factor activator protein-1 (AP-1) to the VEGF promoter (Tsurumi *et al.*, 1997). In their study, VEGF up regulation varies in reciprocal fashion with level of NO secreted from regenerating endothelium.
In summary, a wide array of cytokines, growth factors and other molecules is released in response to vascular injury, which is essential for the repair process. VEGF as a specific endothelial mitogen and survival factor is one of these molecules, which have been shown to release from smooth muscle cells, macrophages and platelet after endothelial injury. So increased VEGF in early atherosclerosis may be regarded as a safeguarding response to endothelial injury, which is responsible for maintaining endothelial integrity.

Acknowledgment

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