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ENOS GLU298ASP POLYMORPHISM IS ASSOCIATED WITH DEVELOPMENT OF COMPLICATED PLAQUES IN PATIENTS FROM SERBIA WITH ADVANCED CAROTID ATHEROSCLEROSIS

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Abstract - Nitric oxide inhibits adhesion of thrombocytes, proliferation and migration of smooth muscle cells and restricts oxidation of atherogenic low-density lipoproteins. Therefore, decreased production or activity of NO may play a role in the initiation, progression or complications of atherosclerosis. The aim of this study was to estimate the effect of Glu298Asp eNOS gene polymorphism on the individual risk for development of complicated carotid atherosclerotic plaque in patients from Serbia with advanced carotid atherosclerosis (CA) who had undergone endarterectomy. The study population included 233 patients. eNOS G894T gene polymorphism was identified by PCR and RFLP methods. Multivariate logistic regression analysis showed that Asp298Asp is an independent risk factor for the presence of complicated plaques in CA patients. Patients who were homozygous for the Asp298 allele had an adjusted OR of 4.36 for the development of complicated plaques compared to those that carry the Glu298 allele. Further validation and replication studies are needed.

Key words: Carotid atherosclerosis, polymorphism, eNOS, oxidative stress

INTRODUCTION

Nitric oxide (NO) is synthesized by the constitutive expressed endothelial NOS (eNOS) encoded by the NOS3 gene, in endothelial cells, platelets and red blood cells ((Radomski et al., 1990; Kleinbongard et al., 2006), as well in certain populations of nerve cells in the brain (Dinerman et al., 1994). NO is a major vasodilative substance released by the endothelium. Nitric oxide has anti-inflammatory properties by inhibiting the synthesis and expression of cytokines and cell adhesion molecules that attract inflammatory cells to the endothelial surface and facilitate their entrance into the vessel wall (Bath et al., 1991; De Caterina et al., 1995). It inhibits adhesion of thrombocytes as well (Randomski et al., 1987), proliferation and migration of smooth muscle cells (Garg and Hassid, 1989; Sarkar et al., 1996), and it restricts the oxidation of atherogenic low-density lipoproteins (LDL) (Hogg et al., 1993). Because of these properties, decreased production or activity of NO, manifested as impaired vasodilatation, may play a role in the initiation, progression or complications of atherosclerosis.

Glu298Asp polymorphism in NOS3 exon 7 was first discovered by a group of French researchers. The G to T nonsynonymous polymorphism at position 894 of the eNOS gene results in an amino acid change from Glu to Asp at codon 298 (Nadaud et al., 1994).

There are several studies showing an association of these polymorphisms with coronary heart

disease (Casas et al., 2006; Cooke et al., 2007). The few studies conducted on different populations reported conflicting results for associations of Asp298Glu polymorphism with intima-media thickness (IMT) (Lembo et al., 2001; Schmoelzer et al., 2003) and the presence of carotid atherosclerotic plaques (Wollf et al., 2005; Djuric et al., 2009). However, data concerning the association of Glu-298Asp eNOS gene polymorphism and a possible effect on the development of complicated carotid plaques has not been evaluated so far. The aim of our study was to estimate the effect of Glu298Asp eNOS gene polymorphism on the individual risk for development of complicated carotid atherosclerotic plaque in patients from Serbia with advanced carotid atherosclerosis (CA) who had undergone endarterectomy.

MATERIALS AND METHODS

Subjects

The 233 patients enrolled in the study were recruited from the Clinic for Vascular and Endovascular Surgery, Clinical Center of Serbia, Belgrade, Serbia (2008-2009) and were comprised of subjects consecutively admitted for carotid endarterectomy with evidence of carotid plaque presence in the internal (ICA) or common (CCA) carotid artery. All of them were Caucasians of European descent from Serbia. Exclusion criteria for all patients were a history of previous carotid endarterectomy (possible restenosis), carotid kinking, carotid aneurism, tumors, chronic inflammatory diseases, autoimmune disease or renal failure. For all individuals enrolled in the study, a complete medical history was taken, including smoking and drinking habits, the presence of diabetes, coronary artery disease, peripheral arterial occlusive disease and drug treatment. Subjects already diagnosed with diabetes mellitus (fasting glucose level of ≥7.0 mmol/l) or taking insulin or oral hypoglycemic drugs were characterized as having diabetes mellitus; those with previous myocardial infarction or with stable angina pectoris evaluated by selective coronarography that confirmed or revealed coronary artery disease were characterized as having coronary heart disease. Peripheral artery disease was diagnosed as an ankle-brachial index (ABI) lower than 0.90. Hypertension was defined as a systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, or current treatment with an antihypertensive drug.

All biochemical analyses were performed at the hospital laboratory by standard procedures.

The study was approved by the Ethics Committees of the participating medical center and participants gave their written informed consent to participate in the study.

Ultrasound assessment of the carotid arteries

Ultrasound assessment of the bilateral carotid arteries was performed by high-resolution B-mode ultrasound Acuson Antares™ system (Siemens, Munich, Germany) and HDL 3500 linear transducer (5 to 12 MHz.; Philips ATL, Bothell, WA). The degree of carotid stenosis was calculated using the European Carotid Surgery Trialists' Collaborative Group method (ECST) (European Carotid Surgery Trial, 1991). The highest peak systolic (PSV) and end-diastolic (EDV) velocities as well as the ICA/CCA carotid artery ratio, were recorded. All patients included in the study had stenosis >85% according to ECST.

Atherosclerotic plaques were defined as focal widening relative to adjacent segments as evidenced by protrusion into the lumen and/or localized roughness with increased echogenicity. For ultrasound carotid measures, the intraclass correlation coefficient for inter-rater and intra-rater reliability was 0.916 and 0.968, respectively. Carotid atherosclerosis was defined as the presence of atherosclerotic plaques in the internal or common carotid artery.

Duplex ultrasound analysis was used to classify different plaque types in terms of hypoechogenic (echolucent and dominantly echolucent) and hyperechogenic (echogenic and dominantly echogenic) according to the Gray-Weale criteria (Gray-Weale et al., 1988). Additionally, B mode was used to ana-

lyze surface irregularity of the plaque (irregular 0.4-2 mm). Plaque ulceration was defined as irregularity greater than 2 mm. Hypoechogenic plaques as well as plaques with ulcerations were defined as complicated, while hyperechogenic plaques were defined as stable plaques.

Genetic analysis

Genomic DNA was isolated from whole blood samples collected with EDTA by the standardized Blood-Prep® DNA Chemistry isolation kit (Applied Biosystems, Forester City, CA) on the ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems, Forester City, CA). eNOS G894T gene polymorphism was identified by PCR and *Mbo*I (MBI Fermentas, Lithuania) restriction digestion, as previously described (Hirata et al., 2002). The digestion products were loaded on an 8 % polyacrylamide gel for genotyping and run for 2 h at 12V/cm. Gels were stained with silver nitrate and visualized using a GDS8000 gel documentation system (Ultra Violet Products Inc, Upland, USA).

Statistical analysis

The allelic frequencies and genotype distribution were estimated by the gene counting method. Differences in allele frequencies and genotype distribution between the cases and controls as well as deviation from Hardy-Weinberg equilibrium were estimated by chi-square (χ^2). Means of normally distributed continuous variables were compared by unpaired t-test and means of skewed continuous variables with the nonparametric Mann-Whitney U Test. Statistical analysis was performed using Statistica Version 8, software package (StatSoft Inc, 2007). As a measure of strength of association between eNOS genotypes and plaque types, multiple logistic regression analysis was used and expressed in terms of crude and adjusted OR and 95% confidence interval (CI). The odds ratio was adjusted for glucose level and active antihypertensive therapy. The genotype effects were examined and represented according to a recessive model (Glu298Glu + Glu298Asp vs. Asp298Asp). In all tests, differences with two-tailed alpha-probability $p<0.05$, were considered significant.

RESULTS

The main characteristics of the patients according to plaque type (stable and complicated) are shown in Table 1. Patients with complicated plaques had a lower glucose serum level and were on active antihypertensive therapy in higher percentage than patients with stable plaques. No other measured parameters were statistically different between the two investigated groups of patients.

The genotype frequencies for eNOS Glu298Asp gene polymorphism did not deviate from the Hardy-Weinberg equilibrium in any of the studied groups. Distribution of eNOS Glu298Asp genotype and allele frequencies were significantly different between patients with stable and patients with complicated plaques (overall $\chi^2 = 9.68$, df=2, $p<0.01$) (Table 2). There was a significantly higher frequency of Asp298Asp genotype in patients with complicated plaques compared to patients with stable plaques, according to the recessive model (16.67% vs. 5.37%, $p<0.01$ respectively) (Table 2). Multivariate logistic regression analysis showed that Asp298Asp is an independent risk factor for the presence of complicated plaques in CA patients. Patients who were homozygous for the Asp298 allele had an adjusted OR of 4.36 for the development of complicated plaques compared to those that carried the Glu298 allele (the OR was adjusted for glucose level and active antihypertensive therapy) (Table 2).

DISCUSSION

Glu298Asp polymorphism in the eNOS gene is still a questionable marker for the formation and progression of atherosclerosis in different populations. So far it has been tested in the population of Serbia as a marker for the development of carotid atherosclerosis (Djurić et al., 2009), but the aim of this study was to investigate the interindividual differences among patients with advanced atherosclerosis at risk for the development of complicated plaque. We found

Table 1. General characteristics of patients according to type of atherosclerotic plaque.

Variable	Stable plaque n=149	Vulnerable plaque n=84	p-Value
Male/Female	81(54.36)/68(45.64)	50(58.82)/35(41.18)	0.51
Age, year	67.0±9.6	66.0±8.0	0.40
BMI (kg/m2) *	26.1±2.7	27.2±3.8	0.07
TC (mmol/L) *	5.81±1.20	5.81±1.78	0.95
TG (mmol/L) *	1.86±0.99	1.81±0.91	0.79
HDL (mmol/L)	1.16±0.34	1.16±0.36	1.00
LDL (mmol/L)	3.89±1.03	3.81±1.04	0.67
DDIM (mmol/L)*	214.8±199.2	235.4±307.3	0.73
Apo A1 (g/L) *	1.59±0.52	1.44±0.36	0.06
Apo B (g/L) *	1.16±0.30	2.72±12.58	0.88
CRP (mg/L) *	9.26±17.90	4.16±6.00	0.18
WBC (cells/μL)*	7.70±1.68	7.57±2.03	0.33
Glucose (mmol/L) *	7.10±2.72	6.26±2.65	<0.01
LDH (U/L)	331.84±75.63	316.00±73.20	0.14
Creatine (mmol/L)*	101.37±99.80	94.67±23.70	0.94
Fibrinogen (g/L)*	4.87±1.54	5.09±1.73	0.64
ASY n (%)	58 (39.29)	25 (29.63)	0.17
CVI n (%)	45 (30.20)	22 (25.88)	0.48
CAD n (%)	47 (31.25)	7 (8.33)	0.10
HLP n (%)	42 (28.19)	32 (38.10)	0.12
DMT II n (%)	43 (28.86)	22 (25.88)	0.62
Hypertension n (%)	125 (83.89)	70 (82.35)	0.76
Smokers n (%)	80 (53.69)	47 (55.29)	0.81
PAD n (%)	21 (14.19)	12 (14.12)	0.99
Actual therapy:			
AAGR n (%)	104 (69.84)	42 (50.00)	0.18
AHT n (%)	86 (57.52)	67 (80.25)	<0.01
ACT n (%)	5 (3.54)	3 (3.70)	0.95
Statins n (%)	26 (17.70)	16 (18.52)	0.88

Values are mean ± SD, for age, Body Mass Index (BMI), Total Cholesterol (TC), Triglycerides (TG), High Density Lipoproteins Cholesterol (HDLC), Low Density Lipoproteins Cholesterol (LDLC), D-Dimer (DDIM), Apolipoprotein A1 (Apo A1), Apolipoprotein B (Apo B), C-Reactive Protein (CRP), White Blood Cells (WBC), glucose, Lactate Dehydrogenase (LDH), creatine and fibrinogen; χ^2-test was used for categorical variables; Student's t-test was used to compare age, HDLC, LDLC and LDH values; * BMI, TC, TG, DDIM, Apo A1, Apo B, CRP, WBC, glucose, creatine and fibrinogen values were compared using Mann-Whitney U-test; p values < 0.05 were considered statistically significant.

ASY - *Asymptomatic* patients, *CVI - cerebrovascular insult,* CAD - *coronary artery disease*, *HLP - hyperlipidemia,* DMT II - *diabetes mellitus type 2*, *PAD - peripheral arterial disease,* AAGR - *antiaggregation therapy*, *AHT - antihypertensive therapy, ACT - anticoagulant therapy.*

that the Asp298Asp genotype was significantly and independently associated with the presence of complicated plaque in Serbian CA patients. Reference data concerning the association of Glu298Asp eNOS gene polymorphism with the risk for development of complicated plaque as such has not been evaluated in any other population yet. Instead, it has been investigated as a marker of a cardiovascular event such as MI or stroke.

Studies on animal and human models show that the function of the endothelial nitric oxide is reduced in proatherogenic conditions, such as hypercholesterolemia, diabetes mellitus and hypertension, which indicates that the reduction of available NO in the cardiovascular system precedes the progression of atherosclerotic lesions (Cooke and Dzau, 1997). NO, thus, inhibits the initiation and progression of atherosclerotic plaque and activation of inflammatory factors that could contribute to plaque instability and rupture (Davignon et al., 2004). A paper by Tesauro et al. (2000) suggests that the eNOS wild-type Glu298 and the Asp298 variant are processed differently in native endothelial cells and vascular tissues. The Asp298 variant undergoes selective proteolysis, which produces smaller fragments that are probably nonfunctional or exert a dominant negative effect, so the steady state level of active eNOS may be reduced in carriers of this allele (Tesauro et al., 2000). Veldman et al. (2002) showed that the Asp298 allele is associated with reduced basal NO production in a healthy population of the Netherlands. Some studies reported that the Asp variant is associated with lower eNOS enzyme activity levels in human placentas and umbilical vein (Wang et al., 2000; Joshi et al., 2007). The Asp298 allele is also associated with reduced production of NO in pregnancy and in male smokers (Cooke et al., 2007). Moreover, in a recent study it was observed that the amount of eNOS enzyme associated with Cav-1 protein is significantly reduced in carriers of the Asp298 allele, either in homozygous or heterozygous state. In accordance with the fact that only Cav-1-associated eNOS can be activated after reception of an activation signal to the cell, it is clear that the presence of an eNOS Asp298 variant may affect the function of the enzyme (Joshi and Bauer, 2008).

This depletion of NO could be associated with thrombus formation in the arterial wall as well as with the higher levels of oxidative stress that are features of complicated plaques formation and rupture. In line with this are the results from the study conducted on healthy nonsmoker Caucasians aged 18 to 45 years. The authors associated genotypes containing Asp298 allele with decreased NO production in platelets. After incubation of the platelet-rich blood with estradiol, which inhibits platelet aggregation through activation of eNOS, they obtained significantly increased platelet aggregation and superoxide synthesis in platelets in individuals homozygous for the Asp298 allele (Tanus-Santos et al., 2002). It is already well known that superoxide contributes to the oxidative modification of LDL (Panasenko et al., 1991), a critical event in development of an atherosclerotic lesion (Schwartz et al., 1991). The presence of oxidized LDL in atheromatous plaques correlates with progression of atherosclerotic carotid disease (Salonen et al., 1992) and promotes the transition from stable plaques to unstable plaques (Xu et al., 1999).

The other negative feature of NO depletion is platelet aggregation. Occlusive or nonocclusive formation of thrombus on atherosclerotic plaque is believed to be of prime importance in the progression of atherosclerosis, as well as the cause of acute coronary syndrome (Kubo-Inoue et al., 2002). It has been shown that platelets from patients with acute coronary syndromes produce significantly less NO compared to patients with stable coronary artery disease (Freedman et al., 1997). In addition, long-term inhibition of nitric oxide synthesis has been shown to increase arterial thrombogenecity in rat carotid artery (Kubo-Inoue et al., 2002). Having in mind that advanced atherosclerosis is a long-term inflammatory disease, patients homozygous for 298Asp allele could be chronically exposed to much lower levels of NO compared with patients carrying genotypes with wild-type 298Glu allele.

Considering all the above facts, we can conclude that the Asp298Asp genotype could be a promising marker for interindividual differences in the risk for complicated plaque development in patients with advanced atherosclerosis from Serbia. Still, we must be aware that the number of patients included in this study was too limited to make a final conclusion. This result must be validated on a larger number of patients from Serbia as well as replicated in other populations.

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