

Certain genetic risk factors of restenosis in peripheral atherosclerotic disease

Theses

of PhD Dissertation

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Introduction

In part of stenosis cases arising from severe atherosclerotic vascular diseases restenosis emerges after surgical intervention. Among the promoting factors of restenosis major risk factors of progressive atherosclerosis (hypertension, diabetes mellitus, hyperlipoproteinemia, genetic predisposition) and coagulopathological alterations (hyperfibrinogenemia, thrombophilia, increased platelet aggregation) can be found.

Recently the investigation of atherosclerotic risk factors was limited to the determination or accidentally to the follow-up of certain metabolic parameters. By the development of genetic methods not only the products and/or functions of genes can be investigated, but their role in atherosclerotic risk can also be elucidated by studying genetic polymorphisms. The genetic polymorphisms of receptors, proteins with several functions, and that of cytokines and intracellular signal molecules may influence atherosclerotic risk.

Following surgical intervention of atherosclerotic occlusions, in a significant part of the cases restenosis occurs within some weeks or months, localized at the site of operation. In one respect restenosis is a consequence of neointimal proliferation from the place of intervention, on the other hand a progression of atherosclerosis can be charged with the generation of reocclusion. It is obvious that inflammatory components play an outstanding role in this process, inducing the migration and adhesion of leukocytes and the aggregation of platelets according to the place of damage. These processes simultaneously lead to the migration of smooth muscle cells and the increased synthesis of extracellular matrix can also be observed. The platelets participate actively not only in the generation of thrombus, but also synthesize proinflammatory mediators, which cause an increased release of adhesion molecules, chemokines, cytokines from leukocytes and endothelial cells. The neointimal proliferation leads to the remodelling of the arterial wall, while the next station of the process is restenosis resulting in clinical complications.

Beside the before mentioned and epidemiologically confirmed risk factors the role of certain gene polymorphisms may be raised. The recognition and clinical consideration of these polymorphisms may have a predictive significance for the

patients before arterial reconstructive intervention. The exact mapping of genetic polymorphisms may provide new possibilities in the development and use of preventive methods.

In the last decades the relationship between atherosclerosis and lipoprotein metabolism became widely verified. However, there are only a few data available on the role of lipoproteins, as well as of the enzymes and their genes participating in lipid metabolism and influencing the development of restenosis after angioplastic surgery.

The exact risk factors of restenosis are still unrevealed. But, as locally generated thrombotic phenomena have a role in the initiation of atherosclerosis, the trigger role of local thrombotic lesions after reconstructive arterial interventions cannot be ignored either in restenosis cases. The relationship of atherosclerosis and thrombophilias has been raised in a number of cases and many studies revealed correlations between the coagulation system and atherogenesis in patients with vascular diseases. A direct link between genetically determined prothrombotic state and cytokines, growth factors playing a role in the generation of restenosis, can not be excluded.

Aims

Apolipoprotein E plays a central role in the lipoprotein metabolic pathway. Beside binding the lipoproteins to the receptors, apolipoprotein E influences the serum total cholesterol level and bears an antioxidant character, influences the endothelial functions and inhibits the formation of restenoses after endothelial injuries.

A lot of observations confirmed that the gene polymorphism of apolipoprotein E plays an important role in the development of ischemic heart disease and carotid atherosclerosis, and is one of the main causes of familiarity. In young people with severe cases of ischemic heart disease the $\epsilon 4$ allele of apolipoprotein E gene was detected in a significantly higher number. In our investigation apolipoprotein E (apoE) alleles in the pathogenesis of accelerated atherosclerosis and restenosis were studied in patients requiring reoperation within five years after

femoropopliteal reconstructive surgical intervention, in comparison with the data of a healthy control group.

The cholesterol ester transfer protein (CETP) is the central player of reverse cholesterol transport and the cholesterol transfer between lipoproteins. The activity of CETP influences the serum HDL-cholesterol level, its increased activity lowers serum HDL-cholesterol concentration. According to epidemiological studies decreasing CETP activity results in increasing HDL-cholesterol level. In the clinical use the CETP inhibitor torcetrapib increased HDL-cholesterol level, but paradoxically the clinical outcomes showed the increase of the incidence of ischemic heart disease. This phenomenon indicates that the development and complications of atherosclerosis is influenced by a factor independent of CETP activity, e.g. the polymorphism of CETP gene.

In our investigation we compared the genotype distributions of CETP Taq1B and I405V polymorphisms of patients with severe femoropopliteal atherosclerosis and restenosis to age and sex matched healthy control subjects.

Following the endothelial injury in the course of atherosclerosis local thrombus generation can be observed. Both the dissolution of the balance between thrombosis and fibrinolysis and the increased prethrombotic state influence the progression of atherosclerosis.

From among the congenital forms of prothrombotic state, the thrombophilias it is the Leiden mutation of coagulation Factor V that is responsible for the majority (40-70%) of venous thromboembolic clinical complications. Based on epidemiological data thrombophilias influence the course of arterial thromboses and have an effect on atherosclerosis, too. Our aim was to compare the frequency of Factor V Leiden in patients with severe atherosclerosis requiring reoperation (redo) due to restenosis to a large number of healthy control subjects.

Methods

Patients

The study population comprised consecutive patients with reconstructive vascular surgery performed within a five years period due to ilio-femoropopliteal atherosclerotic disease or severe restenosis at the Department of Vascular

Surgery, Central Military Hospital, Budapest, Hungary. Patients were contacted by phone and asked to return for blood sampling. Out of 198 patients 109 (73 male, 36 female, aged 34-78, median 58 years) returned for control examinations. Twenty patients died before the study, others did not concede the procedure or moved from their known address. Having got the patients' informed consent 12-hour fasting blood was taken in supine position, for determining routine laboratory and metabolic parameters, as well as for DNA preparation detecting gene polymorphisms. The above mentioned examinations were made separately and at different times, so the respective control groups consisted of healthy, voluntary blood donors and/or healthy office employers participating in their annual screening at the 3rd Department of Internal Medicine, and at the Kútvölgyi Clinical Center of the Semmelweis University, Budapest, Hungary.

Laboratory methods

Routine laboratory clinical chemical tests were performed by a Roche Cobas Integra 700 equipment and internationally validated methods. Serum total cholesterol, triglycerides, HDL-cholesterol and LDL-cholesterol concentrations were determined by enzymatic methods, while the serum level of apolipoprotein A-I, A-II and apolipoprotein B was measured by immunoturbidimetry (Roche Diagnostics, Germany).

DNA analysis for the determination of apolipoprotein E genotypes

Genomic DNA was amplified by polymerase chain reaction (PCR) on a thermal cycler (Perkin Elmer GenAmp 2400, Norwalk, CT, USA) with oligonucleotide primers F-4 and F-6. PCR products were digested with 2,5 U HhaI enzyme (Promega, Madison, WI, USA). Polyacrylamide gel electrophoresis was carried out on the Sturdier vertical electrophoresis unit (Pharmacia, Biotech, Uppsala, Sweden) on 8% polyacrylamide (BioRad, Richmond, CA, USA) gels. Gels were then stained with silver and dried between gel drying films for later analysis.

Determination of CETP Taq1B polymorphism bby PCR-RFLP

For the amplification of the specific section (Figure 1.) of genomic DNA by polymerase chain reaction (PCR) specific oligonucleotide primers were used. The amplified DNA-section was fragmented by TaqI restrictive endonuclease enzyme

(Fermentas International Inc, Burlington, Ontario, Canada), and the fragments were run in polyacrylamide gels stained by 2% ethidium bromide.

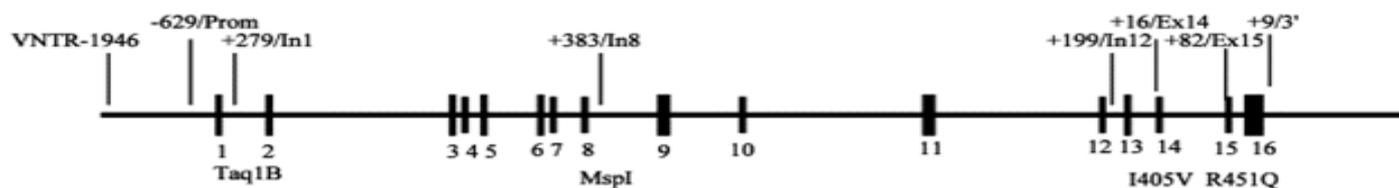


Figure 1. The structure of CETP gene and the localizations of its polymorphisms.

Determination of CETP I405V polymorphism by PCR-RFLP method

DNA was prepared from leukocytes of blood sample with EDTA and the protein product of the exon 14 was investigated. The change of aminoacids isoleucin for valin at the 405th position was determined by using MspI restriction endonuclease enzyme.

Detection of the presence of factor V Leiden mutation by PCR-RFLP

The DNA samples were prepared from the leukocytes of blood with EDTA by salting method. Following the polymerase chain reaction (PCR) the products were digested by 1,0 U MnlI restriction endonuclease enzyme. After digestion the fragments were run on 6% polyacrylamide gels and stained by ethidium bromide.

Statistical analysis

For statistical evaluations the GraphPad Prism version 3.0 for Windows (GraphPad Software, San Diego, USA) was used. Data showing parametric distribution were analysed by two sampled t test, the difference between the means of laboratory parameters were determined by the Mann-Whitney test, while the difference in allele frequencies between groups were calculated by the χ^2 test of Fisher's exact test. Multiple logistic regression analysis was performed using version 0 of the SPSS software (SPSS Inc., Chicago, IL, USA) to eliminate the errors caused by the age-differences between patient and control groups.

Results

The frequency of $\epsilon 4$ allele of apolipoprotein E gene was significantly higher in patients with vascular disease compared to the control subjects (Table 1.)

Apolipoprotein E Genotypes	Patients N = 100	Controls n = 372
$\epsilon 22$	0 (0%)	5 (1%)
$\epsilon 23$	18 (18%)	59 (16%)
$\epsilon 24$	0 (0%)	2 (0.5%)
$\epsilon 33$	57 (57%)	253 (68%)
$\epsilon 34$	25 (25%)	50 (13%)
$\epsilon 44$	0 (0%)	3 (0.8%)

p value = 0,015 (χ^2 test)

Table 1. Distribution of apolipoprotein E genotypes in patients with femoropopliteal restenosis and controls

Since the patients were significantly older than controls the data were re-evaluated by age-adjusted multiple logistic regression. Age-adjusted odds ratio of patients to carry the apolipoprotein E $\epsilon 4$ was determined in 4,264 (CI 2,269-8,013, p = 0,049).

Comparing patients carrying $\epsilon 4$ allele to patients without $\epsilon 4$ allele no difference was found between the two groups in body mass index (BMI), HDL-cholesterol, LDL-cholesterol, apolipoprotein A-I and A-II concentrations, nor in serum creatinine, serum uric acid and transaminase levels. Increased serum triglyceride mean values were found in patients carrying $\epsilon 4$ allele compared to non-carriers ($2,17 \pm 1,94$ vs $2,49 \pm 1,7$ mmol/l mean \pm SD), but the difference was only of a marginal significance (p = 0,092).

There was no difference between vascular patients and age and sex matched controls in the distribution of CETP Taq1B genotypes. Examining CETP 1405V polymorphisms, the number of genotypes carrying V allele was significantly higher (Table 2.).

Taq1B polymorphism	Patients*	Controls
B12	74	75
B22	20	13
B11	15	7

*p= 0,1776

I405V polymorphism	Patients**	Controls
II	44	60
IV	47	29
VV	3	8

** p=0,0425 (OR 1,843, 96% CI 1,036-3,279) /Fisher's exact test/

Table 2. Distribution of genotypes of CETP Taq1B and I405V polymorphisms in vascular patients and control subjects.

There were no differences detected between the CETP I405V polymorphism genotypes in body mass index (BMI), serum cholesterol, triglycerides, HDL-cholesterol, apolipoprotein A-I, A-II and apolipoprotein B concentrations (Table 3.).

I405V polymorphism genotype	II n=44	IV n=47	VV n=3
BMI (kg/m ²)	24,2	25,6	27,5
Koleszterin (mmol/l)	6,26±1,33	6,45±1,55	6,50±0,98
Triglycerid (mmol/l)	2,33±2,29	2,32±1,58	1,66±0,65
LDL-C (mmol/l)	3,9±1,45	4,22±1,41	4,61±1,14
HDL-C (mmol/l)	1,53±0,93	1,30±0,57	1,13±0,35
ApoA-I (g/l)	1,34±0,50	1,39±0,52	1,61±0,46
ApoA-II (g/l)	0,46±0,28	0,39±0,27	0,27±0,09
ApoB (g/l)	1,16±0,43	1,29±0,62	1,14±0,33

Table 3. Lipoprotein lipid and apolipoprotein concentrations in CETP I405V genotypes

The occurrence of factor V Leiden mutation in 100 patients with severe femoro-popliteal atherosclerosis and reoperated due to restenosis was compared to the

occurrence in 445 control subjects. The distribution of homozygote wild, heterozygote and homozygote mutant V genes in patients was found to be significantly ($p = 0,0379$) different compared to that of the control group. Heterozygotes were more prevalent and homozygote Leiden occurred only in patients. Since the mean age of the patients was higher than that of the control group, data were re-evaluated by age-adjusted multiple logistic regression. (Table 4.)

Leiden mutation	Patients n=100*	Controls n=445
0	87 (87%)	411 (92%)
Heterozygote	12 (12%)	34 (8%)
Homozygote	1 (1%)	0 (0%)

* $p = 0,0379$

Table 4. The frequency of Leiden mutation in vascular patients and healthy controls

The odds ratio for Leiden mutation was 1,885 (CI 0,99-5,87, $p = 0,049$). BMI values, serum cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol serum apolipoprotein A-I, A-II, apolipoprotein B concentration, blood glucose, serum creatinine and uric acid level, and serum ALT (SGPT) were compared in vascular patients carrying and those not carrying the Leiden mutation. No significant differences in the above parameters were found between the two groups.

Conclusions

Our observations suggest that the relationship between the apolipoprotein E gene polymorphism and both the process of atherosclerosis and the process and measure of restenosis is only a selective part of a multiple factor process. This relationship presumably depends highly on other risk factors of the given patient and on the localization and type of surgical intervention made on the artery, as well. The results of our investigation emphasize the importance of the detection of $\epsilon 4$ allele in patients with severe atherosclerosis and restenosis in case of reconstructive vascular interventions. The presence of $\epsilon 4$ allele indicates – together with other known risk factors – an increased risk for restenosis after

vascular reconstruction. The recognition of increased risk helps to use specific therapeutic modalities, which may decrease the occurrence of restenosis.

Concerning femoropopliteal atherosclerosis and restenosis, there are no available data regarding nor CETP Taq1B nor I406V genetic polymorphisms. Based on our investigations and observations the common CETP Taq1B polymorphism has no influence on the severity and complications of peripheral vascular disease. It is the case in ischemic heart disease as well, although this fact was confirmed considering also the different, less common haplotypes of CETP gene polymorphisms. The V allele of CETP I405V polymorphism is a proatherogenic risk factor in ischemic heart disease and peripheral vascular disease as well, however, the influence on its risk status of different, rare point mutations has not been recognized yet. It is possible that the further detailed analyses of CETP gene polymorphisms reveal new aspects, which will explain why the cardiovascular risk did increase paradoxically, despite using the effective inhibition of CETP activity by torcetrapib.

In view of the above data we may conclude that the factor V Leiden mutation is not an independent risk factor for atherosclerosis itself, but together with other (genetic as familial hypercholesterolemia, or environmental as smoking) risk factors it does increase the risk and progression of atherogenesis. Our observations proved that it increases the chance of restenoses following arterial vascular reconstruction. The mechanism of its influence can be explained by the increased local thrombus generation first of all. The prolonged administration of low molecular weight heparine in patients with detected Leiden mutation may well prevent restenotic processes after vascular reconstructive surgery. As it is known, the incidence of restenoses after aorto-coronary bypass operation decreased in patinets treated with low molecular weight heparin for several months, despite their other - fully not investigated though - genetic risks.

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