The role and importance of gene polymorphisms in the development of atherosclerosis

Doctor thesis

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Introduction

The development of the atherosclerosis is a multifactorial process, where the clinical pattern is determined by environmental and genetic factors. Except for the classical risk factors of the atherosclerosis (hypertension, lipid-metabolic disorders, diabetes, smoking) the clinical signs can be influenced by the genetic variants (polymorphisms) of the enzymes which are responsible for the endothelial cells function and for the thrombotic factors. Inflammatory answer follows the primary endothelial injury, a generalised process starts, the result is atherosclerotic plack development in the small and large arteries of the organ, finally the occlusion of the artery. Clinical signs of it can be detected, such as myocardial infarction, peripheral occlusion, dysbasia, gangrene. Besides the symptomatic treatment it is important to explore their causes, to know the metabolic, inflammatory processes, the genetic background.

Objective

The purpose of the study was to examine three genetic polymorphisms of the enzymes playing a role in the metabolic processes and to clear their role in the pathology of different illnesses and how they are related to the examined medical groups.

The nitric oxide (NO) produced by the endothelial nitric oxide synthase (eNOS) has an atheroprotective effect. Previous studies have shown, that the eNOS Glu298Asp polymorphism causes low NO production,
which results in hypertension, thrombus development and changes in the vascular tone due to the endothelial dysfunction, and the final outcome is the atherosclerosis. Our aim in this examination was to define eNOS Glu298Asp (GT) polymorphism in healthy, atherosclerotic non-diabetic and diabetic patients. We search for a connection between acute myocardial infarction, stroke, and the polymorphisms of the eNOS 298 Asp/Asp and eNOS 298 Glu/Asp.

The possibility that the high homocysteine level of the plasma can destroy the endothelial cells of the small arteries, which can often be the first step towards atherosclerosis, has been known for a long time. The methylenetetrahydrofolate reductase (MTHFR) C677T enzyme plays an important role in the high plasma homocysteine level and in the folate metabolism. The TT genotype is usually associated with higher homocysteine level, so it is supposed to increase the risk to develop atherosclerosis. In our examination we looked for a link between stroke and myocardial infarction in diabetic and atherosclerotic non-diabetic patients and the occurrence of TT/MTHRF polymorphism.

The TNF-α plays an important role in the pathophysiology of vascular diseases by affecting inflammatory cascade, lipid metabolism, obesity and effect on the insulin resistance. As the degree of the TNF-α expression is highly influenced by genetic factors, it is important to know, how likely atherosclerosis develops in different diseases. The promoter region of this gene contains several polymorphisms, which influences the TNF-α level. In our examination we have set the aim to define the TNF-α 308GA polymorphisms
in atherosclerotic, diabetic and healthy patients. We tried to identify a connection of the frequency of myocardial infarction and stroke in diabetic and atherosclerotic non-diabetic patients.

The aim of the study:
1. eNOS G298T polymorphisms specification in a./ healthy, b./ atherosclerotic diabetic, c./ atherosclerotic non-diabetic patient groups
2. MTHFR C677T polymorphisms specification in a./ healthy, b./ atherosclerotic diabetic, c./ atherosclerotic non-diabetic patient groups
3. TNF-α G308A polymorphisms specification in a./ healthy, b./ atherosclerotic diabetic, c./ atherosclerotic non-diabetic patient groups

Material and methods

In this examination we analysed 992 patients’ data, out of which 608 had been treated at the Cardiovascular Department of the Semmelweis University from November 2003 till June of 2005 period. We compared the data of 348 atherosclerotic non-diabetic patients and 260 diabetic patients with the 384 healthy control samples. We analysed the frequency of myocardial infarction and stroke in the case of different polymorphisms in the atherosclerotic non-diabetic and diabetic group, and it was compared to the healthy group. All the 608 patient was divided into four groups: 1./
atherosclerotic diabetic patients with myocardial infarction, 2./ atherosclerotic diabetic patients with stroke, 3./ atherosclerotic non diabetic patients with myocardial infarction, 4./ atherosclerotic non diabetic patients with stroke. The occurrence of all the hetero- and homozygote variants of the analysed genes were tested in every group, in the relation of the healthy samples. The genotype of the samples was analysed by PCR-RFLP method, and LightCycler real-time PCR melting curve analysis was applied. We analysed the data’s statistically, we used two sides Student t probe, Odds’ ratio, khi-square-probe, average and scattering (SD), graphical presentation. Those difference were significant, where the statistical deviance was p<0,05, and **p<0,005, ***p<<0,001.

![DNA melting curve analysis](image)

1 figure: eNOS 298GT polymorphism specification by DNA melting curve analysis
Results

In this examination the planed aim was reached, positive correlations were proved in every group. It was verified that the lipid (LDL) level is higher in patients who underwent myocardial infarction than those without infarction (4,2 vs. 2,7 SD, p<0,05).

There was no difference in the occurrence of myocardial infarction between the diabetic and atherosclerotic non-diabetic group (39% vs. 38,9%), but the stroke frequency among the diabetic patients was significantly higher (20% vs. 38%, p<0,005).

1. eNOS analysis

We specified the eNOS polymorphisms by DNA melting curve analysis 384 healthy control, 348 atherosclerotic non-diabetic and 260 diabetic patients. A representative DNA melting curve is shown in figure 1, the different genotypes can be identified. Distribution is listed in the table 1. The wild Glu/Glu is GG genotype, the mutant Asp/Asp is TT genotype aminoacide sequence.

We examined the occurrence of the wild GG, the mutant TT and the heterozygote GT allele in different illness-combinations. We verified, that the patient, which is Asp 298 allele homozygote TT, produces less NO, and the endothelial dysfunction can occur often, the risk for atherosclerosis is higher. We justified, that in the case of eNOS 298 mutant TT variant the MI occurrence is significantly higher than the homozygote GG normal or heterozygote GT variant carrying patients. Our tests show the eNOS 298 TT mutant in the control group
occurs 5.7%, among the diabetic patients MI group 14.8% (p<0.005), stroke group 12.9% (p<0.005). In the atherosclerotic non-diabetic patients MI group 16.9% (p<<0.001), in the stroke group 15.09% (p<<0.001), that are all significant difference. At the count of significance we compared the homozygote TT to the homozygote GG frequency.

<table>
<thead>
<tr>
<th>Groups</th>
<th>eNOS298TT Homozygote</th>
<th>eNOS298GT Heterozygote</th>
<th>eNOS298GG Homozygote</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mutant %</td>
<td>Mutant %</td>
<td>Normal %</td>
</tr>
<tr>
<td>1. Control (n=384)</td>
<td>5.7</td>
<td>41.9</td>
<td>52.4</td>
</tr>
<tr>
<td></td>
<td>22/384</td>
<td>161/384</td>
<td>201/384</td>
</tr>
<tr>
<td>2. Diabetes + MI (n=101)</td>
<td>14.8**</td>
<td>44.6</td>
<td>40.6</td>
</tr>
<tr>
<td></td>
<td>15/101</td>
<td>45/101</td>
<td>41/101</td>
</tr>
<tr>
<td>3. Diabetes + stroke (n=108)</td>
<td>12.9*</td>
<td>43.5</td>
<td>43.6</td>
</tr>
<tr>
<td></td>
<td>14/108</td>
<td>47/108</td>
<td>47/108</td>
</tr>
<tr>
<td>4. Atherosclerosis + MI (n=118)</td>
<td>16.9***</td>
<td>49.2</td>
<td>33.9</td>
</tr>
<tr>
<td></td>
<td>20/118</td>
<td>58/118</td>
<td>40/118</td>
</tr>
<tr>
<td>5. Atherosclerosis + Stroke (n=106)</td>
<td>15.1***</td>
<td>48.1</td>
<td>36.8</td>
</tr>
<tr>
<td></td>
<td>16/106</td>
<td>51/106</td>
<td>39/106</td>
</tr>
</tbody>
</table>

1. table: Nitrate oxide synthase (eNOS) 298 Glu/Asp gene polymorphisms distribution in percentage in atherosclerotic diabetic and atherosclerotic non diabetic patients group in the relation of MI and stroke. The mutant eNOS 298TT variant occurs significantly often in MI group than in the control group (*p<0.05, **p<0.005, ***p<<0.001)
The case-control analysis (Odds ratio) shows relative risk. The homozygote TT mutant allele carrying patients in the diabetic group have the risk of MI 3.34 (1.61-6.92), the risk of stroke is 2.72 (1.31-5.66). In the atherosclerotic non-diabetic group the MI risk is higher, 4.56 (2.29-9.08), but the stroke-risks are elevated, also OR 3.74 (1.82-7.71). So high risk affected patients, who were Asp 298 homozygotes (TT) they showed four times higher risk for MI than patients with normal GG allele. One of the important results of our study is that we proved a close connection between Glu298Asp polymorphisms and MI, stroke risk (table 1.)

2. figure: MTHFR C677T polymorphisms DNAmelting curve analysis

We could verify a gene polymorphism on the NOS3 gene, and the Glu298Asp mutation proved to be a
major risk factor for MI. This recognition is important but further confirmation is necessary in other patient groups.

2./ MTHFR analysis

The MTHFR gene polymorphism at 384 control, 260 diabetic, 348 atherosclerotic non-diabetic patients is analysed by melting curve analysis with DNA Light Cycler. Representative sample is shown on the figure 2.

We concluded that in the case of mutant MTHFR C677T variant the MI occurs significantly often. In our sample the MTHFR 677 CT mutant occurs 32,% in the control group. Among the diabetic patients MI group it is 53,5% (p<0,001), in the stroke group 51,8% (p<0,001). Among the atherosclerotic patients MI group the CT comes for in 55,1% (p<0,001), in the stroke patients 53,7%, that is significant difference (p<0,001).

The homozygote TT variant in the diabetic group a bit less but still significantly comes for, so that in the MI group 16,8% (p<0,05), in the stroke group 14,8% (p<0,005). At the atherosclerotic non-diabetic patients group the occurrence is highly significant again, so in MI group 21,2% (p<0,001), in stroke patients 19,8% (p<0,001).

When we exam all the mutant alleles (TT+CT) occurrence in every four patients group in the relation of the normal CC variant, at every count can we get significant high difference (p<001).
<table>
<thead>
<tr>
<th>Groups</th>
<th>MTHFR TT Homozygote Mutant %</th>
<th>MTHFR CT Heterozygote Mutant %</th>
<th>MTHFR CC Homozygote Normal %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control (n=384)</td>
<td>10,9 42/384</td>
<td>32 123/384</td>
<td>57,1 219/384</td>
</tr>
<tr>
<td>2. Diabetes + MI (n=101)</td>
<td>16,8* 17/101</td>
<td>53,5*** 54/11</td>
<td>29,7 30/101</td>
</tr>
<tr>
<td>3. Diabetes + Stroke (n=108)</td>
<td>14,8** 16/108</td>
<td>51,8*** 56/108</td>
<td>33,4 36/108</td>
</tr>
<tr>
<td>4. Atherosclerosis +MI (n=118)</td>
<td>21,2*** 25/118</td>
<td>55,1*** 65/118</td>
<td>23,7 28/118</td>
</tr>
<tr>
<td>5. Atherosclerosis +Stroke (n=106)</td>
<td>19,8*** 21/106</td>
<td>53,7*** 57/106</td>
<td>26,5 28/106</td>
</tr>
</tbody>
</table>

2. table: MTHFR C677T gene polymorphisms distribution in percentage in atherosclerotic diabetic and atherosclerotic non-diabetic patients group in the relation of MI and stroke. The mutant MTHFR 677 CT heterozygote variant occurs significantly often in every group, but the MTHFR 677TT homozygote in atherosclerotic non-diabetic group comes for significantly often in MI and stroke patients group than the control group (*p<0,05,**p<0,005, ***p<<0,001)

In our study we can establish, that the MTHFR C677T allele is the risk factor of stroke. Also relevant difference can be proved for the MI risk at the patients (2. table).

The sample number of this study is not enough to verify the hypothesis, that MTHFR genotypes influences
the development of the coronariasclerosis, more and larger studies are necessary to exam connection of the genetic and environmental factors. In this study we can only establish that the MTHFR C677T polymorphism and the MI and stroke frequency have a significant connection. The case-control analysis the homozygote TT mutant allele carrying patients in the diabetic group shows the risk of MI 2,95 (1,51-5,79), the risk of stroke 2,31 (1,18-4,52). In the atherosclerotic non-diabetic group the MI risk is higher, 4,65 (2,48-8,72), but the stroke-risk is elevated, also OR 3,91 (2,04-7,49). In the case of the heterozygote CT variant in the atherosclerotic diabetic group the risk of MI is 3,21 (1,95-5,25), and of stroke OR 2,79 (1,72-4,43). Carrying CT allele in the atherosclerotic non diabetic group the risk of MI is 4,13 (2,52-6,76), and on the other hand of stroke OR is 3,62 (2,19-5,97)

3./ TNF-α analysis
In our study we can establish that the mutant TNF-α AA allele carried patients has a significant higher risk for cardiovascular events than in the control group (3 table). We could verify that in the healthy control samples the wild TNF-α 308 GG genotype occurrence is 76,7 %, the heterozygote (308 GA) has 21,7%, while the mutant homozygote (308 AA) genotype comes for only in 1,6% (3 table). Our dates shows that diabetic patients MI group (2. examined group) the mutant 308 AA frequency elevated to 9,2% (p<0,05). In the 3. examined group the TNF-α genotype distribution are the followed:
the AA homozygote high-risk mutant in 7,7% (p<0,005), while the GA homozygote in 30,7%, against in the control groups 61,6%. At the patients, who are carried the A allele, the TNF-α level is higher, and this has an elevated risk for cardiovascular events. In the 4. and 5. examination group are the atherosclerotic non-diabetic patients, the MI and the stroke patients. We can establish, that the AA genotype occurrence in this groups are higher (10,7%) (p<0,005), than in control groups (1,6%).

<table>
<thead>
<tr>
<th>Groups</th>
<th>TNF-α Homozygote Mutant 308 AA %</th>
<th>TNF-α Heterozygote Mutant 308 GA %</th>
<th>TNF-α Homozygote Normal 308 GG %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control (n=18)</td>
<td>1,6 3/184</td>
<td>21,7 40/184</td>
<td>76,7 141/184</td>
</tr>
<tr>
<td>2. Diabetes + MI (n=65)</td>
<td>9,2* 6/65</td>
<td>26,2 17/65</td>
<td>64,6 42/65</td>
</tr>
<tr>
<td>3. Diabetes + Stroke (n=65)</td>
<td>7,7** 5/65</td>
<td>30,7 20/65</td>
<td>61,6 40/65</td>
</tr>
<tr>
<td>4. Atherosclerosis + MI (n=75)</td>
<td>10,7** 8/75</td>
<td>28 21/75</td>
<td>61,3 46/75</td>
</tr>
<tr>
<td>5. Atherosclerosis + Stroke (n=45)</td>
<td>11,1* 5/45</td>
<td>28,9 13/45</td>
<td>60,0 27/45</td>
</tr>
</tbody>
</table>

3 table: TNF-α gene polymorphisms distribution in percentage in diabetes and atherosclerotic non diabetic patients group in the relation of MI and stroke (*p<0,05, **p<0,005 vs. control)
The heterozygote GA allele occurrence is elevated at atherosclerotic patients (28%, 28.9%), in contrast to control groups, where this allele is only 21.7% comes for.

At the Odds Ratio examination we found an extreme high risk for MI and stroke in the case of TT mutant allele carry. Among the atherosclerotic non-diabetic patients the MI risk is OR 6,714 (1,725-25,553), the stroke realisation is OR 6,714 (1,725-25,553). Among the atherosclerotic patients the MI risk is high, OR 8,173 (2,242-29,559), while the stroke prevalence is the highest OR 8,703 (2,152-34,988).

Conclusion

The study has the following new statements:

1./ Carrying the eNOS Glu298Asp mutant homozygote TT variant the MI occurrence is significantly higher to the heterozygote GT and normal GG variant (TT allele: control group 5.7%, MI group 16.9%, p<0.001, OR: 4.56).

2./ In the case of MTHFR 677 CT heterozygote variant the MI prevalence is much higher (CT genotype: control group 32.4%, MI group 55.1%, p<0.001, OR: 4.13), and having homozygote TT genotype the difference is still significant (TT allele: control group 10.9%, MI group 21.2%, p<0.001, OR: 4.65).
Among the TNF-α AA allele carried patients the risk of cardiovascular events is significant high (AA allele: control group 1.6%, MI group 10.7%, p<0.005, OR: 8.17).

Screening the endangered or genetically high risk groups is to be considered on the long run - an early detection of a susceptibility of the disease gives better chances for prevention and treatment. Understanding the inflammatory mechanisms of the atherosclerosis gives new therapeutic targets to pharmacologists. Today is available therapeutic possibility the statins, they has anti-inflammatory, antithrombotic and plack-stabilisatory effects. The thrombocyte antiaggregations medicines like acetylsalicylic acid and clopidogrel take part in the anti-inflammatory treatment. Certain cases the high level of plasma homocystein can be reduced with giving acidum folicum. Successful clinical trials were performed to accomplish an affective substance molecule that based on a pharmacophore model. They could identify in different pathomechanisms (tumor, inflammation) leader molecule against validated target molecule.
List of publications

I. Papers in the theme of the study:


II. Papers independent from the study:


III. Bookchapter:


IV. Citable abstracts:


2. Laczkó Á., Nemes B., Simonffy Á., Hüttl K., Bérczi V., Szabó G.V., Turbók E., Acsády Gy.: Streptolysissel szerzett tapasztalataink acut artériás és vénás occlusiok esetében. Érbetegségek, 1999: (suppl.1), 40


4. Szabó G.V., Laczkó Á., Entz L., Windisch M., Brázda E., Acsády Gy.: Carotis műtétek után
kialakult acut neurologiai deficites betegek utánvizsgálata Érbegekégek, 1999: (suppl.2), 23


10. Szabó G.V., Bíró G., Szeberin Z., Acsády Gy.: Végtagmentés homograft erek felhasználásával. Érbegekégek, 2007: (suppl.2), 27


17. Szabó G.V., Daróczy J., Acsády Gy.: Autolog össejtkezelés inoperábilis perifériás típusú
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