ORIGINAL ARTICLE

Resistance to acetylsalicylic acid in patients after ischemic stroke

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KEY WORDS

PFA-100, platelet aggregation, resistance to ASA, stroke

SUMMARY

INTRODUCTION Acetylsalicylic acid (ASA) due to its antiplatelet action is used in ischemic stroke therapy. The platelet response to ASA shows an interindividual variation. Decreased platelet sensitivity to ASA is termed as resistance to ASA.

OBJECTIVES The aim of the study was to assess the prevalence of resistance to ASA in stroke patients and discover dependence between resistance to ASA and stroke recurrence and certain genetic and environmental factors.

PATIENTS AND METHODS 59 patients aged 22–83 years (mean age: 53) who had ischemic stroke within the period of 1 month to 10 years prior to the study were analyzed. 51 patients received ASA in a daily dose of 75 mg, and 8 in a higher dose. ASA had been taken since the stroke episode. Resistance was analyzed using the PFA-100 and optical aggregometer, with adenosine diphosphate, collagen and arachidonic acid as platelet agonists.

RESULTS Resistance to ASA in patients after stroke is observed with frequency ranging from 9% in arachidonic acid-induced aggregometry to 65% in the PFA-100. There were correlations between platelet aggregation in response to various agonists ($r = 0.37–0.77$, $p \leq 0.005$), and between collagen-induced aggregation and the PFA-100 ($r = –0.33$, $p = 0.016$). Platelet aggregation induced by arachidonic acid ($r = 0.39$, $p = 0.029$) correlated with the stroke recurrence ($n = 12$). ASA resistance detected in aggregometry in response to collagen was more common in patients with 807CT genotype for Ia glycoprotein ($p = 0.05$), and in patients with diabetes ($p = 0.039$).

CONCLUSIONS In patients after ischemic stroke resistance to ASA is commonly observed. In patients with diabetes or C807Tglycoprotein Ia gene CT polymorphisms this phenomenon is more frequently detected.

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INTRODUCTION Acetylsalicylic acid (ASA) is a first-line drug used in ischemic stroke prophylaxis and therapy. Its partial effectiveness can be caused by the so-called resistance to ASA, which definition indicates to insufficient platelet activity inhibition, measured by aggregometry, platelet function analyzers (PFA-100, Ultegra RPFA, PlaCorPRT), or flow cytometry. A precise definition based on platelet cyclooxygenase-1 (COX-1) activity maintenance is recommended. Constant thromboxane A_2 synthesis (TxA_2) is probably the main mechanism of resistance to ASA.

The causes of resistance to ASA involve decreased drug bioactivity, drug misuse and its insufficient dose, simultaneous use of other anti-inflammatory drugs, increased platelet activity[1] and their increased formation. Hyperlipidemia, hypercoagulability and TxA_2 biosynthesis with cyclooxygenase-2 (COX-2), cyclic superoxides, G_2 and H_2 prostaglandins (PGG_2/PGH_2) migration from epithelial cells to platelets, and smoking, catecholaminemia, physical exercise, emotional stress, oxidative stress and isoprostane biosynthesis are also of some importance in the occurrence

TABLE 1 Characteristics of the study group (n = 59)

Variable	Number of individuals
Age	Mean age 53 years (min. 22, max. 83)
Women	33
Diabetes	7
Hypertension	26
Smoking	19
Hyperlipidemia	18
Stroke recurrence	12
Myocardial infarction in anamnesis	9
A family history of ischemic stroke	35
Statin intake	12

of resistance to ASA, and multiple genetic factors, in particular polymorphisms in arachidonic acid pathway genes, i.e. polymorphisms of single nucleotides of COX-1, COX-1 gene – A842G and C50T are considered in the pathogenesis of ASA resistance. Platelet receptor glycoprotein (GP) IIb/IIIa polymorphism can also reduce eventual ASA effectiveness. C807T platelet GPIa polymorphism may influence the density of this receptor and in consequence change the speed of platelet adhesion to type I collagen.

Sparse and ambiguous information about the occurrence, genetic and clinical predisposition and methods of resistance to ASA accompanying stroke diagnosis were the reasons for undertaking a multivariable analysis of this phenomenon.

The aim of the study was to assess frequency of resistance to ASA based on our patients with a history of ischemic stroke, and compare frequency of resistance to ASA detected with various analytic methods. We also assessed an association of ASA resistance with 807C/T polymorphism of GPIa, plasma homocysteine levels, and certain individual characteristics (age, sex, body mass index [BMI]), smoking, a family history, more than one ischemic stroke in anamnesis at the time of blood sampling (stroke recurrence), hypertension, diabetes, hyperlipidemia, statin intake as well as duration and doses of ASA.

PATIENTS AND METHODS

We studied 59 patients (33 women and 26 men aged 22–83 years [mean age: 53 years]), after the stroke diagnosed according to the World Health Organization definition within the period of 1 month to 10 years prior to the study. The stroke lesion was documented with computed tomography or magnetic resonance. Stroke was classified according to the Oxfordshire Community Stroke Project: anterior circulation infarction involving the whole middle cerebral artery (MCA) territory – 8 individuals, anterior circulation infarction involving part of the MCA territory – 9 individuals, posterior circulation infarction – 16 individuals; in the remaining individuals lacunar stroke was diagnosed or it was not clearly established. 51 patients received ASA in doses of 75 mg/24 h, in 8 individuals doses were higher (100–325 mg/24 h). The intraplatelet malonyldialdehyde (MDA) level reflecting the ASA intake was <10.8 μmol/10^9 platelets in all patients. Patients did not receive thienopyridines or receptor GPIIb/IIIa inhibitors within 1 month prior to the study. Inclusion criteria were: myocardial infarction within 1 month prior to blood sampling, platelet count <100 G/l, hemoglobin levels <10 g/l and creatinine levels >2.5 mg/dl. The occurrence of at least 2 ischemic strokes (second or next during the ASA intake) before entry to the study was defined as ischemic stroke recurrence. The detailed characteristics of the study group were shown in TABLE 1.

Platelet aggregation with the Born method was examined using the 2-channel optical aggregometer (model 490-2D by Chronolog Company with Agrolink software for Windows system) in platelet-rich plasma with agonists: adenosine diphosphate (ADP) at a level of 3.5 μM and 5 μM, collagen at a level of 2 μg/ml and arachidonic acid at a level of 0.6 mM, and its intensity was presented as a percentage. According to other authors[2,3] resistance to ASA was diagnosed when aggregation was in response to collagen >60%, to 3.5 μM ADP >60%, to 5 μM ADP >80%, to arachidonic acid >20%.

In platelet function analysis using the PFA-100 analyzer (Dade Behring), the closure time of an aperture that pierces a membrane coated with collagen and epinephrine by the platelet thrombus was measured. If closure time was not prolongted (≤165 s), resistance to ASA was diagnosed.[4]

To assess the ASA use objectively, the intraplatelet MDA level in platelet-rich plasma was determined using the method by Paton.[5] MDA levels <10.8 μmol/10^9platelets indicated ASA-inhibited prostaglandin synthesis in platelets.

Genetic analysis of C807T GPIa polymorphism with the allele-specific PCR/RFLP method according to Santoso et al.[6] was performed. The total plasma homocysteine level was analyzed using the high performance liquid chromatography method according to Maansoor et al.[7]

Statistical analysis The statistical analysis was made with the use of the CSS-Statistica program. Quantitative features were characterized by specification of the mean value, median, minimal, maximal and standard deviations. Significance of differences for unrelated samples was estimated with the Mann-Whitney and Kruscall-Wallis tests. Associations between the variables was analyzed with Pearson test. Associations between qualitative variables were tested with the χ^2 and Fisher's test. Correlations between 2 quantitative variables was assessed with the Spearman rank coefficient. The results were considered significant if a $p < 0.05$.

RESULTS

A percentage of individuals resistant to ASA in each test was shown in TABLE 2. It

TABLE 2 Frequency of resistance to ASA in patients after ischemic stroke in optical aggregometry and PFA-100 according to the accepted criteria

Frequency of resistance to ASA in patients after ischemic stroke (n = 59)				
Optical aggregometry				PFA-100
ADP 3.5 μmol/l	ADP 5.0 μmol/l	Collagen 2 μg/ml	Arachidonic acid 0.6 mmol/l	
n = 21 (36%)	n = 9 (15%)	n = 30 (51%)	n = 5 (9%)	n = 38 (65%)

Abbreviations: ADP – adenosine diphosphate, ASA – acetylsalicylic acid

was estimated between 9% in optical aggregometry in response to arachidonic acid to 65% in the PFA-100.

Relationships between laboratory tests and clinical features were shown in TABLE 3. There were positive associations between individual aggregometric tests and between collagen-induced aggregation and results of the PFA-100. There was also the relation between diabetes and resistance to ASA detected based on collagen-induced platelet aggregation (p = 0.039).

In the group of patients after stroke the frequency of CC genotype of GPIa gene was 51.9%, CT genotype – 42.6%, and TT genotype – 3.7%. There was an association between resistance to ASA in collagen-induced aggregation and the 807CT genotype (compated to CC) of GPIa gene (p = 0.05, TABLE 3). Resistance to ASA was observed in 23.3% of patients with CC genotype and in 16% of patients with CT genotype. A statistically significant relationship was found between stroke recurrence and resistance to ASA shown in arachidonic acid-induced platelet aggregation test (r = 0.39, p = 0.029, TABLE 3).

There were no differences in mean plasma homocysteine levels between analyzed subgroups.

In all patients decreased MDA values (<10.8 μmol/10^9 platelets) reflecting platelet blocking by ASA were observed.

A statistically significant relationship between results of platelet aggregation tests and hypertension, smoking, a family history of ischemic stroke, myocardial infarction and the statin intake was not found. Similarly, there were no associations between ASA resistance and age, sex and BMI.

DISCUSSION The highest percentage of patients with resistance to ASA was observed in the PFA-100 test. Among the performed studies arachidonic acid-induced platelet aggregation appeared to be the most useful laboratory test in the assessment of resistance to ASA, because it was the only study showing the association with recurrence of ischemic stroke. However, the relationship of resistance to ASA with other clinical parameters (age, sex, body, smoking, past myocardial infarction, diabetes, hypertension, statin use) and a family history was not confirmed. A relationship between resistance to ASA and the homocysteine level and C807T platelet GPIa polymorphism was not found either.

The occurrence of resistance to ASA in the analyzed group was affected by the used diagnostic method and was estimated between 9% (aggregation in response to arachidonic acid) and 65% (in the PFA-100 test). For comparison, according to Gum et al. resistance in patients with acute coronary syndromes analyzed with the optical agregometry method at a dose of 325 mg of ASA was 5.5%, according to Helgason et al. at the same dose and method resistance was 25%, and according to Szczeklik, ranged 6 to 24%.[8-11] Resistance in the PFA-100 test was according to Gum et al. 9.5%, according to Grundmann – 34%, and according to Chakorun et al. – 50%.[8] Such discrepancies in findings may result from the fact that platelet function tests are only partially dependent on the amount of synthesized TxA_2.[12] This was confirmed by Gonzalez-Conejro et al. and Eikelboom et al., who did not observe significant relationships between *ex-vivo* platelet aggregation tests and TxB_2 synthesis.

Our results suggest that the term resistance should be addressed using a critical approach because the definition of laboratory resistance was developed on the basis of platelet activation tests, only partially dependent on metabolic pathways associated with COX-1.[13,14] The partial inhibition phenomenon can be interpreted ambiguously: ASA does not cause complete COX-1 inhibition, or despite COX-1 inhibition platelet activation is still observed. In the first case no drug action is observed, which is common in clinical practice and may occur concomitantly with resistance to other antiplatelet drugs. The second case indicates to COX-1-independent platelet activation mechanisms, which unable platelets inhibition by ASA in *ex-vivo* tests.[15]

Relationship between the frequency of diabetes and resistance to ASA in aggregation test with collagen as agonist has been shown in the current study. There is no doubt that hyperglycemia alters platelet reactivity. Moreover, it affects the main mechanism of ASA action (competition between acetylation and glycation).[5,12] Glycation may change the effects of cyclooxygenase inhibition and influence functions of other enzymes and receptor proteins.

Despite the ASA intake in 20% of the analyzed patients the recurrences of ischemic stroke were observed. Other authors found that the therapy with ASA in prevention from new strokes ended in failure in 8–18% of patients.[15] A positive relationship between the frequency of stroke occurrence, the grade of neurological deficit and the worse clinical course is commonly observed in resistance tests using aggregation

TABLE 3 Relationships between laboratory tests and clinical features

		Platelet aggregation				PFA-100	Type 2 diabetes	C807T polymorphism	Recurrence of stroke
		ADP 3.5 μmol/l	ADP 5 μmol/l	Collagen	Arachidonic acid				
Platelet aggregation	ADP 3.5 μmol/l								
	ADP 5 μmol/l	r = 0.77 p = 0.0001							
	collagen	r = 0.65 p = 0.0001	r = 0.61 p = 0.0001						
	arachidonic acid	r = 0.37 p = 0.005	r = 0.38 p = 0.004						
PFA-100				r = −0.33 p = 0.016					
Type 2 diabetes				Test χ^2 value = 4.27 p = 0.039					
Glycoprotein Ia gene C807T polymorphism				Test χ^2 value = 1.00 p = 0.05					
Recurrence of ischemic stroke					r = 0.39 p = 0.029				

Abbreviations: see **TABLE 2**

with arachidonic acid.[16] The current study showed a similar dependence between the results of arachidonic acid-induced platelet aggregation test and the recurrence of stroke. Some authors treat with reserve the issue of recognizing resistance as a phenomenon of clinical importance, while in many studies a relationship between laboratory resistance to ASA with increased risk of neurological and cardiac ischemic incidents was described.[17]

The association of resistance to ASA with CT and TT genotypes of GPIa has been confirmed in collagen-induced platelet aggregation. GPIa is a collagen receptor. The GPIa coding gene is located on chromosome 5 and has at least 8 polymorphisms. In patients with the 807T allele, high density of $\alpha_2\beta_1$ receptor on the platelet surface was detected. The $\alpha_2\beta_1$ integrin is one among few platelet receptors for collagen, and the α_2 gene has 3 alleles. Santoso et al. were the first to establish a relationship between the 807T allele and myocardial infarction.[6] The risk of disease (odds ratio = 3.3 [1.23–8.83; p = 0.02]) was observed only in homozygotes, and the number of control cases was limited. It indicates to the possibility of the prothrombotic tendency in individuals with TT genotype. Reports on the influence of this polymorphism on the relationship between aortic media thickness and platelet activation are available.[18,19] The existence of relationship between allele T occurrence and higher frequency of myocardial infarction and stroke episodes, especially in young individuals, was also confirmed in available data; and it was demonstrated that the younger the age, the higher the frequency of allele T occurrence associated with myocardial infarction/stroke.[19]

According to other authors, about 9% of patients do not follow recommendations regarding the use of ASA, which they confess too. Schwartz et al.[12] showed that the analysis performed after the direct administration of 325 mg ASA in presumed resistant patients during its chronic use caused their sensitivity to ASA. In order to confirm ASA use by the patients participating in the current study, the MDA level was measured, because its decrease indirectly speaks for arachidonic acid metabolism inhibition in platelets, catalyzed by cyclooxygenase. Platelet cyclooxygenase inhibition resulting in the MDA level decrease in platelets was found in all study patients.

Resistance to ASA was not associated with age, past myocardial infarction and hypertension coexistence in our patients. Age-related, sex-related and BMI-related resistance to ASA was not found.

The PFA-100 test using a cuvette with collagen and epinephrine, in which resistance to ASA was 65%, did not show the correlation with recurrence of ischemic stroke. The values obtained in the present study have no reference to TxA_2 synthesis assessed on the basis of the urinary excretion of 11-dehydrated thromboxane B_2 (TxB_2) in other studies. Therapy with ASA inhibited TxB_2 formation in the same grade as in patients with short and long closure time of opening by the platelet embolus. On the contrary, other authors consider closure time determination in the PFA-100 test for a good, precise and effective method for resistance to ASA detection,[20] although there are also critical reports.[8,21] The obtained percent of resistance in the PFA-100 test is estimated in other studies at 9.5–50%.[8,21]

A relationship between smoking and increased frequency of resistance to ASA occurrence and

recurrence of ischemic stroke was not observed in the present study. 33% of patients who entered the study were smokers. Smoking increases the number of free oxygen radicals[22,23] and the harmful effects of smoking results from oxidative damage to crucial biological substances (DNA, low-density lipoproteins [LDL] cholesterol fraction, antiproteases). In smoking individuals increased urinary levels of pro-aggregative isoprostane 8-epi prostaglandin $F_{2\alpha}$ and TxB_2 are observed.[22]

The increased homocysteine plasma level is recognized as a risk factor for cardiovascular and neurodegenerative diseases development and an independent risk factor for brain stroke.[7,24-26] The mechanism of prothrombotic and pro-atherosclerotic action of homocysteine is very complicated and not well known.[27] There are reports about the aggregative influence on platelets through decreasing ADPase activity, fibroblast stimulation and oxidation of some lipoproteins (LDL in particular) and the influence on lipoprotein activity. Its increased plasma level predispose arterial and venous thrombosis.[28-31] The data obtained in the current study showed a statistically insignificant increase in the homocysteine level in patients after ischemic stroke compared with the control group. However, a statistically significant increase in the homocysteine level in patients with resistance to ASA and recurrence of ischemic stroke was not demonstrated.

Because of a large scale of resistance to ASA occurrence the use of other antiplatelet drugs in ischemic stroke prophylaxis should be considered. There is an individual variation in a response to platelet inhibition by new-generation antiplatelet drugs.[24,32-34] Studies carried out in Hungary on the group of 1264 patients with vascular diseases showed a high percentage of resistance to different antiplatelet drugs – 49% in the case of ASA in a dose of 100 mg/24 h, 33% in a dose of 325 mg/24 h, 21% in individuals receiving 500 mg of ticlopidine, and in 31% of those treated with clopidogrel in a daily dose of 75 mg. It was demonstrated that the combination of clopidogrel with ASA in patients with acute coronary syndromes causes a 20% decrease in the frequency of complex outcome occurrence including the ischemic incidents.[35,36] Published results of the randomized clinical trial ESPRIT (European/Australia Stroke Prevention in Reversible Ischaemia Trial), performed on group of 2739 patients from 15 countries, showed that antiplatelet therapy with ASA and dipirydamol is more effective than ASA alone in secondary brain stroke prevention.

The current study has demonstrated that the frequency of resistance to ASA in patients after ischemic stroke is dependent on the laboratory method used. The results of aggregation tests with use of the majority of agonists correlated with each other. Arachidonic acid-induced aggregation test should be considered, as its results demonstrated the association with the recurrence of ischemic stroke. The aggregation with collagen showed a relationship between the occurrence of type 2 diabetes, C807T GPIa gene polymorphism and the PFA-100 method. Resistance to ASA is also more commonly observed in patients with diabetes or C807T GPIa gene polymorphism.

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