The role of complement system in the pathomechanism and outcome of ischemic stroke

Doctoral theses

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Budapest
2016

1. Introduction

Cardiovascular diseases are the leading causes of morbidity and mortality in Hungary. The incidence is growing with age, the mortality rate is high. The survivors often live their lives with serious disabilities. The preventive and definitive, curative therapeutic possibilities result in limited effectiveness nowadays. Cerebrovascular diseases are manifested as stroke in most of the cases. A stroke can be defined as a sudden attack of the neurological deficiency caused by a focal vascular reason. Consequently, the definition is based on clinical features proven by other diagnostic tools – such as imaging. The object of our current study is the ischemic type of stroke. However, the initial treatment of stroke does not usually depend on etiology, the identification of the cause and risk factors are essential concerning the secunder prevention. To focus on atherosclerosis is particularly important, since it can be identified by simple diagnostical methods applied in the daily clinical practice. In this aspect, the secunder preventional strategies are proved to be successful. According to the international professional recommendation, the increased intima-media thickness (IMT) of carotis communis arteries (CCA), as the objective sign of

atherosclerosis, can be detected by carotis duplex ultrasound and as the stroke predictor, it can be identified as a causative phenomenon. The detection of IMT by duplex sonography is a generally accepted risk marker of the stroke. The measurement of carotis IMT is an appropriate method in order to aggregate identification of more risk factor exposition. The increased IMT (≥1.0 mm) shows the high risk of stroke and/or coronary disease, particularly in younger patients. Inflammatorical processes play direct and indirect roles during the pathogenesis of stroke. The complement system is activated by the tissue damage. The activation is one of the pathophysiological events, by that the ischemic - reperfusional damage is resulted in an ischemic stroke. Other neuroinflammatorical processes are known during the development of ischemic stroke. The processes related to CRP molecule have been investigated most frequently. The complement system is a cascade system of the protein components circulating in an inactive form in the blood and other body fluids, chain reacted with each other by the appropriate triggers. As the main effector system of natural (innate, not-specific) humoral immune response, they provide the connection between the natural and adaptive immunity. The activation of the complement system can be derived in three

different pathways: classical, alternative and lectin-induced pathway. The process of complement activation is created by the receptors that are able to recognize and connect to Pathogen-Associated Molecular Patterns (PAMPs). Pattern-Recognition Receptors (PRRs) can be found on many protector cells and macromolecules (for example: macrophages, dendritic cells, lectins, C1q, C3, CRP, etc.) Every pathway leads to connection of C5b fragment and C6, C7, C8, C9 proteins by limited proteolysis of C3. The C6, C7, C8 and C9 proteins create the Membrane Attack Complex (MAC), which produces pores in the lipid membrane of cells covered by antibodies, resulting in cell lysis. The lectin activation pathway is called by its initiators, the lectins, such as Mannan-Binding Lectins (MBL) or ficolins, which produces C3-convertase recognizing PAMPs by the pattern recognition molecules (PRMs) and creating complexes with the MBL-Associated Serine Proteases (MASPs).

The C1-esterase inhibitor molecule (C1-INH) supresses proteases of 4 enzyme systems, such as the system of complement system, kinin-kallikrein, coagulation and fibrinolysis. The regulator molecule of the classical and lectin-induced pathways of the complement activation suppresses the

classical pathway by connecting to C1r and C1s and the lectin-induced pathway by the inhibition of MBL-MASP-1/-MASP-2 complex. Among the 4 phenotypes of the hereditary angioedema: type I is based on the decreased production of C1-INH, type II is related to the expression of dysfunctional C1-INH. The classical and lectin-induced pathways are down regulated in cases of C1-INH-HAE disease.

2. Objectives

2.1. Does the complement system play a role in the pathogenesis of atherosclerosis by C1-INH - its regulator molecule?

According to our knowledge, the possible effect of C1-INH - as the regulator of the complement system -, and of the lack of C1-INH have not been investigated concerning the formation process of atherosclerosis, yet. One of my objectives was to detect the clinical presence of atherosclerosis in patients suffering from C1-INH by determination of IMT values in CCA vessels. Then, I examined the role of the complement system in process of atherosclerosis by indirect methods.

2.2. Does the complement activation play an important role in the damage of brain tissue/pathophysiological processes following acute ischemic stroke?

The aim of our study was to determine whether the plasma concentrations of traceable complement activation products are higher in patients suffering from ischemic stroke, then in the controls. Furthermore, we intended to determine the correlation among the levels of complement activation products and the severity of clinical status and the outcome of stroke. The roles of the 3 different pathways (classical, alternative, lectin-induced) of the complement activation were also investigated.

2.3. Does the lectin activation of the complement system play any role in pathophysiological processes following an acute ischemic stroke by MBL and ficolin connections to different ligands?

The relevance of MBL regarding the involvement of lectin pathway has been introduced in ischemic stroke by several studies, nevertheless the role of ficolin molecules have not been analysed yet, consequently, my research focused on these two proteins – ficolin-2, and ficolin-3.

3. Methods

3.1. 57 adult patients cared by the 3rd Department of Internal Medicine of Medical Faculty at the Semmelweis University were involved in our study. The examined patient group consisted of 35 females and 22 males in 16-75 years. 32 patients received durable danazol therapy with a daily doses of 33-200 mg, in line with the Budapest protocol, in order to provide long-term prophylaxis of oedematous episodes. The duration of follow-up was between 20-288 months. The positive control group consisted of 25 patients that had not received long-term danazol prophylaxis. The classification of disease severity based on the “Predicting preventing and treating attacks in patients with hereditary angioedema” (PREHEAT) methods during the first year of the treatment. 20 healthy volunteers created the negative control group – 12 females and 8 males in 25-65 years, the median age was 38.67 years.

We detected anthropometric values (weight, height) and were interested in risk factors (former and current smoking habits, work stress), the highest level of education and the family history (EuroQoL-5D and EQ-VAS as a standardised tool for

use to measure of health outcome with visual analogue scale). We examined all patients by carotis duplex ultrasound. The carotid artery, internal and external carotid artery were examined on both sides with standard methods in spectral measurement of doppler angle between 55°and 60°. The IMT values were identified by Atherosclerosis Risk in Communities Study (ARIC) protocol. Every statistical analysis has two endpoints and $p<0.05$ value was the significant level.

3.2. 28 patients suffered from acute ischemic stroke and admitted to the Neurological Department of Kútvölgyi Clinical Unit of Medical Faculty at Semmelweis University were involved in our study according to the following inclusion criteria: presence of clinical symptoms of acute ischemic stroke, age of 18, more than 3 hours after the appearance of the specific symptoms, cerebral bleeding was not detected by acute CT imaging.

The exclusion criteria were as follow: TIA, acute myocardial infarct in the last 6 months, sepsis/serious acute infection, systemic autoimmune disease, or malignancy in the anamnesis. Finally, 26 patients (16 females and 10 males, in 58-87 years) consisted of the subjects of our study. The severity of stroke in admission and on the 6th days thereafter were evaluated by

neurologist, according to the NIH scale, the functional negative symptoms were analysed by Barthel index and modified by Rankin scale. The patients were classified by clinical symptoms and imaging results and divided into two groups: serious or mild stroke groups.

Blood samples were taken continuously in admission and 24th, 48th, 72th hours and 6 days thereafter. The control group consisted of 26 patients (10 females and 16 males, in 41 -84 years) that suffered from symptomatic or non-symptomatic, serious carotis atherosclerosis (the median stenosis of internal carotid artery: 80%-interquartile range: 80-90%). The alleles of MBL gene (MBL2) were determined by polymerase chain reaction (PCR). The determination of C4d, C3a, SC5b-9 complement activation products, S100B and C1rC1sC1-INH complex were identified by ELISA kit. Every statistical analysis has two endpoints and $p<0.05$ value was the significant level.

3.3. Patients suffered from acute ischemic stroke and admitted to the Neurological Department of Clinical Centre of Medical Faculty at University of Pécs (19 females and 20 males, summarized 39 patients in 49-84 years and to the Neurological Department of Kútvölgyi Clinical Unit of Medical Faculty at

Semmelweis University (16 females and 10 males, summarized 26 patients in 58-87 years) were involved in our study.

Neurological imaging tests were performed for every patient – brain MRI for most of the patients, CT for the others. The severity of stroke was determined by NIH scale, in admission. The blood samples were also taken in admission – the median time was 7 hours after the appearance of the symptoms in the Budapest cohort, 8.5 hours in the cohort from the city of Pécs – and the follow-up samples were taken in 72 and 96 hours later. The outcome of the disease was evaluated by modified Rankin scale. The controls were consisted of serum samples of 100 healthy volunteers and 134 patients suffering from significant atherosclerosis. The concentrations of ficolin-2 and ficolin-3 were detected by ELISA method in Molecular Medicine Laboratory of Righospitalet Clinical Immunology Department in Copenhagen. The human S100B concentrations were measured by ELISA method in the 72th hour. The connection between the serum concentrations of the selected proteins and the outcome of stroke was analysed by multiple logistic regression adjusted to the gender and the age of the patients.

4. Results

4.1.1. A significantly higher BMI was detected in the patients treated with danazol compared to the untreated C1-INH-HAE patients and to the healthy controls. Lower summarized scores of EuroQol-5D and of the EQ-VAS were measured in the C1-INH-HAE patients regardless from the danazol treatment compared to the healthy controls. The C1-INH-HAE patients, regardless from danazol treatment, did not differ from each other and from the healthy control group concerning the risk factors of atherosclerosis.

4.1.2. All the average IMT-values were in the normal range (<1,0mm). We did not find any significant differences among the average IMT-values ,neither the danazol treated and the untreated C1-INH-HAE, nor the healthy control group.

We measured the elevated cholesterin-concentration in the danazol treated patient group, with no significant difference in triglyceride-values. A significantly increased LDL-, and decreased HDL-levels were detected in the danazol treated patient group compared to the untreated and to the healthy controls. Significantly elevated CK, ALT, creatinine, LDL and lower HDL-values were measured in the treated patient group.

Elevated RBC-number, hemoglobin and hematocrit concentration were found in the treated patient group. The CRP-levels were slightly higher compared to the control group.

4.2.1. SC5b-9 and C4d concentrations were significantly higher in stroke-patients than in the control group. While the stroke-patients were significantly older than the controls, the difference between the two groups was analysed by multiple logistic regression. The difference of SC5b-9 concentrations remained significant after the adjustment to the age and gender, however, the difference of C4d lost the significance. The level of SC5b-9 showed a significant correlation with the severity of stroke, the measure of the neurological deficiency and the functional disability.

4.2.2. Samples taken in 24^{th} and 48^{th} hours of the 22 examined patients among 26 patients reached the peaks of C4d-levels, while reduction compared to the baseline could be detected only in cases of 4 patients. In the same evaluation period, growth could be measured concerning C3a and C5a concentrations in cases of 20-20 patients, and C5b-9 concentrations in cases of 19 patients, respectively. The levels of C4d, C3a and C5a significantly increased on the 1^{st} day. Regarding C5b-9 levels, the growth was not significant.

Yet, a significant growth could be measured in the cases of three patients whose C5b-9 levels were not initially detected the highest. We measured a positive correlation among the SC5b-9 and C3a levels taken on admission, however, not any concerning the C4d levels. No correlation was proven among the baseline of SC5b-9, CRP and S-100B values.

4.2.3. We determined the MBL2-genotype of stroke patients. 17 patients were wild-type alleles (A/A) carriers, 9 patients were heterozygous at the variant type of allele (A/0). The C4d and C5b-9 concentrations reached significantly higher values in the cases of MBL2 A/A compared to the variant allele (A/0) carriers.

4.2.4. The SC100B, SC5b-9 and CRP levels were significantly elevated in the patients suffered from a serious stroke (complete or partial cortical arterial infarct) than in the mild forms (incomplete or lacunar infarct).

4.2.5. The SC5b-9 levels showed a significant, positive correlation with the neurological deficiency measured by NIH scale and the negative symptoms analysed by Rankin scale. The SC5b-9 concentrations showed inverse correlation with the functional negative symptoms evaluated by Barthel index, however, on the marginal significance. 1 patient out of 8

subjects with elevated SC5b-9 concentration (>1ug/ml), and 11 patients among the other 18 subjects had the best Barthel index rate.

4.3.1. The ficolin-2 and ficolin-3 levels in the admission and follow-up samples of stroke patients were significantly lower compared to the healthy-, and patient control groups'. Their CRP were significantly higher compared to the admission samples of the healthy controls, but almost the same as the detected levels of serums of patient controls. The CRP levels of follow-up samples were significantly higher than in the two control groups. Concerning the control groups, all the three variables of the patient controls were detected increased more significantly than in the healthy control group, although, the differences of ficolin-3 were slight.

4.3.2. The ficolin-3 concentrations of follow-up samples showed a significantly negative correlation with the indirect values of NIH scale evaluated on admission. Comparing the ficolin-3 levels of subgroups, the subgroup levels with relative favourable prognosis were significantly lower than in the subgroup of patients with unfavourable outcome. In contrast, we did not detect a significant difference between the ficolin-2 levels of the two subgroups.

A significantly negative correlation was found regarding the ficolin-3 and S100B levels in follow-up samples. The ficolin-2 levels were not correlated with the S100B concentrations. The follow-up CRP values were significantly elevated in the patients with high NIH scores, although, we did not find a significant correlation with the S100B levels. The ficolin-3 values were lower in the samples of the subgroup with unfavourable outcomes according to the Rankin scale, but only in cases of follow-up samples. The CRP levels derived from both admission and follow-up samples were significantly higher in the patient subgroup with unfavourable outcomes compared to the subgroup with favourable prognosis.

The ficolin-3 and CRP levels detected in follow-up samples were related to the significant outcome: lower ficolin-3 and higher CRP levels were detected in patient subgroup with unfavourable outcome compared to the patient subgroup with favourable prognosis. Similar, but only marginally significant (ficolin-3) or mildly important correlations were found during the analysis of admission samples. In order to prove the correlations between the low ficolin-3 and high CRP levels; and the unfavourable outcomes, we repeated our analysis detecting the ficolin-3 and CRP levels from follow-up samples. We

commented highly beyond the median ranges of ficolin-3 levels (16ug/ml) or of CRP values (7.7 mg/ml) respectively.

4.3.4. The NIH score and S100B concentration showed a highly significant correlation with the outcome of a stroke: serum concentration of S100B detected from follow-up samples of the patients with unfavourable outcome showed higher values compared to the patients with favourable outcome.

5. Conclusions

5.1. Based on my research, the regulating fault of C1-INH molecule could balance the risk factors of atherosclerosis related to lipid-, and non-lipid metabolism caused by application of long-term danazol. This conclusion might be drawn from the fact that we did not find any difference of IMT values between the danazol treated, and untreated patients suffered from the lack of C1-INH compared to neither each other, nor healthy controls. The values of IMT were detected in normal range. Thus, the complement system may play a relevant role in the pathogenesis of atherosclerosis by its regulator molecule, C1-INH serine protease-inhibitor.

5.2. The results of my research have proven the key role of the activation of the complement system in the pathophysiological

processes of cerebral infarcts. The measurement of the complement activation elements could help determine the clinical prognosis of the acute ischemic stroke. The complement modulation therapy could emerge as one of the potential alternatives in the therapeutic management of acute ischemic stroke. Our current findings might be applied in two possible areas. One of them is the measurement of expansion of complement system taken immediately after admission that might be solved as prognostic factor besides the previous markers. To detect CRP levels increased after a stroke is recommended, but appropriate randomized clinical trials are missing in this respect. The situation is the same concerning the measurements of SC5b-9. Another possibility concerns to the treatment of acute ischemic stroke.

Our current findings, anchoring with other research groups', indicate that the agents hindering the in vivo activation of the complement system might be useful to treat acute ischemic stroke, which assumption might be the most relevant, namely an effective and a special therapy has not existed for the mitigation of ischemic-reperfusion damage of acute ischemic stroke. Several medicines, for instance intravenous gamma-globulin or C1-esterease inhibitor concentration, are used in

clinical practice, then the agent, which is able to down-regulation of in vivo complement activation. Furthermore, newly developed inhibitors are available, such as monoclonal anti-C5 antibodies.

5.3. According to my results, ficolin-derived lectin pathways of the complement activation play a role in the pathophysiology of ischemic stroke, and might be additive effects to the not complement-derived inflammatorical processes. Two different looking, but only partially identified neuro-inflammatoric pathways – the ficolin-3 dependent activation of lectin pathway of the complement system and the CRP dependent processes – play a role in an independent manner form each other, concerning the pathomechanism and unfavourable outcome of acute ischemic stroke. Our findings may contribute to the new therapeutic approach of acute ischemic stroke, for treatment of this disease rather scarce therapeutic alternatives that exist.

6. List of own publications

Publications – serving as the basis for the Thesis

Szegedi R, Széplaki G, Varga L, Prohászka Z, Széplaki Z, Karádi I, Füst G, Farkas H. Long-term danazol prophylaxis does not lead to increased carotid intima-media thickness in hereditary angioedema patients. Atherosclerosis. 2008 May;198(1):184-91. IF: 4.601 (2009)

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Füst G, Munthe-Fog L, Illes Z, Széplaki G, Molnar T, Pusch G, Hirschberg K, Szegedi R, Széplaki Z, Prohászka Z, Skjoedt MO, Garred P. Low ficolin-3 levels in early follow-up serum samples are associated with the severity and unfavorable outcome of acute ischemic stroke. J Neuroinflammation. 2011 Dec 29;8:185. IF: 3.827 (2011)

Summarized impact factors related to publications for the thesis: **12,95**

Further publications

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Summarized impact factors: **12,95**