Molecular pathomechanisms of ischemic stroke

E.A. Arakelova, A.A. Arakelyan, G.M. Mkrtchyan, M.R. Hovsepyan, V.A. Ayvazyan, G.V. Avetisyan, A.S. Boyajyan

Institute of Molecular Biology NAS RA
7, Hasratyan st., 0014, Yerevan

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Stroke is the third most prevalent cause of death and disability worldwide. Ischemic stroke (IS) accounts for 80% of all strokes, 70% of all acute cerebrovascular diseases, and is the most frequent acute neurological disorder. Its incidence is 14 cases/10000 population/year, and 25% of IS occur in working-age people. Only 1/3 of all stroke patients reach full social and professional reintegration, whereas the remainder die or invalid. Yet there is no efficient therapy against IS [5].

IS is mainly caused by intracranial thrombosis or extracranial embolism. It is characterized by a sudden loss of circulation to an area of the brain where crucial artery is narrowed or blocked, resulting in an oxygen cutoff to the brain and corresponding loss of the latest neurological function. The disruption of blood flow to a portion of the brain induces an ischemic cascade, leading to the death of neurons and cerebral infarction. Within minutes of a stroke, an initial cell death zone is surrounded by additional damaged and dying cells forming an ischemic penumbra. This process can continue for hours and days leading to irreversible brain damage. The existence of the ischemic penumbra, a potentially salvageable tissue around a core of irreversibly damaged cells, is a central concept of acute IS therapy, which primary goal is to prevent this not yet irreversibly damaged tissue from proceeding towards infarction, that is to reduce the final size of infarction [17].

Postischemic inflammatory response (PIR) and related aberrant apoptosis are the main pathologic events responsible for progression of penumbra to infarction, increase of infarction size, further development of the inflammatory reactions on a systemic level, and worsening of IS patients. On the one hand, PIR is pointed toward the removal of necrotic tissue from the ischemic area; on the other hand, it enlarges the ischemic area and the severity of IS. Molecular mechanisms of the negative effects of PIR are yet unclear, which makes it difficult to identify targets for therapeutic correction. Clinical trials aimed at limiting PIR have so far had disappointing results, probably due to insufficient preclinical data. The majority of
the data were obtained in animal models of stroke, which do not adequately reflect the pathogenesis of IS in humans. To design clinical trials appropriately, a study of PIR with the involvement of a large number of human subjects is required [7, 17].

The major mediators of the inflammatory response are the cytokine network, complement system and immune complexes. The objective of the present study was to evaluate the functional state, interplay and role of the main inflammatory mediators in PIR progression in humans on a systemic level. For this purpose in the blood serum of patients with IS the levels of cytokines (interleukins and chemokines), activity of the complement system, and composition of pathogenic immune complexes (PICs) and cryoglobulins (Cgs) were determined on days 1, 3, 5, 7, 14 and after 6 months of IS onset.

**Subjects and Methods**

One hundred twenty patients with IS and 80 age- and sex-matched healthy subjects entered the study. In addition, for comparative study of some measured parameters, patients with hemorrhagic stroke, familial Mediterranean fever, schizophrenia, and diabetes mellitus were also involved (in each group n=40). All the patients gave their informed consent to participate in this study. The average clinical and demographic characteristics of IS patients are presented in Table 1.

### Table 1

<table>
<thead>
<tr>
<th>Males/females</th>
<th>54/66</th>
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</thead>
<tbody>
<tr>
<td>Mean age (years, M±S.D.)</td>
<td>65±13</td>
</tr>
<tr>
<td>Stroke type: cardioembolic/large vessel atherosclerosis, n</td>
<td>24/96</td>
</tr>
<tr>
<td>Infarction zone: stem/hemisphere</td>
<td>18/102</td>
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<table>
<thead>
<tr>
<th>Accompanying disorders and risk factors</th>
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<tbody>
<tr>
<td>Hyperlipidemia</td>
<td>84</td>
</tr>
<tr>
<td>Hypertension</td>
<td>54</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>28</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>30</td>
</tr>
<tr>
<td>Current smoking</td>
<td>56</td>
</tr>
<tr>
<td>Heredity: maternal/paternal</td>
<td>18/26</td>
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</table>

Concentrations of interleukins and chemokines were determined by ELISA using commercially available kits (R&D Systems, Inc.), according to manufacturers’ instructions. Complement functional activity was determined by measuring hemolytic activities of its classical and alternative pathways (using erythrocytes as target cells) as well as the levels of its individual components, C1q, C3, and factor B (FB) according to previously described procedures [2, 27]. The hemolytic activi-
ties of the classical and alternative complement pathways were expressed in CH50 and AH50 units, respectively. An AH50 unit is defined as the amount of serum that causes a 50% hemolysis of rabbit erythrocytes in the reaction mixture. A C3H50 unit is defined as the amount of serum that cause a 50% hemolysis of antibody sensitized sheep erythrocytes in the reaction mixture. The hemolytic titer is the number of units per ml of serum, and is calculated as the reciprocal of the serum dilution which gives 50% cell lysis [27]. Analysis of PICs was performed after their isolation from the blood serum followed by separation of its proteins in gradient polyacrylamide gel electrophoresis in the presence of Na-dodecylsulphate (SDS-PAGE) as described earlier [3]. Identification of the nature of PIC-specific proteins was performed after their elution from the gels followed by determination of their N-terminal amino acid sequence and further search in “PROSITE” online database of proteins domains and families according to earlier published approaches [3]. Cgs were isolated from the blood according to earlier published procedure [16]. For characterization of immunoglobulin-composition of Cgs, we used agarose electrophoresis and immunoblotting procedures as described earlier [16]. To reveal the presence of the complement C1q and C3 proteins and their activated split products in Cgs, SDS-PAGE and subsequent Western blotting were carried out according to Bio-Rad Laboratories, Inc. (USA) instruction manuals (catalog numbers 165-3301 and 170-3930). To analyze the presence of LDL (β-lipoprotein and abnormal lipoprotein-X (LP-X)) in Cgs we performed electrophoresis in Bacto agar followed by polyanionic precipitation in situ in gels after electrophoresis [16].

Data analysis included Mann-Whitney U-test. P values < 0.05 were accepted as statistically significant.

Results and Discussion

We focused attention on chemokines (monocyte chemoattractant protein-1 (MCP-1), cytokine inducible neutrophil chemoattractant (CINC)) and interleukins (interleukin-1β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNFα)). MCP-1 and CINC possess proinflammatory properties inducing migration of monocytes and neutrophils in damaged tissues. IL-1β, IL-6, and TNFα possess both pro- and anti-inflammatory activities, and also regulate the expression of MCP-1 and CINC. [7].

According to the results obtained (Fig. 1), a significant increase in the blood levels of all cytokines was detected in IS patients on days 1-3 of IS onset, with the maximum level on day 1. On day 5 concentrations of all cytokines, except MCP-1, reached normal level. The latter remained high even after 6 months of IS onset, when patients suffered from the residual effects of IS.

The results obtained demonstrated that modulation of PIR by targeting cytokines expression might increase the efficiency of IS therapy and that determination of MCP-1 blood level may be used for monitoring of the state of patients with the residual effects of IS.
Investigation of the complement system in PIR in human IS.

The complement system, acting on the interface of innate and adaptive immunity, mediates a large variety of cellular and humoral interactions in the immune response and consists of nearly 60 soluble and membrane-bound proteins including receptors and regulators. It is a cytotoxic host defense system eliminating foreign pathogens, opsonizing immune complexes, apoptotic and necrotic cells facilitating their recognition by macrophages. However, undesirable complement activation contributes to the pathology of many human diseases by damaging tissue and promoting inflammation. Activation of complement by classical, alternative and lectin pathways through a series of proteolytic reactions generates triggers of apoptosis and inflammation, opsonins, chemotactic attractants, anaphylatoxins, and membrane-attack complexes playing an essential role in tissue damage. Overlap of complement cascade with other molecular events occurring in stroke is very complex and is only beginning to be understood [6,19,23]

In our study we evaluate the functional activity of the complement cascade in PRP by measuring hemolytic activities of its classical and alternative pathways as well as the levels of its individual components, C1q, C3, and factor B (FB) in the blood of IS patients and healthy subjects. C1q is an initial point for the classical pathway, FB is an essential component of the alternative pathway, and C3 is an initial point for the alternative pathway and a converge point for all three comple-
ment activation pathways [6,19,23].

According to the results obtained, on day 1 a significant increase was found in hemolytic activity of the classical pathway in patients compared to healthy subjects, but on day 3 the activity sharply decreased below the normal level. On day 5 the activity started to increase again and returned to normal level on day 7. For the alternative pathway, no significant difference in hemolytic activity of IS patients, compared to healthy subjects, was detected on days 1-3 of IS onset, whereas on days 5 and 7 the activity significantly increased. Regarding the levels of the individual components of the complement, the dynamics of C1q changes in PIR progression was the same as detected in case of the hemolytic activity of classical complement pathway, and the dynamics of changes in FB level in PIR progression repeated that of the hemolytic activity of the alternative complement pathway. A more complex picture was detected in case of C3 level. On days 1-3 of stroke onset it changed like the hemolytic activity of the classical pathway did, thereafter the picture was similar to that detected in case of the alternative pathway. The results obtained (Fig. 2) demonstrate that both the classical and the alternative complement pathways are involved in PIR developed after IS.

We concluded that the classical pathway is activated earlier, beginning from the first hours of IS onset, whereas the alternative pathway switches on later, on the third day of IS onset, when the activity of the classical pathway decreases. Here we propose the existence of some compensatory mechanism switching from the classical pathway to the alternative in response to depletion of the classical pathway components. In addition increase in cytotoxicity, autoimmune sensitization, or IS-associated infections [24,25] may be also responsible for activation of the alternative pathway in PIR progression. In summary we suggest that inhibition of the complement on the third day of IS onset by targeting the alternative pathway may sufficiently decrease the negative effects of PIR.

Investigation of composition of PICs and Cgs in PIR progression in human IS.

Formation of immune complexes is a normal reaction of organism to foreign or auto-antigen. In healthy body the immune complexes are easily eliminated from the circulation by phagocytosis. However, in pathological conditions concentration of immune complexes in circulation increases as the efficiency of their elimination decreases. This is very dangerous, since the immune complexes can interact with both humoral and cellular immune recognition systems and significantly affect the immune response at multiple levels. The most aggressive subpopulations of the immune complexes are PICs and Cgs. PICs are originated in the conditions when there is an excess of antigens or antibodies.

They are smaller in size than classical immune complexes, are hardly recognized by macrophages and hardly eliminated worse from circulation [12]. In Cgs both antigens and antibodies are Igs. Another characteristic feature of Cgs is that they reversibly precipitate below the temperature of 37°C [11].
Fig. 2. Changes in hemolytic activities of the classical (a) and alternative (b) complement activation pathways as well as the levels of C1q, C3, and FB complement components in PIR progression.

The ability of PICs and Cgs to precipitate on vascular wall, stimulate the infiltration of macrophages and neutrophils [8,11,12] and expression of TNFα [18] generates our interest to study these formations in IS. In addition, the results of our previous study demonstrated elevated level of total population of the immune complexes and the presence of both PICs and Cgs in the total population of immune complexes in the blood of IS patients [1,15]. In the present study we investigated composition of PICs and Cgs of IS patients.

Figure 3 represents the results of comparative proteome study of PICs in IS, hemorrhagic stroke, schizophrenia, diabetes mellitus, and familial Mediterranean fever. The data obtained suggest that protein composition of PICs in each diseased condition, including IS, is very specific and probably reflects disease-associated pathological processes.
Fig. 3. Frequency distribution of proteins by molecular weight in PICs in different diseased conditions. Histograms were plotted after subjection of PICs to SDS-PAGE in non-reduced conditions.

Figure 4 shows typical SDS PAGE patterns of PICs isolated from the blood of patients with IS, hemorrhagic stroke, familial Mediterranean fever complicated and non-complicated by renal amyloidosis and healthy subjects. As it is seen from the patterns, in all diseased conditions the presence of a polypeptide with an apparent molecular weight of 19 kDa is detected. We isolated this polypeptide from the gels, determined its N-terminal amino acid sequence (12 amino acid residues) and identified its nature through established N-terminal amino acid sequence using PROSITE database – http://us.expasy.org/prosite. According to the results obtained (Fig. 5), in all mentioned diseases the 19 kDa polypeptide represents a subunit of homopentameric C-reactive protein (CRP).

Fig. 4. Electrophoretic patterns of proteins in PICs of patients with IS (1), hemorrhagic stroke (2), Familial Mediterranean fever complicated (3) and non-complicated (4) with...
renal amyloidosis (5), healthy subjects and standard proteins (6) in SDS-PAGE (non-reduced conditions).

Fig. 5. Identification of the nature of 19 kDa polypeptide isolated from PICs of patients with IS, hemorrhagic stroke, and Familial Mediterranean fever.

1 – N terminal amino acid sequence of 19 kDa polypeptide; 2 – full amino acid sequence of C-reactive protein subunit.

CRP, an acute-phase reactant, is an indicator of systemic inflammation [21], and its elevated blood level associates with stroke [22] and familial Mediterranean fever [13]. In this study, we for the first time demonstrated presence of CRP in PICs circulating in the blood of patients with all three mentioned above pathologies, which may be a result of the development of autoimmune reactions caused by increased level of this protein in circulation.

Table 2 summarizes the results of our study of the immunochemical composition of Cgs isolated from the blood of IS patients.

<table>
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<th>Components of Cgs</th>
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<tr>
<td>Complement proteins</td>
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<tr>
<td>C1qα, C1qβ, C1qγ, C3β, C3bi, C3c</td>
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Immunochemical composition of Cgs isolated from the blood of IS patients

As it was revealed by immunoblotting, Cgs isolated from the blood of 80%
of IS patients contained a mixture of polyclonal IgG, IgM and IgA, and Cgs isolated from the blood of 20% of patients contained a mixture of polyclonal IgG and IgA. This finding indicate that Cgs of IS patients belong to type III Cgs, according to Brouet et al. [4], previously described in several infectious and autoimmune diseases [4,11].

With regard to the presence of complement proteins, both C1q (C1qα, C1qβ, C1qγ), C3 (C3β) and its split products (C3bi and C3c) were found. This observation provides strong evidence for the participation of Cgs in activation of both the classical and alternative complement pathways. Moreover, an occurrence of opsonins, C3bi and C3c, suggest about the involvement of Cgs in IS-related aberrant apoptosis.

The results of the experiments on identification of the presence of low density lipoproteins in Cgs demonstrated that all Cgs isolated from the serum of IS patients contained both LP-X and β-lipoprotein. Concerning LP-X, this abnormal subclass of LDL was earlier found in the blood of patients with extreme hypercholesterolemia and cholestasis [26] caused by liver diseases [19] and familial lecithin: cholesterol acyltransferase deficiency [9]. LP-X is a LDL rich in phospholipids and nonesterified cholesterol but poor in cholesteryl ester, triglyceride, and protein [10].

The present study, for the first time, demonstrated the presence of LP-X in the blood of IS patients in a Cg-bound form. Recent study of Lynn et al. [14] has demonstrated that LP-X stimulates monocytes infiltration via a mechanism involving MCP-1 expression, and Mathsson et al. [18] reported a stimulatory effect of Cgs on TNFa production. Therefore, we suggest that Cgs may be involved in PIR through activation of the complement cascade, cytokine production, and apoptosis.

**Conclusions**

1. Modulation of PIR by targeting expression of cytokines may increase the efficiency of IS therapy.
2. Blood MCP-1 level is an informative tool for monitoring of the state of patients with residual effects of IS.
3. Both the classical and alternative complement activation pathways are involved in pathomechanisms of generation and development of PIR in acute IS.
4. Complement-based therapy may positively influence IS progression and outcome through targeted blockade of the alternative pathway on day 3 of IS onset.
5. Cryoglobulins are involved in PIR acting as activators of the alternative and classical complement pathways, apoptosis, and cytokine expression.
6. Cryoglobulins may be considered as targets for therapeutic modulation of PIR upon IS.
Молекулярные патомеханизмы ишемического инсульта

Э.А. Аракелова, А.А. Аракелян, Г.М. Мкртчян, М.Р. Овсепян, В.А. Айвазян, Г.В. Аветисян, А.С. Бояджян

Цель настоящего исследования состояла в оценке функционального состояния, взаимодействия и роли основных медиаторов воспалительных реакций в динамике развития постишемического воспалительного ответа на системном уровне. Для этого в крови 120 больных, перенесших острый ишемический инсульт, определяли уровень провоспалительных и иммунорегуляторных цитокинов, активность системы комплемента, а также уровень и состав патогенных комплексов и криоглобулинов на 1-14-й дни и через 6 месяцев от начала инсульта. Установлено, что уровень цитокинов в крови у больных ишемическим...
инсультом достоверно повышен в первые 3 дня от начала инсульта с максимумом на 1-й день. Показано, что запуск классического пути комплемента происходит после начала инсульта, а на 3-й день наблюдается резкое снижение активности этого пути. Далее включается альтернативный путь в ответ на истощение компонентов классического пути, который находится в активированном состоянии по 7-й день от начала инсульта. Установлено, что патогенез ишемического инсульта характеризуется формированием криоглобулинов типа III, содержащих индукторы воспаления и апоптоза, а также патогенных иммунных комплексов, содержащих С-реактивный белок.

References


