

RESEARCH ARTICLE

# Collectins, C3 complement protein, annexin V and C-reactive protein in acute ischemic stroke: interrelation and implication to upregulated apoptosis and inflammation

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Received: February 02, 2014

Published online: March 15, 2014

**Upregulated inflammatory response and apoptosis, both triggered by ischemia, are the most destructive pathologic processes developed after acute ischemic stroke (IS) at both central and peripheral levels. Identification of molecular mediators of these processes and understanding of their pathomechanisms will support the development of therapies, which can significantly improve outcomes of IS-affected subjects. Mannan-binding lectin (MBL), ficolin L (FL), ficolin H (FH) and C3 complement proteins participate in apoptotic cells recognition and clearance through various mechanisms. In the present study we performed comparative determination of the blood levels of MBL, FL and FH, and hemolytic activity of C3 protein (C3H50) as well as apoptosis marker protein, circulating annexin V (cANXV), and inflammatory marker C-reactive protein (CRP) in 99 patients with acute IS on the first day of stroke onset and 110 healthy subjects. Potential correlation between all measured parameters was analyzed. Methods included enzyme-linked immunosorbent, hemolytic, and immunonephelometric assays; statistics were performed by Student's t-test and Pearson's correlation analysis. The obtained data demonstrated significantly increased and correlated with each other blood levels of MBL and C3H50, cANXV and CRP in patients compared to controls. Slight, while significant, changes in the blood levels of FL and FH were also observed. We concluded that post-ischemic response at the first day of IS onset is characterized by sufficiently elevated and positively correlated with each other blood levels of MBL, C3H50, cANXV and CRP as well as by slightly increased blood levels of FH and FL. The obtained results suggest that MBL and C3 protein are implicated in upregulated inflammatory response and apoptosis developed after IS onset at a peripheral level, and that these pathological processes are interrelated and interdependent.**

**Keywords:** ischemic stroke; inflammation; apoptosis; mannan-binding lectin; ficolin L; ficolin H; C3 complement proteins; annexin V; C-reactive protein

**Abbreviations:** ischemic stroke, IS; Mannan-binding lectin, MBL; ficolin L, FL; ficolin H, FH; hemolytic activity of C3 protein, C3H50; circulating annexin V, cANXV; C-reactive protein, CRP; National Institutes of Health Stroke Scale, NIHSS; Enzyme-linked immunosorbent assay, ELISA

**To cite this article:** Boyajyan A, et al. Collectins, C3 complement protein, annexin V and C-reactive protein in acute ischemic stroke: interrelation and implication to upregulated apoptosis and inflammation. *Inflamm Cell Signal* 2014; 1: e82. doi: 10.14800/ics.82.

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## Introduction

Inflammatory response is a part of the innate immune response and plays a crucial role in the pathophysiology of ischemic stroke (IS). This response is developed to remove a necrotic tissue from an ischemic area. However, it also enlarges the ischemic area, and the disease severity [1]. The detailed mechanism of post-stroke inflammation is unknown. Several studies indicate that neuronal cells apoptosis, which is triggered by ischemia/reperfusion, results to accumulation of the inflammatory cells in the ischemic brain and induces the development of postischemic inflammatory response [2, 3]. Administration of kallikrein encoding human gene into rat brain immediately after middle cerebral artery occlusion protects against ischemic brain injury by inhibiting apoptosis [4]. Within minutes of a focal IS onset, a part of brain tissue, which is exposed to a maximum dramatic blood flow reduction, becomes irreversibly damaged and cells in this zone then die by necrosis. This necrotic core is surrounded by a zone of less severely injured and potentially recoverable tissue known as the "ischemic penumbra" [5]. The cells in this zone are characterized by the increased apoptotic rate and during the initial stages of ischemia may constitute as much as half of the total lesion [2, 5-7]. Recent studies have clearly indicated that many neuronal cells in the ischemic penumbra may undergo apoptosis after several hours or days [6, 7]. These cells are potentially recoverable for some time after the onset of IS [6, 7]. Therefore, a primary aim of acute stroke therapy is to prevent these not yet irreversibly damaged cells from death [6]. Upregulated inflammation and apoptosis after stroke onset are observed not only in the brain but as well in peripheral tissues and circulation [8-10]. Cerebral ischemia triggers two general pathways of apoptosis: the intrinsic pathway, which originates from mitochondrial release of cytochrome *c* and associated activation of caspase-3; and the extrinsic pathway, which originates from the activation of cell surface death receptors and stimulates caspase-8 [7, 11, 12]. Further progress in our knowledge on molecular mediators and pathomechanisms of upregulated apoptosis and inflammation triggered by ischemia will support the development of therapies, which can significantly improve patient outcomes after IS.

Mannan-binding lectin (MBL), ficolin L (FL) and ficolin H (FH) are multivalent serum proteins, components of the complement lectin pathway, recognizing microbial and apoptotic cells. These collectins either act as opsonins facilitating the uptake of apoptotic cells by phagocytes or participate in the clearance of apoptotic cells through activation of the complement lectin pathway [13-18]. C3 is another multi-functional component of the complement system. This protein initiates the alternative pathway, is the

main component of the classical pathway, and represents a converge point of all three pathways of the complement activation, the classical, alternative and lectin [19, 20]. The C3 protein consists of two polypeptide chains,  $\alpha$  and  $\beta$ . Hydrolysis of a short fragment (C3a) from the amino terminus of the  $\alpha$ -chain, which is catalyzed by the enzyme C3 convertase, generates C3b split product. C3b covalently binds to a surface of apoptotic cell and acts as an opsonin activating leukocytes bearing complement receptors. Cleavage of C3b produces iC3b, which is more effective opsonin. Further splitting of iC3b generates C3c and C3d. Fragments of C3 deposited on the apoptotic cells bind the complement receptors (CR1, CR3, and CR4) on phagocytes to facilitate cell recognition and clearance [20-22]. Disturbances in the levels of MBL, FL and FH are detected in pathogenesis of diseased conditions characterized by upregulated inflammation and/or apoptosis [23, 24].

In the present study we performed comparative determination of the blood levels of MBL, FL, FH, and hemolytic activity of C3 protein as well as apoptosis marker protein, circulating annexin V (cANXV), and inflammatory marker C-reactive protein (CRP) in patients with acute IS and healthy subjects. Potential correlation between all measured parameters was analyzed.

## Materials and methods

### Study population

A total of 94 patients with the first-episode IS (mean age  $\pm$  SD: 67  $\pm$  9 years, females/males: 59/35) and 110 healthy subjects (mean age  $\pm$  SD: 57  $\pm$  9 years, females/males: 62/48) were enrolled in this study. All subjects were unrelated Caucasians of Armenian ancestry. Patients were recruited among those, whose stroke occurred within the prior 24 hours (before any medication was applied), who were consecutively admitted to the Emergency Department of the "Armenia" Republican Medical Center of the Ministry of Health (MH) of RA. Diagnosis of IS was based on clinical history and neurological examination and was confirmed by brain computer tomography (CT) imaging and standard laboratory analyses. Patients with signs of brain trauma, cerebral hemorrhage or tumors were excluded from the study group. Stroke subtype was assessed according to definitions of TOAST [25]. Patients with large vessel atherothromboembolic stroke (n=77) and cardioembolic stroke (n=17) were selected for this study; those with lacunar stroke syndromes were excluded from the study group. Severity of neurological deficit was defined using the National Institutes of Health Stroke Scale (NIHSS). In the present study, patients with a moderate to severe impairment (average NIHSS score 17) were involved. Healthy subjects (controls) were volunteers from the institutes of NAS RA reported no personal or family

**Table 1. Levels of MBL, FH, FL, cANXV, CRP and C3H50 (mean ± SD) in the blood serum of IS patients and controls.**

Parameter	Study group		p
	IS patients	Controls	
[MBL], µg /ml	4.6 ± 1.6	1.6 ± 0.5	0.001
[FH], µg /ml	13.6 ± 1.1	13.3 ± 1.1	0.035
[FL], µg /ml	4.5 ± 2.3	4.3 ± 2.2	0.049
[cANXV], ng/ml	4.7 ± 1.5	1.8 ± 0.5	0.0001
[CRP], mg/l	21.9 ± 8.6	3.6 ± 1.4	0.0001
C3H50, U/ml	140.2 ± 58.3	96.4 ± 39.8	0.001

**Table 2. Results of correlation analysis between blood serum levels of MBL, FH, FL, cANXV, CRP, and C3H50 in IS patients and controls.**

Parameter	Study group			
	IS patients		Controls	
	r	P	r	P
[MBL] vs. [FH]	-0.5	0.05	-0.10	0.10
[MBL] vs. [FL]	-0.5	0.05	-0.50	0.05
[MBL] vs. [cANXV]	0.47	0.002	0.14	0.46
[MBL] vs. [CRP]	0.62	0.001	0.18	0.37
[MBL] vs. C3H50	0.85	0.0001	0.26	0.06
[FH] vs. [FL]	0.38	0.25	0.34	0.2
[cANXV] vs. [FH]	-0.22	0.32	-0.20	0.39
[cANXV] vs. [FL]	-0.14	0.58	-0.16	0.56
[cANXV] vs. [CRP]	0.45	0.0020	0.20	0.24
[cANXV] vs. C3H50	0.32	0.0070	0.12	0.32
[CRP] vs. [FH]	0.49	0.0300	0.14	0.39
[CRP] vs. [FL]	0.44	0.0400	0.24	0.26
[CRP] vs. C3H50	0.7	0.0002	0.20	0.065
C3H50 vs. [FH]	0.24	0.4900	0.27	0.52
C3H50 vs. [FL]	0.34	0.3500	0.37	0.40

history of IS, myocardial infarction, and any other cerebrovascular or cardiovascular disease. They had no serious medical disorder or treatment during the past 12 months. Exclusion criteria for all subjects included chronic inflammation or/and infectious diseases, present or past history of metabolic (diabetes mellitus, etc.), neuropsychiatric, immune system and oncological disorders, myocardial infarction or any other serious medical conditions. All subjects or their legal representatives gave their informed consent to participate in the study, which was approved by the Ethical Committee of the Institute of Molecular Biology NAS RA (IRB #00004079).

#### Collection of blood and preparation of serum

Practically fasting blood samples were collected by venipuncture in appropriate tubes and kept on ice for 60 min. After that the coagulated blood was centrifuged at 3000 g for 15 min at 4°C to separate serum from blood corpuscles. The obtained serum samples were stored in aliquots at -30°C and thawed immediately prior to use.

#### Enzyme-linked immunosorbent assay (ELISA) based measurements of MBL, FH, FL, and cANXV levels in the blood serum

A sandwich ELISA was used to measure concentrations of MBL, FH, FL, and cANXV in the blood serum of study subjects. Concentration of MBL was determined by the earlier described procedure in which ELISA plates were coated with mannan [26]. Concentration of MBL was expressed in µg per ml of serum (µg/ml). Concentrations of FH, FL and cANXV were determined using commercially available kits (Hycult Biotech and Usen Life Science, respectively) according to manufacturers' instructions. Concentrations of MBL, FH, and FL were expressed in µg per ml of serum (µg/ml) and in case of cANXV in ng per ml of serum (ng/ml). In each assay, we run duplicates of each sample, standard, and blank control (zero standard) on the same microplate. Cases and controls were run on the same microplate. Also, duplicates of the same cases and controls (three of each) were run in each assay/on each microplate. The calculated overall intra-assay coefficient of variation was 5%, and the calculated overall inter-assay coefficient of variation was 8%. Standard curves were reproducible with coefficient of variation <4.

#### Determination of the C3 hemolytic activity in the blood serum

A hemolytic assay of the C3 protein (C3H50) was based on the standard test for the classical pathway of human serum complement. Sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte antibodies were used as target cells. The hemolytic activity was expressed in units per ml of serum (U/ml). One unit of hemolytic activity is defined as an amount of serum that causes a 50% hemolysis of erythrocytes in an incubation mixture. The hemolytic titer, a number of units in 1 ml of serum, is calculated as the reciprocal of the serum dilution giving 50% cell lysis. C3-deficient human serum was obtained by the affinity chromatography [27, 28]. In this assay triplicates of each sample and control were analyzed.

#### Determination of CRP levels in the blood serum

Concentration of CRP in the serum samples was measured by immunonephelometric method on a BN II automated analyzer (Dade Behring) using commercially available kit (Dade Behring) as described by the manufacturer. Measurements were performed in "Siemens N Cuvette Segments for BN II" at wavelength of 840 nm against a buffer. In this assay duplicates of each sample and control were analyzed. Concentration of CRP was expressed in mg per liter of serum (mg/l). In this assay duplicates of each sample and control were analyzed.

#### Statistical analysis

For data analysis Student's unpaired two-tailed t-test and

Pearson's correlation analysis with calculation of correlation coefficient ( $r$ ) were performed using GraphPad Prism 3.03 (GraphPad Software Inc) software. Data was presented as mean  $\pm$  SD. P values  $< 0.05$  were considered significant.

## Results

According to the results obtained, statistically significant 2.9-fold increase of the mean serum level of MBL in IS patients compared to controls was detected, and statistically *significant, but negligible*, increase in the mean serum levels of FH and FL was found. The mean serum levels of apoptotic marker, cANXV protein, as well as the inflammatory marker, CRP, in patients were significantly 2.6 and 6.0 times, respectively, higher than in controls. Regarding the activity of C3 complement protein, we revealed statistically significant elevation of the mean value of C3H50 in the serum of IS patients as compared to controls. The results obtained are presented in Table 1. We detected no difference in the mean values measured parameters when comparing females and males or non-smokers and smokers in both study groups. Statistically significant positive correlations between all measured parameters, excluding FH and FL blood levels, in case of IS patients were detected (Table 2). In controls no significant correlations between measured parameters were observed (Table 2).

## Discussion

The results of this study clearly demonstrated the excessive production of proinflammatory and proapoptotic mediator, MBL, as well as hyperactivation of the C3 complement protein in acute IS in human. The increased blood levels of MBL and C3 hemolytic activity positively correlated with each other as well as with elevated cANXV and CRP blood levels of IS-affected subjects. In addition, the present study revealed slight, while significant, changes in the blood levels of FL and FH on the first day of IS onset.

A number of animal and human studies [29-39], including our own [40-42], demonstrated that activation of the complement system play a decisive role in the pathophysiology of IS. No study has reported data on functional state of FH and FL in this disorder. Few reports have explored the role of MBL in IS progression [42-46]. In our previous study [42], we demonstrated the involvement of the complement lectin pathway in pathomechanisms of IS: the increased blood levels of MBL and activities of MBL-associated serine proteases, including MASP-1 and MASP-2, were detected in IS patients (from day 1 to day 14 of the onset of stroke) compared to healthy subjects [42]. Our present study confirmed the association of the increased blood levels of MBL with IS, and, in addition, revealed positive correlation between the levels of MBL, cANXV,

CRP and C3 complement protein hemolytic activity in the blood of IS-affected subjects. Interestingly, recent study indicated that elevated MBL levels could be considered as an independent stroke risk factor in Chinese population [46]. The elevated blood levels of C3 complement protein in IS patients have been reported earlier by us [47], as well as by other research groups [48-52]. However, this is the first study demonstrated the increased hemolytic activity of C3 in the blood of IS-affected subjects.

It has to be also mentioned that whereas the elevated levels of CRP in circulation of IS-affected individuals has been demonstrated in many studies [reviewed in 53], increased blood levels of cANXV in IS-affected individuals has been reported here for the first time. ANXV belongs to a recently discovered family of proteins, the annexins, is a convenient instrument for the apoptotic cells detection, because in the presence of  $Ca^{2+}$  this protein preferentially binds to phosphatidylserines and other anionic phospholipids (PLs) [54, 55]. ANXV is an important modulator of the immune response against PS-exposing particles like apoptotic cells. Anionic PLs exposure on the cell surface, due to loss of plasma membrane asymmetry, is an early sign of apoptosis and is observed before apoptosis-specific changes in cell morphology as well as loss of the cell plasma membrane integrity have occurred. The apoptotic cells are cleared *via* an anti-inflammatory pathway, and the above mentioned sign is a major "eat-me" signal that they send to phagocytes [54, 55]. ANXV may interfere *in vivo* with the immunosuppressive effects of apoptotic cells, since it binds PS with high selectivity and inhibits the uptake of apoptotic cell by phagocytes [54, 55]. This protein is expressed by many types of cells and is an *in vivo* marker of cellular injury and death. cANXV can be released from smooth muscle cells and endothelial cells of the vascular wall, hepatic and splenic secretory cells or from apoptotic particles derived from injured tissues, lymphocytes, monocytes, etc [56, 57]. High blood levels of cANXV indicate high rate of apoptosis and the severity of cell damage [56, 57]. Therefore, the present study demonstrating elevated levels of cANXV in the blood of IS-affected individuals provide additional evidence on increased rate of apoptosis after IS on a systemic level.

Further, positive correlation between MBL, C3H50, cANXV, and CRP levels detected in this study in IS-affected subjects strongly suggest interrelation between upregulated inflammation and apoptosis in IS progression.

## Conclusions

In summary we concluded that post-ischemic response at the first day of stroke onset is characterized by sufficiently elevated and positively correlated with each other blood levels of MBL, C3H50, cANXV and CRP as well as by slightly increased blood levels of FH and FL.

The results obtained suggest that MBL and C3 are implicated in upregulated inflammatory response and apoptosis developed after IS onset at a peripheral level, and that these pathological processes are interrelated and interdependent. The latest should be taken into consideration upon developing therapeutic measures pointed towards neutralization of the negative effects of stroke-induced post-ischemic response.

### Conflicting interests

The authors have declared that no competing interests exist.

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