

Thrombin antithrombin complex and IL-18 serum levels in stroke patients

Ornella Piazza, Giuliana Scarpati,
Simona Cotena, Maria Lonardo,
Rosalba Tufano

Università degli Studi di Napoli Federico II, Anestesiologia e Rianimazione, Napoli, Italy

Abstract

The complex picture of inflammation and coagulation alterations comes to life in acute stroke phases. Increasing evidence points to a strong interaction and extensive crosstalk between the inflammation and coagulation systems: the interest towards this relationship has increased since recent experimental research showed that the early administration of antithrombin III (ATIII) decreases the volume of ischemia in mice and might be neuroprotective, playing an antiinflammatory role.

We aimed to establish the extent of the relationship among markers of inflammation (S100B and IL-18) and procoagulant and fibrinolytic markers (ATIII, thrombin-antithrombin III complex (TAT), Fibrin Degradation Products (FDP), D-dimer) in 13 comatose patients affected by focal cerebral ischemia.

Plasma levels of TAT, D-dimer and FDP, IL18 and S100B were increased. IL-18 and S100B high serum levels in ischemic patients suggest an early activation of the inflammatory cascade in acute ischemic injury.

The basic principles of the interaction between inflammatory and coagulation systems are revised, from the perspective that simultaneous modulation of both coagulation and inflammation, rather than specific therapies aimed at one of these systems could be more successful in stroke therapy.

Introduction

Thrombin antithrombin complex in brain ischemia

Procoagulant or impaired fibrinolytic states as well as inflammatory reactions mediated by cytokines are very likely involved in the pathogenesis of acute ischemic stroke¹ (Table 1).

Toll-like receptors (TLRs) are critical components of the innate immune system that have been shown recently to mediate ischemic injury.^{7,8} Host-endogenous molecules associated with damaged cells and tissues have been

demonstrated to activate TLRs. TLRs are located on antigen presenting cells such as B cells, dendritic cells, monocytes/macrophages and microglia.⁹⁻¹¹ The TLRs signal through common intracellular pathways leading to transcription factor activation and the generation of cytokines and chemokines.^{12,13} TLR2 and TLR4 have been shown to play a role in cerebral ischemic damage. The TLR endogenous ligands HSP 60, HSP70 and HMGB1 have been found in the brain following injury,¹⁴⁻¹⁶ hence, these molecules may activate TLR2 and TLR4 within the brain itself, leading to the generation of inflammatory mediators such as TNF α , IL1, IL6, and I NOS, all known to be associated with stroke damage.

During focal cerebral ischemia, alterations in the hemostatic system, platelet activation and changes in the plasminogen activator-inhibitor axis take place, particularly in the acute phase when there is an elevation of thrombin activity and a depressed fibrinolytic activity.^{17,18}

The conversion of prothrombin into active thrombin is a key event within the coagulation cascade. Thrombin is a serine protease and an essential component in the coagulation cascade. Direct infusion of large doses of thrombin into the brains of animals causes inflammatory-cell infiltration, mesenchymal-cell proliferation, scar formation, brain oedema formation, and seizures.^{19,26} Thus, early oedema formation involves activation of the coagulation cascade and thrombin production.²²

Thrombin acts on various physiological substrates (fibrinogen, protein C, platelets, etc.) and is inhibited by antithrombin III, resulting in an inactive proteinase/inhibitor complex (TAT complex) (Figure 1).

ATIII is an important endogenous inhibitor of serine proteases that are generated within the coagulation cascade. Clinically, it has been widely used in patients admitted to ICU as treatment for ATIII deficiency, treatment of sepsis or disseminated intravascular coagulation. Recently, it has been demonstrated that ATIII may have other functions in addition to its role in inhibiting clotting. O' Reilly *et al.*²⁷ have shown its remarkable antiangiogenic and antitumor activities. In addition, some investigators have reported that ATIII suppresses leukocyte infiltration and subsequent tissue damage in endotoxin-induced vascular injury²⁸ or ischemia-reperfusion injury.^{29,30} In the field of septic shock, the suppressive effects of ATIII on inflammation have been highlighted.^{31,32} In patients with severe sepsis, treatment with ATIII improved lung function and prevented septic liver and kidney failure.³³ ATIII has attracted a great deal of attention as a possible treatment for these various conditions; because no adverse side effects have been reported in human trials.³⁴ However, little information is available about the effects of

Correspondence: Ornella Piazza, Università degli Studi di Napoli Federico II, Anestesiologia e Rianimazione, Via Pansini 5, 8013 Napoli, Italy
Tel.: +39.0817463542, Fax +39.0817463554
E-mail: orpiazza@unina.it

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ATIII on cerebral ischemia and the mechanisms of its antiinflammatory action.

When generated endogenously or administered intravenously, ATIII is tethered to the wall of the microvasculature with the help of heparin and it is then bound to the active site on thrombin to suppress its functions. In addition to its hemostatic role, thrombin has the ability to induce interactions between endothelial cells and blood-flowing cells, such as leukocytes³⁵⁻³⁷ or tumor cells.^{38,39} Because these interactions are thought to be the initial step in leukocyte infiltration, tumor invasion, or metastasis,^{40,41} the antiinflammatory and antitumor properties of ATIII may be based partially on its suppressive effects on these interactions.

In addition, recent experimental research showed that the administration of ATIII decreases the volume of ischemia in mice and might be neuroprotective.⁴² Detecting and monitoring activation of the coagulation system in ischemic stroke with conventional laboratory tests is difficult because the tests lack adequate sensitivity and specificity. The recent development of immunochemical assays has allowed the detection of intermediate breakdown products of fibrin formation and fibrinolysis.^{43,44} Among these, thrombin-antithrombin III (TAT) complex reflects thrombin activation and fibrin formation, while D-dimer marks plasmin activity and fibrinolysis.⁴⁵⁻⁴⁸

IL18

IL-18, previously termed IFN-alpha-inducing factor, is a member of the IL-1 family, a large group of pro-inflammatory cytokines. It is synthesized as an inactive 24-kDa precursor protein (pro-IL-18) that is subsequently processed by caspase-1 into its mature and biologically active form, which has a molecular weight of 18kDa. The secreted pro-IL-18 can also be processed into its active form by various extracellular enzymes constitutively expressed by leukocytes, such as proteinase-3. The active

form of IL-18 induces signal transduction by binding to its heterocomplex IL-18 α / β receptor expressed on diverse cell-types. These include cells resident in the CNS, such as hypothalamic neurons and murine glia.

Beyond the CNS, the biological effects of IL-18 binding to IL-18R include induction of Th1 and Th2 helper T-cell responses and of cytotoxic activity by natural killer cells, in addition to propagation of intrinsic and extrinsic pathways of apoptosis. IL-18 is a 'key' cytokine in the CNS, controlling two distinct immunological regulatory pathways of cytotoxic and inflammatory responses under neuropathological conditions (Table 2).

S100B

S100B, a low-molecular-weight calcium-binding protein, is most abundant in glial cells of the central nervous system, predominately in astrocytes. Initially these proteins were thought to be brain-specific, but later studies have shown that S100 proteins are found in other tissues, albeit in much lower concentrations than those found in the CNS⁵³ (Table 3).

Astrocytes dominate the cellular regulation of homeostasis and are immediately activated after primary brain injury. S100B is involved specifically in this process by regulation of calcium fluxes and stimulation of astrocyte proliferation.

Intracellularly, S100B is involved in signal transduction via the inhibition of protein phosphorylation, regulation of enzyme activity and by affecting the calcium homeostasis. S100B serum level is increased in a variety of CNS disorders: in cerebral ischemia it has been associated to infarct size, neurovascular status on admission and outcome.⁵⁴ Although in many instances its release may be an effect of the condition rather than the cause, it is nonetheless strongly implicated, and can be considered to be a strong candidate as a surrogate marker of CNS injury.

S100B, is involved in cytokine cascade⁵³ (Figure 1). Secreted S100B can therefore be compared to the circulating pro-inflammatory cytokines, since, like interleukin 1 (IL1), it promotes neuronal survival at low concentrations but is neurotoxic at high levels.

Materials and Methods

We aimed to establish the relationship between markers of the inflammatory response (IL-18 and S100B) and procoagulant and fibrinolytic markers (AT III, TAT, D-dimer, FDP), in patients with cerebral ischemia, and to investigate the association of brain inflammatory markers (S100B and IL-18) and severity of coma. Only a few markers were chosen because of particular interest, but this choice

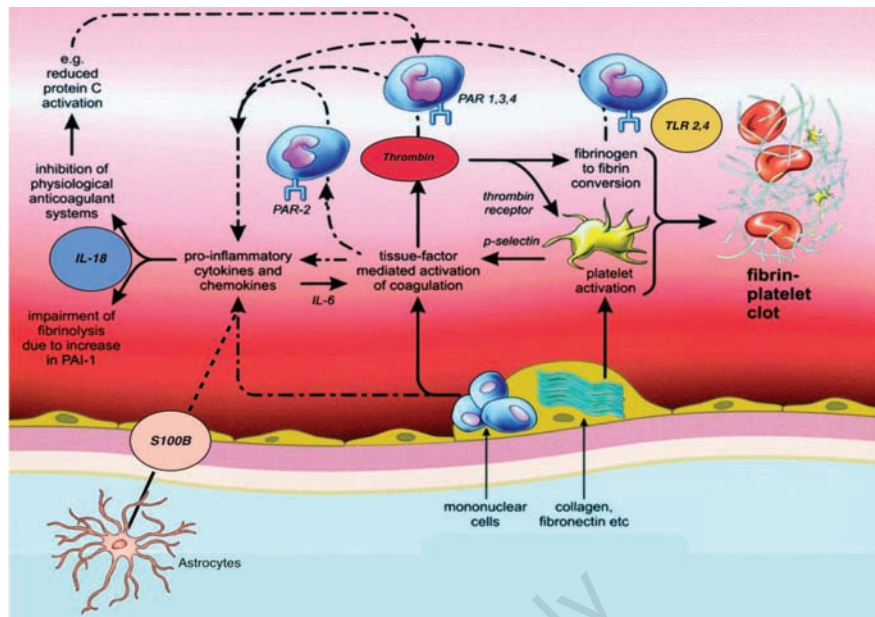


Figure 1. Schematic representation of activation of coagulation and inflammation during cerebral ischemia. Exposure of tissue factor-bearing inflammatory cells to blood results in thrombin generation and subsequent fibrinogen to fibrin conversion. Simultaneously, activation of platelets occurs, both by thrombin and by exposure of collagen (and other subendothelial platelet-activating factors) to blood. Binding of tissue factor, thrombin, and other activated coagulation proteases to specific PARs on inflammatory cells may affect inflammation by inducing release of proinflammatory cytokines (such as IL-18), which will subsequently further modulate coagulation and fibrinolysis. Fibrinogen and fibrin can directly stimulate expression of proinflammatory cytokines on mononuclear cells and induce production of chemokines by endothelial cells and fibroblast. The effects of fibrin (and fibrinogen) on mononuclear cells are at least in part mediated by toll-like receptors (TLR2-4), which are also the receptors of endotoxin. S100 β at supraphysiological concentration can stimulate astrocytes and microglia to produce proinflammatory cytokine. Coagulation pathways are indicated by straight arrows, inflammatory mechanisms by dashed arrows.

Table 1. Thrombin-antithrombin III complex in literature.

Authors	Journal	Type of study	Results	Conclusions
Kataoka S ²	Neurol Sci (2000)	Coagulation markers in atherothrombotic infarct (ATI), cardioembolic infarct (CEI) and lacunar infarct (LI)	CEI: >TAT, FpA, D-dimer, <ATIII ATI: >TAT, FpA, D-dimer, ATIII normal LI: No significant >TAT, d-dimer, FpA	Alterations in thrombotic and fibrinolytic markers should contribute to the clinical diagnosis of brain infarct subtypes
Ince B ³	Thromb Res (1999)	Coagulation and fibrinolysis in ischemic stroke of undetermined etiology	>TAT and FpA >>D-dimer	Hemostatic abnormalities have a primary role in the pathogenesis
Haapaniemi E ⁴	Acta Neurol Scand (2004)	Coagulation and fibrinolytic markers in acute and convalescent phase of ischemic stroke	>D-dimer TAT stable	Minor changes of the fibrinolytic and coagulation system activity in the patients with mild ischemic stroke
Ilzecka S ⁵	Neurol Neurochir Pol (2001)	TAT in ischemic stroke patients	>>TAT	Intense thrombinogenesis
Haapaniemi E ⁶	Acta Neurol Scand (2002)	ATIII in mild and moderate ischemic stroke	>>ATIII No correlation with etiology of stroke, any stroke risk factor or neurological scores	Natural anticoagulants levels did not deliver useful information

FpA, fibrinopeptide A.

cannot imply a completely exhaustive presentation of the topic.

An audit of stroke outcome and procedures was designed in ICU patients; the study protocol was reviewed and approved by the Hospital Ethics Committee (University hospital of Naples, "Federico II").

Thirteen comatose patients following acute stroke admitted in our ICU in one year time were studied.

The inclusion criteria were the following:

- no prior history of ischemic stroke (defined as a rapid developing focal neurological deficit with no apparent signs other than a vascular origin);
- ICU admission within 24h after stroke onset;
- lesion confirmed by neuroradiological examination (brain CT).

To assess the natural course of coagulatory changes following brain infarct, and to avoid the enrolment of patients with concurrent diseases or conditions interfering with the expression of inflammatory mediators, the following exclusion criteria were applied:

- presence of infections, inflammatory, autoimmune, hematologic or malignant diseases;
- severe renal or liver failure;
- thrombolysis,
- abnormalities induced by deep vein thrombosis, disseminated intravascular coagulation and collagen vascular disease.
- thrombolytic treatment.

In all patients, we measured the plasma levels of: thrombin-antithrombin III complex (TAT), D-dimer, Fibrin Degradation Products (FDP), percent activity of ATIII and IL-18 at the 1st and 5th (two time points) day after the stroke onset, S100B serum levels within the first 24 h (one time point). All patients were evaluated using Simplified Acute Physiologic Score (SAPS) III on admission day, Sequential Organ Failure Assessment (SOFA) and Glasgow Coma Scale (GCS) every day.

Patients were divided in two groups according to coma severity:

- Group 1) GCS \leq 8
- Group 2) GCS>8

Data were expressed as mean \pm standard deviation; the significance of the differences among the groups was assessed using ANOVA test and a randomness level of P<0.05 was considered statistically significant.

Measurement of coagulation markers

Plasma concentrations of TAT were determined by enzyme-linked immunoassay (EIA)/Enzygnost TAT micro, Behring, Marburg, Germany). The concentrations of D-dimer and FDP were determined by latex photometry immunoassay (LPIA) (DIA-IATRON, Tokyo, Japan). The measurements of ATIII biological

Table 2. IL18 in literature.

Authors	Journal	Type of study	Results	Conclusions
Zaremba J ⁵⁰	Neurol Sci (2003)	IL-18 in stroke patients	Elevated serum IL18 levels in stroke patients	IL-18 is involved in stroke-induced inflammation
Mallat Z ⁵¹	Circulation (2001)	IL18 mRNA in stable and unstable human carotid atherosclerotic plaques	Significantly higher levels of IL-18 mRNA were found in symptomatic (unstable) plaques than asymptomatic (stable) plaques	Major role for IL-18 in atherosclerotic plaque destabilization leading to acute ischemic syndromes
Yuen CM ⁵²	Circ J (2007)	IL-18 in ischemic stroke patients	Elevated IL18 levels	Evaluation of circulating IL-18 level might improve the prediction of unfavorable clinical outcome following ischemic stroke

Table 3. S100 β in literature.

Authors	Journal	Type of study	Results	Conclusions
Jackson RG ⁵⁴	Clin Chem Lab Med (2000)	S100 β in traumatic brain injury	Levels of S-100 β fell rapidly after its release following traumatic brain injury	S100 β is released after brain insults and serum levels are positively correlated with the degree of injury
Piazza O ⁵⁵	Minerva Chir (2005)	S100B in cardiac arrest	S100 β levels are elevated after cardiac arrest	Increased levels of S100 β 12 h after a cardiac arrest might be expression of a still amendable brain damage
Piazza O ⁵⁶	Br J Anaesth (2007)	S100B in severe sepsis	Elevated S100 β levels	An increase in S100 β does not allow the physicians to distinguish patients with severe impairment of consciousness from those with milder derangements or to prognosticate neurological recovery

activity were performed by the amidolytic method. Venous blood was sampled from the antecubital vein and then measured within 3h after blood sampling. TAT, ATIII and D-dimer were collected in tubes containing 3.8% trisodium citrate. These plasma samples were separated by centrifugation at 2300xg for 15 min at room temperature and then stored at -80° until needed for assay.

Measurement of IL-18

The samples obtained by the antecubital venipuncture were allowed to clot at room temperature for 30 min, and after being cen-

trifuged for 10 min, the serum was immediately frozen. IL-18 serum levels were quantified by ELISA (MBL, Naka-ku Nagoya, Japan) according to the manufacturer's instructions (normal value 155.7 \pm 51.4 pg/mL⁵⁰ Value of our control group of 10 volunteers, age 28 \pm 1.3, 20% women, is 168.9 \pm 40 pg/mL).

S100B analysis

A peripheral blood sample of S100B was analyzed in duplicate using a monoclonal two-site immunoradiometric assay (LIAISON® Sangect® 100). The lower limit detection was 0.02 μ g/L (normal value <0.15 μ g/L).¹⁷

Table 4. Demographic data and severity scores.

Mortality % (dead/alive)	58/42
SAPSIII	63.9±7.23
SOFA day 1	6.90±3.04
SOFA day 5	6.88±3.51
GCS day 1	7.25±3.30
S100B day 1 (g/mL)	1.11±1.48
IL18 day 1 (pg/mL)	355.42±105.94
IL18 day 5 (pg/mL)	350.42±236.36
ATIII day 1 (%)	92.08±10.63
ATIII day 5 (%)	89.30±12.80
TAT day 1 (ng/mL)	11.63±13.67
TAT day 5 (ng/mL)	8.37±10.38
D-d day 1 (ng/mL)	4.54±6.00
D-d day 5 (ng/mL)	6.24±6.99
FDP day 1 (ng/mL)	2.09±0.70
FDP day 5 (ng/mL)	1.70±0.82

Results

The study group was composed of 13 patients with atherothrombotic infarcts (7 males/6 females, age 73.6±6.75 years, SAPS III 63.9±7.23). Demographic data, mortality and biochemical markers of study groups are shown in Table 4.

In all the patients plasma levels of TAT increased at all time points, but no significant differences were observed between day 1 and day 5. These constantly elevated levels were associated with increased D-dimer and FDP plasma levels at all time points.

Percent activity of ATIII was not decreased in ischemic patients on day 1 and 5 after the ischemic onset.

No correlation was found between inflammatory response (IL-18 and S100B) and procoagulant and fibrinolytic markers (ATIII, TAT, D-dimer and FDP).

IL-18 plasma levels were significantly ($P<0.05$) higher at day 1 (309.4±58.4 pg/mL) and at day 5 (246.7±73.6 pg/mL) compared to reference value.

S100B serum levels were significantly higher ($P=0.004$) in stroke patients compared to normal value.

No correlation was found between IL-18 serum levels in focal ischemic patients and GCS on admission. S100B serum levels were higher (1.5772 µg/mL) in those patients who had the poorer neurological examination ($GCS\leq 8$) ($P=0.003$) (Table 5).

Discussion

In our case series, in contrast to other available literature data,^{49,52} the increase of TAT is

Table 5. Biomarkers in the two GCS-related groups.

	N° of patients	S100 β	IL-18	Mortality (dead/alive)
GCS ≤ 8	9	1.5772 µg/mL	296 pg/mL	78%
GCS > 8	4	0.2005 µg/mL	334 pg/mL	0%

not followed by the reduction of plasma ATIII activity. The elevation of D-dimer and FDP without a depletion of ATIII-activity may indicate the presence of active thrombotic lesions promoting the endogenous fibrinolysis.

Increase in serum IL-18 levels of ischemic stroke patients within the first 24-48 hours after disease onset may suggest cytokine upregulation during acute phase of ischemia.

No correlation was found between IL-18 serum levels in focal ischemic patients and GCS on admission, suggesting that IL-18 could be considered an early but aspecific marker of brain injury and its role as a predictive factor for ischemic outcome should be better investigated.

High S100B serum levels are correlated with the GCS score.⁵⁸ The ischemic-induced cerebral S100B release could be the result of a passive release by damaged cerebral cells and of an active release by stimulated cerebral cells initiating repair mechanisms,^{55,59} but S100B serum levels might not reflect the "cerebral" S100B levels because of extra-cerebral sources of this protein.⁶⁰

Conclusions

Thrombin production may be directly related to thrombus formation or may reflect secondary hemostatic activation due to inflammation and endothelial injury. Antithrombin III is the most important physiological inhibitor of blood coagulation as it interferes with the clotting process at various levels and its deficiency has been suggested to have an important role in the pathogenesis and in the evolution of cerebral ischemia and might be neuroprotective, playing an anti inflammatory role.

Activation of coagulation and impairment of fibrinolysis is mediated by cytokines, with an enhancement of coagulation. IL-18 is a pro-inflammatory cytokine considered an important marker for monitoring severe inflammation conditions and it is involved in early stroke-induced inflammation: increase in serum IL-18 levels of ischemic stroke patients within the first 24-48 hours after disease onset suggests cytokine upregulation during acute phase of ischemia. The use of biochemical markers of cerebrovascular injury in ischemia, such as the Calcium-binding protein S100B, could improve patients care but, unfortunately, up to now, none of these biochemical markers

can be considered specific enough to guide clinical decisions.

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