**International Journal of Chemical and Biomolecular Science**
Vol. 1, No. 4, 2015, pp. 278-283
http://www.aiscience.org/journal/ijcbs

# Influence of IgG Separated from Blood Plasma of Patients with Ischemic Stroke on the Process Platelet's Proteins Secretion

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## Abstract

Influence of IgG accumulated in the blood plasma of patients with different types of ischemic stroke (atherothrombotic and cardioembolic) and with another neurological diseases (without stroke in anamneses) on the platelets was investigated. Influence of thrombin and collagen was tested too. Presence in the medium of incubation of most common proteins like Plasminogen activator inhibitor (PAI-1), von Willebrand factor (vWF), Heat shock proteins (Hsp-60, Hsp-70) were detected through the Western blot method.

## Keywords

IgG, PAI-1, FvW, Hsp-60, HsP-70, Ischemic Stroke

Received: September 8, 2015 / Accepted: September 24, 2015 / Published online: November 11, 2015



## 1. Introduction

Today stroke is a global problem, that hit a big part of the population even in the most developed countries [1]. According to the latest data of the World Health Organization each year 300 cases of stroke recorded for every 100 thousand of population [3, 4]. During the past 10 years the prevalence of this disease increased by 5%, mainly due to people under the 40 [7]. According to many experts, the incidence of stroke will grow in the next 25 years [2, 5]. Among all vascular diseases 85% belong to acute ischemic cerebrovascular accident [6]. As known stroke provoke activation of the coagulation process, thrombus formation and vascular occlusion [7, 8]. The most important role in the thrombus formation belongs to platelets. They secrete substances needed to attract other platelets, activates coagulation process, produces the promoters of endothelial healing and blood vessels [9]. Today we know that platelets are involved in the development of inflammatory reactions and immune response [10, 11]. In parallel, it is known that the process of a diseases development, including ischemic stroke may be associated with the appearance in the blood circulation of immunoglobulin G that are capable of activating of certain links of hemostasis [12, 13].

Considering this fact the great interest today provoke examination of the functioning of platelets under the influence of IgG formed in the bloodstream of patients with the most common subtypes of stroke such as atero- and cardioembolic ischemic stroke.

## 2. Materials and Methods

Blood samples were taken from the cubital vein from the patients. For plasma excretion and removal fibrinogen and related proteins blood samples were centrifuged around 2 thousand RPM for 40 minutes. The supernatant was used for the experiment.

Three factions of IgG were used in the experiment: the first one was obtained from the plasma of patients with atherothrombotic (AII), the second one from the plasma of patients with cardioembolic ischemic stroke (KII) and the third one from the plasma of patients with other neurological

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diseases (OND).

To isolate IgG plasma fraction was salting out by the saturated solution of ammonium sulfate ((NH4) 2SO4) to the final concentration equal to 45%. Then centrifugation was performed for 1,500 g duration 30 min at 4°C. The precipitate was dissolved in half the original volume of 50 mM Tris-NCl buffer containing 0.13 M NaCl, pH 7.4 and removed the remains with ammonium sulfate by chromatography on Sephadex G-25 column.

Then solution was applied to the column of Protein A Sepharose HP. The column was washed with 10 volumes of 50 mM Tris-NCl buffer, pH 7.4, containing 0.13 M NaCl and eluted with 100 mM glycine-HCl buffer, pH 2.0. The eluate was collected, by controlling the absorption (wavelength was equal 280 nm) and immediately neutralized with 1 M Tris to pH 7,6. Fractions containing immunoglobulins, were transfer in 50 mM Tris-HCl buffer pH 7.4 containing 0.13 M NaCl by chromatography on Sephadex G-25 column [18]. Quality of immunoglobulin fractions was controlled by disk-electrophoresis in 10% PAGE with SDS-Na [20].

For extracting of platelets reach plasma blood was taken from the cubital vein, with the addition of sodium citrate (38 g/L) in the final proportion 9: 1, then 20 min of centrifugation was performed with 150 g and a temperature 20˚C. The supernatant contain platelets rich plasm were taken for further experiment. Activity of platelets were tested by adding an equal volume of 50 mM Tris HCl buffer, pH 7,4 containing 0.13 M NaCl, 0.1 mM of CaCI2 and ½ volume solution of 10 mM ADP. Platelet aggregation was observed after 2 minutes of incubation 37°C [19].

For the purification of platelet was done by using of chromatography that divide particle to the size on Sepharose 4B column. The column was equilibrated of 50 mM Tris-NCl buffer, pH 7.4, containing 0.13 M NaCl, then platelets rich plasma was applied. The eluate was collected in 2 ml plastic eppendorfs and visually controlled presence of cells. For control quality platelets were centrifuged for 10 min. for 300g. Then supernatant were analyzed by disc electrophoresis in polyacrylamide gel in the presence of 0.1% SDS-Na [3]. Chromatography allowed us to make platelets clean (second fraction) without protein impurities that was in the third fraction. (Fig. 1).

Pure platelets were incubated with presence of the fraction of IgG from all experimental groups in a final concentration of 0.7 mg/ml for 30 min. then centrifugation 300 g for 15 min. and a temperature of 20°C was performed. Protein content in the medium after incubation was analyzed by method of Western blotting [21]. Specific antibodies against target proteins were used for the Western blot. Results of incubating platelets with IgG were compared with the results obtained by analogical incubation platelet with thrombin, collagen which are standart inducers of platelet aggregation.

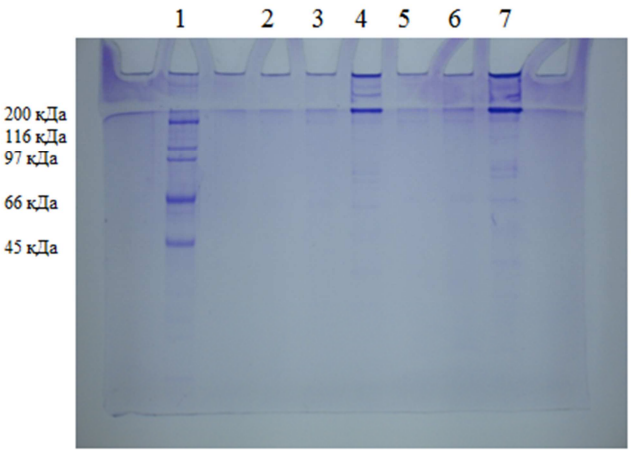


**Fig. 1.** Gel-elektroforesis of purified platelet on Sepharose 4B column.

1. Leader of Molecular Weight (120 kDa, 116 kDa, 97 kDa, 66 kDa, 45 kDa)
2. Fraction 1; 5 µl
3. Fraction 2; 5 µl (purified platelet)
4. Fraction 3; 5 µl (protein impurities)
5. Fraction 1; 10 µl
6. Fraction 2; 10 µl (purified platelet)
7. Fraction 3; 10 µl (protein impurities)

## 3. Results and Discussion

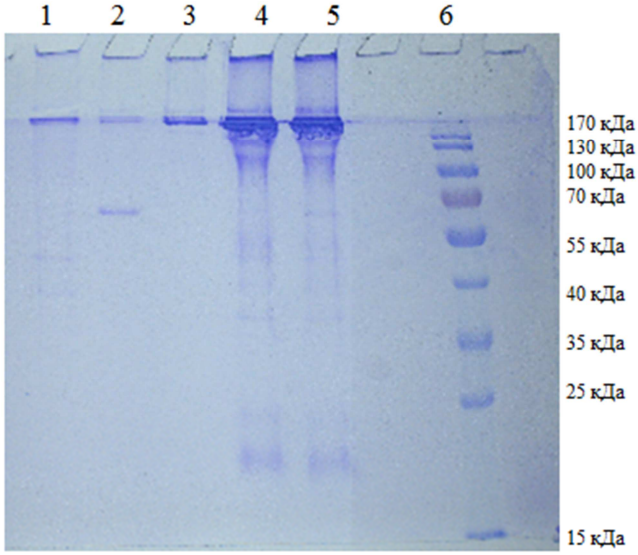


**Fig. 2.** Electrophoresis of mudium after.

1. Incubation platelets with collagen
2. Incubation platelets with thrombin
3. Incubation platelets with IgG separated from plasma of patients with other neurological diseases
4. Incubation platelets with IgG separated from plasma of patients with cardioembolic ischemic stroke
5. Incubation platelets with IgG separated from plasma of patients with atherothrombotic ischemic stroke
6. Leader of Molecular Weight (170 kDa, 130 kDa, 100 kDa, 70 kDa, 55 kDa, 40 kDa, 35 kDa, 25 kDa, 15 kDa)

We have shown that incubation of platelet with IgG fractions of all studied groups, causes the release of some proteins and complexes of protein in a wide range of molecular weight start from 13 kDa to 230 kDa. IgG separated from the plasma of patients with other neurological diseases have not effected the secretory function of platelets. Presence only one complex with a molecular weight of 190 kDa in the platelet incubation medium was shown in this case (Fig. 2).

In the same conditions, collagen and thrombin have caused the secretion of one protein or complex of protein, which corresponded to molecular weight in the region of 186-192 kDa. (Fig. 2 / Table. 1).

**Table 1.** The molecular weight of proteins detected in the incubation medium after influence of studied platelet inductors.

| M.w. kDa | IgG from AII | IgG from CII | IgG from OND | Thrombin | Collagen |
|---|---|---|---|---|---|
| 230-210 | + | + | - | - | - |
| 186-192 | + | + | + | + | + |
| 147-170 | + | + | - | - | - |
| 120 | + | + | - | - | - |
| 104 | + | + | - | - | - |
| 61 | + | + | - | - | - |
| 54 | + | + | - | - | - |
| 49 | + | + | - | - | - |
| 40 | + | + | - | - | - |
| 36 | + | - | - | - | - |
| 21 | + | + | - | - | - |
| 13 | + | + | - | - | - |

The next stage of the experiment was to investigate the influence of IgG to the secretion of Plasminogen activator inhibitor type 1 (PAI-1). Today the complex forms of PAI-1 are well characterized and are known molecular weight, including isolated complex shape PAI-1 with tissue plasminogen activator (t-PA) and vitronectin (over 700 kDa) complex PAI-1 with vitronectin (450 kDa ) and functionally inactive free form PAI-1 (52 kDa) [14]. However, proteins or complexes of proteins that we detect are not recorded in the literature data and according to this fact require further study. As a result of our study complex of PAI-1(with a molecular mass of about 170 kDa.) was detected under the influence of collagen and thrombin. Moreover collagen provokes releasing of this complex of PAI-1 from platelet on 40% intensively comparing with the same by thrombin. IgG patients with AII and KII causing the secretion of small amounts of the inhibitor's complex with molecular weight equal to 210 kDa and 280 kDa. IgG from the plasma of patients with OND did not cause the secretion of PAI-1 (Fig. 3 / Table 2).

The next step of the experiment was detection of the influence of IgG on the process of vWF releasing from platelets. The Fig. 4 and the Table.3 clearly shows that all IgG fractions, and thrombin cause secretion of vWF (with a molecular weight 270 kDa.), and complex forms of vWF with molecular weight equal to 300 kDa. Besides IgG separated from the plasma of patients with both subtypes of stroke caused appearance in the platelets medium of incubation other complex forms of vWF with molecular weights 333 kDa, 240 kDa, 167 kDa, 97 kDa and 54 kDa. Some of the detected complexes were identified in the literature data. As you know vWF was synthesized as a predecessor the processing of which takes place in EPR and in the Golgi complex of endothelial cells. Then multimerization oh this product and cutting to the mature product (240 kDa) and propeptide (97 kDa) [15, 16].

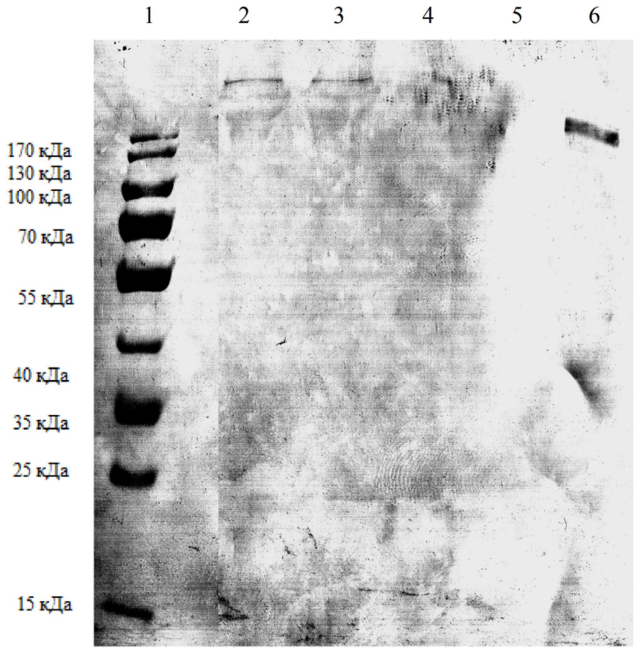


**Fig. 3.** Western Blot of PAI-1 identification in the medium of platelets incubation with.

1. Leader of Molecular Weight (170 kDa, 130 kDa, 100 kDa, 70 kDa, 55 kDa, 40 kDa, 35 kDa, 25 kDa, 15 kDa)
2. IgG separated from plasma of patients with atherothrombotic ischemic stroke
3. Incubation platelets with IgG separated from plasma of patients with cardioembolic ischemic stroke
4. IgG separated from plasma of patients with other neurological diseases
5. Thrombin
6. Collagen

**Table 2.** The molecular weight of PAI-1 complexes detected in the incubation medium after influence of studied inductors on the platelets.

| M.w. kDa | IgG from AII | IgG from CII | IgG from OND | Thrombin | Collagen |
|---|---|---|---|---|---|
| 280 | + | + | - | - | - |
| 210 | + | + | - | - | - |
| 170 | - | - | - | + | + |

In contrast, collagen caused secretion from the platelets of one fragment of vWF in the area of 246 kDa. It is important to emphasize that von Willebrand factor is a multidimeric plasmatic glycoprotein, the molecular weight of one subunit is approximately 240-250 kDa [14]. It should be noted that most intensive impact was made by IgG separated from the plasma of patients with KII. There was shown that IgG separated from

the plasma of patients with AII provoke on the 20% intensive secretion of vWF (with a molecular weight of 270 kDa) in respect with the same influence of thrombin. IgG from the plasma of patients with KII up then 44% and IgG from the plasma of patients with OND up then 53% compared to thrombin. A similar situation was observed with a complex of vWF with molecular weight 300 kDa. The most effective impact was done by IgG from patients with KII compared with other factions IgG (30% relative fraction obtained from the plasma of patients with AII and 83% relative OND) and thrombin (79%).

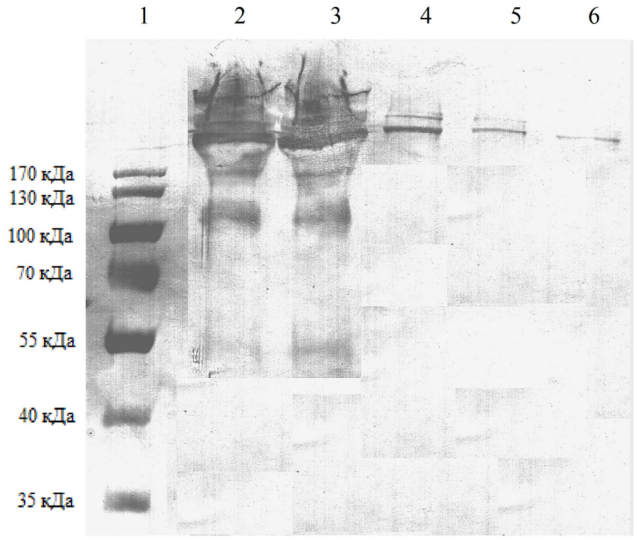


**Fig. 4.** Western Blot of von Willebrand factor identification in the medium of platelets incubation with.

1. Leader of Molecular Weight (170 kDa, 130 kDa, 100 kDa, 70 kDa, 55 kDa, 40 kDa, 35 kDa, 25 kDa)
2. IgG separated from plasma of patients with atherothrombotic ischemic stroke
3. Incubation platelets with IgG separated from plasma of patients with cardioembolic ischemic stroke
4. IgG separated from plasma of patients with other neurological diseases
5. Thrombin
6. Collagen

**Table 3.** The molecular weight of von Willebrand factor complexes detected in the incubation medium after influence of studied inductors on the platelets.

| M.w. kDa | IgG from AII | IgG from CII | IgG from OND | Thrombin | Collagen |
|---|---|---|---|---|---|
| 333 | + | + | - | - | - |
| 300 | + | + | + | + | - |
| 270 | + | + | + | + | - |
| 246 | - | - | - | - | + |
| 240 | + | + | - | - | - |
| 167 | + | + | - | - | - |
| 97 | + | + | - | - | - |
| 54 | + | + | - | - | - |

The next step of the experiment was detection of the influence of IgG on the process of heat shock proteins (HSP-60, HSP-70) releasing from platelets. HSP-60 secretion was recorded only under the influence of IgG separated from patients with AII and KII. AII patients IgG caused about 44% intense secretion of HSP-60 in respect with KII patients. All IgG factions induced appearance in the platelets medium of incubation complex forms of HSP-60 with a molecular weight equal to 270 kDa, 192 kDa, 164 kDa and 104 kDa. It should be noted that the main products of platelet secretion under the influence of thrombin were components with molecular masses of 224 kDa (similar fragments were recorded under the influence of IgG from plasma KII and OND) and 139 kDa (similar fragments were recorded under the influence of IgG from plasma AII and KII). About the collagen, it caused the secretion of two complex forms of HSP-60, 224 kDa (IgG from plasma KII, OND and thrombin showed a similar effect) and 164 kDa (like all IgG factions) (Figure 5 / Table 4).

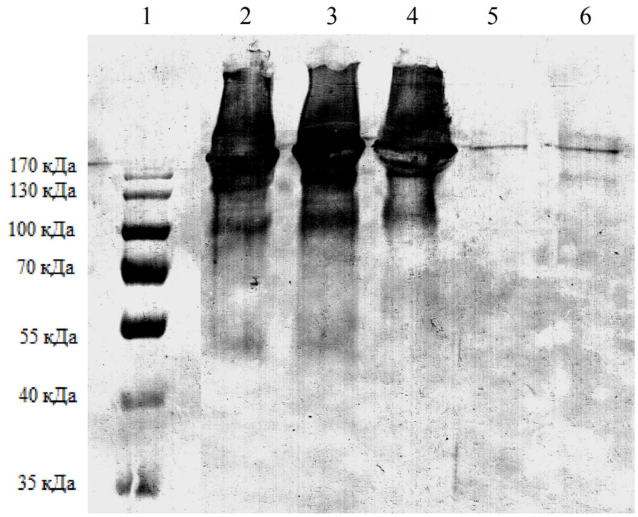


**Fig. 5.** Western Blot of HSP-60 identification in the medium of platelets incubation with.

1. Leader of Molecular Weight (170 kDa, 130 kDa, 100 kDa, 70 kDa, 55 kDa, 40 kDa, 35 kDa, 25 kDa)
2. IgG separated from plasma of patients with atherothrombotic ischemic stroke
3. Incubation platelets with IgG separated from plasma of patients with cardioembolic ischemic stroke
4. IgG separated from plasma of patients with other neurological diseases
5. Thrombin
6. Collagen

**Table 4.** The molecular weight of HSP-60 complexes detected in the incubation medium after influence of studied inductors on the platelets.

| M.w. kDa | IgG from AII | IgG from CII | IgG from OND | Thrombin | Collagen |
|---|---|---|---|---|---|
| 270 | + | + | + | - | - |
| 224 | - | + | + | + | + |
| 192 | + | + | + | - | - |
| 164 | + | + | + | - | + |
| 139 | + | + | - | + | - |
| 104 | + | + | + | - | - |

A similar situation was relatively secretion of HSP-70. According to numerous literary sources of heat shock proteins are multifunctional proteins. We know that they are

involved in assembly, stabilization, folding and translocation of proteins and mediate the degradation of damaged proteins. That there is a great variety of protein complex component which can be heat shock proteins, including HSP-60 and HSP-70 [17]. IgG caused a release of all fractions of complex forms of HSP-70 with a molecular weight of about 147 kDa and 99 kDa. The highest concentration of the complex with a molecular weight of 147 kDa was observed under the influence of IgG isolated from the plasma of patients with AII 91% intense compared to IgG isolated from the plasma of patients with KII and 60% compared to IgG isolated from the plasma of patients with OND. The complex of 99 kDa was fixed in the platelets incubation medium on 7% intense by the action of the IgG isolated from the plasma of patients with KII compared with the same influence of IgG isolated from the plasma of patients with AII and by 48% compared with IgG isolated from the plasma of patients with OND. Besides the influence of IgG from the plasma of patients with AII and KII provoked the appearance of the incubation medium 120 kDa complex. Thrombin caused the secretion of the protein complex of 216 kDa (and IgG isolated from the plasma of patients with both types of stroke), and provoked the appearance of collagen in the incubation medium 186 kDa complex (like IgG isolated from the plasma of patients with both types of stroke). (Fig. 6/Table. 5).

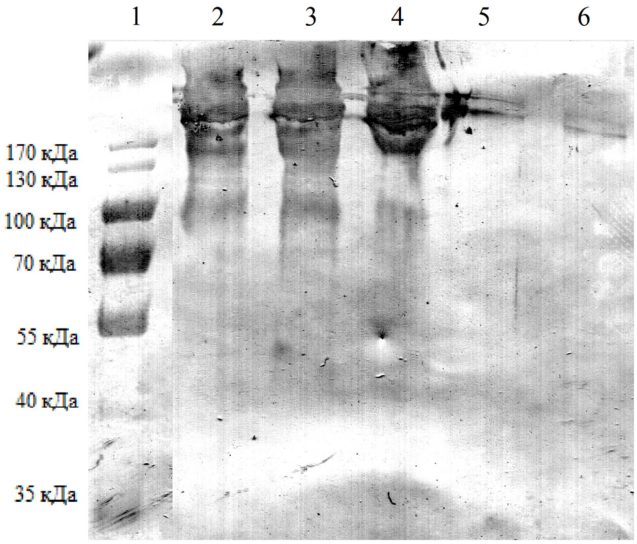


**Fig. 6.** Western Blot of HSP-70 identification in the medium of platelets incubation with.

1. Leader of Molecular Weight (170 kDa, 130 kDa, 100 kDa, 70 kDa, 55 kDa, 40 kDa, 35 kDa, 25 kDa)
2. IgG separated from plasma of patients with atherothrombotic ischemic stroke
3. Incubation platelets with IgG separated from plasma of patients with cardioembolic ischemic stroke
4. IgG separated from plasma of patients with other neurological diseases
5. Thrombin
6. Collagen

**Table 5.** The molecular weight of HSP-70 complexes detected in the incubation medium after influence of studied inductors on the platelets.

| M.w. kDa | IgG from AII | IgG from CII | IgG from OND | Thrombin | Collagen |
|---|---|---|---|---|---|
| 216 | - | - | - | + | - |
| 186 | - | - | - | - | + |
| 147 | + | + | + | - | - |
| 120 | + | + | - | - | - |
| 99 | + | + | + | - | - |

## 4. conclusions

We conducted a platelet incubation with immunoglobulin G isolated from blood plasma of patients with atherothrombotic and cardioembolic ischemic stroke and blood plasma of patients with other neurological diseases without a history of stroke. By western blot of the incubation medium was found a series of proteins: plasminogen activator inhibitor type 1, heat shock protein with a molecular weight 60 kDa and complex forms of von Willebrand factor and heat shock protein with a molecular mass of 70 kDa. It was established that the presence in the incubation medium IgG isolated from the blood plasma of patients with both subtypes of stroke cause the appearance of the incubation medium large number of protein fragments and protein complexes, in contrast to thrombin, collagen and IgG isolated from blood plasma of patients with other neurological diseases without a history of stroke. Moreover, immunoglobulin G isolated from blood plasma of patients with cardioembolic stroke provoke releasing of components intense an average on 20-40% than class G immunoglobulins isolated from blood plasma of patients with atherothrombotic stroke.

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