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# MORPHOLOGICAL AND IMMUNOENZYMATIC CHARACTERISTICS OF ASPIRATED THROMBI IN PATIENTS WITH ST-ELEVATION MYOCARDIAL INFARCTION

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***Abstract***

*The aim of the study was to evaluate the morphological and immunohistochemical characteristics of aspirated thrombi in patients with ST-elevation myocardial infarction undergoing percutaneous coronary intervention to find the possible platelet activation and inflammatory cell involvement in coronary thrombi.*

*Thrombi collected from 67 patients were studied. Macroscopic analysis was performed and immunoenzymatic methods were used to reveal the cellular components of thrombi. The peroxidase-antiperoxidase immunohistochemical method was applied with monoclonal antibodies to CD4, CD8, CD15, CD63 and CD105 using diaminobenzidine as a visualization agent to investigate the potential involvement of different cellular subpopulations.*

*The macroscopic examination revealed friable thrombi with apparent laminations (lines of Zahn), which represent pale platelet and fibrin deposits alternating with darker red blood cell-rich layers. The microscopic examination of thrombi revealed a loose meshwork of fibrin fibers, with compact accumulation of activated platelets. The platelets were surrounded by neutrophil granulocytes. The immunophenotyping analysis confirmed cell aggregates consisting of CD63+ activated platelets. The surrounding neutrophils were also CD63+. The granulocytes also tested positive for CD15 antigen. The immunohistochemical analysis revealed the presence of CD8+ cytotoxic T-lymphocytes and CD4+ helper T-lymphocytes. No expression of iNOS, CD105 and VEGF was revealed in the analyzed thrombi.*

*The histopathological evaluation of thrombi in patients with acute ST-elevation myocardial infarction revealed the presence of activated CD63+ platelets and CD15+ neutrophilic granulocytes. Activated platelets and neutrophils may play a role in thrombo-inflammatory activation process leading to destabilization of atherosclerotic plaque and development of acute thrombosis in the patients with ST-elevation myocardial infarction.*

***Keywords:*** *myocardial infarction, coronary thrombus, platelet aggregation, thrombectomy.*

## *Introduction*

Acute occlusion of coronary artery due to atherosclerotic plaque rupture, erosion with subsequent superimposed thrombus formation cause ST-elevation myocardial infarction (STEMI) [*Davies MJ, Thomas AC, 1985*]. Several cellular mechanisms play a key role in the progression of local inflammation, platelet activation and thrombus formation [*Srikanth S, Ambrose J, 2012*]. The rupture of plaque enriched by lipids, which is a highly thrombogenic event, promotes rapid platelet recruitment to the site. Platelet adhesion and aggregation, together with formation of fibrin polymeric network, result in a thrombus formation which further limits the blood flow around the atherosclerotic plaque. It is also known that local inflammatory stimuli might contribute to plaque vulnerability [*Hansson GK et al., 2015*] and that red blood cells, neutrophils, monocytes and T- and B-lymphocytes are present in these thrombi within hours after symptom onset [*Silvain J et al., 2011; Yunoki*

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*K et al., 2012; Sadowski M et al., 2014; Ramaiola I et al., 2015*], but the involvement and pathophysiological role of different cell subpopulations in the process of coronary thrombus formation are not yet completely clear. The morphological evaluation of cellular composition of coronary artery thrombi is therefore an important step towards the understanding of the pathophysiology of plaque destabilization and formation of occlusive thrombi.

Percutaneous coronary intervention is considered to be the gold-standard therapeutic procedure in the patients with STEMI. Adjunctive aspiration of thrombus (thrombectomy) is performed when necessary and gives the possibility to carry out morphological assessment of retrieved thrombi. In this study, we aimed to evaluate the morphological and immunohistochemical characteristics of aspirated thrombi in the STEMI patients undergoing percutaneous coronary intervention, and to reveal the possible pathophysiological mechanisms leading to platelet activation and inflammatory cell involvement in the formation of coronary artery thrombi.

### *Material and methods*

We prospectively screened all the patients with acute STEMI referred to catheterization laboratory for percutaneous coronary intervention from January 2013 to April 2014. All the patients had STEMI lasting >30 *min* and arrived to University Hospital within 12-hours after the chest pain onset, had ST-elevation >2 *mm* in two contiguous leads. All percutaneous coronary interventions were performed according to current standard guidelines for percutaneous coronary interventions [*Authors/Task Force members et al., 2014; Ibanez B et al., 2017*]. Those patients, in whom aspirated material could not be interpreted immunohistochemically, or no material could be obtained, were excluded from this study. All the patients received aspirin initially at 250 *mg* and clopidogrel loading dose of 300-600 *mg*, 5000 *IU* of heparin intravenously (iv) on admission or prehospitally. The culprit artery was determined as coronary artery perfusing the area related to ST-segment elevation area on resting 12-lead electrocardiogram.

Thromboaspiration was performed in all the patients with thrombolysis in myocardial infarction, flow grade 0 or 1, if thrombus was visible after coronary angiography, before any interventional devices other than guidewires and thrombectomy devices were used. None of the patients received thrombolysis therapy or glycoprotein IIb/IIIa receptor inhibitors before thrombus aspiration.

Collected thrombi were immediately fixed in a 10% neutral buffered formalin solution. The paraffin-embedded material was serially sectioned and placed on glass slides. The sections were stained with hematoxylin and eosin for light microscopy. The visualization of fibrin was performed by Picro-Malory's staining method. To perform the investigation of cellular components and activated thrombocytes, the peroxidase-antiperoxidase immunohistochemical method was used with monoclonal antibodies to CD4, CD8, CD15, CD63 and CD105 using diaminobenzidine as a visualization agent of Spring Bioscience Roche.

The slides were stained by Picro-Malory's method for fibrin visualization. We used the following primary antibodies (Spring Bioscience): CD15 (dilution 1:50, clone SP159, rabbit monoclonal), CD105 (dilution 1:50, clone – N/A, rabbit polyclonal), vascular endothelial growth factor antibody (dilution 1:100, clone SP28, rabbit monoclonal), inducible nitric oxide synthase antibody (dilution 1:100, clone SP28, rabbit monoclonal), CD63 (dilution 1:200, clone SPM524, mouse monoclonal), CD4 (dilution 1:50, clone SP35, rabbit monoclonal) and cytotoxic T-lymphocyte (CD8) (dilution 1:100, clone SP16, rabbit monoclonal) by the standard peroxidase-antiperoxidase method, followed by the visualization with detection of horseradish peroxidase activity based the action of diaminobenzidine using Polyvalent horseradish peroxidase diaminobenzidine Detection system. The anti-nitric oxide synthase and anti-vascular endothelial growth factor antibodies were also used in the assessment.

***Statistical analysis.*** Categorical variables were expressed as percentages. The study protocol was approved by Yerevan State Medical University Ethic Committee.

### *Results*

Overall, thrombi collected from 67 patients were investigated. The clinical characteristics of patients are presented in table. The images of morphological characteristics of thrombi were compatible with those commonly forming in the arte-

rial wall. The macroscopic examination revealed friable thrombi, with apparent laminations (lines of Zahn), which represent pale platelet and fibrin deposits alternating with darker red blood cell-rich layers. The microscopic examination of thrombi revealed a loose meshwork of fibrin fibers, with compact accumulation of CD63-positive activated platelets (Fig.1).

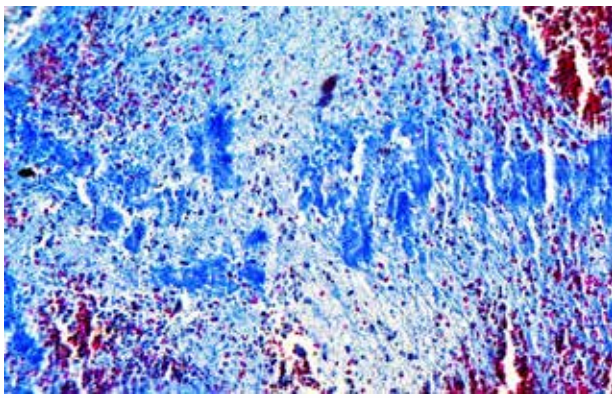

***Figure 1.*** *The loose meshwork of blue fibrin. Picro-Malory. *100*

The platelets were surrounded by neutrophil granulocytes, in some areas with foci of extensive, "microabscess-like" accumulation. The aggregates also contained a small number of degenerated erythrocytes, which, on a fibrin meshwork, were sometimes found as areas of accumulated cell groups with preserved morphology (Fig. 2).

The immunophenotyping analysis revealed that aggregates of cells consisted of CD63-positive activated platelets. The surrounding neutrophils also showed cytoplasmic positivity for CD63, which represents azurophilic granules of neutrophil granulocytes [*Cham B et al., 1994*]. Apart from CD63 positivity, the granulocytes were also positive for CD15 antigen, also known as Leu-M1 and Lewis X antigen (Figure 3), which plays a role in the activation of phagocytosis and chemotaxis [*Kerr M, Stocks S, 1992*].

***Table***

Clinical characteristics of patients.

| | | |
|---|---|---|
| Total number of patients | 67 | |
| Age, yrs (mean±SD) | 58.4±12.9 | |
| Male gender (%) | 40 | (59.7%) |
| Diabetes mellitus, n (%) | 17 | (25.4) |
| Dzslipidemia, n (%) | 49 | (73.1) |
| Smoking, n (%) | 37 | (55.2) |
| Previous stroke, n (%) | 2 | (2.9) |
| Prior myocardial infarction, n (%) | 7 | (10.4) |
| Prior PCI, n (%) | 5 | (7.4) |
| Previous CABG, n (%) | 2 | (2.9) |
| Prior systolic heart failure, n (%) | 6 | (8.9) |
| Acute heart failure (Kilip II), n (%) | 21 | (31.1) |
| Acute heart failure (Kilip II), n (%) | 5 | (7.4) |
| Shock, n (%) | 2 | (2.9) |
| Infarct-related artery | | |
| Left anterior descending | 31 | (46.2) |
| Left circumflex | 14 | (20.9) |
| Right coronary artery | 22 | (32.8) |
| Postprocedural TIMI 3 flow | 49 | (73.1) |
| Multivessel disease | 23 | (34.3) |

The immunohistochemical analysis for CD8 and CD4 revealed a positive result, indicating the presence of CD8-positive cytotoxic T-lymphocytes and CD4-positive helper T-lymphocytes (Fig. 4). No expression of inducible nitric oxide synthase, CD105 and vascular endothelial growth factor were revealed in the analyzed thrombi.

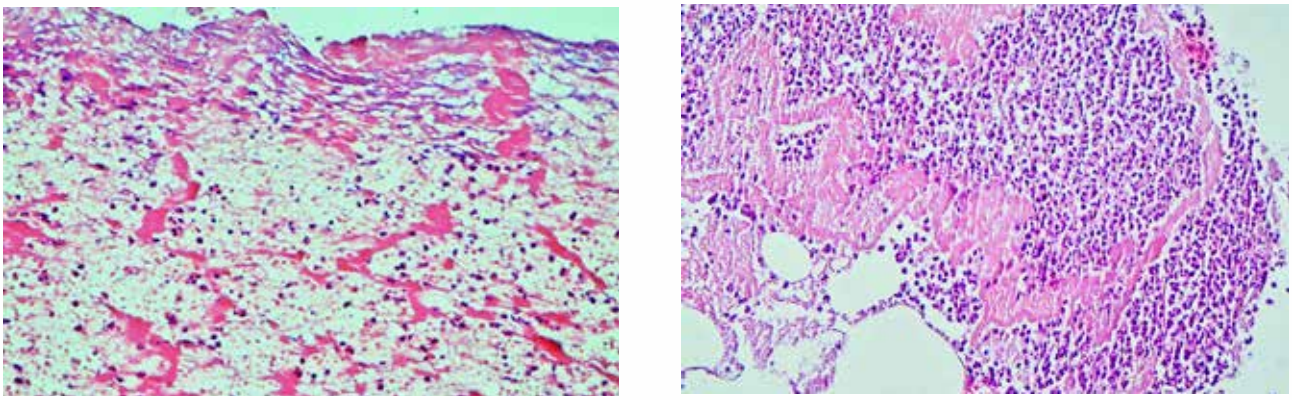

***Figure 2.*** *Panel A. Pink platelet aggregates surrounded by granulocytes. H@E. *100.*
*Panel B. Extensive, "microabscess-like" accumulation of neutrophil granulocytes. H@E. *100*

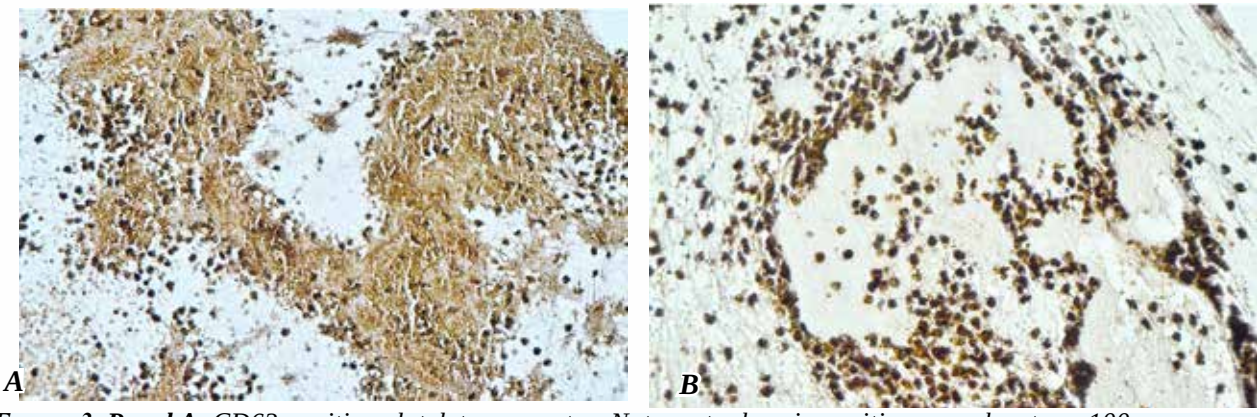

***Figure 3. Panel A.*** *CD63-positive platelet aggregates. Note: cytoplasmic-positive granulocytes. x100*
***Panel B.*** *Platelet aggregates surrounded by CD15-positive granulocytes. x100*

### *Discussion*

The pathophysiological pathways leading to the development of atherosclerosis and thrombosis represent complex mechanisms involving inflammation, endothelial dysfunction and platelet activation. Platelet-leukocyte interaction and consecutive formation of platelet-leukocyte aggregates may determine the clinical outcome in patients with acute myocardial infarction [*Furman M et al., 200; Michelson A et al., 2001*]. The activation of thrombo-inflammatory response may result in different cell interplay and formation of coronary thrombus, with consecutive generation of tissue factors, cytokines and other contributors of thrombi formation. Platelet activation followed by the release of biological active substances can provoke rolling and adhesion of neutrophils to inflamed endothelium [*Duerschmied D et al., 2013*]. Furthermore, previous studies have shown that platelet alpha granules contain adhesive proteins, coagulation and angiogenic factors, such as platelet factor 4 and P-selectin, [*Shi G et al., 2013*] which may contribute or even play a major role in thrombus formation. However, the majority of previous studies have focused on the histological aspects of thrombi [*Rittersma S et al., 2005; Kramer M et al., 2009; Yunoki K et al., 2012*], whereas the immunohistochemical assessment has not been performed to reveal the possible cellular factors involved in thrombogenesis.

Considering the above-mentioned cellular pathophysiological mechanisms, we studied the morphological characteristics of thrombi in patients with coronary artery thrombosis leading to STEMI. The findings of this study demonstrate the presence of activated thrombocytes and erythrocyte components in the thrombi aspirated from the STEMI patients by using the immunohistochemical staining analysis for several cellular antigens. Though previous studies have revealed the presence of platelets, meshwork of fibrin, erythrocytes, and leucocytes in coronary thrombi [*Silvain J et al., 2011; Srikanth S, Ambrose J, 2012; Kovacs A et al., 2015*], most of them focused on the interrelations of structural features of thrombi, laboratory

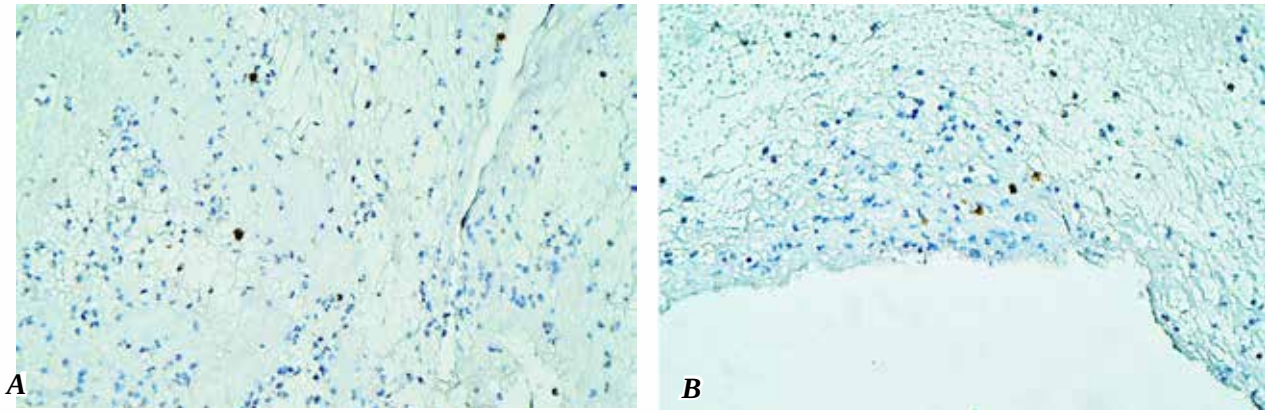

***Figure 4. Panel A.*** *CD8-positive cytotoxic T-lymphocytes. x100*
***Panel B.*** *CD4-positive helper T-lymphocytes. x100*

parameters and clinical findings, which didn't allow for precise evaluation of cellular and antigenic components of thrombi. The recent advancements in the thrombectomy technique allowed us to perform an improved assessment of morphological components of thrombi.

The role of activated platelets in the formation of coronary thrombi has not been previously studied. The current study demonstrated that coronary artery thrombi are rich with platelets and erythrocytes. More specifically, the positive staining of CD63 antigen in thrombi clearly demonstrates the involvement of megakaryocytes, particularly, activated platelets, in coronary thrombus formation. In resting, inactive platelets CD63 are present on the membrane of dense granules and lysosomes, and relocates to the plasma membrane in result of platelet activation and exocytosis, where it is associated with platelet integrin alpha-IIBbeta3-CD9 complex and actin cytoskeleton in the alpha-IIBbeta3-dependent manner [*Nishibori M et al., 1993*]. This process enables the function of CD63 expressed on the plasmatic membrane as a modulator of platelet spreading on immobilized fibrinogen [*Israels S, McMillan-Ward E, 2005*].

In a study by Arakawa et al. the degree of accumulated neutrophils in aspirated thrombi of patients with STEMI was an independent predictor of poor myocardial blush grade, ST –segment resolution, and left ventricular function at 6 months [*Arakawa K et al., 2009*]. In our study we found positivity of granulocytes for CD15 in aspirated thrombi, which promotes phagocytosis and chemotaxis.

Our immunohistochemical analysis showed presence of CD4- and CD8-positive lymphocytes in coronary thrombi, indicating possible involvement of activated lymphocytes in thrombus formation. No evidence was found on any role of lymphocytes in the formation of coronary thrombi. The presence of T-lymphocytes (both helper CD4+ and cytotoxic CD8+) has been determined in atherosclerotic plaque along with macrophages. The evidence derived from animal studies has demonstrated that specialized T- and B-cells may play either provoking or stimulating effects on atherosclerosis [*Ammirati E et al., 2015*]. In particular, T-cells may play an important role in the interaction between vascular and immune systems by enhancing inflammatory effect on vascular wall and development of atherosclerosis. The theory that vascular inflammation is considered an important driver of atherosclerosis led to the implementation of several clinical trials to influence the production of inflammatory cytokines, such as interleukin 1b (IL-1β) (targeted with Canakinumab, a human monoclonal anti-IL-1β antibody in CANTOS trial), IL-6 and tumor necrosis factor-α (TNF-α) (targeted with low dose methotrexate in Cardiovascular Inflammation Reduction Trial [*Sparks J et al., 2017*].

***Study limitations:*** The decision to perform thromboaspiration was made by interventional cardiologists at the Clinic of General and Invasive Cardiology, University Hospital No 1, and. therefore, was no protocol for preliminary selection of candidate patients among all the patients with STEMI. The complete medical history of the onset of angina symptoms was based on patients' subjective description thereby complicating the determination of exact timing of thrombus formation**.** However, despite of the fact that electrocardiographic pattern of ST-elevation is considered a more objective marker of MI onset and coronary artery occlusion, thrombus age is difficult to determine, as the exact time from non-occlusive to occlusive thrombus formation may not be related to clinical manifestation of STEMI.

In conclusion, the histopathological evaluation of thrombi in patients with acute STEMI revealed presence of activated CD63+ platelets and CD15+ neutrophilic granulocytes. The presence of CD4+ and CD8+ cells may provide evidence that lymphocyte activation is involved in the pathophysiology of thrombus formation and requires further investigation. Our findings show that activated platelets and neutrophils may be significant factors involved in the process of thrombo-inflammatory activation leading to destabilization of atherosclerotic plaque and development of acute thrombosis in patients with STEMI. Future studies should further investigate the potential role of activated platelets in the induction of thrombo-inflammatory response which will create an insight of thrombus formation. The unraveling of the mechanisms underlying thrombus formation will optimistically pave the way for developing novel therapeutic approaches to prevent thrombosis in patients with coronary artery disease.

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