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РАЗМЕР ТРОМБОЦИТАРНО-ЛЕЙКОЦИТАРНЫХ АГРЕГАТОВ У ПАЦИЕНТОВ С РАЗЛИЧНОЙ ВЫРАЖЕННОСТЬЮ КОРОНАРНОГО АТЕРОСКЛЕРОЗА

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Резюме. Повышенное содержание тромбоцитарно-лейкоцитарных агрегатов является отражением возрастания протромбогенной и провоспалительной активности клеток периферической крови. Целью данного исследования стало изучение содержания и свойств тромбоцитарно-моноцитарных и тромбоцитарно-лимфоцитарных агрегатов у пациентов с коронарным атеросклерозом. В исследование вошло 19 пациентов с ишемической болезнью сердца и коронарным атеросклерозом (15 мужчин; 4 женщины; 59,0 (55,0; 69,0) лет). Группу сравнения составили 8 пациентов высокого сердечно-сосудистого риска без коронарного атеросклероза. Выраженность атеросклероза оценивали по величине индекса Gensini Score, рассчитанного по данным ангиографии. Для изучения тромбоцитарно-лейкоцитарных агрегатов применяли метод проточной цитометрии с визуализацией. Оценивали относительное количество тромбоцитарно-моноцитарных и тромбоцитарно-лимфоцитарных агрегатов от общего количества моноцитов и лимфоцитов соответственно; долю агрегатов, образованных посредством Р-селектина (CD62P); количество тромбоцитов, агрегированных с каждым отдельным лейкоцитом (моноцитом или лимфоцитом). По результатам исследования среди пациентов с наличием коронарного атеросклероза (Gensini Score > 0 баллов) значимо меньшее количество моноцитов образовывало небольшие агрегаты, в состав которых входил 1 моноцит и 1 тромбоцит (78,8 (68,1; 86,2) против 84,7 (83,8; 87,1) % у пациентов без атеросклероза (p = 0,039)). При этом у пациентов с более выраженным атеросклерозом (Gensini Score ≥ 42,5 баллов) мы выявили тенденцию к увеличению доли агрегатов лимфоцитов с более чем 3 тромбоцитами (0,6 (0,3; 1,6) против 0,1 (0; 0,8) % у пациентов с Gensini Score < 42,5 баллов (p = 0,075)). Доля крупных тромбоцитарно-лимфоцитарных

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агрегатов (с 3 и более чем с 3 тромбоцитами) прямо коррелировала с величиной Gensini Score, концентрацией IL-1β, системными воспалительными индексами, отношением концентрации триглицеридов к глюкозе и триглицеридов к холестеролу липопротеинов высокой плотности (индексы инсулинорезистентности), и обратно – с содержанием холестерола липопротеинов высокой плотности. Для доли мелких агрегатов (1 лимфоцит с 1 тромбоцитом) были характерны обратные корреляции с выраженностью коронарного атеросклероза, концентрации IL-1β и индексом инсулинорезистентности. Таким образом, отличительной чертой пациентов с коронарным атеросклерозом является не количество тромбоцитарно-лейкоцитарных агрегатов, а размер гетеротипических агрегатов. Неблагоприятным признаком является образование крупных агрегатов, в состав которых входит 3 и более тромбоцита, что также взаимосвязано с интенсивностью системного воспаления и метаболическим дисбалансом.

Ключевые слова: тромбоцитарно-лейкоцитарные агрегаты, проточная цитометрия с визуализацией, атеросклероз, P-селектин, IL-1β, липиды

SIZE OF PLATELET-LEUKOCYTE AGGREGATES IN PATIENTS WITH VARIOUS DEGREE OF CORONARY ATHEROSCLEROSIS

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Abstract. An increased content of platelet-leukocyte aggregates suggests an elevated thrombogenic and inflammatory activity of peripheral blood cells. The aim of this study was to investigate the ratios and properties of platelet-monocyte and platelet-lymphocyte aggregates in patients with coronary atherosclerosis. The study included 19 patients with coronary artery disease and coronary atherosclerosis (15 men; 4 women; their mean age was 59.0 (55.0; 69.0) years old. The comparison group consisted of eight patients at high cardiovascular risk without coronary atherosclerosis. The atherosclerosis severity was assessed by coronary angiography and Gensini Score of ≥ 42.5 points. Platelet-leukocyte aggregates were analyzed by imaging flow cytometry. We assessed the percentage of platelet-monocyte and platelet-lymphocyte aggregates; the percentage of P-selectin $(CD62P)^+$ aggregates; the number of platelets aggregated with each individual leukocyte (either monocyte or lymphocyte). In patients with coronary atherosclerosis, a significantly lower number of monocytes formed small aggregates of 1 monocyte and 1 platelet as compared to patients without atherosclerosis (Gensini Score > 0), i.e., 78.8% (68.1; 86.2) versus 84.7% (83.8; 87.1) ($p = 0.039$). At the same time, in patients with more severe atherosclerosis (Gensini Score ≥ 42.5), the percentage of lymphocyte aggregates with more than 3 platelets tended to increase to 0.6% (0.3; 1.6) compared to patients with Gensini Score < 42.5 with 0.1% (0; 0.8) ($p = 0.075$). The proportion of large platelet-lymphocyte aggregates (with 3 or more platelets) directly correlated with Gensini Score, IL-1β concentration, systemic inflammatory indices, as well as with ratios of triglycerides/glucose and triglycerides/high-density lipoprotein cholesterol (insulin resistance indices), and showed inverse correlation with high-density lipoprotein cholesterol concentration. The percentage of small aggregates (1 lymphocyte with 1 platelet) inversely correlated with severity of coronary atherosclerosis, IL-1β concentration, and insulin resistance index. Thus, the larger size of heterotypic aggregates but not the increased number of platelet-leukocyte aggregates seems to be a distinguishing feature between patients with coronary atherosclerosis. Formation of large aggregates with 3 or more platelets is an unfavorable sign which is also associated with intensity of systemic inflammation and metabolic imbalance.

Keywords: platelet-leukocyte aggregates, imaging flow cytometry, atherosclerosis, P-selectin, IL-1β, lipids

The study was performed in the framework of fundamental research No. 122020300043-1.

Introduction

The increased lifespan together with the spread of unhealthy lifestyle and stress pressure has facilitated propagation of atherosclerosis and coronary artery disease (CAD), despite the vast implementation of new therapeutic approaches. The decreased age of patients affected also gives rise to a global concern [11].

Platelets represent unique anucleated cells, about 2-3 μm in diameter, linking the processes of inflammation and thrombogenesis, and, thus, playing an indispensable role in atherogenesis [16]. Large-scale studies have demonstrated that activation of platelets is associated with calcification of the arterial wall and narrowing of the arterial diameter [3].

Platelet-leukocyte aggregates are formed as a consequence of thrombotic or immune reactions and represent complexes of at least one leukocyte with one platelet. Platelet-leukocyte aggregates may be formed both with and without involvement of P-selectin [7]. All the types of leukocytes are capable of interacting with platelets. However, platelet-monocyte aggregates remain the most studied ones, and platelets' P-selectin preferentially binds to P-selectin glycoprotein ligand 1 (PSGL-1) expressed on monocytes [15]. Factors inducing the formation of platelet-leukocyte aggregates include standard pro-thrombotic substances (ADP, collagen, thrombin), lipopolysaccharide and interleukin-1β (IL-1β) at low concentration, decreased pH, shear stress and exposure to danger-associated molecular patterns (DAMPs) during vessels' damage. In contrast, C-type natriuretic protein (CNP) and nitric oxide (NO), released by normal endothelial cells, inhibit the formation of platelet-leukocyte aggregates, as well as methylation of platelet endothelial aggregation receptor-1 (PEAR-1) [16].

Even though platelet-leukocyte aggregates have proven to be the biomarkers of high cardiovascular risk, efficacy of antiplatelet therapy and development of complications during interventional therapy [16], there is still much to be understood about platelet-leukocyte interactions. The established protocol to study platelet-leukocyte aggregates is missing. Meanwhile, new emerging methods allow for more in-depth analysis than was possible years before. For example, the method of imaging flow cytometry permits not only to explore the numbers of aggregates, but also to identify their size and precise number of platelets tethered to a certain leukocyte [7].

The concentration of platelet-leukocyte aggregates has been demonstrated to correlate with inflammation intensity during certain pathologies [1, 5]. A standard biomarker of the residual inflammatory risk in CAD patients, high-sensitive C-reactive protein (hsCRP) is not always effective for detecting the presence of low-grade chronic inflammation [11]. Systemic inflammatory indices, based on the basic parameters of blood cell count (numbers of neutrophils, monocytes, lymphocytes, and platelets) are widely studied as promising biomarkers of cardiovascular risk and predisposition to plaque formation [4], but their interconnection with the ability of platelets to form aggregates with leukocytes has never been explored.

The aim of the present study was to investigate the interconnection between the formation of platelet-monocyte and platelet-lymphocyte aggregates and the severity of coronary atherosclerosis, taking into consideration the size of the aggregates and the severity of inflammation in patients at high cardiovascular risk.

Materials and methods

Patients

An observational single-centered comparative study was performed in the Cardiology Research Institute, Tomsk NRMC (Director – academic S.V. Popov). In total, 27 patients were recruited.

All the procedures were conducted in accordance with the Declaration of Helsinki with amendments as of 2000 and “Rules of Clinical Practice in the Russian Federation” approved by the Order of the Ministry of Health of the Russian Federation in 19 June 2003, No. 266. The protocol of the study was approved by the Biomedical Ethics Committee of Cardiology Research Institute (protocol No. 210 of February 18, 2021). All the recruited patients signed an informed consent before participation in the study.

All the patients received standard conventional therapy, including RAAS inhibitors, Ca^{2+} channels blockers, beta-blockers, and statins. Patients diagnosed with type 2 diabetes mellitus (T2DM) received standard glucose-lowering therapy.

Inclusion criteria were as follows: the presence of the confirmed coronary artery disease or high cardiovascular risk; indications to perform coronary angiography; age 40-80 y.o.; consent to participate in the study. Exclusion criteria were as follows: the presence of the acute cardiovascular event or revascularization in the 6 months preceding the study; an acute inflammatory condition in the last 30 days; the presence of any other serious disorder other than atherosclerosis (autoimmune or hematological disorder; cancer; hepatic or kidney disorders); refusal to participate in the study.

All the patients underwent selective coronary angiography on an Artis one angiographic complex and Digitron-3NAC computer system (Siemens Shenzhen Magnetic Resonance Ltd., Shenzhen, China). The patients were divided into two groups based on the results of the selective coronary angiography: those with coronary atherosclerosis (n = 19) and those without (n = 8). Gensini Score was calculated based

on the results of coronary angiography to evaluate the severity and spread of atherosclerosis [6].

All the patients underwent clinical and anthropometric examination. The anthropometric obesity parameters, including body mass index (BMI), waist circumference (WC) and waist-to-hip ratio (WHR), were evaluated.

Flow cytometry

Fasting blood was collected in vacuum tubes with ethylenediaminetetraacetic acid (EDTA). The buffy coats obtained during density gradient centrifugation (Histopaque 1077, Sigma Aldrich, USA), containing both mononuclear leukocytes and platelets, were collected and washed twice with phosphate buffer saline (PBS, Sigma Aldrich, USA). A 100 μl aliquot of cell suspension was stained with monoclonal antibodies, conjugated to fluorochromes: anti-CD45-allophycocyanin (APC), anti-CD49b-phycoerythrin (PE), andi-CD62P-fluorescein isothiocyanate (FITC) (all reagents: Becton Dickinson, USA). Cells were lysed to remove the remaining erythrocytes, washed and fixed (all buffers: Becton Dickinson, USA). Samples were analyzed on an Amnis FlowSight imaging flow cytometer (Cytek Biosciences, Fremont, CA, USA) equipped with 488 nm and 642 nm lasers. INSPIRE software version 100.3.218.0 (Amnis Corporation, Seattle, DC, USA) was used for analysis of the collected data. Populations of monocytes and lymphocytes were identified based on the parameters of side scatter (SSC) and cell area, detected in the bright field channel [14]. Platelet-monocyte and platelet-lymphocyte aggregates were gated as cells positive both for CD45 and CD49b antigens. To identify the true aggregates of leukocytes and platelets and to exclude concomitant events, the dilate mask (plus 1 pixel) was applied to the bright field image of leukocytes, followed by calculation of the Internalization Feature of the CD49b-PE signal within the CD45-APC leukocytes' mask. Events with the score of the Internalization Feature equal to or more than 0 were considered to be true aggregates, while those with the negative score were discarded as concomitant events. The percentage of true aggregates out of the total number of monocytes or lymphocytes was calculated. The percentage of P-selectin positive ($CD62P^+$) aggregates of the total number of aggregates was also calculated. The Spot count feature was used to calculate the number of platelets bound to leukocytes.

ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed in fasting serum. In particular, the concentrations of high-sensitive C-reactive protein (hsCRP, Biomerica, USA), IL-1β, IL-10 (all cytokine kits – VECTOR-BEST, Novosibirsk, Russia) were measured.

Systemic inflammatory indices

The systemic inflammatory indices were calculated based on the results of the complete blood count obtained on the automatic hematological analyzer and included: Neutrophil/Lymphocyte Ratio (NLR) = neutrophil count ÷ lymphocyte count; Monocyte/Lymphocyte Ratio (MLR) = monocyte count ÷ lymphocyte count; Platelet/Lymphocyte Ratio (PLR) = platelet count ÷ lymphocyte count; Systemic Inflammation Response Index (SIRI) = neutrophil count × monocyte count ÷ lymphocyte count; Aggregate Index of Systemic Inflammation (AISI) = neutrophil count × monocyte count × platelet count ÷ lymphocyte count; Systemic Inflammation Index (SII) = neutrophil count × platelet count ÷ lymphocyte count.

Biochemical assays

The analyzed biochemical parameters included the concentrations of fasting glucose, the percentage of glycated hemoglobin, and the concentrations of total cholesterol, triglycerides (TG), and high-density lipoproteins cholesterol (HDL-C), which were measured by Cobas 6000 C501 (Roche, Mannheim, Germany) automatic analyzer. The concentration of low-density lipoproteins cholesterol (LDL-C) was calculated by the Friedwald equation: LDL-C (mM) = total cholesterol (mM) – (HDL-C (mM) + TG (mM)/2.2). The TG/glucose ratio (TyG index) was calculated as a measure of insulin resistance using the formula: ln (TG (mg/dl) × fasting glucose (mg/dl)/2) [9]. The ratio TG/HDL-C was also calculated as a measure of insulin resistance.

Statistics

The data were processed using Statistics 10.0 software (StatSoft Inc., USA). The normality of the distribution of the tested parameters was checked via the Shapiro-Wilk test. The results were represented as median (Me) and interquartile interval ($Q_{0.25}$-$Q0_{.75}$). Categorical data were represented as n and %, when appropriate. The Mann-Whitney Rank Sum Test was used to evaluate the presence of differences between independent samples; the Wilcoxon Signed Rank Test was used to evaluate the presence of differences between paired samples. The Spearman correlation coefficient (rs) was calculated to assess the presence of correlations between parameters. A two-sided p value < 0.05 was considered to be significant.

Results

As expected, patients with atherosclerosis had higher values of Gensini Score and a higher intake of statins (Table 1). All the other basic clinical parameters, including the presence of comorbidities and anthropometric characteristics of obesity, were comparable between patients with and without atherosclerosis (Table 1).

TABLE 1. BASIC CHARACTERISTICS OF PATIENTS

Parameters	Patients with coronary atherosclerosis (n = 19)	Patients without coronary atherosclerosis (n = 8)	p
Sex (men/women)	15/4	4/4	0.183
Age, years	59.0 (55.0-69.0)	66.0 (62.0-67.0)	0.534
Patients with hypertension, n	18	8	0.999
Hypertension duration, years	20.0 (15.0-20.0)	13.0 (10.0-15.0)	0.120
Patients with diabetes mellitus type 2, n	7	1	0.365
Atherosclerosis severity (Gensini Score, points)	42.5 (19.0-75.0)	1.0 (0-3.5)	<0.001
Body mass index, kg/m^2	27.0 (25.0-32.2)	32.0 (29.2-34.3)	0.120
Waist circumference, cm	100.0 (93.0-108.0)	113.0 (112.0-119.0)	0.087
Waist-to-hip ratio	0.99 (0.92-1.03)	0.97 (0.93-1.09)	0.857
Statins intake, n	18	4	0.017

TABLE 2. PROPERTIES OF PLATELET-LEUKOCYTES AGGREGATES IN PATIENTS DEPENDING ON THE PRESENCE OF ATHEROSCLEROSIS

Parameters	Patients with coronary atherosclerosis (n = 19)	Patients without coronary atherosclerosis (n = 8)	p
Monocytes, %	12.3 (9.2-15.1)	10.8 (8.9-13.3)	0.360
Platelet-monocytes aggregates (PMA), %	21.7 (8.8-40.3)	20.1 (14.4-26.5)	0.481
P-selectin$^+$ PMA, %	96.1 (89.0-99.0)	92.4 (81.0-95.9)	0.333
PMA – 1 platelet, %	78.8 (68.1-86.2)	84.7 (83.8-87.1)	0.039
PMA – 2 platelets, %	13.3 (8.8-22.7)	11.8 (10.7-14.0)	0.549
PMA – 3 platelets, %	1.8 (0.6-4.8)	0.3 (0.1-1.1)	0.449
PMA > 1 platelets, %	16.4 (10.3-31.2)	15.0 (12.2-16.1)	0.481
PMA > 3 platelets, %	0.4 (0.0-1.6)	0.3 (0.1-1.1)	0.979
Lymphocytes, %	68.6 (60.0-76.1)	76.3 (64.5-80.8)	0.197
Platelet-lymphocytes aggregates (PLyA), %	4.2 (2.0-8.8)	2.7 (2.2-6.9)	0.658
P-selectin$^+$ PLyA, %	70.0 (48.1-80.0)	56.8 (37.8-65.8)	0.217
PLyA – 1 platelet, %	87.8 (81.7-92.8)	94.4 (87.2-95.3)	0.238
PLyA – 2 platelets, %	5.2 (2.6-12.5)	4.0 (2.6-7.4)	0.549
PLyA – 3 platelets, %	0.6 (0.2-2.1)	0.3 (0.2-0.5)	0.658
PLyA > 1 platelets, %	5.9 (2.6-16.9)	4.2 (3.0-10.7)	0.658
PLyA > 3 platelets, %	0.4 (0.0-1.3)	0.3 (0.2-0.5)	0.621

Note. PMA, platelet-monocytes aggregates; PLyA, platelet-lymphocytes aggregates; % of monocytes and lymphocytes is indicated relating to total fraction of mononuclear leukocytes; % of PMA and PLyA is indicated out of all monocytes and lymphocytes; % of P-selectin$^+$ aggregates is indicated out of all aggregates.

The percentage of both platelet-monocyte and platelet-lymphocyte aggregates did not differ depending on the presence of atherosclerosis (Table 2). Meanwhile, patients with atherosclerosis had a lower percentage of small aggregates (composed of 1 monocyte and 1 platelet) compared to patients without atherosclerosis (Table 2). There were significantly more P-selectin$^+$ platelet-monocyte aggregates compared to P-selectin$^+$ platelet-lymphocyte aggregates in both patients with ($p < 0.001$) and without atherosclerosis ($p = 0.012$). Also, monocytes formed large aggregates with platelets at a higher frequency than lymphocytes, based on the percentage of aggregates with more than

TABLE 3. INFLAMMATORY AND METABOLIC BIOMARKERS IN PATIENTS DEPENDING ON THE PRESENCE OF ATHEROSCLEROSIS

Parameters	Patients with coronary atherosclerosis (n = 19)	Patients without coronary atherosclerosis (n = 8)	p
NLR	1.6 (1.1-2.5)	1.4 (1.1-1.7)	0.307
MLR	0.3 (0.2-0.3)	0.2 (0.2-0.3)	0.389
PLR	95.1 (65.1-154.4)	106.4 (79.9-139.7)	0.621
SIRI	1.0 (0.6-1.5)	0.8 (0.6-0.9)	0.163
AISI	229.5 (110.7-386.7)	193.3 (128.3-250.2)	0.585
SII	407.5 (241.9-480.1)	333.7 (246.6-432.0)	0.621
hsCRP, mg/L	3.8 (1.1-5.2)	8.8 (2.1-36.1)	0.307
IL-1β, pg/mL	0.9 (0.7-1.3)	1.2 (0.5-1.2)	0.814
IL-10, pg/mL	2.0 (1.7-3.2)	1.8 (0.4-4.3)	0.740
Glucose, mM	6.0 (5.3-6.8)	5.7 (5.4-5.9)	0.418
HbA1c, %	6.2 (5.8-6.6)	5.5 (4.5-5.8)	0.010
Total cholesterol, mM	3.8 (3.0-4.6)	5.2 (3.2-5.6)	0.208
TG, mM	1.5 (1.0-1.9)	1.5 (1.1-1.9)	0.999
HDL-C, mM	1.1 (1.0-1.3)	1.4 (1.1-1.5)	0.160
LDL-C, mM	2.0 (1.4-2.5)	2.9 (1.4-3.6)	0.489
TyG	3.9 (3.7-4.1)	3.8 (3.8-4.0)	0.638
TG/HDL-C	1.3 (0.8-2.0)	1.3 (0.8-1.6)	0.581

Note. NLR, Neutrophil/Lymphocyte Ratio; MLR. Monocyte/Lymphocyte Ratio; PLR, Platelet/Lymphocyte Ratio; SIRI, Systemic Inflammation Response Index; AISI, Aggregate Index of Systemic Inflammation; SII, Systemic Inflammation Index; hsCRP. high-sensitive C-reactive protein; IL, interleukin; HbA1c, glycated hemoglobin; TG, triglycerides; HDL-C, high-density lipoproteins cholesterol; LDL-C, low density lipoproteins cholesterol; TyG. TG/glucose ratio.

1 platelet ($p < 0.001$ and $p = 0.012$ for patients with and without atherosclerosis, respectively).

The systemic inflammatory indices and concentrations of cytokines and metabolic parameters did not differ between the groups of patients, except for the level of glycated hemoglobin, which was higher in patients with atherosclerosis (Table 3). Of note, the frequency of patients with type 2 diabetes mellitus was comparable between the groups (Table 1).

The median of Gensini Score, reflecting the severity of coronary atherosclerosis, in patients with atherosclerotic plaques constituted 42.5. Patients with coronary atherosclerosis were divided into two groups based on their Gensini Score values. Patients with Gensini Score ≥ 42.5 tended to have more large platelet-lymphocyte aggregates (with more than 3 platelets associated with 1 lymphocyte) compared to patients with Gensini Score < 42.5 (Table 4).

The frequency of small platelet-lymphocyte aggregates (composed of 1 lymphocyte and 1 platelet) inversely correlated with Gensini Score, the concentration of IL-1β, and ratio TG/HDL-C in the total group of patients. At the same time, frequencies of large platelet-lymphocyte aggregates (composed of 1 lymphocyte with 3 or more platelets) positively correlated with several systemic inflammatory indices (SII, NLR, PLR) and concentration of IL-1β, while correlation with the concentration of atheroprotective HDL-C was negative (Table 5). We also observed the correlation between frequencies of platelet-lymphocyte aggregates with more than 3 platelets and Gensini Score and parameters of insulin resistance (TyG and TG/HDL-C ratios) (Table 5).

Discussion

According to our results, the main difference between patients with and without atherosclerosis may be not the frequency of platelet-leukocyte aggregates, but rather the size of aggregates. The smaller aggregates formed by one leukocyte and one platelet appeared to be typical of patients without coronary atherosclerosis, while higher Gensini Score values were associated with the increased percentage of larger aggregates formed by several platelets attached to a single leukocyte. Of note, large platelet-lymphocyte aggregates rather than platelet-monocyte aggregates

TABLE 4. PROPERTIES OF PLATELET-LEUKCOCYTES AGGREGATES IN PATIENTS WITH CORONARY ATHEROSCLEROSIS DEPENDING ON ITS SEVERITY

Parameters	Patients with Gensini Score < 42.5 (n = 8)	Patients with Gensini Score ≥ 42.5 (n = 11)	p
Monocytes, %	13.5 (11.1-17.2)	11.4 (8.5-15.0)	0.351
Platelet-monocytes aggregates (PMA), %	21.4 (15.9-27.7)	28.8 (7.1-43.2)	0.967
P-selectin⁺ PMA, %	96.3 (91.6-98.0)	96.1 (87.8-99.0)	0.904
PMA – 1 platelet, %	79.2 (70.5-84.6)	78.3 (59.5-86.3)	0.717
PMA – 2 platelets, %	14.3 (10.6-20.8)	11.2 (8.2-25.7)	0.600
PMA – 3 platelets, %	1.3 (0.5-3.9)	2.2 (0.6-6.3)	0.395
PMA > 1 platelets, %	18.5 (11.4-25.7)	12.6 (9.4-38.0)	0.903
PMA > 3 platelets, %	0.5 (0.2-1.5)	0.3 (0-1.6)	0.778
Lymphocytes, %	69.4 (68.3-74.3)	66.0 (59.7-77.4)	0.840
Platelet-lymphocytes aggregates (PLyA), %	4.4 (3.0-8.4)	4.2 (1.7-9.3)	0.492
P-selectin⁺ PLyA, %	70.0 (65.9-77.9)	74.7 (45.0-80.1)	0.999
PLyA – 1 platelet, %	90.9 (85.4-95.4)	84.9 (79.1-92.0)	0.109
PLyA – 2 platelets, %	4.7 (2.8-10.2)	8.8 (1.5-13.9)	0.717
PLyA – 3 platelets, %	0.5 (0.2-1.4)	0.8 (0.3-2.2)	0.442
PLyA > 1 platelets, %	5.2 (2.9-12.4)	10.1 (2.3-17.1)	0.717
PLyA > 3 platelets, %	0.1 (0.0-0.8)	0.6 (0.3-1.6)	0.075

Note. PMA, platelet-monocytes aggregates; PLyA, platelet-lymphocytes aggregates; % of monocytes and lymphocytes is indicated relating to total fraction of mononuclear leukocytes; % of PMA and PLyA is indicated out of all monocytes and lymphocytes; % of P-selectin⁺ aggregates is indicated out of all aggregates.

appeared to be more significant for distinction of patients with more severe atherosclerosis.

The use of imaging flow cytometry allowed us to eliminate the problem of coincident events, which could interrogate the results of conventional flow cytometry of platelet-leukocyte aggregates and lead to as many as 30% of false positive results [7].

Platelets play an indispensable role in immune responses, both binding definite pathogens and activating immune cells. Even though platelets are anucleated cells, they were shown to contain mRNA for all types of Toll-like receptors (TLRs) and NF-κB. Platelet α-granules contain a vast variety of cytokines, immune molecules and growth factors, including CD40L, CD62P, transforming growth factor β (TGF-β), macrophage inflammatory protein 1α (MIP-1α), regulated and normal T cell expressed and secreted (RANTES), IL-1β, and many others. Platelet microvascular vesicles are the most abundant in the peripheral blood and represent potent inflammatory mediators in both infectious and non-infectious inflammatory disorders, including CAD [12]. Relationships between platelet-lymphocyte aggregates and markers of systemic inflammation (values of inflammatory indices and concentration of IL-1β), revealed in our study, further confirm close association between inflammation and platelet function.

The receptors enabling interaction between platelets and leukocytes include P-selectin (CD62P) and CD40 ligand (CD40L) on platelets, and PSGL, CD40 and CD11b/CD18 on leukocytes [5].

The interaction between platelets and leukocytes has several consequences. On the one hand, aggregates are formed as a result of the release of biologically active substances from platelet granules, and hence, may be a marker of predisposition to atherothrombosis, acute cardiovascular events, and inefficacy of anti-platelet therapy [16]. On the other hand, binding platelets to white blood cells induces activation of the latter and may contribute to inflammation development [18]. Thus, the formation of platelet-leukocyte aggregates may play an indispensable role in the development of atherosclerosis, which was proven to be an inflammatory disorder long ago.

Platelets attached to monocytes have been shown to secrete alpha-granules, containing several biologically active substances, including β2-microglobulin, which may favor differentiation of monocytes into $CD16^+$ cells [18]. Intermediate monocytes expressing both

TABLE 5. CORRELATIONS BETWEEN PLATELET-LEUKOCYTES AGGREGATES, BIOCHEMICAL PARAMETERS AND CLINICAL CHARACTERISTICS

Parameters	PLyA-1, %	PLyA-3, %	PLyA>3, %
Gensisni Score, points	r_s = -0.529; p = 0.009		r_s = 0.453; p = 0.030
IL-1β, pg/mL	r_s = -0.661; p = 0.001	r_s = 0.544; p = 0.011	r_s = 0.708; p < 0.001
SII		r_s = 0.449; p = 0.019	r_s = 0.386; p = 0.047
NLR		r_s = 0.458; p = 0.016	r_s = 0.487; p = 0.010
PLR		r_s = 0.506; p = 0.007	r_s = 0.392; p = 0.043
HDL-C, mM		r_s = -0.517; p = 0.010	r_s = -0.557; p = 0.005
TyG			r_s = 0.503; p = 0.014
TG/HDL-C	r_s = -0.426; p = 0.038		r_s = 0.498; p = 0.013

Note. PLyA-1, platelet-lymphocytes aggregates with 1 platelet; PLyA-3, platelet-lymphocytes aggregates with 3 platelets; PLyA > 3, platelet platelet-lymphocytes aggregates with more than 3 platelets; SII, systemic inflammatory index; NLR, neutrophil/lymphocytes ratio; PLR, platelet/lymphocytes ratio; HDL-C, high density cholesterol; TyG, index triglycerides/glucose; TG/HDL-C, triglycerides/high density lipoproteins cholesterol ratio.

CD14 and CD16 were associated with increased severity of atherosclerosis, especially in metabolically compromised patients [9]. Binding of platelets to monocytes induced polarization of the latter towards inflammatory phenotype and increased production of inflammatory cytokines: TNFα, IL-1β, IL-12, IL-8, IL-6, MIP-1β [15, 12].

Platelet-lymphocyte aggregates are less studied than platelet-monocyte aggregates. According to the available data, platelets are more readily bound to previously activated enlarged T lymphocytes, both helper and cytotoxic, and NK cells. Binding to B cells appeared to be only residual [10]. Platelets were required to prime T lymphocyte to differentiation towards Th1 and Th17 lineages. Platelet-depleted animals did not develop experimental autoimmune encephalomyelitis (EAE) upon immunization with myelin oligodendrocyte glycoprotein (MOG)35-55 peptide [17]. Co-incubation of platelets with $CD4^+$ lymphocytes skewed their differentiation towards Th1/Th17 axis, reducing the frequency of Th2 cells in the culture. This may be mediated by the production of serotonin (5HT), CXCL4 and CCL5 by platelets, and by the release of these substances via the interaction between platelets and lymphocytes in a manner similar to that in the neuron synapse [17]. However, the formation of platelet-lymphocyte aggregates via interaction between CD40-CD40L and P-selectin-PSGL was associated with diminished activation of Th1 and Th17 cells and rather led to resolution of inflammation. Normally, such close interaction between platelets and lymphocytes was observed after exhaustion of platelets and their inability to secrete biologically active substances in granules [17]. The possibility exists that the direction of T cell differentiation might also be dependent upon the number of adhered platelets, which was not studied in the previous works. This might be the focus of our further research.

We have revealed close relationships between the frequency of platelet-lymphocyte aggregates with various number of adhered platelets and such metabolic parameters as HDL-C and metabolic indices. Of note, the concentration of atheroprotective HDL-C was inversely associated with the percentage of large aggregates, while proatherogenic indices demonstrated direct correlations. The fact that metabolic reprogramming of immune cells occurs during the immune response became widely accepted, and even led to the appearance of a new branch of science – immunometabolism. Since platelets are involved in inflammation development, it is reasonable to assume that their activity is dependent on the metabolic profile [19]. This was confirmed in patients with sickle-cell anemia, who demonstrated an impaired platelet-specific metabolic dysfunction [2]. The disturbed lipid profile in patients with cardiovascular disorders was associated with increased platelet absorption of cholesterol from lipid-rich environment and subsequent hyperactivation of platelets [13].

Agents, controlling platelets functions, such as cyclooxygenase and P2Y12 inhibitors, not only suppressed thrombotic functions, but also dampened their inflammatory potential [12].

One of the limitations of our work is a relatively low number of patients enrolled. Also, the distinction between platelet-monocyte and platelet-lymphocyte aggregates was made based on the parameters of cell size and SSC intensity. The use of the specific markers to distinguish between different monocyte and lymphocyte subsets, such as CD14, CD16 (for monocytes), CD3, CD4, CD8, and specific

chemokine receptors (for lymphocytes), could have yielded more specific results.

Conclusions

In our study, we have demonstrated for the first time that the presence of coronary atherosclerosis is associated with the decreased number of small aggregates formed between monocytes and platelets. The increase in coronary atherosclerosis severity correlates with elevation in the frequency of large aggregates between lymphocytes and platelets, which is also interconnected with the intensity of systemic inflammation and parameters of metabolic disturbances.

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