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• *CLINICAL RESEARCH* •

Blood platelet and monocyte activations and relation to stages of liver cirrhosis

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Abstract

AIM: Blood platelets (plt) and monocytes are the cells that play a crucial role in the pathogenesis of liver damage and liver cirrhosis (LC). In this paper, the analysis of mutual relationship between platelets and monocytes activation in LC was conducted.

METHODS: Immunofluorescent flow cytometry was used to measure the percentage of activated platelet populations (CD62P, CD63), the percentage of plt-monocyte aggregates (pma) (CD41/CD45), and activated monocytes (CD11b, CD14, CD16) in the blood of 20 volunteers and 40 patients with LC. Platelet activation markers: sP-selectin, platelet factor 4 (PF4), beta-thromboglobulin (βTG) and monocyte chemotactic peptide-1 (MCP-1) were measured and compared in different stages of LC.

RESULTS: Platelet activation with the increase in both βTG serum concentration and elevation of plt population (CD62P and CD63 as well as MIF CD62P and CD63) is elevated as LC develops and thrombocytopenia rises. There is a positive correlation between medial intensity of fluorescence (MIF) CD62P and MIF CD63 in LC. We did not show any relationship between monocyte activation and pma level. SP-selectin concentration correlates positively with plt count and pma, and negatively with stage of plt activation and MIF CD62P and MIF CD63. There was no correlation between MCP-1 concentration and plt, monocyte activation as well as pma level in LC. CD16 monocytes and MIF CD16 populations are significantly higher in the end stage of LC. A positive correlation occurs between the value of CD11b monocyte population and MIF CD14 and MIF CD16 on monocytes in LC.

CONCLUSION: Platelet and monocyte activation plays an important role in LC. Platelet activation stage does not influence monocyte activation and production of plt aggregates with monocytes in LC. With LC development, thrombocytopenia may be the result of plt consumption in platelet-monocyte aggregates.

INTRODUCTION

There are many reports presenting multipotential properties of blood plt, e.g. in inflammatory and immunological processes. It is connected with the plt secretory reaction, interaction with inflammatory cells, immunological cells and with plt abilities to phagocytosis. Thrombotic and inflammatory processes have common biochemical links and the same cellular elements, which are the part of the immune system, are engaged in them[1,2]. Platelet co-operation with neutrophils and monocytes can be observed in many inflammatory, thrombotic processes and autoimmune diseases[3]. Two mechanisms of plt activation are taken into consideration. One of them is the release of soluble substances capable of plt activation (such as proteases, thromboxan A2, arachidonic acid metabolites, peroxides, PAF, and catepsin G) in pathological conditions. The second one is a direct contact of inflammatory cells with plt. The result is the increase in expression of superficial adhesive molecules[4]. Such interaction is possible thanks to membrane receptors such as GPIIb/IIIa on plt and CD11/CD18 on leukocytes[5]. In inflammatory processes, activated plt can module functions of cells that participate in inflammation[6]. Platelet agonists (e.g. collagen from extracellular matrix) lead to the rise of expression of CD11b antigen on leukocytes and vice versa, leukocytes incubation with CD11b agonists decreases P-selectin expression on plt. GPIIb/IIIa or P-selectin blockage on plt decreases leukocyte activation with drop of CD11b expression (Li 2000). Substances that activate binding of plt and monocytes originate from damaged vessel endothelium, activated leukocytes, macrophages or thrombocytes. These are thrombin, collagen, thromboxan A2, serotonin, plt activating factor, catecholamines, and others[7,8]. Recent studies have proven that plt have a significant role in pathogenesis of liver damage. Blood plt while activated in inflammatory and immune processes and in the hemostatic disorders in the liver, release active compounds (PF4, βTG, PDGF, *etc*)[9]. Biological significance of these proteins is stimulating influence on fibrinogenesis

and mitogenesis of Ito cells in the liver. *In vivo* studies prove that after selective decrease in plt count, liver tissue damage is smaller depending on neutrophils. The influence of activated neutrophils is significant, at the time of "oxygen outburst"; active substances activating plt are released, such as cathepsin G, which has stronger activating properties than thrombin[10]. Platelet activating factor (PAF) is an important link mediating intercellular contacts. It is produced in acute and chronic inflammatory conditions by e.g. plt, monocytes, and plays a key role in plt leukocyte-dependent activation[11]. P-selectin, present in activated plt, and soluble (s) P-selectin in the blood play a role in plt-blood cells interactions[12,13]. Platelet activation promotes binding with leukocytes, granulocytes, and monocytes producing aggregate complexes with these cells[14,15]. P-selectin, a component of alpha-granules internal membrane, appears on plt surface after its activation and is also released as soluble to the surrounding microenvironment. In dense granules there are proteins specific for plt: Plt factor 4 (PF4) and β-thromboglobulin (βTG), secreted outside from activated plt as a consequence of release reaction. PF4 has chemotactic properties towards neutrophils, influences fibroblasts and inhibits endothelial cell proliferation[16,17]. High concentrations of PF4 occur in conditions with increased plt activation, such as after myocardiac infarction, operations exertion, and in chronic hepatitis. βTG is a sensitive marker of plt activation, comparable to P-selectin[18]. After thrombocyte activation, other receptors (CD63, CD107b (LAMP-2)), originating from lyzosomal granules, are transported to the surface[19,20].

There are not many studies concerning the relationship between plt and monocytes activation in liver cirrhosis (LC) and therefore an attempt to solve this problem is justified. Platelet activation was analyzed by estimating their surface receptors and molecules soluble in peripheral blood. Cytometric estimation of circulating plt-monocyte complexes in patients with various stadiums of LC was performed.

MATERIALS AND METHODS

Patients

The studies were conducted in the group of 40 patients with advanced LC (28 men and 12 women), aged 50±13 years and in 20 healthy volunteers (12 women and 8 men), aged 40±7 years. Liver cirrhosis was confirmed clinically and histologically. Patients in the course of immunosuppression, anti- or pro-coagulant therapy and with neoplasmatic diseases were excluded from the study. None of the patients suffered from coronary heart disease or heart infarction. Patients with LC were divided into groups (B and C) in accordance with the classification of liver failure according to Child-Pugh[21]. Ethical approval for research was obtained from local ethics committee in Medical University.

Blood preparations for cytometric examination

The blood was collected before breakfast. Pressure bandage was not used and massage was avoided not to stimulate blood plt. We used needles of the diameter bigger than 0.8 mm, blood was let due to venal pressure, hypotension was not used neither were syringes. The first 2 mL of blood was used for biochemical examination, next 2 mL for plt examination. Platelets were prepared for cytometric studies according to recommendations of the European Working Group on Clinical Cell Analysis protocol[22].

Resting platelets

Venal blood was collected in plastic tubes, which contained EDTA K_2 1.5 mg/mL of blood. Immediately after collection, 1% solution of paraformaldehyde in phosphate buffered saline (PBS) deprived of Ca^{2+} and Mg^{2+} ions was added to 100 μL of blood. Monoclonal antibodies (mAbs) were used against CD61 and CD41 marked by fluorescein isothiocynate isomer 1 (FITC) and against CD62P marked by phycoerythrin (PE), CD63PE (Becton Dickinson). Plastic tubes were filled with 20 μL of whole blood with EDTA K_2 each. Ten microliters of mAbs CD61/FITC and CD62P/PE or CD63PE and 0.5 mL of PBS (without Ca^{2+} and Mg^{2+} ions) were added to each tube. After mixing (vortex), the suspension was incubated for 30 min in dark place and at room temperature. Blood plt were examined in flow cytometer Coulter EPICS XL (Coulter Electronics Corporation, Fl, USA). We analyzed per sample approximately 10 000 blood plt. by dividing the population into normal plt, microplatelets and macroplatelets. Parameters obtained from the gate of normal plts were taken for the analysis.

Monocyte population

Peripheral blood monocyte and plt population were determined using cytometric method with the flow cytometer equipped with an argon ion laser. Monocytes were identified by their characteristic forward and orthogonal light scatter properties. Circulating monocytes with adherent plt were identified by positivity for RED670 with 670-nm band-pass filter and FIC with a 525-nm band-pass filter. Monoclonal antibodies stained with fluoresceine (FITC) or phycoerythrin (PE), (Dako, Denmark) were used. The following monocytes populations were tested: CD11b, CD14, CD16 and CD45. Venal blood was collected in the mornings in plastic tubes containing EDTA K_2 of 1.5 mg/mL of blood. Cytometric analysis was performed within 2 h after blood collection. The amount of 5 μL of stained monoclonal antibodies was added to 50 μL of whole blood. Afterwards, it was incubated in room temperature for 15 min. Erythrocytes were eliminated by addition of diluted lysin fluid (ImmunoPrep-Reagenziensysem ABC, Coulter). We analyzed per sample approximately about 2 000 monocytes. Platelet-monocyte complexes were determined using mAb CD41 PE (blood plt) and CD45 FITC (monocyte population) as the percentage of double positive cells. Platelet and monocyte count were carried out in hematological analyzer Sysmex (Japan).

MCP-1, sP-selectin

Using ELISA method we measured the level of sP-selectin (sP-selectin, R&D System, Oxon, UK), MCP-1 (R&D System, Oxon, UK). The blood was centrifuged at 2 000 r/min for 15 min. at 4 ℃ and all sera were stored at a temperature of -76 ℃. Immunoenzymatic studies were carried out simultaneously.

β-Thromboglobulin, platelet factor 4

A sample of 4.5 mL of venal blood were taken, in the manner described above (in blood preparation for flow cytometry), to plastic tubes in ice containing heparin (Vacutainer CTAD, Becton Dickinson, UK). Tubes were centrifuged in 3 500 r/min for 30 min at the temperature of 4 ℃. Plasma was examined and βTG and PF4 were examined by means of immunospectrophotometric method (Asserachrom, Diagnostica Stago).

Statistical analysis

The results were presented as mean±SD and medians. Statistical analysis was performed by non-parametrical Mann-Whitney *U*-test. Result correlation was calculated with the use of Spearman correlation test. Statistically significant values were considered for $P<0.05$.

RESULTS

Platelet count drops and plt secretion activity can be observed together with the stage of liver failure advancement in LC (Table 1). There is a negative correlation between plt amount and PF4 concentration in stadium B and C according to Child-Pugh classification as well as βTG in patients in stadium C (Table 2). LC reveals the increase in plt activation with the rise in population of CD62P and CD63 phenotypes despite thrombocytopenia intensification. Moreover, there is a higher expression on plt of receptors MIF (medial intensity of fluorescence) CD62P and CD63 (statistically significant) in cases of LC. A negative correlation between MIF CD62P and MIF CD63 and plt count was observed in the stadium Child-Pugh C. A positive correlation between MIF CD62P and MIF CD63 ($r = 0.51$, $P<0.01$) occurs in LC, there was no such relationship in the control group. There is also a positive correlation between plt activation stage and PF4 concentration in blood sera in LC. PF4 level correlates positively with plt CD62P population ($r = 0.53$, $P<0.02$), CD63 ($r = 0.66$, $P<0.04$), MIF CD62P ($r = 0.54$, $P<0.02$), and MIF CD63 ($r = 0.46$, $P<0.005$) in patients with LC.

In LC, monocyte percentage increases in peripheral blood. Cytometric analysis shows that monocytes CD16 population is raised significantly with LC advancement. MIF CD16 on monocytes also increases (statistically significant). The population of CD11b monocytes significantly drops with cirrhosis advancement. However, a significant increase in MIF CD11b receptor expression on monocytes (MIF) can be observed.

Circulating plt-monocyte complexes tend to increase in LC. In C stage of LC, CD45 receptor expression on monocytes in plt-monocyte aggregates is markedly higher. A strongly positive correlation ($r = 0.9$) between MIF CD45 on plt aggregates and monocytes is observed in LC as in healthy liver. There is a positive correlation between plt count and the level of aggregates plt-monocytes. Platelet activation stage (CD62P, CD63, PF4, βTG) correlates negatively with plt-monocytes aggregates (Table 3). Monocyte CD16 population is significantly higher in LC as compared to the healthy group and depends on liver failure stage. There is a positive correlation between monocyte CD16 percentage and receptor (MIF) CD45 expression in plt-monocyte aggregates ($r = 0.56$, $P<0.02$). A positive correlation also occurs between MIF CD16 and MIF CD45 on monocytes in aggregates plt-monocytes ($r = 0.53$, $P<0.03$). There is a positive correlation between CD11b monocyte population and MIF CD14 receptor expression on monocytes ($r = 0.45$, $P<0.05$) and MIF CD16 ($r = 0.35$, $P<0.04$) in LC. It is also observed that MIF CD14 values on monocytes are comparable in B and C stage of LC and healthy subjects.

The level of MCP-1 chemokine, released by activated monocytes, is much lower in LC than in healthy subjects. There is a negative correlation between MCP-1 concentration

Table 1 Blood plt, monocyte serum and cytometric parameters in clinical stages of LC according to Child-Pugh classification

	Healthy	Cirrhosis hepatitis stage (Child-Pugh)	
		B	C
Blood plt			
Blood plt ($1\times10^3/\mu L$)	219±25	124±77[a]	115±66[a]
sP-selectin (pg/mL)	133±70	74±42[a]	69±35[a]
PF4 (IU/mL)	36±7	30±9	26±16
βTG (IU/mL)	128±45	137±40	101±62
CD62P (%)	6±1.8	10±4[a]	9.3±3[a]
MIF CD62	0.47±0.08	0.66±0.2[a]	0.66±0.2[a]
CD63 (%)	0.7±0.3	2.2±1.5	1.5±0.9
MIF CD63	0.3±0.04	0.4±0.09[a]	0.4±0.07[a]
Monocyte			
Monocyte (%)	5.7±3.1	6.8±3.3	7.0±2.9
MCP-1 (pg/mL)	218±129	150±93	153±81
MIF CD14	26±2	27±7	26±4
CD16 (%)	2.9±1.6	7.9±7.8[a]	8.1±5.2[a]
MIF CD16	1.5±0.5	1.9±1.1	2.9±1.5[a]
CD11b (%)	100±0.1	99.5±0.8	99.5±2.0[a]
MIF CD11	17.4±5.8	18.5±4.8	21.2±5.9[a]
Aggregates			
Plt–monocytes (%)	13.5±4.0	10.7±5.6	13.6±16.8
MIF CD45 on plt–monocyte aggregates	11.8±3.6	11.6±3.5	15.1±4.5

[a]$P<0.05$ *vs* Healthy statistical significance in healthy subjects, Mann–Whitney *U*-test. MIF-medial intensity of fluorescence.

Table 2 Correlations (*r*) of blood plt count and their activation markers in clinical stages of LC

	Cirrhosis hepatitis stage (Child-Pugh)	
	B	C
sP-selectin	0.61	0.58
MCP-1	0.41	0.39
PF4	–0.42	–0.62
βTG	NS	–0.45
CD62P	–0.53	–0.52
MIF CD62P	NS	–0.52
CD63	–0.58	–0.41
MIF CD63	–0.56	–0.3
Plt–monocyte aggregates	0.77	0.81

There was no correlation in the controls. Spearman's test, NS – no statistical differences.

and monocyte count in peripheral blood (r = -0.56, P<0.0001), MIF CD14 on monocytes (r = -0.4, P<0.01), and albumin concentrations (r = -0.58, P<0.01) in LC.

Soluble P-selectin concentration in LC correlates positively with plt count, plt-monocyte aggregates and biochemical liver injury stage (ALT, AST, ALP, GGT). However, it correlates negatively with plt activation and receptors CD62P and CD63 expression (Table 4). A negative correlation of sP-selectin concentration and monocyte (MIF CD14 and MIF CD16) activation can be also observed.

Table 3 Correlations (r) of plt-monocytes aggregate level and markers of plt activation (Spearman's test)

	Healthy	Cirrhosis hepatitis stage (Child–Pugh)	
		B	C
Plt CD62P (%)	NS	–0.47[b]	–0.58[b]
MIF CD62P	NS	–0.45[b]	–0.58[b]
Plt CD63 (%)	NS	–0.3[a]	–0.49[b]
SP-selectin	NS	0.6[b]	0.51[b]
PF4	NS	NS	–0.68[b]
βTG	NS	NS	–0.44[a]

[a]P<0.05, [b]P<0.01 *vs* Healthy NS – no statistical correlations.

Table 4 Correlations (r) of sP-selectin value with monocyte and plt activation markers and other parameters in LC

	Cirrhosis hepatitis stage (Child–Pugh)	
	B	C
AST (U/L)	NS	0.56[b]
ALT (U/L)	NS	0.41[a]
ALP (U/L)	NS	0.42[a]
GGT (U/L)	NS	0.54[b]
Leukocyte ($1\times10^3/\mu L$)	0.6[a]	0.58[b]
Monocyte (%)	NS	–0.37[a]
MIF CD14	–0.73[b]	NS
MIF CD16	–0.3[a]	NS
CD62P (%)	–0.57[b]	NS
MIF CD62P	–0.53[b]	NS
CD63 (%)	–0.54[b]	NS
MIF CD63	–0.5[a]	NS

In healthy subjects we observed no statistical correlations (Spearman's test). [a]P<0.05, [b]P<0.01 *vs* other groups NS – no statistical correlations.

DISCUSSION

Blood plt, by connecting hemostasis and inflammatory processes, participate in pathogenesis of chronic liver diseases. Thrombocytes play an important role in intensification of damage and fibrosis of liver tissues. The level of plt damage depends on severity of the course of liver disease, and injury is reversible with liver efficiency improvement. The causes of plt activation in LC are complex and not yet fully understood. In LC, thrombocytopenia is connected with shorter life span of plt and is a result of constant plt activation by cytokines (IL2, IL6, TNF α), use up in subclinical DIC, intensified elimination by the reticulo-endothelial system of the spleen and liver[23]. Hyperdynamic portal circulation and retention in the spleen microcirculation in LC act to stimulate plt. Immunological and inflammatory phenomena in liver tissues and their influence are the other causes of plt activation. P-selectin expression on plt is elevated in the microcirculation at inflammation sites[24]. Endotoxemia often accompanies severe LC. In experimental liver injury with lipopolysaccharides (LPS), plt and neutrophil counts in peripheral blood are significantly lower and are increased in the liver tissue[25].

Endotoxemia activates plt and causes their migration from blood to sinusoidal and perisinusoidal spaces[26]. Platelets removed under experimental conditions protect from focal liver necrosis[27]. Platelets with P-selectin, undergoing adhesion to vascular endothelial cells, facilitate the stop of circulating neutrophils in small vessels and initiate inflammatory and thrombotic processes. P-selectin may represent a possible link between inflammation and thrombosis. P-selectin correlated with plt counts, but not with endothelial injury and tissue factor or coagulation factors in chronic liver disease[28].

The results of our investigations showed that the more developed thrombocytopenia, the stronger plt activation in LC. With developed thrombocytopenia in LC, plt CD63+ population increases, which reflects strong plt activation with elevated MIF CD62P and CD63. As cirrhosis develops and plt count diminishes, plt aggregates with monocytes increase. Our studies showed a negative correlation between the stage of plt activation (CD62P, CD63, PF4, βTG) and plt aggregates creation with monocytes in LC. It proves that plt activation influence binding with monocytes. It seems that thrombocytopenia in LC may be the result of plt consumption of plt in aggregates with other cells. There was no relationship between monocyte activation and the level of plt-monocyte aggregates.

CD62P and CD63 receptor expression on plt (both MIF and population percentage) as well as PF4 and βTG release are sensitive markers of plt activation. It is significant that plt activation was connected with the increase of plt aggregates with monocytes. There was a negative correlation between plt activation (MIF CD62P, MIF CD63) with sP-selectin concentration in blood serum. Our studies suggest that in LC plt are not the main source of the soluble form of P-selectin and it does not reflect plt activation.

Monocyte activation is connected with CD11b population elevation that presents β2-integrin, which enable binding with ICAM-1, fibrinogen, and factor *x*. Activated CD14/16 monocyte population has proinflammatory properties and produces large amount of TNF-α. Activated plt are bound to PMN with the involvement of P-selectin and activated β_2-integrin CD11b/CD18[29]. Monocytes adhere to activated plt, mediated by P-selectin. Thrombin-activated plt induce secretion of monocyte chemotactic protein-1 by monocytes. It is suggested that activated plt regulate chemokine secretion by monocytes in inflammatory sites[30]. Hyperdynamic condition in portal hypertension may be important in triggering the monocyte and plt activation. Platelet-monocyte complexes have been implicated in inflammatory processes. Microenvironment conditions in liver tissue regulate monocyte differentiation to become dendritic cells despite the presence of fibroblasts. Monocyte chemoattractant protein 1 (MCP-1) has chemotactic properties, and recent evidence suggests that they might participate in the fibrotic process by inducing the secretion of extracellular

matrix components.

Our studies revealed that MCP-1 level did not reflect inflammatory activity in LC, as it was lower in blood serum than in healthy subjects. There was no relation observed between MCP-1 concentration and activation of plt, monocytes, (population CD16, CD11b) and predisposition for plt-monocyte complexes creating. Thus, MCP-1 is not a mediator in plt-monocyte contacts in LC. Secretion of MCP-1 correlated with intensity of the inflammatory infiltrate in chronic liver disease. In active cirrhosis, MCP-1 expression was present in the portal tract, in regenerating bile ducts, and in inflammatory infiltration sites. In the group of patients in Child-Pugh C stage, CD45 receptor expression on monocytes in plt-monocyte aggregates is much higher which proves that complex creation happens due to $CD45^+$ monocyte activation and/or there is an increase in CD45 receptors on monocytes (difference statistically insignificant). It seems that although thrombocytopenia is a non-desired effect in LC, it is at the same time, a protective phenomenon against intensification of inflammatory processes in the liver. Monocyte and plt activation in LC may contribute to progressive liver injury. Thus, further investigations are needed to check pharmacological effect on plt and monocyte activation blockade and intensification of liver regeneration.

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