Status of Platelet-Lymphocyte Aggregation in Circulating Blood of Patients with Type 1 Diabetes with and without Diabetic Nephropathy

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Abstract

Background: Platelets can modulate the role of lymphocytes in the development of micro- and macrovascular complications in type 1 diabetes mellitus.

Objectives: To clarify the status of platelet-lymphocyte aggregation in circulating blood in patients with T1DM, as well as the differences in the platelet-lymphocyte aggregation in T1DM patients with and without diabetic nephropathy.

Methods: We recruited 115 T1DM patients (47 men and 68 women) aged 15–52 years. The subjects with mean albumin excretion ≥ 5 µg/mg creatinine comprised group 1, and those with < 5 µg/mg creatinine comprised group 2. The matched healthy participants (n=50) served as the control group. Detection of LPA was achieved using a light microscope after Ficoll-gradient centrifugation. Immunophenotyping of lymphocytes was performed by flow cytometry.

Results: Significantly more LPA (430.4 ± 20.6/µl) were observed in group 2 compared with group 1 (223.9 ± 12.8/µl, $P < 0.001$) and the control group (296.1 ± 22.6/µl, $P = 0.027$). In group 1 significantly more LPA/CD4 (21.1 ± 1.6%) and LPA/(CD4 + NK) (17.8 ± 1.7%) were found than in group 2 and the control group.

Conclusion: T1DM with diabetic nephropathy is associated with higher levels of LPA than T1MD without diabetic nephropathy. The role of LPA in microvascular complications in diabetes should be elucidated in further studies.

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Vascular complications are the leading cause of morbidity and mortality in diabetic patients [1]. Platelets have a key function in thromboembolic complications in diabetes mellitus. Their hyperactivation could play a central role in the pathogenesis of diabetic micro- and macroangiopathies, as they express and secrete a wide array of factors that affect intercellular interactions, adhesion, signaling events in target cells, and facilitate trans-endothelial cell migration [2]. Platelet hyperactivity was shown in diabetic patients with vascular complications [3].

Type 1 diabetes mellitus is an organ-specific autoimmune disease manifested by significant dysfunction of cell-mediated immunity. Naive as well as effector T cells constitutively express lymphocyte function-associated antigen-1, and intercellular adhesion molecule expression is found on renal endothelial, epithelial, and mesangial cells [4,5]. It is likely that this interaction will play a significant role during T cell migration into the kidney. Activation of $CD4^+$ and $CD8^+$ T cells by advanced glycosylation end-products initiate interferon-gamma secretion by T cells [6], which will induce further inflammation and oxidative stress within the target tissues. T cell accumulation is also found in the juxtaglomerular apparatus of patients with T1DM [5].

Platelets are involved in the pathogenesis of diabetic micro-angiopathy. Their membrane glycoprotein Ia/IIa, α2β1 integrin, serves as a platelet receptor for collagen [7]. It mediates platelet primary adhesion to sub-endothelial tissues, which is an essential first step in thrombus formation [8]. Moreover, evidence indicating the beneficial effect of antiplatelet therapy on retinopathy and nephropathy suggests the involvement of platelets in the pathogenesis of micro-angiopathy in diabetes [9].

Little is known about lymphocyte-platelet interactions during the development and progression of microvascular complications in T1DM. Platelets can modulate lymphocyte function, supporting lymphocyte rolling and adhesion via P-selectin bridging [10], enhancing lymphocyte adhesion to the endothelium [11] and consequently facilitating lymphocyte homing in high endothelial venules [12]. Recently we described the ability of activated platelets to promote activated T cell adhesion to fibronectin under flow via integrins, CD40-CD40L and P-selectin-PSGL-1 mediated interactions [13].

The purpose of the present study was to determine the number of lymphocyte-platelet aggregates in type I diabetic patients with or without diabetic nephropathy.

Patients and Methods

The study protocol was approved by the Regional Bioethical Commission at Chita Medical Academy, Chita, Russia. All subjects gave informed consent. The study groups comprised patients with diabetes type I from the Diabetology Center of the Chita Medical Academy. The inclusion criterion was T1DM diagnosed ≥ 5 years earlier. Exclusion criteria were any active infectious disease, malignancy, pregnancy, or treatment with aspirin, phosphodiesterase inhibitors, calcium channel blockers, or non-steroidal anti-inflammatory drugs for at least 2 weeks before study entry. The control group included healthy people attending

T1DM = type 1 diabetes mellitus
LPA = lymphocyte-platelet aggregates

the local family medicine clinics for routine blood examination. To test urinary albumin, 24 hour urine samples were collected on 2 consecutive days from all subjects and the mean of albumin excretion was taken for statistical evaluation.

Laboratory analyses

- *Blood hematology and chemistry analyses*

Blood hematology and chemistry analyses were performed at the Central Clinical Laboratory of Chita Medical Academy. Complete blood count was performed using the Cell Dyn 3000 automated counter (Abbott, USA). Diagnostic assays for hemoglobin HbA1C and other biochemical parameters were done on a Hitachi 917 biochemical analyzer using the spectrophotometric method (Boehringer Mannheim, USA). Immunoglobulin classes were quantitated by the single radial immunodiffusion method.

- *Flow cytometry*

Monoclonal antibodies for flow cytometry studies were purchased from Becton Dickinson (San Jose, CA, USA). Mature human T cells were identified by PC5-CD3 (clone HIT3a). T helper (CD4+) and T cytotoxic (CD8+) cells were further separated with PC5-CD4 (clone RPA-T4) and PC5-CD8 (clone HIT8a) respectively. Natural killer cells were identified by double staining with PC5-CD56 (clone B159) and PC5-CD16 (clone 3G8). B lymphocytes were identified by CD19 staining (HIB19 clone, format PE-Cy5). Aliquots of 8 µl blood were added to 45 µl of Hepes-buffered saline (150 mM NaCl, 5 mM KCl, 1 mM MgSO4, 10 mM Hepes, pH 7.4) containing appropriately diluted fluorescent monoclonal antibodies. The samples were incubated at room temperature for 20 minutes. The samples were diluted and mildly fixed with 0.5% (v/v) formaldehyde saline, and analyzed with a Beckman-Coulter EPICS XL-MCL flow cytometer (Beckman-Coulter Corp., USA).

- *Visual assessment of lymphocyte aggregation with platelets*

This method was described previously by Vitkovsky et al. [14]. Briefly, peripheral blood was drawn between 8:30 and 10:30 a.m. into preservative-free heparin (25 USP U/ml). The heparinized blood was placed on a Ficoll (density 1077) gradient and centrifuged with 1500 r/min for 40 min. After centrifugation the interphase ring, which contains predominantly lymphocytes and thrombocytes, was drawn off by a Pasteur pipette and washed using the phosphate salt buffer (pH 7.4), then centrifuged at 1000 r/min for 3–4 min. Supernatant liquid was poured and the sediment was analyzed with a light microscope (Olympus, Tokyo, Japan). In each case, 10 regions were analyzed (x100 magnification) and the absolute number and percentage (number of lymphocyte-platelet aggregates to 100 lymphocytes) were calculated.

Statistical analysis

All data are expressed as means ± SE. The differences between means from healthy and diabetic patients were tested using a two-tailed Student's *t*-test for unpaired data. $P < 0.05$ was considered to be statistically significant. Calculations were performed with the Statistica 6 Software (StatSoft Inc., USA).

Results

The study population included 115 T1DM patients (47 men and 68 women) aged 15–52 years. The subjects with mean albumin excretion ≥ 5 µg/mg creatinine comprised group 1, and those with < 5 µg/mg creatinine comprised group 2. The two groups were similar with respect to basic demographic characteristics and laboratory values [Table 1]. The main differences were a longer duration of T1DM in patients with albuminuria (13.9 ± 4.1 vs. 7.7 ± 2.6 years, $P = 0.038$) [Table 1]. Fifty matched healthy participants served as the control group. Fasting plasma glucose, blood HbA1C and fibrinogen levels, and systolic and diastolic blood pressure were higher in both diabetic groups than in healthy participants [Table 1].

There was no significant difference in immunological parameters between the study groups [Table 2]. More platelet-bound lymphocytes (430.4 ± 20.6/µl; 20.0 ± 0.7) were found in group 2 than in group 1 (223.9 ± 12.8/µl; 10.2 ± 0.7%) and the control

Table 1. Demographic and basic laboratory data of healthy and diabetic participants

	Group 1 (Type 1 DM with albuminuria)	Group 2 (Type 1 DM without albuminuria)	Healthy controls	*P**	*P***	*P****
Number	57	58	50	NS	NS	NS
Age (yrs)	41.9 ± 6.3	43.1 ± 7.2	43.5 ± 8.1	NS	NS	NS
Gender (M/F)	20/37	27/31	28/32			
Body mass index (kg/m^2)	26.9 ± 1.2	27.1 ± 0.8	27.4 ± 1.7	NS	NS	NS
Disease duration (yrs)	13.9 ± 4.1	7.7 ± 2.6	0	0.038	<0.001	<0.001
Total daily insulin (units)	36.4 ± 2.9	32.7 ± 3.2	0	NS	<0.001	<0.001
Smoking N (%)	21 (36.8)	24 (41.4)	23 (46)	NS	NS	NS
Systolic blood pressure (mmHg)	137.1 ± 9.3	143.4 ± 8.1	121.3 ± 11.2	NS	0.043	0.018
Diastolic blood pressure (mmHg)	77.2 ± 4.1	76.3 ± 6.2	76.7 ± 4.5	NS	NS	NS
Total cholesterol (mg/dl)	186.9 ± 9.7	187.4 ± 12.8	174.3 ± 10.4	NS	NS	NS
LDL-cholesterol (mg/dl)	124.7 ± 7.1	121.0 ± 8.5	118.2 ± 9.3	NS	NS	Ns
Fasting glucose (mg/dl)	185.7 ± 16.6	179.3 ± 19.2	91.6 ± 5.1	NS	<0.001	<0.001
HbA1C (%)	8.7 ± 0.8	8.4 ± 0.6	4.7 ± 0.4	NS	<0.001	<0.001
White blood cells (/µl)	6156 ± 197	6337 ± 134	6120 ± 234	NS	NS	NS
Lymphocytes (/µl)	2156 ± 99	2366 ± 94	2117 ± 210	NS	NS	NS
Platelets (x104/ µl)	251 ± 97	263 ± 84	249 ± 91	NS	NS	NS
Urine albumin (µg/mg of creatinine)	13.8 ± 4.5	2.6 ± 2.3	1.9 ± 2.1	<0.001	<0.001	NS
Fibrinogen (g/L)	3.7 ± 0.2	3.8 ± 0.3	2.9 ± 0.3	NS	0.024	0.006

Data are means ± SD

*P= between group 1 and group 2; **P= between group 1 and healthy control; ***P= between group 2 and healthy control.

LDL = low density lipoprotein

Table 2. Immunologic parameters of healthy and diabetic participants

	Group 1 Type 1 DM with albuminuria	Group 2 Type 1 DM without albuminuria	Healthy controls	*P**	*P***	*P****
WBC (/µl)	6156 ±197	6337 ± 134	6120 ± 234	NS	NS	NS
Lymphocytes (abs)	2156 ± 99	2366 ± 94	2117 ± 210	NS	NS	NS
Lymphocytes (%)	32.4 ± 2.3	31.1 ± 2.5	34.5 ± 2.8	NS	NS	NS
B lymphocytes (abs) (CD3–, CD19+) (/µl)	259 ± 23	233 ± 34	221 ± 33	NS	0.07	0.036
B lymphocytes (CD3-, CD19+) (%)	12.1 ± 2.3	10.8 ± 2.1	10.4 ± 1.9	NS	NS	0.082
T lymphocytes (CD3+) (/µl)	1714 ± 71	1658 ± 68	1502 ± 96	NS	NS	NS
T lymphocytes (CD3+) (%)	79.5 ± 9.4	70.1 ± 7.9	70.9 ± 6.8	NS	NS	NS
CD3+, CD4+ (/µl)	1062 ± 78	1120 ± 96	1016 ± 82	NS	NS	NS
CD3+, CD4+ (%)	49.2 ± 9.2	47.3 ± 11.1	49.9 ± 5.3	NS	NS	NS
CD3+, CD8+ (/µl)	562 ± 68	699 ± 46	611 ± 82	NS	NS	NS
CD3+, CD8+ (%)	26.1 ± 4.2	29.5 ± 5.1	30.8 ± 4.3	NS	NS	NS
Natural killers (CD3+, CD16+, CD56+)(/µl)	197 ± 26	207 ± 31	213 ± 47	NS	NS	NS
Natural killers (CD3+, CD16+, CD56+)(%)	9.1 ± 2.6	8.7 ± 3.1	10.6 ± 2.2	NS	NS	NS
NKT cells (CD3-, CD16+, CD56+) (/µl)	36.6 ± 12.3	40.2 ± 11.8	38.5 ± 15.9	NS	NS	NS
NKT cells (CD3-, CD16+, CD56+)(%)	0.5 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	NS	NS	NS
IgA (g/L)	1.83 ± 0.09	1.91 ± 0.12	2.06 ± 0.05	NS	NS	NS
IgM (g/L)	2.01 ± 0.07	1.95 ± 0.03	1.92 ± 0.06	NS	NS	NS
IgG (g/L)	7.23 ± 1.23	7.14 ± 1.34	8.19 ± 1.63	NS	NS	NS

Data are means ± SD
**P* = between group 1 and group 2
***P* = between group 1 and healthy control
****P* = between group 2 and healthy control.
abs = antibodies, Ig = immunoglobulin

Table 3. Lymphocyte-platelet aggregates in healthy and diabetic participants

	Group 1 Type 1 DM with albuminuria	Group 2 Type 1 DM without albuminuria	Healthy controls	*P**	*P***	*P****
LPA (/µl))	223.9 ± 12.8	430.4 ± 20.6	296.1 ± 22.6	<0.001	NS	0.027
CD3+, CD4+ (/µl)	1062 ± 78	1120 ± 96	1016 ± 82	NS	NS	NS
_____LPA_____ (CD3+, CD4+) cells (%)	21.1 ± 1.6	38.4 ± 2.1	29.1 ± 2.8	0.002	0.058	0.011
(CD3+, CD4+) + (NK cells) (/µl)	1259 ± 75	1327 ± 92	1229 ± 79	NS	NS	NS
_____LPA_____ (CD3+, CD4+) + NK cells (%)	17.8 ± 1.7	32.4 ± 2.1	24.1 ± 2.6	0.007	0.028	0.001

Data are means ± SD
**P* = between group 1 and group 2
***P* = between group 1 and healthy control
****P* = between group 2 and healthy control.
NK cells = (CD3+, CD16+, CD56+)

group (296.1 ± 22.6/µl; 14.1 ± 1.1%) [Table 3]. Our previous observations established that mainly CD4+ and NK cells participate in lymphocyte-platelet aggregation. The role of other lymphocytes in the formation of platelet-lymphocyte aggregation is negligible [14]. That is why we calculated not only a percentage and absolute quantity of LPA, but also a percentage of LPA/CD4 and LPA/(NK + CD4) [Table 3].

Discussion

In the present study we determined the number of lymphocyte-platelet aggregates in type I diabetic patients with or without diabetic nephropathy. Our findings clearly demonstrate that in patients with T1DM at earlier stages of the disease (shorter disease duration, absence of diabetic nephropathy), platelets bind more intensively to lymphocytes and form platelet-lymphocyte aggregates.

As a rule, platelets in diabetic patients circulate in an activated state and are larger in size [15]. They are characterized by increased adhesiveness and aggregation, both spontaneous and in response to stimulating agents, due to increased expression of activated glycoprotein complex IIb-IIIa, P-selectin, lysosomal and GP53 thrombospondin [16]. Diabetes is also associated with lymphocyte redistribution, such as T cell lymphocytosis and T cell lymphopenia [17], while glycemic control changes lymphocyte distribution between blood and tissues [18].

Platelet-leukocyte cross-talk in T1DM and its contribution to diabetic micro-angiopathy were intensively studied by Hu and co-workers [16]. The phenomenon of platelet-leukocytes cross-talk involves both heterotypic cell-cell contact and soluble mediators released from platelets and leukocytes, which may prime both platelets and leukocytes and enhance platelet and leukocyte reactivity [16].

Our study shows that, in T1DM, lymphocytes readily adhere to platelets, forming LPAs. Previously we demonstrated that interleukin-2-activated lymphocytes are the main contributors of LPA construction via enhanced adhesive molecule ICAM-1 expression [14,19]. Since the number of circulating LPA is related to T lymphocyte activation and reflects T lymphocyte activity rather than

NK = natural killer
ICAM = intercellular adhesion molecule

platelet activation *in vivo*, LPA may be used as a simple and novel marker of T cell activation [19,20].

The role of these LPA in diabetes mellitus is largely speculative. Our study did not examine the role of lymphocyte-platelet aggregates in the patient groups. Activated platelets can transiently form a bridge between lymphocytes and endothelial cells, thereby enabling the lymphocytes (in LPA) to undergo subsequent beta-2 integrin-dependent firm adhesion, contributing to the "pro-inflammatory condition" in diabetes [21]. The transmigrated LPAs may promote the microvascular complications (nephropathy) through several possible mechanisms: a) the migrated activated lymphocytes within the LPA complexes may secrete an increased amount of transforming growth factor-beta 1 [22]; b) increased quantity of endoglycosidase, an enzyme splitting heparan sulfate of the subendothelial basal membranes of the glomeruli, thereby interfering with the negative charge of the glomerular filter and the local anticoagulation potential [23]; and c) LPA produce procoagulant, oxidative and mitogenic substances, which can cause microembolism in capillaries as well and ischemic damage of tissues [24]. At the earlier stages of T1DM, platelet and lymphocyte activation is reflected in an increased number of LPA in peripheral blood. As diabetes progresses, LPA migrate to the tissues (and their number in peripheral blood decreases) and micro-angiopathy worsens.

It would be interesting to examine whether platelet membrane glycoprotein (GPIa/IIa, GPIba, GPIIb/IIIa) polymorphisms influence LPA formation and the development of microvascular complications in diabetes, although such polymorphisms were not found to be associated with macrovacular diseases [25].

If a role of LPA in microvascular complications in diabetes is substantial, pharmacological interventions aimed at interrupting their action will provide hope for the future treatment of microvascular disease in diabetes mellitus.

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