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ORIGINAL ARTICLE

The formation of platelet–leukocyte aggregates varies during the menstrual cycle

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Abstract
Platelet–leukocyte aggregates are considered to play a significant role in blood coagulation and inflammatory processes. We hypothesized that hormonal changes during the menstrual cycle affect the formation of heterotypic aggregates and therefore may constitute cycle-dependent variations of the susceptibility for thromboembolic events and inflammatory disease. We therefore measured platelet–leukocyte interaction by the determination of platelet–leukocyte aggregates (PLA), platelet P-Selectin expression, and platelet fibrinogen receptor activation by PAC-1 binding in 20 healthy women during their menstrual cycle by flow cytometry. The number of platelet–granulocyte aggregates (PGA) and platelet–monocyte aggregates (PMA) was higher at ovulation compared to any other time-point of the menstrual cycle ($p = 0.005$, $p = 0.022$, respectively). Likewise, P-Selectin expression peaked on day 14 ($p = 0.040$). The course of PLA formation during the menstrual cycle followed the course of estrogen levels, strongly suggesting direct effects of estrogen on platelet–leukocyte interaction. The susceptibility to form platelet–leukocyte aggregates that are inducible in vitro by a suboptimal concentration of thrombin receptor activating peptide-6 decreased slightly during the transition from day 1 to 14 ($p = 0.040$). These data indicate that platelet function varies during particular phases of the normal menstrual cycle.

Keywords: *Menstrual cycle, platelet-leukocyte aggregates, P-Selectin, PAC-1*

Introduction

Platelets cooperate in a variety of clinical syndromes, like inflammatory disorders, which are known to wax and wane during the menstrual cycle [1, 2]. Further, platelets play a significant role in blood coagulation, which also changes during the cycle [3–5]. Whether or not the menstrual cycle, and thus presumably changes of hormonal levels, have an influence on platelet function has been addressed in the past by studies on platelet aggregation. No changes in primary aggregation were found in two studies [6, 7]. Agonist induced secondary aggregation was recorded more frequently during the follicular phase than during menstruation or luteal phase [6]. In contrast to these findings, an increase of agonist induced platelet aggregation [8] and a greater sensitivity to ADP [9] during ovulation and premenstrual compared to menstruation have been described. Thus, it remains unclear whether or not platelets are more ready to participate in primary homeostasis at any particular phase of the menstrual cycle.

Platelet–leukocyte aggregates (PLA) are considered to be the most sensitive marker for platelet activation [10]. Upon activation, platelet express surface P-Selectin (CD62P), the most important ligand for P-Selectin glycoprotein ligand 1 (PSGL-1) on leukocytes. PLA have been shown to be increased in various disorders associated with thrombophilia, such as myeloproliferative disorders [11], unstable angina [12], myocardial infarction [13], chronic venous insufficiency [14] or associated with autoimmune inflammatory disease, such as systemic lupus erythematosus and rheumatoid arthritis [15]. As the risk for thromboembolic events varies premenopausal from that during later life periods, and autoimmune disorders affect women most often in their reproductive age, it is of particular interest

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to evaluate whether or not the menstrual cycle influences the formation of heterotypic aggregates. We show that ovulation is indeed marked by peak levels of heterotypic aggregates. However, there is no increased susceptibility for further platelet activation, as estimated by platelet responsiveness in vitro to thrombin receptor activating peptide-6.

Materials and methods

Study population

Twenty healthy non-hormonal-contraceptive-taking women with a median age of 27 years (range 25–38 years) gave their informed written consent for the study. A clinical history was obtained from each individual in form of a written consult and checked for exclusion criteria, like a family history of thromboembolic disease, high blood pressure or abnormal lipid profile. Physical examination included blood pressure and laboratory screening included evidence for liver or renal disease and an abnormal lipid or coagulation profile. Only non-smoking, non-pregnant women without a history of acute or chronic disease or bleeding abnormalities were included. They were advised not to take any medication, nor to change their usual dietary or sporting habits. The Institutional Review Board of the University Vienna Hospital has approved the study protocol.

All women reported regular menstrual cycles of 28 ± 2 days without menstrual abnormalities. Based upon the length of their last cycles and the date of the last menstruation blood samples were drawn on day 1 (onset of menstruation), day 7, ovulation day (which was day 14 in most women but day 15 or 16 in four women, as documented by serum concentrations of follicle stimulating hormone and luteinizing hormone, data not shown) and day 21. The follicular phase is the time before ovulation and the luteal phase the time after ovulation until the onset of the next menstruation.

Blood collection

After applying a light tourniquet, which was immediately released, blood was drawn using a 21-gauge butterfly needle. The first 5 ml of blood were drawn into a sterile Vacutainer® (Becton-Dickinson Immunocytometry Systems (B.D.), San Jose, CA) tube to collect serum; the next tube contained 0.054 ml of EDTA to determine the platelet count. The next 5 ml were drawn into a Vacutainer® tube containing trisodium citrate (9 parts of whole blood, 1 part of trisodium citrate 0.108 mol/L) and used for flow cytometry evaluations. Plasma from the sample was also saved for the determination of fibrinogen. Blood samples were used within 20 min after sampling.

Materials

All antibodies were used at optimal concentrations as determined by titration. Anti-CD61 (clone RUU-PL 7F12), perdidine chlorophyll Protein (PercP) conjugated, anti-CD45 (clone 2D1), fluoresceine isothiocyanate (FITC) conjugated, anti-CD14 (clone MΦP9), allophycocyanin (APC) conjugated and PAC-1 monoclonal antibody (MoAb), FITC-conjugated, were purchased from B.D. Anti-CD62p (anti-P-Selectin) (clone CLB-Thromb6), PE-conjugated, was purchased from Immunotech (Marseille, France).

Thrombin receptor activating peptide (TRAP-6) was from Bachem (Bubendorf, Switzerland). In titration experiments we evaluated that 10 μL of 50 μM is the smallest dose that still induces 30–60% of platelets to express P-Selectin and was therefore used at this concentration.

Detection of platelet–leukocyte aggregates

A modified method was used for the evaluation of PLA, based on minimal sample manipulation, as published previously [16, 17]. In brief, 5 μL of trisodium citrate anticoagulated whole blood were aliquoted in polystyrene test tubes containing 55 μL Hepes-buffered saline (Hepes 20 mmol/L, NaCl 137 mmol/L, KCl 2.7 mmol/L, MgCl 1 mmol/L, glucose 5.6 mmol/L and BSA 1 g/L, pH 7.4). Three μL of anti-CD45-FITC, anti-CD61-PerCP and anti-CD14-APC moAbs were added. Corresponding isotype-matched control antibodies were used to detect non-specific bindings. Either 10 μL of TRAP-6 (50 μM) or Hepes-buffered saline were added to determine the amount of PLAs with or without agonist. After 20 min of undisturbed incubation in the dark at room temperature, samples were diluted with 0.5% formaldehyde in Hepes and immediately analyzed using a FACSCalibur flow cytometer (B.D.) with excitation by an argon laser at 488 nm and a red diode laser at 635 nm. The cytometer was calibrated daily with standard B.D. Calibrite beads containing specific amounts of 'mean equivalent soluble fluorescein molecules'.

The cytometer was triggered by FITC (CD45) fluorescence, i.e. the pan leukocyte label, obtained in linear mode. A gate was set and at least 5000 CD45+ events were acquired. Granulocytes, lymphocytes and monocytes were then discriminated based on their forward scatter versus side scatter characteristics as well as side scatter versus Fl-1(CD45). Only events that were found in both corresponding subpopulation gates were further analyzed. They were then subjected to two color analysis and divided into CD45+ events and CD45+CD61+ events. The latter population represents the PLAs. Monocytes were further identified by their staining with CD14.

Detection of P-Selectin and PAC-1

P-Selectin (CD62p) expression and PAC-1 binding were determined before and after the addition of TRAP-6 as follows: citrate anticoagulated whole blood was diluted 1:10 in phosphate-buffered saline (PBS) and 20 μL were aliquoted into polystyrene test tubes. Five μL of anti-CD61-PerCP and either PAC-1-FITC, or anti-CD62p-PE, or the corresponding isotype matched controls were added. Ten μL of TRAP-6 (50 μM) or PBS were added. After 15 min incubation in the dark the reaction was stopped with 500 μL PBS. To minimize in vitro artifacts fixation and lysing procedures were omitted.

All samples were analyzed within 20 min. Platelets were gated in the forward scatter vs. side scatter based on the characteristic platelet cloud. To exclude all CD61 negative events a list mode gate was set in FL-3. Ten thousand events were acquired. The gated events were further analyzed in histograms for FL-2 for the detection of P-Selectin and Fl-1 for PAC-1, respectively. Mean fluorescence intensities (MFI) were used for statistical analyses.

Determination of platelet counts, fibrinogen and estrogen

Platelet counts were determined using a Coulter Counter T-540 (Coulter, Miami, FL).

Plasma and serum, respectively, were stored at −20°C until the determination of plasma fibrinogen according to Clauss (Diagnostica Stago, Asnieres, France); serum estrogen, luteinizing hormone and follicle stimulating hormone were measured on an E-170 Modular Analytics (Roche Diagnostics, Penzberg, Germany). All samples were run within the same assay.

Statistical analyses

The evaluation of PLA formation was the main outcome variable. Results are expressed as mean, and 95% confidence interval (CI) (Table I) and median, first and third quartiles together with the range of data in Figures 1 and 2. Data were assessed by analysis of variance with the fixed factor time-point (days 1, 7, 14 and 21) and the random factor individual woman. *Post hoc* comparisons between time-points were performed by Tukey's multiple comparison procedure. We tested our hypothesis that PLAs peak at ovulation (day 14) by contrasting day 14 in comparison to the other three time points. A p value <0.05 was considered to indicate statistical significance. Calculations were performed using the SAS software system V8.2 (SAS Institute Inc., 2002, Cary, NC).

Results

Median platelet counts were 216×10^9/L (141–404 $\times 10^9$/L), 244×10^9/L (155–371 $\times 10^9$/L), 220×10^9/L (136–367 $\times 10^9$/L), 228×10^9/L (125–417 $\times 10^9$/L) on days 1, 7, 14, 21 of the menstrual cycle, respectively. These changes were not significant.

Changes of PLAs formation during the menstrual cycle are shown in Figure 1. In addition we depicted the variation of plasma fibrinogen levels and serum estrogen. By the latter we document ovulation in all women (Figures 1 and 2). We first tested our hypothesis that platelets are more activated and therefore form more PLAs on day 14 in comparison to the other three time points. Indeed, platelet activation peaked at ovulation, marked by higher expression of P-Selectin. P-Selectin is regarded the major ligand for PLA formation due to its binding to PSGL-1, but the total number of heterotypic aggregates did not change significantly. However, PLA subpopulation analyses revealed peak formation of platelet–granulocyte and platelet–monocyte aggregates at ovulation (Figure 1). The increase of platelet–granulocyte aggregates occurred during the transition from day 1 to 14.

PAC-1 binding, which corresponds to the fibrinogen binding site that is exposed by activated platelets due to the conformational change to allow interaction with plasma fibrinogen [18], however, did not change during the cycle (Table I), while plasma fibrinogen levels increased during the luteal phase (Figures 1 and 2). We hypothesized that low dose TRAP-6 is additive or even co-stimulatory to the effects of the hormonal changes during the cycle on

Table I. P-Selectin expression and PAC-1 binding in 20 healthy women during their menstrual.

	Day 1	Day 7	Day 14	Day 21
P-Selectin expression				
Unstimulated	8.64(7.46–9.83)	7.86(6.72–8.99)	9.99(8.74–11.26)*	8.28(7.34–9.2)
With TRAP-6	255.57(157.9–353.2)	187.64(130.6–244.6)	221.24(128.3–314.2)	294.69(201.2–388.2)
% Increase after stimulation	3088.4(1815–4361)	2398.5(1614–3182)	2236.5(1209–3263)	3776.7(2356–5193)
PAC-1				
Unstimulated	6.26 (5.37–7.15)	6.2(5.61–6.79)	7.28(6.17–8.4)	5.99(5.39–6.61)
With TRAP-6	101.75(79.33–124.16)	108.42(81.46–135.37)	128.44(103.54–153.3)	91.5(70.19–112.81)
% Increase after stimulation	1564.81(1190–1938)	1635.17(1200–2069)	1742.03(1384–2099)	1452.23(1068–1835)

Data represent the mean (CI 95%).
*Significant difference between day 7 and 14 ($p = 0.038$), and compared to the other three time-points ($p = 0.040$).

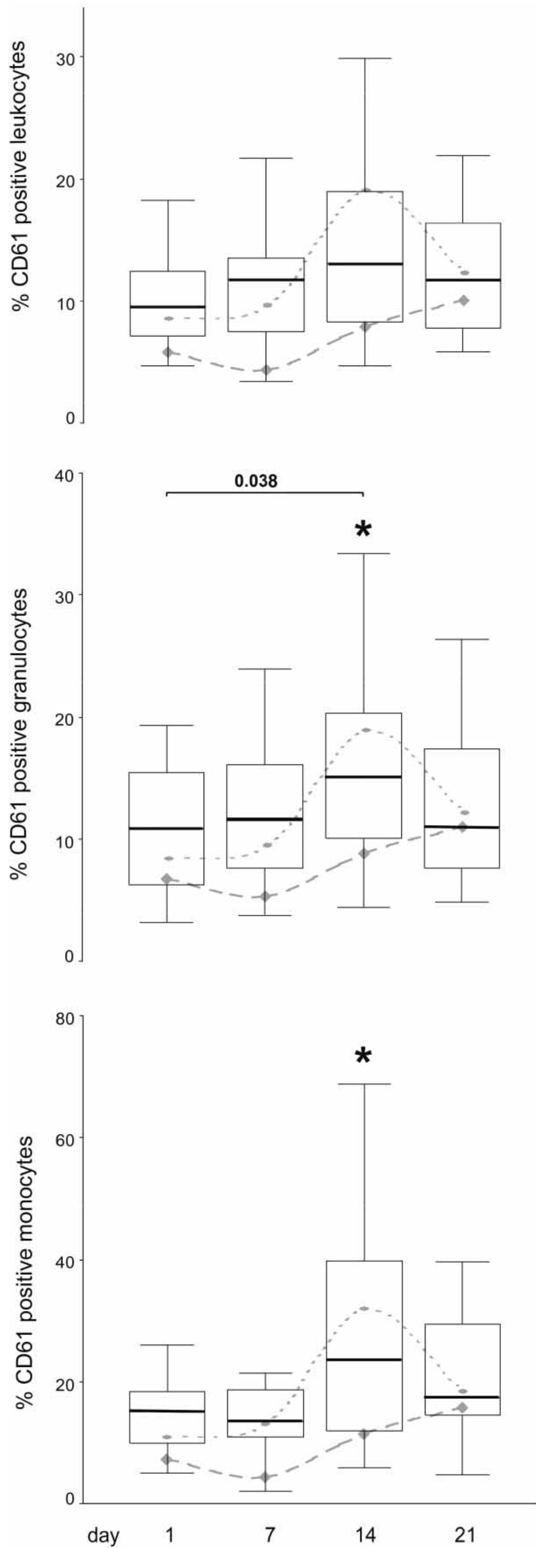

Figure 1. Formation of platelet–leukocyte aggregates during the menstrual cycle in 20 women. The figure shows pan-leukocyte–platelet aggregates, platelet–granulocyte aggregates, and platelet–monocyte aggregates, from top to bottom. Results are presented as box plots that show the median as a horizontal bar, the range between the first and the third quartiles as a box, and the total range of data as whiskers. Dotted and dashed line show the profile (median values) of estrogen and plasma fibrinogen, respectively (without correspondence to the *y*-axis). Asterix to indicate a significant difference at day 14 compared to the other time-points (platelet–granulocyte and platelet–monocyte aggregates; $p = 0.005$ and $p = 0.022$, respectively).

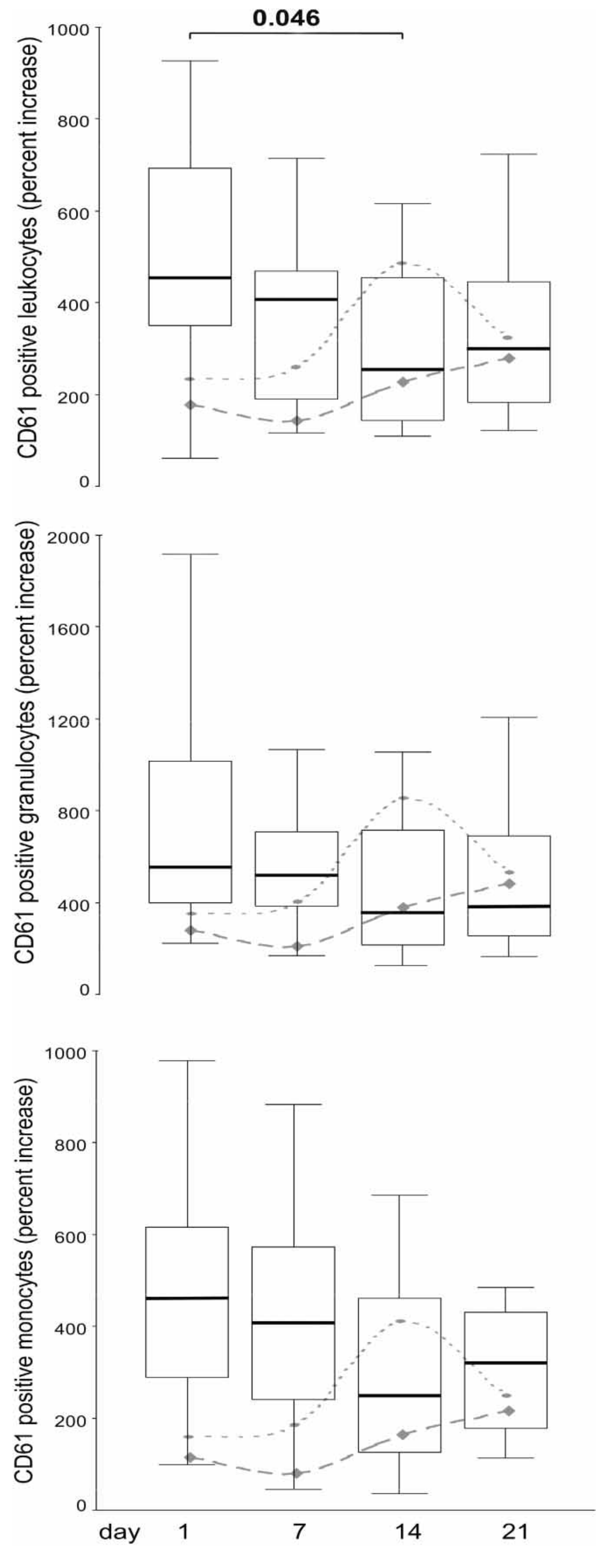

Figure 2. Percent increase of TRAP-6 induced platelet–leukocyte aggregates. Design as in Figure 1.

platelets, and thus will allow disclosing in vivo low grade platelet activation that is not characterized by PAC-1 expression. In contrast to our expectations, however, the in vitro inducible amount of platelet–leukocyte aggregates decreased slightly during the transition from day 1 to 14, and was not different from day 21 (Figure 2).

Discussion

We have addressed whether or not the formation of PLAs changes during the menstrual cycle. Our data show a cycle-dependent variation of the formation of heterotypic aggregates. Thereby, the number of PGA and PMA peaked on day 14 of the menstrual cycle. These findings suggest a hormonal influence on PLA formation. We further investigated if platelets are more susceptible to in vitro activation at any particular time point during the menstrual cycle, but such an increased 'activatablity' could not be documented.

Whether or not particular phases of the menstrual cycle affect platelet reactivity has been addressed in the past by studies using the platelet count [19], platelet aggregation [6, 7] and plateletfibrinogen [20], but results remain overall controversial. Platelet–leukocyte aggregates (PLA) are considered to be a very sensitive marker for platelet activation [10], and we therefore studied their formation during the cycle. We addressed two different aspects of platelet reactivity, namely detectable changes that occur in vivo, and, by exogenous addition of suboptimal concentrations of TRAP-6, whether or not platelets change their susceptibility for activation. Thereby we determined P-Selectin expression as well as the conformational change by PAC-1 binding, together with the formation of heterotypic aggregates. Overall, we observed cycle-specific changes, as P-Selectin and PLA formation peaked at mid-cycle. These changes may be attributable to ovulation accompanied inflammation, or due to direct effects of estrogen, or their combination.

Previous studies [21, 22] and our data show that plasma fibrinogen is higher in the luteal than during the follicular phase of the menstrual cycle. Consequently, platelet-fibrinogen levels increase during the luteal phase [20]. We therefore were interested to see if PAC-1 binding also increases after ovulation. However, PAC-1 binding did not change significantly during the menstrual cycle. We therefore hypothesized that exogenous addition of suboptimal concentrations of TRAP-6 will result in an augmented recruitment of PAC-1 binding platelets, which are already poised for activation. However, an increased 'activatability' was not discernable by the determination of PAC-1 binding (Table I), and the susceptibility to induce PLA formation even decreased slightly (Figure 2). Thus, at mid-cycle platelets may become less responsive to stimuli that lead to the formation of heterotypic aggregates, possibly to prevent coagulation and allow menstruation to occur at the end of the cycle.

The course of the PLA formation strongly suggests a relation to changes of sex hormone levels during the menstrual cycle. As depicted in Figure 1, the course of estrogen was strongly correlated with the levels of PLA, PGA, and PMA.

In the past, the impact of estrogen on platelets has been discussed controversially. It has been shown that platelets express the estrogen receptor β [23, 24], confirming that platelets are a potential target for direct estrogen effects [25]. Further, estrogen replacement therapy [26] as well as in vitro adding of 17β-estradiol to platelets inhibit agonist induced platelet aggregation [27, 28]. Yet, it is well established that oral contraception pills containing estrogen increase the risk for thromboembolic events. Thus, while estrogens inhibit platelet aggregation, they may enhance PLA formation, which play a major role for the development of thrombosis and inflammation [3]. The higher amounts of platelet–granulocyte and platelet–monocyte aggregates as well as higher expression of P-Selectin at ovulation compared to the other time points support the hypothesis that higher concentrations of estrogen increase platelet reactivity. These findings are further substantiated by the results from the Heart and Estrogen/Progestin Replacement Study (HERS [29]), showing that hormone-replacement therapy has no long-term benefits in women with established coronary artery disease. It has been even suggested that hormone-replacement therapy may enhance the platelet component of the pathophysiology of coronary artery disease [23].

In summary, we show a cyclic pattern of platelet activation, particularly affecting PLA formation with a peak at ovulation. These findings corroborate the clinical observations that the susceptibility to thromboembolic events and chronic inflammatory diseases vary during the menstrual cycle.

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