Thrombopotein: its ups and downs
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Chapter 10

Effects of Endotoxemia on Thrombopoiesis in Men
ABSTRACT

Febrile conditions are often associated with increased platelet turnover and refractoriness to platelet transfusions, although several pyrogenic cytokines enhance thrombopoiesis. This study aimed to characterize the effects of experimental human endotoxemia on platelet turnover and thrombopoiesis.

Endotoxin (4 ng/kg) was infused into 30 healthy men to study the regulation of thrombopoiesis in systemic human inflammation. Platelet counts, plasma thrombopoietin (Tpo) and glycoplasmin levels, and reticulated platelets (RP) were measured to evaluate the effect of acute endotoxemia on thrombopoiesis. Ten subjects received pretreatment with 1000 mg aspirin po. to evaluate possible effects of aspirin on platelet turnover, and ten subjects received paracetamol to control for effects of antipyresis.

Platelet counts dropped by about 15% (p<0.001) one hour after LPS infusion, began to recover at 24h, and exceeded baseline values by 8% (CI: 5 - 12; p<0.001) at 7 days after LPS iv.. Reticulated platelet counts increased from 1.62% (CI: 1.24 - 2.0) to a maximum of 2.39% (CI: 1.81 - 2.98; p=0.003) at 6h. Tpo levels increased from baseline values of 10 A.U/mL (CI: 8.8 - 11.2) to 15.5 A.U/mL (CI: 13.6 - 17.3) at 24h (p<0.001), whereas plasma glycoplasmin did not change (p>0.05). The number of circulating platelet-neutrophil aggregates increased more than 100% at 6h (p<0.001). Neither aspirin nor paracetamol affected changes in any of the parameters measured.

Low grade endotoxemia induces a rapid fall of platelet counts, which is followed by an early increase in reticulated platelets and Tpo levels but not of glycoplasmin levels. Finally peripheral platelet counts increase several days after LPS infusion.
INTRODUCTION

Platelets are released into the peripheral blood as a consequence of megakaryocytic fragmentation. Normally, platelet production is a self-regulating process: induction of thrombocytopenia results in an increase of thrombopoietin (Tpo) [1], the key regulator of thrombopoiesis. Tpo enhances size, number and ploidy of megakaryocytes and consequently the number of peripheral platelets [2]. Thrombopoietin is the ligand for the Mpl receptor, which is found on all cells of the megakaryocytic lineage, including platelets. Via binding of Tpo to its receptor and subsequent uptake, Tpo can be effectively removed from the circulation as the platelet count rises [3,4].

However, Tpo- or Mpl-deficient knock-out mice still have platelet counts of approximately 10% [5,6], supporting the concept that other cytokines are also involved in the regulation of thrombopoiesis. In particular interleukin-6 (IL-6) [7,8] among other cytokines, such as IL-11 and IL-3, stimulates thrombopoiesis [3]. Therefore, elevated IL-6 generation has been implicated to cause secondary thrombocytosis in patients with inflammatory diseases [9,10] or cancer [11]. Hence, reactive thrombocytosis may be due to overproduction of cytokines, other than Tpo, such as IL-6. In contrast to IL-6, tumor necrosis factor (TNF-α) suppresses megakaryopoiesis [12].

However, febrile conditions, which are mediated by pyrogenic cytokines such as IL-6 and TNF-α, are long known to be associated with increased platelet turnover or refractoriness to platelet transfusions [13]. Hence, the regulation of thrombopoiesis in febrile conditions appears to be relatively complex, likely because of the simultaneous action of cytokines that enhance and suppress platelet counts.

To directly characterize the regulation of thrombopoiesis in systemic human inflammation, we infused endotoxin into healthy volunteers, which represents an established and frequently used inflammation model [14]. We measured peripheral platelet counts, plasma Tpo and glycocalcin levels and young, mRNA-containing, reticulated platelets (RP) [15] to evaluate the effect of acute endotoxemia on thrombopoiesis. Further, we aimed at studying the effects of aspirin on platelet turnover, and the role of antipyresis by use of paracetamol on endotoxin-induced platelet turnover.

METHODS

Study design and study subjects

The study was randomized, double-blind, placebo-controlled, in 3 parallel groups (n=10/group) in 30 healthy male subjects (mainly students); a report of the effects of endotoxin on adhesion molecules is published elsewhere [16]. The study was approved by the Ethics Committee of the Vienna Medical School and all participants gave written informed consent prior to enrollment in the study. Mean age of the study subjects was 27.5±5 years (±SD) and the body mass index averaged 24 ± 2.3 kg/m² (±SD).
Health status was determined by a battery of laboratory and clinical tests, including medical history, physical examination and hematological, biochemical, virological and drug screening as previously described [17]. Exclusion criteria were hypersensitivity to either aspirin or paracetamol and regular or recent (within 3 weeks) intake of any drugs, including over-the-counter medication.

**Study protocol**

Volunteers reported at the study ward at 8:00 am after an overnight fast. Throughout the entire study period they had to stay in bed in supine position and were kept fasting for 8.5 hr. after endotoxin infusion. A 5% glucose infusion (Leopold Pharma) was started at 8:30 a.m. and continued over 8.5 hours at 3 mL/kg/h to ascertain constant blood glucose levels and adequate urinary output. Placebo, 1000 mg paracetamol (Paracetamol Genericon Pharma, Lannach, Austria) or 1000 mg of acetylsalicylic acid (ASS Genericon; each dissolved in 100 mL mineral water to guarantee blinding) were administered p.o. immediately after the start of the glucose infusion. At 9:00 am, the study subjects received 4 ng/kg lipopolysaccharide (National Reference Endotoxin, E. coli; United States Pharmacopoeial Convention Inc., Rockville, 20852 MD, USA) as an intravenous bolus infusion over 1-2 minutes. Vital parameters were monitored continuously (ECG, heart rate and oxygen saturation) or at 20-min intervals (blood pressure) on a Care View System™ (Hewlett Packard, Böblingen, Germany). Tympanic temperature was recorded hourly with an electronic thermometer (Thermoscan™, San Diego, CA, USA) and urine was collected throughout the entire study period. For safety reasons, study subjects had to stay at the research ward overnight until 24 hours after endotoxin infusion. A final physical examination and check of laboratory parameters, including a differential blood count, were performed 7 days after LPS administration.

**Sampling and analysis**

Venous blood was drawn into Vacutainer tubes before administration of any drug and thereafter as indicated in the figures, until 8 hours after endotoxin injection by repeated venipunctures (except platelet counts, which were obtained from an indwelling venous line) on the contralateral arm from where LPS had been administered. EDTA-plasma was obtained by centrifugation at 2000 g for 15 min at 4°C and stored at -80°C until analysis as duplicates, and all samples from individual subjects were run in the same assay. Tpo plasma levels were determined by a sensitive enzyme immunoassay, recently described by Folman et al. [18] and glycocalcin levels were determined as described by Porcelijn et al. [19]. IL-6 was determined by ELISA (Quantikine HS®, R&D Systems, Oxon, UK) and C-reactive protein (CRP) values were determined by nephelometry (Tina-quant® CRP by Boehringer Mannheim/Hitachi) [20]. Prothrombin fragment F1+2 (Enzygnost F1+2, Behringwerke [21]) was measured at times when maximal levels were expected [22]. Platelet counts were obtained with a cell counter (Sysmex, Japan), and were corrected for the small changes in hemodilution (<5%).
Measurement of reticulated platelets (RP)

Venous whole blood was collected into citrated tubes (Becton Dickinson, Vacutainer Systems, Rutherford, NJ) (0,129M). In contrast to our previous studies [23,24] but in accordance with a recent consensus protocol [25] formaldehyde-fixed platelets were used. Venous whole blood was collected in Vacutainer tubes containing trisodium citrate. The whole blood sample was mixed by inverting the tube gently, and 5 µl of blood sample was added to 1 mL of 1% paraformaldehyde in phosphate-buffered saline (Gibco, NY, USA); this mixture was incubated for a minimum of 30 min to fix the platelets. To flow cytometrically analyze the platelets alone, we performed a double-labeling technique with every blood sample. Fixed platelets were pelleted by centrifugation (1000g, 5 min) and washed once with PBS before labeling with a Phycoerythrin-conjugated monoclonal antibody against glycoprotein IIb/IIIa (CD41; Immunotech) for 30 min at room temperature. Afterwards samples were incubated with 1 mL of thiazole orange (TO; ReticCOUNT™, Becton Dickinson, San Jose, CA) for 60 min at room temperature in the dark and acquisition on a flow cytometer (FACScscan, Becton Dickinson) was started immediately thereafter. In the whole-blood acquisition, platelets were first selected by setting a first gate that separated platelets from erythrocytes, white cells and debris in logarithmic amplification of forward scatter (FSC) versus side scatter (SSC). 10,000 cells within the gate were acquired for each sample. To accurately identify the platelet population, we used a second gate setting in logarithmic amplification of the FSC versus FL2 fluorescence, and the CD41PE-gated platelet population was evaluated for TO staining. The highly TO-positive platelets were identified in logarithmic amplification of SSC versus FL1 of the CD41+ platelet population. Compared to our old method, which yielded a normal 90% range of 0.2-2.3% RP (n=100), the normal range of our currently used method is 0.4-2.5% RP (n=100). Pairwise correlation of values obtained with both methods with the Spearman ranks correlation test of ten samples in the normal range was r=0.8 (p=0.005); however, when samples from ten patients with ITP (range: 9-39% RP) were included in the correlation analysis the correlation coefficient was r=0.96 (p<0.001). When digestion of RNA by RNAsa was performed on samples from patients with ITP according to the methods described by Ault et al. [26] the mean percentage of Thiazole Orange* platelets decreased from 17% to 4% (n=10; p<0.01).

The flow cytometer was calibrated daily before each run with 2-micron beads (DNA QC particles: Vial C, Becton Dickinson) for fluorescence and light scatter.

Flow cytometric analyses of platelet leukocyte aggregates

Platelet-leukocyte aggregates were evaluated by measuring CD41+ neutrophils as described previously [27]. Because all samples required instantaneous processing to avoid artificial activation of leukocytes or platelets, only samples taken at -30 min, 90 min, 6 h and 24 h were stained. Antibodies were fluoro-isothiocyanate labeled and purchased from Immunotech (CD41, isotypic IgG control). Flow cytometric analysis was performed as described previously [17].
Thrombopoiesis in endotoxemia

LPS i.v.

-0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0

0 10 20 30 40 50

-50 -40 -30 -20 -10 0

decrease in platelet counts (10^9/L)

-0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0

0 10 20 30 40 50

-50 -40 -30 -20 -10 0

decrease in platelet counts (10^9/L)

0 120 240 360 480 1440 minutes

Figure 1 Absolute decrease in platelet counts after an iv. injection of 4 ng/kg LPS (p<0.001 Friedman ANOVA vs baseline). Subjects received placebo (O), 1000 mg paracetamol (Δ), or 1000 mg aspirin (■) 30 min before LPS administration. Data are expressed as mean and SEM. No differences between groups were seen.

Data analysis

Data are expressed as the mean and the 95 % confidence intervals for description in the text (n=30). Non-parametric statistics were applied. All statistical comparisons within groups were done with the Friedman ANOVA and the Wilcoxon signed ranks test for post-hoc comparisons. To test changes in endpoints between groups for statistical significance, the Kruskal- Wallis ANOVA was used and post-hoc comparisons were performed by the Mann-Whitney U-test. A two tailed p-value of ≤0.05 was considered significant.

RESULTS

Platelet counts

Baseline levels of platelets averaged 215 x 10^9/L (95% CI: 187 - 243) in the placebo group, 242 x 10^9/L (CI: 212 - 272) in the paracetamol group and 244 x 10^9/L (CI: 201 - 286) in the aspirin-treated subjects (p>0.05 between groups). Platelet counts dropped by about 15% in all three groups at 75 min. after LPS infusion (p<0.001 vs. baseline; Fig. 1). There was a slight recovery at the end of the observation period at 24 hours and platelet counts were 8% (CI: 5 - 12; p<0.001) higher than baseline values at 7 days after LPS administration.
Figure 2 Increase in relative reticulated platelet counts (left), plasma levels of thrombopoietin (middle) and glycocalcin (right) after an iv. injection of 4 ng/kg LPS. Subjects received placebo (O), 1000 mg paracetamol (Δ), or 1000 mg aspirin (■) 30 min before LPS administration. Data are expressed as mean and SEM. **p<0.01 vs baseline. No differences between groups were seen.

Reticulated platelets
Reticulated platelet counts increased from 1.62% (CI: 1.24 - 2.0) to a maximum of 2.39% (CI: 1.81 - 2.98; p=0.003) at six hours (Fig. 2).

Tpo and glycocalcin levels
As depicted in Fig. 2, Tpo levels started to increase from baseline values of 10 A.U./mL (CI: 8.8 - 11.2) at 3 hours and reached a maximum of 15.5 A.U./mL (CI: 13.6 - 17.3) at 24 hours (p<0.001). Plasma glycocalcin levels averaged 352 A.U./mL (CI: 322-382) and did not change significantly during the observation period (p>0.05).

Platelet-neutrophil aggregates
The number of circulating platelet-neutrophil aggregates, as measured by the number of CD41+ neutrophils (Fig. 3), decreased from 233 /μL (CI: 182-284) at baseline to 120/μL (CI: 91-150) at 90 min and then increased to 522/μL (CI: 424-621; p<0.001) at 6 hours. CD41+ neutrophils were still increased at 24 hours (425/μL, CI: 319-530).

Plasma levels of IL-6, CRP and F$_{i+2}$
IL-6 levels increased from an average of 2.8 ng/L (range: 0.2 - 9.8) more than 1000-fold at 3 hours (range: 220-15819 ng/L), and serum levels of C-reactive protein increased from below 0.5 mg/dL to 5.9 mg/dL (CI: 5.1 - 6.7; p<0.001) at 24 hours. (p>0.05 between groups for both parameters). As expected, baseline F$_{i+2}$ levels (0.8 ng/mL; CI: 0.3 to 1.3) did not change during the first hour after LPS administration. F$_{i+2}$ levels averaged 13.8 ng/mL (CI: 10.3-17.3; p<0.001) at 4 hours (p>0.05 between groups).
DISCUSSION

Infusion of endotoxin caused an abrupt 15% decrease in peripheral platelet counts after 45 to 80 min, which is in good agreement with a previous study [28] (Fig. 1). For comparison, platelet counts dropped by about 50 to 60% in lethal septicemia in baboons [29]. First, a low-grade disseminated intravascular coagulation could have induced platelet consumption. This is unlikely to occur within the first hour in this experimental setting, because no increase in F1,2 levels was observed at that time, in agreement with a previous study [22]. Second, the platelets could have been removed from the circulation by adhesion to either microvascular endothelium or leukocytes, which is supported by the >100% higher number of platelet-neutrophil aggregates seen at 6 hours. These heterotypic aggregates are generated after platelet or leukocyte activation. Increased numbers of platelet-neutrophil aggregates, as observed in septicemia, may serve as a marker for ongoing inflammation [27]. Since platelets co-migrate with leukocytes as platelet/leukocyte aggregates from the vasculature into tissues [30], this process could conceivably account for the decrease in platelet counts.

Platelet counts started to recover after 24 hours and were significantly higher than baseline values after 7 days. These data suggest that low grade endotoxemia stimulates the release of platelets from the bone marrow. This is in good agreement with the rise of reticulated platelets. Reticulated platelets contain some residual RNA and represent platelets recently released from the bone marrow [15,26,31-33]. Reticulated platelets increased at 6 and 24 hours after infusion of LPS. The magnitude of increase in %RP is in agreement with the effects of a 30% diminution in platelet counts by apheresis on the peripheral %RP counts [26]. First, this could be due to a relative increase in RP, indicating platelet consumption, and would be in line with the increased RP counts observed in thrombotic thrombocytopenic purpura or disseminated intravascular coagulation [33]. Alternatively, RP may be redistributed from storage pools.
such as the bone marrow, because %RP are higher in the bone marrow than in the peripheral blood (Stohlawetz et al. unpublished).

However, the early rise in %RP was unlikely to be due to an effect of Tpo on the production of RP, because administration of Tpo or IL-6 elevates %RP with a delay of 3 days [34,35], with an even later increase in total platelet counts [34,36]. Tpo plasma values were about 50-75% higher than baseline values 24 hours after LPS infusion. First, the early decrease in platelet counts and hence diminished available receptors for Tpo, could have increased Tpo levels, because Tpo levels are regulated primarily by the peripheral platelet counts and the number of megakaryocytes [1,37,38]. This notion is supported by a study showing that an acute antibody-induced thrombocytopenia is followed by an increase in Tpo four hours later [1]. Alternatively, the increase in Tpo could be due to an intravascular consumption of platelets and subsequent release of Tpo from sequestered platelets. This is in good agreement with the increased Tpo levels found in patients with disseminated intravascular coagulation [39,40], and is in line with in-vitro experiments showing that strong platelet agonists induce a Tpo release from platelets [39]. In contrast to Tpo, glycocalcin levels were not affected by LPS infusion. This indicates that plasma glycocalcin levels are not cytokine inducible, and may serve as a stable surrogate marker of platelet mass [41] even in systemic inflammatory diseases such as septicemia.

Endotoxemia-induced effects are mediated by a number of cytokines, such as TNF, IL-6, IL-8 and IFN-γ. Many of those cytokines may act in concert and synergy with Tpo to increase thrombopoiesis [2,3]. In particular, IL-6 enhances megakaryocytosis and leads to an increase in RP counts in animals [36]. This mechanism may be causative in reactive thrombocytosis, because elevated serum IL-6 levels have been found in secondary but not in primary thrombocytosis [9]. Therefore, an increase in cytokine production, such as that observed for IL-6, could also be responsible for the increase of RP. Such direct effects of IL-6 on platelets seem to depend on arachidonic acid metabolism in-vitro [42]. Further, aspirin has been shown to inhibit nuclear translocation of NF-κB, which transcriptionally regulates several „immediate early genes“ such as TNF [43]. Thus, a secondary aim was to examine whether the administration of aspirin influences the LPS-induced changes in outcome variables. Our data indicate that neither a single dose of 1000 mg aspirin nor paracetamol, influenced peripheral platelet counts, RP or Tpo plasma levels. Paracetamol was used to study a potential effect of an anti-pyretic agent without an inhibitory action on platelet cyclooxygenase. Hence, it appears that the acute inhibition of cyclooxygenase had no detectable effect on markers of platelet turnover and thrombopoiesis after LPS administration. In addition, aspirin did not inhibit the acute phase response as measured by serum IL-6 or CRP levels. Conceivably, this can be explained by the fact that supra-pharmacological concentrations of aspirin were necessary to inhibit NF-κB in vitro, which exceed pharmacological plasma levels in humans [43]. However, we cannot exclude that this may be different after long-term intake of very high doses of aspirin, which lead to drug accumulation. This must be regarded as a limitation of our study.
In conclusion, low-grade endotoxemia induces a rapid fall of platelet counts, which is followed by an early increase in reticulated platelets and thrombopoietin. The fact that reticulated platelets increase rapidly after LPS iv. indicates that endotoxemia induces platelet release. LPS also enhances Tpo plasma levels. This could be due to the decreased platelet counts or may reflect delayed release of Tpo from sequestered platelets. In contrast plasma glycopilicin levels are not affected by systemic inflammation, and may therefore serve as a marker of platelet mass irrespective of inflammation. Finally, an increase in peripheral platelet counts occurs several days after LPS administration.

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REFERENCES

Chapter 10


