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ORIGINAL CONTRIBUTION

Endotoxin-induced effects on platelets and monocytes in an in vivo model of inflammation

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Abstract *Aims* Chronic inflammation is a major contributing factor to atherosclerosis and various markers of inflammation, fibrinolysis and coagulation are upregulated in patients with established atherosclerotic disease. The aim of this study was to investigate the direct and short-term effects of inflammation on platelet and monocyte activation with an in vivo model of endotoxemia in healthy volunteers. *Methods and results* In this study, 13 healthy male subjects with a mean age of 29.5 ± 5.4 years received intravenous administration of lipopolysaccharide (LPS; 20 IU/kg IV). The kinetics of CD40-ligand and CD62P expression on platelets, tissue-factor binding on monocytes and platelet-monocyte aggregates were measured by whole blood flow cytometry at baseline and at 1, 2, 4, 6 and 24 hours after LPS administration. Plasma levels of soluble CD40-ligand were measured with an ELISA over the same time course. Platelet-monocyte aggregates, tissue-factor binding on monocytes and surface expression of platelet CD40L significantly increased in experimental endotoxemia in vivo, reaching peak values 1 hour after LPS administation. All values returned to baseline after 24 hours. Surface expression of CD62P on platelets and plasma levels of sCD40L did not change significantly in response to LPS. *Conclusions* In vivo administration of endotoxin leads to an activation of platelets and monocytes with an upregulation of proatherogenic CD40L on platelets. These findings underpin the role of inflammation in early atherogenesis through platelet and monocyte activation in an in vivo model.

Key words endotoxemia – atherogenesis – inflammation – platelets – monocytes

Introduction

Smoking, obesity, hypertension, diabetes, physical inactivity and hypercholesterolemia are established atherogenic risk factors. Influenced by these risk factors, atherosclerosis is considered a chronic inflammatory disease [3, 5, 16, 22, 33, 40, 41], associated with endothelial dysfunction and other profound changes in the vascular system [6, 8, 16, 40, 41]. In the initiation and progression of atherosclerosis under a chronic inflammatory condition, activated platelets and monocytes play a prominent role. On account of this, antiplatelet and antiinflammatory therapy with acetylsalicylic acid is widely accepted in various atherosclerotic diseases. Several clinical trials have established an additional role for statin therapy besides its lipid lowering properties due to direct antiinflammatory effects in atherosclerosis [34, 35, 37, 38].

Platelet stimulation and subsequent aggregation as well as monocyte activation involve the expression or release of several factors that affect vascular pathology: These include P-selectin, an α-granule protein that mediates platelet rolling, leukocyte adhesion and coagula-

tion, tissue factor expression on monocytes, platelet-monocyte aggregates, and CD40L, a member of the tumor necrosis factor-α family of proteins [1, 7, 19, 21, 37]. The CD40L protein appears to be particularly relevant because an enhanced expression of CD40L on platelets and elevated plasma levels of soluble CD40 ligand (sCD40L) are prothrombotic [2], play a proven role in atherosclerotic lesion progression [36] and are a risk factor for cardiovascular events [10, 12, 19–21, 26, 28, 39]. In the pathogenesis of atherosclerosis, the CD40-CD40L dyad indeed plays a pivotal role. Binding of CD40L to its CD40-receptor on cell membranes induces an enhanced synthesis of inflammatory cytokines and chemokines (e. g., interleukin-8 and monocyte chemotactic protein-1), tissue factor, upregulation of adhesion molecules as well as activation of matrix metalloproteinases [13, 26, 27, 32] with relevance to atherogenesis.

All these factors involved in atherosclerosis are predominantly observed under chronic inflammatory conditions and primarily have been examined in patients with established cardiovascular diseases. Little is known about the direct and early effects of inflammatory stimultation and the kinetics of the described markers on platelets and monocytes in healty persons without established atherosclerotic diseases. In particular, the role of CD40L in early atherosclerotic disease is mostly unknown.

We already studied the effects of in vitro whole blood LPS stimulation, in coherence with alimentary lipemia, on membrane expression of platelet CD40L and on levels of soluble CD40L in apparently healthy subjects and in patients with a history of acute myocardial infarction, complicated by ventricular fibrillation [20].

In the present study, we sought to investigate the early phase of systemic inflammation and atherogenesis with regard to parameters of platelet and monocyte activation and coagulation activation in an in vivo model of experimental endotoxemia by LPS administration in healthy volunteers.

Methods

Study subjects

In this study, 13 healthy, male subjects with a mean age of 29.5 ± 5.4 years (range 24 to 43 years) were included. All subjects were clinically asymptomatic at the time of blood sampling. Persons with known cardiovascular diseases or diabetes were excluded from the study. Written informed consent for the participation in the study was obtained from each subject. The study was approved by the local ethics committee.

All subjects presented fasting in the morning. A glucose 5 % infusion was administered over a time period of 8.5 hours with an infusion rate of 3 ml/kg/hour. Over this time period, all subjects were asked to keep bed rest. A dose of oral paracetamol between 500 and 1000 mg was given to prevent a possible headache as a side effect of endotoxin. Half an hour after the glucose infusion initiation, intravenous administration of lipopolysaccharide with a bolus of 20 IU (2 ng) per kg bodyweight (National reference endotoxin, E. coli; The United States Pharmacopeial Convention) was performed. Blood samples were drawn at baseline (30 min before LPS-injection) and at 1, 2, 4, 6 and 24 hours after LPS administration. Vital parameters were continuously measured over 24 hours.

Experimental design

Flow cytometric analysis

Blood samples of heparinized blood (15 IE heparin per ml blood) (Sarstedt AG & Co, Nuembrecht, Germany) were obtained at baseline (30 min before LPS-injection) and at 1, 2, 4, 6 and 24 hours after in vivo LPS administration. To avoid aggregation and activation of platelets, the blood samples were shaken over the time until measurement. We performed all flow cytometric measurements shortly after each blood sampling in order to avoid potential ex vivo effects of LPS. All whole blood samples were subsequently investigated on activation of monocytes and platelets as well as platelets binding on monocytes. Surface expression of CD62P and CD40L on platelets were measured by flow cytometry. Platelet-monocyte aggregates were measured by CD41 (GPIIb/IIIa receptor) surface expression on platelets adherent to monocytes. Optilyse C (Beckman Coulter, Krefeld, Germany) was used for a complete lysing of the red blood cells as well as fixing of cells.

For the analysis of platelets, 100 µl of each sample were stained for 30 min at room temperature with 10 µl aliquots of mouse anti-human CD62P-PE antibodies (CLB-Thromb/6) (Coulter Immunotech, Krefeld, Germany) and mouse anti-human CD40L-FITC antibodies (P2) (BD Pharmingen, Heidelberg, Germany). For the analysis of monocytes, 100 µL of each sample were stained for 30 min at room temperature with 10 µl aliquots of PE-conjugated murine antibody against CD41 (Coulter Immunotech, Krefeld, Germany) and murine FITC-conjugated antibody against tissue-factor (American Diagnostica, Pfungstadt, Germany). Isotype matched mouse anti-human IgG1 PE/FITC antibodies (Beckman Coulter, Marseille, France) were used as a control. After incubation, erythrocytes were lysed with 500 µL Optilyse C (Coulter Immunotech, Krefeld, Germany). After 15 minutes, cells were resuspended in 500 µL PBS and were then ready for flow cytometric analysis.

Flow cytometric measurement of CD62P and CD40L expression on platelets, by executing a gating for for-

ward and sideward scatter, applied to all monocytes and platelets in the whole blood samples. For measurement of CD41 on platelets adherent to monocytes to determine platelet-monocyte-aggregates and for measurement of membrane bound tissue factor on monocytes, a previous gating for CD14 positivity (mouse anti-human CD14-ECD (RM052) from Coulter Immunotech, Krefeld, Germany) and sideward scatter was performed to identify monocytes. Platelet-monocyte aggregates were strictly defined by a combined flow cytometric CD41 and CD14 positivity (Fig. 1).

All flow cytometry analysis was performed on an EPICS XL-MCL machine (Coulter Immunotech, Krefeld, Germany) equipped with an argon laser tuned at 488 nm. Mean fluorescence intensity (MFI) was measured and all FACS data are expressed as MFI in this manuscript. System II Version 3.0 software was used for data acquisition and evaluation. Compensation of the four channel fluorescence was precisely adjusted using Cyto-Comp™ reagents and Cyto-Trol™ control cells (Coulter Immunotech, Krefeld, Germany).

Enzyme linked immuno sorbent assay (ELISA)

Plasma levels of soluble CD40 ligand (sCD40L) were measured with an ELISA kit (Quantikine®) from R&D Systems GmbH (Wiesbaden-Nordenstadt, Germany) according to the manufacturer's instructions.

Routine laboratory methods

For measurement of leucocyte cell count, red blood cell count and platelet count, blood was drawn into tubes for serum preparation at baseline and 24 hours after LPS infusion and analyzed with established routine laboratory methods.

Thrombin time (INR) (MDA Simplastin) and activated partial thromboplastin time (aPTT) (MDA Platelin LS) were measured using a MDA II coagulation autoanalyzer from Biomérieux (Nuertingen, Germany).

Statistical analysis

The calculations were performed using SPSS for Windows Version 11.5.1. Numerical data were expressed as means ± SD. A Wilcoxon test was applied as a nonparametric test for comparison between all values of each patient. A two-tailed probability value < 0.05 was considered significant.

Results

Demographic characteristics of all participants are shown in Table 1. No volunteer experienced serious side effects after the administration of LPS. Angina pectoris or other cardiovascular complications were not observed during the entire study period.

Table 2 depicts the biological and experimental parameters, indicating that leucocytes, platelet and red blood cell count, thrombin time, INR, aPTT, body temperature, heart rate and blood pressure remained unchanged after 24 hours.

LPS-induced effects on cellular coagulation

Platelet-monocyte aggregates significantly increased one and two hours after intravenous administration of Endotoxin (20 IU (2 ng) per kg bodyweight) (Fig. 2). Tissue-factor on monocytes significantly rose from

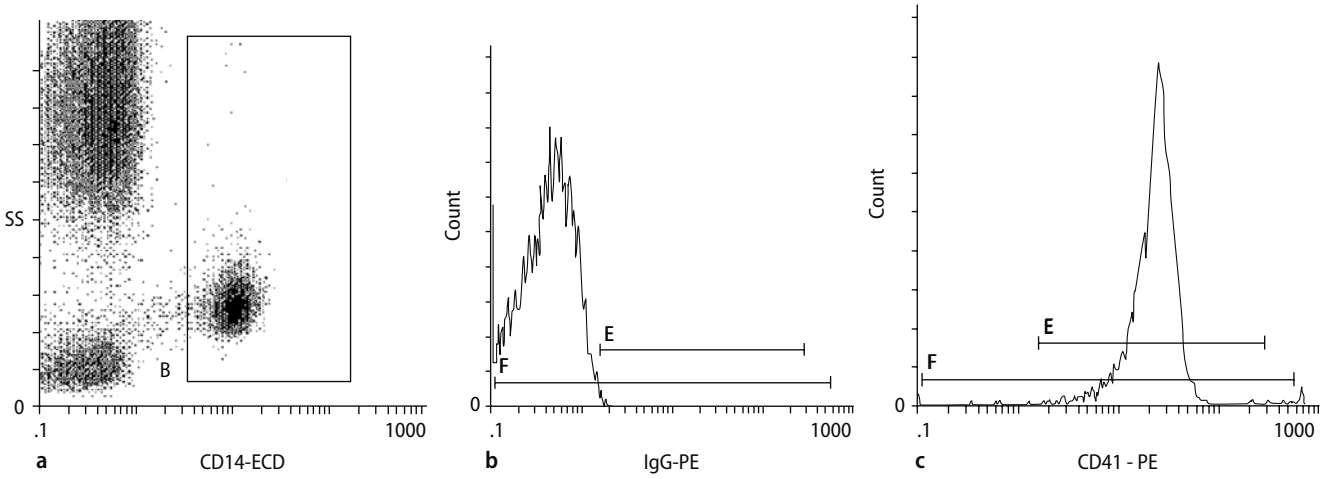

Fig. 1 Flow cytometric measurement (FACS analysis) of platelet-monoyte aggregates, illustrated by an exemplary FACS plot. CD41 expression on platelets adherent to monocytes was measured (**C**) after a previous gating for CD14 positivity and sideward scatter had been performed to identify monocytes (**A**). Platelet-monocyte aggregates are defined by a combined flow cytometric CD41 and CD14 positivity. Negative control measurement is depicted in plot (**B**)

Table 1 Demographic characteristics

	(% or mean ± SD)
Mean age (yr)	29.5 ± 5.4
Male sex (%)	100.0
Smoking (%)	30.8
Hyperlipidemia (%)	0.0
Hypertension (%)	0.0
Diabetes (%)	0.0
Mean body weight (kg)	73.8 ± 8.8
Mean height (cm)	179.3 ± 7.7
Body mass index (kg/m^2)	22.9 ± 1.8

Table 2 Biological and experimental parameters

	0 hours (mean ± SD)	24 hours (mean ± SD)	p-value
Platelets (10E3/µl)	244.7 ± 39.6	215.4 ± 31.2	NS
Leucocytes (10E3/µl)	6.6 ± 1.9	5.9 ± 1.0	NS
Red blood cell count (10E6/µl)	5.0 ± 0.5	4.8 ± 0.7	NS
Thrombin-time (%)	84.0 ± 15.5	80.9 ± 14.0	NS
INR	1.1 ± 0.2	1.1 ± 0.2	NS
aPTT (s)	30.8 ± 4.7	30.2 ± 4.7	NS
Heart rate (BPM)	69.9 ± 11.6	77.2 ± 8.2	NS
Systolic blood pressure (mmHg)	113.2 ± 11.4	111.5 ± 9.3	NS
Diastolic blood pressure (mmHg)	73.6 ± 9.1	71.4 ± 10.6	NS
Temperature (°C)	36.7 ± 0.4	36.7 ± 0.6	NS

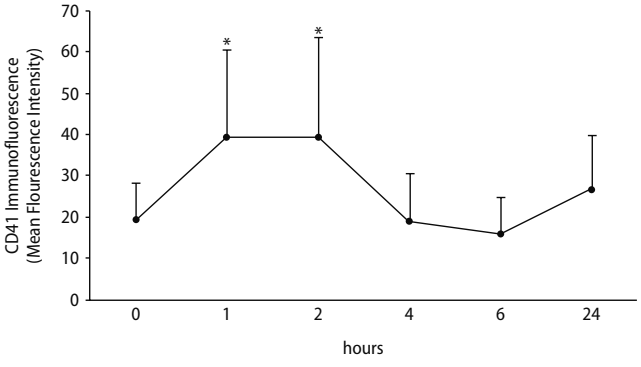

Fig. 2 After LPS administration, platelet-monocyte aggregates significantly increased from 19.38 ± 8.65 to 39.20 ± 21.09 (p = 0.003) after one hour and to 39.02 ± 24.53 (p = 0.019) after two hours. Data are expressed as mean ± SD (* p < 0.05)

1.56 ± 0.55 to a peak value of 2.90 ± 1.78 (p = 0.001) after one hour and stayed significantly elevated 2 hours (2.84 ± 1.88; p = 0.003) and 4 hours (1.97 ± 0.81; p = 0.025) after LPS administration (Fig. 3). All values returned to baseline after 24 hours (all p-values > 0.05 against baseline value).

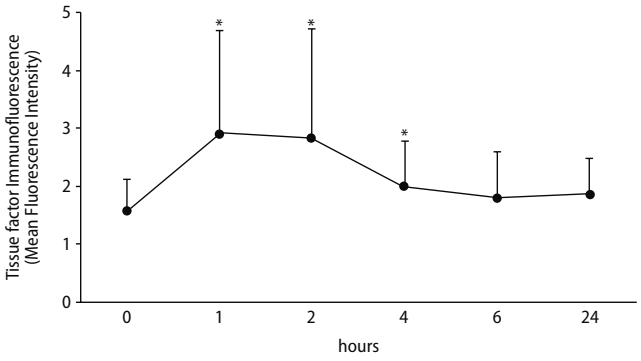

Fig. 3 Tissue-factor on monocytes significantly rose from 1.56 ± 0.55 to a peak value of 2.90 ± 1.78 (p = 0.001) after one hour and stayed significantly elevated 2 hours (2.84 ± 1.88; p = 0.003) and 4 hours (1.97 ± 0.81; p = 0.025) after LPS administration. Data are expressed as mean ± SD (* p < 0.005)

LPS-induced effects on platelet activation

Surface expression of CD40L on platelets significantly increased from 1.70 ± 0.33 to 1.91 ± 0.31 (p = 0.006) after one hour, to 1.86 ± 0.29 (p = 0.011) after 2 hours and to 1.87 ± 0.35 (p = 0.007) after 6 hours, and returned to a value of 1.80 ± 0.33 (p = 0.12), 24 hours after experimental endotoxemia (Fig. 4). Surface expression of CD62P on platelets and plasma levels of sCD40L did not change significantly in response to LPS within the observation time of 24 hours. All values returned to baseline after 24 hours (all p-values > 0.05 against baseline value).

Discussion

Activated platelets and monocytes play an established and pivotal role in atherogenesis and are regarded as cellular mediators of atherosclerosis [9, 13, 15, 22, 23, 27, 28, 36]. Predominantly under chronic inflammatory conditions, the release of several factors affecting vascular pathology such as C-reactive protein, P-selectin, inter-

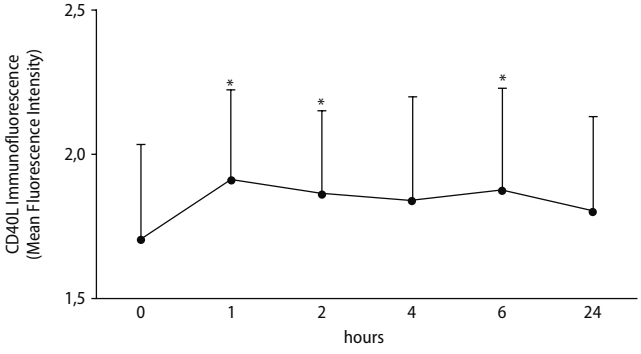

Fig. 4 Surface expression of CD40L on platelets significantly increased one hour after LPS administration and returned to baseline values after 24 hours. Data are expressed as mean ± SD (* p < 0.05)

leukins (IL-1beta, IL-6, IL-8), CD40L, matrix metalloproteinases (MMP-1, MMP-2, MMP-9) and monocyte chemoattractant protein-1 (MCP-1) is observed upon platelet and monocyte activation [3, 4, 8, 11, 14, 18, 22, 24, 25, 33]. Little is known about the direct and early effects of inflammatory stimulation and the kinetics of the described markers on platelets and monocytes in atherogenesis.

In the present study, in an in vivo model of inflammation, endotoxin-induced and acute inflammatory effects were studied. The early phase of systemic inflammatory response with regard to parameters of platelet and monocyte activation and coagulation activation in an in vivo model of experimental endotoxemia by LPS administration in into healthy volunteers was studied.

In vivo administration of endotoxin leads to an early and significant activation of platelets and monocytes with a procoagulant response. Endotoxemia, one hour after in vivo administration, resulted in an upregulation of CD40L on platelets, an enhanced expression of tissue factor on monocytes and an elevated number of platelet-monocyte aggregates. These findings support earlier studies describing direct effects of in vivo inflammatory stimulation on platelets and monocytes [16, 17, 31, 37]. LPS-induced induction of monocyte tissue factor expression via platelet-leucocyte crosstalk were previously described and affirmed by our study [1, 29, 37]. In contrast to previous findings, with earliest measurements performed not until four hours after in vivo administration of LPS [37], we observed a significant increase in platelet-monocyte aggregates and in the expression of CD40L on platelets with peak values measured only one hour after LPS injection. Since former in vivo studies did not perform measurements in this early time period, the present study is the first to reveal changes in platelet and monocyte activation upon inflammatory stimulation in this phase.

Henn et al. observed that CD40L is maximally expressed on the platelet surface within 1 minute of platelet activation [13]. Regarding the time course of solubilization of CD40L from the platelet surface, soluble CD40L (sCD40L) is gradually released over a period of 90–120 minutes after platelet stimulation reaching peak serum levels after 2 hours [30]. This represents a long lasting process compared to the secretion and surface exposure of integran α-granule membrane proteins [30]. Secondary to surface exposure of CD40L, microparticles with membrane-bound CD40L are shed from activated platelets with intracellular processes seeming to be involved in the solubilization of CD40L. Interestingly, in contrast to the observed increase of CD40L expression on platelets, in our study, there was no significant change in plasma levels of soluble CD40L over the time period of 24 hours after LPS administration, validating earlier findings [37].

Chronic inflammation has a well known persisting influence on platelet activity, but the findings of this study furthermore point out that direct inflammatory stimulation may have an acute and short term activating impact, within one hour of stimulation, on platelets, especially concerning proatherogenic CD40L and prothrombotic platelet-monocyte-interaction.

In summary, our results underpin the role of acute inflammation in atherogenesis through early platelet and monocyte activation. By means of an in vivo model in apparently healthy subjects, we were able to reveal the kinetics of known proatherogenic markers on platelets and monocytes after inflammatory stimulation. Although the importance of a lipid lowering and antiinflammatory statin therapy in established atherosclerosis is well described and several studies have established an additional role for statin therapy besides its lipid lowering properties due to direct antiinflammatory effects in atherosclerosis [34, 35, 37, 38], our present results may also illuminate possible antiinflammatory and antiatherogenic implications of a statin therapy in early subclinical atherosclerotic disease since atorvastatin directly reduces platelet expression of CD40 ligand [34] and simvastatin attenuates endotoxin-induced tissue factor formation [37]. Further studies will be needed to address this issue.

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