In vivo effects of lipopolysaccharide and TLR4 on platelet production and activity: implications for thrombotic risk

Muthuvel Jayachandran,1,2 Gregory J. Brunn,1,6 Krzysztof Karnicki,3 Randall S. Miller,4 Whyte G. Owen,4,5 and Virginia M. Miller1,2

Departments of 1Surgery, 2Physiology and Biomedical Engineering, 3Medicine, 4Biochemistry and Molecular Biology, 5Section of Hematology, and 6Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic Rochester, Rochester, Minnesota

Submitted 15 December 2005; accepted in final form 9 August 2006

Infection is a risk factor for thrombosis in humans (6). LPS is a principal outer membrane component of gram-negative bacteria. LPS initiates inflammation, including innate immune responses through activation of Toll-like receptor-4 (TLR4), a transmembrane glycoprotein expressed by leukocytes, lymphocytes, vascular endothelium, and platelets (2–4, 7, 23, 34). Platelets respond within seconds to signals generated by invading pathogens, thus contributing to the inflammation response, initiation of hemostasis/thrombosis, and, ultimately, tissue repair (17). Effects of LPS on platelet functions are controversial and seem to depend on the platelet preparation and whether platelets are exposed to LPS in vitro or in vivo (28, 29). For example, in vitro incubation of rabbit platelet-rich plasma with LPS enhanced aggregation (9, 20). In contrast, incubation of human washed platelets with LPS did not induce aggregation (28, 29). Administration of LPS to mice decreases the number of platelets in the blood within minutes, perhaps by stimulating accumulation of platelets in the liver and lungs (24). The studies that examined effects of LPS on platelet functions using short incubations or exposures (minutes to few hours; Refs. 2, 24) define nongenomic responses of platelets produced under steady-state conditions. However, “priming” animals with a sublethal dose of LPS in vivo could alter gene expression in nucleated cells, affecting functions such as maturation and expression of membrane adhesion receptors defining an activated phenotype (14). Therefore, the present study was designed to test the hypothesis that sublethal in vivo exposure to LPS would increase, through TLR4, platelet aggregation and secretion.

METHODS AND MATERIALS

Experiments were approved by the Institutional Animal Care and Use Committee, Mayo Clinic College of Medicine, Rochester, MN. Adult (4–5 mo old) female wild-type mice (WT; C57BL/10SnJ) and mice with TLR4 homzygous deletion of a 74,723-bp DNA fragment in the third exon of TLR4 gene (dTLR4; C57BL/10ScSn) were obtained from Jackson Laboratories and housed in stainless steel cages with groups of five animals per cage. These mice do not express the IL-12Rβ2 mutation that was originally described for this strain (26). Animals had free access to food (laboratory mouse chow) and water; the dark-light cycle was 12:12 h. The long-term goal of this research program is to evaluate sex differences in thrombotic propensity. Female animals were used to demonstrate proof-of-principle so that other experiments evaluating sex differences and hormonal status could be designed to target specific responses.

Phenotypic evidence for TLR4 gene deletion. Groups of WT or dTLR4 mice were injected with either sterile saline or purified sterile LPS in sterile saline for injection into animals (1, 5, 10, or 20 μg). Blood (50–75 μl) was collected from the tail vein at 30-min intervals prior to and following the LPS injection. Serum samples were prepared by centrifugation at 3,500 rpm for 10 min and immediately frozen at −20°C for subsequent determination of serum tumor necrosis factor (TNF)-α by enzyme-linked sandwich ELISA (R&D Systems, Minneapolis, MN).

Evaluation of platelet phenotype. Mice were injected with a single dose of either LPS (5 μg/animal or 0.2 mg/kg body wt) or equal volume of sterile saline through the tail vein. After 7 days, blood (300–600 μl/mouse) was collected from the retro-orbital sinus under isoflurane anesthesia through heparin or hirudin plus tick anticoagulant peptide (TAP) coated capillary tubes into tubes containing 10 μl 0.4% citrate dextrose solution formula A (Baxter Healthcare). This time period was chosen because the life span of platelets in mice is 4–6 days, thus allowing turnover of the platelet population (unpublished observation by our group and Ref. 33). Platelets were counted

http://www.jap.org 8750-7587/07 $8.00 Copyright © 2007 the American Physiological Society 429
in whole blood diluted in physiological saline (1:10 dilution) using a three-part differential Coulter counter (model T660, Coulter, Miami, FL). The number of platelets containing RNA (reticulated) was determined by flow cytometry as previously described (10, 13). The number of circulating reticulated platelets in WT and dTLR4 mice is presented as percentage of total number of platelets in the blood.

Platelet aggregation was determined in whole blood (1:2 diluted in physiological saline) containing a fixed number of platelets by electrical impedance method (whole blood aggregometer, model 560-VS, Chrono-Log; Havertown, PA) as described previously (10). Collagen (6 μg/ml) was used to produce maximal aggregation.

Secretion of ATP from dense granules (blood diluted 1:1,000 in sterile Hanks’ balanced salt solution) was measured by luciferin bioluminescence in response to mouse (0.1 U) thrombin (10, 31). Data were acquired for 2–5 min at 1-s intervals, and the rate of ATP release is expressed as nanomoles per platelet per minute, whereas total ATP release to mouse thrombin (plateau response) is expressed as nanomoles per platelet.

For analysis of membrane protein expression in activated platelets, blood was mixed with either thrombin (0.1 U) or collagen (6 μg/ml) and incubated at room temperature for 10 min. Membrane adhesion molecule (P-selectin), fibrinogen receptor (fibrinogen binding) expression, and annexin V (membrane phosphatidylserine expression) binding were determined using rat anti-mouse P-selectin-FITC, chicken anti-human fibrinogen-FITC antibodies, and annexin V-FITC, respectively (10, 13). Log forward scatter (for size characteristic) and log side scatter (for granularity) were used to identify platelets and confirmed using the platelet marker CD61-PE. Ten thousand events acquired through forward light scatter and side light scatter were analyzed by CellQuest software. P-selectin, fibrinogen, and annexin V-positive platelets are presented as percentage of total number of platelets in the blood (10, 13).

All values are presented as means ± SE. Statistical significance was evaluated by one-way analysis of variance followed by Bonferroni multiple comparison test and Student’s t-test for paired and unpaired observations. Differences at a level of P < 0.05 were considered to be significant. Two separate sets of experiments were performed using LPS from two different sources. Results of each set of experiments were not statistically different from each other and, therefore, were combined. All experiments were carried out independently using 12–17 individual mice from the WT and dTLR4 colonies.

Materials. Antibodies were purchased as follows: phycoerythrin-conjugated hamster anti-mouse CD61-PE, rat anti-mouse CD62P-FITC monoclonal antibodies, and recombinant annexin V-FITC from BD PharMingen International, San Diego, CA. Chicken anti-human fibrinogen FITC polyclonal antibody was purchased from Accurate Chemical and Scientific, Westbury, NY. Collagen (equine tendon) was purchased from Helena Laboratories, Beaumont, TX. LPS (Escherichia coli strain 0111:B4) was obtained from Sigma Chemical (St. Louis, MO; product #L4391 is purified by chloroform-phenol-petroleum ether extraction and further purified by gel-filtration chromatography) or from InvivoGen (strain-TLR4-Ligand, product number tlr4-pelps), San Diego, CA. HEPES, Hanks’ balanced salts, prostaglandin E1, mouse thrombin, and other analytical/reagent grades were purchased from Sigma.
RESULTS

Phenotypic evidence for TLR4 gene deletion. Serum TNF-α showed a dose- and time-dependent increase following an intravenous injection of LPS in WT but not dTLR4 mice (Fig. 1). These results also demonstrate that the LPS preparation did not activate other inflammatory receptors.

Evaluation of platelet phenotype. Under control/baseline conditions (prior to treatment with LPS), total number of circulating platelets and percentage of reticulated platelets (those containing RNA) were significantly less in dTLR4 compared with WT mice (Fig. 2, control). Collagen induced aggregation and thrombin induced dense body ATP secretion similarly in both WT and dTLR4 mice (Fig. 3; control). Basal (unstimulated) expression of P-selectin, fibrinogen receptors, and phosphatidylserine (annexin V binding) were comparable between WT and dTLR4 mice (Table 1; control). Under control conditions, only thrombin-activated expression of P-selectin was less in dTLR4 compared with WT mice (Table 1, control). Activated expression of fibrinogen and annexin V binding were comparable in each group (Table 1, control).

One week following a single sublethal injection of LPS, platelet number but not turnover (reticulated platelets) increased significantly in both groups of mice (Fig. 2). In addition, aggregation but not dense body ATP secretion (Figs. 3 and 4) increased to the same extent in both groups of mice. Neither basal P-selectin expression nor fibrinogen receptor expression was altered significantly 1 wk following the sublethal injection of LPS. Only basal annexin V binding increased significantly in platelets from dTLR4 mice after LPS treatment. Thrombin or collagen stimulated expression of P-selectin, annexin V, and fibrinogen increased comparably after LPS treatment (Table 1). Responses of platelets from control mice

Table 1. Expression of platelet membrane adhesion molecules

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LPS In Vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n = 12)</td>
<td>dTLR4 (n = 12)</td>
</tr>
<tr>
<td>P-selectin, % of positive platelets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>1.37±0.13</td>
<td>2.12±0.27</td>
</tr>
<tr>
<td>Thrombin activation</td>
<td>62.7±2.26</td>
<td>53.3±3.87*</td>
</tr>
<tr>
<td>Fibrinogen, % of positive platelets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>2.86±0.28</td>
<td>3.25±0.42</td>
</tr>
<tr>
<td>Collagen activation</td>
<td>8.56±1.28</td>
<td>9.04±0.82</td>
</tr>
<tr>
<td>Annexin V, % of positive platelets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>2.26±0.23</td>
<td>2.91±0.32</td>
</tr>
<tr>
<td>Thrombin activation</td>
<td>5.18±0.90</td>
<td>5.09±0.46</td>
</tr>
</tbody>
</table>

Data [percentage (%)] of positive platelets] are shown as means ± SE; n = number of animals. WT, wild type; dTLR4, Toll-like receptor 4 deleted. *Denotes statistical significance from WT P<0.05. †Denotes statistical significance from control, P < 0.05.
injected with saline were not different from those of unmanipulated mice (data not shown).

Although collagen induced platelet aggregation, collagen did not stimulate expression of P-selectin significantly or phosphatidylserine in mouse platelets under any condition (data not shown).

DISCUSSION

Results of this study define the platelet phenotype resulting from TLR4 gene deletion and identify consequences of a single sublethal injection of LPS on platelet turnover and reactivity. Specifically, deletion of a 74,723-bp DNA fragment in the third exon of the TLR4 gene decreased platelet number, turnover, and thrombin-stimulated expression of P-selectin.

Decreases in platelet number and the percentage of reticulated platelets suggest a requirement of TLR4 for genomic regulation of platelet production (turnover) from megakaryocytes, increased degradation of mature platelets, or decreased stability of platelet RNA in dTLR4 mice. Furthermore, decreased P-selectin expression following thrombin stimulation suggests that TLR4 regulates an intracellular mechanism(s) leading to α-granule secretion.

Changes in platelet phenotype to a sublethal dose of LPS is most likely through genomic effects at the level of the platelet precursors, megakaryocytes, as circulating platelets, do not contain nuclei. This conclusion is supported by the observation that increases in platelet number and sensitivity to aggregation were observed 1 wk after the single injection of LPS, the time it takes for the circulating platelet pool to turnover in mice (19). This observation contrasts observed effects observed immediately after a sublethal injection of LPS, where the number of circulating blood platelets decreases due to increased formation of platelet aggregates (5, 24, 30). Therefore, increases in platelet number and reactivity (aggregation) 7 days after a single injection of LPS most likely involves altered gene transcription and protein synthesis in the megakaryocytes, producing a platelet pool with altered protein capacity reflected as increased thrombin-activated expression α-granule P-selectin. In addition, the difference in basal expression of phosphatidylserine (annexin V binding) between WT and dTLR4 mice supports a contribution of TLR4 to the response to LPS. However, it is unlikely that these genomic effects of LPS are mediated solely through TLR4 in megakaryocytes as changes in P-selectin were observed in both WT and dTLR4 mice.

Some have argued that responses to LPS are in part due to activation of other Toll-like receptors resulting from contaminants in the LPS (8, 18, 32). We used ultra-pure LPS that activates specifically only TLR4 (Fig. 1 and unpublished results) from two different suppliers and produced the same results. Therefore, it is unlikely that impurities in the LPS account for the phenotypic changes in platelets observed in the dTLR4 mice.

Expression of platelet α-granule P-selectin and fibrinogen and membrane phosphatidylserine is necessary for platelets to interact with endothelial cells, neutrophils, monocytes, and a subpopulation of T cells in the early stages of inflammation (21, 22, 35, 36). Therefore, changes in platelet phenotype following a single, sublethal injection of LPS to one of increased aggregation and agonist invoked expression of these membrane proteins may provide a possible explanation for how infection may increase risk for thrombosis to other stimuli.

In conclusion, following TLR4 gene deletion, platelet number and turnover (platelet production) decreased and the resulting platelet phenotype had a decreased α-granular response to thrombin. However, LPS affects phenotypic changes in platelet thrombin-induced expression of P-selectin by mechanisms other than TLR4 signaling. Thrombin is the rate-limiting factor in thrombosis. As elevated platelet counts are associated with increased platelet-rich thrombus formation injured arteries (15), these results suggest that increased platelet activation following LPS treatment may represent a mechanism by which infection increases thrombotic risk. One limitation of this study is that only adult female mice were used. As sex-steroid hormones affect platelet characteristics (1, 10–13), it remains to be seen if changes in TLR4 affect platelet characteristics in males similarly as females and whether these responses would change with hormonal status (puberty or reproductive senescence) in each sex. In humans, Asp299Gly polymorphism in TLR4 is associated with a lower risk of carotid atherosclerosis and less intimal media thickness (16, 25). Platelet characteristics in individuals with this polymorphism are not known, and it is also not known if platelet characteristics in individuals with Asp299Gly polymorphism would be the same as those with gene deletion as we describe here. Therefore, these studies in mice provide the basic science evidence to develop similar studies of platelets in humans with infection and/or genetic variation in Toll-like receptors (2–4, 7, 23, 27, 33, 34).

GRANTS

This work was supported by the Mayo Foundation and National Heart, Lung, and Blood Institute Grant HL-78638–01.

REFERENCES


