Peptidoglycan from Staphylococcus aureus Induces Tissue Factor Expression and Procoagulant Activity in Human Monocytes

Eva Mattsson,1,2* Heiko Herwald,2 Lars Björck,2 and Arne Egesten3

Department of Medical Microbiology, Dermatology, and Infection1 and Department of Cell and Molecular Biology,2 Lund University, Lund, and Department of Medical Microbiology, Lund University, Malmö University Hospital, Malmö,3 Sweden

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Staphylococcus aureus is one of the most significant pathogens in human sepsis and endocarditis. S. aureus can initiate blood coagulation, leading to the formation of microthrombi and multiorgan dysfunction in sepsis, whereas in endocarditis the bacterium induces fibrin clots on the inner surface of the heart, so-called endocardial vegetations. In the present study, we show that live and heat-killed S. aureus bacteria are potent inducers of procoagulant activity in human peripheral blood mononuclear cells. Furthermore, purified peptidoglycan, the main cell wall component of S. aureus, induced procoagulant activity in mononuclear cells in a concentration-dependent fashion. The procoagulant activity in these cells was dependent on expression of tissue factor, since antibodies to tissue factor inhibited the effect of peptidoglycan. In mononuclear cells stimulated with peptidoglycan, reverse transcription-PCR showed tissue factor gene expression, and the gene product was detected by enzyme-linked immunosorbent assay. Finally, flow cytometry identified tissue factor at the surface of CD14-positive monocytes. Peptidoglycan is known to induce proinflammatory cytokine production in monocytes. The present investigation shows that peptidoglycan also activates the extrinsic pathway of coagulation by inducing the expression of tissue factor in these cells. This mechanism helps to explain the procoagulant activity, which plays such an important role in the pathogenicity of severe S. aureus infections.

* Corresponding author. Mailing address: Department of Cell and Molecular Biology, BMC B14, Tornav, 10, SE-221 84 Lund, Sweden. Phone: 46-46-2220720. Fax: 46-46-157756. E-mail: fam.mattsson@delt.telenordia.se.

Further, TF activity has been reported during experimental peritonitis and in monocytes from patients with bacterium-induced DIC (1, 11, 22).

The molecular interactions required for the induction of TF expression in monocytes by gram-positive bacteria are unclear. The cell wall of S. aureus is composed mainly of peptidoglycan (PG) (50 to 60% by weight), teichoic acid (TA), and lipoteichoic acid (LTA). PG has a rigid structure and consists of repeating units of N-acetylmuramyl and N-acetyl-muramic acid, to which are linked amino acid residues. TA and LTA are built of phosphorus, and ribitol or glycerol, and in LTA fatty acids are also included (15). PG, TA, and LTA can independently induce an inflammatory response in human monocytes, measured as release of tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β), and IL-6 (17, 32).

Inflammatory responses and procoagulant mechanisms play important roles in the pathogenesis of severe infectious disease. However, the molecular basis for PCA is not well understood, and the present investigation was stimulated by the hypothesis that the staphyloccocal cell wall component PG could induce TF production in human monocytes. Our results show that PG has this effect, which supports the notion that inflammatory responses and PCA are intimately connected in S. aureus infections.

MATERIALS AND METHODS

Materials. Polymyxin B (PMB), LPS from Escherichia coli strain O111:B4, LTA from S. aureus, and FVII-deficient plasma were purchased from Sigma, St. Louis, Mo. Plasma was obtained from healthy volunteers after informed consent and stored at −70°C. Goat anti-human TF immunoglobulin G (IgG) was a kind gift from Marianne Kjalke, Copenhagen, Denmark, and IgG purified from a goat immunized with human β2-microglobulin was used as a control. IL-1β was from...
Strains of bacteria. The bacteria used in the study were a clinical isolate of S. aureus (strain 5120) derived from a patient with septic shock and a laboratory strain, S. aureus WOOD. The bacteria were cultured on blood agar plates. Single colonies were picked and grown in brain heart infusion (Difco, Detroit, Mich.) at 37°C overnight. Prior to incubation with peripheral blood mononuclear cells (PBMC), the bacteria were washed three times and thereafter resuspended in phosphate-buffered saline, pH 7.4. In some experiments heat-killed (80°C for 30 min) bacteria were used.

Preparation of staphylococcal PG. PG was prepared from S. aureus WOOD according to the method of Peterson et al. (24). Bacteria were grown in PYK medium (containing 0.5% [wt/vol] yeast extract, 1.3 mM KH₂PO₄, and 1.1 mM glucose [pH 7.2 to 7.4]) and incubated at 37°C for 24 to 2 h. Log-phase bacteria were added to 10 liters of PYK medium and incubated at 37°C for 18 h in a shaking incubator. The bacteria were harvested by centrifugation (5,000 × g, 4°C, 10 min), and the white top layer of the pellet, containing bacteria, was removed. The remaining cells were resuspended in distilled water and washed. Gram staining was confirmed to obtain the presence of staphylococcal cells. The cell walls were resuspended in sodium dodecyl phosphate (2% [wt/vol]; BDH Chemicals, Ltd., Poole, England) and incubated overnight. The material was washed twice with distilled water and thereafter with 0.05 M NaH₂PO₄ (pH 7.0) and 0.05 M Tris-HCl (pH 7.5). Cell walls were resuspended in 200 ml of 0.05 M Tris-HCl containing 5 mM MgCl₂, DNase (5 μg/ml; Boehringer GmbH, Mannheim, Germany), and RNase (5 μg/ml; Boehringer) and slowly stirred at 37°C for 1 h. Subsequently, trypsin (200 μg/ml; Sigma) was added, and the mixture was stirred for an additional 4 h. After centrifugation, the pellet was resuspended in 50 ml of distilled water, mixed with 50 ml of phenol (80%; Merck, Darmstadt, Germany), and stirred at room temperature for 30 min. After subsequent centrifugation, the cell wall fraction was carefully collected from the interface, resuspended in distilled water, and washed five times with cold distilled water. The fraction was dissolved in 10 to 20 ml of 100 μg/ml trichloroacetic acid to remove TA and stirred at 4°C for 24 h. A crude PG preparation was then obtained by centrifugation, and the supernatant was retained. The trichloroacetic acid extraction procedure was repeated once. To obtain purified PG, the crude PG preparation was resuspended in 10% (wt/vol) trichloroacetic acid and heated at 60°C for 90 min to ensure complete removal of TA. PG was then washed four to six times with cold distilled water, lyophilized, and weighed. Before use PG was resuspended in phosphate-buffered saline containing 50 mM EDTA, and 0.1% lysozyme (Biospec Products, Bartlesville, Okla.) containing sterile glass beads (diameter, 0.1 mm) for 10 to 20 min on an ice bath (12, 15, 16) containing 10% (wt/vol) Triton X-100 (vol/vol) for 20 min. The lysates were centrifuged for 30 min to remove cellular debris, and the supernatant was collected and stored at -70°C until used. Preparation of PG was performed using a commercially available TF enzyme-linked immunosorbent assay (ELISA) reagent (American Diagnostica, Greenwich, Conn.), or, as a control, DIAD antibody and an isotopically matched FITC-conjugated irrelevant mononuclear antibody (mononuclear antibody IgG1; Immunotech, Marseille, France) at the same concentration as the TF antibody. Monoctyes were grafted by their characteristic side scatter and forward scatter, and their identity was further confirmed by their characteristic CD14 signal. The mean fluorescence intensity (MFI) for the control antibody, representing background, was subtracted from the MFI for the TF antibody.

Detection of TF by ELISA. PBMC were incubated with LPS, PG, or medium alone as described above. After 4 h of incubation, the cells were centrifuged at 9,000 × g, the supernatant was removed, and the remaining cell pellet was lysed on ice by the addition of 1% (vol/vol) Triton X-100 for 20 min. The lysates were centrifuged for 30 min to remove cellular debris, and the supernatant was collected and stored at -70°C until used. TF content was measured with ELISA as previously described (24). The limit in the samples was 50 pg/ml. For the detection of TF expression by RT-PCR, PBMC were incubated as described above in medium alone or in the presence of PG (100 μg/ml), LPS (100 ng/ml), IL-1β (5 ng/ml), or TNF-α (5 ng/ml) for 4 h at 37°C. Total RNA was isolated from the cells with a kit based on guanidine thiocyanate and treatment with DNase (Absolutely RNA RT-PCR Miniprep Kit; Stratagene, La Jolla, Calif.), according to the provider’s manual. Reverse transcription-PCR (RT-PCR) was performed essentially as described previously (30, 34). The primer sequences were intron spanning to ascertain that genomic DNA was not amplified. The following primers were used: 5'-ATG AAG ACC CCT GCC TGG-3' (sense) and 5'-CCA GTA GCA CGG CGC GTG CTC-3' (antisense) (TF), 5'-TGG CCA TTT GCT TGG ATC CGC CAG CCT-3' (sense) and 5'-TAG CCA CAC TCA AGA ATG GGA GAA CAT-3' (antisense) (growth-related oncogene α [GRO-α]), and 5'-ACC ACC ATG AGA AAC GTG GG-3' (sense) and 5'-CAC AGT GTA GCC CAG CAT GC-3' (antisense) (glyceraldehyde-3-phosphate dehydrogenase) (RT-PCR). The RT-PCR reactions were performed with treated, predeuterated, preamplification solutions to 100 ng of RNA with 100 ng of random primers (Ready-To-Go RT-PCR Beads; Pharmacia). Ten-microliter samples of the PCR mixtures were put on a 2% agarose gel and stained with ethidium bromide. The PCR products were detected by UV light in a computer-based gel documentation system (Gel Doc 2000; Bio-Rad Laboratories, Hercules, Calif.).
RESULTS

*S. aureus* bacteria and PG induce PCA in PBMC. A possible induction of PCA in PBMC by whole *S. aureus* bacteria was investigated. Live or heat-killed *S. aureus 5120* and WOOD bacteria were incubated with human PBMC (5 × 10⁶ cells/ml) at various ratios for 4 h. Subsequently, the cell-bacterium suspension was added to recalcified human plasma, and the clotting time was determined with a coagulometer. Figure 1 shows that PCA was induced in a concentration-dependent manner by live and heat-killed *S. aureus 5120* and WOOD bacteria at a concentration range of 10⁵ to 10⁶ CFU/ml. At concentrations above 10⁷ CFU/ml, the PCA reached a plateau when heat-killed bacteria were used and decreased when live *S. aureus* bacteria were used. Maximal induction of PCA was reached at bacterium/monocyte ratios of 1:1 and 10:1 for live and heat-killed *S. aureus*, respectively. Clotting time for control cells without stimuli was 335 ± 93 s. Bacteria alone did not affect the clotting time (data not shown).

To study whether PG, the main cell wall component of *S. aureus*, could also induce PCA, it was incubated with PBMC. Subsequently, the mixtures were added to healthy human plasma, and the clotting time was determined. LPS from *E. coli* was used as a positive control. Both PG and LPS showed induction of PCA in PBMC in a concentration-dependent fashion (Fig. 2A). Compared to LPS, a thousandfold-higher amount of PG was needed to reach the same effect on clotting time.

To investigate the kinetics of the PCA induced by PG and LPS, PBMC were incubated with PG (100 μg/ml) or LPS (100 ng/ml) for different time intervals before the cell suspensions were added to human plasma and the clotting time was analyzed. After a 2-h incubation, the PCA increased in both PG- and LPS-stimulated PBMC compared to control cells (Fig. 2B). After 4 h, a plateau for the PCA was reached for both stimuli. The data show that the induction of PCA in human PBMC by PG and LPS is a rapid process, with similar kinetics for PG and LPS. The PG preparation was free of endotoxin as tested by the *Limulus* amebocyte lysate assay (described in Materials and Methods). To exclude the possibility that endotoxin contamination occurred during the experimental procedure, PG was preincubated with PMB (an antibiotic that neutralizes endotoxin) before it was added to the PBMC. No difference in the induction of PCA by PG was obtained in the presence of PBMC, whereas the induction of PCA by LPS was blocked following preincubation with PMB (Fig. 2C).

Expression of TF by monocyes is necessary for the PCA induced by PG in PBMC. To investigate whether TF was responsible for the PCA, IgG blocking TF activity or control IgG was added to PBMC after 4 h of incubation with PG or LPS and incubated for another 30 min. Subsequently, PCA was determined. IgG directed against TF effectively inhibited the increased PCA of LPS- and PG-stimulated cells, showing that the PCA was dependent on TF expression (Fig. 3A). Control IgG did not affect PCA.

TF initiates the coagulation cascade via activation of FVII, thereby forming a potent procoagulant, which activates the extrinsic coagulation pathway. We therefore compared FVII-depleted plasma with normal plasma in the PCA assay. Because trace amounts of FVII are present in the FVII-deficient plasma, it was diluted four times and compared with normal control plasma identically diluted. No clotting was obtained in FVII-depleted plasma by PBMC stimulated with PG or LPS, whereas a marked reduction in clotting time was obtained for normal plasma incubated with cells stimulated by 100 μg of PG/ml or 100 ng of LPS/ml (152 ± 16 or 149 ± 48 s, respectively [mean ± SD]). This further underlines the finding that TF and its activation of FVII are crucial for the activation of the extrinsic coagulation pathway by PG-stimulated PBMC.

TF RT-PCR was used to detect TF gene expression in PBMC. PG induced TF expression at levels similar to those obtained by stimulation with LPS after 4 h of incubation (Fig. 3B-I). PG and LPS induce expression of TNF-α and IL-β in monocytes, raising the possibility that PCA is induced as a
consequence of cellular stimulation by these cytokines. However, no TF expression was detected in cells incubated in the presence of IL-1β (5 ng/ml) or TNF-α (5 ng/ml). GRO-α, a proinflammatory CXC chemokine, expressed by stimulation with LPS, IL-1β, and TNF-α, served as a control to confirm that the cytokines included were biologically active (Fig. 3B-II) (33). Moreover, high levels of TF (1,102 ± 695 or 950 ± 384 pg/ml [mean ± SD]) were detected by ELISA in cell lysates obtained from PBMC stimulated with 100 µg of PG/ml or 100 ng of LPS/ml, respectively (the level of TF in unstimulated cells was <50 pg/ml). Using flow cytometry, it was found that PG- and LPS-stimulated cells expressed similar amounts of TF on their surface (Fig. 3C), and when CD14 was used as a cellular marker, the analysis showed that only CD14-positive cells, i.e., monocytes, expressed TF after stimulation by PG or LPS.

**PCA of adherent monocytes in the absence and presence of lymphocytes.** The PBMC preparations used here contained both monocytes and lymphocytes. Lymphocytes do not express TF, but previous work has shown that they can modulate TF expression in human monocytes (27, 29). For this reason, the PG- and LPS-induced PCA of adherent monocytes was investigated in the absence or presence of lymphocytes. The cells were incubated with medium alone, PG, or LPS for 4 h. Supernatants were removed, and human plasma was added to the wells, followed by incubation for 5 min in order to activate coagulation. Plasma supernatants were then aspirated, recalci
cified, and analyzed for clotting time. However, the presence or absence of lymphocytes did not significantly influence the PCA of the monocyte monolayers (Fig. 4), suggesting that lymphocytes do not contribute to the PCA in our assay.

**DISCUSSION**

The nature of the gram-positive bacterial components responsible for PCA and TF expression in monocytes is unknown. The present study demonstrates that heat-inactivated *S. aureus* bacteria were as effective as live *S. aureus* in inducing PCA, suggesting that staphylococcus-generated PCA requires only bacterial surface components. Highly purified PG, one of the main cell wall components of *S. aureus*, was investigated for its TF-inducing capacity in human PBMC. PG generated a concentration- and time-dependent PCA in PBMC. The threshold concentration of PG needed to generate PCA was 100 ng/ml, which corresponds to 10⁵ CFU of *S. aureus*/ml (26). Recently, similar concentrations of PG were detected in plasma from patients with bacterial sepsis (14). Compared to LPS, a thousandfold-larger amount of PG was required to reach similar levels of PCA. This ratio between PG and LPS is similar to their cytokine-inducing potency in PBMC in the absence of serum (17) and to the difference in LPS and PG concentrations in plasma of patients with sepsis (LPS, 5 to 500 pg/ml; PG, 10 to 190 ng/ml) (7, 14).

PG-induced PCA in PBMC was dependent on TF expression by monocytes, and anti-TF IgG reduced PCA levels to background levels (Fig. 3A). Furthermore, RT-PCR could de-PBMC. After 4 h of incubation, PBMC were mixed with recalcified human plasma, and the clotting time was determined. Values are means ± SDs (n = 3).
FIG. 3. TF expressed by monocytes is responsible for the PCA induced by PG in PBMC. (A) PBMC were incubated with medium alone (control), PG (100 μg/ml), or LPS (100 ng/ml). After 4 h of incubation, TF-neutralizing goat IgG or control goat IgG was added to the cells, followed by another 30 min of incubation. Subsequently, the PBMC were mixed with recalciﬁed human plasma, and the clotting time was determined. Values are means ± SDs (n = 3). (B) Total RNA was isolated from PBMC incubated in medium (control) or in the presence of PG (100 μg/ml), LPS (100 ng/ml), IL-1β (5 ng/ml), or TNF-α (5 ng/ml) for 4 h. RT-PCR was performed on the isolated RNA to detect transcripts for TF, GRO-α, and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GADPH). PG and LPS both induced TF expression in PBMC (I). IL-1β and TNF-α induced expression of GRO-α but not TF (II). (C) PBMC were stimulated with PG (100 μg/ml) or LPS (100 ng/ml) for 4 h. Cells were then incubated with phycoerythrin-conjugated CD14, FITC-conjugated TF, or isotype-matched control antibodies and assayed by flow cytometry (upper panels). CD14-positive monocytes showed similar increases in TF expression after incubation with PG and with LPS (lower panel). Data represent means ± SDs (n = 4) for MFI (mean ﬂuorescence intensity), detected in the FITC channel, which is expressed on the y axis.
tect TF gene expression, and TF was detected in cell lysates and at the surface of monocytes by ELISA and flow cytometry, respectively (Results and Fig. 3B and C). In accordance with their PCA, LPS and PG induced TF in monocytes. We feel that the functional assay that measures PCA is an important complement to the analyses by flow cytometry and TF ELISA, since levels of TF antigen do not always correlate with TF functional activity. Lymphocytes do not express TF but may modulate TF expression by monocytes under certain conditions (29). The results of this study demonstrate that PG- and LPS-induced PCA was not influenced by lymphocytes. These findings are in agreement with the data of Shands, who found that lymphocytes were not required for the induction of PCA in endotoxin-stimulated macrophages (28).

Stimulation by IL-1β or TNF-α did not induce TF gene expression in PBMC as detected by RT-PCR. Therefore, autocrine or paracrine effects of these cytokines are not responsible or sufficient for the induction of TF expression in monocytes under the conditions used. However, it cannot be ruled out that cytokines can potentiate the cellular response to PG, as in the case of whole staphylococcus bacteria in endothelial cells (31). The failure of PG- and LPS-stimulated PBMC to induce PCA in FVII-deficient plasma shows that the PCA was initiated by TF expression, since FVII is required for the procoagulant effect of TF. It is interesting that TF together with FVIIa, in addition to being the major in vivo initiator of coagulation, also induces proinflammatory responses in macrophages, i.e., production of reactive and tissue-toxic oxygen species and expression of major histocompatibility complex class II antigens and cell adhesion molecules (6).

Previous work has shown that TF expressed by monocytes is crucial for the formation of endocardial vegetations in bacterial endocarditis (9). However, the role for monocytes in endocarditis is probably complex. In rabbits suffering from S. aureus endocarditis, monocytopenia induced by etoposide treatment led to rapid death (30), and in these rabbits no endocardial vegetations were found. Thus, monocytes, despite promoting the formation of endocardial vegetations, appear to have an overall beneficial role during S. aureus endocarditis. Other cell components of S. aureus may also be of interest in the pathogenesis of endocarditis and septicemia, for example, LTA. Therefore, commercial LTA was tested in our assays and was found to be unable to induce PCA or TF expression in human monocytes. However, recently it was shown that commercial LTA is of poor quality and purity and may not be biologically relevant (20). It is, therefore, still unclear whether LTA can activate the extrinsic pathway of the coagulation system. Further, coagulase, a secreted protein from S. aureus, has prothrombin-activating properties converting fibrinogen to fibrin, but its contribution to bacterial virulence is uncertain (16). Clumping factor, however, significantly contributes to the pathogenesis of S. aureus endocarditis in a rat model (21), and alpha-toxin alone induces many of the findings typical of sepsis in animal models (3). Therefore, it is more than likely that a number of different S. aureus components induce mediators participating in pathophysiological mechanisms of sepsis.

TF is the main initiator of blood coagulation, and when it comes to bleeding disorders seen in cases of severe S. aureus sepsis (especially DIC), the induction of TF should represent an important mechanism. The finding that PG can induce TF production in monocytes is therefore of theoretical and clinical interest. It represents a novel biological effect of PG, which connects inflammatory and procoagulant cascades, and further underlines the highly complex molecular host-microbe interplay in sepsis.

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