Endotoxin-induced migration of monocytes and PECAM-1 phosphorylation are abrogated by PAF receptor antagonists

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Endotoxin-induced migration of monocytes and PECAM-1 phosphorylation are abrogated by PAF receptor antagonists. Am. J. Physiol. 275 (Endocrinol. Metab. 38): E479–E486, 1998.—The trafficking of monocytes across the endothelial lining of the blood vessel increases in response to bacterial infection at sites of inflammation. However, the molecular events involved in the diapedesis of monocytes in response to endotoxin are not completely understood. Our studies revealed that signaling by lipopolysaccharide (LPS) in human umbilical vein endothelial cells (HUVEC) resulted in a threefold increase in the transendothelial migration of monocyte-like HL-60 cells and a sevenfold increase in the phosphorylation of platelet endothelial cell adhesion molecule-1 (PECAM-1). The transmigration induced by LPS was inhibited by an antibody to PECAM-1. Both the phosphorylation of PECAM-1 and transendothelial migration of monocytes were inhibited by a platelet-activating factor (PAF) receptor antagonist, indicating the autocrine effect of PAF in these events. Treatment of HUVEC with LPS caused a fourfold increase in PAF receptor mRNA expression that was completely blocked by the PAF receptor antagonist. We conclude that PAF, generated by HUVEC in response to LPS or gram-negative bacterial infection, acts in an autocrine manner, causing PECAM-1 phosphorylation and thus the transendothelial migration of monocytes.

endothelial cells; lipopolysaccharide; platelet endothelial cell adhesion molecule-1; platelet-activating factor; transendothelial migration; diapedesis

BACTERIAL INFECTION, leading to septicemia, is the major cause of death among patients in intensive care units (24). The annual incidence of sepsis for all pathological diseases in the United States is estimated to be in excess of one-half million people, with an estimated mortality rate of ~35% (20, 24). Approximately 50% of all cases of septic shock are associated with gram-negative bacteria. Lipopolysaccharide (LPS), released from growing or dying gram-negative bacteria, is thought to play a crucial role in eliciting pathophysiological changes culminating in the systemic and local inflammation seen in gram-negative sepsis and meningitis (3, 20). Mononuclear phagocytes, polymorphonuclear leukocytes, and endothelial cells are the primary cells for the initiation and potentiation of the inflammatory response. Endothelial cells that form the lining of the blood vessel are activated by released LPS, leading to the secretion of cytokines (3) and surface expression of cell adhesion molecules (CAMs) (6). Changes in the expression of CAMs [P-selectin, E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular CAM (VCAM-1)] in response to inflammatory mediators on the endothelium have been implicated in the process of rolling and adhesion of leukocytes (6, 7). After adhesion, these leukocytes undergo diapedesis (27). However, the mechanisms of diapedesis or transendothelial migration of leukocytes in response to LPS are poorly understood. Because platelet endothelial CAM-1 (PECAM-1), concentrated at endothelial cell-to-cell junctions, has been previously observed to participate in mediating the transmigration of leukocytes, including monocytes and neutrophils both in vivo and in vitro (15, 21, 22, 25, 28), we determined whether PECAM-1 played a role in LPS-mediated transendothelial migration of monocytes. We utilized vitamin D3-differentiated HL-60 monocytic cells (referred to in this study as HL-60 cells) to study the transendothelial migration of monocytes, as this cell line has been shown to be a reliable model for transendothelial migration studies and in vivo monocyte function (18).

We report that LPS (25–100 ng/ml) causes an increase in the migration of monocyte-like HL-60 cells across the human umbilical vein endothelial cell (HUVEC) monolayer. The results presented in this study show the role of PECAM-1 in mediating the transmigration of monocytes. We also observed a qualitative parallel relationship between LPS-mediated increase in the phosphorylation of PECAM-1 and concomitant migration of monocytes across the endothelial cell monolayer. The data show that LPS-augmented phosphorylation of PECAM-1 and concomitant transendothelial migration of monocytes may be due to the autocrine effect of platelet-activating factor (PAF), because PAF receptor antagonists blocked both of these events.

METHODS

Reagents. LPS preparation (Escherichia coli 0111:B4) was obtained from List Biologic Labs (Campbell, CA). PAF receptor antagonists CV-6209 and CV-3988 were obtained from Biomol Research Laboratories (Plymouth Meeting, PA); WEB-2086 was a kind gift from Boehringer Ingelheim Pharmaceuticals (Ingelheim am Rhein, Germany); Calcybin A was purchased from LC Laboratories (Woburn, MA); GF-109203X was obtained from Calbiochem (La Jolla, CA); antibody XVII to bovine PECAM-1 was developed against a bovine aortic endothelial cell antigen (13) and found to cross-react with PECAM-1 antigen in HUVEC; monoclonal antibody to human PECAM-1 (clone 5.6E) was obtained from...
were washed with PO₄-free RPMI-1640 (GIBCO BRL) and

cultured in RPMI-1640 medium

(13), which cross-reacts

with PECAM-1 (SEW-16) was kindly provided by Dr. Peter New-

man, Blood Research Institute (Milwaukee, WI); polyclonal

antibody to human CD14 was obtained from Accurate Chemical

(Westbury, NY). The human recombinant soluble CD14 was a kind gift from

Dr. Sanna M. Gyorgy (Northshore University Hospital/Cornell

University Medical College, Manhasset, NY).

Cell cultures. HUVEC were harvested from umbilical cord veins by collagenase digestion, as previously described (16).

Endothelial cells were identified by their cobblestone morphol-

gy, immunofluorescence staining with factor VIII-related

antigen and uptake of diacylated low-density lipoprotein (Biomed-

tical Technologies, Stoughton, MA). Cells were pas-
saged by trypsinization with 1% trypsin-EDTA (GIBCO BRL,

Grand Island, NY) every 4–5 days. HUVEC were used from passages 2 to 6. The human promyelocytic cell line HL-60

(ATCC, Rockville, MD) was cultured in RPMI-1640 medium

(13, 15, 25). Briefly, to the precleared superna-

tant, 10 µl of a monoclonal antibody to bovine PECAM-1

was added, and the mixture was incubated for an

additional 60 min at 4°C. The immunocomplex was collected

by 10,000 g for 10 min. The pellets were washed three times

with cold PBS, then suspended in 1 ml lysis buffer (50 mM

Tris·HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 0.5% sodium

deoxycholate, and 0.1% SDS), and then the pellet was subjected to electrophoresis on 10% SDS-polyacrylamide gels.

The gels, after drying, were exposed to Kodak X-AR-5 X-ray film

at −80°C and developed after 4 h. The radioactivity incorpo-

rated in the 130-kDa gel band identified as PECAM-1 was

quantitated by scanning with an Ambis Radioanalytic Imaging

Systems scanner (San Diego, CA).

Assay for transendothelial migration of monocytes. HUVEC

monolayers were grown to confluence on fibronectin-coated porous membranes (Biocoat cell culture inserts, 3.0 µm;

Collaborative Biomedical Products, Bedford, MA) for 5–6
days as described (25). Transendothelial resistance was mea-

sured daily on the filters with sterilized electrodes and an

EVOM voltmeter (World Precision Instruments, Sarasota, FL). At day 5 or 6, the HUVEC monolayer exhibited total electrical resistance (endothelial cell monolayer filter) of

58 ± 6 Ω·cm² (n = 13), with net transendothelial electrical

resistance of 30 ± 4 Ω·cm², and was used at that time for

migration studies. The values for transendothelial electrical

resistance for HUVEC cultivated on fibronectin-coated porous membranes are similar to literature values of 28 ± 11

Ω·cm² for a HUVEC monolayer cultured on polyethylene terephthalate micropore membranes (17). The confluent mono-

layer of HUVEC was treated with 100 ng/ml of LPS, unless

otherwise indicated, in the absence and presence of either

soluble CD14 (sCD14, 100 ng/ml) or 5% human serum,

followed by the addition of an aliquot (0.1 × 10⁶ cells per well) of differentiatated HL-60 cells to the upper compartment of

the Transwell chamber for indicated time periods. The lower compartment of the Transwell chamber contained 1 ml of

RPMI-1640 with 5% FCS, whereas the upper compartment

contained 0.5 ml of the same medium as well as monocytes. At

the indicated time point, a 50-µl aliquot containing the

migrated HL-60 cells was removed from the bottom compart-

ment of the well. Cells were counted microscopically using a

hemacytometer grid as described (25). To keep the volume

constant in the lower compartment, an equal amount (50 µl)

of medium was added at removal of monocytes. In inhibition

experiments, antibodies and inhibitors were added 30 min

before treatment of HUVEC with LPS.

Isolation of RNA and Northern analysis. Confluent HUVEC

were treated with LPS (100 ng/ml) for time periods ranging

from 2 h to 6 h. Where indicated, PAF receptor antagonist

CV-3988 was added to HUVEC for 30 min before treatment

with LPS. Total RNA was extracted using TRizol reagent (GIBCO BRL), according to the manufacturer’s instructions. Total RNA was

size-fractionated on 1.2% agarose-formaldehyde gels, trans-

ferred to nylon membranes (Hybond N+, Amersham, Airth-

ton Heights, IL), and covalently linked by ultraviolet irradia-

tion with a Stratalinker ultraviolet cross-linker (Stratagene,

La Jolla, CA). The nylon membranes were prehybridized in

10 ml of QuickHyb hybridization solution (Stratagene) for 30

min at 68°C. A cDNA probe for the PAF receptor (PAF R, 1.1 kb),
cut from the plasmid vector by BamHI and PstI (plasmid was

kindly provided by Dr. Richard Ye, Scripps Research

Institute, La Jolla, CA) (30), was radioabeled utilizing a nick-translation kit (Boehringer Mannheim, Indianapolis,

IN). The hybridization was carried out for 1 h at 68°C in

QuickHyb buffer containing 100 µg/ml of sheared denatured

salmon sperm DNA and radiolabeled probe (5 × 10⁶ cpm/ml)

according to the manufacturer’s instructions. This was fol-

lowed by two successive washings of the filter with 2× standard sodium citrate (SSC) containing 0.1% SDS at room

temperature for 15 min and then one washing in 0.1× SSC

containing 0.1% SDS at 60°C for 30 min. The filters were

subjected to autoradiography at −70°C overnight. The mem-

branes were stripped by boiling in 0.1 M Tris·HCl, pH 7.5, 1 M

glycine, and 0.1% SDS and subjected to autoradiography at −70°C overnight. The mem-

branes were stripped by boiling in 0.1× SSC containing 0.1% SDS before rehybridization with the control human β-actin

probe (Clontech, San Diego, CA). The intensities of the bands

on the autoradiograph were quantitated using Digital Imaging

and Analysis Systems (Alpha Innotech, San Leandro,

CA). The ratio of intensity of the PAF receptor band to the

β-actin band was determined for each sample in the lane. The

data were normalized, with the value obtained for untreated

HUVEC used as a control.
Statistical analysis. The statistical significance of difference in a treatment series was determined by ANOVA. Individual treatment in the experimental series was compared with the control using Dunnett’s test. Statistical analysis was performed using Instat-2 software program (Graphpad, San Diego, CA).

RESULTS

Effect of LPS on the transendothelial migration of monocytes. Previous studies have shown that LPS caused increased expression of CAM and concomitantly increased adherence of leukocytes to the endothelial cells (6). We investigated the effect of LPS on the monocyte migration across the endothelial cell monolayer. We utilized vitamin D3-differentiated HL-60 monocytic cells (referred to in this study as HL-60 cells) to study the transendothelial migration of monocytes, because this cell line was previously shown by us to be a reliable model for transendothelial migration studies of peripheral blood monocytes (15). As shown in Fig. 1, addition of LPS (100 ng/ml) alone to the confluent monolayer of HUVEC cultured in a Transwell chamber resulted in a modest (25–30%) increase in the transmigration of HL-60 cells. Several studies (4, 10), including ours, have shown that HUVEC lack membrane CD14 and sCD14 or that human serum was required for endothelial cell response to LPS. We examined the effect of sCD14 on LPS-mediated transendothelial migration of HL-60 cells. As shown in Fig. 1, the addition of sCD14 (100 ng/ml) or human serum (5%) to HL-60 cells in the presence of LPS resulted in a time-dependent (30 min to 4 h) increase in the migration of monocyte-like HL-60 cells. There was an approximately two- and threefold increase in the migration of monocyte-like HL-60 cells with sCD14 and human serum, respectively, compared with basal level of migration. Human serum (5%) in the absence of LPS did not significantly affect the transendothelial migration of HL-60 cells (Fig. 1). A 4-h time period was used in subsequent experiments to assess the effect of antibodies and inhibitors on the transendothelial migration of HL-60 cells. Of interest, when HL-60 cells were pretreated with LPS (100 ng/ml) for 1 h, washed, and then added to the HUVEC monolayer, no significant enhancement on the transendothelial migration of monocytes was observed (data not shown). These findings suggest that the effect of LPS might be primarily on the endothelial cells and not on the monocytes for transmigration to ensue.

We next examined the dose-response (1, 10, 25, and 100 ng/ml) effect of LPS on the transendothelial migration of HL-60 cells. As shown in Fig. 2, LPS at concentrations of 1 and 10 ng/ml in the presence of 5% human serum did not affect the transendothelial migration of HL-60 cells above the basal level. However, LPS at concentrations of 25 and 100 ng/ml increased the transendothelial migration of monocytes by 65 and 190%, respectively, relative to untreated HUVEC. Of interest, when HL-60 cells were preincubated with LPS (100 ng/ml) for 1 h, washed, and then added to the HUVEC monolayer, no significant enhancement on the transendothelial migration of monocytes was observed (data not shown). These findings suggest that the effect of LPS might be primarily on the endothelial cells and not on the monocytes for transmigration to ensue.
necrosis factor-\(\alpha\) (10 ng/ml) and interleukin (IL)-1\(\alpha\) (10 ng/ml) augmented the transendothelial migration of monocytes to almost the same levels as those observed with LPS (100 ng/ml) (data not shown).

Effect of antibody to CD14 on the transendothelial migration of monocytes. Anti-human CD14 monoclonal antibody (CD14 MAb) has been shown to completely block LPS-induced IL-6 release from endothelial cells in the presence of sCD14 (4). Thus, we examined the effect of CD14 MAb on the serum-mediated LPS effect on transendothelial migration of monocytes. As shown in Fig. 3, preincubation of HUVEC with MAb to sCD14 (clone TUK4, DAKO A/S, Denmark) for 30 min before the addition of LPS reduced by \(\sim 75\%\) the transendothelial migration of HL-60 cells induced by LPS in the presence of human serum. Similar results were obtained with anti-CD14 MAb (clone My4) (4). As shown in Fig. 3, the addition of isotype-matched control antibody (MSIgG2b; Coulter Immunology, Hialeah, FL) did not affect transmigration of HL-60 cells.

Involvement of PECAM-1 in LPS-mediated transendothelial migration of monocyte-like HL-60 cells. Previous studies have shown that PECAM-1 localized at endothelial cell-to-cell junctions plays a role in the transendothelial migration of leukocytes (21, 25, 28). Thus, we examined the effect of the addition of PECAM-1 antibody on LPS-mediated transendothelial migration of HL-60 cells in the presence of 5% human serum. As shown in Fig. 3, the addition of MAb (5 \(\mu\)g/ml) to PECAM-1 reduced by \(62 \pm 7\%\) the transendothelial migration of HL-60 cells. Doubling the amount of antibody did not produce additional inhibition (data not shown). Similarly, polyclonal antibody to PECAM-1 (SEW-16) also displayed \(\sim 70\%\) inhibition in the transendothelial migration of monocytes induced by LPS (100 ng/ml) (data not shown). However, the addition of an antibody to vWF, used as a negative control, had no effect on the transendothelial migration of HL-60 cells.

Effect of LPS on the phosphorylation of PECAM-1. Recent studies (23, 25) have shown that, in endothelial cells and platelets, the PECAM-1 molecule expressed on the cell surface undergoes phosphorylation. Furthermore, the PECAM-1 phosphorylation is augmented severalfold in platelets by thrombin (23). Similarly, cigarette smoke condensate (25) and hypoxia (15) have been shown to augment PECAM-1 phosphorylation in HUVEC. Thus, we examined whether PECAM-1 is phosphorylated in response to treatment with LPS. As shown in Fig. 4, treatment of \(^{32}\)P-labeled HUVEC with LPS (100 ng/ml) in the presence of serum resulted in a time-dependent (15–20 min) increase in \(^{32}\)P incorporation into PECAM-1. At 30 min, there was a 720 \(\pm 105\%\) incorporation of \(^{32}\)P into PECAM-1 in endothelial cells treated with LPS compared with phosphorylation of PECAM-1 in control untreated cells. The phosphorylation of PECAM-1 increased to a maximal level within 30 min of exposure to LPS and then decreased by 60- and 120-min points. The incorporation of \(^{32}\)P into PECAM-1 at 120 min was \(\sim 200\%\) relative to untreated HUVEC.

Effect of inhibitors on PECAM-1 phosphorylation. The cytoplasmic tail of 118 amino acid residues in the PECAM-1 molecule has been shown to undergo phosphorylation at serine/threonine but not at tyrosine residues in response to thrombin (23), indicating the role of protein kinase C (PKC) in PECAM-1 phosphorylation. As shown in Fig. 5, the addition of GF-109203X (20 nM), a selective PKC inhibitor, reduced by \(\sim 90\%\) the LPS-induced incorporation of \(^{32}\)P into PECAM-1. Recent studies (8) have shown that LPS augments the synthesis and release of PAF from endothelial cells. As shown in Fig. 6, PAF receptor (PAFR) antagonists CV-6209 (300 nM) and WEB-2086 (10 \(\mu\)M) completely inhibited the phosphorylation of PECAM-1 induced by LPS. As previously observed in endothelial cells exposed to hypoxia (15), the addition of protein phosphatase inhibitor Calyculin A (2 nM) further enhanced LPS-mediated \(^{32}\)P incorporation into PECAM-1 in HUVEC (1,000 \(\pm 45\%\) vs. 100% incorporation in untreated HUVEC (Fig. 5). Of interest, treatment of \(^{32}\)P-labeled HUVEC with Calyculin A (2 nM) alone, without LPS, resulted in 250% incorporation of \(^{32}\)P into PECAM-1, an increase of 150% above the basal (100%) level of PECAM-1 phosphorylation in HUVEC (data not shown). This experiment shows that inhibition of endogenous protein phosphatase activity in HUVEC results in an increased PECAM-1 phosphorylation.

Effect of inhibitors on LPS-induced transendothelial migration of monocytes. To examine whether there is a parallel relationship between LPS-induced PECAM-1 phosphorylation and transendothelial migration of
HL-60 cells, we examined the effects of the PKC inhibitor and the PAFR antagonist on transendothelial migration of monocytes. As shown in Fig. 6, preincubation of HUVEC with GF-109203X (20 nM) for 30 min, followed by the addition of LPS (100 ng/ml) and 5% serum, resulted in an ~70% decrease in the transendothelial migration of HL-60 cells. Additionally, pretreatment of HUVEC with PAFR antagonists CV-6209 (300 nM) and WEB-2086 (10 µM) resulted in 86 ± 6% and 80 ± 10% reduction in the transmigration of HL-60 cells, respectively. Both PKC inhibitor GF-109203X and PAFR antagonist CV-6209 alone, in the absence of LPS, had a minimal (<10% inhibition) effect on the basal level of migration of monocyte-like HL-60 cells (data not shown). Studies were undertaken to determine whether conditioned medium derived from HUVEC (2 × 10⁶ cells) treated for 1 h with LPS (100 ng/ml), which presumably contains PAF (8), could support the migration of HL-60 cells. The conditioned medium (50 µl from a total of 1 ml) augmented twofold the migration of monocytes (Fig. 6). Moreover, the conditioned medium-augmented transendothelial migration of monocytes was inhibited 80% by a PAFR antagonist (CV-6209) (Fig. 6). LPS in the 50 µl of conditioned medium is estimated to be ~5 ng, and the latter concentration of LPS is not sufficient to induce migration of monocytes, as shown earlier in this study (Fig. 2). Taken together, these findings suggest that PAF,
elaborated from HUVEC in response to LPS, mediates the migration of monocytes across the endothelial cell monolayer.

Furthermore, the inhibition of PECAM-1 phosphorylation and transendothelial migration of monocytes by a PKC inhibitor in response to LPS shows that both PECAM-1 phosphorylation and transendothelial migration of monocytes may have either a direct or causal relationship. To substantiate the relationship between the extent of PECAM-1 phosphorylation and transendothelial migration of monocyte-like HL-60 cells, HUVEC were preincubated with Calyculin A, a protein phosphatase inhibitor, followed by the addition of LPS along with 5% human serum. As shown in Fig. 6, Calyculin A (2 nM) caused an ~30% increase in the transendothelial migration of HL-60 cells relative to endothelial cells exposed to LPS and serum.

Effect of LPS on expression of PAFR mRNA. Northern analysis was performed to determine whether the autocrine effect of PAF generated in response to LPS also caused increased transcription of the PAFR. As shown in Fig. 7, incubation of HUVEC with LPS (100 ng/ml) for 2–6 h resulted in a time-dependent increase in PAFR mRNA levels relative to control, utilizing a 1.1-kb fragment of human PAFR cDNA, with a single transcript of ~4 kb. There was a fourfold increase in the expression of PAFR mRNA at a 4-h time period, as determined by the ratio of PAFR to β-actin. At a 6-h time period, there was a decline in the PAFR mRNA expression almost to the basal level. Preincubation of HUVEC with a PAFR antagonist (CV-3988, 300 nM) before treatment with CoCl₂ for 4 h resulted in an ~90% reduction in the induction of PAFR mRNA.

These experiments suggest that PAFR antagonist CV-3988 blocks the LPS-induced autocrine effect of PAF on PAFR mRNA expression. Furthermore, actinomycin D (5 µg/ml), a transcription inhibitor, blocked the expression of the PAFR, suggesting that LPS may affect PAFR gene expression in endothelial cells through transcriptional regulation.

**DISCUSSION**

It is well established that inflammation due to bacterial infection or other proinflammatory mediators leads to increased accumulation of leukocytes at the site of injury, stemming from the adhesion and diapedesis of both polymorphonuclear neutrophils (PMN) and monocytes. The transendothelial migration of those leukocytes involves at least three steps: the initial rolling of leukocytes mediated by members of the selectin family (e.g., P-selectin and E-selectin) of adhesion molecules; the firm adhesion of leukocytes mediated by integrin ligands (e.g., β₂ and VLA-4) expressed on leukocytes with the counter receptors of CAM (iCAM-1, E-selectin and VCAM-1) on endothelial cells; and, finally, movement of leukocytes through the endothelial intercellular junctions (6, 7, 12, 18, 19, 25). At present, relatively less information is available regarding the molecular events involved in the process of transendothelial migration of PMN and monocytes under steady-state conditions and during inflammation (11, 18, 27).

In the present study, we examined the mechanism(s) by which LPS induced migration of monocytes across the endothelial cell monolayer. In this study we utilized the vitamin D₃-differentiated human HL-60 cell line to study the transendothelial migration of monocytes. We (4) and others (10) have previously shown that serum or sCD14 is essential for LPS-mediated endothelial cell responses, i.e., release of IL-6. In agreement with previous studies, we also observed that human serum or sCD14 was essential for LPS-mediated transendothelial migration of HL-60 cells.

We also observed that antibody to human PECAM-1 blocked (~70%) LPS-mediated transendothelial migration of monocyte-like HL-60 cells. In our earlier studies, we showed that the PECAM-1 antibody was equally effective in blocking the transendothelial migration of both monocyte-like HL-60 cells and peripheral blood human monocytes induced by cigarette smoke condensate (25) and hypoxia (15), suggesting that LPS-induced migration of monocytes involves the PECAM-1 molecule. Because LPS-induced transendothelial migration of monocyte-like HL-60 cells was not completely inhibited by an antibody to PECAM-1, we suspect that other adhesion molecules concentrated at endothelial cell-to-cell junction (5) may also play a role in transendothelial migration of monocytes.

We also show that LPS in the presence of serum causes a severalfold increase in the phosphorylation of PECAM-1 in HUVEC. Because the PECAM-1 molecule is concentrated at intercellular junctions of endothelial cells (1, 14), we sought to determine whether there was a relationship between the phosphorylation of PECAM-1 and the migration of monocytes through the PECAM-1...
junctons. Our studies show that pretreatment of HUVEC with a PKC inhibitor, GF-109203X, reduced the phosphorylation of PECAM-1 and transendothelial migration of monocyte-like cells by ~90 and 70%, respectively. Furthermore, our studies show that pretreatment of HUVEC with a phosphatase inhibitor, Calyculin A, increased ~30% the phosphorylation of PECAM-1 and also augmented by 30% the transendothelial migration of HL-60 cells above the level induced by LPS in the presence of human serum. These findings led us to propose that phosphorylation of PECAM-1 in HUVEC may lead to an increase in the transendothelial migration of monocytes across the endothelial cell monolayer, whereas inhibiting the phosphorylation of PECAM-1 causes a reduction in the migration of monocytes across the endothelial cell monolayer. However, it is not clear whether phosphorylation/dephosphorylation events in PECAM-1 localized at endothelial cell junctions have a direct or causal effect on the transendothelial migration of monocytes. There is precedent in the literature (26) showing that phosphorylation of ZO-1 protein, localized in mammary epithelial cell junctions, leads to an increase in the junction permeability. Further studies are needed to characterize the specific amino acid residue(s) in the cytoplasmic domain of PECAM-1 that undergoes phosphorylation and the role of such phosphorylated amino acid residue(s), if any, in the migration of leukocytes across the endothelial cell monolayer.

Because exposure to gram-negative sepsis or meningitis results in the production of numerous chemokines and PAF (2, 3, 9) by the host, we investigated the role of PAF, if any, in LPS-induced signaling, leading to PECAM-1 phosphorylation and the concomitant increase in migration of monocytes across the HUVEC monolayer. Recent studies have shown that LPS in the presence of sCD14 results in the biosynthesis of PAF by HUVEC (8). The levels of PAF synthesized in HUVEC treated with LPS (100 ng/ml) in the presence of sCD14 have been shown to be ~4.5 ng/10^6 cells compared with almost none in untreated HUVEC (8). Our studies show that PAFR antagonists CV-6209 and WEB-2086 completely inhibited the PECAM-1 phosphorylation and abrogated by ~80% the migration of monocyte-like HL-60 cells induced by LPS in the presence of serum. Hill et al. (11) have shown that HUVEC-associated PAF does not affect the adhesion of neutrophils but causes an increase in the transendothelial migration of PMN. The role of PAF in increasing phosphorylation of PECAM-1 and transendothelial migration of HL-60 cells is shown by our recent studies (15), wherein we demonstrated that PAF, at a concentration of 100 nM, resulted in a severalfold (~12-fold) increase in PECAM-1 phosphorylation and a threefold increase in the rate of migration of monocytes across the HUVEC monolayer. Moreover, PAF-induced phosphorylation of PECAM-1 and concomitant transendothelial migration of monocytes were inhibited by PKC inhibitor and PAFR antagonists. These results support the contention that the LPS-induced effect on PECAM-1 phosphorylation and transendothelial migration of monocytes, observed in the present studies, occurs as a result of the autocrine effect of PAF.

The autocrine effect of PAF in endothelial cells occurs through the PAFR, which undergoes increased transcription in response to LPS. An increase in PAFR expression in response to LPS has also been noted in rat ileum in response to LPS (29). The increase in the transcription of the PAFR in HUVEC, in response to LPS, was inhibited both by the PAFR antagonist and the transcriptional inhibitor actinomycin D. We suggest that LPS-mediated flux of monocytes across the vascular endothelium is possibly regulated by the expression of the PAFR. However, further studies are needed to determine the cellular signaling pathways involved in the upregulation of PAFR in endothelial cells in response to LPS.

In conclusion, the results of this study show that LPS causes the phosphorylation of cell-to-cell junction molecule PECAM-1 in the endothelium. As a consequence of PECAM-1 phosphorylation, the transendothelial migration of monocytes increases. The increases in both the phosphorylation of PECAM-1 and migration of monocytes may occur as a result of LPS-induced release of PAF followed by the autocrine effect of PAF on HUVEC. It is suggested that phosphorylation/dephosphorylation events in PECAM-1 localized at endothelial cell-to-cell junctions may have a direct or causal effect in regulating the trafficking of monocytes across the endothelium. The LPS-induced cellular signaling leading to the phosphorylation of endothelial cell junction molecule PECAM-1, and concomitantly increased migration of leukocytes (e.g., monocytes), may play a vital role in the extravasation of PMN and monocytes that is seen commonly in gram-negative bacterial infection such as sepsis or meningitis. Furthermore, PAFR antagonists may be useful in ameliorating clinical manifestations of septic shock in gram-negative infections.

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