Lipopolysaccharide Mediates Endothelial Apoptosis by a FADD-dependent Pathway*

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Endothelial cells play a pivotal role in the inflammatory process by coordinating the recruitment of inflammatory cells to sites of tissue injury. Lipopolysaccharide (LPS) activates many of the proinflammatory and procoagulant responses of endothelial cells, and endothelial injury is thought to play a crucial role in the pathogenesis of septic shock due to Gram-negative bacteria. The receptor used by LPS to signal endothelial responses has not been identified. It is also not known how LPS induces endothelial injury/death. In this study, we demonstrate that LPS mediates endothelial apoptosis by a FADD-dependent pathway. FADD is a death domain-containing protein that binds to certain members of the tumor necrosis factor receptor family, namely TNFR1, Fas, and DR3. However, none of these receptors appear to be involved in LPS-mediated death, suggesting that LPS may utilize a novel death domain-containing protein to transduce a death signal.

Lipopolysaccharide (LPS) is a critical glycolipid component of the outer wall of Gram-negative bacteria, and many of the cellular signals activated by Gram-negative bacteria are attributed to LPS (1). Several responses are evoked in endothelial cells by LPS, including up-regulation of adhesion molecules and expression of tissue factor. The endothelial cell is a prime target of the LPS molecule, and vascular complications of septic shock due to Gram-negative bacteria are related to endothelial injury (2, 3). Whereas LPS directly induces apoptosis of sheep leukocytes, it is only toxic to human endothelial cells when the expression of new genes is blocked (4, 5).

Several intracellular molecules have been implicated in transducing LPS signals. Activation of NF-κB, the Jak-STAT pathway, mitogen-activated protein kinases, and phosphatidylinositol 3-kinase have all been demonstrated to play a role in the intracellular signaling of LPS-mediated events (6–8). LPS complexed with a serum protein, LPS-binding protein, signals through membrane-bound CD14 on monocytes and myeloid cells. In contrast, endothelial and epithelial cells, which are CD14-negative but still respond to LPS, require soluble CD14 present in serum in order to transduce LPS signals (6, 9, 10). It is still unclear how the LPS-soluble CD14 complex actually transmits a signal across the cell membrane. Evidence has been presented to suggest the presence of a signaling transmembrane receptor recognizing the LPS-CD14 complex (11). However, others (12, 13) have postulated that LPS is internalized by a vesicular transport mechanism and mediates signals, at least partly, by structurally mimicking ceramide. Transmembrane signaling by LPS has also been shown to be mediated by CD11/CD18 integrins independently of CD14 (14, 15).

How LPS activates the death pathway in endothelial or other cell types has not yet been investigated. On the other hand signaling of apoptosis by the TNF receptor 1 (TNFR1) and Fas has been extensively studied (16–19). Engagement of TNFR1 or Fas results in cell death by the recruitment of a complex of proteins to the cell membrane. In their cytoplasmic regions, both these transmembrane receptors contain an 80–100-amino acid motif called the death domain (DD), which acts as a protein-interacting domain (18). Upon receptor ligation, a cytoplasmic DD-containing protein, FADD/Mort1, is recruited to the plasma membrane (20). In the case of TNFR1, FADD associates with the receptor via a docking protein, TRADD, whereas FADD directly binds the DD of Fas (20, 21). Activation of caspases, a family of cysteine proteases that act as the final common pathway of apoptosis, occurs following recruitment of caspase 8 (FLICE/MACH) or caspase 10 to the cell membrane, by FADD (22–24). Recently, a third member of the TNFR family, DR3/wsl-1, has also been shown to mediate apoptosis by recruiting FADD to the cytoplasmic face of the receptor (25, 26). It is important to note, however, that other members of the TNFR family can engage the death pathway independently of FADD (27, 28).

In this report we demonstrate that LPS stimulates a caspase-mediated death pathway in a human microvascular endothelial cell line, HMEC-1. We show that the LPS-induced apoptosis in endothelial cells is mediated through FADD. However, the LPS death signal does not appear to be transduced by any of the known FADD-interacting transmembrane receptors. These findings suggest that the LPS death signal may be transmitted by a novel DD-containing transmembrane receptor.

MATERIALS AND METHODS

Reagents—LPS (Escherichia coli 0111:B4) and isotype control antibodies were purchased from Sigma. TNF was purchased from R & D Systems. C2-ceramide and anti-PARP antibody was obtained from Biomol. Neutralizing anti-TNFR1 antibody was obtained from Bender MedSystems, and anti-Fas antibody was from MBL Co. Ltd. Anti-CD14 antibody was a gift of R. Todd. AU1 antibody and anti-Myc antibody was obtained from Babco. ZVAD-fmk was purchased from Kamiya. The
horseradish peroxidase-conjugated secondary antibodies used were purchased from Bio-Rad.

**Cell Culture**—The human dermal microvascular cell line, HMEC-1 (29), was cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 20 μg/ml bovine brain extract (Sigma). The PA317 and PES01 packaging lines (30) (provided by A. D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA) and BHK cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. All cells were maintained at 37 °C in 5% CO₂.

**Gene Transfer**—The AU1-FADD-DN cDNA was ligated into the HindIII/Hpal I sites of the replication-deficient retroviral vector, pLNCX (31). The viral long terminal repeat drives expression of neoR, whereas the cytomegalovirus promoter drives transgene expression in pLNCX. Generation of retroviral producer cell lines was performed as described (31). The pLNCX-FADD-DN construct or pLNCX construct was transiently transfected into the ecotropic packaging line, PES01, by calcium-phosphate precipitation. Viral supernatants were harvested and used to transduce the amphotropic line PA317 in the presence of 4 μg/ml Polybrene. Retroviral producer cell lines were obtained by selection in 1 mg/ml G418 (Life Technologies, Inc.). Retroviral supernatants from the PA317 cell lines were used to transduce HMEC-1 cells. Following selection in 200 μg/ml G418 and expansion, HMEC-1 cells were used in survival studies. Polyclonal HMEC-1 lines were used in order to avoid artifacts due to retroviral integration.

To generate stable Myc-tagged DR3 lines, 2 × 10⁶ BHK cells were cotransfected with 2 μg of the expression vector encoding a human Myc-DR3 fusion construct (or the empty vector pSecTagA) and 300 ng of an expression vector encoding human dihydrofolate reductase, using LipofectAMINE. Stable transformants were selected in 1 μM methotrexate in Dulbecco's medium supplemented with 5% dialyzed fetal bovine serum. After 10–12 days, individual clones were isolated and expanded or pooled to generate polyclonal lines.

**Western Blotting**—Total cellular extracts from the transduced cells were prepared by lysing cells in 20 mM Tris, 140 mM NaCl, 1% Triton X-100, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Protein from 1 × 10⁶ cells was fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes over 1 h at 4 °C. Filters were blocked for 2 h with Tris-buffered saline (TBS) containing 5% skim milk. Immunostaining steps were performed in TBS with 0.05% Tween 20 and 3% bovine serum albumin at room temperature. Filters were incubated with primary and secondary antibodies for 1 h each. Filters were washed in TBS and 0.05% Tween 20 four times for 10 min between each step and were developed by chemiluminescence. Cleavage of PARP was demonstrated by immunoblotting with monoclonal antibody, C-2-10, as described previously (32).

**Viability Assay**—For viability assays, transduced or wild type HMEC-1 cells were seeded on 96-well plates at a density of 15,000 cells/well. The following day cells had reached confluence and were incubated for 15 h in the various conditions specified. When neutralizing antibodies were used, HMEC-1 cultures were pretreated with the relevant antibody for 1 h prior to LPS stimulation. Viable cell numbers were estimated by an assay using (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT) (33). Briefly, medium was removed and replaced with medium containing 1 mg/ml MTT (Sigma) and incubated for 5 h. The medium was then aspirated and the formazan product solubilized with dimethyl sulfoxide, and absorbance at 570 nm was measured for each well. Viability was expressed as a proportion of CHX-only-treated cells for LPS and TNF stimulation experiments. In the case of ceramide, viability was expressed as a proportion of vehicletreated cells.

**RESULTS AND DISCUSSION**

LPS Activates a Caspase-mediated Death Pathway in Endothelial Cells—The endothelium, by virtue of its location between blood and tissue, plays a central role in inflammatory and infectious processes (5). The integrity of the endothelium is crucial during sepsis, and endothelial death and loss of its barrier function may play a key role in the pathogenesis of septic shock (2). As with TNF, LPS does not induce death of human endothelial cells unless new gene expression is blocked (5, 31, 34–36). Although, LPS in the presence of CHX has been demonstrated to cause toxicity of human endothelial cells, it has not been shown whether a caspase pathway is engaged (5). A commonly used assay to show that cysteine proteases of the caspase family are activated is the demonstration of cleavage of the nuclear substrate, poly(ADP-ribose) polymerase (PARP) (37). When HMEC-1 microvascular endothelial cells are exposed to LPS (100 ng/ml) and cycloheximide (CHX) (50 μg/ml), there is cleavage of PARP to an 85-kDa form as demonstrated with other inducers of endothelial apoptosis (32). As shown in Fig. 1A, there is a time-dependent increase in the cleaved form of PARP over a 12-h period, following exposure to LPS and CHX. Furthermore, the cell-permeable tripeptide caspase inhibitor, ZVAD-fmk, is able to abrogate LPS-triggered death in a dose-dependent fashion (Fig. 1B). These findings indicate that in the presence of CHX, LPS activates a caspase-mediated apoptotic pathway in human endothelial cells.

The LPS-mediated Apoptotic Pathway Is Dependent on FADD—Many of the signaling pathways activated by LPS are shared by TNF (3, 6). Thus we attempted to determine whether LPS shared the proximal signaling molecule of the TNF death pathway. It has previously been shown in other cell types that the DD-containing cytoplasmic protein, FADD, is the central adaptor molecule utilized in transmitting the death signal by TNFR1 and Fas (20). However, not all receptor-mediated apoptotic signals are transduced by FADD. Of note, death mediated by at least one of the TRAIL receptors, members of the TNFR family, occurs independently of FADD (27). It has been shown that truncation of the N terminus of FADD results in a dominant negative (DN) cDNA. HMEC-1 cells were transduced either with the FADD-DN construct or the empty vector. To avoid artifacts due to integration site, polyclonal cell lines were used in all experiments. As demonstrated in Fig. 2A, overexpression of FADD-DN in HMEC-1 cells (HMEC-FADD-DN) protects these cells from death induced by LPS as compared with cells transduced with the empty vector (HMEC-Neo). As a control, we confirmed that HMEC-FADD-DN cells were also protected from TNF-mediated death (Fig. 2B). These results indicate that LPS activates a FADD-dependent apoptotic pathway.

To confirm that HMEC-FADD-DN cells were not protected from apoptosis in a nonspecific manner, these cells were also induced to undergo apoptosis by exposure to ceramide (31). Fig. 2C demonstrates that both HMEC-FADD-DN and HMEC-Neo cells were killed to a similar extent by ceramide. This finding is
important for two reasons. First, it demonstrates that FADD-DN does not protect HMEC-1 cells from apoptosis indiscriminately. Second, Joseph and colleagues (12) have shown that although LPS does not cause sphingomyelin hydrolysis, it can stimulate ceramide-activated protein kinase. Molecular modeling showed strong structural similarity between ceramide and a region of the bioactive moiety of LPS, lipid A, prompting the suggestion that LPS can signal by mimicking the second messenger activity of ceramide (12). Our findings suggest that the death activity of LPS is not mediated by molecular mimicry of ceramide but rather by a DD-containing receptor-mediated pathway. Staurosporine-initiated death of HMEC-1 cells was also not blocked by FADD-DN (data not shown).

The LPS-mediated Death Pathway Is Independent of TNFR1, Fas, or DR3—To determine whether LPS might utilize TNFR1 or Fas to transduce the apoptotic signal, neutralizing antibodies were used against these receptors (Fig. 3A). As previous studies had suggested that Fas ligation does not induce apoptosis in endothelial cells (38), we did not expect that an anti-Fas antibody would block LPS-initiated death. As expected, at concentrations that were effective in abrogating Fas-induced vascular smooth muscle death (data not shown), an anti-Fas neutralizing antibody (1 μg/ml) did not abrogate LPS-initiated death. Similarly, an anti-TNFR1 neutralizing antibody (5 μg/ml) did not block LPS-induced death. In contrast, LPS-mediated death was inhibited by an anti-CD14 neutralizing antibody, confirming a previous report demonstrating the

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requirement of soluble CD14 in LPS-mediated endothelial injury (39). In all cases an isotype-matched control antibody had no effect on cell viability (Fig. 3A).

Since TNF can kill HMEC-1 cells when the expression of new genes is inhibited (31), it was important to verify that TNFR1 was not involved in LPS-signaled death. To this end, HMEC-1 cells were exposed to various concentrations of LPS in the presence or absence of TNF (10 ng/ml). As seen in Fig. 3B, the presence of TNF elicits a synergistic decrease in viability of LPS-stimulated HMEC-1 cells. As shown in Fig. 2B, TNF-mediated HMEC-1 death is abrogated by FADD-DN. Others have shown that the FADD-mediated death pathway is the dominant death pathway engaged by TNF (28, 40). Thus, although both TNF and LPS can activate death via FADD, the synergistic increase in cell death by TNF and LPS suggests that the signaling molecules upstream of FADD are distinct for each pathway.

Recently, a novel member of the TNFR family, DR3/wsl-1, was cloned independently by several groups. DR3-dependent death is also signaled by FADD, since FADD-DN blocks DR3-triggered apoptosis. DR3 is not expressed in vascular tissues, however, whereas DR3-transfected cells were more sensitive to death is also signaled by FADD, since FADD-DN blocks DR3-dependent death. We postulated that if DR3 were involved in the LPS death signal that overexpression of this molecule would sensitize BHK cells to the effect of LPS. How-

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