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Neru Munshi, Aaron Z. Fernandis, Rama P. Cherla, In-Woo Park and Ramesh K. Ganju

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*Lipopolysaccharide-Induced Apoptosis of Endothelial Cells and Its Inhibition by Vascular Endothelial Growth Factor*

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Lipopolysaccharide-Induced Apoptosis of Endothelial Cells and Its Inhibition by Vascular Endothelial Growth Factor

Neru Munshi, Aaron Z. Fernandez, Rama P. Cherla, In-Woo Park, and Ramesh K. Ganju

Endothelial injury is a major manifestation of septic shock induced by LPS. Recently, LPS was shown to induce apoptosis in different types of endothelial cells. In this study, we observed that pretreatment with vascular endothelial growth factor (VEGF), a known cell survival factor, blocked LPS-induced apoptosis in endothelial cells. We then further defined this LPS-induced apoptotic pathway and its inhibition by VEGF. We found that LPS treatment increased caspase-3 and caspase-1 activities and induced the cleavage of focal adhesion kinase. LPS also augmented expression of the pro-apoptotic protein Bax and the tumor suppressor gene p53. The pro-apoptotic Bax was found to translocate to the mitochondria from the cytosol following stimulation with LPS. Pretreatment of endothelial cells with VEGF inhibited the induction of both Bax and p53 as well as the activation of caspase-3. These data suggest that VEGF inhibits LPS-induced endothelial apoptosis by blocking pathways that lead to caspase activation. The Journal of Immunology, 2002, 168: 5860–5866.

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he vascular and pulmonary endothelia are critical components within the circulatory and alveolar compartments. Damage to large vessel and microvascular endothelium is an important event in many forms of heart and lung injuries (1–3). LPS, a complex glycoprotein constituent of the outer cell wall of Gram-negative bacteria, has been implicated in endothelial injury that leads to septic shock and its associated syndromes (4–6).

Recent studies have shown that LPS induces apoptosis in different types of endothelium, including HUVEC and lung-derived normal human microvascular endothelial cells (6–15). Previous studies have also reported that release of LPS into the circulation induces endothelial apoptosis in vivo and thus causes microvascular injury in numerous tissues, including lung, gut, and liver, during sepsis (4–5). LPS administration has also been shown to cause apoptosis in B cells (16), CD4+8+ T lymphocytes, and lymphoid organs (17). Enhanced apoptotic cell death has also been shown in various tissues derived from patients who have died due to sepsis or multiorgan failure. Apoptotic endothelial cells have also been detected in murine models of sepsis (4, 18).

In the present studies, we observed that vascular endothelial growth factor (VEGF) pretreatment of HUVEC protected the cells against LPS-induced apoptosis. VEGF treatment could therefore act as a potential therapeutic and counteractive strategy that might protect vascular integrity against LPS-induced damage. VEGF has been shown to be a critical mediator of angiogenesis, growth, vascular permeability, and cell migration (19–23). VEGF, which exhibits its biological effects by binding to VEGF receptor 1 (Flt-1) and VEGF receptor 2 (Flk-1/KDR) (24, 25), has also been shown to act as a survival factor for endothelium (26–29). The survival effects of VEGF appear to be mediated through the expression of the anti-apoptotic proteins A1 and Bcl-2 (30) and via activation of the AKT/PKB pathway (31). AKT, upon activation, phosphorylates and inactivates components of the apoptotic machinery, including Bad and caspase-9 (32–33). Recently, Brunet et al. (34) found that AKT also regulates the activity of PKHRL-1, a member of the forkhead family of transcription factors.

The molecular pathways of apoptosis in endothelial cells are only just being deciphered (7, 35). Choi et al. (7) recently reported that LPS induced apoptosis in microdermal endothelial cells via recruitment of the adaptor Fas-associated death domain. Administration of a broad-spectrum caspase inhibitor in mice was shown to decrease LPS-induced endothelial cell apoptosis in the lung, resulting in a higher survival rate (36). In this study, we further characterized the mechanisms of LPS-induced endothelial apoptosis. We observed that caspase-1, caspase-3, pro-apoptotic Bax, and the tumor suppressor gene p53 are induced upon LPS treatment.

Activation of caspases is modulated by several mechanisms (37–39). The most studied mechanism is caspase regulation by two families of downstream mediators, the anti-apoptotic Bcl-2 family and the pro-apoptotic Bax family (39). Recently, several studies have shown that the tumor suppressor gene p53, which is known to participate in cell death in response to a variety of stimuli, also regulates caspase-mediated apoptotic mechanisms (40–43). However, the signaling pathways whereby p53 activates caspases remain somewhat uncharacterized. Transcriptional activation of Bax and caspase-1 has been suggested as one of the possible mechanisms (43–45). It has been proposed that the p53-mediated activation of Bax may trigger its translocation to the mitochondria where it leads to a decline in mitochondrial membrane potential, followed by the cytosolic release of cytochrome c (44–46). In turn, cytochrome c might amplify the apoptotic signaling by activating various caspases. In this report, we have investigated the mechanism of LPS-induced endothelial apoptosis and have shown that p53, Bax, caspase-1, and caspase-3 may participate in this process. Furthermore, our studies reveal that VEGF treatment may protect endothelial cells against LPS-induced apoptosis.
Materials and Methods

Cell culture

HUVECs were purchased from Clonetics (San Diego, CA). Cells were grown at 37°C in 5% CO2 in endothelial growth medium (EGM-2-MV) containing 2% FBS, 12 μg/ml bovine brain extract, 10 ng/ml human recombinant epidermal growth factor, 1 μg/ml hydrocortisone, GA-1000 (gentamicin and amphotericin B, 1 μg/ml), according to the recommendations of the supplier.

Reagents

LPS (Escherichia coli 0111:B4) and the protease inhibitors aprotinin, leupeptin, and pepstatin, as well as the trypsin inhibitor, were obtained from Sigma-Aldrich (St. Louis, MO). Abs for Bax, Bcl-2, and Bcl-xL were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the caspase-1, caspase-8, and caspase-9 substrates were from Calbiochem (San Diego, CA). The paxillin and p53 Abs were obtained from Upstate Biotechnology (Lake Placid, NY). The focal adhesion kinase (FAK) Ab was a gift from Dr. H. Avraham (Beth Israel Deaconess Medical Center, Boston, MA). The HRP-conjugated secondary Abs were purchased from Bio-Rad (Hercules, CA). Recombinant VEGF was obtained from Genentech (South San Francisco, CA). The caspase inhibitors and the caspase substrate were purchased from Enzyme System Products (Livermore, CA). Electrophoresis reagents and nitrocellulose membrane were obtained from Bio-Rad.

Sandwich ELISA for histone-associated DNA fragments

Endothelial cell death was assessed by ELISA using a death detection kit from Boehringer Mannheim (Indianapolis, IN). HUVECs were plated at 2 × 104 per well in a flat-bottom 96-well assay plate, and the cells were grown to 90% confluence. The cells were then treated with VEGF (100 ng/ml) for 2 h in endothelial growth medium containing 0.5% FCS. Controls consisted of cells in the low serum (0.5%) medium without VEGF. The cells were then incubated with 100 ng/ml LPS for 24 h at 37°C. At 24 h, the cells were harvested in lysis buffer, and the cytoplasmic and nuclear fractions were separated by centrifugation at 200 × g. Twenty microliters of supernatant (cytoplasmic fraction) was added to a streptavidin-coated microtiter plate. Biotin-labeled anti-histone Ab was added, followed by HRP-conjugated anti-DNA Ab. Photometric analysis of the colorimetric reaction produced between the peroxidase and substrate (2,2'-azino-di-[3-ethyl-thiazolium-sulfonate]) permitted quantification of the bound nucleosome DNA fragments. The fold increase in nucleosome degradation was calculated by comparing the values with that of the serum-starved cells not treated with LPS. Statistical analysis was done by using the Student’s t test.

Caspase inhibitor assay

HUVECs were grown in 96-well plates. At confluence, the cells were serum starved or treated with caspase inhibitors for 4 h. The caspase inhibitors used were: the broad-spectrum cell permeable caspase inhibitor, Z-valine-alanine-aspartate-fluoromethyl ketone (Z-VAD-FMK) and the specific caspase-3 inhibitor, Z-Asp-Glu-Val-Ala-Asp-fluoromethyl ketone (Z-DEVD-FMK). Both of these inhibitors were used at 20 μM concentrations. Z-phenoxyalanine-alanine-fluoromethyl ketone (Z-FA-FMK) at 20 μM was used as a control for the general inhibitor, as the inhibitor sequence (VAD) is replaced by FA and therefore does not cause the inhibition of caspase activity. DMSO was used as a diluent control. The assays were done in duplicates and were repeated three times.

TUNEL

The level of chromatin cleavage due to apoptosis in HUVEC was quantified by using the Fluorescein In Situ Cell Death Detection kit (Boehringer Mannheim). Briefly, HUVECs were plated in 75-cm2 flasks (Corning Glass, Corning, NY) and grown to 90% confluence. The cells were then subjected to low serum treatment with or without 100 ng/ml VEGF for 4 h. This treatment was followed by stimulation with 100 ng/ml LPS for 24 h as described above. At 24 h, the treated cells were removed from the tissue culture by a gentle scraping, centrifuged, washed with 1× PBS, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. The cells were then washed twice with 1× PBS, resuspended in TUNEL reaction mixture or in Label solution as a negative control, incubated for 60 min at 37°C in humidified atmosphere in the dark, washed twice with PBS, and analyzed by flow cytometry or visualized under a fluorescence microscope.

Caspase activity

To determine the activity of caspase-3, the cells were grown in a 24-well plate, then serum starved and stimulated as described above. Cells were scraped in PBS containing 0.05% Triton X-100 and lysed by three freeze-thaw cycles in a dry ice/ethanol bath. The lysates were centrifuged for 5 min at maximum speed and 50 μl of the supernatant was added to 495 μl assay buffer containing 0.1 M HEPES (pH 7.4), 2 mM DTT, 0.1% 3-[3-cholamidopropyl(dimethylammonio) -l-propanesulfonate (CHAPS), and 1% sucrose. The peptide substrate for caspase-3, Ac-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC), was then added at a final concentration of 20 μM. The reaction was allowed to proceed for 30 min at room temperature. The release of AFC was measured by using a fluorometer set at 400 nm excitation and 505 nm emission. A standard curve was generated with free AFC. The specific activity was determined by analyzing the protein concentration of each sample using a protein quantification method supplied by Bio-Rad. The specific activity of caspase-3 was determined by comparing the results of the LPS-treated samples with that of the serum-starved controls.

For the caspase-1, caspase-8, and caspase-9 assays, the cells were grown and stimulated as described above, then scraped in cell lysis buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, 10% glycerol, and 0.2 mM of the pNA-conjugated substrate. The substrates used for the caspase-1, caspase-8, and caspase-9 assays were acetyl-Tyr-Val-Asp-p-nitroanilide, acetyl-Ile-Glu-Thr-Asp-p-nitroanilide, and acetyl-Leu-Glu-His-Asp-p-nitroanilide, respectively. The assays were conducted at 37°C for 1 h and the colored product was read at 405 nm. The specific activity of each caspase was calculated from a standard graph generated using free pNA.

Isolation of cytosolic and mitochondrial fractions

The cytosolic and mitochondrial fractions were extracted as described elsewhere (47).

Cytosolic fractions. The cells were harvested and washed with 1× PBS, then lysed in 100 μl of lysis buffer (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 250 mM sucrose). The lysed cell pellet was homogenized (five strokes) and spun at 1000 rpm for 5 min. The resulting supernatant was centrifuged at 50,000 rpm for 30 min at 4°C and was then used as the soluble cytosolic fraction.

Mitochondrial fractions. Briefly, after treatment with LPS, cells were harvested and spun at 5000 rpm for 5 min. The cell pellet was washed with 1× PBS and resuspended in 2.5 ml of H-medium (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, and 1 mM EGTA). Five microliters of digitonin (1 M) was added and the cells were homogenized (seven strokes) in a glass homogenizer. The cells were spun at 4000 rpm for 5 min and the supernatant was saved. The pellet was then resuspended two to three times in H-medium and respun. The supernatants were collected and centrifuged at 9800 × g for 10 min, and the pellet was dissolved in lysis buffer (1% Nonidet P-40, 0.1% deoxycholate, 0.05% SDS, 0.1 mM PMSF, and 10 μg/ml each of leupeptin, aprotinin, and pepstatin).

Western blotting

Total cellular extracts from the LPS-treated cells were prepared by lysing the cells in radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin, leupeptin, and pepstatin, 10 mM sodium vanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate). Proteins were size fractionated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked for 2–3 h with 5% nonfat milk and then incubated with the respective primary and secondary Abs for 2–3 h each. The membranes were washed three to four times for 15 min each with TBS and 0.05% Tween 20, and later developed by chemiluminescence (ECL System; Amersham Pharmacia Biotech, Piscataway, NJ). The densitometric scanning of films was done by using Bio-Rad model GS–700 or Alpha Imager 2000 (Alpha Annotate, San Leandro, CA) image densitometers. Relative density values were calculated by densitometric scanning of Bax and p53 and then by dividing the values by their corresponding paxillin density values for each time period. The values shown are an average of two independent experiments.
Results

LPS has been shown to cause endothelial injury by inducing apoptosis (3–7). However, the molecular mechanisms of this effect are not well understood. In the present studies, we characterized apoptotic signaling molecules activated upon LPS treatment of HUVEC. Furthermore, we studied the effects of VEGF, a critical cell survival factor and angiogenesis mediator, on LPS-induced apoptosis.

**VEGF pretreatment protects cells against LPS-induced apoptosis**

LPS has been shown to induce the apoptosis of HMVEC-L in the presence of cycloheximide. In this study, we used a low serum (0.5%) concentration. LPS treatment under these conditions led to a significant induction of apoptosis in HUVEC (data not shown). Next, we sought to determine whether VEGF, an endothelial mitogen that has been shown to block apoptosis of HUVEC upon serum starvation and TNF-α treatment (27, 28), could also modulate LPS-induced apoptosis. As shown in Fig. 1A, LPS was found to induce apoptosis over a concentration range of 10–1000 ng/ml. Furthermore, VEGF pretreatment of HUVEC resulted in an inhibition of the LPS-induced apoptosis. VEGF treatment at various concentrations revealed that 10 ng/ml VEGF was sufficient to block endothelial cell death initiated by LPS at 100 ng/ml. However, VEGF pretreatment was less protective against apoptosis induced by higher concentrations of LPS (1000 ng/ml). To further confirm the apoptotic inhibitory effect of VEGF observed using the nucleosome ELISA (Fig. 1A), we used the TUNEL method. As shown in Fig. 1, Bb and C, in the presence of LPS (100 ng/ml) at 24 h, ~50% of the cells were TUNEL positive. However, upon VEGF pretreatment, only 10–15% of the cells were found to be TUNEL positive at 24 h of LPS treatment (Fig. 1, Bc and C). VEGF, alone or with low serum, resulted in around 5–10% TUNEL-positive cells, respectively (Fig. 1, Bd and C). Similar results were obtained by analyzing TUNEL-positive cells by FACS analysis (data not shown).

**LPS activates caspase-3 and caspase-1**

LPS has been reported to activate caspase-mediated death signaling pathways in endothelial cells (7). Caspases are a large family of proteases (37, 38), and the specific caspase family members activated by LPS have not previously been well characterized. HUVEC were treated with LPS in the presence of either a broad-spectrum caspase pathway inhibitor or a specific caspase-3 pathway inhibitor, and the degree of apoptosis was assessed by ELISA. The general caspase inhibitor (GI) Z-VAD-FMK, at a concentration of 20 μM, markedly reduced the degree of apoptosis. However, use of the inhibitor control (IC) Z-FA-FMK, at the same concentration, had no effect on apoptosis. This inhibitor was used as the control because its caspase inhibitor sequence (VAD) is replaced by FA, thereby yielding a specific inhibitory effect on cysteine proteases such as cathepsin B, but no effect on caspase activity. Further use of different caspase inhibitors with LPS pointed toward the involvement of a caspase-3 pathway. As shown in Fig. 2A, the specific caspase-3 inhibitor (C-3I), Z-DEVD-FMK (20 μM), inhibited the LPS-induced apoptosis. The diluent control, DMSO, had no such abrogating effect. The role of caspase-3 was further assessed by measuring its enzymatic activity following LPS treatment by using a specific caspase-3 substrate, Ac-DEVD-AFC. This activity was quantified by measuring the release of AFC. As shown in Fig. 2B, the caspase activity increased over time (from 3 to 12 h) after LPS treatment. Low levels of caspase-3 induction were observed under the control conditions. However, the addition of LPS increased caspase-3 activity by 1.7-fold at 12 h (p < 0.05).

Caspase-3 is present as an inactive form, pro-caspase 3 (32 kDa), and upon activation is cleaved to a 17-kDa active form. To confirm this enhanced enzymatic activity of caspase-3, HUEVC were serum starved for 4 h, treated with LPS (100 ng/ml) for various time periods, and then lysed. The cell lysates were immuno blotted with anti-caspase-3 Ab. As is evident from Fig. 2C, LPS treatment induced the cleavage of caspase-3 to a pro-active 17-kDa form at 12 h, further indicating that LPS exerts its apoptotic effect by specifically activating caspase-3.

We also determined the effect of LPS on caspase-1, caspase-8, and caspase-9 activities, which have been shown to play an important role in the apoptosis induced by various stimuli (37, 38). As shown in Fig. 2D, LPS treatment resulted in the enhancement of caspase-1 activity by 12 h. However, no significant effect on caspase-8 or caspase-9 activity was observed under similar conditions (Fig. 2, E and F). Furthermore, we did not find any active forms of these caspases upon Western blot analysis (data not shown).

**FIGURE 1.** VEGF inhibits LPS-induced apoptosis. Apoptosis was assessed by either sandwich ELISA for histone-associated DNA fragments (A) or the TUNEL method (B). For ELISA, HUVEC were grown in a 96-well assay plate. The cells were serum starved with or without VEGF (100 ng/ml) for 4 h before LPS (10–1000 ng/ml) stimulation for 24 h. At 24 h, the cells were centrifuged at 3000 rpm for 10 min, the supernatant was aspirated off, and the cell pellets were lysed. The lysates were analyzed for nucleosome degradation as described in Materials and Methods. *p < 0.05. B, For the TUNEL method, cells were transferred to endothelial growth medium containing 0.5% serum for 4 h and were untreated (a), treated with LPS (100 ng/ml) (b), or VEGF (100 ng/ml) (c), or pretreated with VEGF (100 ng/ml) followed by LPS treatment (100 ng/ml) (d) for 24 h. The cells with green fluorescence represent apoptotic cells. C, Graph represents TUNEL assay results obtained from three independent experiments.
FIGURE 2. HUVEC apoptosis by LPS is mediated by caspase-3. A, HUVEC were serum starved (0.5% FBS), then untreated (Control) or preincubated with caspase inhibitors for 4 h. Next, LPS (100 ng/ml) was added to the cells along with either the general caspase pathway inhibitor (GI) Z-DEVD-FMK, inhibitor control (IC) Z-FA-FMK, or a specific caspase-3 pathway inhibitor (C-3) Z-DEVD-FMK for 24 h at a concentration of 20 μM. At 24 h, apoptosis was assessed by an ELISA nucleosome method as described in Materials and Methods. *, p < 0.001; **, p < 0.0005. B, HUVEC were serum starved for 4 h and then treated with LPS (100 ng/ml). Cell lysates were harvested at various time points, and caspase-3 activity was assessed by using Ac-DEVD-AFC as a substrate at 20 μM concentration. The reaction was allowed to proceed at room temperature. The release of AFC was measured at 400 nm excitation and 505 nm emission.

LPS induces FAK degradation
To further establish the role of caspase-3 in LPS-induced apoptosis, we investigated the involvement of FAK. FAK is an important nonreceptor protein tyrosine kinase activated in several signal transduction events in multiple cell types. These signaling processes lead to cell survival, proliferation, and cell migration (48). FAK is a component of focal adhesions, which consist of complete assemblies of cytoskeletal proteins. It has been demonstrated to be cleaved by caspase-3 at two distinct sites during apoptosis (49–52). As shown in Fig. 3A, we observed FAK degradation upon LPS treatment. Reduced amounts of FAK were present in the LPS-treated samples as compared with the untreated samples. The results were further confirmed by confocal microscopy. Lower FAK content (Fig. 3Bb) was observed in cells treated with LPS as compared with the untreated cells (Fig. 3Ba).

LPS modulates the expression of apoptotic regulatory molecules
Pathways of programmed cell death are mediated by the pro-apoptotic Bax family and anti-apoptotic Bcl-2 family of proteins (39). LPS has been shown to trigger the concurrent activation of pro-apoptotic and anti-apoptotic pathways (35, 53, 54). To further explore the mechanisms of endothelial apoptosis mediated by LPS, we studied the expression of the pro-apoptotic protein, Bax. This protein has been found to be the predominant pro-apoptotic family member present in HUVEC (55). As shown in Fig. 4A, cell lysates were analyzed by Western blotting with anti-Bax Ab (top panel); anti-paxillin Ab (bottom panel) was used to quantitate the amount of protein. The expression of Bax was detected by 6 h after the addition of LPS. Quantitative analysis revealed that LPS-induced Bax expression was ~1.4-fold higher at 3 h and ~2.2-fold higher at 6 h than its expression in the serum-starved control samples. We also observed a slight decrease in the expression of the anti-apoptotic molecule Bcl-2 over an increased time course of exposure to LPS (Fig. 4C, top panel). Equal amounts of protein were shown to be present upon reprobing the blot with paxillin Ab (Fig. 4C, bottom panel). Of note, the increase in Bax expression or decrease in Bcl-2 expression was not paralleled by any change in expression of the other predominant Bcl-2 family member, Bcl-xL. The Bcl-xL protein levels remained constant over 6–12 h of LPS stimulation (data not shown), indicating that LPS may induce apoptosis in endothelial cells in part by activating certain pro-apoptotic family members.

Apoptotic stimuli are known to cause the translocation of cytosolic pro-apoptotic Bax to mitochondrial membranes (44, 45). Fig. 4B depicts the localization of Bax upon treatment with LPS. HUVEC were treated with LPS (100 ng/ml) for 24 h and the cytosolic and mitochondrial fractions were separated as described in Materials and Methods. At 24 h, Western blot analysis showed that the Bax levels were clearly higher in the mitochondrial than in the cytosolic fractions.
VEGF INHIBITS LPS-INDUCED ENDOTHELIAL APOPTOSIS

We next investigated the mechanisms of how VEGF might protect HUVEC from LPS-mediated apoptosis. We observed that VEGF pretreatment blocked the induction of p53 (Fig. 6A) and Bax (Fig. 6B) expression by LPS. Equal amounts of protein were present in each lane, as detected by blotting with anti-paxillin Ab (Fig. 6, A and B, bottom panels). VEGF pretreatment also inhibited the LPS-induced cleavage of caspase-3 to its proactive form (Fig. 6C) as well as the degradation of FAK (Fig. 6D). These data suggest that VEGF may counteract LPS-induced endothelial cell apoptosis by blocking p53 and Bax induction as well as caspase activation.

Discussion

LPS has been shown to contribute to the damage observed in various types of endothelium (3–7, 11). Recent studies have shown that LPS induces apoptosis in vitro and in vivo, a condition that may lead to many diseases including septic shock and its associated syndromes (3–7, 9–11). However, the molecular mechanisms of LPS-induced apoptosis are not well characterized. In the present studies, we have explored LPS-induced apoptotic signaling mechanisms and have shown that VEGF pretreatment protects endothelium against LPS-induced apoptosis.

We have shown that LPS induces caspase-3 and caspase-1 activities in HUVEC. Similarly, in bovine glomerular endothelial cells, LPS has been demonstrated to enhance caspase-3 activity (15). LPS has also been shown to activate caspase-1 in monocytes and endothelium (59). Interestingly, it was reported that knockout mice lacking caspase-1 exhibit resistance toward the induction of septic shock and show a partial defect in apoptosis (36). Furthermore, we observed the cleavage of FAK, a substrate of caspase-3 that has been found to be important for the assembly and disassembly of focal adhesion contacts. FAK consists of an N-terminal domain, kinase domain and focal adhesion targeting (FAT) domain in the C-terminal half of the protein. It has been demonstrated that cleavage of FAK by caspase results in separation of the kinase domain from the FAT domain (51, 60–61). The FAT domain-containing fragment known as FAK-related nonkinase has been shown to inhibit FAK activity and acts as a competitive inhibitor of full-length FAK. Therefore, a decrease in the total amount of FAK and its activity leads to inhibition of the survival promoting activity of FAK and to enhancement of apoptosis.

In addition to caspase-3 and caspase-1, our data also indicate the involvement of p53 and Bax in the LPS-induced apoptosis of endothelial cells. We observed an increase in p53 and Bax expression...
and the translocation of Bax to the mitochondria. It has been demonstrated that p53 is required for apoptosis in various cell types and that one of the possible mechanisms for this cell death is transcriptional activation of the pro-apoptotic Bax (44, 45). Activation of Bax in turn leads to its translocation to the mitochondria, where it promotes the release of cytochrome c from the mitochondrial intermembrane space. Cytochrome c release facilitates activation of the effector caspases, which then cleave their substrates, leading to apoptotic cell death (40–42, 44). In our experiments, LPS treatment of HUV-EC led to an induction of p53 and Bax expression by 3–6 h. However, significant caspase-3 activation was observed only after 6–12 h. These results indicate that p53 is possibly required for the activation of Bax, which in turn, via the release of cytochrome c, may lead to the induction of caspases.

We also observed that VEGF has the potential to inhibit endothelial cell apoptosis initiated by LPS. VEGF has previously been found to act as a survival factor for endothelium. Anti-apoptotic proteins A1, Bcl-2, phosphatidylinositol 3-kinase, and AKT/PKB have previously been shown to mediate the survival effects of VEGF (27, 30, 31). AKT inhibits apoptosis by phosphorylating and inactivating components of the apoptotic machinery, such as Bad and caspase-9 (32, 33), which in turn regulate the activation of other caspases. We observed the inhibition of p53 and Bax induction, caspase-3 activation, and FAK degradation upon pretreatment of cells with VEGF. VEGF has been shown to activate FAK, which in turn promotes cell survival pathways (62, 63) known to antagonize p53-mediated apoptosis (64, 65).

Taken together, our studies suggest that expression of Bax and its translocation to the mitochondria may provide a link between the p53 expression and caspase-3 activation observed in LPS-treated endothelial cells. Additional studies are required to confirm this possibility. Elucidating the molecular mechanisms of endothelial cell apoptosis induced by LPS may lead to the development of novel strategies for the treatment of septic shock. In this regard, our findings also provides a rationale for studying the application of VEGF therapy in sepsis and other related syndromes.

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References


