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Molecular Mechanisms in Peptidoglycan-Induced Human Umbilical Vascular Endothelial Cell Apoptosis

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ABSTRACT

Peptidoglycan (PGN), a component of the outer membrane of Gram-positive bacteria has been implicated in the pathological process of sepsis. However, the molecular mechanism of PGN-induced vascular endothelium dysfunction has not been fully elucidated. Apoptosis signal regulating kinase 1 (ASK1) has been recently reported to play a crucial role in cell apoptosis under various cellular stresses. The purpose of this study was thus to investigate whether PGN-activated ASK1 results in cell apoptosis in human umbilical vascular endothelial cells (HUVECs). PGN was shown to cause a decrease in cell viability in a concentration-dependent manner. Flow cytometric analysis demonstrated that PGN increased the percentage of apoptotic cells. PGN induced ASK1 activation was accompanied by the increased phosphorylation of p38MAPK, a major downstream signaling molecule of ASK1. In addition, PGN was shown to increase apoptotic protein, Bax, expression in HUVECs. Inhibitor of p38MAPK signaling abrogated the PGN-increased DNA fragmentation and Bax expression, suggesting functional crosstalk. The toll-like receptor 2 (TLR2) agonist, Pam3CSK4, was also shown to induce ASK1 activation and p38MAPK phosphorylation, and subsequent cell apoptosis in HUVECs. Our data suggest that ASK1-p38MAPK cascade activation, followed by Bax expression, contributes to PGN-induced cell apoptosis.

Key words: sepsis, vascular leak syndromes, peptidoglycan (PGN), ASK1 (Apoptosis signal regulating kinase 1)

INTRODUCTION

Sepsis and subsequent multiple organ dysfunction remain a leading cause of death among severely ill patients[1-3]. The occurrence of gram-negative sepsis has diminished over the years to about 25-30% while gram-positive infections accounted for 30-50%[4]. PGN, the main cell-wall component of gram-positive bacteria, is composed of alternating *N*-acetyl glycosamine and *N*-acetyl muramic acid long sugar chains that are interlinked by peptide bridges, resulting in a large, complex macromolecular structure[5]. PGN contributes to most of the clinical manifestations of gram-positive bacterial infections including inflammation, fever, and septic shock[4]. Many lines of evidence demonstrated that invading pathogens trigger sequential intracellular events in immune cells, epithelium and endothelium via pattern-recognition receptors (PRR)[6]. Among PRRs, the toll-like receptors (TLRs) play a crucial role in mediating inflammatory responses[7]. Endothelial dysfunction plays a causal role in the pathogenesis of sepsis[8]. In sepsis, the vascular endothelium barrier undergoes functional changes such as inflammatory cytokines release that contribute to vascular collapse and subsequent multiple organ failure[3,9]. PGN activates the innate immune system of a host through toll-like receptor (TLR)-2 and cluster of differentiation (CD)-14, leading to the generation of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6[10-12]. PGN was also shown to induce cyclooxygenase-2 expression in human polymorphonuclear leukocytes[13]. Although it is well known that bacterial products have multiple effects on the regulation of host defense and immune responses by macrophages, little is known about the actions of PGN on vascular endothelial cells.

Among the critical pathway mediating the inflammatory response is the mitogen-activated protein kinase (MAPK) cascade, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38MAPK[8,14]. JNK and p38MAPK have also been shown to regulate the expression of a variety of genes that contribute to cell apoptosis[15-17]. In addition, the B-cell lymphoma 2 (Bcl-2) family comprises proteins with antiapoptotic and proapoptotic function, which regulated the mitochondrial apoptotic pathway. Bax, a proapoptotic member of Bcl-2 family, plays an important role in promoting apoptosis[18]. We have

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previously demonstrated that p38MAPK-Bax signaling cascade contributed to the apoptotic paradigm of cerebral endothelial cells exposure to amyloid[17]. Apoptosis signal-regulating kinase 1 (ASK1) belongs to the MAPK kinase kinase family and activates the JNK and p38MAPK pathways via MAPK kinase (MKK)4/7 and MKK3/6, respectively[19]. ASK1 is a multifunctional serine/threonine protein kinase and has been recently highlighted to play a major role in regulating diverse physiological processes, including cell differentiation and apoptosis[17,20]. ASK1 has been reported to be activated by many stress signals or proinflammatory cytokines[21,22]. We recently demonstrated that PGN may activate ASK1 through TLR2 resulting in macrophage activation[10]. Into and Shibata[23] also demonstrated that ASK1 may contribute to the TLR2 signaling cascade. However, the TLR2 signaling in vascular endothelial cells exposed to PGN remains unknown. Furthermore, little information is available about the role of ASK1 in PGN actions in vascular endothelial cells. Therefore, we attempted to elucidate the role of ASK1 and MAPK signaling cascade in PGN-induced vascular endothelial cell dysfunction. In this study, we demonstrated that PGN caused cell apoptosis in human umbilical vascular endothelial cells (HUVECs). PGN induced ASK1 activation may occur through the induction of ASK1 Ser845 phosphorylation. In addition, PGN activation of ASK1 was accompanied by the increased phosphorylation of p38MAPK in HUVECs.

MATERIALS AND METHODS

I. *Reagents*

PGN purified from *Staphylococcus aureus* (*S. aureus*) was purchased from Fluka (Buchs, Switzerland). Selective TLR2 ligand, $Pam_3Cys\text{-}Ser\text{-}(Lys)_4$ (Pam_3CSK_4), p38 inhibitor III, was obtained from Calbiochem (San Diego, CA, USA). M199 medium, fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Antibodies specific for α-tubulin were purchased from Transduction Laboratories (Lexington, KY, USA). Anti-mouse and anti-rabbit immunoglobulin G (IgG)-conjugated alkaline phosphatase antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies specific for p38MAPK phosphorylated at Thr180/Tyr182, and ASK1 phosphorylated at Ser967 or Thr845 were purchased from Cell Signaling (Beverly, MA, USA). All materials for immunoblotting were purchased from Bio-Rad (Hercules, CA, USA) and GE Healthcare (Little Chalfont, UK). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

II. *Cell Culture*

HUVECs were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan), and cells were maintained in M199 containing vascular endothelial cell growth supplement (ECGS) (Millipore), 10% FBS, 5 U/mL heparin, 20 mM HEPES, 100 U/mL of penicillin G, and 100 μg/mL streptomycin in a humidified 37°C incubator.

III. *Cell Viability Assay*

Cell viability was measured by a previously described colorimetric MTT assay[24]. Briefly, cells (2×10^5 cells/well) were cultured in 24-well plates and incubated with vehicle or various concentrations of PGN or Pam3CSK4 for 48 h. After various treatments, 1 mg/mL MTT was added to the culture plates and incubated at 37°C for an additional 4 h. Then cells were lysed in 500 μL dimethyl sulfoxide. The absorbance at 550 nm was measured on a microplate reader

IV. *Flow Cytometric Analysis*

HUVECs were cultured in 6 cm dishes. After reaching confluence, cells were treated with vehicle, PGN or Pam3CSK4. At the end of the experiments, cells were washed twice with PBS (in mM: 137 NaCl, 2.7 KCl, 4.3 Na_2HPO_4, and 1.5 KH_2PO_4, pH 7.4) and resuspended in ice-cold 70% ethanol at 0°C overnight. Cells were washed for 5 min with 0.4 mL phosphate-citric acid buffer, pH 7.8, containing 50 mM Na_2HPO_4, 25 mM citric acid, and 0.1% Triton X-100 and subsequently stained with 1.5 mL of propidium iodide (PI) staining buffer containing 0.1% Triton X-100, 10 mM PIPES, 100 mM NaCl, 2 mM $MgCl_2$, 100 μg/mL RNase A, and 50 μg/mL PI for 30 min in the dark before flow cytometric analysis. Cells were filtered on a nylon mesh filter. The samples were analyzed by the FACS CantoII and FACS Diva program (BD Biosciences, San Jose, CA, USA). Each experiment was repeated at least three times.

V. *Immunoblot Analysis*

Immunoblot analyses were performed as described previously[17]. Briefly, cells were lysed in extraction buffer containing 10 mM Tris (pH 7.0), 140 mM NaCl, 2 mM PMSF, 5 mM DTT, 0.5% NP-40, 0.05 mM pepstatin A, and 0.2 mM leupeptin. Samples of equal amounts of protein were subjected to SDS-PAGE and transferred onto a PVDF membrane which was then incubated in TBST buffer (150 mM NaCl, 20 mM Tris-HCl, and 0.02% Tween 20; pH 7.4) containing 5% non-fat milk. Proteins were visualized by specific primary antibodies and then incubated with horseradish peroxidase- or alkaline phosphatase-conjugated secondary antibodies. Immunoreactivity was detected using NBT/BCIP following the manufacturer's instructions. Quantitative data were obtained using a computing densitometer with a scientific imaging system (Kodak, Rochester, NY).

VI. *DNA Fragmentation ELISA*

Cellular DNA fragmentation ELISA assay kit (Roche Diagnostics GmbH, Mannheim, Germany) was applied to measure apoptotic cell death by detection of

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5'-bromo-2'-deoxy-uridine (BrdU)-labeled DNA fragments in cytoplasm of cell lysates, according to manufacturer's instructions.

VII. *Statistical Analysis*

Results are presented as the mean ± SE from at least three independent experiments. One-way analysis of variance (ANOVA) followed by, when appropriate, the Newman-Keuls test to determine the statistical significance of the difference between means. A p value < 0.05 was considered statistically significant.

RESULTS

I. *PGN Induced Cell Apoptosis in HUVECs*

To determine whether PGN affects cell viability in HUVECs, MTT assay was used. As shown in Figure 1, treatment of HUVECs for 48 h with PGN (10 - 100 μg/mL) significantly decreased cell viability in a concentration-dependent manner. PGN at 30 μg/mL and 100 μg/mL significantly decreased the cell viability by 23.5 ± 4.1% and 43.4 ± 4.5% (n = 4) (Figure 1). We next investigated whether PGN-decreased cell viability is attributable to apoptosis. Flow cytometric analysis was thus employed for the detection of the extent of apoptosis. As shown in Figure 2A, the percentage of propidium iodide (PI)-stained cells in the apoptotic region (Apo) was significantly increased following PGN treatment. The compiled data are shown in Figure 2B. The percentage of apoptotic cells was significantly increased from 15.8 ± 0.3% to 43.0 ± 5.9% and 61.4 ± 9.8% after 48 h exposure to PGN at the concentrations of 30 and 100 μg/mL, respectively (Figure 2B).

II. *PGN Induced ASK1, JNK and p38MAPK Activation in HUVECs*

To explore whether ASK1 activation is involved in the signaling cascade of PGN-induced apoptosis, ASK1 activity was measured after PGN exposure. ASK1 phosphorylation at Thr845 is correlated with ASK1 activity[25]. We thus determined whether the extent of ASK1 Thr845 phosphorylation is altered by PGN in HUVECs. Treatment of cells with PGN increased ASK1 Thr845 phosphorylation significantly at as early as 10 min, and this sustained to 30 min after PGN exposure (Figure 3A). We next determined whether p38MAPK, a critical downstream molecule of ASK1[17,19,26], was activated in PGN-stimulated HUVECs. Results from Figure 3B illustrate that PGN time-dependently induced p38MAPK phosphorylation within 60 min after PGN exposure. The increase of p38MAPK phosphorylation was 2.2 ± 0.4 folds (n = 4) after 60 min exposure to PGN (Figure 3B). It is conceivable that PGN may affect apoptotic protein expression, leading to cell apoptosis. Several lines of evidence demonstrated that Bax, a proapoptotic Bcl-2 family protein, was shown to

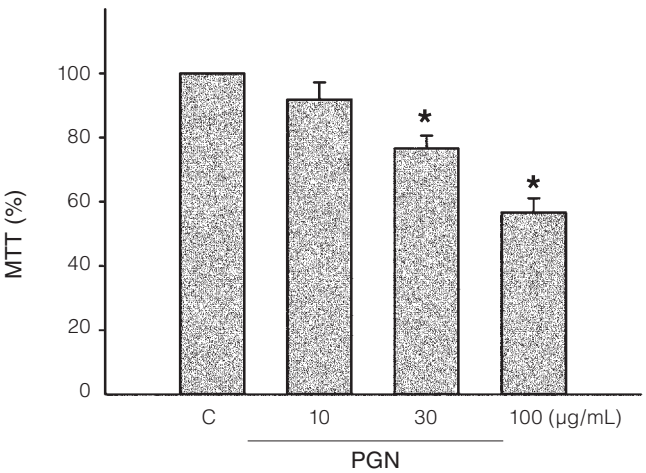

Figure 1. PGN decreased cell viability in HUVECs. Cells were treated with vehicle or PGN at indicated concentrations for 48 h. Each column represents the mean ± SEM of four independent experiments performed in duplicate. * $p < 0.05$, compared to the control group. C: vehicle-treated control group.

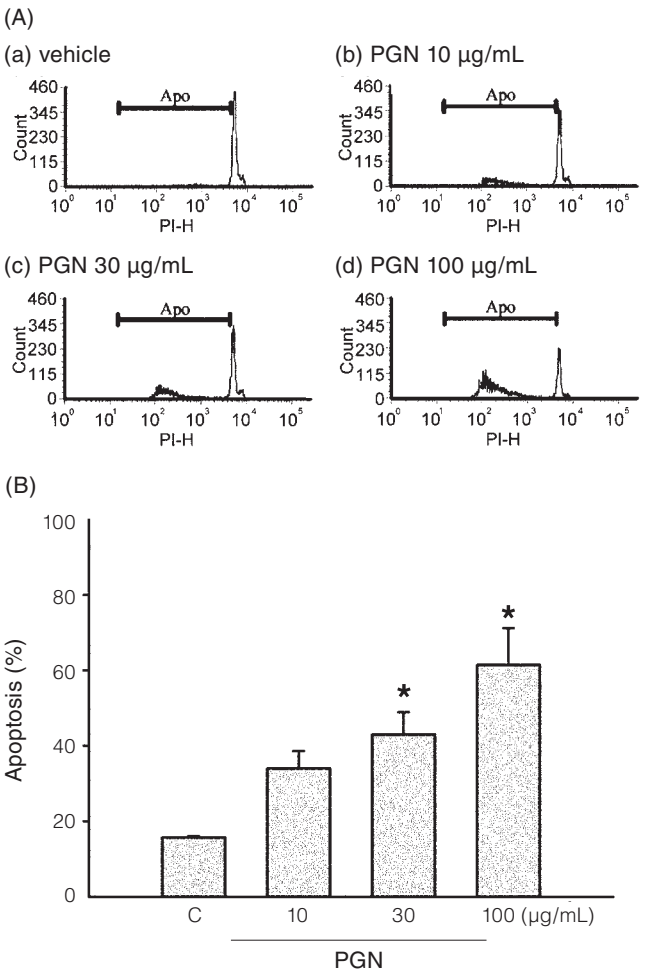

Figure 2. PGN induced cell apoptosis in HUVECs. (A) Cells were treated with vehicle or PGN at indicated concentrations for 48 h. Compiled results are shown in (B) Each column represents the mean ± SEM of at least three independent experiments. * $p < 0.05$, compared to the vehicle-treated group. C: vehicle-treated control group.

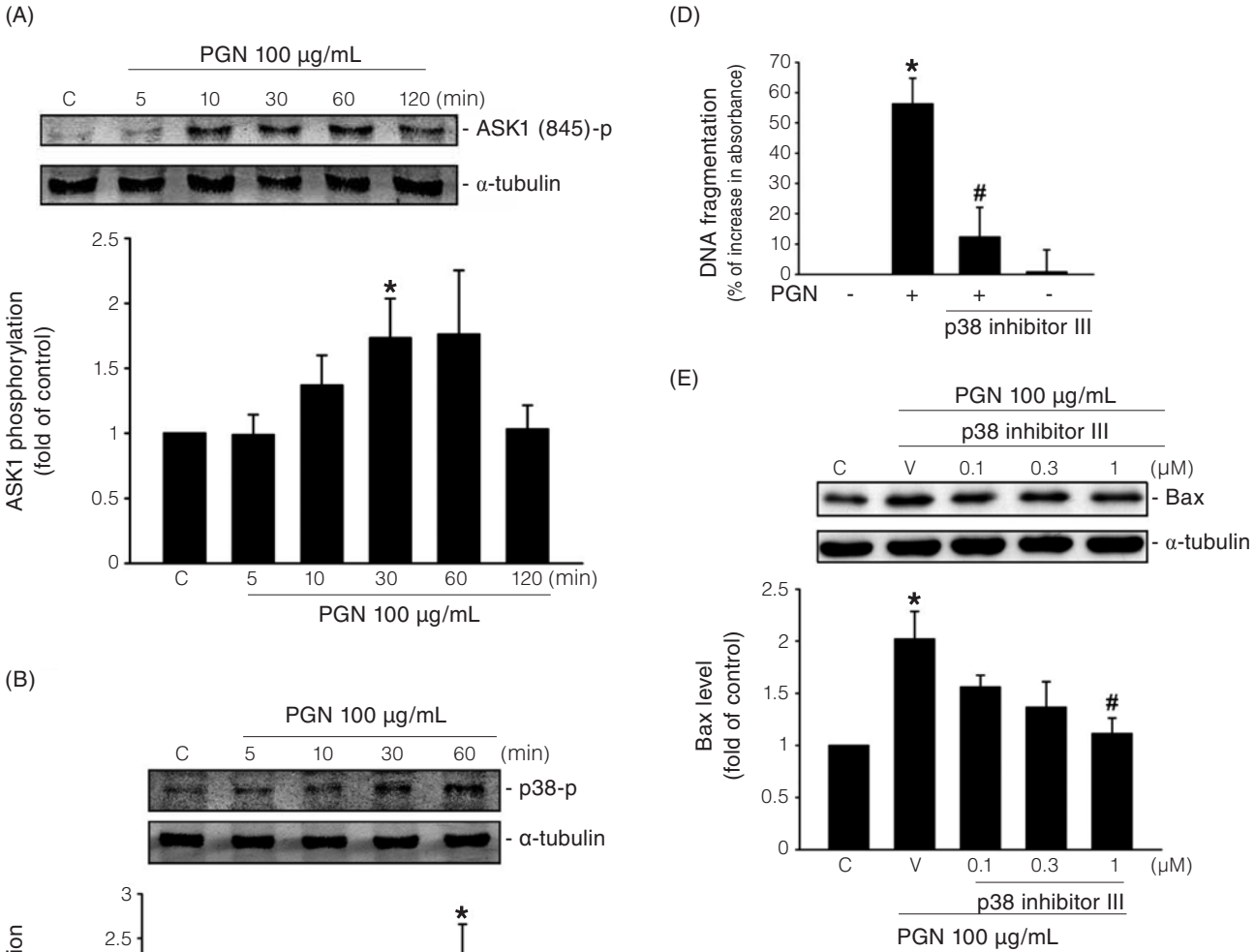

Figure 3. Effects of PGN on ASK1 and p38MAPK activation in HUVECs. (A) Cells were treated with PGN (100 μg/mL) for the indicated time periods. Cells were then harvested and ASK1 phosphorylation at Thr845 was determined by immunoblotting. Compiled results are shown at the bottom. Each column represents the mean ± SEM of four independent experiments. * $p < 0.05$, compared to the control group. (B) Cells were treated with PGN (100 μg/mL) for the indicated time periods. Cells were then harvested and p38MAPK phosphorylation at Thr180/Tyr182 was determined by immunoblotting. Compiled results are shown at the bottom. Each column represents the mean ± SEM of at least three independent experiments. * $p < 0.05$, compared to the control group. (C) Cells were treated with PGN at indicated concentrations for 48 h. The extent of Bax was then determined by immunoblotting. Compiled results are shown at the bottom. Each column represents the mean ± SEM of four independent experiments. * $p < 0.05$, compared to the control group. (D) Cells were pretreated for 30 min with vehicle or 1 μM p38 inhibitor III followed by treatment with 100 μg/mL PGN for another 48 h. Each column represents the mean ± SEM of four independent experiments. *$p < 0.05$, compared with the control group. $^{\#}p < 0.05$, compared to the group in the presence of PGN. (E) Cells were pretreated for 30 min with vehicle or p38 inhibitor III (0.1 - 1 μM) followed by treatment with 100 μg/mL PGN for another 48 h. The extent of Bax was then determined by immunoblotting. Compiled results are shown at the bottom. Each column represents the mean ± SEM of three independent experiments. * $p < 0.05$, compared to the control group. $^{\#}p < 0.05$, compared to the group in the presence of PGN. C: vehicle-treated control group; V: vehicle-treated group in the presence of PGN.

play a pivotal role in promoting apoptosis[18]. We have also demonstrated recently that p38MAPK activation may lead to Bax expression and subsequent cell apoptosis[17]. We thus determined whether Bax level is altered in PGN-stimulated HUVECs. As shown in Figure 3C, PGN significantly induced Bax expression in HUVECs after PGN exposure for 48 h. A P38MAPK signaling inhibitor, p38 inhibitor III, was used to determine whether p38MAPK signaling contributes to PGN-induced Bax expression and subsequent cell apoptosis. A hallmark of apoptosis is the degradation of DNA into a specific fragmentation pattern. BrdU was thus used to label DNA fragments in elucidating the extent of DNA fragmentation. As shown in Figure 3D, BrdU-labeled DNA fragments were significantly increased in samples from cells exposure to PGN (100 μg/mL). In addition, p38 inhibitor III significant inhibited PGN-induced DNA fragmentation (n=4) (Figure 3D). Furthermore, PGN-increased Bax expression was attenuated by the presence of p38 inhibitor III (Figure 3E). Taken together, these results suggested that PGN may activate ASK1-p38MAPK signaling cascade resulting in Bax expression and cell apoptosis in HUVECs.

III. *TLR2 Agonist, Pam3CSK4, Induced Cell Apoptosis in HUVECs*

Many lines of evidence demonstrated that the ability of PGN to induce a proinflammatory response. PGN has been reported to act as a TLR2 agonist[27,28], although recent studies suggested that the reported TLR2-activating activity of PGN may be a result of contamination of lipoprotein or superantigen-like activity[29,30]. However, Dziarski and Gupta[31] re-evaluated and concluded that PGN is a TLR2 activator. To determine whether TLR2 contributes to PGN actions on HUVECs as described above, we used a selective TLR2 agonist Pam3CSK4 to activate TLR2 signaling cascade. As shown in Figure 4A, treatment of cells with Pam3CSK4 (1 - 10 μg/mL) for 48 h markedly decreased cell viability in HUVECs. Pam3CSK4 at 10 μg/mL significantly decreased the cell viability by 28.6 ± 9.5% (n = 3). We next determined whether Pam3CSK4 causes apoptosis in HUVECs using flow cytometric analysis. As shown in Figure 4B, the percentage of PI-stained cells in the apoptotic region (Apo) was significantly increased following Pam3CSK4 treatment. The compiled data are shown in Figure 4C. The percentage of apoptotic cells was significantly increased by 22.6 ± 2.7% and 25.7 ± 5.1% after 48 h exposure to Pam3CSK4 at the concentrations of 3 and 10 μg/mL, respectively (Figure 4C).

IV. *Pam3CSK4 Induced ASK1, JNK and p38MAPK Activation in HUVECs*

Activation of ASK1 is regulated by the phosphorylation of its threonine or serine residues. ASK1 phosphorylation at Thr845 is correlated with ASK1 activity[25]. However, phosphorylation of the ASK1 Ser967 residue was shown to suppress the ASK1 function[22]. We recently demonstrated that ASK1 Ser967 dephosphorylation resulted in its dissociation from 14-3-3, leading to ASK1 activation in cerebral endothelail cells[17]. We thus examined whether the extent of ASK1 Ser967 phosphorylation is altered after Pam3CSK4 exposure. As shown in Figure 5A, Pam3CSK4 caused ASK1 Ser967 dephosphorylation at as early as 10 min, and this was sustained to 30 min after Pam3CSK4 exposure. Similar to PGN, Pam3CSK4 also markedly increased

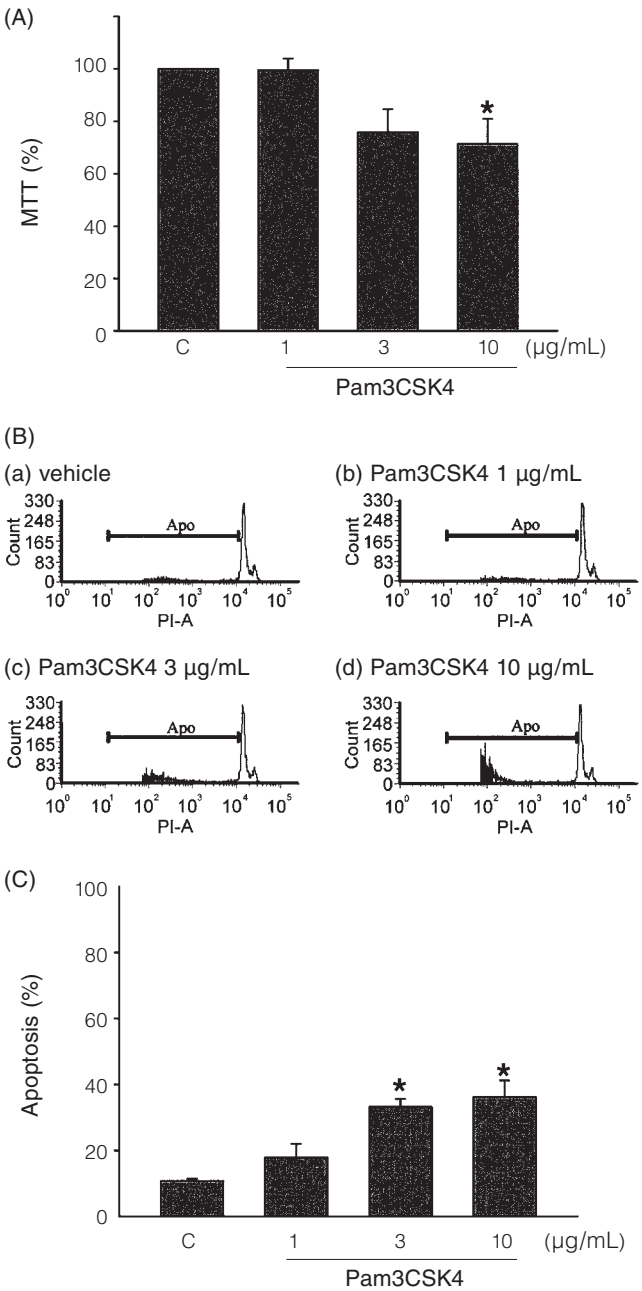

Figure 4. Pam3CSK4 induced apoptosis in HUVECs. (A) Cells were treated with vehicle or Pam3CSK4 at indicated concentrations for 48 h. Each column represents the mean ± SEM of four independent experiments performed in duplicate. * $p < 0.05$, compared to the control group. (B) Cells were treated with vehicle or Pam3CSK4 at indicated concentrations for 48 h. Compiled results are shown in (C) Each column represents the mean ± SEM of at least three independent experiments. * $p < 0.05$, compared to the vehicle-treated group. C: vehicle-treated control group.

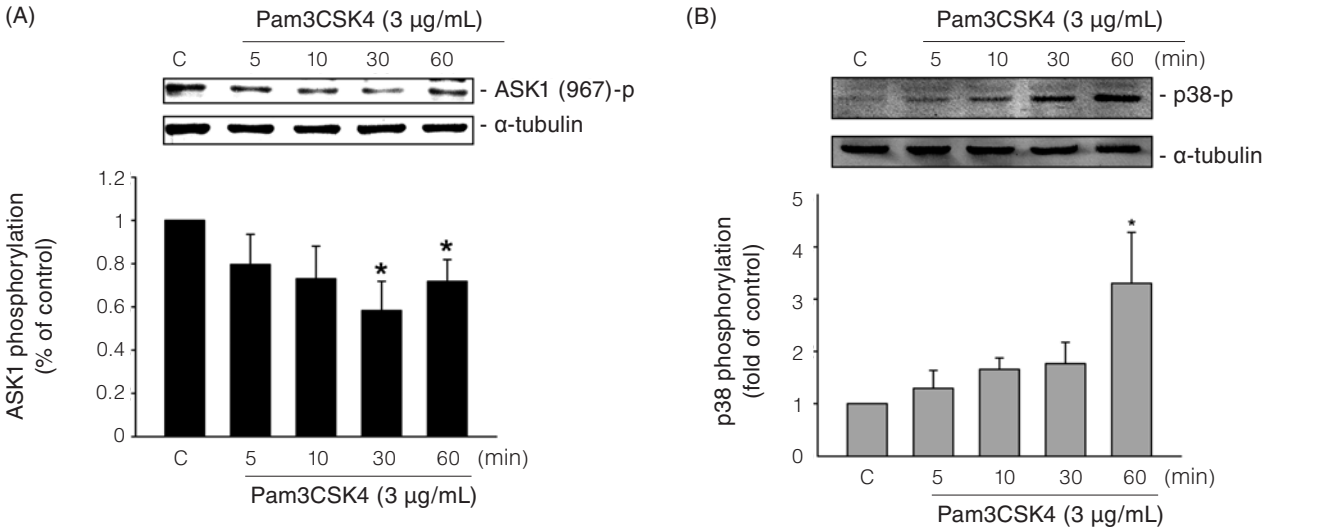

Figure 5. Effects of Pam3CSK4 on ASK1 and p38MAPK activation in HUVECs. (A) Cells were treated with Pam3CSK4 (3 μg/mL) for the indicated time periods. Cells were then harvested and ASK1 phosphorylation at Ser967 was determined by immunoblotting. Compiled results are shown at the bottom. Each column represents the mean ± SEM of four independent experiments. * $p < 0.05$, compared to the control group. (B) Cells were treated with Pam3CSK4 (3 μg/mL) for the indicated time periods. Cells were then harvested and p38MAPK phosphorylation at Thr180/Tyr182 was determined by immunoblotting. Compiled results are shown at the bottom. Each column represents the mean ± SEM of at least three independent experiments. * $p < 0.05$, compared to the control group. C: vehicle-treated control group.

p38MAPK phosphorylation in a time-dependent manner within 60 min after Pam3CSK4 exposure (Figure 5B). These results suggested that Pam3CSK4, like PGN, may activate the ASK1-JNK or p38MAPK signaling cascade, leading to cell apoptosis in HUVECs.

DISCUSSION

We demonstrated in this study that PGN induced HUVEC death by apoptosis. The apoptotic mechanism of PGN may involve the activation of AK1 and p38MAPK. In addition, selective TLR2 agonist, Pam3CSK4, was also shown to activate the ASK1-p38MAPK signaling cascade, leading to cell apoptosis in HUVECs. These observations suggested that TLR2-mediated signaling may cause HUVEC dysfunction by apoptotic mechanisms. Thus, specific therapeutic strategies target on decreasing apoptosis could form a basis for treatments of a variety of vascular diseases, such as sepsis[33].

The molecular mechanism involved in TLR2-mediated ASK1 activation in HUVECs remains unresolved. We noted in this study that PGN increased ASK1 Thr845 phosphorylation while Pam3CSK4 caused ASK1 Ser967 dephosphorylation. It raises the possibility that unknown protein kinase or protein phosphatase may contribute to TLR2-mediated ASK1 Thr845 phosphorylation or Ser967 dephosphorylation and subsequent ASK1 activation. We recently demonstrated that TLR2-mediated ASK1 activation through the induction of ASK1 Ser967 dephosphorylation via protein phosphatase 2A (PP2A) activation in RAW264.7 macrophages[26]. Further investigation is needed to clarify whether PP2A contributes to ASK1 Ser967 dephosphorylation and to characterize the protein kinase involved in ASK1 Thr845 phosphorylation in HUVECs.

We found in this study that p38MAPK signaling may be causally related to PGN- or Pam3CSK4-induced cell apoptosis in HUVECs. The molecular mechanism involved in p38MAPK activation by PGN and Pam3CSK4 remains unresolved. Recent study has demonstrated that ASK1 signaling may contribute to PGN-triggered cell death in human embryonic kidney (HEK) 293 cells[23]. ASK1 was also found to activate p38MAPK in cerebral endothelial cells[17] and to activate JNK in HUVECs[36]. Whether ASK1 contributes to PGN activation of p38MAPK in HUVECs has not previously been demonstrated. In agreement with those observations, we noted that PGN and Pam3CSK4 significantly activated ASK1 and accompanied by the activation of p38MAPK in HUVECs. Together, these findings raise the possibility that PGN activation of ASK1 may regulate at least two separate pathways in HUVECs: one on the JNK signaling cascade and another on the p38MAPK signaling cascade. The differential mechanisms of PGN actions in driving these two signaling pathways remain to be elucidated. It is likely that the two pathways may culminate in inducing cell apoptosis in HUVECs.

In the present study, we found that PGN may induce Bax expression through ASK1 and p38MAPK activation. Furthermore, we also noted that PGN-induced Bax expression was accompanied by cell apoptosis. Whether other Bcl-2 family proteins are involved in PGN apoptotic actions in HUVECs remains to be investigated. Our findings revealed, at least, that ASK1-p38MAPK-Bax cascade may contribute to PGN-induced cell apoptosis in HUVECs. Pharmacological

approaches to diminish the effects of pathogens by modulation of ASK1-p38MAPK cascade may provide new strategies for managing gram-positive infection-associated vascular diseases.

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