Mini Review

Effect of antimicrobial cathelicidin peptides on the endothelial cell apoptosis

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Endothelial cells function as a barrier between the intravascular compartment and extravascular tissues. At the site of inflammation, endothelial cells are impaired through the apoptosis, and vascular integrity is disrupted. Thus, apoptosis of endothelial cells triggers the microcirculatory disorder and organ dysfunction, and its attenuation is considered to be one of the therapeutic strategies for inflammatory diseases, including sepsis. Cathelicidins are the family of antimicrobial peptides found in several mammalian species. They are constitutively expressed by neutrophils and epithelial cells in normal condition, and abundantly induced upon infectious or inflammatory stimuli. In addition to the broad spectrum of bactericidal activities, cathelicidins diversely affect biological functions such as chemotaxis and cytokine production, thereby modulating inflammatory or immune responses. Moreover, some cathelicidins exhibit angiogenic activity against ischemic endothelial cells, suggesting their therapeutic role for vascular damage. Consistently, two cathelicidins (porcine PR-39 and human LL-37) are reported to inhibit the apoptosis of endothelial cells. In this review, we introduce the effects of cathelicidins on the apoptosis of endothelial cells.

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Introduction

Endothelial cells function as a barrier between the intravascular compartment and extravascular tissues, and regulate the flow of nutrient substances, diverse biologically active molecules and the blood cells[1]. Damage of the endothelial barrier causes exudation of blood cells, blood flow obstruction and eventually organ dysfunction. Mounting evidence strongly suggests that at the site of inflammation, endothelial cells are impaired through the apoptosis and vascular integrity is disrupted[2,3]. Various stimuli such as bacterial substances, hypoxia and inflammatory mediators induce the apoptosis of endothelial cell during inflammation[4-6].

Cathelicidins are family of antimicrobial peptides found in several mammalian species[7]. They consist of a highly conserved N-terminal domain and a variable C-terminal short peptide (12~100 amino acids), which is proteolytically cleaved and released by epithelial and myeloid cells (Fig.1). The C-terminal peptide exerts broad spectrum of antibacterial activity. The most

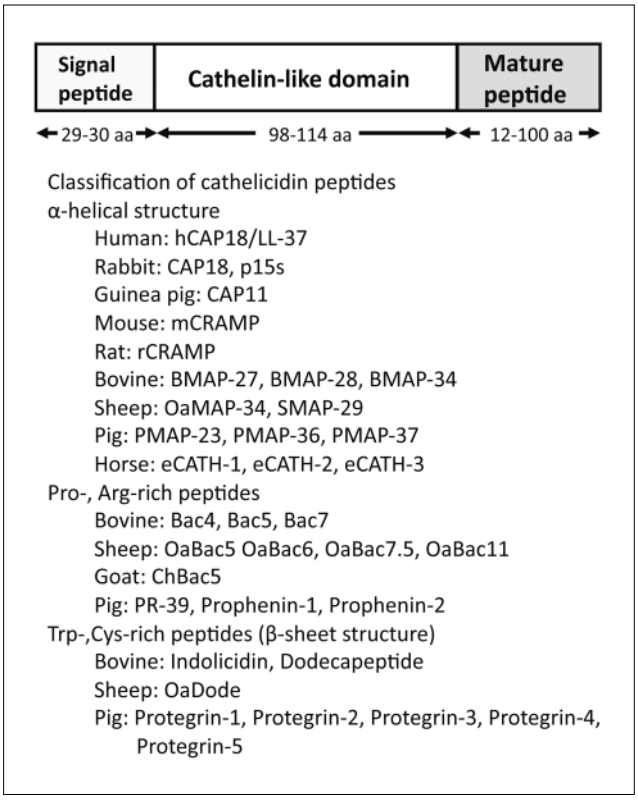

Fig.1 Structure of cathelicidin family of antimicrobial peptides

Cathelicidins are characterized by the highly conserved cathelin-like pro-sequences and variable C-terminal sequences that correspond to the mature antibacterial peptides. About 30 cathelicidins are isolated from mammalian species, including bovine, sheep, goat, pig rabbit, guinea pig, mouse, horse and human.

widespread peptides belong to cathelicidins are those with α-helical conformation[8], and LL-37 is the human α-helical cathelicidin originally isolated from neutrophils[9]. Mouse cathelin-related antimicrobial peptide (CRAMP) and guinea pig cationic antibacterial polypeptide of 11-kDa (CAP11) also have α-helical conformation. In contrast, proline-rich linear cathelicidins such as porcine PR-39 and bovine Bac5, Bac7[8] are isolated from neutrophils and macrophages of only artiodactyl animals. Additionally, β-sheet cathelicidin such as porcine protegrins[10] are also involved in this family. As well as broad spectrum of bactericidal activities, cathelicidins diversely affect biological functions such as chemotaxis and cytokine production to modulate inflammatory or immune responses[11]. Moreover, previous studies demonstrated that some cathelicidins exhibit angiogenic activity against ischemic endothelial cells, suggesting their therapeutic role for vascular damage[12,13]. Thus, inhibition of endothelial cell apoptosis is essential during angiogenesis[14], and it is reasonable to speculate that cathelicidins exert an antiapoptotic activity against endothelial cells. In this review, we introduce the effects of cathelicidins on the apoptosis of endothelial cells.

Antiapoptotic effect of PR-39 on hypoxic endothelial cells

Although several studies revealed the angiogenic role of cathelicidins[12,13], the effect of those peptides on the apoptosis of endothelial cells has been barely reported. PR-39 is the only cathelicidin that was demonstrated to have a direct antiapoptotic effect to endothelial cells[15]. In brief, Wu et al. revealed that PR-39 is induced by hypoxia in bovine aortic endothelial cells and inhibits the hypoxia-induced apoptosis of the cells. They speculate that PR-39 inhibits the apoptosis by increasing the level of inhibitor of apoptosis protein-2 (IAP-2) through the augmentation of its promoter activity and mRNA stability, thereby suppressing the caspase activity in the endothelial cells[15]. This capacity of PR-39 is provided by the potential that it rapidly penetrates the cell membrane and localized to the cytoplasm in the endothelial cells[16]. PR-39 also has an angiogenic activity against endothelial cells[12]. These antiapoptotic and angiogenic activities of PR-39 may synergistically contribute to maintain vascular integrity and prevent blood flow from inflamed damage.

LPS-induced endothelial cell apoptosis in sepsis

Sepsis is a systemic inflammation resulting from harmful host response to bacterial infections[17]. Lipopolysaccharide (LPS), an outer membrane component of the Gram-negative bacteria, functions as a major virulence factor for the pathogenesis[17,18]. Binding of LPS to the LPS receptor complex consisting of CD14, toll-like receptor 4 (TLR4) and adaptor molecule MD-2 induces diverse cellular responses to various host cells by activating downstream intracellular signaling of TLR4[19]. Although endothelial cells express CD14 and TLR4 much less compared to monocytes or macrophages[20], it is demonstrated that LPS binds to endothelial cells through these molecules and upregulates adhesion molecules[21] and coagulation factors[22,23], and eventually induces apoptosis[4,24]. In severe sepsis or septic shock, endothelial cell apoptosis is considered to be crucial for the exacerbation, since endothelial cell apoptosis induced in the susceptible organs such as lung and liver triggers microcirculatory disorder and organ dysfunction[25,26]. This was confirmed by the experimental evidence that administration of a caspase inhibitor or

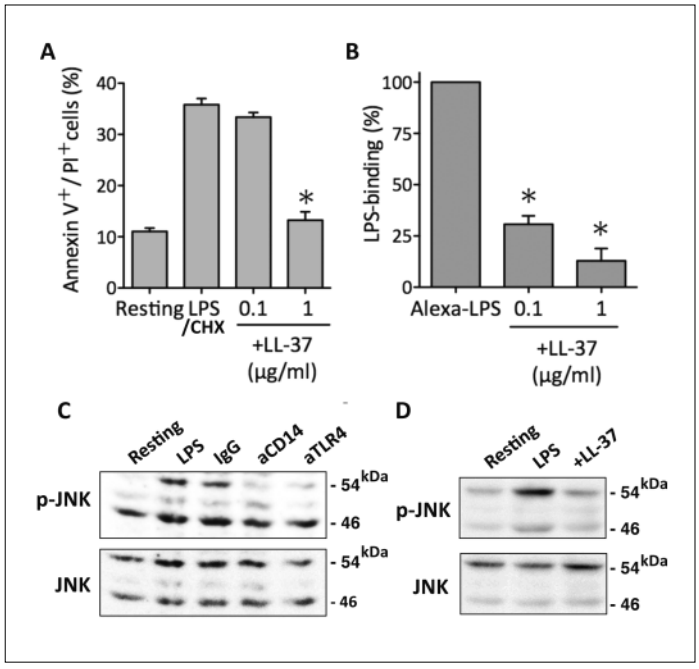

Fig.2 Effects of LL-37 on the LPS/CHX-induced apoptosis and LPS-binding to HMVEC-Ls

(A): HMVEC-Ls were incubated without (Resting) or with LPS (100 ng/ml) and CHX (10 μg/ml) (LPS/CHX) for 24 h in the absence or presence of LL-37 (+LL-37; 0.1 or 1 μg/ml). Apoptosis was determined with Annexin V/PI staining. Values were compared between the incubation with LPS/CHX in the absence and presence of LL-37.

(B): HMVEC-Ls were incubated with Alexa Fluor 488-labeled LPS (Alexa-LPS, 1 μg/ml) at 37℃ for 15 min in the absence or presence of LL-37 (+LL-37; 0.1 or 1 μg/ml). The LPS-binding was analyzed by flow cytometry. The mean fluorescent intensity was measured in each group, and the LPS-binding was expressed as a percentage of that with Alexa-LPS alone. Values were compared between the incubation with Alexa-LPS in the absence and presence of LL-37. Data are the mean ± SE of three independent experiments. * $p<0.05$.

(C): HMVEC-Ls were incubated with neutralizing antibodies for CD14, TLR4 or isotype IgG (20 μg/ml) for 30 min, and then incubated with LPS/CHX for 2 h. Phosphorylated JNK (p-JNK) and total JNK (46 and 54 kDa) were detected by western blot analysis.

(D): HMVEC-Ls were incubated without (Resting), with LPS/CHX in the absence or presence of LL-37 (+LL-37; 1 μg/ml) for 2 h. p-JNK and total JNK (46 and 54 kDa) were detected by western blot analysis.

vascular endothelial growth factor (VEGF) inhibits the LPS-induced endothelial cell apoptosis and improves the survival rates of murine models with acute lung or liver injury[27,28].

Inhibitory effect of LL-37 on the LPS-induced endothelial cell apoptosis in culture

Since the endothelial cell apoptosis is a key event triggering organ dysfunction during sepsis[29], its attenuation is considered to be one of the therapeutic strategies[25]. Human cathelicidin LL-37 was previously shown to induce angiogenesis in the models of physiologic and pathologic angiogenesis[13], suggesting its protective effect on the apoptosis of endothelial cells.

We evaluated the effect of LL-37 on the LPS-induced apoptosis of endothelial cells *in vitro*. Incubation of human lung-derived microvascular endothelial cells (HMVEC-Ls) with LPS (100 ng/ml) and cycloheximide (CHX, 10 μg/ml) resulted increased apoptotic cells (about 40% of the cells), as previously reported[30]. Notably, LL-37 (1 μg/ml) significantly reduced the LPS/CHX-induced apoptosis assessed by Annexin V/PI staining (Fig.2A), TUNEL staining and caspase activity.

We further clarified the mechanism for the suppressive action of LL-37 on the LPS/CHX-induced apoptosis by examining the effect of LL-37 on the LPS-binding to HMVEC-Ls. The cells were incubated with fluorescence-labeled LPS in the absence or presence of LL-37, and cell-associated LPS was analyzed. The LPS-binding was obviously inhibited by LL-37 (0.1, 1 μg/ml) (Fig.2B), suggesting that LL-37 suppresses the LPS/CHX-induced apoptosis of HMVEC-Ls by inhibiting LPS-binding to the cells. We next examined the effects of neutralizing antibodies for LPS receptors on the LPS/CHX-induced apoptosis and the LPS-binding. Anti-CD14[20] and anti-TLR4[31] mAbs significantly reduced the LPS/CHX-induced apoptosis as well as the LPS-binding. These observations suggest that LL-37 suppresses the LPS/CHX-induced apoptosis possibly by inhibiting the binding of LPS to CD14/TLR4, thereby preventing the activation of downstream apoptotic signaling in HMVEC-Ls.

Previous studies indicated that LPS-induced apoptosis is mediated by the activation of c-Jun N-terminal kinase (JNK) in dermal microvascular endothelial cells[39]. To clarify the involvement of JNK in the LPS/CHX-induced apoptotic signaling in HMVEC-Ls, we examined the effect of a JNK inhibitor

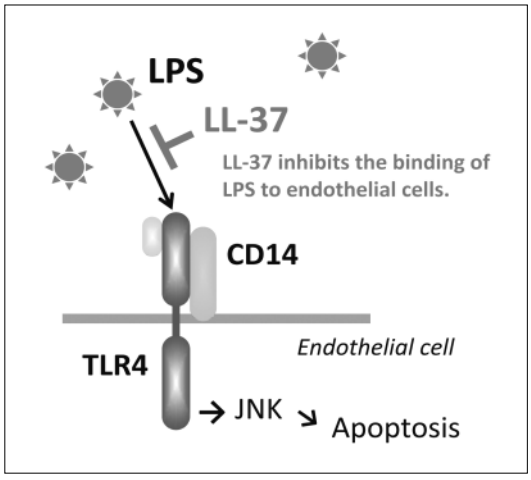

Fig.3 Schematic mechanism for the suppression of LPS-induced endothelial cell apoptosis by LL-37

LL-37 suppresses the LPS-induced apoptosis of endothelial cells by inhibiting the binding of LPS to CD14/TLR4, and preventing the activation of JNK that lies downstream of CD14/TLR4.

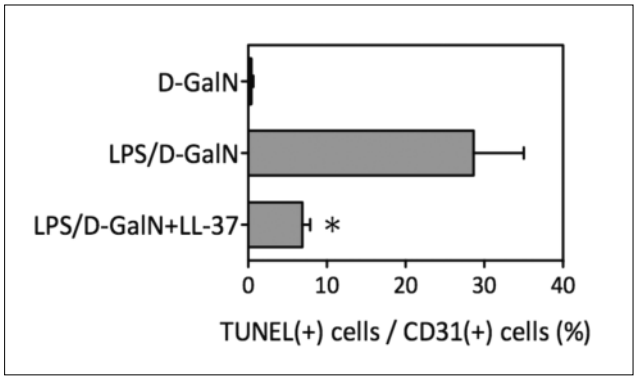

Fig.4 Effect of LL-37 on the apoptosis of endothelial cells in the liver of D-GalN-sensitized endotoxin shock mice

Mice were intraperitoneally injected with LPS (100 ng/mouse) and D-GalN (18 mg/mouse) (LPS/D-GalN), and LL-37 (50 μg/mouse) was simultaneously injected with LPS/D-GalN (LPS/D-GalN+LL-37). Mice injected with D-GalN alone were used as a control (D-GalN). Liver frozen section was stained with TUNEL reagent and anti-CD31 mAb (an endothelial cell marker), and the percentage of TUNEL-positive cells among CD31+ endothelial cells was calculated and shown as TUNEL(+) cells/CD31(+) cells. Data are the mean ± SE of three mice. Values were compared between the LPS/D-GalN without and with LL-37 injection. * $p<0.05$.

SP600125 on the apoptosis of HMVEC-Ls. SP600125 dose-dependently suppressed not only the phosphorylation of JNK but also the apoptosis induced by LPS/CHX, suggesting that JNK activation is involved in the LPS/CHX-induced apoptosis of HMVEC-Ls. Moreover, neutralizing antibodies for CD14 and TLR4 reduced the level of LPS/CHX-induced phosphorylation of JNK (Fig.2C). Since neutralizing anti-CD14 and anti-TLR4 mAbs suppressed the LPS/CHX-induced apoptosis and the binding of LPS, it is suggested that the binding of LPS to CD14/TLR4 activates the JNK-mediated apoptotic signaling in CHX-treated HMVEC-Ls. Next, we examined the effect of LL-37 on the level of LPS/CHX-induced phosphorylation of JNK and found that LL-37 (1 μg/ml) reduced the level of LPS/CHX-induced phosphorylation of JNK almost to that of resting control (Fig.2D). Based on these observations, LL-37 is assumed to suppress the LPS/CHX-induced apoptosis by inhibiting the binding of LPS to CD14/TLR4 and preventing the activation of JNK that lies downstream of CD14/TLR4 in HMVEC-Ls (Fig.3).

Effect of LL-37 on the endothelial cell apoptosis in D-GalN-sensitized endotoxin shock mice

We further evaluated the effect of LL-37 on the LPS-induced endothelial cell apoptosis *in vivo* using a D-galactosamine (GalN)-sensitized endotoxin shock model[32]. Previous our study revealed that survival rate considerably increased from 10% in LPS/D-GalN mice to 80% in LPS/D-GalN+LL-37 mice[34], indicating that the administration of LL-37 protects mice from lethal endotoxin shock. Mice were intraperitoneally injected with LPS (100 ng) and D-GalN (18 mg)[33] without or with LL-37 (50 μg). We evaluated the apoptosis of hepatic endothelial cells by staining liver section with TUNEL reagent and anti-CD31 antibody. LPS/D-GalN-injection induced apoptosis (TUNEL$^+$ cells) in a large number of CD31$^-$ cells in the liver, and the most of apoptotic cells were hepatocytes[35]. In addition, we found that TUNEL$^+$ apoptotic cells were detected among CD31$^+$ endothelial cells lining blood vessels. These observations indicate that apoptosis is induced in not only hepatocytes but also endothelial cells in the liver of this model. Notably, LL-37 administration drastically reduced the number of TUNEL$^+$ apoptotic cells among CD31$^+$ endothelial cells (Fig.4) as well as CD31$^-$ cells (mostly hepatocytes) in the liver of LPS/D-GalN mice. Thus, it is demonstrated that LL-37 is able to suppress the apoptosis of both hepatic endothelial cells and hepatocytes *in vivo* in this endotoxin shock

model. Since the cause of death is due to the liver failure in this model[32], our observations strongly suggest that LL-37 protects mice from lethal endotoxin shock by suppressing the apoptosis of hepatocytes and hepatic endothelial cells.

Apoptosis was induced in a large number of hepatocytes and endothelial cells in the liver of LPS/D-GalN mice, and LL-37 administration markedly reduced the apoptosis of both cells. It is interesting to know whether the suppressive effect of LL-37 on the endothelial cell apoptosis contributes to the protection from hepatocyte apoptosis (liver failure) in LPS/D-GalN mice. Importantly, it has been reported that endothelial injury precedes parenchymal cell damage in LPS-injected mice, and loss of vascular integrity plays a role in tissue damage and multiple-organ dysfunction[36]. Furthermore, the suppression of hepatic endothelial cell apoptosis by the administration of recombinant VEGF protected LPS/D-GalN mice from the destruction of sinusoidal architecture and hepatic injury[27]. Consequently, the maintenance of vascular integrity is crucial for the protection of parenchymal cells during the progression of organ dysfunction in sepsis. Of note, present our study revealed that LL-37 suppresses the apoptosis of hepatic endothelial cells as well as hepatocytes. In addition, we found that LPS was predominantly bound to the surface of blood vessels in the liver of LPS/D-GalN mice, and the binding was suppressed by the administration of LL-37. Together these observations likely suggest that the suppression of hepatic endothelial cell apoptosis by LL-37 not only prevents vascular damage but also attenuates hepatocyte apoptosis, thereby protecting mice from lethal liver failure.

Conclusion

Endothelial cell apoptosis is one of the common events caused by various kinds of inflammatory stimuli. In this review, we introduced the effects of antimicrobial cathelicidins on the inflammation-induced apoptosis of endothelial cells.

PR-39 enters the cells and inhibits the hypoxia-induced apoptosis of endothelial cells by increasing the level of IAP-2, thereby suppressing the caspase activity[15]. Similarly, PR-39 exhibits angiogenetic activity[12], in which the penetrated PR-39 increases several angiogenesis-related genes including VEGF and VEGF receptor 1 by inhibiting the degradation of hypoxia-inducible factor-1α (HIF-1α). As the common mechanism for these effects, it is speculated that PR-39 alters the gene expression of endothelial cells by penetrating into the cells and binding to the signaling molecules in the cytoplasmic component[16]. Such function of PR-39 likely depends on its conformation. Although PR-39 and LL-37 are cationic and play angiogenetic role, their structures (proline-rich PR-39 vs. α-helical LL-37) and angiogenic mechanism are different: LL-37 increases the proliferation and formation of vessel-like structure of endothelial cells by interacting with the cell surface receptor, formyl peptide receptor-like 1 (FPRL1)[13]. Although LL-37 was reported not to affect the apoptosis of endothelial cells in healthy condition[13], LL-37 possibly regulates the apoptosis of endothelial cells under pathogenic condition.

Recently, we have revealed that LL-37 suppresses the LPS-induced endothelial cell apoptosis via the inhibition of LPS-binding with the cells[37]. This effect is exerted by the potent LPS-neutralizing activity of LL-37, but not the direct interaction with endothelial cells. Such LPS-neutralizing activity is specific for α-helical cathelicidins[38]. In this context, we previously demonstrated that the LL-37 and CAP11 potently suppresses the production and release of septic mediators from LPS-stimulated macrophages[33,34]. Thus, LL-37 could be expected as a therapeutic agent for Gram-negative bacterial sepsis/endotoxin shock, because LL-37 could attenuate the progression of LPS-induced multiple cellular responses by its potent LPS-neutralizing activity.

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