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［原著］

Human neutrophil elastase enhances tissue factor expression by peripheral blood monocytes

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Abstract

Neutrophil elastase plays an important role in the development of tissue injury in patients with acute pancreatitis. Disseminated intravascular coagulation (DIC) is one of the life-threatening complications of acute pancreatitis. Peripheral blood monocytes (PBMCs) and subendothelial structures are potential sources of tissue factor (TF), which is the primary initiator of coagulation.

The expression of TF messenger RNA (mRNA) was investigated in PBMCs and THP-1 monocytic cells after stimulation with lipopolysaccharide (LPS) or human neutrophil elastase (HNE). TF mRNA expression was assessed by RT-PCR in PBMCs or THP-1 cells with or without induction of differentiation by phorbol-12-myristate-13-acetate (PMA). The expression of TF protein by PBMCs and THP-1 cells was also analyzed by immunofluorescence microscopy.

LPS and HNE enhanced TF mRNA expression in PBMCs, while immunofluorescence revealed that HNE augmented the TF protein level in PBMCs, as did LPS. HNE increased TF mRNA expression in THP-1 cells treated with PMA (macrophages), as well as in THP-1 cells without PMA treatment (monocytes). In addition, immunofluorescence showed that TF protein levels were increased in THP-1 cells with or without PMA treatment.

Thus, HNE upregulated TF mRNA and protein expression, as did LPS. In addition, HNE enhanced TF mRNA and protein expression in both differentiated and undifferentiated THP-1 cells. These results indicate that LPS and HNE both upregulate TF mRNA expression, which could contribute to the development of disseminated intravascular coagulation (DIC).

Keywords: neutrophil elastase, tissue factor, THP-1 cell

Ⅰ. Introduction

Acute pancreatitis is a potentially lethal disease and the sepsis syndrome has been reported in patients with severe acute pancreatitis[1]. There is accumulating evidence to support the concept that the intestines have a key role in the pathophysiology of severe acute pancreatitis. Because intestinal permeability increases early in the course of pancreatitis and this change is associated with sepsis and organ failure, bacterial translocation has been implicated in the infective complications associated with acute pancreatitis[2]. In fact, it has been demonstrated that necrotising pancreatitis is accompanied by persistent endotoxemia with prolonged elevation of anti-endotoxin antibodies, and transient endotoxemia has been reported in patients with edematous pancreatitis[3].

Disseminated intravascular coagulation (DIC) can occur in patients with severe pancreatitis[4].

LPS is a major trigger of DIC in patients with sepsis via the tissue factor/factor VIIa-dependent coagulation pathway. Tissue factor (TF) is a transmembrane glycoprotein that activates the extrinsic coagulation pathway and is the primary cellular initiator of blood clotting, with peripheral blood monocytes and subendothelial structures being the potential sources of TF. Systemic inflammatory response syndrome, disseminated intravascular coagulation, and sepsis are accompanied by hyperactivation of TF in circulating monocytes[5]. In addition, elevation of plasma TF levels has been detected in patients with severe acute pancreatitis[6]. Moreover, it has been reported that TF is an early marker of severe acute pancreatitis which is superior to CRP, but inferior to IL-6[7].

Neutrophil activation plays an important role in the pathogenesis of acute pancreatitis accompanied by endothelial cell damage and hypercoagulability. Neutrophils are an essential component of host defenses against invading pathogens and the granules of these cells contain serine proteases, including cathepsin G, neutrophil elastase, and proteinase 3. These neutrophil serine proteases may also be key regulators of the inflammatory response [8) 9)].

Human neutrophil elastase (HNE) is a 29 KDa serine endoprotease from the proteinase S1 family. It is an important mediator of inflammatory tissue damage, being involved in the degradation of extracellular matrix components such as elastin, fibronectin, proteoglycans, and collagen. Plasma levels of leukocyte proteases like HNE have been reported to be persistently increased in patients with acute pancreatitis[10]. Accordingly, the present study was undertaken to investigate the influence of HNE and LPS on TF expression by human peripheral blood mononuclear cells (PBMCs) and a human acute monocytic leukemia cell line (THP-1).

Ⅱ. Materials and Methods

1. Ethics statement

Human peripheral blood was obtained from healthy volunteers after informed consent in accordance with procedures approved by the Kumamoto Health Science University Institutional Review Board (No.24-14).

2. Chemicals and reagents

Human neutrophil elastase (HNE) was purchased from Elastin Products Co., Inc. (Owensville, MO), and was used to study intracellular signal transduction related to TF mRNA expression. The HNE solution was negative for endotoxin according to the Endospecy test[11) 12)].

LPS (Escherichia coli serotype 0111:B4) and phorbol myristate acetate (PMA) were obtained from Sigma Chemical Co. (St Louis, MO).

3. Isolation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated as described previously[13]. Briefly, heparinized blood was obtained from nonsmoking healthy volunteers and blood samples were diluted 1:1 in pyrogen-free saline. Then PBMCs were isolated by density gradient centrifugation over Ficoll-Hypaque and washed three times. The viability of the harvested PBMCs was greater than 95% as determined by trypan blue dye exclusion. These cells were resuspended in RPMI-1640 medium supplemented with HEPES (100 mM), L-glutamine (100 U/mL), penicillin (100 U/mL), streptomycin (100 μg/ml) d, and 10% fetal calf serum. PBMCs (at a density of 1×10^6 cells/well) were stimulated with LPS (1 μg) or HNE (5 μg), and were incubated at 37 ℃ under a humidified atmosphere of 5% CO_2 and 95% air.

Preparation of THP-1 human acute monocytic leukemia cells

The THP-1human monocytic cell line (American Type Culture Collection, Manassas, Va.) was maintained in RPMI 1640 medium supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) , HEPES (25 mM), and 5% fetal bovine serum. For the induction of differentiation, cells (5×10^5 to 10^6/ml) were incubated in macrophage serum-free medium

with phorbol 12-myristate 13-acetate (PMA; 50 nM) for 24 h[14) 15)]. Then, the nonadherent cells were removed by aspiration and the adherent cells were washed with complete-RPMI medium three times. THP-1 cells (1×10^6 cells/well) with or without PMA pretreatment were stimulated by HNE (5 μg) or LPS (1 μg), and were incubated at 37℃ under a humidified atmosphere of 5% CO_2 and 95% air.

4. Immunofluorescent staining

Immunofluorescent staining was carried out as described previously[16)]. Briefly, immunofluorescent analysis of TF expression by PBMCs was performed with or without HNE (5 μg) pretreatment for 6 hrs. The cells were incubated with mouse anti-human TF (CD142) antibody, followed by incubation with appropriate secondary antibodies conjugated with Alexa Fluor 555 fluorescent dye (red). Nuclei were stained with DAPI (blue) for 10 min. Then the cells were photographed under a Leica DM5000B fluorescence microscope. Each treatment was replicated three times and the experiment was repeated twice.

5. RNA extraction, reverse transcription, and real-time quantitative PCR

Cells from 3 wells (3×10^6 cells in total) were extracted with 500 μL of TRIzol reagent (Invitrogen). After total RNA was isolated and precipitated according to the manufacturer's instructions, 1 μg of total RNA was reverse-transcribed using random heptamer primers with MMLV (Invitrogen). Then 1 μl of reverse-transcribed RNA was amplified by PCR using an ABI PRISM 7000 thermal cycler (Applied Biosystems) and the Taqman Master Mix Kit.

Ⅲ. Results

RT-PCR revealed that LPS and HNE both stimulated TF mRNA expression in PBMCs (Fig. 1). Immunofluorescent staining demonstrated that the TF protein level was elevated in PBMCs by HNE, as well as by LPS. Representative confocal images of TF expression on the surface of PBCs are shown in Fig. 2. In THP-1 cells with

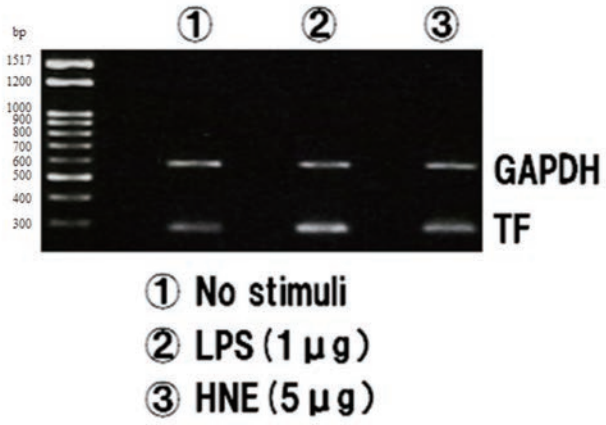

Fig.1 TF mRNA expression by PBMCs after stimulation with HNE or LPS. LPS and HNE stimulate TF mRNA expression by PBMCs. No stimuli also shows TF mRNA expression. Mononuclear cell adherence may be associated with the regulation of gene expression. TF; tissue factor, PBMCs; peripheral blood mononuclear cells, HNE; neutrophil elastase, LPS: lipopolysaccharide.
A representative result of three experiments is shown.

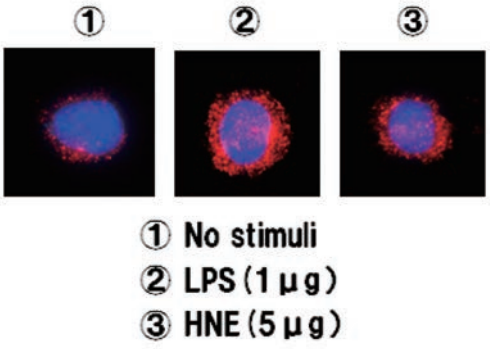

Fig.2 Immunofluorescent staining of PBMCs. TF protein expression on the surface of PBMCs (red) is enhanced by LPS (1 μg), as well as by HNE (5 μg). THP-1 cells treated PMA produce small amounts of TF protein without stimulation. However, LPS-or HNE-induced accumulation of TF protein by THP-1 cells are shown. TF; tissue factor, PBMCs; peripheral blood mononuclear cells, HNE; neutrophil elastase, LPS: lipopolysaccharide, PMA; phorbol myristate acetate. Representative confocal images of PBMCs after immunofluorescent staining for TF (red) are shown.

or without PMA pretreatment, TF mRNA expression was increased by HNE, as well as by LPS (Fig. 3). HNE and LPS enhanced TF protein expression in undifferentiated and differentiated THP-1 cells according to immunofluorescence analysis. Representative confocal images obtained by immunostaining THP-1 cells with anti-human tissue factor antibody with or without PMA pretreatment are shown in Fig. 4.

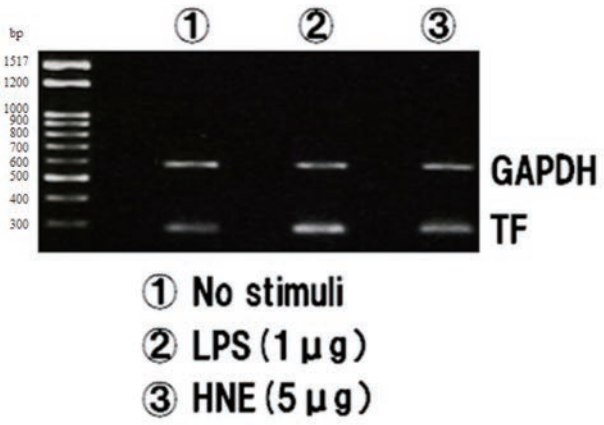

Fig. 3 RT-PCR for TF mRNA expression by THP-1 cells. No stimuli also shows TF mRNA expression. THP-1 cells with or without PMA pretreatment were stimulated by LPS or HNE and TF mRNA expression was upregulated. TF; tissue factor, PBMCs; peripheral blood mononuclear cells, HNE; neutrophil elastase, LPS: lipopolysaccharide. A representative result of three experiments is shown.

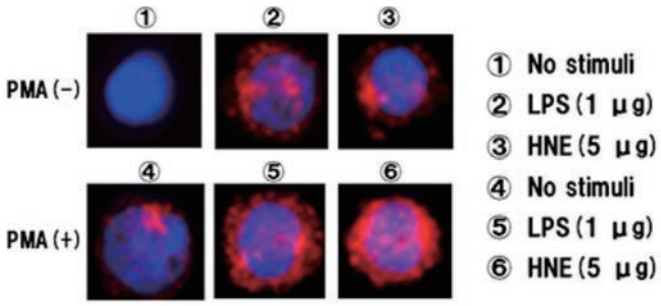

Fig. 4 Immunofluorescent staining of THP-1 cells. THP-1 cells increase TF production after treatment with PMA. TF protein expression on the surface on THP-1 cells with or without PMA pretreatment was increased by LPS as well as HNE. TF; tissue factor, HNE; neutrophil elastase, LPS: lipopolysaccharide, PMA; phorbol myristate acetate. Representative confocal images of THP-1 cells with immunofluorescent staining for TF (red) are shown.

Ⅳ. Discussion

The present study demonstrated that LPS stimulates TF mRNA expression in PBMCs. TF is an essential component of the factor VIIa-TF enzyme complex that triggers activation of the coagulation cascade. HNE also activates the extrinsic clotting pathway by inducing TF expression on PBMCs. Although TF is expressed on the surface of monocytes either constitutively or in response to stimulation, its catalytic activity is only about 10% of the activity of lysed cells[17]. TF is exclusively associated and expressed by circulating monocytes in healthy individuals[18]. However, there are several pathological conditions in which activated monocytes shed TF-rich microparticles (MPs) that are taken up by activated platelets, neutrophils, and endothelial cells[19]. It has been reported that patients with meningococcal sepsis have comparatively high levels of mainly monocyte-derived circulating TF-containing MPs[20]. These MPs can be derived from the vascular endothelium or from blood cells and circulate in the peripheral blood[19]. MPs are tiny (sub-micron) vesicles that are shed by activated or apoptotic cells, including platelets and monocytes. An increase of circulating MPs is associated with thrombosis, and MPs derived from monocytes have been shown to increase thrombin generation and fibrin formation, suggesting a unique contribution to thrombosis[21].

The potentially fatal condition of disseminated intravascular coagulation (DIC) is initiated in patients with bacterial sepsis when LPS induces TF expression on monocytes. Toll-like receptor-4 (TLR-4) mediates the cytokine response to LPS in immune cells[22], and intracellular signal transduction is directly activated by the TLR4 receptor complex[23]. LPS induces the expression of various proinflammatory mediators by human

monocytes, including the procoagulant molecule tissue factor (TF) and the cytokine tumor necrosis factor-alpha.

It has been reported that TF gene expression can be transiently induced in human monocytic THP-1 cells by LPS[24]. Differentiation of THP-1 cells into macrophage-like cells is induced by exposure to phorbol myristate acetate (PMA). In this study, we used the THP-1 cells with or without PMA treatment to identify the surface marker expression of TF by undifferentiated (monocytic) and differentiated (macrophagocytic) cells. The present study demonstrated that TF mRNA expression was induced by HNE, as well as LPS, in both undifferentiated and differentiated THP-1 cells.

Polymorphonuclear neutrophils form part of the primary defenses against bacterial infection, using complementary oxidative and non-oxidative pathways to destroy phagocytized pathogens. Three serine proteases (elastase, proteinase 3, and cathepsin G) are major components of primary neutrophil granules that are involved in the non-oxidative pathway of intracellular pathogen destruction[25]. These proteases also have an important role as specific regulators of the immune response, since they control cellular signaling through the processing of chemokines, modulate the cytokine network, and activate specific cell surface receptors. HNE proteolytically degrades endothelial cell-bound tissue factor pathway inhibitor-1 (TFPI), and TFPI mRNA expression is downregulated by HNE and cathepsin G (CG). Proteinase 3, but not HNE or CG, increases the surface expression of TF and TF mRNA by human umbilical vein endothelial cells[26]. However, the present study showed that exposure to HNE led to an increase of both TF mRNA and protein levels in PBMCs. Monocytes and neutrophils are activated during the microvascular disturbances associated with disseminated intravascular coagulation (DIC). Thus, neutrophil elastase is one of the factors that promotes organ dysfunction in patients who have disseminated intravascular coagulation associated with sepsis[27].

Ⅴ. Conclusion

In conclusion, the present study demonstrated that HNE may promote TF expression by PBMCs, as does LPS.

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