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Mechanism of tissue factor production by monocytes stimulated with neutrophil elastase

Jin Kawata, Manabu Aoki, Yasuji Ishimaru, Tomomichi Ono, Katsurou Sagara, Shinji Narahara, Tamami Matsmoto, Eiji Hirose, Yasuo Yamaguchi *

Department of Medical Technology, Kumamoto Health Science University, Kumamoto, Japan

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ABSTRACT

Background: Monocytes and neutrophils are activated during disseminated intravascular coagulation. Tissue factor, the main initiator of coagulation, is expressed by monocytes, while elastase is released by neutrophils.
Aims: This study investigated tissue factor production by peripheral monocytes after stimulation with human neutrophil elastase.
Methods: Tissue factor mRNA levels were investigated by the reverse transcriptase-polymerase chain reaction and tissue factor protein production was assessed by western blotting when monocytes were exposed to neutrophil elastase with or without preincubation using various inhibitors.
Results: Neutrophil elastase upregulated tissue factor mRNA and protein levels in monocytes. Both U73122 (phospholipase C inhibitor) and TMB-8 (intracellular calcium antagonist) prevented the upregulation of tissue factor mRNA. SB203580 (p38 mitogen-activated protein kinase inhibitor) suppressed this response, but PD98059 (extracellular signal-regulated kinase inhibitor) did not. Ro-318425 (ATP-competitive and selective protein kinase C (PKC) inhibitor) and Go 6976 (inhibitor of conventional PKCs and PKCμ) blocked the upregulation of tissue factor mRNA expression. Go 6983 (broad-spectrum PKC inhibitor) and CGP 4125 (staurosporine analog) partially attenuated it, as did a PKC theta/delta inhibitor.
Conclusions: Neutrophil elastase mainly enhances tissue factor production by monocytes via the phospholipase C/conventional PKC/p38 MAPK pathway, although a novel PKC is also involved.

Introduction

Tissue factor (TF) is a membrane-bound glycoprotein that is induced in circulating monocytes by various inflammatory mediators and triggers the extrinsic pathway of blood coagulation. Disseminated intravascular coagulation (DIC) is characterized by systemic activation of coagulation, and is a life-threatening condition. Exposure of TF to the blood is the event that usually initiates DIC [1]. Peripheral blood mononuclear cells (PBMCs) and subendothelial structures are both potential sources of TF. Since TF can be expressed on the cell membrane by monocytes, these cells are thought to be responsible for extrinsic activation of the coagulation cascade during DIC [2]. Surface expression of TF by monocytes can be stimulated in response to agents such as endotoxin [3], phorbol ester [4], lipoteichoic acid [5], and C-reactive protein [6], thereby generating TF activity. In addition, microvascular damage leads to activation of monocytes and neutrophils in patients with DIC [7]. Activated neutrophils release the contents of their granules during migration to sites of inflammation and the various granule proteins play a central role in the early inflammatory response. The serine proteases cathepsin G, human leukocyte elastase, and proteinase 3 are major proteins found in neutrophil granules [8]. Among them, several observations have indicated that human neutrophil elastase (HNE) is important in the establishment of DIC. HNE has been reported to play a key role in the onset of symptomatic DIC and multiple organ failure in patients with head injury [9]. HNE has also been suggested to be important for the development of acute respiratory distress syndrome and DIC in patients with sepsis [10]. These findings raise the question of how HNE stimulates TF production by PBMCs, especially the intracellular signal transduction pathways involved.

Materials and methods

Reagents

Human neutrophil elastase (HNE) was purchased from Elastin Products Co., Inc. (Owensville, MO). Lipopolysaccharide (LPS) (*Escherichia coli* serotype 0111:B4) was obtained from Sigma Chemical Co. (St Louis, MO). U73122 (Merck Millipore, Bedford, MA), Rottlerin (Merck Millipore), TMB-8 (Sigma-Aldrich, Oakville, Ontario, Canada),

* Corresponding author at: Department of Medical Technology, Kumamoto Health Science University, Kitaku Izumi-machi 325, Kumamoto 861-5598, Japan. Fax: +81 96 245 3126.
E-mail address: yamaguti@kumamoto-hsu.ac.jp (Y. Yamaguchi).

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Table 1
Characteristics of the reagents used.

Reagents	Action
U73122	Phospholipase C inhibitor
Rottlerin	Protein kinase C inhibitor
TMB-8	Intracellular calcium antagonist
SB 203580	p38 mitogen-activated protein kinase inhibitor
PD98059	Extracellular signal-regulated kinase inhibitor
TAPI-1	Disintegrin and metalloproteinase inhibitor

SB203580 (Wako, Kanagawa, Japan), and PD98059 (Wako) were employed to study the intracellular signal transduction pathways involved in the expression of TF mRNA. The characteristics of these reagents are summarized in Table 1.

In addition, the protein kinase C (PKC) inhibitors Ro-318425 (Merck Millipore), Go 6976 (Tocris Bioscience, Bristol, UK), Go 6983 (Tocris Bioscience), and CGP 41251 (Tocris Bioscience), as well as a PKC theta/delta inhibitor (Merck Millipore), were utilized to investigate the roles of various PKC isoforms in TF production. The different PKC isoforms inhibited by these reagents are summarized in Table 2.

Xestospongin C, which antagonizes the calcium-releasing action of inositol-1,4,5-trisphosphate (IP_3) at the receptor level, was obtained from Sigma-Aldrich. All reagent solutions were negative for endotoxin according to the Endospecy test [22,23].

Isolation of peripheral blood mononuclear cells

PBMCs were isolated as described previously [24]. Briefly, heparinized blood samples were obtained from nonsmoking healthy volunteers and were diluted 1:1 with pyrogen-free saline, after which PBMCs were isolated by density gradient centrifugation with Ficoll-Hypaque and washed three times. The viability of the cells thus obtained exceeded 95% in the trypan blue dye exclusion test. PBMCs were resuspended in an RPMI-1640 medium supplemented with 25 mM HEPES, 100 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum. PBMCs were stimulated by incubation with HNE for 6 h with or without preincubation using the reagents mentioned above. LPS was employed as the positive control.

Extraction of RNA, reverse transcription, and real-time quantitative polymerase chain reaction (PCR)

Cells from 3 wells (3×10^6 cells in total) were extracted with 500 μL of TRIzol™ reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated and precipitated according to the manufacturer's instructions, after which 1 μg of total RNA was reverse-transcribed using random heptamer primers with Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen, Carlsbad, CA). Then 1 μL of the reverse-transcribed RNA was amplified by PCR using an ABI PRISM 7000 thermal cycler (Applied Biosystems) and the Taqman™ Master Mix Kit. The 18S ribosomal RNA (rRNA) gene was employed as a reference and quantification of the target mRNA was done by comparing the number of cycles required to reach the reference and target thresholds [25].

Table 2
Isoform specificity of the protein kinase C inhibitors.

Reagents	PKC isoforms inhibited						Reference no.
Ro 31-4425	PKCα	PKCβI	PKCβII	PKCγ			[11–13]
Go 6976	PKCα	PKCβI					[14,15]
Go 6983	PKCα	PKCβI		PKCγ	PKCδ		[16,17]
CGP41251	PKCα	PKCβI	PKCβII	PKCγ	PKCδ		[18,19]
PKCθ/δinhibitor					PKCδ	PKCθ	[20,21]

PKC: protein kinase C.

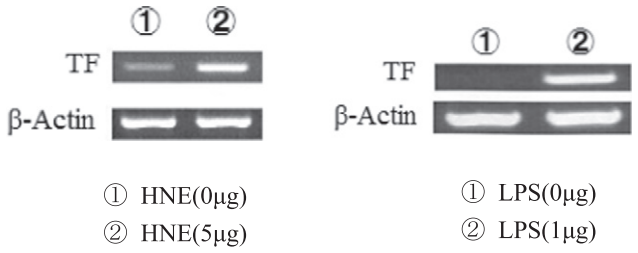

Fig. 1. Lipopolysaccharide (LPS) upregulates tissue factor (TF) mRNA expression by peripheral blood monocytes (PBMCs) and human neutrophil elastase (HNE) also upregulates TF mRNA expression. A representative result of three experiments is shown.

Western blotting for TF

TF was assayed by western blotting as described previously [26]. In brief, equivalent amounts of cell lysates were subjected to electrophoresis and the products were transferred to polyvinylidene difluoride membranes. The membranes were incubated with 0.2 μg/mL mouse anti-human TF IgG (American Diagnostica, Greenwich, NY), washed, and incubated with alkaline phosphatase-conjugated guinea pig anti-mouse IgG (Sigma) diluted to 1:5000. Then the membranes were incubated with chemiluminescent enhancer (Immun-Star, Bio-Rad, Hercules, California) and exposed to XAR film (Kodak, Rochester, NY). After the film was developed, bands were quantified with a densitometer and ImageQuant software (Molecular Dynamics, Sunnydale, CA).

Results

Incubation with LPS regulated TF mRNA expression by PBMCs. Similarly, incubation with HNE stimulated TF mRNA expression by PBMCs (Fig. 1). When the TF concentrations in cell lysates were assayed by western blotting, it was shown that HNE promoted TF production by PBMCs (Fig. 2). This increase of HNE was markedly inhibited by preincubation with U-73122 (a phospholipase C inhibitor). In addition, TMB-8 (an intracellular calcium antagonist) blunted HNE-induced TF mRNA expression. The stimulatory effect of HNE on TF mRNA expression in PBMCs was also reduced by SB 203580 (a p38 mitogen-activated protein kinase inhibitor), but not by PD98059 (an extracellular signal-regulated kinase inhibitor). Rottlerin (a protein kinase C inhibitor) did not inhibit the response of TNF mRNA to HNE. Similarly, TAPI-1

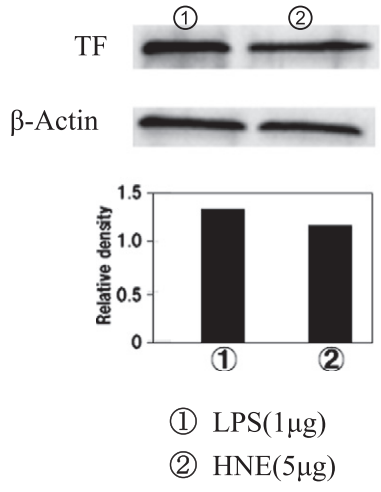

Fig. 2. TF protein production by PBMCs after stimulation with HNE or LPS. TF levels in cell lysates were assayed by western blotting. HNE (5 μg) and LPS (1 μg) both increased TF production by PBMCs after 6 h of stimulation. A representative result of three experiments is shown.