Thrombin Induces the Expression of Tissue Factor in Human Aortic Endothelial Cells

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Background: Atherosclerotic plaque disruption and subsequent thrombosis formation is responsible for many thrombotic complications in cardiovascular disease. Tissue factor (TF) exposure upon plaque rupture can trigger thrombin formation. Thrombin, on the other hand, can induce tissue factor expression in human umbilical vein endothelial cells or saphenous vein endothelial cells. The impact of thrombin in the endothelial cells of arterial side was less known.

Methods: Human aortic endothelial cells (HAEC) were cultivated and methoxyphenyl tetrazolium inner salt assays were used to determine the non-toxicity of thrombin to endothelial cells. Real-time PCR was used to determine the relative TF mRNA quantity. Western blot was used to determine the relative protein level. TF functional activity was determined by a chromogenic assay.

Results: Thrombin was non-toxic to the HAEC. TF mRNA relative quantity was enhanced 390 ± 114% by thrombin (P = 0.034). TF protein relative quantity was enhanced 332 ± 39% by thrombin (P = 0.01). TF activity was enhanced 547 ± 54% by thrombin (P < 0.001).

Conclusion: Thrombin is a sufficient stress to induce TF expression in HAEC. The transcriptional control by thrombin causes an increase in TF mRNA. This increase in mRNA is modestly paralleled by an increase in protein level and functional activity.

Key Words: Human aortic endothelial cell • Thrombin • Tissue factor

INTRODUCTION

Atherosclerotic plaque disruption and subsequent thrombosis formation is responsible for the onset of most cardiovascular events. The magnitude of the thrombotic processes triggered upon plaque disruptions is dependent upon tissue factor (TF). TF is the principal initiator and propagator of thrombus formation in the extrinsic coagulation system. In blood vessels, TF is detectable in adventitial fibroblasts under normal circumstance. Atherosclerotic plaque, however, contains significant TF mRNA and antigen, which is associated with monocytes and smooth muscle cells within the plaque. TF in plaque can bind to factor VIIa and is functional. Once formed in situations of plaque rupture, TF:VIIa can activate factor IX or factor X. The Xa can assemble with factor Va on phospholipid surface to catalyze the formation of thrombin from prothrombin. Thrombin then initiates clot formation by converting fibrinogen to fibrin. These chain reactions of coagulation cascades will result in thrombus formation at the ruptured plaque site.

Previous reports have documented that thrombin could elicit tissue factor production in endothelial cells coming from human umbilical cord vein and human saphenous vein.

The endothelium is a special organ and performs a...
complex function. A unique characteristic of endothelial cell is their remarkable heterogeneity in different organs. Because of such heterogeneity, we are interested in determining whether endothelial cells from an arterial side (i.e. human aortic endothelial cells, HAEC) still preserve the same response to human thrombin regarding to its ability to induce tissue factor expression. The purpose of the current study was to examine whether exposure of HAEC to human thrombin could cause sufficient perturbation to alter the expression of tissue factor.

MATERIALS AND METHODS

Cell culture

HAEC were purchased from Cell Applications, Inc. (San Diego, CA). The HAEC were cultured in endothelial cell growth medium (Cell Applications, Inc.) according to the supplier’s recommendation. Cells were grown to near confluence in 10-cm culture dishes before experiment. Before thrombin stimulant was added, the HAEC were rendered quiescent in Medium-199 supplement with 1% fetal bovine serum (HyClone) for 24 hours. Human thrombin (Sigma-Aldrich) stimulation (1 unit/ml) up to 3 hours (for TF mRNA expression) and 5 hours (for TF protein and activity expression) was done.

Methoxyphenyl tetrazolium inner salt (MTS) cell viability assay

MTS assays were performed to monitor conversion of methoxyphenyl tetrazolium inner salt to formazan by mitochondrial dehydrogenase in viable cells. Briefly, HAEC were seeded in a 96-well plate with final cell concentration $1 \times 10^4$/well. After serum starvation for 24 hours, the HAEC were treated according to the protocol. Twenty µl MTS solution (0.5 mg/ml, Promega) was then added to each well and the cells were cultivated at 37 °C for two hours. The absorbance was then read at 490 nm.

Real-time polymerase chain reaction analysis

Endothelial cells were harvested by trypsinization and pelleted. Total RNA was extracted from the pellet using Micro-to-Midi total RNA purification system (Invitrogen). cDNA was synthesized from total RNA using M-MLV reverse transcriptase (Superscript II, Invitrogen), and PCR was performed using cDNA as the template in a 25-µl reaction mixture containing a specific primer pair of each cDNA. The total cDNA pool obtained served as template for subsequent PCR amplification using the SYBR PCR kit (Applied Biosystems) according to the manufacturer’s protocols for the ABI PRISM 7900 HT sequence detection system. The primers were designed using Primer Express 2.0 (Applied Biosystems) and are synthesized by Blossom Biotechnologies Inc. (Taiwan). The primer sequences were as follows:

Tissue factor

forward primer: 5'-TCCCCGAACAGTTAACC CGAA-3'
reverse primer: 5'-GACCACAAATACCCACAGCTCA-3'

GAPDH

forward primer: 5'-ATCCCTTCAAAATCAAGTG GG-3'
reverse primer: 5'-TGAAAGCGCCAGTGGA CTC-3'

The real-time PCR was performed using the following cycling parameters: 95° for 10 minutes for 1 cycle; 95° for 30 seconds, 60° for 1 minute, and 72° for 1 minute, for a total of 40 cycles. Expression of GAPDH mRNA served as loading control, and a melting curve analysis was performed after amplification to verify the accuracy of the amplicon.

Tissue factor activity assay

TF activity was analyzed in HAEC by a colorimetric assay (Actichrome TF, American Diagnostica) according to the supplier’s recommendations. Briefly, HAEC were grown, serum-starved, and stimulated in 6-well plates according to the treatment protocols. After stimulation, cells were washed twice with phosphate-buffered saline (PBS) and lysed by sonications in a buffer of 50 mM Tris-HCl, 100 mM NaCl, 0.1% Triton X-100, pH 7.4 for 30 minutes at 37 °C. The extracted tissue factors were placed in a 96-well microplate and incubated with human Factor VIIa, and human Factor X at 37 °C for 15 minutes. The formed FVIIa/FX complex could cleave a chromogenic substrate Spectrozyme FXa, which was added to each well and incubated at 37 °C for 20 minutes. This chromogenic reaction was stopped after
20 minutes by adding glacial acetic acid. The data were read by the absorbance of the reaction solutions at a 405 nm wavelength. A standard curve with different concentrations of lipidated human TF was constructed for interpolating the TF concentrations of the test samples.

**Western blotting for tissue factor**

Protein was quantified with BCA™ protein assay kit (PIERCE), and 35 μg protein were then loaded and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein was transferred onto PVDF membrane by semi-dry transfer at 5 volts for 100 minutes. Antibodies against tissue factor (American Diagnostica), GAPDH (Santa Cruz Biotechnology) were used at 1:1500 and 1:3000, respectively. Results were quantified by densitometry analysis of the bands and then normalization to GAPDH.

**Statistical analysis**

All data were presented as mean ± SEM. The significance was determined by unpaired t-test. A P < 0.05 was considered statistically significant.

**RESULTS**

**Lack of effects of thrombin stimulation on the morphology and cell viability of HAEC**

HAEC were cultivated in endothelial cell growth medium until the experiment was done. The HAEC were serum-starved in M-199 supplement with 1% fetal bovine serum for 24 hours and stimulated with human thrombin (1 unit/ml) for 5 hours. The morphology was not changed upon thrombin treatment (Figures 1A, 1B). Cell viability was also not changed by thrombin, as measured using MTS assay (P=NS). This result revealed that thrombin did not cause damage to HAEC during the experiments (Figure 1C).

**Thrombin enhanced TF mRNA expression in HAEC**

To determine whether human thrombin could affect TF mRNA expression, we treated the HAEC with thrombin (1 unit/ml) for 3 hours. We determined the TF/GAPDH mRNA relative quantity compared to control. The thrombin stimulation induced the relative mRNA quantity to increase 390 ± 114% (n = 5, P = 0.034) (Figure 2).

**Thrombin enhanced TF protein production in HAEC**

As expected from the TF mRNA up-regulation by
the thrombin, TF protein expression could be induced under thrombin treatment for 5 hours. The relative TF expression normalized to GAPDH increased $332 \pm 39\%$ ($n = 4$, $P = 0.01$) (Figure 3).

**Thrombin enhanced functional TF activity**

To determine whether thrombin could affect TF activity, we treated HAEC with thrombin for 5 hours to determine if their extracted proteins could trigger TF activity. As anticipated, the TF chromogenic assay showed enhancement of TF activity by $547 \pm 54\%$ ($n = 5$, $P < 0.001$) (Figure 4).

**DISCUSSION**

The central player of thrombotic complications in cardiovascular disease is the generation of thrombin through the tissue factor pathway. Ruptured plaque with exposure of underlying tissue factor is responsible for many atherothrombosis events, culminating in ischemic stroke, myocardial infarction, peripheral arterial occlusive diseases, and death. Thrombin, on the other hand, can exert a positive feedback to augment the tissue factor production. This vicious cycle was shown by our data, with thrombin proved to be a stress sufficient to trigger TF activity in endothelial cell origin from arterial side.

Previous study utilizing human saphenous vein endothelial cells (HSVEC) showed that thrombin-induced TF mRNA expression was time-dependent, with significant mRNA expression from 2 to 4 hours after thrombin stimulation and rapid decrease to baseline levels after 6 hours. The optimal tissue factor activity and protein expression in human umbilical vein cells (HUVEC), however, was 4-6 hours after thrombin stimulation. Based on the above time-course studies, we selected the 3-hour time point for TF mRNA study and the 5-hour time point for TF activity and protein expression study. A previous HSVEC study showed that thrombin treatment for 4 hours could increased TF mRNA to 8 times

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**Figure 2.** TF/GAPDH mRNA relative expression level upon thrombin stimulation. Real-time PCR demonstrated that relative quantities of TF mRNA level was enhanced $390 \pm 114\%$ (*$P = 0.034$ vs control). Data are shown as mean $\pm$ SEM from five independent experiments.

**Figure 3.** Western blot of TF protein expression upon thrombin stimulation. The results were quantified with densitometry analysis of the bands and then normalization to GAPDH. The TF protein was enhanced $332 \pm 39\%$ in TF protein expression (*$P = 0.01$ vs control). Data are shown as mean $\pm$ SEM from four independent experiments. Bolt was representative of four different experiments.

**Figure 4.** Functional TF activity upon thrombin stimulation. Human thrombin significantly increased the relative TF activity to $547 \pm 54\%$ (*$P < 0.001$ vs control). Data are shown as mean $\pm$ SEM from five independent experiments.
the control level, assessed by RT-PCR. Our HAEC data also showed a 3.9-fold significant increase in mRNA level upon thrombin stimulation. This difference in the fold changes was likely due to different cell sources, detection methods, and time course. Furthermore, cultured HUVEC could respond to different thrombin concentrations (10^{-2} – 10 NIH u/ml) with a 2- to 5-fold increase in TF activity. This result was consistent with our HAEC result of 5.47-fold increase in TF activity upon 1 unit/ml thrombin stimulation.

Expression of tissue factor in the endothelium is very rare in vivo, found, for example, in the splenic microvasculature in a baboon septic shock model or vascular endothelial cells in patients with invasive breast cancer. In-vitro studies, however, occasionally noted variable TF mRNA and activity expressions, even in non-perturbed conditions. Those stresses incurred during cell cultures were rendered minimally by 1% fetal bovine serum starvation to decrease the interference of endothelial cell culture medium and preserve the viability of endothelial cells in our study design. Therefore, the tissue factor activity was not totally absent in our control group, even after 24 hours’ starvation. However, thrombin stimulation could significant up-regulate the TF induction in HAEC. This increase in TF mRNA, protein, and functional activity supported that thrombin can up-regulate the TF gene expression via a transcriptional control.

Cultured endothelial cells can be induced to express tissue factor by a variety of agents, including tumor necrosis factor-α, interleukin-1β, CD40 ligand, serotonin, histamine, oxidized LDL, vascular endothelial growth factor, and thrombin. Thrombin can induce activation of c-Jun-N-terminal kinase and NF-κB activation in endothelial cells. Both JNK and NF-κB pathways are important transcription pathways for tissue factor expression in endothelial cells. The reported transcription factors involved in the tissue factor expression of HUVEC are c-Fos/c-Jun and c-Rel/P65. Since thrombin also induced TF mRNA expression in thrombin-stimulated HAEC, both c-Fos/c-Jun and c-Rel/P65 NF-κB transcription factors were considered to be involved.

We conclude that thrombin is a sufficient stress to induce TF expression in HAEC. Transcriptional control by thrombin causes an increase in TF mRNA. This increase in mRNA is modestly paralleled by an increase in protein level and functional activity.

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