Introduction

Tissue factor (TF) is an initiator in physiologic coagulation and pathologic thrombosis.¹² We had observed that the elevation of TF in plasma was associated with the onset of acute cerebrocardiac thrombotic diseases.² TF not only participates in the activation of blood coagulation, but also can mediate a series of cell responses to proinflammatory factors in the inflammation process.¹ Considerable evidence has been accumulated to indicate that there is a coagulation–inflammation network in vivo in which the expression of TF in activated vascular endothelial cells may present a major link between inflammation and thrombosis.³ Vascular endothelial cells participate in the regulation of hemostasis by controlling the expression of procoagulant factors as well as anticoagulant factors. TF is of major biologic significance for the procoagulant properties of endothelial cells and monocytes when they are activated by proinflammation cytokines, and functions as a membrane glycoprotein receptor that specifically binds coagulation factor VII or VIIa. Production of TF in endothelial cells and monocytes
has been associated with coagulopathy and thrombosis in irritated vessels in a number of diseases, such as septic shock, atheroma, and disseminated intravascular coagulation. Many pathologic agents are important in causing cell TF expression. Among them, proinflammatory tumor necrosis factor alpha (TNFα) is most strongly implicated in vascular disease.

Cinnamic acid (CINN) is the major component extracted from the Chinese herb cinnamon. CINN is demonstrated to be able to exert cytoprotective and antioxidant effects on endothelial cells, and anti-tumor effects in experimental animal models. However, whether it affects the procoagulant activity of endothelial cells remains unclear.

In this study, we investigated the effects of CINN on the expression of TF induced by TNFα in human umbilical vein endothelium-derived cell line ECV304 and the role of NF-κB in the down-regulation of TF expression caused by CINN.

Methods

Materials
Citrated normal human plasma was obtained from Changsha Red Cross Blood Center, Hunan, China. Human polyclonal anti-TF antibody was generously provided by Dr. J.H. Morrissey (Department of Biochemistry, College of Medicine/University of Illinois, Urbana-Champaign, IL, USA). DEGR-FVIIa was purchased from American Diagnostica Inc. (Stamford, CT, USA). RPMI-1640 cell culture medium and fetal bovine serum were from Gibco (Gaithersburg, MD, USA). Recombinant human TNFα came from Dingguo (Beijing, China). Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) cDNA probe was purchased from Sangon (Shanghai, China). Classic total RNA minipreps kit (Hyclone-Pierce) according to the manufacturer’s manual. The purity and integrity of total RNA were confirmed by the absorbance ratio of A260/A280 ( > 1.8) and agarose electrophoresis.

Culture of human umbilical vein endothelial cells
A human umbilical endothelium-derived cell line (ECV304) from the Chinese Type Culture Collection (Wuhan, China) was used in this study. The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and 50 µg/mL gentamycin, under an atmosphere of 95% air and 5% CO₂. Cells were maintained by subculturing 2 or 3 times per week. Before experiments, cells were washed twice with RPMI and cell viability was checked by trypan blue exclusion (viable cells > 95%).

Determination of ECV304 TF activity
After incubation of the cells (24-well plates, 1.5 × 10⁵ cells/well) with TNFα in the presence or absence of CINN, the cells were washed 3 times with D-Hank’s solution and scrape-harvested into 300 µL of D-Hank’s. The cells were frozen in liquid nitrogen and thawed in a water bath at 37°C for 3 cycles. The TF activity of the cell lysate was determined with 1-stage clotting assay that measures total cellular procoagulant activity as described previously. Briefly, 50 µL of lysate was added to 50 µL of citrate-anticoagulated pooled human normal plasma or factor VII-deficient plasma, and clotting was initiated by the addition of 50 µL of 25 mmol/L CaCl₂. The clotting time at 37°C was recorded. Each sample was run in duplicate. TF procoagulant activity (PCA) was quantitated by reference to standard curves constructed with rabbit brain thromboplastin as the TF source. The TF activity of 1 µL thromboplastin was arbitrarily defined as 1 × 10⁻³ mU. The standard curves were linear in the range of 0–1000 mU of TF activity (r² = 0.99).

Analysis of TF mRNA
Total RNA of ECV304 was prepared by Classic total RNA minipreps kit (Hyclone-Pierce) according to the manufacturer’s manual. The purity and integrity of total RNA were confirmed by the absorbance ratio of A260/A280 (> 1.8) and agarose electrophoresis.

The ECV304 cells were treated with TNFα in the presence or absence of CINN. TF mRNA was assessed using the RT-PCR method. Briefly, total RNA (1 µg) was reverse transcribed with the superscript preamplification system (Gibco BRL, Gaithersburg, MD, USA) using random hexamer primers, and the reverse-transcribed cDNA was then amplified by PCR using the primers prepared based on the mRNA sequences of TF or GAPDH. GAPDH gene is a housekeeping gene that is not affected by the used stimulants and was used as the control in this study. The primers used for TF mRNA amplification were the same as those described by Iochmann et al.
TF specific forward primer (5'-CTACTGTTCAGTTCA- 
GTGTTCAAGCGTAGA-3') and reverse primer (5'-CAGTGCAATAGCATTTGGCAGTAG C-3') 
correspond to positions 759–784 and 1016–1041 of 
TF cDNA, respectively, and amplify a 282-bp region 
comprising the 5th and 6th exons of TF. Primers 
were incubated in diaminobenzidine for color 
development. The ECV304 cells were washed once in phosphate-
buffered saline (PBS) and were lysed in ice-cold buffer 
containing 50 mM Tris (pH 8.0), 110 mM NaCl, 
5 mM EDTA, 1% Triton X-100, and 10 μg/mL 
phenylmethylsulfonyl fluoride (PMSF). Protein 
concentrations were determined using a Bio-Rad 
assay. Fifty micrograms of cell lysates were boiled in 
equal volumes of loading buffer (125 mM Tris-HCl, 
ph 6.8, 4% SDS, 20% glycerol, 2% β-mercaptoethanol, 
and 0% bromophenol blue) and were subjected to 
5–12% gradient gel electrophoresis in Tris/glycine 
buffer. Following electrophoretic separation, proteins 
on the gel were transferred to nitrocellulose membranes 
(Novex) using the Novex X Mini Gel system (Promega) 
for Western immunoblotting. Membranes were 
blocked with 5% nonfat dried milk in Tris-buffered 
saline (TBS) and 0.1% Tween 20 for 1 hour and then 
incubated with a rabbit polyclonal anti-IκB-α antibody 
at a dilution of 1:300 for 2 hours 
followed by washing twice with TBS containing 0.1% Tween 20, peroxidase-
conjugated antirabbit immunoglobulin G (IgG) 
antibody. After repeated washing, a strepavidin– 
biotin complex system with diaminobenzidene as 
chromogen was used for color development. Sections 
were finally counterstained with hematoxylin and 
examined under a light microscope.

Statistical analysis
Results are expressed as mean ± SD. Comparison 
between data from control and treatment groups was 
performed by Mann-Whitney rank-sum test. Correlation 
data from different groups was analyzed with Spearman rank correlation. Results 
were considered statistically significant at \( p < 0.05 \).
Effects of CINN on ECV304 TF mRNA expression induced by TNFα

After 2 hours of TNFα treatment, 1,000 U/mL of TNFα markedly induced TF mRNA expression. CINN significantly inhibited TF mRNA expression induced by TNFα. These effects of CINN on the TF mRNA expression appear to correlate well with those CINN exhibited on the total TF activity in ECV304, as shown in Figure 2.

Dynamic observation of the changes of I-κB protein in cytoplasm

The dynamic observation of the changes of I-κB protein in cytoplasm showed that I-κB protein began decreasing at 15 minutes after TNFα (1,000 U/mL) was added to ECV304 (n = 3, p < 0.05); I-κB decreased to its lowest point at 45 minutes and started recovery at 60 minutes and completely returned to the basic level at about 120 minutes (Figure 3). Figure 4 shows CINN might alleviate the decrement of I-κB protein in cytoplasm induced by TNFα.

The effect of CINN on the translocation of NF-κB from the cytoplasm to the nucleus

ECV304 cells without TNFα treatment were not stained in the nucleus after immunostaining. In contrast, the nuclei of the cells treated with TNFα for 45 minutes showed highly positive staining. The cells pretreated with CINN 15 minutes before the addition of TNFα to the cell culture showed much weaker staining compared with the cells treated with TNFα alone (Figures 5 and 6) (n = 4, p < 0.01).

Discussion

Numerous experimental and clinical studies have shown a variety of detrimental stimulations which could cause an inflammation reaction that was frequently followed by hypercoagulation.⁹,¹⁰ Although the mechanism by which inflammation and coagulation are linked is not fully elucidated, it is speculated to be related to the changes of the coagulation system, anticoagulation system, fibrinolysis system, antifibrinolysis and platelets, etc. Those system changes in turn influence the functions of vascular endothelium cells (VEC), especially the TF expression of VEC. Little TF is expressed in VEC under physiologic conditions, but when VEC is injured or challenged with inflammatory factors, a high level of TF might be expressed. The TF expression on VEC may be relevant to numerous conditions related to clinical medicine.
Cinnamic acid inhibits expression of tissue factor

Proinflammatory cytokines play a critical role during an inflammatory reaction. Inflammatory cytokines including TNFα, interleukin-1 (IL-1) and IL-8 may induce ECV304 and monocytes to express TF, and play an important role in TF expression of atherosclerosis plaque. Numerous studies have demonstrated that most cardiocerebral vascular thrombotic events are related to TF procoagulatory activity released from the broken atherosclerotic plaque. Therefore, the inhibition of coagulation initiated by inflammatory cytokines is an important strategy for prevention and treatment of thrombotic diseases.

CINN is known to exert cytoprotective and antioxidant effects on endothelial cells and is widely used in clinical practice of traditional Chinese medicine. These findings on CINN prompt us to investigate the effect of CINN on the procoagulant activity of human umbilical vein endothelial cells (HUVECs) stimulated with TNFα. The results of the present study showed that CINN significantly inhibited the expression of TF in ECV304 cells, and suggested that CINN does have an antithrombotic effect.

There is a coagulation–inflammation network formed by inflammatory factors and coagulant factors and their interactions. The TF expression on VEC is regulated by this coagulation–inflammation network. The inflammatory cytokines such as TNFα are stimulators of TF expression on VEC. On the other hand, TF belongs to the type II cytokine receptor family and may mediate the signal transduction involved in the inflammatory process. This study demonstrated that CINN was able to inhibit TNFα-induced TF expression on VEC, suggesting its potential anti-inflammatory effect.
While the study conclusively demonstrated the induction of TF expression in endothelial cells by TNFα, it was not designed to assess the cellular mechanisms for the inhibition of CINN on TF expression in HUVECs. TNFα has been shown to increase the TF mRNA level in endothelial cells predominantly by increasing transcription. In addition, TNFα-mediated activation of protein kinase C (PKC) was in general not required for NF-κB activation or for protein expression in endothelial cells. It is clearly explicated that the TNFα-induced TF expression of ECV304 mainly involves PKC, protein tyrosine kinase (PTK), and mitogen-activated-protein kinase (MAPK) pathways, but a co-pathway is exerted through NF-κB/IκB. The inactivated form of NF-κB is a heterodimer usually consisting of 2 subunits, the p65 and p50 proteins. In unstimulated cells, NF-κB is found in the cytoplasm and is bound to IκB, which prevents NF-κB from entering the nucleus. When these cells are stimulated by TNFα, specific kinases phosphorylate IκB, causing its rapid degradation by proteasomes. The release of IκB from NF-κB/IκB complex results in the passage of NF-κB from cytoplasm to the nucleus, where it binds to specific DNA sequences in the promoter regions of target genes. The potential implication of NF-κB activation in the TF expression involved in the pathogenesis of atherosclerosis is an attractive emerging idea, but there is evidence that intracellular signaling pathways in endothelial cells, initiated by diverse agonists that induce TF expression, converge in a common pathway involving NF-κB.

In the current study, the analysis of the TF mRNA expression, together with the finding that the changes of the amount of TF mRNA were transient and positively correlated with that of the TF activity, suggest that the inhibitory effect of CINN on TNFα-induced TF expression in HUVECs was probably a result of the interference of CINN in TF gene transcription. In addition, we also found that CINN could inhibit TNFα-induced TF expression of vascular endothelium cells by blocking NF-κB activation.

High expression of TF by endothelial cells and monocytes driven by pro-inflammatory substances, such as TNFα, may be responsible for some thrombotic diseases, and TF expression may also be involved in some cancer diseases. Our present study showed that CINN might influence the expression of TF locally, suggesting that CINN might have potential application in the prevention and therapy of systemic inflammation in addition to acting as an anticoagulant agent in vivo. However, the precise role of CINN in regulation of TF expression requires further elucidation, since multiple agonists are quickly generated and are present simultaneously with TF during an inflammation-associated thrombotic event.

In brief, the present study showed for the first time that CINN strongly inhibits TNFα-induced TF expression in endothelial cells. The results are favorable to understand the antithrombotic effect of CINN, and offer new therapeutic applications of CINN.

References