Cdc2-Like Kinases and DNA Topoisomerase I Regulate Alternative Splicing of Tissue Factor in Human Endothelial Cells

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Abstract—Tumor necrosis factor (TNF-α)–stimulated human umbilical vein endothelial cells express 2 naturally occurring forms of tissue factor (TF), the primary initiator of blood coagulation: the soluble alternatively spliced isoform and the full-length TF isoform. The regulatory pathways enabling this phenomenon are completely unknown. Cdc2-like kinases and DNA topoisomerase I regulate alternative splicing via phosphorylation of serine/arginine-rich proteins. In this study, we examined effects of serine/arginine-rich protein kinases on TF splicing following stimulation with TNF-α. Human endothelial cells were pretreated with specific inhibitors or small interfering RNAs against Cdc2-like kinases and DNA topoisomerase I before stimulation with TNF-α. TF levels were determined by semiquantitative RT-PCR, real-time PCR, and Western blotting. Cellular procoagulant activity was analyzed in a chromogenic TF activity assay. All 4 known Cdc2-like kinases forms were expressed in human endothelial cells. Selective inhibition of Cdc2-like kinases and DNA topoisomerase I elicited distinct changes in TF biosynthesis in TNF-α–stimulated endothelial cells, which impacted endothelial procoagulant activity. This study is the first to demonstrate that serine/arginine-rich protein kinases modulate splicing of TF pre-mRNA in human endothelial cells and, consequently, endothelial procoagulant activity under inflammatory conditions. (Circ Res. 2009;104:589-599.)

Key Words: cardiovascular research ■ endothelial cells ■ tissue factor ■ tumor necrosis factor ■ vascular biology

Endothelial cells (ECs) are crucial regulators of vascular wall homeostasis and blood thrombogenicity. inflammatory cytokines switch the endothelial phenotype from an anti-toward prothrombogenic. Tissue factor (TF) is the primary initiator of the coagulation cascade. We recently showed that the cytokine TNF-α induces human ECs to express 2 TF forms, the membrane bound full-length TF (fTF); and the soluble alternatively spliced TF (sHTF). fTF originating from ECs and the soluble, secreted asHTF circulate in blood and participate in thrombogenesis. Alternative pre-mRNA splicing is essential for the plasticity of the proteome in response to environmental changes. Serine/arginine-rich (SR) proteins are a family of highly conserved factors required for constitutive and alternative pre-mRNA splicing. The Cdc2-like kinase (Clk) family, comprising 4 members (Clk1/Sty and Clks 2 to 4), and DNA topoisomerase I (DNA topo I) are known to phosphorylate SR proteins. Phosphorylation-dependent activity of SR proteins is critical to alternative splicing. In human endothelial cells, TF pre-mRNA processing is induced by cytokines, and in human platelets, it is evidently induced by thrombin: inhibition of Clk1 in thrombin stimulated platelets reduced phosphorylation of the essential SR protein SF2/ASF and inhibited splicing of TF pre-mRNA into fTF mRNA, thereby reducing fTF protein expression.

Despite extensive literature on EC biology, it is completely unknown whether SR protein kinases are expressed in ECs and/or involved in regulated pre-mRNA splicing in this cell type. We assessed the role of Clks and DNA topo I in regulation of TNF-α–induced expression of TF in human ECs, with a special focus on EC procoagulant activity and the phosphorylation state of SR proteins.

Materials and Methods

Cell Culture

For details regarding the cell culture maintenance, treatment with pharmacological agents, and experimental conditions, see the expanded Materials and Methods section in the online data supplement, available at http://circres.ahajournals.org.
Semiquantitative RT-PCR
Total RNA was reverse transcribed using AMV (Roche Diagnostics GmbH, Mannheim, Germany), and cDNAs encoding Clk forms 1 to 4, DNA topo I, and the splice variant TF-A were amplified. See the online data supplement for primers and PCR conditions. iTF- and asHTF-specific primers were described.17

TF Isoform–Specific Real-Time PCR
Real-time PCR (TaqMan) using iTF-, asHTF-, and GAPDH-specific primers and probes was performed as described.15

Transfection Experiments
Human umbilical vein endothelial cell (HUVECs) (1×10⁶) were transfected with 75 pmol of small interfering (si)RNA (online data supplement). Forty-eight hours after transfection, cells were stimulated with TNF-α, total RNA collected, reverse-transcribed, and subjected to real-time PCR.

Western Blotting
Analyses of inhibited, stimulated, and unstimulated ECs were performed as described2; the used antibodies are listed in online data supplement.

Measurement of TF Activity
Determination of total procoagulant activity of stimulated and nonstimulated ECs and ECs pretreated with inhibitors was performed as described.2

Quantification of Western Blots and RT-PCR
The results of Western blot and RT-PCR experiments were quantified using Gel-Pro Analyzer software version 4.0.00.001 (Media Cybernetics, Bethesda, Md).

Flow Chamber Experiments
ECs were grown on fibronectin-coated cover slips to confluence and then placed into a flow chamber with parallel-plate geometry. ECs were pretreated with inhibitors, stimulated for 8 hours with TNF-α, and then placed into the flow chamber. Perfusions were performed for a period of 15 minutes at the flow rate of 116 mL/min (calculated shear rate: 366 sec⁻¹). The flow chamber temperature was set at 37°C throughout the entire experiment; perfusion buffer composition: 20 mmol/L Hepes, 150 mmol/L NaCl, 2 mmol/L CaCl₂, pH 7.4, supplemented with physiological concentrations of FVIIa (0.1 mmol/L) and FX (170 mmol/L) (American Diagnostica, Greenwich, Conn).8 To exclude possible influence of the intrinsic pathway on FXa generation, 50 μg/mL corn trypsin inhibitor (Calbiochem, Darmstadt, Germany) was added to the perfusion buffer prewarmed to 37°C. The flow rate was controlled using Harvard syring pump (Harvard Apparatus Co Inc, Dover, Mass). Aliquots (100 μL) of the perfusion buffer were removed at 10 and 15 minutes, and FXa generation was stopped by adding EDTA (25 mmol/L). After that, perfusion buffer/EDTA mixtures were supplemented with 2.5 U/mL recombinant hirudin (Sigma Aldrich, St Louis, Mo) to inhibit thrombin, and FXa levels were determined by adding the chromogenic FXa substrate Spectrozyme FXa (American Diagnostica); reactions were read at 405 nm as described.17

Statistical Analysis
All data were expressed as mean±SEM. Data were analyzed by Student’s t test or 1-way ANOVA. A probability value ≤0.05 was deemed significant.

Results
Detection of SR Protein Kinases in HUVECs
Alternative splicing of human Clk1 to 4 pre-mRNA generates catalytically inactive, truncated Clk isoforms lacking the protein kinase domain.9,20 To analyze Clk expression in HUVECs, RT-PCR using primers specific for each of the Clk-family members, and Western blotting of HUVEC lysates were performed. Using PCR primers spanning the known alternatively spliced regions of Clk1, -2, -3, and -4, we detected all 4 Clks, and the alternatively spliced truncated forms of Clk1, -2, and -4, but not Clk3, in untreated HUVECs (Figure 1A and 1D). Stimulation with TNF-α led to an appreciable increase in mRNA levels of all 4 full-length Clks, whereas the expression of the truncated forms was decreased. Protein levels of all 4 Clks were also increased 5 hours after stimulation with TNF-α (Figure 1B).

DNA topo I mRNA and protein were also detected in nontreated HUVECs, and stimulation with TNF-α increased DNA topo I expression (Figure 1C).

Modulation of Alternative TF Pre-mRNA Splicing in Human ECs
Unstimulated HUVECs do not constitutively express TF.2 Treatment of resting cells with Clk or DNA topo I inhibitors had no effect on TF expression (data not shown). Stimulation with TNF-α led to a 14-fold increase in asHTF mRNA, and a 24-fold increase in iTF mRNA levels compared to nontreated controls (Figure 2A, 2B, and 2E).

Pharmacological inhibition of Clks lowered TNF-α–induced expression of asHTF mRNA to baseline, whereas inhibition of DNA topo I led to a 3-fold increase in asHTF mRNA expression compared to TNF-α–stimulated HUVECs (Figure 2A). Inhibition of Clks also reduced the TNF-α induced increase in iTF mRNA levels by ≈50% (Figure 2B). In contrast to the observed effect on asHTF upregulation by TNF-α, upregulation of iTF was reduced to near-baseline levels when HUVECs were pretreated with a DNA topo I inhibitor (Figure 2B). Transfection of cells with siRNA against Clk1, Clk4, and DNA topo I yielded results analogous to those observed after pharmacological inhibition (Figure 2C and 2D).

Inhibition of SR protein kinases was associated with the generation of a third, noncoding TF mRNA form: on TNF-α stimulation, we observed a PCR product (140 bp) corresponding to exon 1 normally spliced to exon 2; however, pretreatment of TNF-α–stimulated cells with the Clk inhibitor resulted in appearance of a larger PCR product (Figure 2F). The length (635 bp) and sequence of this product corresponded to the noncoding mRNA form TF-A, in which a 495 bp segment of intron 1 is included between exon 1 and 2 because of alternative splicing (Figure 2G and Figure I in the online data supplement).21,22 Searches in various databases, performed to ascertain whether TF-A like variants exist in other species, produced no significant matches, indicating that this splicing event may be unique to human TF pre-mRNA processing (data not shown). Cumulatively, this data indicates that Clks and DNA topo I modulate the splicing pattern of TF pre-mRNA in TNF-α–stimulated HUVECs.
To verify that these findings are not unique to HUVECs, we used human aortic endothelial cells (HAoECs) and performed mRNA expression analyses for SR protein kinases and TF mRNA isoforms as above. We found that mRNA expression of asHTF, flTF, TF-A, Clks, and DNA topo I is analogous to that observed in HUVECs (supplemental Figure II): stimulation of HAoECs with TNF-α/H9251 induced asHTF and flTF expression, Clk inhibition reduced the expression of both TF isoforms, and inhibition of DNA topo I significantly induced asHTF and reduced flTF mRNA expression (Figure 3A and 3C). Unlike Clk inhibition, but not by DNA topo I inhibition in TF-α-stimulated HAoECs (supplemental Figure II, C), DNA topo I and Clk isoforms were also expressed in HAoECs, as seen in HUVECs after TNF-α induction; TF-α stimulation induced the expression of all full-length Clks, as well as DNA topo I, and reduced the expression of “truncated” Clks after 1 hour (supplemental Figure II, D). Thus, the effects of Clk and DNA topo I inhibition on TF pre-mRNA splicing are similar in venous and arterial human ECs.

Reduction of TNF-α-Induced Procoagulant Activity

Western blotting was performed to assess the effect of Clks and DNA topo I inhibition on protein levels of the 2 TF isoforms, flTF and asHTF were elevated 5 and 8 hours after stimulation with TNF-α (Figure 3A and 3C). Clk inhibition did not affect TNF-α-induced increase in flTF protein, whereas DNA topo I inhibition markedly reduced flTF upregulation. Compared to flTF, levels of asHTF were affected by the inhibitors in the opposite fashion: Clk inhibition reduced asHTF protein levels, whereas DNA topo I inhibition resulted in increased asHTF levels compared to TNF-α-stimulated controls (Figure 3A and 3C).

To determine the effect of SR protein kinases on EC procoagulant activity, a chromogenic TF activity assay was performed.

Figure 1. Expression of Clk forms and DNA topo I in human ECs. A, Expression of mRNA encoding Clks (Clk1 to 4) and their alternatively spliced isoforms in untreated controls (control) and TNF-α-stimulated cells 60 minutes posttreatment. The shown PCR products are Clk1 (347 and 437 bp), Clk2 (199 and 286 bp), Clk3 (300 bp), Clk4 (447 and 520 bp), and GAPDH (185 bp). B, Western blot of Clk1 (57 kDa), Clk2 (60 kDa), Clk3 (59 kDa), and Clk4 (58 kDa) proteins 5 hours after TNF-α stimulation. C, mRNA (291 bp) and protein (37 kDa) expression of DNA topo I 60 minutes and 5 hours after stimulation with TNF-α. GAPDH, loading control. A through C are representative of at least 3 independent experiments. D, Schematic representation of the Clk forms and DNA topo I in 5’ to 3’ orientation of the sense strand, including the corresponding accession numbers, positions of the alternatively spliced regions (darkened domains), and the primers used in the study.
performed (Figure 3B and 3D). Five and 8 hours after stimulation with TNF-α, TF activity was increased up to 5-fold. In line with the protein expression data (Figure 3A and 3C), Clk inhibition did not affect TF activity 5 hours after TNF-α stimulation (Figure 3B), yet after 8 hours, procoagulant activity was reduced (Figure 3D). In contrast to Clk inhibition, DNA topo I inhibition significantly decreased TNF-α–induced upregulation of TF activity at 5 and 8 hours. 

Figure 2. Inhibition of Clks and DNA topo I impacts alternative TF pre-mRNA splicing. A and B, Ratio of the TF mRNA expression normalized to GAPDH, $5 \times 10^6$ HUVECs 1 hour after TNF-α stimulation. A, asHTF mRNA expression in nonstimulated cells (control), TNF-α–stimulated cells (TNF-α), stimulated cells pretreated with Clk inhibitor (Clk Inh), or cells pretreated with DNA topo I inhibitor (DNA topo I Inh). B, itTF mRNA expression. C and D, TF isoform mRNA expression 48 hours after transfection of $1 \times 10^6$ HUVECs with siRNAs against Clk1 (siClk1), Clk4 (siClk4), and DNA topo I (siRNA topo I) 1 hour after TNF-α stimulation. C and D, asHTF (C) and itTF (D) mRNA expression. In A through D, the means±SEM of at least 14 (A and B) or 3 (C and D) independent experiments are shown. +P<0.05, #P<0.01, §P<0.0001; i.t. indicates P<0.15 (in trend, comparison of unstimulated controls with TNF-α–stimulated cells); n.s. indicates no significant difference. E and F, RT-PCR of itTF, asHTF, and TF-A mRNA 1 hour after TNF-α stimulation. Marker, 100-bp DNA ladder. The displayed amplicons are itTF: 931 bp (E) or 140 bp (F); asHTF: 771 bp (E); TF-A: 635 bp (F). GAPDH (185 bp), loading control. The results are representative of at least 3 independent experiments. G, Schematic representation of TF pre-mRNA, itTF, asHTF, and TF-A mRNA and the corresponding splicing events.
These observations suggest that Clks and DNA topo I regulate surface bound and secreted TF activity of TNF-α-stimulated ECs.

Effects of Clk and DNA Topo I Inhibition on SR Protein Phosphorylation

To assess the phosphorylation state of SR proteins, Western blotting was performed, and results were quantified. The antibody mAb1H4 selectively detects phosphorylated SR proteins as efficiently as the related, isotypically distinct anti-SR protein antibody mAb104 that recognizes hypophosphorylated SR proteins. SRp75, SRp55, SC35, and SF2/ASF were detected in HUVECs (Figure 4A). Compared to TNF-α-stimulated cells, Clk inhibition followed by TNF-α stimulation decreased phosphorylation of SRp75, SRp55, and SF2/ASF (Figure 4B, 4C, and 4E). Inhibition of DNA topo I decreased phosphorylation of SRp55 and SF2/ASF, whereas phosphorylation of SRp75 was not affected. Neither Clk nor DNA topo I inhibition affected the expression levels of SR proteins (Figure 4A). These results indicate that in ECs, Clks and DNA topo I modulate the phosphorylation state of SR proteins, but not their expression levels.
Figure 4. Inhibition of the Clk-family and DNA topo I impacts the phosphorylation state of SR proteins in human ECs. A, Left, Western blots, SR protein phosphorylation pattern in resting HUVECs, 2 minutes after TNF-α stimulation, 2 and 20 minutes without stimulation. Right, SR proteins detected using a phosphorylation-independent anti-SR protein antibody. Displayed are nonstimulated cells (control), TNF-α–stimulated cells (TNF-α), and stimulated cells pretreated with Clk inhibitor (Clk Inh) and DNA topo I inhibitor (DNA topo I Inh). The results are representative of least 3 independent experiments. B through E, Western blot quantification. Shown is the total band density as mean±SEM. *P<0.001, #P<0.01, +P<0.05; i.t. indicates P<0.15 (in trend); n.s. indicates no significant difference.
Impact of siRNA-Mediated Silencing of SRp75 and SF2/ASF on ECs Procoagulant Activity

The results of SR phosphorylation prompted us to evaluate the effect of downregulating SRp75, a structurally divergent SR protein whose phosphorylation level was uniquely affected by Clk inhibition, and SF2/ASF, an essential SR protein whose phosphorylation state was similarly affected by inhibition of Clk and DNA topo I (Figure 4A). The role of SR proteins SRp75 and SF2/ASF in modulating TF expression and activity was assessed as follows. We transfected HUVECs with siRNA against SRp75 alone, SF2/ASF alone and against both SR proteins (Figure 5). Transfection with siRNA against SF2/ASF increased asHTF mRNA and reduced iTF mRNA levels 1 hour after TNF-α stimulation (Figure 5A and 5B), anti-SRp75 siRNA alone had no effect on asHTF mRNA expression, yet it reduced iTF mRNA expression. Transfection of cells with anti-SF2/ASF and anti-SRp75 siRNA in combination resulted in reduced asHTF and iTF mRNA expression. Transfection of cells with control (nonsense) siRNA had no significant effect. The obtained data for the TF activity of siRNA-transfected HUVECs was in agreement with the mRNA data (Figure 5C and 5D); treatment with TNF-α increased TF activity 5 and 8 hours after stimulation, transfection of cells with nontarget siRNA had no impact on the TNF-α-induced increase in TF activity, and inhibition of SF2/ASF alone or SRp75 and SF2/ASF together reduced total TF activity 5 hours after stimulation. Eight hours after TNF-α induction, total TF activity of HUVECs transfected with siRNA against both SR proteins alone or in combination was significantly reduced.

As shown in Figure 5E through 5G, mRNA expression levels of SF2/ASF and SRp75 mRNA were significantly decreased 48 hours after siRNA transfection, and protein levels of SF2/ASF and SRp75 were also significantly reduced 48 hours after siRNA transfection and 5 hours after TNF-α stimulation (Figure 5E). siRNA-mediated inhibition of SF2/ASF or SRp75 was highly specific in that it only influenced the expression of the target SR protein. This data indicate that SF2/ASF and SRp75 participate in differential TF isoform expression, thereby modulating TF-mediated procoagulant activity of TNF-α-stimulated ECs.

Inhibition of SR Protein Kinases in ECs Decreases TF-Dependent FXa Generation Under Flow Conditions

To assess the possible physiological relevance of blocking Clks or DNA topo I in ECs, we used a reconstitutive flow chamber in which a HUVEC monolayer was perfused with a physiological buffer containing biologically relevant concentrations of FVIIa and FX.18 To assess the impact of Clk or DNA topo I inhibition on TNF-α induced TF activity under flow conditions, we measured the rate of FXa generation 8 hours after stimulation, at 10 and 15 minutes of perfusion (Figure 6A and 6B). The baseline rate of FXa generation was ~4 nmol/L in a volume of 100-µL perfusion solution. As expected, TNF-α stimulation led to a 2.5-fold increase of FXa generation 10 and 15 minutes postperfusion. Inhibition of Clks significantly reduced the TNF-α-induced increase of FXa generation at 10 and 15 minutes postperfusion. Inhibition of DNA topo I also reduced FXa generation at both time points. These observations indicate that under physiologically relevant flow conditions, Clks and DNA topo I modulate the TF-mediated procoagulant potential on the surface of TNF-α-stimulated ECs.

Discussion

Regulation of Alternative TF Pre-mRNA Splicing in Human ECs

Alternative pre-mRNA splicing is a fundamental mechanism for posttranscriptional regulation of gene expression.10,26 Clks and DNA topo I modulate phosphorylation of SR proteins, thereby regulating alternative splicing.15,27,28 The expression pattern of all 4 Clk-family members in human ECs is reported here for the first time. Further, we demonstrate that human ECs express full-length Clk1 to -4 and the alternatively spliced truncated forms of Clk1, -2, and -4, but not of Clk3. Stimulation of cells with TNF-α increased the expression of full-length Clk isoforms, and decreased the expression of the truncated isoforms of Clk1, -2, and -4. The full-length Clk1 is known to form heterodimers with the truncated Clk1 form in vitro,19 and it was suggested that Clk1 heterodimerization may be involved in the regulation of Clk1 activity.19 Thus, TNF-α-induced changes in the splicing pattern of active full-length Clks and their inactive truncated counterparts are likely to reflect regulation of Clk activity in ECs under normal and proinflammatory conditions. We also find that human ECs express abundant levels of DNA topo I, as previously reported by other groups.14

Our study demonstrates that Clks and DNA topo I regulate differential expression of TF mRNA isoforms in TNF-α-stimulated ECs. Additionally, we found that Clks, but not DNA topo I, are involved in the generation of a noncoding TF mRNA splice variant (TF-A) after TNF-α induction. This variant was previously detected only in cancer cell lines,21 and although its biological role is unknown, generation of TF-A is likely to be a feature of the cellular regulatory machinery controlling the levels of the produced TF proteins. Together, our observations support the notion that Clks and DNA topo I regulate alternative splicing of human TF pre-mRNA via distinct intracellular pathways.

Modulation of TF-Mediated Procoagulant Activity of Human ECs

The observation that Clks and DNA topo I modulate TF mRNA and protein isoform expression is consistent with reports of other groups.15,17,20 Schwertz et al showed that inhibition of Clk1 reduces iTF mRNA expression and protein synthesis in platelets; however, asHTF mRNA was not observed in resting or thrombin stimulated human platelets.16 Our data demonstrate only a partial reduction of iTF expression in ECs following Clk inhibition. One possible explanation of incomplete iTF reduction by Clk inhibition is the fact that all 4 Clks are expressed in ECs, whereas platelets possess exclusively Clk1. The specific Clk inhibitor used in this study, named Tg003, blocks Clk1, -2, and -4, but not Clk3, at the concentrations used here.13 Thus, it is possible that iTF biosynthesis was only partially inhibited in our experiments because of a lack of Clk3 inhibition. More-
Figure 5. SRp75 and SF2/ASF silencing elicits changes in TF mRNA expression. A and B, asHTF and flTF mRNA expression levels 48 hours after siRNA transfection and 60 minutes after TNF-α/H9251 stimulation: shown are nonstimulated cells (control), TNF-α/H9251–stimulated cells (TNF-α), cells transfected with nontarget control siRNA (siNon-target control), and cells transfected with siRNA against SF2/ASF (siASF), SRp75 (siSRp75), and both SR proteins (siASF + siSRp75). A, asHTF mRNA expression. B, flTF mRNA expression. C and D, TF activity 48 hours after siRNA transfection and 5 hours (C) or 8 hours (D) after TNF-α stimulation. The data are displayed as arbitrary units of TF activity per 10⁶ cells. E and F, Transfection efficiency of siRNAs. In A through F, the means ± SEM of at least 5 independent experiments are shown. +P<0.05, #P<0.01, *P<0.001, §§P<0.0001; i.t. indicates P<0.15 (in trend) compared to nonstimulated controls or TNF-α-stimulated cells; n.s. indicates no significant difference. G, Western blot, SF2/ASF, and SRp75 in HUVECs transfected with specific siRNA as indicated.
over, Clk inhibition led to reduced asHTF protein expression. Thus, Clks appear to regulate expression of both TF isoforms in ECs.

DNA topo I was proposed to be essential for exonic splicing enhancer (ESE)-dependent splicing events. We show that DNA topo I inhibition strongly reduces flTF protein expression, leading to a drastic reduction of total TF activity. In contrast to flTF, asHTF protein expression was significantly increased. Although asHTF promotes FXa generation in the presence of phospholipids, the procoagulant activity of asHTF is much lower than that of flTF. Thus, the TF activity assay used here may not be sensitive enough to assess relative contribution of asHTF and flTF to the total TF activity in the same sample. At the present time, there is no TF activity assay available that is optimized to delineate the relative impact of asHTF and flTF on procoagulant activity; we note, however, that asHTF is a secreted protein released by ECs, and asHTF-associated procoagulant activity in the vasculature is thus expected to circulate in plasma, rather than be localized in ECs. Another noticeable point is a slight divergence between the effects of Clks or DNA topo I on TF mRNA and protein expression level. Clk inhibition reduced flTF mRNA levels by 50% and asHTF almost completely 60 minutes after TNF-α stimulation. Inhibition of DNA topo I reduced flTF mRNA by 75% and increased asHTF. flTF protein expression after Clk inhibition was reduced later (8 hour), compared to DNA topo I inhibited HUVECs (5 and 8 hours after TNF-α induction). Also, the intensity of TF activity reduction by Clk inhibition was found to be 30%, whereas inhibition of DNA topo I led to a more pronounced reduction of TF activity. A possible explanation for this divergence is the dramatically increased ratio of asHTF to flTF in DNA topo I inhibited cells, compared to Clk inhibited HUVECs, which in turn could favor the expression of secreted asHTF and thus additionally reduce flTF protein expression and total TF activity on EC surface. Alternatively, flTF protein expression may be modified earlier (5 hours after stimulation) in DNA topo I–inhibited cells, compared to cells treated with the Clk inhibitor. In sum, we demonstrate that selective inhibition of Clks and DNA topo I in ECs before TNF-α stimulation modulates the ratio of the expressed TF isoforms, thereby dramatically impacting TF-mediated surface and secreted procoagulant activity of human ECs.

The Role of SR Protein Phosphorylation

Our results demonstrate that, in TNF-α–stimulated ECs, Clks and DNA topo I alter the phosphorylation pattern of SR proteins, but not their expression levels. SR proteins are essential regulators of alternative splicing. Splice site selection is largely determined by cis-acting regulatory sequences, such as ESE, whereas activity, localization, and RNA binding affinity of SR proteins is determined by their reversible phosphorylation. Exon 5 of the human TF gene contains functional ESE for SF2/ASF, an essential SR protein. In line with our data, inhibition of Clk1 was found to repress SF2/ASF phosphorylation, which was associated with a significant reduction in flTF expression in thrombin stimulated platelets. In ECs, Clks and DNA topo I appear to participate in alternative splicing of TF pre-mRNA, most likely by modulating the phosphorylation state of SR proteins. Binding affinity of SR proteins to target ESE is dependent on SR protein phosphorylation. Thus, it is feasible that altered ESE recognition elicits changes in the structure and the levels of multiple mRNA species generated from a single pre-mRNA.
We demonstrate here that SF2/ASF and SRp75 participate in the regulation of differential TF isomform expression in human ECs, thereby modulating their TF-mediated procoagulant activity. SF2/ASF and SRp75 are known to regulate alternative pre-mRNA splicing.\textsuperscript{12,16,29} Simultaneous silencing of both SR proteins lowers asHTF and fITF mRNA expression levels to those observed following pharmacological inhibition of Clks, when SF2/ASF and SRp75 are hypophosphorylated. Total TF activity is reduced in such treated HUVECs in a time-dependent manner 8 hours after, but not 5 hours after, TNF-α stimulation, in line with pharmacological Clk inhibition. Silencing of SF2/ASF alone increases asHTF mRNA but reduced fITF mRNA expression to the levels comparable to those observed following pharmacological inhibition of DNA topo I, when SF2/ASF phosphorylation is reduced. Cellular procoagulant activity is reduced 5 and 8 hours after stimulation, as also observed in cells treated with the DNA topo I inhibitor. These results suggest that Clks and DNA topo I, and the SR proteins SF2/ASF and SRp75, participate in the regulation of alternative splicing of TF pre-mRNA and, consequently, modulate the nature of TF expressed by human ECs. A possible mechanistic explanation could be that Clks and/or DNA topo I regulate the phosphorylation state of SF2/ASF, SRp75, or both, thereby controlling their ability to modulate TF pre-mRNA splicing.

**Possible Physiological Relevance of SR Protein Kinases in Regulated TF Expression**

The data from our flow chamber experiments demonstrates that inhibition of Clks and DNA topo I reduces the surface EC procoagulant activity in a system approximating physiological conditions, albeit with different intensities. Reduction of FXa generation on the EC surface was more striking after DNA topo I inhibition, compared to inhibition of Clks. These observations are in line with the data obtained from static assessment of TF activity (Figures 2 and 3). We note that, although DNA topo I inhibition yields a more profound reduction in surface TF activity, it also potentiates production of secreted asHTF and is thus likely to increase TF activity of circulating plasma.

In summary, our findings reveal that regulation of the differential TF isomform expression in ECs modulates the activation of the coagulation cascade under static and flow conditions and therefore constitutes a previously unknown regulatory mechanism of EC procoagulant activity. Our study also offers principally novel insights into the regulatory network of alternative splicing processes in human ECs, essential regulators of vascular wall homeostasis and blood thrombogenicity. Inflammatory processes that impair these functions contribute to pathogenesis of cardiovascular diseases such as arteriosclerosis.\textsuperscript{30} Based on our observations, we propose that alternative TF splicing is likely critical to maintenance of endothelial homeostasis.

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**Disclosures**

None.

**References**


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Supplement Material.

**Cell culture.** Human aortic endothelial cells (HAoEC; kindly provided by Dr. M. Lorenz, Charité – Universitätsmedizin Berlin, Germany) and pooled HUVEC (PromoCell, Heidelberg, Germany) were cultured in EC growth medium containing 5% fetal calf serum at 37°C in a humidified incubator (5% CO₂, 95% air). Cells from passages 3 to 6 were used. For inhibition experiments, HUVEC were switched to EC basal medium (without fetal calf serum) for 1 h, pre-treated with Clk inhibitor Tg003 (10 µM) or DNA topo I inhibitor camptothecin (100 nM) (both from Calbiochem, Darmstadt, Germany) for 1 h, and then stimulated with TNF-α (Sigma Aldrich, St Louis, USA); cells were stimulated with TNF-α (10 ng/mL) for 1 h for mRNA analysis, 5 and 8 h for protein analysis to ensure that the mRNA and protein levels are sufficient for reliable detection, or 2 min for assessment of the phosphorylation state of SR proteins. Positive controls were stimulated only with TNF-α, and negative controls were non-treated.

**Semi-quantitative RT-PCR:** For RT-PCR the following primers were used: Clk1 For (5’-GAACAAGCGCTGCAAATACA-3’), Clk1 Rev (5’-TACCTCCCCGCTTTATGATCG-3’), Clk2 For (5’-AATATCAGCGGGAGAACAGC-3’), Clk2 Rev (5’-ATGATCTTCAGGGCAACCTCG-3’), Clk3 For (5’-TTCACGTTTCTCAGTCATCGTC-3’), Clk3 Rev (5’-TTCAGGGCAACCTGAGACTT-3’), Clk4 For (5’-AAGTCACAAGCGGAGAGGA-3’), Clk4 Rev (5’-CACGGTAACGGCCTACTTT-3’), DNA topo I For (5’-CTCCACAACAGATCCCA GAT-3’), DNA topo I Rev (5’-TATTTTTGCACTCCGAGAGG-3’), hTF E1 For (5’-CTCGGCTGGGTCTTC-3’) and hTF-E2 Rev (5’-GTGTTAGCTTTGAGC GGTTG-3’). PCR conditions: 94°C, 2 min, and 36 cycles of 94°C, 30 sec; 58°C, 25 sec; and 72°C, 1 min. PCR products were separated on 1.5% agarose gels, excised, purified, and their identity confirmed by automated sequencing.

**Transfection Experiments:** HUVEC were transfected with siRNAs against Clk1, Clk4, and DNA topo I (Applied Biosystems, Darmstadt, Germany), or against SF2/ASF and SRp75 (Dharmacon, Chicago, USA) using a HUVEC Nucleofector® Kit (Amaza Biosystems, Cologne, Germany).
**Western Blotting:** For protein detection, primary monoclonal antibodies recognizing flTF (clone TFE, Enzyme Research Laboratories, South Bend, USA), Clk 1, 2, 3 and 4 (ABGENT, San Diego, USA), SF2/ASF, phosphorylated SR proteins - clone 1H4, and total SR proteins - phosphorylation-independent clone 16H3E8 (Invitrogen GmbH, Karlsruhe, Germany), SC35 - clone SC-35 (Abcam, Cambridge, UK), DNA topo I - clone 1A1 (Abnova GmbH, Heidelberg, Germany), and asHTF (custom polyclonal antibodies raised against the unique C-terminal asHTF domain, Pineda Antikoerper-Service, Berlin, Germany), and the corresponding horseradish-peroxidase conjugated secondary antibodies were used.
Online Figure I

Exon 1

Exon IA

Exon 2

TF-E1 for

gttggg tagaggcaat

gc

TF-E2 rev
Online Figure II
Online Figure I. Results of the sequence analysis of the TF-A amplicon. Sequence analysis of the TF-A amplicon, corresponding to the known sequence (GenBank accession no. AY940729); 5'-3' orientation of the sense strand is shown. The primer locations are underlined, the exonic sequences are in bold letters, and the sequence corresponding to TF exon 1A is in italics.
Online Figure II. Effects of Clk and DNA topo I inhibition on TF mRNA expression and expression of Clks or DNA topo I in HAoEC. (A, B) Shown is the fold induction of TF mRNA expression normalized to GAPDH and referred to non-treated controls (set as “1”) of 5 x 10^5 HAoEC 1 h post TNF-α stimulation. (A) asHTF mRNA expression (B) flTF mRNA expression. Shown are non-treated cells (control), TNF-α stimulated cells (TNF-α), and TNF-α stimulated cells pre-treated with Clk inhibitor (Clk Inh) or DNA topo I inhibitor (DNA topo I Inh). (+) p<0.05; (#) p<0.01; compared to non-stimulated controls or TNF-α stimulated cells; (n.s.) no significant difference. (C) RT-PCR of flTF and TF-A mRNA 1 h post TNF-α stimulation of HAoEC. Marker, 100 bp DNA ladder. The displayed amplicons are flTF (140 bp), TF-A (635 bp), and GAPDH (185 bp). (D) Expression of mRNA encoding Clks (Clk 1-4) and their corresponding alternatively spliced isoforms, DNA topo I, and GAPDH (loading control) in non-stimulated (control) and TNF-α induced (TNF-α) HAoEC. The sizes of all PCR products are shown in brackets. In A – D, all analyses were performed 60 min post TNF-α treatment and are representative of at least three independent experiments.