TRAIL/Apo2L Mediates the Release of Procoagulant Endothelial Microparticles Induced by Thrombin In Vitro A Potential Mechanism Linking Inflammation and Coagulation

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A Potential Mechanism Linking Inflammation and Coagulation

Stéphanie Simoncini,* Makon-Sébastien Njock,* Stéphane Robert, Laurence Camoin-Jau, José Sampol, Jean-Robert Harlé, Catherine Nguyen, Françoise Dignat-George, Francine Anfosso

Abstract—Microparticles are small vesicles playing a crucial role in cell communication by promoting prothrombotic and proinflammatory responses. However, the molecular mechanisms underlying their release are still elusive. We previously established that thrombin promoted the generation of endothelial microparticles (EMPs). In the present study, gene profiling identified *TRAIL/Apo2L*, a cytokine belonging to the tumor necrosis factor-α superfamily, as a target of thrombin. Thrombin increased the expression of cell-associated and soluble forms of TRAIL (sTRAIL) in HMEC-1 cells and human umbilical vein endothelial cells (HUVECs). Blocking TRAIL by specific antibodies or by small interfering RNA reduced both the number and the procoagulant activity of EMPs released by thrombin. Consistent with an involvement of sTRAIL in thrombin-induced EMP release, we showed that (1) exogenously added sTRAIL generated procoagulant EMPs; (2) supernatants from thrombin-stimulated endothelial cells induced EMP release by HMEC-1 cells and HUVECs, whereas those recovered from TRAIL knockdown endothelial cells displayed no effect. TRAIL/TRAIL-R2 complex mediated EMP release by initiating the recruitment of adaptor proteins and the activation of nuclear factor κB. Moreover, sTRAIL modulated intercellular adhesion molecule-1 and interleukin-8 expression induced by thrombin by a downstream pathway involving nuclear factor κB activation. Our data reveal a novel mechanism controlling EMP release and identify TRAIL as a key partner in the pathway linking coagulation and inflammation elicited by thrombin. **(*Circ Res*. 2009;104:943-951.)**

Key Words: endothelium ■ vesiculation ■ cell signaling ■ inflammation ■ coagulation ■ thrombosis

Thrombin is a serine protease that plays a key role in the pathogenesis of vascular diseases. It is generated at sites of vessel damage and controls the formation of a hemostatic plug. Thrombin plays a central role in the crosstalk between inflammation and coagulation by inducing the synthesis of proinflammatory cytokines that activates the coagulation cascade[1] and by impairing anticoagulant mechanisms.[2] It also exerts pleiotropic effects on endothelium.[3] Recent data from our laboratory indicated that thrombin induces the release of microparticles by endothelial cells (endothelial microparticles [EMPs]), an effect mediated by the receptor PAR-1.[4]

Microparticles (MPs) are a heterogeneous population of small membrane–coated vesicles resulting from disruption of the membrane phospholipid asymmetry, leading to phosphatidylserine exposure in response to cell activation or apoptosis. Their capacity to carry surface antigens, cytoplasmic proteins, or nucleic acids from their parent cells confers to MPs a major role in cell communication by binding to their targets or facilitating cell–cell interactions.[5] In theory, all cells have the capacity to form MPs and endothelial cells generate EMPs that provide procoagulant surfaces able to trigger coagulation activation.[6] Indeed, once available at the surface of the MP, phosphatidylserine exposure promotes the assembly of the enzymes of the clotting system and provides a catalytic surface for tissue factor (TF) activity. Moreover, binding of EMPs to THP1 cells induces the synthesis of TF contributing to amplify a procoagulant pathway.[7] In addition, EMPs also provide catalytic surfaces for the conversion of plasminogen into plasmin.[8] The expression of other proteolytic systems such as matrix metalloproteinases confers to MPs a putative role in vascular remodeling and angiogenesis in vitro.[9]

In human diseases, elevated levels of EMPs are found in disorders associated with thrombotic and/or inflammatory events such as diabetes,[10] antiphospholipid syndrome,[11] acute renal failure,[12] sickle cell anemia,[13] coronary syndrome,[14] or preeclampsia.[15] It is now obvious that elevated EMP levels are

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From the Institut National de la Santé et de la Recherche Médicale UMR608 (S.S., M.-S.N., S.R., L.C.-J., J.S., F.D.-G., F.A.), Physiopathologie de l'Endothelium, Universite Mediterranee, Faculte de Pharmacie Marseille, France; Hopital de la Conception (L.C.-J., J.-R.H., F.D.-G.), Assistance Publique-Hopitaux de Marseille, France; and Institut National de la Santé et de la Recherche Médicale U928 (C.N.), Technologies Avancées pour le Génome et la Clinique, Marseille, France.

*Both authors contributed equally to this work.

Correspondence to Dr F. Anfosso, INSERM UMR608, Physiopathologie de l'Endothélium, Faculté de Pharmacie, 27 Bd Jean Moulin 13385 Marseille cedex 05, France. E-mail francine.anfosso@pharmacie.univ-mrs.fr

associated with most of the cardiovascular risk factors, are often correlated with endothelial dysfunction, and are associated with a poor clinical outcome. Although EMPs raised considerable interest as markers of vascular damage, the mechanisms leading to their generation are poorly understood.

We previously showed that the release of EMPs by HMEC-1 cells required the recruitment of caspase-2 in the absence of an apoptotic cell death. Caspase-2, in turn, activated the Rho-kinase II by its proteolytic activity.[4] In the present study, using gene expression profiling, we identified TRAIL, a cytokine belonging to the tumor necrosis factor (TNF)-α superfamily, as a target of thrombin in HMEC-1 cells. The upregulation of the soluble form of TRAIL (sTRAIL) increases the release of EMPs carrying a procoagulant activity by a signaling network involving the interaction with the receptor TRAIL-R2, the recruitment of the adaptor proteins TRADD, TRAF2 and the kinase RIP1, and the activation of the transcription factor nuclear factor (NF)-κB in HMEC-1 cells and in the human umbilical vein endothelial cells (HUVECs). Moreover, sTRAIL participates to the thrombin-mediated upregulation of the inflammatory mediators intercellular adhesion molecule (ICAM)-1 and interleukin (IL)-8 by a pathway requiring NF-κB. Our data indicate a novel mechanism linking TRAIL/TRAIL-R2 system to the amplification of the endothelial vesiculation induced by thrombin and identify TRAIL as a key partner in the crosstalk between inflammation and coagulation.

Materials and Methods

Details of endothelial cultures, transfections, flow cytometry, and ELISA are described in the online data supplement at http://circres.ahajournals.org.

Results

Thrombin Generates Procoagulant EMPs

Thrombin induced the release of EMPs by HMEC-1 cells.[4] We investigated whether these EMPs carried a procoagulant activity in a clotting assay measuring their capacity to convert FX into FXa. Thrombin induced a dose-dependent increase in FXa generation by HMEC-1 and HUVEC-derived EMPs (Figure 1A). Preincubation of EMPs with blocking monoclonal antibody against TF inhibited the procoagulant activity induced by thrombin (Figure 1B, lanes f versus d). These data indicate that thrombin generated EMPs carrying a TF-dependent procoagulant activity.

TRAIL Is Modulated by Thrombin

Thrombin modulates genes linked to inflammation and coagulation.[4] By cDNA microarray, we identified a cluster belonging to the TNF-α superfamily (Figure I, A, in the online data supplement) and including *TNFSF10* (*TRAIL*), a cytokine that displays multiple effects on endothelial cells.[16] Relative quantification of changes in gene expression indicated a long-lasting upregulation of *TRAIL* in response to thrombin stimulation (Online Figure I, B).

Validation of microarray data by real-time PCR confirmed the upregulation of *TRAIL* expression that peaked at 6 hours in HMEC-1 and HUVECs (Figure 2A). Western blot indicated an increased protein expression peaking at 10 hours (Figure 2B). TRAIL is a type II transmembrane protein.[16]

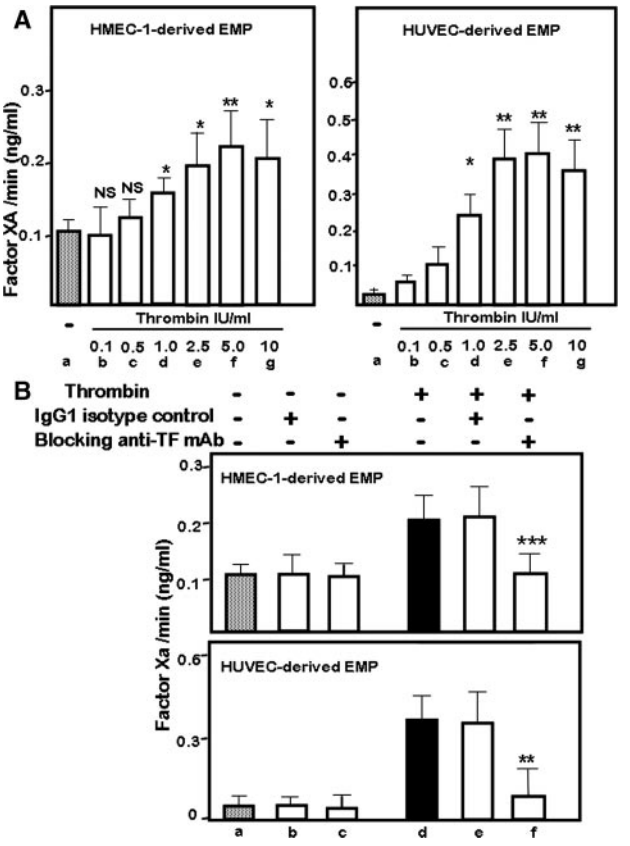

Figure 1. TF-dependent procoagulant activity of EMPs generated by thrombin. HMEC-1 or HUVECs were kept untreated or were stimulated with thrombin. EMP recovery was determined as described in Materials and Methods. Procoagulant activity was assayed by release of FXa from FX in the presence of EMPs (A) or EMPs preincubated 30 minutes with 20 μg/mL of blocking TF monoclonal Ab (mAb) or IgG1 isotype control (B). Bars represent means±SEM (n=9).

Thrombin induced a moderate increase in its transmembrane form in HMEC-1 and HUVECs (Online Figure II).

TRAIL interacts with 5 receptors, including 2 decoy receptors, a soluble receptor (osteoprotegerin), and 2 transmembrane receptors (DR4 [TRAIL-R1] and DR5 [TRAIL-R2]).[17] Because these latter mediate signaling pathways, we determined whether thrombin modulated their expression. Thrombin upregulated *TRAIL-R2* mRNA in HMEC-1 and HUVECs (Figure 2A) and also increased TRAIL-R2 protein (Figure 2B) and its membrane-bound form (Online Figure II). In contrast, TRAIL-R1 expression was unaffected by thrombin (Figure 2A and 2B and Online Figure II). TRAIL also exists as a full-active soluble protein (sTRAIL) and thrombin increased its release in cell-free supernatants (Figure 2C).

We previously showed that ROCK-II mediated EMP generation by thrombin.[4] Stimulation of HMEC-1 or HUVECs by thrombin in the presence of the selective inhibitor of the Rho-kinase Y27632 (1 μmol/L) did not inhibit the release of sTRAIL (Online Table I) and did not modify TRAIL-R2 and TRAIL protein expression (Online Figure II, B). TRAP, the agonist peptide mimicking the effects of thrombin, induced EMP generation by HMEC-1[4] and HUVECs but did not modify sTRAIL levels (Online Table I). These findings showed that

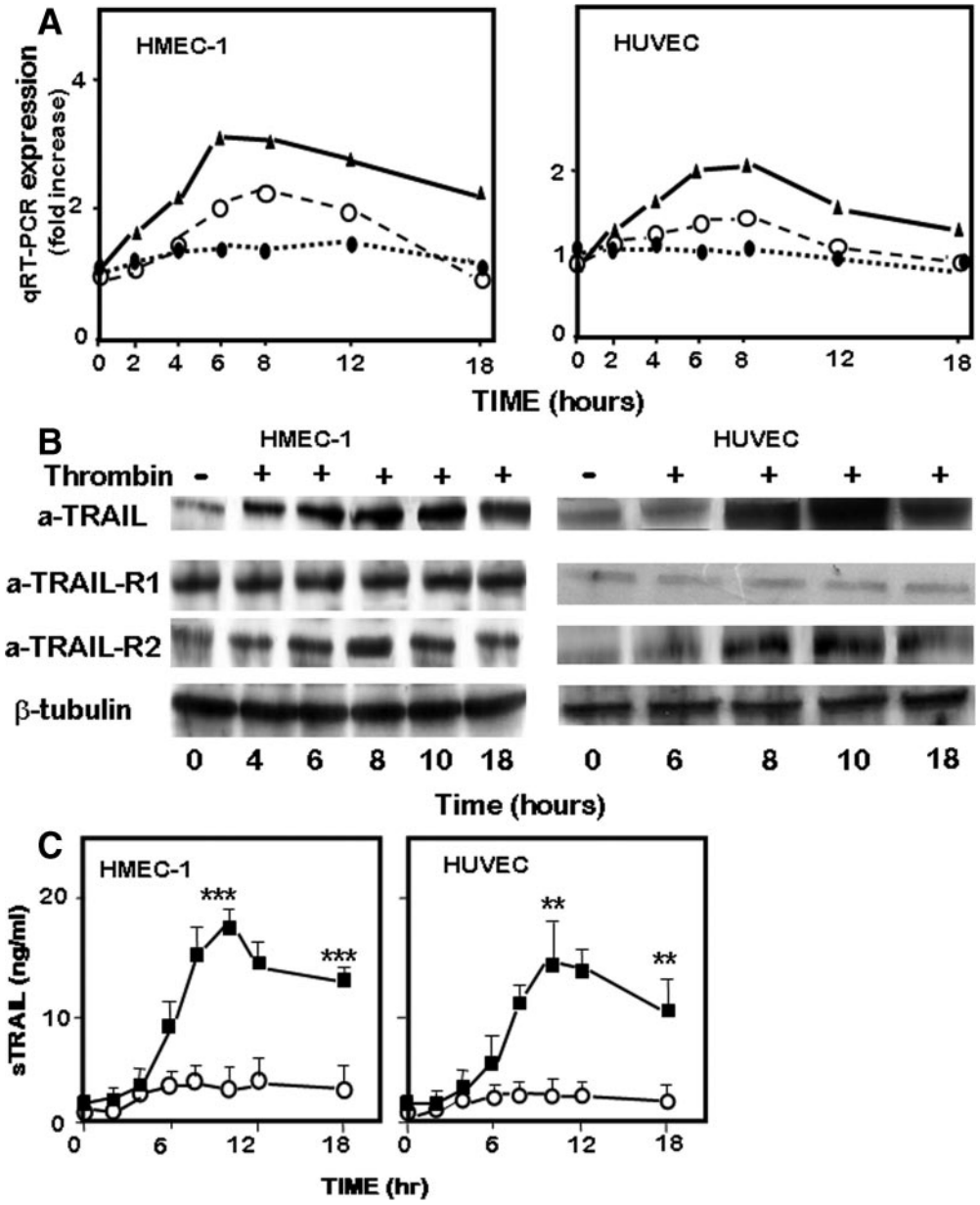

Figure 2. Thrombin-induced changes in TRAIL and TRAIL receptors in ECs. A, Real-time quantitative PCR analysis of the expression of TRAIL (▲—▲), TRAIL-R1 (●—●), and TRAIL-R2 (○—○) mRNA in HMEC-1 or HUVECs. B, Time-course analysis of TRAIL and TRAIL receptors by Western blot in HMEC-1 and HUVECs: 40 μg of cell lysates were analyzed on 12% SDS-PAGE. The blots were probed with human anti-TRAIL, anti–TRAIL-R1, or anti–TRAIL-R2 monoclonal Abs and anti–β-tubulin as loading control. C, sTRAIL was assayed by ELISA in cell-free supernatants of HMEC-1 or HUVECs kept untreated (○—○) or stimulated with thrombin (■—■). Curves represent means±SEM (n=9). $^{**}P<0.03$, $^{***}P<0.001$.

thrombin upregulated the TRAIL/TRAIL-R2 system at the gene and protein levels and triggered the release of sTRAIL.

TRAIL and *TRAIL-R2* Are Involved in the Generation of Procoagulant EMPs by Thrombin

The involvement of TRAIL/TRAIL-R2 complex in the generation of EMPs by thrombin was investigated by antibody blockade or specific cell silencing. Stimulation with thrombin in the presence of neutralizing anti-TRAIL or anti–TRAIL-R2 antibodies (Abs) resulted in an inhibition of EMP release (Online Figure III, A and B) that reached 40% and 32% inhibition in HMEC-1 and HUVECs, respectively (Figure 3A, lanes f and g versus e). Addition of neutralizing anti-TRAIL Abs at different times after the onset of thrombin stimulation, showed a significant inhibition of thrombin-induced EMP release beginning at 4 hours (Online Figure IV, A). When anti-TRAIL Abs were added at early times (2 or 3 hours), the inhibition only began after 4 hours of stimulation whereas anti-TRAIL Abs rapidly prevented the rise in EMP release between 4 and 10 hours. The data indicated that TRAIL did not control the early phases of EMP release.

The involvement of TRAIL and TRAIL-R2 in thrombin-induced EMP generation was confirmed by gene knockdown experiments. Three different small interfering (si)RNAs for each gene were tested for their capacity to reduced EMP release. Among them, TRAIL siRNA1 and TRAIL-R2

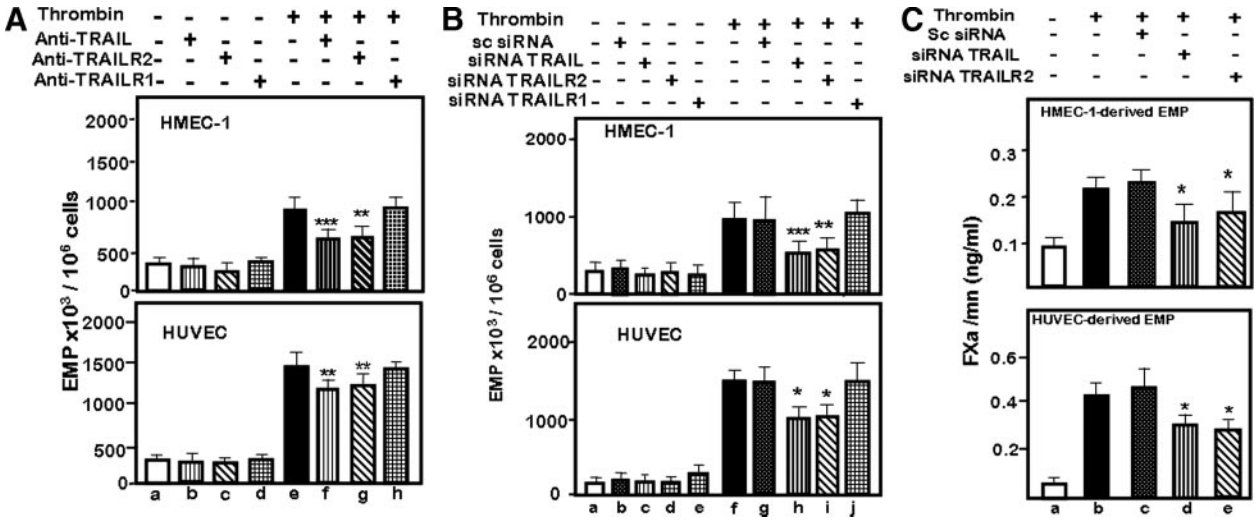

Figure 3. Involvement of TRAIL and TRAIL-R2 in thrombin-induced EMP generation. A, Effect of neutralizing Abs. HMEC-1 or HUVECs were incubated without or with thrombin (5 IU/mL) in the presence of neutralizing Abs (100 μg/mL) against TRAIL, TRAIL-R2, or TRAIL-R1 for 18 hours. Bars represented means±SEM (n=6). $^{**}P<0.01$, $^{***}P<0.001$ vs thrombin (lane e). B, Effect of TRAIL and TRAIL-R2 gene silencing. EMPs were quantified in 18-hour supernatants from HMEC-1 or HUVECs silenced with scrambled siRNA (scsiRNA) or specific siRNA. The cells were then kept untreated or were stimulated for 18 hours with thrombin. Bars represent means±SEM (n=4). $^{*}P<0.01$, $^{**}P<0.05$, $^{***}P<0.001$ vs thrombin (lane f). C, Effect of TRAIL and TRAIL-R2 gene silencing on the generation of procoagulant EMPs. EMPs were isolated from supernatants of knockdown HMEC-1 or HUVECs and stimulated with thrombin (5 IU/mL), and the FXa generation was quantified. Bars represent means±SEM (n=6). $^{**}P<0.01$ vs thrombin (lane b).

siRNA3 gave the more pronounced reduction of EMPs (Online Figure V, A), protein of interest, and mRNA expression (Online Figure V, B and C). They were used for the subsequent experiments. Knockdown of TRAIL or TRAIL-R2 mRNA decreased the level of EMPs released by thrombin by HMEC-1 and HUVECs (Online Figure VI, A; Figure 3B, lanes h and i versus f) in a time-dependent manner (Online Figure VI, B). Specific silencing of TRAIL or TRAIL-R2 siRNA also reduced the procoagulant activity of EMPs mediated by thrombin (Figure 3C, lanes d and e versus b). These findings showed that TRAIL/TRAIL-R2 complex mediated the release of procoagulant EMPs initiated by thrombin.

sTRAIL Mediated the Generation of Procoagulant EMPs by Thrombin

To ascertain the role of sTRAIL in EMP generation, we investigated the capacity of supernatants from thrombin-stimulated HMEC-1 or HUVECs to induce EMP release by naive cells.

Cell media from HMEC-1 or HUVECs stimulated with thrombin were ultracentrifuged to eliminate cell debris or small vesicles carrying TRAIL molecules. sTRAIL levels determined in the remaining supernatants were not modified by the ultracentrifugation (Figure 4A, lanes d versus c) but were greatly reduced in supernatants from TRAIL knockdown HMEC-1 or HUVECs (lanes e versus c) compared to TRAIL-R2 knockdown cells (lanes g versus c).

The transfer of these ultracentrifuged supernatants to untreated monolayers increased both the number (Figure 4B, upper pattern) and the procoagulant activity (Figure 4B, lower pattern) of EMPs released by untreated HMEC-1 (Figure 4B, lanes c versus a) or HUVECs (Figure 4B, lanes k versus i). This increase was not modified by addition of hirudin (20 mg/mL), an inhibitor of thrombin (Figure 4B, lanes d versus c and l versus k). EMP and FXa levels were significantly reduced with supernatants from TRAIL-silenced HMEC-1 (lanes e versus c) or HUVECs (Figure 4B, lanes m versus k), whereas supernatants from TRAIL-R2 knockdown cells had no effect (Figure 4B, lanes g versus c and o versus k).

Recombinant human TRAIL (rhTRAIL) was tested for its capacity to generate EMPs. rhTRAIL dose-dependently increased EMP numbers (Online Figure VII, A). At a dose of 20 ng/mL, a concentration of sTRAIL found in thrombin-stimulated supernatants, rhTRAIL significantly increased EMPs carrying a procoagulant activity dependent of TF (Online Figure VII, B). Both the increase in EMP levels and procoagulant activity were inhibited in TRAIL-R2 knockdown HMEC-1 or HUVECs. These data showed that sTRAIL contributed to the generation of procoagulant EMPs induced by thrombin and that the selective interaction between TRAIL and TRAIL-R2 controlled this release.

Signaling Pathways Involved in TRAIL-Mediated EMP Release

Following engagement by TRAIL, TRAIL receptors form a complex with TRADD or FADD, 2 adaptors, respectively, involved in survival or death signaling pathway.[18] We investigated which of TRADD or FADD adaptors mediated the signaling events that controlled TRAIL-induced EMP release. Coimmunoprecipitations with anti–TRAIL-R2 indicated that on thrombin stimulation, TRADD, but not FADD, was recovered in the immunoprecipitates of HMEC-1 and HUVECs. TRADD recruitment was prevented when TRAIL or TRAIL-R2 were silenced (Figure 5A, lanes d and e). TRADD is an adaptor protein that recruits other internal adaptors such as TRAF2 and the kinase RIP1.[18] Coimmuno-

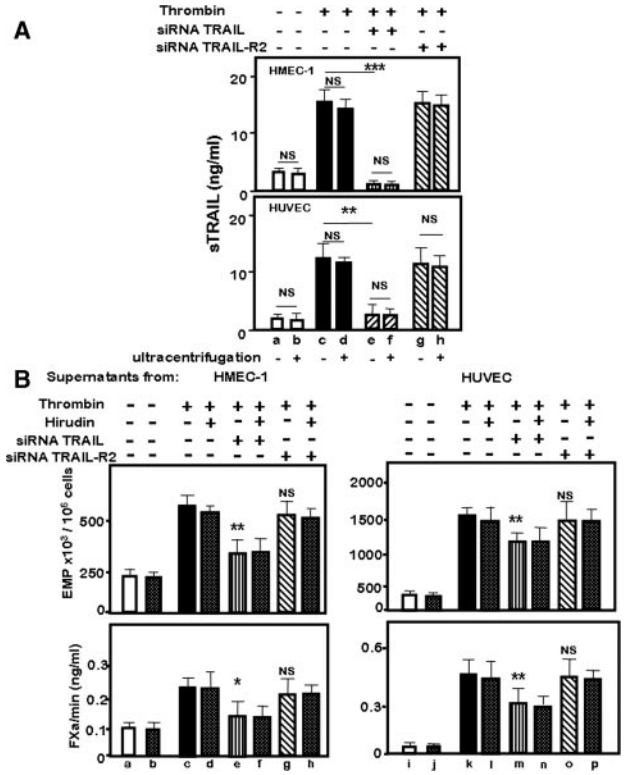

Figure 4. Involvement of sTRAIL in the release of procoagulant EMPs. A, Effect of ultracentrifugation on the release of sTRAIL. HMEC-1 or HUVECs were stimulated with thrombin and culture media were ultracentrifuged at 75 000*g* for 90 minutes. sTRAIL was assayed in the ultracentrifuged supernatants by ELISA. Bars represent means±SEM (n=4). NS indicates not significant, $^{**}P<0.05$, $^{***}P<0.001$ vs thrombin (lane c). B, Generation of EMPs by thrombin-induced sTRAIL. Untreated HMEC-1 or HUVECs were incubated, in the presence or absence of hirudin (20 mg/mL), with ultracentrifuged supernatants obtained from TRAIL or TRAIL-R2 knockdown HMEC-1 or HUVECs stimulated with thrombin. EMPs were numerated in the cell media after an 18-hour incubation with the supernatants (upper graph), and their procoagulant activity was determined (lower graph). Bars represent means±SEM (n=4). NS indicates not significant, $^{*}P<0.01$, $^{**}P<0.05$ vs thrombin (lanes c and k, respectively).

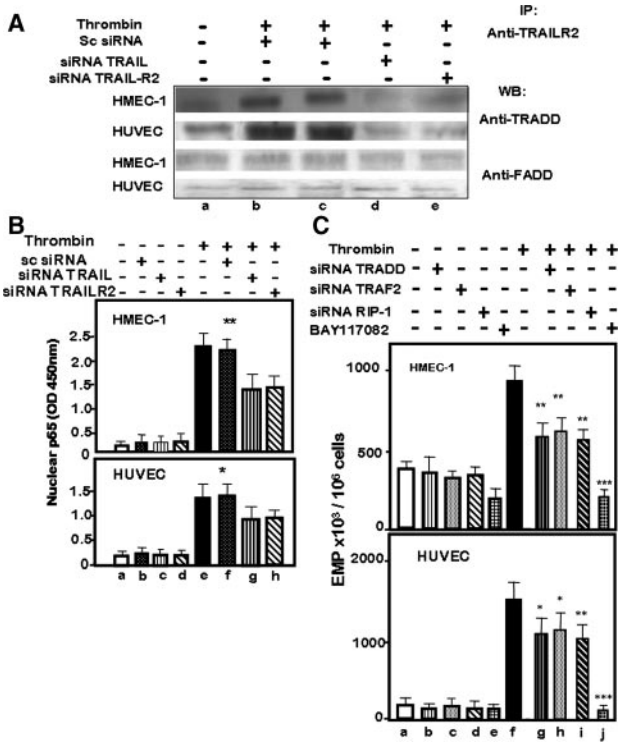

Figure 5. Signaling pathways involved in TRAIL-mediated EMP generation in response to thrombin stimulation. A, Recruitment of TRADD to TRAIL-R2. Cell lysates from HMEC-1 or HUVECs (200 μg) were immunoprecipitated with anti–TRAIL-R2 and protein G-agarose beads, resolved by 12% SDS-PAGE, and blotted against anti-TRADD or anti-FADD. Representative pattern of 3 independent experiments. B, Time-course activation of NF-κB. TRAIL or TRAIL-R2 knockdown HMEC-1 and HUVECs were stimulated with thrombin for 18 hours. After lysis, p65 was assayed in the nuclear fraction by TRANS-AM ELISA. Bars represent means±SEM (n=6). $^{*}P<0.04$, $^{**}P<0.07$ vs thrombin stimulation (lane e). C, Involvement of the signaling pathway in EMP generation. HMEC-1 or HUVEC knockdown for TRADD, TRAF2, or RIP-1 were kept untreated and stimulated with thrombin during 18 hours. In separate experiments, HMEC-1 or HUVECs were stimulated with thrombin in the presence of BAY117082 (20 μmol/L). Bars represented means±SEM (n=4). $^{*}P<0.01$, $^{**}P<0.05$, $^{***}P<0.001$ vs thrombin (lane f).

precipitation showed the recruitment of both TRAF2 and RIP1 to TRAIL-R2 (Online Figure VIII, A) that was inhibited when TRAIL or TRAIL-R2 were silenced in HMEC-1 cells. These data indicated that TRAIL/TRAIL-R2 interaction induced a signaling complex at the plasma membrane.

Translocation to the nucleus of the transcription factor NF-κB occurs downstream the engagement of TRADD.[19] We investigated the involvement of NF-κB in the TRAIL-mediated EMP release by thrombin. Thrombin induced a time-dependent increase in p65 nuclear translocation that was reduced in HMEC-1 incubated with thrombin in the presence of neutralizing anti-TRAIL Abs (Online Figure IX) and in TRAIL or TRAIL-R2 knockdown cells (Figure 5B, lanes g and h). Addition of BAY117082 (20 μmol/L), a selective inhibitor of NF-κB, prevented the increase in p65 over the entire course of thrombin stimulation (Online Figure IX, A). Addition of anti-TRAIL inhibited the p65 increase only for times greater than 4 hours of stimulation (Online Figure IX, B). Nevertheless, the addition of BAY1170082 at different times of the thrombin stimulation, inhibited early and late phases of EMP release (Online Figure X). All of these data indicated that TRAIL controlled the late phases of NF-κB activation mediated by thrombin, whereas EMP release depended on NF-κB activation for all the thrombin stimulation.

The role of the signaling complex in EMP generation was then investigated. Silencing of TRADD, TRAF2, and RIP1 (Online Figure VIII, C) resulted in a reduction of EMP numbers in HMEC-1 cells and HUVECs. The extent of the inhibition was similar to that observed when TRAIL or TRAIL-R2 were knocked down (Figure 5C, lanes g h and i). These results indicated that TRAIL/TRAIL-R2 interaction triggered a signaling complex that participated to the thrombin-induced EMP release.

Participation of sTRAIL to the Inflammation Induced by Thrombin

We investigated whether TRAIL and its downstream pathway participated to the thrombin-induced inflammation. Thrombin

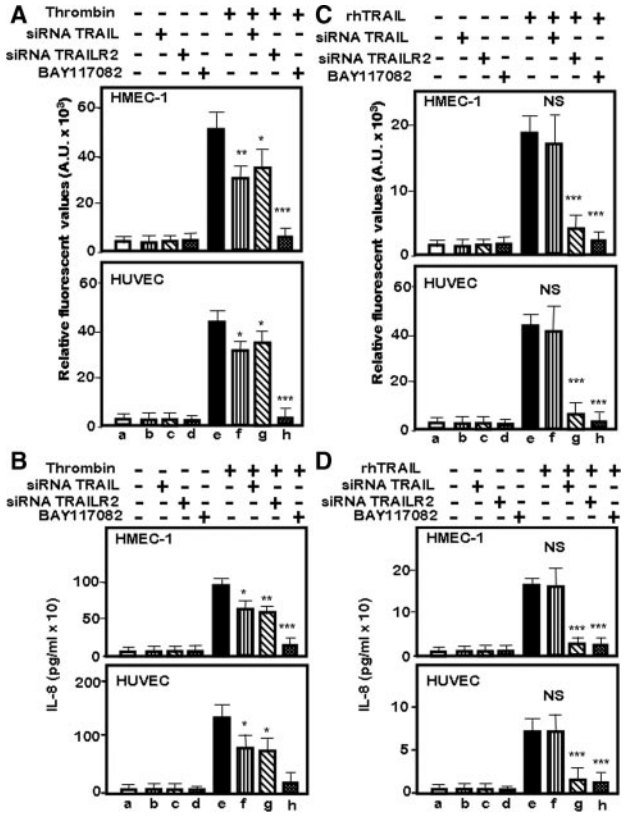

Figure 6. Involvement of sTRAIL in the proinflammatory phenotype induced by thrombin. Role of TRAIL, TRAIL-R2, or NF-κB in ICAM-1 expression (A) and IL-8 secretion (B). TRAIL or TRAIL-R2 knockdown HMEC-1 or HUVECs were kept untreated or stimulated with thrombin for 18 hours. In separate experiments, cells were stimulated with thrombin in the presence of BAY117082. ICAM-1 and IL-8 were assayed, respectively, by fluorescent or colorimetric ELISA. ICAM-1 (C) and IL-8 (D) were assayed in HMEC-1 or HUVECs treated as above and stimulated with rhTRAIL (20 ng/mL) for 18 hours. The bar graphs represent means±SEM (n=6). NS indicates not significant, $^{*}P<0.7$, $^{**}P<0.05$, $^{***}P<0.001$ vs thrombin (lane e).

upregulated ICAM-1 membrane expression both in HMEC-1 and HUVECs (Figure 6A, lanes e versus a) and the release of IL-8 (Figure 6B, lanes e versus a). These increases were reduced in TRAIL and TRAIL-R2 knockdown cells (Figure 6A and 6B, respectively, lanes f and g versus e). In addition the inhibition of NF-κB prevented both the dependent of thrombin increases in ICAM-1 and IL-8 (lanes h versus e). The blockade of TRAIL/TRAIL-R2 interaction by anti-TRAIL Abs inhibited IL-8 release for times longer than 4 hours of thrombin stimulation (Online Figure XI, A). In contrast, the addition of BAY117082 completely inhibited IL-8 release at early and late phases (Online Figure XI, B). These data indicated that TRAIL controlled IL-8 release during the late phases of thrombin stimulation, whereas NF-κB was involved throughout all the thrombin stimulation. In HMEC-1 and HUVECs, rhTRAIL (20 ng/mL) increased the membrane expression of ICAM-1 and the release of IL-8 (Figure 6C and 6D, respectively, lanes e versus a), an increase inhibited in TRAIL-R2 knockdown cells or cells treated with the inhibitor of NF-κB (lanes g, h). These data confirmed the role of TRAIL in the inflammation mediated by thrombin and indicated that TRAIL/TRAIL-R2 interaction modulated the inflammatory phenotype by a downstream pathway involving the activation of NF-κB.

Discussion

The present study defines a novel mechanism controlling the in vitro release of procoagulant EMP generation in response to thrombin. For the first time, to our knowledge, we identify *TRAIL* as a target of thrombin. We demonstrate that the soluble form of TRAIL contributes to the release of procoagulant EMPs by thrombin. The interaction between sTRAIL and its receptor TRAIL-R2 initiates the recruitment of downstream adaptor proteins TRADD, TRAF2, RIP1, and NF-κB. Moreover, the engagement of this signaling pathway controlled the thrombin-mediated upregulation of the inflammatory mediators ICAM-1 and IL-8. Thus, the present study provides insight into the mechanisms of EMP generation and unravels a new function of TRAIL as a mediator between coagulation and inflammation in response to thrombin.

Thrombin induced EMP generation in HMEC-1 and HUVECs. The higher capacity of HUVECs than HMEC-1 to form EMPs in response to thrombin would represent either an increased responsiveness of their plasma membrane to thrombin or structural differences with respect to the localization of these cells on the vascular tree, respectively, on macro- and microvessels.[20]

TRAIL secretion in the cell culture medium was increased in HMEC-1 and HUVECs and participated to the thrombin-induced EMP release both in the transformed endothelial cell line or cells directly extracted from the vessel. Inhibition of TRAIL by blocking Abs or by silencing TRAIL mRNA reduced by ≈40% EMP release by thrombin in HMEC-1 cells and ≈32% in HUVECs. The higher inhibition in HMEC-1 cells than in HUVECs would reflect the higher TRAIL secretion by HMEC-1 cells compared to HUVECs. Because the inhibition was not complete, other pathways may be additionally involved in EMP generation by thrombin.

We postulate that the soluble form of TRAIL mediates EMP release. In accordance with a role of sTRAIL, we demonstrated that (1) thrombin increased TRAIL levels in cell-conditioned medium. (2) Exogenous rhTRAIL induced EMP release at a concentration found for sTRAIL in culture medium of cells stimulated with thrombin. (3) EMP release by HMEC-1 and HUVECs incubated with culture media from thrombin-treated identical endothelial cells was abolished with supernatants from TRAIL-silenced endothelial cells. This latter experiment supported the importance of sTRAIL in EMP release by thrombin and excluded an effect of residual thrombin because hirudin[21] prevented EMP generation and did not modify EMP numbers generated by the supernatants. Another important finding in this work is the hitherto undescribed role of sTRAIL in the mediation of procoagulant EMPs generated by thrombin. Indeed, thrombin elicited the expression of TF in endothelial cells,[20,22] and EMPs displayed a procoagulant activity dependent on TF. When TRAIL was silenced, the EMP procoagulant activity was reduced. Moreover, the generation of TF-dependent procoagulant EMPs by exogenous rhTRAIL confirmed the ability of sTRAIL to generate procoagulant EMPs. Therefore,

we propose that sTRAIL mediates the amplification of procoagulant EMP release by thrombin.

Among the receptors interacting with TRAIL, TRAIL-R1 and TRAIL-R2 are involved in the signaling pathway of apoptotic cell death.[17] Our study indicated a critical role of TRAIL-R2 in EMP release by thrombin. The upregulation of TRAIL-R2 by thrombin and the fact that the inhibition of TRAIL-R2 with blocking Abs or with its specific siRNA decreased EMP generation demonstrated that the interaction of TRAIL-R2 with TRAIL was a key element in EMP release. Moreover, TRAIL-R1 receptor displayed a low expression in HMEC-1 and HUVECs[17] and an absence of modulation by thrombin. These data indicated an absence of redundancy between the two receptors or a different sensitivity toward TRAIL[23,24] in the control of thrombin-induced EMP release. The generation of EMPs and the modulation of TRAIL/TRAIL-R2 by thrombin depended on different pathways. Indeed, (1) Rho-kinases did not control their expression although modulating EMP release, and (2) TRAP did not increase TRAIL or TRAIL-R2, indicating that the proteolytic activity of thrombin was necessary for their modulation.

The interaction of TRAIL with TRAIL-R2 triggered the recruitment of the death domain–containing adapter protein TRADD. TRAIL-R2 has 2 overlapping signaling pathways. It associates with FADD or TRADD adapter molecules.[18] FADD initiates a downstream pathway linked to apoptosis in *FADD*$^{-/-}$ mice[25] and to caspase-8 activation.[26] Nevertheless, FADD did not associated with TRAIL-R2 in response to thrombin. The recruitment of TRADD, TRAF2, and RIP1 promoted the formation of the membrane-bound complex 1 associated with the nuclear translocation of NF-κB and the promotion of antiapoptotic responses.[27] In addition, TRADD engages the cells toward a survival pathway by the recruitment of molecular complexes linked to NF-κB activation and TRAIL-R2 mediates NF-κB activation when cell death pathways are inhibited.[28,29] Moreover, thrombin induced the nuclear translocation of p65.[30] In our work, the use of TRAIL and TRAIL-R2 knockdown cells and the stimulation with rhTRAIL clearly conferred a role of TRAIL in the activation of NF-κB by thrombin. The blockade of TRAIL/TRAIL-R2 interaction clearly showed that TRAIL mediated NF-κB activation during the late phases of thrombin stimulation. Taken together, the recruitment of complex 1 molecules and NF-κB activation controlled the generation of EMPs by thrombin.

Thrombin and TRAIL increased the expression of ICAM-1[3] and the inflammatory cytokine IL-8[31] in endothelial cells.[32] One important finding in this work is the role played by TRAIL in the upregulation of inflammatory mediators by thrombin. Indeed, silencing TRAIL or TRAIL-R2 reduced the thrombin-mediated increase in ICAM-1 and IL-8, and the stimulation with rhTRAIL confirmed the involvement of TRAIL in the elicitation of an endothelial proinflammatory phenotype by thrombin. NF-κB plays a key role in inflammatory processes, and TRAIL also induces NF-κB activation.[28,33] In our work, NF-κB prevented the increase in ICAM-1 or IL-8 mediated by rhTRAIL, demonstrating an involvement of the NF-κB pathway in the control of the inflammatory phenotype mediated by TRAIL. The inhibition of NF-κB prevented the increase in IL-8 over the entire course of the stimulation, and TRAIL only controlled the late phases of IL-8 secretion. These data suggested that 2 phases controlled EMP release or IL-8 secretion during thrombin stimulation. One occurred early and involved NF-κB activation, and during the course of thrombin stimulation, a second occurred lately and was mediated by TRAIL and NF-κB.

Despite its apoptotic role of TRAIL in cancer cells,[34] TRAIL also signals for nonapoptotic responses on multiple cells, including endothelial cells.[35,36] Although there is controversial evidence regarding the potential protective[17] or detrimental role of TRAIL on the endothelium,[32,37,38] our data suggest a dual role of TRAIL on endothelial cells. The engagement of signaling molecules involved in survival could protect the cells from apoptosis, but the amplification of the thrombin-induced release of procoagulant EMPs and proinflammatory mediators could be deleterious for endothelial cells.

sTRAIL is present in the plasma,[39] and increased levels are associated with inflammatory processes characterized by a dysregulation of the coagulation and the inflammation such as systemic lupus erythematosus,[40] sepsis,[41] and rheumatoid arthritis.[42] In these pathologies, plasma EMP levels are also increased.[6,43,44] These data suggest a relationship between increased TRAIL levels and EMP release that remains to be proven.

Data based on in vivo and in vitro experiments indicated that EMPs behave as pathogenic vectors modulating cellular processes that underlie inflammation and thrombosis.[45] Procoagulant microparticles originating from endothelial cells and other cell types are sequestered within the atherosclerotic plaque[46,47] and could contribute to the rupture of the plaque. TRAIL is also present in the atherosclerotic plaque,[48] and its expression is increased in vulnerable plaques.[49,50] Because inflammation plays a role in plaque destabilization, and extensive thrombin generation occurs within plaques,[47] thrombin would upregulate the local expression of TRAIL, thereby triggering an amplification loop of vesiculation by an autocrine and/or paracrine mechanism. TRAIL also acts on lymphocytes or smooth muscle cells. It could potentiate apoptosis or the release of inflammatory mediators, thereby contributing to plaque rupture.

In conclusion, the present study identifies sTRAIL/TRAIL-R2 system as a critical pathway involved in the late phases of the generation of procoagulant EMPs induced by thrombin in vitro. It establishes that TRAIL could take part in an inflammatory process and emphasizes its role in the interplay between coagulation and inflammation. Elucidation of the mechanisms underlying microparticle formation may help to design therapeutic approaches for a better control of EMP release.

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Disclosures

None.

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