Rickettsia rickettsii Infection of Cultured Human Endothelial Cells Induces Tissue Factor Expression

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Microvascular thrombi underlie many of the clinical manifestations of Rocky Mountain spotted fever (RMSF), a disease characterized by Rickettsia rickettsii infection of vascular endothelial cells. Studies were designed to determine whether R. rickettsii infection of cultured human umbilical vein endothelial cells results in tissue factor (TF) induction, a process that could directly activate coagulation in infected vessels. Whereas uninfected endothelial cell cultures showed essentially undetectable TF mRNA and activity, both TF mRNA and activity were present after R. rickettsii infection. TF mRNA levels were transient, peaking at 4 hours after the initiation of infection, whereas the peak of TF activity occurred at 8 hours. Induction of the TF response requires the intracellular presence of R. rickettsii organisms, because uninfected rickettsia were ineffective and the response was blocked by inhibiting rickettsial entry using cytochalasin B. TF induction was not mediated by endothelial cell release of soluble factor, because no response was induced using culture medium conditioned by R. rickettsii-infected cells. Furthermore, preadsorption of suspensions of R. rickettsii with polymyxin B to remove contaminating lipopolysaccharide did not eliminate the TF response. Induction of TF in vital endothelial cells during R. rickettsii infection could be the trigger for vascular thrombus formation of RMSF.

THE SPOTTED FEVER diseases, of which Rocky Mountain spotted fever (RMSF) is the most virulent (caused by Rickettsia rickettsii), result from infection with insect-vector-transmitted rickettsial organisms. These diseases are manifest by fever, papular skin rash, and microvascular occlusive disease with multiple organ involvement and tissue ischemia that can lead to a fatal outcome.1-4 Rao et al5 showed evidence of platelet activation as well as activation of the extrinsic coagulation, contact, and fibrinolytic enzyme systems within hours after infection of human volunteers inoculated with R. rickettsii as part of a vaccine-assessment study. The organism infects endothelial cells of small vessels in regions of ischemic skin necrosis early in the illness6-8 and of larger vessels in patients with advanced disease.9 Because the vascular endothelial cell is a primary target of infection by this organism, development of a prothrombotic phenotype before loss of cell viability could be responsible for much of the pathologic change in this disease. In studies of cultured endothelial cells infected in vitro with R. rickettsii, we have previously demonstrated changes resulting from in vitro infection and before cell death, including increased platelet adherence to the surface of infected cells,10 increased release of large von Willebrand factor multimers,11 and enhanced E-selectin cell surface expression and neutrophil adherence,12 and others have shown increased secretion of plasminogen activator inhibitor in R. rickettsii and R. conorii-infected endothelial cells.13 Rickettsial organisms likely enter the endothelial cell through a process of induced phagocytosis, after which exit from the phagosome presumably involves rickettsial phospholipase A activity.14-17 Entry of the organisms requires both rickettsial energy and endothelial cell participation. Pretreatment of endothelial cells with cytochalasin B, which disrupts the actin-containing cellular cytoskeleton, inhibits rickettsial entry as does inactivation of rickettsia with N-ethylmaleimide or formalin.18 The presence of calcium ionophores inhibits entry of rickettsia, suggesting that formation of a calcium gradient is involved in this process.18 The cholesterol receptor binding drugs digitonin and amphoteracin B reduced plaque formation by R. rickettsii on endothelial cells, implicating involvement of this receptor in the infection process.19 R. rickettsii replicates in both the nucleus and the cytoplasm of endothelial cells and exhibits early cell to cell spread without detectable host cell injury.19

Tissue factor (TF) is a membrane-bound glycoprotein that is the essential cofactor involved in activation of the extrinsic pathway of coagulation.20,21 TF forms complexes with factors VII and VIIa, which leads to activation of factors IX and X, resulting in the conversion of prothrombin to thrombin and, finally, of fibrinogen to fibrin. Increased TF expression in cultured endothelial cells has been induced by a variety of perturbations, including exposure to tumor necrosis factor-α (TNF-α),22,23 interleukin-1 (IL-1),24 phorbol 12-myristate 13-acetate (PMA),25 or bacterial lipopolysaccharides (LPS).26 Such expression is transient and regulated at the mRNA level due to both enhancement of transcriptional rate and enhancement of mRNA stability.27,28 In the present study, we have investigated the possibility that R. rickettsii infection of cultured endothelial cells results in TF expression. Changes in both TF activity and steady state TF mRNA levels were monitored during the course of infection. In addition, we have explored whether or not TF induction requires the intracellular presence of the rickettsia. We hypothesize that TF expression may occur in R. rickettsii-infected endothelial cells in vivo and may be

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the fundamental component that initiates local thrombotic events that underlie the pathologic changes of RMSF.

**MATERIALS AND METHODS**

**Endothelial cell culture and infection.** Primary cultures of human umbilical vein endothelial cells were prepared as previously described after isolation from human umbilical cord veins by mild proteolytic digestion. Second passage cells, used in experiments, were cultured in McCoy’s 5a medium (Flow Laboratories, McLean, VA) with 20% (vol/vol) fetal bovine serum, 50 μg/mL endothelial cell mitogen (Biomedical Technologies, Inc, Stoughton, MA), 100 μg/mL heparin (Sigma Chemical Co, St Louis, MO), and 25 μg/mL insulin. A plaque-purified seed of *R rickettsii* with a titer of \(2 \times 10^7\) pfu/mL was prepared in VERO (African green monkey kidney) cells (American Type Culture Collection, Rockville, MD) as previously described. Endothelial cells were infected by placing suspensions of *R rickettsii* contained within lysates of VERO cells diluted in complete culture medium without antibiotics onto near-confluent cultures and allowing them to incubate at 37°C in a 5% CO₂ atmosphere for specified times. For control experiments, VERO were cultured in the absence of *R rickettsii*, lysed, and diluted to the same concentration as used during infection. *Escherichia coli* LPS, serotype 0111:B4 (final concentration, 1 μg/mL), cytochalasin B (final concentration, 10 μmol/L), and TNF-α (final concentration, 10 ng/mL) were obtained from Sigma.

**TF activity and antigen measurement.** Endothelial cells, cultured in 12-well culture plates for subsequent TF activity determination, were washed twice with TBS (0.05 mol/L Tris, 0.1 mol/L NaCl, pH 7.5) and lysed by scraping and repeated freeze-thawing in 0.16 mL TBS with 10 mg/mL bovine serum albumin. TF activity of the lysed cell samples was then determined using a two-stage clotting assay calibrated with a sample of pure human brain TF reconstituted into phospholipid vesicles as previously described. TF antigen in lysed cell samples was determined by enzyme-linked immunosorbent assay (ELISA) using pure human brain TF to construct a standard curve.

**RNA isolation, Northern blotting, and hybridization.** RNA was extracted from cultured cells using a modification of previously described methods. Briefly, one T75 flask of endothelial cells per condition was washed twice using ice-cold phosphate-buffered saline (PBS) then 5 mL RNA lysis buffer (4 mol/L guanidine thiocyanate, 0.5% [wt/vol] Na-N-lauroyl sarcosine, 0.1 mol/L β-mercaptoethanol, 0.1% [vol/vol] Antifoam A [30% emulsion, Sigma], 5 mmol/L MgCl₂) was added and agitated until viscous. Flasks were then rinsed with an additional 5 mL RNA lysis buffer. Samples were then frozen at −70°C and thawed before RNA isolation to complete cell lysis. To isolate RNA, samples were layered onto a 3 mL cushion of CsCl (5.7 mol/L CsCl, 1 mmol/L EDTA) and then centrifuged at 25,000 rpm in an SW41 rotor at 18°C for 18 hours. RNA pellets were resuspended four times in 125 μL aliquots of sterile water, after which the RNA was extracted once or until clear with an equal volume of phenol:chloroform:isoamyl alcohol (50:48:2). Ethanol precipitated in 0.2 mol/L potassium acetate, and dissolved in 20 to 50 μL water. Twenty micrograms of total RNA per condition was denatured with glyoxal/dimethylsulfoxide (DMSO) and then resolved by electrophoresis in 1% agarose gels in 10 mmol/L sodium phosphate buffer, pH 7.0. Total RNA was prepared for Northern blot analysis by electrophoretic transfer to Zeta-probe membranes (BioRad, Centreville, NY). To prepare TF or γ-actin probes, we used the EcoRI-HindIII fragment corresponding to bases 1 to 1358 subcloned from pKS20 (a kind gift from Dr W.H. Konisberg, Yale University, New Haven, CT) into pGEM7Zf(-) (Promega, Madison, WI). The human γ-actin probe, pHFY A1, was obtained from Dr L. Kedes (Stanford University, Palo Alto, CA). The 2,200-bp BamHI fragment of γ-actin was subcloned into the BamHI site of pGEM7Zf(-). Probes were labeled with [α-32P]dCTP using the random primer labeling kit from Life Technologies (Gaithersburg, MD). To determine steady state levels of TF or γ-actin, hybridization was performed at 65°C with 0.5 mol/L Na₂HPO₄, pH 7.2, 1 mmol/L EDTA, 7% [wt/vol] sodium dodecyl sulfate (SDS) with final stringency washes performed as described.

**Immunofluorescence staining for R rickettsii organisms.** Endothelial cells cultured on glass coverslips were fixed and permeabilized as previously described. To detect the presence of *R rickettsii* organisms, endothelial cells were stained using 20 μL of fluorescein isothiocyanate (FITC)-labeled anti-*R rickettsii* antibody (Centers for Disease Control, Atlanta, GA) on the coverslip for 30 minutes, rinsed in PBS, and then mounted in Fluoromount-8 (Fisher Scientific, Orangeburg, NY).

**Inactivation and sonication of *R rickettsii* and adsorption of LPS.** *R rickettsii* organisms were inactivated by adding 10 μg/mL tetracycline (Sigma) and incubated for 1 hour before placement on cells. Alternatively, *R rickettsii* were UV-inactivated by exposure 5 cm from a germicidal light in an open container for 30 minutes. *R rickettsii* were disrupted by sonication using a Sonifier R Cell Disruptor, Model W140 (Heat Systems-Ultrasonics, Inc, Plainview, NY) at full power for 30 seconds. LPS was adsorbed from solutions containing *R rickettsii* or disrupted *R rickettsii* by incubation with polymyxin B-agarose (Sigma; binding capacity, 200 to 500 μg LPS from *E coli* serotype 0128:B12/mL, 10 μL/4.5 × 10⁶ pfu *R rickettsii*). Polymyxin B-agarose was allowed to settle and supernatant was placed on the endothelial cell cultures.

**RESULTS**

Endothelial cell cultures were infected using 6 × 10⁴ pfu/cm² *R rickettsii* organisms or incubated with 1 μg/mL LPS for 6 to 8 hours, then lysed for TF activity determination (Fig 1). The amount of TF activity used to infect the endothelial cells was based on previous studies indicating that such amounts result in uniform infection without loss of cell...
viability during the first 48 hours of infection.\textsuperscript{11} Untreated endothelial cells possessed nearly undetectable amounts of TF activity (averaging less than 1 ng/3 \times 10^6 cells) as measured using a two-stage clotting assay. TF activity of \textit{R. rickettsii}-infected endothelial cells increased to 7.0 \pm 1.7 ng/3 \times 10^6 cells compared with 16 \pm 2.2 ng/3 \times 10^6 cells after exposure to LPS. Increases in endothelial cell TF activity was paralleled by increases in TF antigen, as determined by ELISA (data not shown). Incubation of endothelial cells with suspensions of lyzed VERO cells (the vector in which the rickettsia organisms are propagated) containing no \textit{R. rickettsii} did not result in increases in TF activity (not shown).

Steady state TF mRNA levels and TF activity of cells infected with \textit{R. rickettsii} or treated with LPS for varying times between 2 and 24 hours were monitored by Northern blot analysis using a TF cDNA probe (Fig 2A). TF mRNA was essentially undetectable in untreated cultures. Infection with \textit{R. rickettsii} or LPS treatment resulted in a dramatic increase in the 2.2-kb translatable TF mRNA. Increases of a 3.4-kb mRNA also occurred (amounts varying among cell cultures), but this untranslatable mRNA species, representing the presence of an unspliced copy of intron 1, always increased in proportion to the 2.2-kb form. The increase in TF mRNA levels occurring in response to \textit{R. rickettsii} infection was transient, peaking at 4 hours and declining by 8 hours after the initiation of infection. The increase in mRNA occurring in response to LPS treatment was also transient, peaking at 3 hours after initial stimulation. Amounts of \gamma-actin mRNA remained relatively stable during the time course, indicating that there was very little loss of cell viability during the time course of infection. TF activity also increased transiently (Fig 2B), peaking at approximately 8 hours. Therefore, a lag period of approximately 4 hours existed between peak TF mRNA expression and peak TF activity expression.

The increases in TF activity and mRNA levels observed after \textit{R. rickettsii} infection required the presence of viable,
infected organisms (Fig 3). *R. rickettsii* organisms were either pretreated with tetracycline or exposed to a germicidal lamp to render the organisms noninfective. Endothelial cell cultures were then exposed to these organisms for 8 hours, at which time cultures were lysed and TF activity determined. Treatment with UV (RR<sub>UV</sub>) or tetracycline (RR<sub>Tet</sub>) eliminated the cells' response to *R. rickettsii*. Tetracycline (5 μg/mL) did not eliminate the response of cells to LPS (RR<sub>Tet</sub> + LPS).

Polymyxin B-agarose was used to adsorb LPS from solutions containing *R. rickettsii* before placing them on the cells (Fig 4). The adsorption process did not affect infectivity of *R. rickettsii* as monitored by immunofluorescence staining. Adsorption using polymyxin B did not inhibit the activity response to infective *R. rickettsii*. On the contrary, adsorption of sonicated *R. rickettsii* with polymyxin B eliminated the response of the cells. Thus, rickettsial LPS plays a role in the stimulation of endothelial cells to express TF only when the organisms are disrupted. Polymyxin B completely eliminated the stimulatory effect of LPS (1 μg/mL), but there was no inhibitory effect on TNF (10 ng/mL), another known inducer of endothelial cell TF activity.

To determine whether *R. rickettsii*-infected endothelial cells could be restimulated to induce TF using another agent, endothelial cells were infected for 24 hours, during which time TF levels increased and returned to near-baseline levels. At this time, endothelial cells were restimulated using LPS in parallel with endothelial cells that were previously uninfected (Fig 5). Preinfected endothelial cells responded to LPS stimulation by expressing TF levels equivalent to those of LPS-stimulated endothelial cells not previously infected. These results indicate that not only were cells capable of undergoing a secondary response and were therefore viable, but, furthermore, that the cells likely had not responded to LPS during the infection.

Endothelial cells were incubated with cytochalasin B (10 μmol/L) during *R. rickettsii* exposure to inhibit internalization.
Incubation period. Thus, infection with Rickettsia were likely equivalent, given that the endothelial cells were not uniformly infected with Rickettsia during the 8-hour incubation period. Thus, infection with Rickettsia organisms is a potent inducer of in vitro endothelial cell TF expression, thus providing a potential explanation for the striking thrombotic manifestations of RMSF.

The increases in TF activity and TF mRNA levels in infected endothelial cells were transient, with the peak of TF mRNA occurring at 4 hours after the initiation of infection and the peak of TF activity lagging by approximately 4 hours. Although peak TF activity varies depending on the stimulant used, the later appearance of peak TF after Rickettsia infection could have been caused by the time required for organisms to gain entry into the endothelial cells. Prior studies of in vitro infection of endothelial cells have shown that the percentage of infected cells increases during the first 1 to 2 hours of incubation with Rickettsia organisms, plateauing with further incubation. TF activity and TF mRNA levels returned to near-baseline levels by 12 hours after both LPS stimulation and Rickettsia infection. The TF response of infected cells was truly a transient phenomenon, because there was no loss of cell viability or general decrease in recovery of intact mRNA as reflected by constant levels of actin mRNA during the 24-hour infection. Furthermore, endothelial cells infected with Rickettsia for 24 hours were also capable of undergoing a second, maximal TF response on subsequent stimulation using LPS (Fig 5).

The regulation of TF mRNA in response to LPS, PMA, or TNF has been studied by other investigators. PMA and TNF exposure were shown, by nuclear run-off assays, to increase the rate of TF mRNA transcription, suggesting that PMA and TNF are capable of increasing the rate of TF mRNA transcription by stimulating TF promoter activity. This stimulation was apparently not due to an increased rate of TF mRNA transcription, as the rate of TF mRNA transcription would be expected to decrease with time due to the decrease in the concentration of PMA and TNF. However, there was no significant change in the rate of TF mRNA transcription during the 24-hour incubation period. Thus, the increase in TF activity and TF mRNA levels in infected endothelial cells was likely due to an increase in the number of TF molecules per cell, rather than an increase in the rate of TF mRNA transcription. This increase was small and significantly less than that induced by infective Rickettsia or LPS.

**DISCUSSION**

In response to infection with Rickettsia organisms, cultured human umbilical vein endothelial cells express TF. Although the overall magnitude of this TF response by the aggregate endothelial cell culture was approximately half that observed with LPS (Fig 1), the individual cell responses were likely equivalent, given that the endothelial cells were not uniformly infected with Rickettsia during the 8-hour incubation period. Thus, infection with Rickettsia organisms...
to result in increased transcription of the TF gene, whereas the increase in mRNA content resulting from LPS stimulation was shown to depend largely on increased mRNA stability rather than on increased transcription rate. Studies of both transcription rate and changes in mRNA stability would be required to show the mechanism(s) responsible for the increased steady-state TF mRNA levels found in endothelial cells after *R. rickettsii* infection.

Several results presented here suggest that the TF response observed requires the infection of endothelial cells with *R. rickettsii* organisms as well as intracellular uptake of the organisms. Rendering *R. rickettsii* organisms noninfective by treatment with tetracycline or UV exposure before placement on cells eliminated the TF response. Incubation of endothelial cells with cytochalasin B, which inhibits internalization of *R. rickettsii* without eliminating adherence of the organisms to the cell surface by disrupting the endothelial cells' actin-containing cytoskeleton, also obliterated the TF response (Fig 6). These results suggest that, even in the presence of infective *R. rickettsii*, the TF response cannot be elicited unless the organisms are able to enter the cells. The data are also consistent with the observation that tetracycline-treated or UV-treated *R. rickettsii* fail to induce the TF response (Fig 3), because only viable organisms are internalized within endothelial cells.

Because LPS is a potent inducer of endothelial cell TF activity in vitro, and because rickettsial organisms possess LPS, which is similar in chemical composition and biologic properties to that derived from *E. coli*, the possibility exists that TF was induced by rickettsial LPS released into the medium. This is unlikely, because preadsorption of suspensions of intact, viable *R. rickettsii* using polymyxin B-agarose, which binds to the lipid A component of LPS responsible for TF induction, did not eliminate the TF response (Fig 4). Furthermore, endothelial cells did not exhibit a statistically significant increase in TF activity in response to the presence of medium conditioned by infected endothelial cells (Fig 7).

However, rickettsial LPS is a potent inducer of endothelial cell TF expression. When suspensions of tetracycline-treated *R. rickettsii* were sonicated before placement on cells, thus releasing LPS, TF was induced and was inhibitable by preincubation with polymyxin B-agarose. This indicated that the lipid A component of rickettsial LPS, like LPS from *E. coli*, is the portion of the molecule responsible for the cellular response. We hypothesize that rickettsial LPS is not involved in TF induction during an actual infection for several reasons. Cells exposed to LPS remain refractory to secondary stimulation using LPS. The observation that infected endothelial cells responded to subsequent LPS stimulation suggests that they had not been previously exposed to LPS during the infection process (Fig 5). *R. rickettsii* is an obligate intracellular parasite that must replicate within the host cell cytoplasm or nucleus. Therefore, it is unlikely that the organisms are lysed to release LPS after entry into the cells. It is possible that a molecule(s) present on the rickettsial surface interacts directly with intracellular components of the endothelial cell and results in activation of intracellular signalling pathways, as has been described for other pathogenic bacteria, including *Yersinia*. It is also possible that the endothelial cell responds to oxidative injury, which has been implicated in endothelial cell injury caused by *R. rickettsii* infection. Infection of endothelial cells with *R. rickettsii* likely represents a novel stimulus for induction of TF, yet the exact nature of the stimulus and the intracellular signaling mechanisms involved are not yet known.

It has been demonstrated that the procoagulant properties of cultured endothelial cells are enhanced in response to other infective agents. For example, infection of endothelial cells with herpes simplex virus (HSV) results in TF mRNA transcription. The time course of TF mRNA expression after HSV infection was also transient, peaking at 4 hours after the initiation of infection and increasing with the magnitude of infection. In addition to increased TF expression, prothrombinase complex assembly is enhanced on HSV-infected endothelial cells. Presumably because of expression of HSV gC, a viral protein that can act as a binding site for factor X and prothrombinase complex assembly. The procoagulant phenotype of HSV-infected endothelial cells is further enhanced by decreased expression of the protein C anticoagulant cofactor, thrombomodulin, and enhanced platelet adhesion. Furthermore, HSV-infected endothelial cells exhibit decreased synthesis of plasminogen activator inhibitor type 1 (PAI-1) and tissue plasminogen activator (t-PA) at the level of transcription. Procoagulant activity is enhanced on cytomegalovirus-infected endothelial cells, but is independent of increased TF expression and occurs even using heat-inactivated virus and is therefore likely to be independent of active infection. von Willebrand factor release, decreased thrombomodulin expression, and transient increases in TF expression have also been demonstrated in *R. conorii*-infected endothelial cells.

This study reports that cultured vascular endothelial cells infected with *R. rickettsii* rapidly and transiently express TF. Because RMSF and other spotted fever diseases caused by infection of vascular endothelial cells with rickettsial organisms result in microvascular thrombosis, this finding may shed light on the possible function of the perturbed endothelial cell in the development of thrombotic disease. Such endothelial cell-derived TF could directly activate coagulation and could explain localized thrombus formation in infected vessels. However, there has been no clear demonstration of the inducibility of TF by endothelial cells in vivo, much less the role of endothelial cell TF in thrombotic formation. Detailed studies of animal model systems are needed to assess the importance of endothelial cell TF in pathologic thrombosis.

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