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Infection of vascular endothelial cells with herpes simplex virus enhances tissue factor activity and reduces thrombomodulin expression

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ABSTRACT **Latent infection of vascular cells with herpesviruses may play a pathogenic role in the development of human atherosclerosis. In a previous study, we found that cultured human umbilical vein endothelial cells (HUVECs) infected with herpes simplex virus 1 (HSV-1) became procoagulant, exemplified both by their enhanced assembly of the prothrombinase complex and by their inability to reduce adhesion of platelets. We now report two further procoagulant consequences of endothelial HSV infection: loss of surface thrombomodulin (TM) activity and induction of synthesis of tissue factor. Within 4 hr of infection of HUVECs, TM activity measured by thrombin-dependent protein C activation declined 21 ± 3% ($P < 0.05$) and by 18 hr, 48 ± 5% ($P < 0.001$). Similar significant TM decrements accompanied infection of bovine aortic endothelial cells. Identical TM loss was induced with HSV-2 infection but not with adenovirus infection. Decreased surface expression of TM antigen (measured by the specific binding of a polyclonal antibody to bovine TM) closely paralleled the loss of TM activity. As examined by Northern blotting, these losses apparently reflected rapid onset (within 4 hr of HSV infection) loss of mRNA for TM. In contrast, HSV infection induced a viral-dose-dependent increase in synthesis of tissue factor protein, adding to the procoagulant state. The results indicate that loss of endothelial protein-synthetic capacity is not a universal effect of HSV infection. We suggest that the procoagulant state induced by reduction in TM activity and amplified tissue factor activity accompanying HSV infection of endothelium could contribute to deposition of thrombi on atherosclerotic plaques and to the "coagulant-necrosis" state that characterizes HSV-infected mucocutaneous lesions.**

From an earlier suggestion that herpes simplex virus 1 (HSV-1) may be involved in human atherosclerosis (1), we became interested in the effects of HSV-1 infection on endothelial cells *in vitro*. The possibility that such infection might induce a "procoagulant state" is suggested from the histology of HSV-1 mucosal lesions, which commonly reveals leukocytoclastic vasculitis and intense intravascular fibrin deposition (2, 3). Additional clinical support for the concept of hypercoagulability in HSV-1 infections is the severe, often fatal, intravascular coagulation that often occurs in disseminated HSV infection of neonates (4).

We demonstrated that HSV-infected human umbilical vein endothelial cells (HUVECs) became prothrombotic by virtue of enhanced prothrombinase complex assembly on their membrane surfaces. In addition, thrombin-stimulated platelets exhibited enhanced adhesiveness to HSV-infected cells (5). However, we acknowledged that the possible procoagulant effects of thrombin generated on the endothelial cell surface by an amplified prothrombinase complex system might be offset by upregulation of a major anticoagulant pathway involving the thrombomodulin (TM)/protein C system. Here we demonstrate the converse: namely, that the potential ameliorating effects of TM are actually lost in HSV infections. Thus, TM surface expression and mRNA are rapidly reduced in endothelial cells that are infected with HSV-1. Superimposed upon this potentially procoagulant event, we found a significant increase in the expression of tissue factor (TF) by infected endothelial cells. We conclude that these procoagulant events may aggravate the thrombotic diathesis associated with HSV vascular infections.

MATERIALS AND METHODS

Reagents. Bovine protein C and bovine antithrombin III were purchased from Enzyme Research Laboratories (South Bend, IN). Protein C lacking γ-carboxyglutamic (Gla) residues was prepared as described (6). The chromogenic substrate S2238 (D-Phe-pipecolyl-Arg-*p*-nitroanilide) was from Helena Laboratories. Tunicamycin, human α-thrombin, polymyxin B, bovine serum albumin, *Escherichia coli* lipopolysaccharide (LPS), and Hepes were from Sigma. Trypsin/EDTA solution and Hanks' balanced salt solution were from GIBCO. Rabbit brain thromboplastin (thromboplastin C) was from Baxter, Dade Division (Miami). The RIA kit for interleukin 1α (IL-1α) was from Genzyme.

Endothelial Cell Cultures. Primary HUVEC cultures were grown to confluence as described (7). Bovine aortic endothelial cells (BAECs; passages 3–6) were used in some experiments. These cells demonstrated the typical cobblestone morphology of endothelial cells and stained positive for factor VIII-related antigen by indirect immunofluorescence.

Virus. HSV-1 strain 17^+ was propagated in rabbit skin cells and virus was titrated by standard methods (8). Confluent monolayers of endothelial cells were generally infected with 10 plaque-forming units (pfu) per cell (5, 6). Neither the HSV-infected nor the mock vehicle contained >0.05 ng of endotoxin per ml as assayed by the *Limulus* amoebocyte lysate assay (Associates of Cape Cod). Where indicated, UV irradiation was performed by exposing the virus suspension in open-topped 35-mm dishes to a germicidal 30-watt UV strip light (General Electric) at a distance of 20 cm for the appropriate length of time.

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Abbreviations: HSV, herpes simplex virus; TM, thrombomodulin; TF, tissue factor; HUVEC, human umbilical vein endothelial cell; BAEC, bovine aortic endothelial cell; LPS, lipopolysaccharide; pfu, plaque-forming unit(s).

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TF and TM Activity Assay. Total cellular TF activity was measured in HUVECs by a modified one-stage clotting assay (9).

For assay of TM activity, human α-thrombin (0.15 NIH unit/ml) and bovine protein C (27.5 μg/ml) were added to buffer over washed monolayers. The cells were incubated for 60 min at 37°C in 5% CO_2. The supernatants were then removed and residual thrombin activity was quenched with excess antithrombin III (600 nM). In the second stage of the assay, conditioned medium was added to a cuvette containing buffer and the amidolytic substrate S2238 (0.2 mM). The rate of increase in A_{405} was taken as a measure of activated protein C enzymatic activity and was expressed per μg of endothelial cell protein (10). The mean activity for triplicate wells was expressed as a percentage of control wells that had been incubated with medium.

Measurement of TM Antigen. Polyclonal rabbit anti-bovine TM was prepared as described (11). Iodination with $Na^{125}I$ (15 mCi/μg, Amersham; 1 mCi = 37 MBq) was performed by the iodogen method (12).

Monolayers of mock-infected or HSV-infected BAECs were washed three times with ice-cold Hepes-buffered 0.9% NaCl containing 0.2% bovine serum albumin. A saturating concentration (0.4 μg/ml) of the iodinated anti-TM antibody was then added together with normal human immunoglobulin (50 μg/ml). Cells were incubated with the labeled antibody for 4 hr at 4°C, after which the monolayer was washed and the cells were solubilized with 1 M NaOH before measurement of radioactivity in a γ counter. Nonspecific binding was determined by adding a 200-fold molar excess of unlabeled antibody to duplicate wells. Specific binding was then calculated as the difference between total and nonspecific binding.

Analysis of TM mRNA. Total cellular RNA from control and HSV-1 infected HUVECs was isolated (13). Northern analysis was performed by standard techniques (14). Briefly, 10 μg of total RNA was fractionated by electrophoresis in 2.2 M formaldehyde/1% agarose. RNA was visualized by ethidium staining and UV transillumination. RNA was then transferred to a nitrocellulose membrane (Schleicher & Schuell), hybridized to ^{32}P-labeled human TM cDNA (15), washed, and autoradiographed. Radioactive DNA probes were labeled by nick-translation in the presence of [α-^{32}P]dCTP (16). The TM cDNA was removed by washing the membrane in water at 75°C for 120 min, and the membrane was hybridized to a ^{32}P-labeled mouse actin cDNA (17) by the same methods. Molecular sizes of the transcripts were determined by comparison with the electrophoretic mobility of commercial RNA molecular size markers (BRL).

Assay of IL-1α. HUVECs in 35-mm dishes were infected with HSV as described for 18 hr, except that medium 199/2% fetal bovine serum was substituted for the usual tissue culture medium. These conditioned supernatants (1 ml per well) were removed, filtered (0.2 μm), and assayed by RIA for IL-1α. The cells were removed from the culture dish by scraping and suspended in 1 ml of medium 199/2% fetal bovine serum. These suspensions were then freeze–thawed three times, filtered, and assayed for IL-1α as described by the test kit manufacturer; the Genzyme kit allows measurement of IL-1α at 79–2500 pg/ml. As a positive control, some cells were exposed to LPS (10 μg/ml) in the same medium for 18 hr.

Statistical Methods. All results are reported as means ± SEM. When more than one time point following infection was compared with baseline (time 0), one-way analysis of variance was employed. When matched mock- and HSV-infected samples (at a single time point) were compared, the paired *t* test was used. In both instances, $P < 0.05$ was considered significant.

RESULTS

Characteristics of HSV-1 Infection of Endothelial Cells. Both HUVECs and BAECs can be productively infected with HSV-1. Viability (as assessed by trypan blue and ^{51}Cr-release methods) is maintained in both for at least 36 hr (6). The maximum period of infection reported in these studies is 18 hr, at which time cell viability is >95% and lactate dehydrogenase release does not differ significantly in uninfected or HSV-infected HUVECs. Viral replication (measured as virus recovery in supernatant plus removed adherent cells) is more efficient in HUVECs than in BAECs (Fig. 1), and after 24 hr, HUVECs are 50 times more heavily infected.

Activation of Protein C. Protein C activation was significantly reduced on HUVECs after as little as 4 hr of HSV infection, and progressive reduction in activity occurred with more prolonged (18-hr) infection (Fig. 2; ◆); mock infection up to 18 hr provoked no loss of thrombin-mediated protein C activation when compared to untreated endothelium (data not shown). Significant, although lesser and more delayed, reductions of protein C activation also accompanied HSV infection of BAECs (Fig. 2; □) perhaps reflecting the difference in viral replication in the two endothelia (Fig. 1).

Since protein C activation requires thrombomodulin, we assessed the surface expression of TM antigen on BAEC by a specific binding assay using a polyclonal anti-bovine TM. After 18 hr of HSV infection the TM activity of BAECs as measured by protein C activation (74 ± 3% of mock-infected control) exactly reproduced the surface TM antigen (75 ± 4% of control). Notwithstanding this parallelism, we explored the possibility that loss of surface TM activity might also involve membrane changes that could conceivably alter the ability of TM to recognize the protein C substrate. This seemed a reasonable possibility since we previously demonstrated that HSV infection leads to surface perturbations, including phospholipid rearrangements, in endothelial plasma membranes (5). To test this hypothesis, we compared the capacity of TM to activate either intact bovine protein C or its proteolytically modified derivative lacking Gla residues. Membrane-bound TM activates native protein C much more readily than the Gla-less form, whereas soluble TM shows no such discrimination (7). No apparent alteration in this discrimination was observed in infected cells. That is, in BAECs

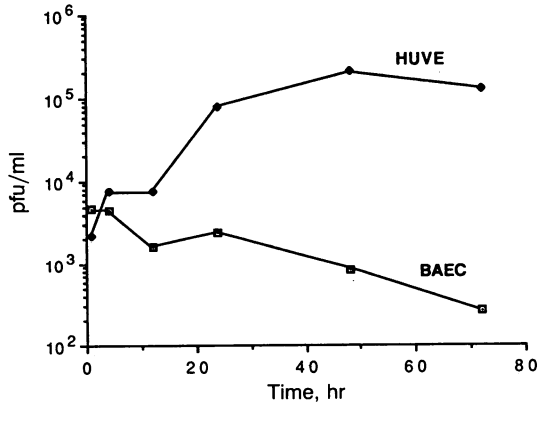

FIG. 1. HSV-1 multiplies more efficiently in HUVECs than in BAECs. HUVEC (◆) or BAEC (□) cultures were infected by incubation with HSV-1 (10 pfu per endothelial cell) for 1 hr at 37°C. The virus suspension was then removed, and the monolayers were washed before the addition of fresh medium. At various times after infection, the medium from duplicate wells was removed and pooled together with the endothelium, which was removed from the plastic wells by scraping. The virus was then titered on rabbit skin cells by using an overlay containing 0.3% human immune serum globulin (8) and results are presented as pfu. Experiment depicted is representative of three performed.

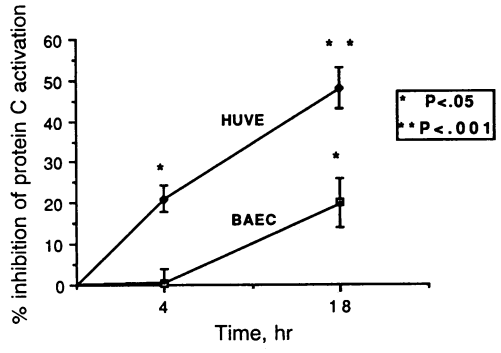

FIG. 2. Protein C activation is reduced on endothelium infected with HSV-1. HUVEC (◆) or BAEC (□) cultures were infected with HSV-1 (10 pfu per cell). Endothelial TM activity, measured by the activation of protein C in the presence of thrombin, was assayed at various times after infection. Values are expressed as percent inhibition compared to control wells (incubated with medium), mean ± SEM. *P* values were calculated by one-way analysis of variance, comparing 4- and 18-hr time points to time 0. Protein C activation on mock-infected HUVECs (data not shown) did not differ significantly from control (medium treated). Results are means ± SEM of six experiments.

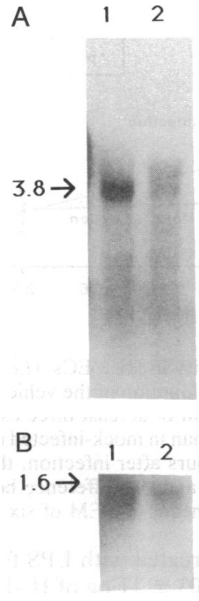

FIG. 3. HSV-1 infection markedly reduces TM mRNA levels in HUVECs. TM transcripts in control (lane 1) and HSV-1-infected (lane 2) cells were studied by Northern blot analysis. HSV-1 infection was associated with a marked decrease in TM mRNA of 3.8 kilobases (*A*). Actin transcripts at 1.6 kilobases also decreased severalfold (*B*). Visualization of the RNA by UV transillumination confirmed identical lane loading and intactness of rRNA between HSV-1-infected and control samples.

infected for 18 hrs, the rate of activation of Gla-less protein C decreased in exact parallel with that of native protein C so that the native/Gla-less ratio of activation was not significantly different from that seen in uninfected cells (ratio of 6.39 for unincubated control, 6.70 for mock-infected, and 6.04 for HSV-infected cells).

These results favor the proposition that loss of TM activity in HSV-infected cells reflects direct loss rather than a critical change in membrane TM/protein C topography. This loss can conceivably result from bulk depletion of membrane surface. In agreement with this hypothesis is our previous observation of budding and vesiculation of HSV-infected cells by electron microscopy (6). However, no significant difference in TM activity was demonstrated in ultracentrifuged (100,000 × *g*, 60 min) pellets of supernatants from infected, mock-infected, or untreated endothelium. Moreover, we detected no secreted inhibitor of TM in conditioned cell-free medium from infected HUVECs, nor was such conditioned medium capable of inhibiting activated protein C activity. Alternatively, direct loss of TM in HSV-infected endothelium could result from reduced synthesis of the protein. In support, Northern blots showed a marked loss of TM mRNA within 4 hr of HSV infection (Fig. 3*A*), suggesting that decreased *de novo* synthesis of TM at least partially underlies HSV-mediated TM loss.

Several experiments were performed to determine what other characteristics of the infectious process might contribute to the loss of TM. First, we reasoned that reduced TM activity on HSV-infected HUVECs could result from a virally encoded glycoprotein expressed on the host cell surface that might "cover up" TM in such a way as to interfere with protein C activation. Endothelium infected with HSV-1 expresses a variety of virally encoded glycoproteins at the cell surface; for example, viral glycoprotein C functions as a receptor for complement component C3b, and glycoprotein E can act as an Fc receptor (18). To test this possibility, HUVECs were incubated with tunicamycin (2 μg/ml) (which inhibits N-linked glycosylation of viral glycoproteins) during the period of infection (19). Incubation with tunicamycin had no effect on the endothelial activation of protein C, nor did it reverse the HSV-induced decrement in TM expression (Table 1), implying that expression of viral glycoproteins does not underlie TM loss. Second, to test whether the effect of TM depends on viral replication, HSV was rendered incapable of replication by exposure to short-wave UV light. Infection of endothelium for 18 hr with virus exposed to UV light at 5 J/mm^2 (equivalent to a 30-min exposure at a distance of 20 cm from the UV source)—a maneuver that interferes with replication but not with viral penetration—provoked no loss of TM activity at 18 hr when compared to mock infection (Table 1).

Finally, other large DNA viruses known to infect human endothelium were assessed for their ability to alter TM expression. With a similar multiplicity of infection and an 18-hr infection time, HSV-2 was at least as effective as HSV-1 in suppressing TM (78 ± 1% loss vs. control), whereas adenovirus type 4 had no significant effect (6 ± 3% loss vs. control).

TF Expression. HSV induced TF synthesis in HUVECs in a time-dependent fashion; no activity was noted until 2 hr after infection, and maximal expression occurred at 4 hr (Fig. 4 *Left*). TF expression decreased thereafter, returning to baseline by 20 hr. Mock-infected HUVECs showed minimal TF activity, probably not related to contamination by endotoxin, since the preparations of rabbit skin cells used were negative by the *Limulus* amoebocyte lysate test, and polymyxin B had no effect on TF induction. The degree of TF expression increased with the magnitude of infection (Fig. 4 *Right*) so that TF expression at the highest multiplicity of infection used (20 pfu per cell) was approximately half that maximally induced by high concentrations of LPS (0.1 μg/ml).

Table 1. UV light, but not tunicamycin, renders HSV-1 incapable of inhibiting TM activity

Treatment	Protein C activation, % of control	*P*
Mock infection	105 ± 7	NS
Mock + tunicamycin*	95 ± 5	
HSV infection	63 ± 4	NS
HSV + tunicamycin*	59 ± 8	
UV-exposed HSV†	98 ± 2	<0.001‡

Results are means ± SEM and are expressed as a percentage of control values in native endothelial cells. NS, not significant.
*HUVECs were incubated with or without tunicamycin (2 μg/ml) during the 18-hr period of infection with HSV-1. Mock-infected cells were similarly treated.
†HUVECs were infected for 18 hr with HSV-1 that was previously exposed to UV light at 5 J/mm^2.
‡Compared to unirradiated HSV.

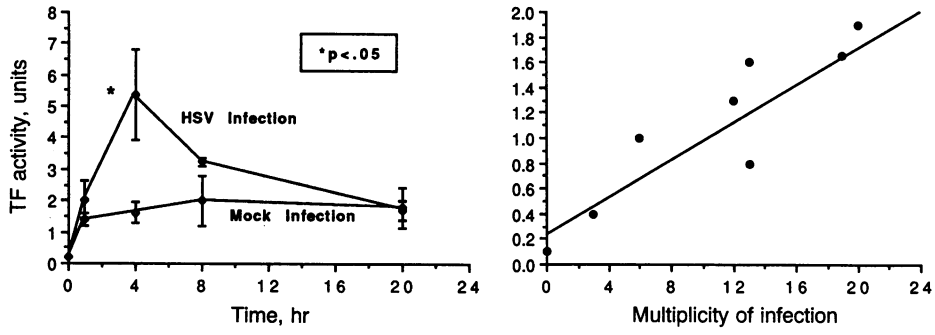

FIG. 4. HSV-1 infection induces TF activity in HUVECs. (*Left*) Cells were infected with HSV-1 at a multiplicity of infection of 10 pfu per cell or were mock-infected with an equivalent dilution of the vehicle. At various times after infection, the cells were harvested and total cellular TF activity was determined. The mean ± SEM of at least three experiments at each time point is shown. TF activity was significantly greater ($P \leq 0.05$) in HSV-infected cells (upper line) than in mock-infected cells (lower line) at 4 hr postinfection. (*Right*) Cells were infected with HSV-1 at various multiplicities of infection. Four hours after infection, the cells were assayed for total cellular TF activity. Control (mock-infected) cells were similarly assayed for TF activity, and the difference between HSV-infected and mock-infected cells is expressed as (specific) TF activity per tissue culture well. Results are means ± SEM of six experiments.

IL-1α Assay. By RIA, the HUVECs treated with LPS for 18 hr (as a positive control) contained 203 ± 11 pg of IL-1α per 35-mm dish. However, the supernatant fraction of LPS-treated cells, as well as the cell and supernatant fractions of untreated and 18-hr HSV-infected HUVECs contained no detectable IL-1α.

DISCUSSION

These results demonstrate that early in the course of infection with HSV-1, two potentially additive procoagulant phenomena are engendered: namely, HUVECs exhibit a progressively diminishing capacity to activate protein C and also transiently express TF activity. Cultured endothelial cells do not constitutively express TF but may synthesize the protein *de novo* when appropriately stimulated, for instance, by inflammatory mediators such as endotoxin (LPS) or by monokines such as tumor necrosis factor and IL-1 (20–22). However, the biological significance of these observations with cultured cells is uncertain, since efforts to demonstrate TF within endothelial cells of atherosclerotic plaques by *in situ* hybridization and immunohistochemical techniques have been unsuccessful. In contrast, TF can be shown to be present in macrophages and other cells within the plaque (23). It is not clear whether the failure to detect endothelial TF *in vivo* is simply a question of sensitivity; however, from a functional point of view, even minute quantities of exposed TF may be highly prothrombotic. Of interest, Etingin *et al.* (24) recently have shown binding and activation of factor X via glycoprotein C on HSV-infected HUVECs.

The second abnormality demonstrated in HSV-infected endothelium—loss of TM and concomitant decrease in protein C activation—may more adversely affect vascular homeostasis. We previously showed that assembly of the prothrombinase complex is greatly facilitated on HSV-infected endothelium (5); the increase in surface thrombin predicted by these previous findings and its inefficient binding by decreased TM (present studies) might not only promote thrombosis at infected endothelial sites but also provoke monocyte attachment (24). If monocytes/macrophages can adhere to the surface without inhibition, they may undergo transition into foam cells, characteristic of those which are found in atherosclerotic plaques.

The interest in herpesvirus infections of the vascular wall as it may relate to atherogenesis has been summarized recently by several investigators (1, 25, 26). Herpesviridae have been detected in endothelial and smooth muscle cells of the arterial wall in patients with atherosclerosis (1, 27, 28). Cytomegalovirus infection is associated with accelerated coronary artery disease in recipients of cardiac transplants (29). In chickens, Marek disease, which is caused by an avian herpesvirus, promotes atherosclerosis (30). HSV-1-infected arterial smooth muscle cells accumulate significantly greater amounts of cholesterol esters and triacyglycerols than uninfected cells, primarily due to decreased synthesis of cholesterol esterase in infected cells (31, 32). Not only may HSV be involved in atherogenesis, but it may promote thrombosis on atherosclerotic lesions as well.

Regarding the mechanism responsible for the loss of thrombomodulin in HSV-infected endothelial cells, it appears from our data that shedding of TM by membrane vesiculation is unlikely to be important and that virally encoded surface glycoproteins do not interfere with TM expression. Using conditioned supernatants, we were also unable to demonstrate that infected endothelium secretes an inhibitor of activated protein C or of surface TM. A more likely possibility is that diminished synthesis of TM occurs due to shutoff of host cell protein synthesis induced by HSV-1. Previous studies have shown that an early (within 2 hr), as well as late, suppression of host protein synthesis occurs with this virus. The early shutoff is virion-protein-dependent and is due to dispersion of host cell polyribosomes and degradation of host mRNA; this does not require the synthesis of new viral RNA or protein (33). In contrast, late (delayed) inhibition does require viral protein synthesis (34). Our data demonstrating a decrease in TM mRNA after 4 hr of infection with the live virus suggest that reduced endothelial synthesis of TM is the major mechanism responsible for the reduced TM activity in HSV-infected cells. It is unclear from these data whether the altered TM mRNA levels are the result of reduced mRNA stability, reduced transcription, or both. The reported effect of HSV infection on actin mRNA (32) is similar to that observed in this experiment (Fig. 3*B*). Consistent with the interpretation that the measured loss of TM activity reflects reduced TM synthesis are previous TM kinetic data. That is, although the half-life of TM in HUVECs is not known, the half-life of TM in mouse hemangioma cells is about 19.8 hr (35). This closely matches the results in Fig. 2, which show an ≈50% decrement in HSV-infected cells after 18 hr, a coincidence that might also suggest that TM loss is mainly due to shutoff of host protein synthesis. However, we emphasize that the shutoff of host protein synthesis, at least in the early hours following infection, is not universal since levels of another protein, TF, increase in HUVECs infected with HSV-1. We considered the possibility that HSV infection might also induce synthesis of another protein, IL-1, in the host cell, which in turn could account for the changes in TM transcription and TF activity observed. This would be a

plausible explanation, since others have demonstrated that IL-1 activity is induced in HUVECs exposed to tumor necrosis factor or endotoxin and that this activity remains membrane-associated (36). By RIA, we also detected cell-associated IL-1α in LPS-treated HUVECs; however, we found no IL-1α in HSV-infected cells. Nevertheless, we cannot exclude the possibility that the viral genome encodes other regulatory proteins that can secondarily alter transcription of TM, and possibly TF, genes.

In summary, our data provide further evidence that HSV-infected endothelium may actively promote coagulation. Not only does it manifest the seemingly procoagulant combination of enhanced TF expression and prothrombinase complex assembly, but it is also deficient in the antithrombotic moieties prostacyclin and TM. We suggest that these changes in endothelial cell thrombotic tendency may result in the extensive fibrin deposition that is seen in acute mucocutaneous herpetic lesions, and might also be germane to thrombosis associated with atherosclerotic plaques.

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