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Enhanced thrombin generation and platelet binding on herpes simplex virus-infected endothelium

(thrombosis/atherosclerosis)

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ABSTRACT **Atherosclerotic lesions have been reported to contain herpes simplex virus 1 (HSV-1) genomic material. This, and other previous evidence, suggests that latent viral infection may be an atherogenic trigger. Moreover, active HSV-1 lesions manifest marked fibrin deposition in microvessels. In this report we show that very early infection of human endothelial cells with HSV-1 appears to alter surface conformation as detected by merocyanine 540 staining. Concomitantly, the efficiency of prothrombinase complex assembly increases, resulting in a 2- to 3-fold accelerated rate of thrombin generation on the cell surface. Increased thrombin generation is probably doubly procoagulant, since we also demonstrate that thrombin-induced platelet accumulation on HSV-infected endothelium (50.7 ± 9.3%) is increased compared to uninfected endothelium (9.5 ± 2.1%; $P < 0.002$). Associated with HSV infection, prostacyclin secretion in response to thrombin is diminished by a factor of 20, probably explaining the enhanced platelet attachment. We conclude that HSV infection shifts endothelial cell properties from anticoagulant to procoagulant, both by promoting prothrombinase complex formation and function and by increasing platelet binding, well before cell disruption takes place. Virus-induced changes in the endothelial plasma membrane and diminished prostacyclin secretion are suggested as the pathways for this pathophysiologic mechanism, which may be germane to atherosclerotic thrombosis as well as HSV-mediated tissue necrosis.**

An intact vascular endothelium is actively antithrombotic. Rather than forming a passive vascular lining, endothelium has a variety of natural anticoagulant properties that are able to exert a dampening effect upon the coagulation cascade (1, 2): heparin-like species, thrombomodulin, plasminogen activator, and prostacyclin are endothelial constituents thought to contribute to vascular thromboresistance. Consequently, a breach of this barrier by mechanical disruption of the endothelial lining with frank endothelial loss and exposure of the subendothelium can lead to massive coagulation activation and platelet adherence. More subtle injury to endothelium that might alter its thromboresistant properties has only recently emerged as a topic of inquiry (3). Theoretically, several different mechanisms may be involved in diminished endothelial thromboresistance: (*i*) decreased production or exhibition of the above-mentioned antithrombotic factors; (*ii*) increased production or secretion of thrombogenic factors such as tissue factor, platelet-activating factor, von Willebrand factor, and plasminogen-activator inhibitor; and (*iii*) changes in the endothelial cell surface, leading to altered exposure of proteins or procoagulant phospholipids.

With regard to mechanism *iii*, endothelial cell surface alterations can result from viral infection, which readily occurs with several viruses *in vitro*. Diverse viruses replicate readily in cultured endothelium, including herpesviruses, adenoviruses, and enteroviruses, among others (4, 5). Herpes simplex virus (HSV) efficiently infects human endothelium (4) and induces a number of surface changes in these cells: the expression of an Fc receptor and a complement receptor (6) and diminished exhibition of the extracellular matrix proteins fibronectin and type IV collagen (7). That these alterations might promote coagulation is suggested in that HSV-induced mucosal lesions manifest fibrin deposits in microvessels (8, 9), and newborns with fatal systemic HSV infection characteristically have terminal courses dominated by disseminated intravascular coagulation; moreover, autopsy histology reveals fibrin deposition upon, and HSV replication in, endothelial cells (10). The authors of one such report (8) speculated that viral-induced vascular damage may have set the stage for disseminated intravascular coagulation by exposing subendothelial collagen.

Although this was a plausible explanation, we wondered whether more subtle endothelial pertubations, for example in the plasma membrane, might be involved as the trigger of increased coagulation. For instance, during endothelial cell infection with enterovirus there is a restructuring of the plasma membrane, which is associated with enhanced adhesion of neutrophils to the virus-infected cells (11). Using different techniques, we have recently demonstrated altered membrane topography in HSV-infected endothelial cells as well; that is, we have preliminarily reported data (12)—expanded in the present report—that outer-leaflet membrane conformation may be abnormal in HSV-infected endothelium as reported by the membrane probe merocyanine 540 (MC 540).

For the present studies we hypothesized that virus-induced changes in endothelial cell phospholipid exhibition might alter their natural thromboresistance. To begin to test this hypothesis, we assessed the ability of HSV-infected endothelium to support prothrombinase complex assembly using purified prothrombin and factors Va and Xa. Finding enhanced thrombin generation, we studied thrombin-induced platelet accumulation on HSV-infected endothelium and found this also to be enhanced—a finding associated with defective prostacyclin production by infected endothelial cells. The relevance of virus-induced coagulant proclivity may not be limited to acutely infected tissue but may extend to more latent infected vascular tissue as well. In this regard, the demonstration by Benditt *et al.* (13) of the HSV genome

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Abbreviations: HSV, herpes simplex virus; MC 540, merocyanine 540.

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in atherosclerotic plaques, but not in surrounding normal vascular tissue, suggests that latent HSV infection may be an atherogenic trigger. If so, the present results imply a potential amplification of vascular occlusion in atherosclerosis, that of facilitated thrombosis.

MATERIALS AND METHODS

Reagents. Trizma, EDTA, human thrombin (for the platelet adhesion studies), MC 540, and indomethacin were obtained from Sigma. Trypan blue and trypsin were obtained from Gibco Laboratories, Chagrin Falls, OH. Fatty acid-free bovine serum albumin was obtained from Miles Scientific, Naperville, IL. The chromogenic thrombin substrate S2238 was obtained from Helena Laboratories, Beaumont, TX. The coagulation proteins used in this study were purified from human plasma, as described (14).

Virus. HSV-1 strain 17+ was used (15). Rabbit skin cells were used to propagate and titrate the virus by standard methods. Confluent monolayers of primary endothelial cells (generally 5–10 days after plating) were infected with 10 plaque-forming units per cell. After 1 hr of adsorption at 37°C with intermittent shaking, the virus suspension was replaced by tissue culture medium and the cells were incubated for the indicated time. Mock-infected controls were treated with the same dilution of a preparation of uninfected rabbit skin cells. As another control, adenovirus 4-infected endothelium was used; in this case, a similar plaque-forming unit/cell ratio was used and infection was documented by immunofluorescence assay.

Endothelial Cells. Human umbilical cord endothelial cells were grown to confluence as described (16). Primary cell cultures were used in all experiments. Endothelial cells contained von Willebrand factor as determined by immunofluorescence assay (17). In experiments where virus-infected and uninfected endothelium were compared, cells obtained from the same umbilical cords were used.

Prothrombinase Complex Assembly. The formation of a functional prothrombinase complex on the surface of the endothelial cells was assessed by minor modifications of a published assay for cells in suspension (14). In brief, endothelial cells, in 12-well plates (5 cm^2 per well), were either untreated, HSV-1-infected, mock-infected, adenovirus 4-infected, or preincubated with endotoxin (1 μg/ml for 8 hr; Difco). After washing, they were overlaid with 2 ml of Hepes/Tyrode/albumin buffer and warmed to 37°C. Factors Va and Xa were then added to a final concentration of 5 nM, which is rate-saturating with respect to thrombin generation. After 3 min at 37°C, prothrombin was added to a final concentration of 1.39 μM to initiate thrombin generation. Exactly 2, 4, 6, 8, and 10 min after the initiation of the reaction, samples (100 μl) were removed from each well and the reaction was terminated by the addition of an equal volume of cold 0.02 M Tris·HCl/0.15 M NaCl/0.05 M EDTA/0.1% PEG 8000, pH 7.4. Thrombin concentrations in each sample were determined by chromogenic assay using S2238 as substrate and compared to a thrombin standard curve prepared daily. The rate of thrombin generated per well was calculated by linear regression analysis of the different samples per well. The correlation coefficient of the samples was generally higher than 0.990. After completion of the reaction, the cells were detached from the well with trypsin/EDTA and counted. Results are expressed as rate of thrombin generated per endothelial cell.

MC 540 Binding to Endothelial Cells. MC 540 was dissolved in 50% ethanol at a stock concentration of 1 mg/ml. HSV-infected or uninfected endothelial cell monolayers were washed with a balanced salt solution containing 2% calf serum, exposed to MC 540 (10–40 μg/ml) at room temperature under reduced lighting conditions, and then washed extensively and examined by fluorescence microscopy. To quantify dye binding, MC 540 was extracted from similarly treated endothelial cells with ethanol as described (18) and measured spectrophotometrically at 555 nm.

Platelet–Endothelial Cell Binding Assay. Platelet attachment to endothelial cells was measured as described by Cervionke *et al.* (19), by using ^{51}Cr-labeled platelets and infected or mock-infected endothelial cells grown to confluence in 24-well plates. In some experiments endothelium was pretreated with 20 μM indomethacin for 30 min at 37°C and then washed once. Thrombin (final concentration, 0.5 unit/ml) or buffer was added to endothelial wells, followed 5 min later by 2×10^8 platelets to give a final platelet/endothelial cell ratio of 1000:1. After 30 min of incubation at 37°C while shaking at 100 rpm, the wells were washed, the adherent cells lysed, and nonadherent and adherent cell fractions counted, from which the percentage of platelet binding was calculated.

Prostacyclin Assay. Endothelial cells were cultured, washed, and pretreated when appropriate with thrombin and indomethacin as described above for the platelet adherence assay. After incubation for 5 min at 37°C, the cell-free supernatant fluid was aspirated and assayed without extraction for the stable prostacyclin metabolite, 6-keto-prostaglandin $F_{1\alpha}$, by radioimmunoassay (New England Nuclear). Total cellular protein was measured by the method of Lowry *et al.* (20), and variation between wells was less than 20%.

Statistical Analysis. The standard error was taken as an estimate of variance. Statistical differences were determined by the *t* test.

RESULTS

MC 540 Staining. Fluorescence microscopy of uninfected human endothelial cells, as well as endothelium infected for 4 hr with 10 plaque-forming units per cell, demonstrated no staining with MC 540, a dye thought to intercalate in membranes when lipids become loosely packed. In contrast, 12- and 18-hr-infected cells were strikingly fluorescent with MC 540; uninfected cells remained unstained. To assay dye binding more quantitatively, the absorbed dye was extracted and measured at 555 nm. MC 540 binding doubled after 18-hr HSV infection of endothelium (1.16 ± 0.24 μg per 10^6 cells for HSV-infected vs. 0.52 ± 0.04 for uninfected endothelium; $P < 0.05$).

Thrombin Generation Elicited by Prothrombinase Complex Assembly. Associated with the possible alteration in surface lipid conformation, the rate of thrombin generation on HSV-infected endothelial cells becomes significantly greater than on uninfected cells. Excessive thrombin generation rates were first detected after 4 hr of infection and progressively increased with duration of virus infection, reaching a 2- to 3-fold increase after 18 hr (Table 1). In contrast, infection of

Table 1. Rate of thrombin generation on HSV-1-infected and uninfected human endothelium

Treatment of endothelium	*n*	Thrombin, mol $\times 10^{-17}$ per cell per min
None	8	8.0 ± 1.1
Mock-infected 4 hr	4	12.2 ± 1.7
Mock-infected 18 hr	18	10.2 ± 0.6
HSV-1-infected 4 hr	8	15.8 ± 1.1
HSV-1-infected 18 hr	21	21.7 ± 0.8*
Endotoxin	14	6.1 ± 1.6

Monolayers were preincubated with factors Va and Xa (5 nM), followed after 5 min by prothrombin (1.39 μM). Samples were taken every 2 min, and the thrombin generated was measured by using the chromogenic substrate S2238.

*$P < 0.001$ compared to 18-hr mock-infected and untreated cells.

endothelial monolayers with another large DNA virus, adenovirus 4, did not significantly enhance prothrombinase activity after either 4 or 18 hr of infection (data not shown). The enhanced thrombin generation rate with HSV-infected endothelium is maximal, since saturating concentrations of reaction constituents (factor Va and factor Xa) were employed in these studies.

These results suggest that the surface of HSV-infected endothelium may more efficiently promote prothrombinase assembly than the surface of uninfected cells. In support, data depicted in Fig. 1*A* show that the rate of thrombin generated on either infected or uninfected endothelium is dependent on the concentration of added factor Va and that the maximum rate of Va-driven thrombin generation is greater on infected cells. Analogous titrations with factor Xa demonstrated an identical concentration-dependent and amplified response (Fig. 1*b*), which indicates that the surface of HSV-infected endothelium is also more catalytic for thrombin generation with Xa as the limiting substrate; moreover, we calculate that Va and Xa form a 1:1 stoichiometric complex on this surface.

We emphasize that these studies were performed with viable endothelial cells; trypan blue and ^{51}Cr-release studies indicated >98% viability. Moreover, removal of the supernatant fluid from an ongoing reaction terminated thrombin formation, indicating that thrombin generation was entirely cell-dependent and not due to free membrane fragments of virus-infected cells. In addition, preincubation of endothelium with endotoxin, which is known to induce a number of other coagulant properties in endothelial cells, did not increase formation of the prothrombinase complex (Table 1).

Platelet Adherence and Aggregation. Thrombin can induce aggregation and endothelial adhesion of platelets as well as endothelial cell prostacyclin production; thus the degree of thrombin-induced platelet binding to endothelial monolayers reflects a balance between these opposing effects. Preincubation of uninfected endothelial cells with thrombin, followed by the addition of platelets, resulted in only a small increase in radiolabeled-platelet attachment to endothelium, unless prostaglandin synthesis was inhibited by preincubation of the endothelium with indomethacin (Fig. 2). In striking contrast, thrombin induced marked platelet binding to HSV-infected cells ($P < 0.001$ compared to uninfected) even in the absence of indomethacin, whose addition resulted in little further increase in monolayer radioactivity (stippled bars, Fig. 2). When examined by transmission electron microscopy, platelets attached to thrombin-exposed HSV-infected endothelium appeared both as spread, adherent single cells and as aggregates of 5–10 cells. These data suggest prostacyclin production is deficient in infected endothelium. In confirmation, baseline prostacyclin production by uninfected endothelium was low (<0.3 ng per well) but increased with the addition of thrombin (13.7 ± 0.9 ng per well; $P < 0.001$); as expected, indomethacin inhibited this response (<0.3 ng per well; $n = 9$). In contrast, 18-hr HSV-infected cells only slightly increased their prostacyclin release with thrombin stimulation (0.7 ± 0.3 ng per well) above baseline (<0.3 ng per well)—a significantly deficient response compared to uninfected cells ($P < 0.0001$).

DISCUSSION

These results demonstrate that HSV infection of human endothelium promotes excessive thrombin generation on endothelial surfaces and enhances platelet attachment thereto. In the former instance, this potentially procoagulant phenomenon would seem to reflect more efficient assembly of the prothrombinase complex on infected monolayers by an unknown mechanism. It is tempting to speculate that virus-induced cell surface alterations may be involved. Perhaps negatively charged phospholipids (particularly phosphatidylserine), which are critical in mediating factor Va binding to phospholipid vesicles (14), are more readily exhibited on

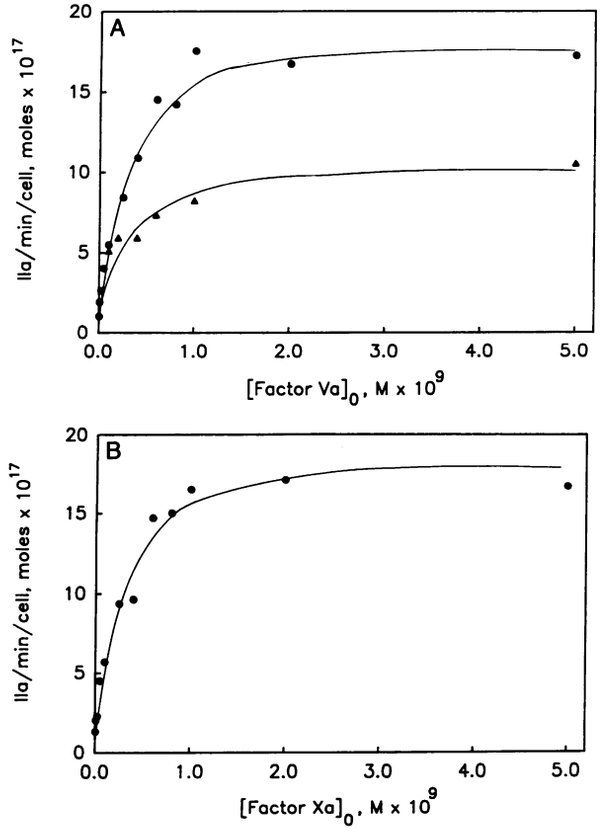

FIG. 1. Rate of thrombin generation on 18-hr HSV-infected (●) and mock-infected (▲) human endothelial cell monolayers. (*A*) Monolayers were preincubated with 0.005–5 nM factor Va and 5 nM factor Xa. After 5 min, 1.39 μM prothrombin was added. Samples were taken every 2 min, and the thrombin (factor IIa) generated was measured by using the chromogenic substrate S2238. (*B*) Parallel experiments were performed as in *A* except that factor Xa was varied from 0.005 to 5 nM and factor Va was held constant at 5 nM.

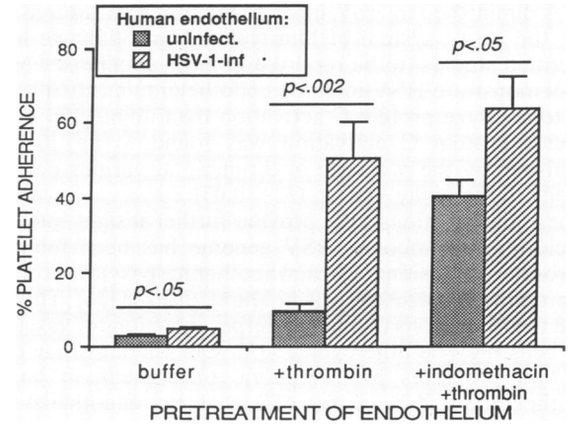

FIG. 2. Adherence of radiolabeled platelets to uninfected and 18-hr HSV-infected endothelial cell monolayers. Endothelium was pretreated with indomethacin (20 μM) or buffer for 30 min, followed if appropriate by replacement with thrombin (0.5 unit/ml) for 5 min. Radiolabeled platelets were added, and after 30 min of incubation the percentage of adherent platelets was determined. Bars represent means (+ SEM) of three experiments with three replicates per experiment.

surfaces of infected endothelial cells; of note, these phospholipids are generally not significantly present in the external bilayer leaflet of most cells (21). Equally plausible is that altered membrane proteins may affect prothrombinase assembly. Viruses, including HSV, have been shown to induce diverse changes in the proteins exhibited on the external plasma membrane during cell infection; these changes include the appearance of neoreceptors for immunoglobulin Fc portions and for complement component C3b (6, 11, 22). In partial support of a hypothesis that the hypercoagulability described in the present studies may reflect outer-leaflet conformational changes in HSV-infected endothelium, we found excessive uptake of the membrane probe MC 540. This probe is thought to intercalate in the external bilayer leaflet when lipids become relatively loosely packed (18). Although the exact cause of the increased binding of MC 540 to infected endothelial membranes is unknown, we believe that this phenomenon reports a critical surface conformational alteration that somehow promotes increased assembly of the prothrombinase complex.

A clearer picture emerges regarding the mechanism by which thrombin might induce excessive platelet attachment to HSV-infected endothelium (Fig. 2). By using a similar endothelial cell model, Hoak and coworkers (19, 23) have shown that thrombin induces prostacyclin release from endothelial cells that blunts thrombin-induced platelet adherence and aggregation. In this report, we show that HSV infection of endothelial cells results in a marked abrogation of prostacyclin synthesis, thereby lessening this endothelial cell-dependent inhibition of thrombin-induced platelet stickiness. Although deficient prostacyclin production by infected endothelium would seem to importantly underlie its avidity for activated platelets, additional factors may be involved as well. That is, even in the presence of indomethacin, platelets adhere significantly more to infected endothelium than to uninfected cells (Fig. 2).

Our data imply, but do not prove, that HSV-infected endothelium may be prothrombotic. We acknowledge that the extra thrombin generated on surfaces of infected endothelium might be neutralized under *in vivo* conditions by stimulated anticoagulant mechanisms, including enhanced activation of protein C (or S) or more robust antithrombin III modulation. The latter possibility seems unlikely, since Kaner *et al.* (24) have reported that synthesis of heparan sulfate proteoglycan (required for antithrombin III activation) is diminished in HSV-infected endothelium. Moreover, using the assay system of Maruyama *et al.* (25), we have performed studies (to be reported in more detail elsewhere) that demonstrate HSV-infected endothelium is actually less able to catalyze protein C activation than uninfected endothelium; Protein C activation was found to be 30–40% diminished by the presence of thrombin at various concentrations in the assay system ($P < 0.05$).

These observations may provide further insight into atherosclerotic thrombosis. HSV genome has been found in atherosclerotic lesions (13), and another herpesvirus in chickens promotes atherosclerosis in Marek disease (26). This has led to the hypothesis that latent herpes infections of vascular-wall cells may be atherogenic (13). Our findings that HSV infection promotes prothrombotic phenomena in human endothelium, and a report (27) that rat endothelial cell infection with a herpesvirus, rat cytomegalovirus, shortens the clotting time on the surface of these cells, prompt this construct: herpes infection not only damages endothelium as an initiating event in atherosclerotic plaque formation but promotes thrombosis on these lesions as well.

We conclude that HSV infection shifts endothelial cell properties from anticoagulant to procoagulant by promoting prothrombinase complex formation and function and by increasing platelet adherence to endothelial cells well before cell disruption takes place. Virus-induced changes in the endothelial plasma membrane and diminished prostacyclin secretion are suggested as the pathways for this pathophysiologic mechanism, potentially involved in both atherosclerotic thrombosis and acute HSV-induced tissue necrosis.

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