von Willebrand factor mediates platelet adhesion to virally infected endothelial cells

(adhesion molecule/herpes simplex virus)

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ABSTRACT von Willebrand factor is an adhesive glycoprotein critical to normal hemostasis. It is stored in the Weibel–Palade body of endothelial cells and upon release may mediate platelet adhesion. Herpesvirus-infected endothelium is known to be prothrombotic and to support enhanced platelet adherence. We previously identified P-selectin as a monocyte receptor that is translocated from the Weibel–Palade body to the endothelial cell surface in response to the local generation of thrombin on herpesvirus infected cells. In this study, we show that viral injury to vascular endothelial cells induces secretion of von Willebrand factor which mediates enhanced platelet adhesion to these cells.

Vascular endothelial cells and megakaryocytes synthesize von Willebrand factor (vWF), an adhesive glycoprotein which circulates in plasma (1–3). Activated platelets secrete vWF and express at least two receptors for vWF. Interactions of vWF with platelet glycoprotein IIb/IIIa may regulate platelet-to-platelet contact and platelet adhesion to subendothelial matrix. vWF interaction with platelet glycoprotein IIb is instrumental in mediating platelet adhesion to subendothelial collagen in vessels at high shear rates (4–6). These processes are critical to hemostasis, since qualitative or quantitative defects of vWF result in a mild to severe bleeding diathesis.

Endothelial vWF is both secreted constitutively and stored in intracellular organelles known as Weibel–Palade bodies (7). The stored vWF is predominantly in the form of high molecular weight multimers that are biologically potent and are more active in vitro platelet binding assays than circulating forms. Upon release, this vWF may mediate platelet adhesiveness to the vascular endothelium (8, 9).

Herpes simplex virus (HSV)-infected endothelium has been shown to be prothrombotic and to support enhanced platelet adherence (10). We have previously shown that HSV-infected endothelial cells generate significantly increased amounts of thrombin (11) and that increased thrombin generation promotes monocyte adhesion to the surface of infected endothelial cells. We identified P-selectin as a monocyte receptor on these cells, and postulated that the receptor translocated from the Weibel–Palade bodies to the external plasma membrane in response to the localized generation of thrombin on these cells (12). Since vWF and P-selectin colocalize to this intracellular organelle, we hypothesized that endothelial cells may comitochondriate secrete increased amounts of vWF in response to HSV infection. In this study, we show that subacute injury to vascular endothelial cells caused by HSV infection induces secretion of vWF which mediates increased platelet adhesiveness to the endothelial cell.

Materials and Methods

Culture of Endothelial Cells. Human umbilical vein endothelial cells (HUVECs) were isolated as described (13). Cells were routinely grown in RPMI 1640 medium (GIBCO) with 20% fetal bovine serum, heparin (100 µg/ml; Sigma), and 1% penicillin/streptomycin solution (Sigma). Cells were confirmed to be HUVECs by immunofluorescent staining with anti-vWF antisera. All cells in a single experiment were obtained from the same umbilical cord. Endothelial cell monolayers were prepared as described (14).

Viruses. HSV-1 (strain F) and adenovirus type 2 (Ad2) were purchased from the American Type Culture Collection. Confluent monolayers of HUVECs were infected with 0.1–1.0 plaque-forming unit of virus per cell. After 2 hr of adsorption at 37°C, the virus was removed and the cells were washed and refed with fresh medium for 24 hr prior to each experiment. Control studies were performed on mock-infected and Ad2-infected cells. In selected studies, HUVECs were infected with a previously characterized mutant of HSV-1, NS-1, which does not express herpesviral glycoprotein C (gC) on the infected endothelial cell surface (11). In these experiments, the number of virus plaque-forming units used was lower due to the toxicity of this mutant virus for endothelial cells (12).

Platelet Adhesion Assay. Platelets were freshly isolated from the blood of normal volunteers by gel filtration. Platelet adhesion was measured by using 51Cr-labeled platelets, which were prepared by the addition of 5 125CrCl3 to a suspension of platelets (106 per ml) at 22°C for 90 min. After they were washed in phosphate-buffered saline, labeled platelets (106 per well) were added to monolayers of cells in 24-well plates and incubated at 37°C. After 2 hr, culture medium, containing nonadherent platelets, was removed and the cells were gently rinsed once with culture medium. NaOH (0.2 M) was added to each well for 1 hr to solubilize the cells, and radioactivity was measured in a γ counter. In some experiments, antibodies to vWF (American Diagnostica, Greenwich, CT), HSV gC (courtesy of Harvey Friedman, University of Pennsylvania, Philadelphia), or platelet glycoprotein Ib or IIb/IIIa (courtesy of Adam Asch, Cornell Medical College) were added 1 hr prior to the addition of platelets. Hirudin (Sigma) and PPACK (d-Phe-L-Pro-L-Arg-CH2Cl), which are thrombin inhibitors, were added separately to designated wells 1 hr before the labeled platelet adhesion assay was done. In separate experiments, thrombin generation was measured with the chromogenic substrate S-2268 (Kabi Diagnostica, Stockholm) to confirm inhibition of thrombin generation in the presence of inhibitors. In specific

Abbreviations: Ad2, adenovirus type 2; gC, glycoprotein C; HSV, herpes simplex virus; HUVEC, human umbilical vein endothelial cell; PPACK, d-Phe-L-Pro-L-Arg-CH2Cl; vWF, von Willebrand factor.

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experiments, platelet adhesion to endothelial cell-derived extracellular matrices was similarly measured. Finally, vWF antigen levels were measured in conditioned media from control and HSV-infected endothelial cells with a vWF ELISA kit (American Diagnostica). A standard curve was generated with commercial vWF (American Diagnostica).

Statistics. Data were examined by analysis of variance.

RESULTS

HSV-Infection Induces vWF Secretion by Cultured HUVEC. HSV-infected HUVECs secreted 5-fold more vWF than uninfected cells (Fig. 1). Ad2-infected cells (a DNA virus control) secreted vWF at similar levels as control cells (data not shown). vWF secretion required surface expression of HSV gC, since specific murine monoclonal antibodies to gC inhibited vWF secretion of HSV-infected cells by >90% when added at least 1 hr prior to removal of cell conditioned medium for vWF measurement (Fig. 1). Nonimmune serum treatment did not inhibit vWF secretion of HSV-infected cells. In addition, cells infected with HSV mutants that do not express gC did not secrete increased amounts of vWF (Fig. 1). HSV-infected cells similarly treated with the thrombin inhibitor PPACK (10 μM) did not secrete vWF above the level of uninfected cells (Fig. 1). These data suggest that enhanced vWF secretion by HSV-infected HUVECs was dependent on both the local generation of thrombin and the surface expression of HSV gC.

Endothelial cell secretion of vWF significantly increased by 4–6 hr. Release appeared to decrease by 24 hr (Fig. 2). This time course parallels that of platelet adhesion, where maximal adhesion is seen 24 hr postinfection, with a significant increase in platelet adhesion seen as early as 4–6 hr postinfection (10). vWF secretion also paralleled the course of P-selectin expression that we previously reported (12). Specifically, increments in P-selectin and vWF expression were seen by 4 hr and remained elevated up to 24 hr.

![](image)

**Fig. 1.** vWF release by HSV-infected endothelial cells. HUVECs were cultured to confluence in 24-well plates (Costar). Cells were infected with HSV-1 or with the gC-negative (gC−) HSV mutant, NS-1. Sixteen to 24 hr after infection, antibodies to HSV gC (α-gC, 1:500), or control nonimmune serum IgG (ctrl, 1:500), or PPACK (10 μM) was added separately to specific wells. Cells were incubated for 1–2 hr after antibody addition, and then the conditioned medium was removed and frozen at −80°C until the vWF assay was performed. vWF was measured by ELISA (American Diagnostica) in which a standard curve was constructed by using pooled human plasma. One unit of activity corresponds to the amount of vWF in 1 ml of plasma. Data represent the mean ± SD from three experiments each performed in quadruplicate. Control cells (black bar) were mock-infected.

Platelet Adherence to HSV-Infected Endothelium Is Mediated by vWF. HSV infection induced a 4-fold increase in platelet adhesion as compared with uninfected cells or cells infected with the non-gC expressing HSV mutant NS-1 (Fig. 3). Target-cell viability was from 90–95% as assessed by trypan blue exclusion. This increased adhesion was inhibited 90% by goat anti-human vWF antibodies, whereas nonimmune serum did not inhibit adhesion to infected or uninfected cells. Noninfected endothelial cells exposed for 30 min to thrombin (3 units/ml) supported an 8-fold increase in platelet adhesion compared with nontreated cells (data not shown). Microscopic examination of the cells revealed that the platelets were adherent to the HUVECs and not to exposed extracellular matrix. In addition, extracellular matrices prepared from HSV-infected and uninfected cells did not significantly support differential platelet adhesion, further supporting the hypothesis that the platelets were adhering to endothelial cells and not to the underlying matrix (data not shown). The binding of platelets to HSV-infected cells was

![](image)

**Fig. 2.** Time course of vWF release by HSV-infected endothelial cells. Conditioned medium from HSV-infected (■) and mock-infected (□) HUVECs was collected at intervals 0–24 hr after infection. Conditioned media were stored at −80°C before vWF ELISA. Data represent the mean ± SD from two experiments each performed in triplicate.

**Fig. 3.** Platelet adhesion to HSV-infected endothelial cells. HUVECs were mock-infected (control, black bars) or were infected with HSV-1 (open bars) or the gC-negative HSV mutant NS-1 (hatched bar). Cells were preincubated with polyclonal goat anti-human vWF or control antisera (Ctrl IgG) for 30 min. Radiolabeled platelets were added and after 2 hr of incubation, the percentage of adherent platelets was measured. Data represent the mean ± SD from three experiments each performed in quadruplicate.
most likely mediated by platelet glycoprotein Ib–vWF interaction since polyclonal antibodies to glycoprotein Ib completely inhibited adherence of platelets to virally infected cells (Figs. 4 and 5). Furthermore, antibodies to platelet glycoprotein IIb/IIIa or IV did not significantly inhibit platelet adhesion to HSV-infected endothelium, demonstrating specificity (Fig. 4).

Taken together, our findings suggest that platelet adhesion to infected endothelial cells was dependent upon expression of vWF, which in turn was mediated by microenvironmental generation of thrombin.

**DISCUSSION**

HSV infection of cultured endothelial cells is known to promote a prothrombotic endothelial cell phenotype by virtue of increased tissue-factor and thrombin generation, decreased prostacyclin production, and the adhesion of circulating platelets, monocytes, and neutrophils (10, 15, 16). Thrombin may act in an autocrine fashion by promoting localized cell–cell adhesion by expressing new adhesion molecules (12). In this study, we demonstrate that increased vWF expression by HSV-infected endothelial cells promotes platelet adhesion to these cells. Our data further implicate the autocrine role of thrombin in this system, since the mechanism for vWF expression is a thrombin-inducible one. In our model put forth earlier, the Weibel–Palade body, a source of preformed vWF, translocates to the endothelial cell membrane when stimulated by thrombin, resulting in expression of P-selectin and monocyte adhesion. We now extend this hypothesis to include simultaneous vWF secretion by HSV-infected cells, with resultant increased platelet adhesion. In cells with acute herpesvirus infection, protein synthesis and mRNA transcription are down-regulated because the virus degrades total RNA transcripts in the cell (17). However, HSV infection can nevertheless promote novel expression of adhesion molecules on the endothelial surface by promoting translocation of the endothelial storage-granule membrane contents to the surface of these cells. This is also consistent with generalized HSV-induced cellular actions, since it does not require de novo protein synthesis of either P-selectin or vWF.

![Platelet adhesion](image)

**FIG. 4.** Platelet glycoprotein Ib mediates platelet adhesion to HSV-infected endothelial cells. HUVECs were mock-infected (black bar) or were infected with HSV-1 or the gC-negative mutant NS-1. HSV-infected cells were preincubated for 30 min with polyclonal antibodies to platelet glycoprotein Ib, IIb/IIIa, or IV (1:500). Platelet adherence was measured as described earlier. Data represent the mean ± SD from three experiments each performed in quadruplicate.

![Glycoprotein Ib](image)

**FIG. 5.** Glycoprotein Ib mediates platelet adhesion to HSV-infected cells. Endothelial cells grown in 24-well plates were infected with HSV. Platelet adherence was measured 18 hr after infection. (a and b) Platelet adherence to control and HSV-infected cells, respectively. (c) Platelet adherence to HSV-infected cells in the presence of anti-glycoprotein Ib. (×140.)

The concept of adhesion molecules mediating pathogen-induced host-cell injury is not derived solely from vWF-platelet interaction on HSV-infected endothelium. Rickettsial infection of cultured cells similarly induces vWF release which is thought to participate in the pathogenesis of Rocky Mountain spotted fever (18). Bacterial endotoxin has also been shown to enhance vWF release from endothelial cells, suggesting a common pathway for pathogen-mediated thrombocytopenia (19). Hostetter and coworkers (20) have described an integrin analogue on *Candida albicans* as a possible mediator of yeast–endothelial adhesion. This yeast surface protein is structurally and antigenically homologous to the α subunit of the leukocyte adhesion molecule Mac-1 found on neutrophils and monocytes. Mac-1, a receptor for complement component C3bi, also has a functional homolog on HSV-infected cells—namely, gC, which can bind and activate coagulation factor X, leading to thrombin generation and monocyte adhesion on infected cells (12). In this study, we have shown that platelet adhesion to virally injured endothelial cells is mediated by surface expression of vWF, which depends on thrombin generation on these cells. Visser et al. (10) postulated that decreased prostacyclin synthesis may account in part for increased platelet adherence to HSV-infected endothelial cells. However, indomethacin treatment did not abolish the observed increase in platelet adhesion, suggesting that other factors, such as vWF, may be involved.

Platelet adhesion to the endothelium is an early event in some animal models of inflammation and atherosclerosis. Intravascular coagulation which occurs clinically in sepsis and thrombotic thrombocytopenic purpura (TTP) is also characterized by diffuse platelet–endothelial adhesion. The
role of large vWF multimers in mediating such platelet–endothelial adhesion in TTP has been postulated by Moake et al. (21). In *vitro*, large vWF multimers have been shown to mediate increased adhesion of sickle erythrocytes to endothelial cells (22).

Platelet adhesion may also play an important role in tumor metastasis. In animal models, thrombocytopenia and impaired platelet function have been shown to correlate with reduced tumor metastasis. vWF, platelet glycoprotein IIb/IIIa, and fibronectin have all been implicated *in vitro* in platelet–tumor cell interactions and metastasis (23).

In summary, we propose that HSV-infected endothelium is prothrombotic, in part due to expression of adhesion molecules for circulating platelets. One mechanism for such expression may be virus-induced translocation of a pre-formed pool of these molecules to the endothelial cell surface. This type of pathophysiologic mechanism may also be involved in virus-mediated atherogenesis and virus-induced thrombocytopenia.

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