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Herpes Simplex Virus Inhibits Endothelial Cell Attachment and Migration to Extracellular Matrix Proteins

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Herpes simplex virus (HSV) infection may be involved in various endothelial-injury syndromes, including vasculitis and atherosclerosis. In a previous study, it was reported that HSV-infected human umbilical endothelial cells are more vulnerable to detachment mediated by granulocyte-secreted proteases. To elucidate the molecular basis of this observation, the authors examined the interaction of infected endothelial cells with the purified basement membrane proteins, fibronectin, laminin, and type IV collagen. HSV-infected endothelial cells exhibited defects in their ability to adhere, spread, and migrate on all three matrix components. This defective adhesion could be partially overcome by increasing concentrations of fibronectin; in contrast, no abrogation of deficient binding occurs with increased levels of laminin or collagen type IV. This suggests that endothelial cells may use different surface constituents for binding to the three proteins and use multiple "receptors" for adhesion to the fibronectin molecule—"receptors" that are variably affected by HSV infection. The authors investigated this supposition by assaying adhesion of normal and infected endothelial cells to two nonoverlapping cell-adhesion promoting fragments of fibronectin: 1) a 75 kd motility-promoting fragment which contains the arginyl-glycyl-aspartyl-serine (RGDS) adhesion sequence, and 2) a 33 kd carboxyl-terminal heparin binding fragment, which promotes cell adhesion by an RGDS-independent mechanism. Normal endothelial cells adhered and spread on both purified fragments. In contrast, while infected endothelial cells could adhere, albeit rather poorly, to high coating concentrations of the 75 kd fragment, these cells did not bind to the 33 kd heparin binding fragment of fibronectin at all. These results support the concept that endothelial cells adhere to multiple domains of fibronectin, and that HSV infection preferentially abrogates binding to the heparin-binding domain, while leaving relatively intact receptors for the RGDS-containing domain. In support, soluble RGDS significantly blocked fibronectin adhesion of infected, but not control, endothelial cells. It is concluded that HSV infection inhibits the interaction of endothelial cells with basement membrane proteins and weakens their tethering to substratum. This tethering is inadequate for proper cell spreading or movement to occur and may result in both excessive endothelial lift-off and impaired vascular repair in HSV infections. (Am J Pathol 1989, 134:223–230)

A continuous lining of endothelial cells separates the vascular lumen from underlying tissues. This endothelial cell layer is in intimate contact with a basement membrane, which is important for the maintenance of cellular continuity and phenotype. Traumatic disruption of the continuous endothelial lining with concomitant exposure of the subendothelial basement membrane activates a number of pathophysiologic mechanisms that are intended to limit and repair vascular damage. Normally, influx of inflammatory cells, activation of blood coagulation, and endothelial cell proliferation and migration all act in concert to restore a confluent endothelial lining. More subtle endothelial damage induced by chemical, immunologic, or viral agents may not only result in the disruption of the endothelial lining, but also may hinder subsequent endothelial repair mechanisms, resulting in the continuous activation of inflammatory or coagulation systems.

As a possible example of this more subtle injury, her-

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pes simplex virus (HSV) has been shown to infect endothelial cells *in vitro*[1] and has been found in endothelial cells in disseminated infections.[2] In addition, HSV lesions often show signs of leukocytoclastic vasculitis, in which granulocytes are found in and around vessel walls together with thrombi and fibrin deposits.[3] Consistent with these *in vivo* observations, we and others have shown that granulocytes are attracted to, and damage, HSV-infected endothelium *in vitro*.[4,5] Our own interest in this phenomenon has been energized by suggestions that herpes virus infection of vascular structures may be involved in atherogenesis.[6] To review: Benditt et al[7] found HSV RNA in the intima of atherosclerotic plaques, but not in normal surrounding tissue of patients undergoing coronary bypass surgery; moreover, herpes virus particles have been observed by others in atherosclerotic lesions[8]; in addition, the Marek's disease herpes virus induces atherosclerotic lesions in chickens[9]; and last, HSV infection may induce lipid accumulation in arterial smooth muscle cells, somewhat analogous to the lipid accumulation observed *in vivo* during human atherosclerosis.[10]

Taken together, these observations suggest HSV could be an important contributing factor in vascular damage and have prompted the present studies. We reasoned that the repair and maintenance of the endothelial layer following injury should be intimately related to the ability of endothelial cells to adhere and migrate on components of the underlying basement membrane. We recently showed that HSV-infected endothelial cells are excessively detached from their matrix proteins by granulocyte-released proteinases as compared to their uninfected counterparts.[11] This observation raised the question whether HSV infection might diminish the interactions between endothelial cells and matrix proteins which would seem important in mediating cell adhesion, spreading, and motility.

In the present studies we examined the ability of normal and HSV-infected endothelial cells to adhere, spread, and migrate on the basement membrane proteins, fibronectin, laminin, and collagen type IV. We found these functions to be markedly abnormal with infected cells and speculate that faulty tethering and movement of endothelium on basement membrane proteins may have a pathophysiologic role in HSV-induced tissue necrosis and perhaps in atherogenesis as well.

Material and Methods

Reagents

Ethylenediamine tetraacetic acid (EDTA) was obtained from the Sigma Chemical Company, St. Louis MO. Human plasma fibronectin and 75 kd and 33 kd fibronectin fragments were purified from a byproduct of Factor VIII production by gelatin and ion exchange chromatography as described previously.[12,13] Laminin and collagen type IV were isolated from Englebreth-Holm-Swarm sarcoma grown in lathrytized animals as described.[14] RGDS and RGES were synthesized and HPLC purified by the microchemical facility at the University of Minnesota.[13]

Virus

Herpes simplex virus type 1 strain 17 syn+ was used. Rabbit skin cells were used to propagate and titrate the virus by standard methods described previously.[15]

Endothelial Cells

Human umbilical cord endothelial cells (HUEC) were separated and grown to confluence as described.[16] Care was taken to use endotoxin-free materials and media; the absence of endotoxin was validated by limulus assay (Pyrotell, Associates of Cape Cod, Inc., Woods Hole, MA). Primary cell cultures were used except in the endothelial cell migration assay, where subconfluent secondary passage cells were used. Endothelial cells contained von Willebrand factor antigen by immunofluorescence assay.[17] In experiments where virus-infected and uninfected endothelium were compared, cells obtained from the same umbilical cords were used.

Endothelial Cell Adhesion to Matrix Proteins

Solutions of fibronectin, collagen type IV and laminin were added to microtiter wells (Removacell, Dynatech Laboratories, Alexandria, Virginia) for 12 hours at 37 C at the indicated concentrations in carbonate buffer (pH 9.6). The wells were then coated with fatty acid free bovine serum albumin (Pentex fraction V; Miles Scientific, Naperville, IL) to block nonspecific binding sites for cells on plastic.

Human umbilical vein endothelial cells were grown to confluence in tissue culture dishes (10 cm; Becton Dickinson Labware, Oxnard, CA). After inoculation of some monolayers with 5–10 PFU per cell of HSV for various periods up to 18 hours, the dishes were washed and incubated with 100 μCi $Na_2\ ^{51}CrO_4$ in RPMI 1640 at 37 C for 1 hour, at which time they remained completely confluent. The monolayers were washed with Ca/Mg-free Hank's balanced salt solution (HBSS) (Gibco), and taken into single cell suspension by incubation in Ca/Mg-free HBSS with 10 mM EDTA for 10–20 minutes, followed by three washings in RPMI with 0.5% albumin. In some experiments, the suspended endothelial cells were incubated

for 45 minutes with the indicated concentrations of RGDS or RGES. The infected or uninfected cell suspensions, (50 μl containing 10^4 cells in RPMI with 0.5% albumin; >95% viable by trypan blue exclusion) were added to matrix-protein coated wells, and the microtiter plate was incubated for 45 minutes at 37 C. After vigorous shaking (200 rpm during 30 seconds) the wells were washed twice and the total and the adherent cell fractions were counted on a gamma counter. Spontaneous ^{51}Cr release from endothelial cells was less than 20% and did not differ between infected and uninfected cells. Unless noted otherwise, the mean of five replicate samples is presented. Endothelial cell viability remains normal (>95% trypan blue exclusion) for 48 hours after HSV infection; maximum infection time in the present studies was 18 hours. After 18 hours of infection, minimal cytopathic changes are noted by light microscopy. Rounding up of the cells is first seen 30–48 hours after infection and eventually the cells do detach from the tissue culture plate.

Endothelial Cell Migration Assay

Migration assays were performed as previously described[12] with subconfluent (60–95%) primary or first-passage human endothelial cells that were infected and taken in single-cell suspension as described for the endothelial cell adherence assay. The cells were resuspended at a final concentration of 4×10^5 cells/ml in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) containing 20 mM HEPES, pH 7.4. Putative attractants were added at various concentrations in a volume of 25 μl to the lower wells of modified Boyden microchemotaxis chambers (Neuroprobe, Bethesda, MD). The lower wells were then overlaid with an 8 micron pore size polycarbonate filter without polyvinyl pyrollidone coating (Nucleopore Corp., Pleasanton, CA), and the upper half of the chamber was secured into place. Each of the upper wells received 50 μl of cell suspension and the chambers were incubated at 37 C in a humid atmosphere for 6 hours. At the end of the assay period the filters were removed, fixed, stained (Diff-Quick fixative, American Scientific Products, McGaw Park, IL) and mounted on a glass slide. The migrated cells were quantitated by viewing in a Zeiss Universal microscope integrated with an Optomax Image Analysis System equipped with an Apple IIe computer. The data are presented as the number of migrated cells/mm^2 filter surface area and unless otherwise indicated, the mean of triplicate determinations is presented.

Measurement of Cell Spreading

The area of endothelial cells adherent on matrix coated substrata was determined directly by using a Nikon Diaphot inverted phase microscope connected to an Optomax Image Analysis System (Optomax, Inc., Hollis, NH) that was integrated with an Apple IIe Computer. The conversion factor used to convert pixels to actual cell area (sq μ) was determined using a stage micrometer. For the determination of area, unlabeled endothelial cells were allowed to adhere to 24-well tissue culture plates that had been coated with various matrix proteins as described above. After a 90 minute to 2 hour incubation, the medium was aspirated, and the cells fixed with the addition of 1–2% glutaraldehyde in PBS. The cells were incubated in this solution for at least 45 minutes, at which time the wells were washed with PBS and incubated overnight in Wright's stain. The cultures were then washed and immersion oil was added to each well to help prevent leaching of the stain. Cell area was determined for at least 30 randomly selected cells per culture, and each experimental condition was in duplicate.

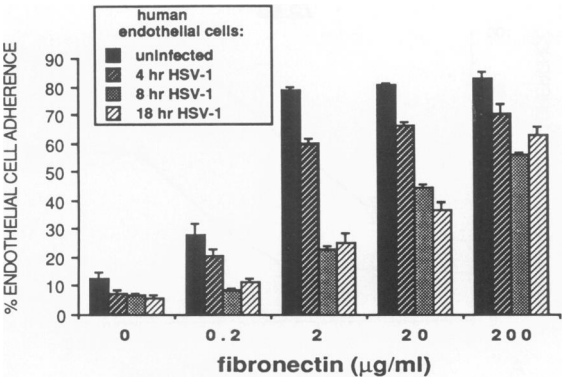

Figure 1. *Endothelial cell adherence to fibronectin coated wells. Human endothelial cells were infected with HSV for various time periods and cell adhesion to tissue culture wells pretreated with RPMI and 0.5% albumin (○) or various concentrations of fibronectin assessed as in the Methods section. Results are expressed as the mean percentage adherent endothelial cells ± SE.*

Statistical Analysis

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

Results

Endothelial Cell Attachment to Fibronectin

As shown in Figure 1, binding of human endothelial cells is dependent on the concentration of fibronectin used to coat the microtiter wells. HSV infection of endothelial cells diminishes their reattachment to fibronectin, which is particularly apparent at lower coating concentrations of fi-

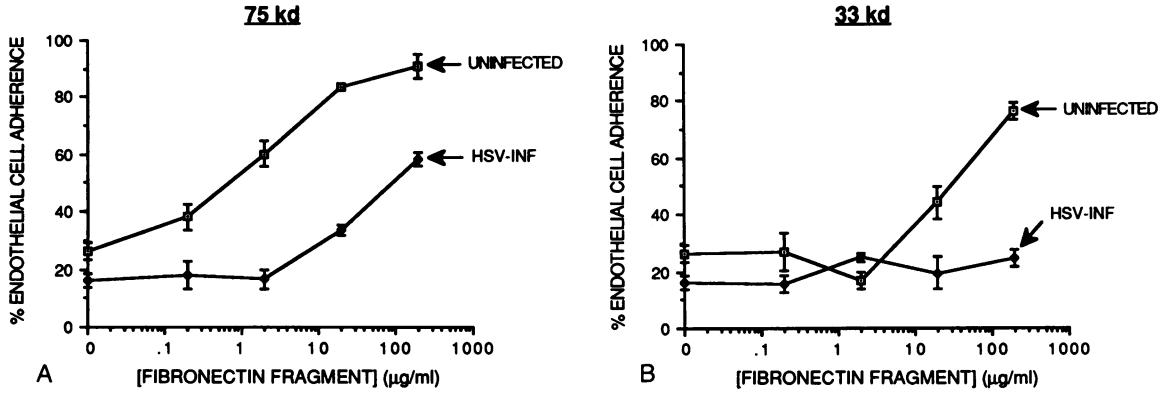

Figure 2A, B. *Endothelial cell adhesion to fibronectin fragments. Tissue culture wells were pretreated with buffer (○) or various concentrations of the 75 kd (A) or 33 kd (B) fibronectin fragments; uninfected and HSV-infected endothelial cell adhesion to these fragments were assessed as in the Methods section. Results are expressed as the mean percentage adherent endothelial cells ± SE.*

bronectin (0.2–20 μg/ml). Defective adhesion can be detected after only four hours of infection and binding is virtually maximally inhibited after 8 hours of infection.

Because fibronectin contains multiple cell-binding domains on different proteolytically-cleaved fragments, we coated wells with either a 75 kd or a 33 kd fibronectin fragment (Figures 2A, B).[12] Uninfected cells bind and spread in a dose-dependent fashion to either fragment (90 ± 4% to 200 μg/ml 75 kd and 76 ± 3% to 200 μg/ml 33 kd fragment). In contrast, HSV-infected cells do not increase binding to the 33 kd fragment (solid circles, Figure 2B) and attach poorly to the 75 kd fragment—mainly at very high coating concentrations (>20 μg/ml) (Figure 2A). Because this 75 kd fragment contains the cell-binding RGDS sequence, the effect of exogenous RGDS on cell binding was studied. As shown in Table 1, attachment of HSV-infected endothelial cells to fibronectin is highly susceptible to inhibition by RGDS, whereas their uninfected counterparts are not inhibited by the soluble peptide; addition of RGES as a control did not inhibit binding of either cell preparation, even when used at ×10 the concentration of RGDS.

Endothelial Attachment to Type IV Collagen and Laminin

HSV infected endothelial cells also bind poorly to the other major extracellular matrix proteins, collagen type IV and laminin. Unlike with fibronectin, where very high concentrations of coating protein can partially improve the defective adherence, binding to collagen is only minimally, (if at all) improved by increasing adhesogen concentrations (Figure 3). Moreover, infected cells also bound poorly to 200 μg/ml laminin (32 ± 6%) as compared to uninfected cells (72 ± 1.6%, $P < 0.01$, Figure 4).

Table 1. *Endothelial Cell Adherence to Fibronectin Coated Wells*

	Buffer	RGDS (100 μM)	RGES (1000 μM)
Uninfected	75 ± 2%	74 ± 1%	73 ± 1%
HSV-infected	36 ± 4%	17 ± 1%*	33 ± 2%

Endothelial cells (uninfected) or HSV-infected) were pretreated with Buffer (RPMI with 0.5% albumin), RGDS (100 mM) or RGES (1000 mM) and reattachment to fibronectin coated wells (200 mg/ml) assessed as in the Methods section. Results are expressed as mean % adherent cells ± SE.

* $P < 0.05$ infected cells RGDS vs. buffer.

Endothelial Cell Spreading and Migration to Matrix Proteins

Under light microscopy uninfected endothelial cells manifest obvious spreading after 45 minutes of contact with fibronectin, collagen type IV or laminin (Figure 5); in contrast, infected endothelial cells (although >95% viable by trypan blue exclusion) remain rounded. Because with other motile cells, spreading usually precedes cell migra-

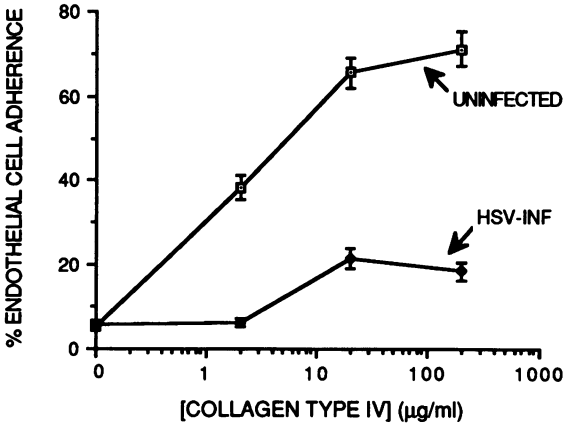

Figure 3. *Endothelial cell adhesion to collagen type IV. Tissue culture wells were pretreated with buffer (○) or various concentrations of collagen type IV; uninfected and HSV-infected endothelial cell adhesion was assessed as in the Methods section. Results are expressed as mean percentage endothelial cells adherent ± SE.*

tion, we studied chemotaxis of endothelial cells. Fibronectin or type IV collagen efficiently stimulate migration of uninfected endothelial cells in Boyden chambers and do so at similar doses (Figure 6); diminished migration noted at the highest concentrations probably reflects cell aggregation. As might be expected, virus-infected endothelial cells are severely defective in migration to the gradients. That spreading may be required, but is not sufficient, to induce motility, is suggested by our findings with laminin (Figure 6); that is, neither uninfected or infected cells migrate to laminin despite the fact that spreading occurs in the former instance.

Discussion

The adhesion of many normal and transformed cell types to extracellular matrix proteins has been intensively studied in recent years.[18-20] These studies have demonstrated that cell adhesion to components of the extracellular matrix has a complex molecular basis, involving multiple determinants within matrix molecules that interact with distinct receptors on the surface of cells. Fibronectin, laminin, and collagen type IV have all been demonstrated to promote cell adhesion by interacting with distinct cell receptors.[21] Furthermore, adhesion to any single extracellular matrix protein may also involve multiple determinants which interact with distinct cell surface receptors. The protein most intensively studied in this regard is fibronectin, which is present in plasma, connective tissues, and in certain basement membranes as well.[18-20] The best characterized adhesion promoting determinant within fibronectin is the RGDS adhesion sequence.[22] This sequence, which is also present in a variety of other adhesion-promoting proteins such as von Willebrand factor, fibrinogen, and vitronectin, promotes cell adhesion as a result of interacting with a family of adhesion promoting receptors termed integrins.[21] Despite the importance of this ligand/receptor interaction in cell adhesion, recent evidence from a number of laboratories has implicated additional determinants and cell surface receptors in adhesion to fibronectin. For example, purified heparin-binding fragments of fibronectin, which lack the RGDS sequence, promote the adhesion and spreading of a number of normal and transformed cells *in vitro*.[20] Furthermore, the formation of focal adhesions and stress fibers in cells adherent on fibronectin requires the participation of both the RGDS containing region of fibronectin as well as the heparin binding region of this protein.[20] These results are consistent with the hypothesis that cell surface proteoglycans are important in the adhesion of a variety of cell types.

The current studies demonstrate that uninfected human umbilical vein endothelial cells adhere and spread in a concentration dependent manner on substrata coated

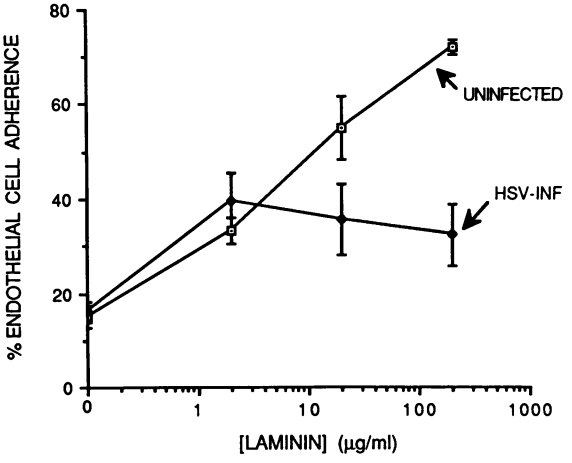

Figure 4. *Endothelial cell adhesion to laminin. Tissue culture wells were pretreated with buffer (○) or various concentrations of laminin; uninfected and HSV-infected endothelial cell adhesion was assessed as in the Methods section. Results are expressed as mean percentage endothelial cells adherent ± SE.*

with purified fibronectin, laminin, or collagen type IV; however, laminin is much less effective than either fibronectin or collagen type IV at stimulating adhesion and spreading of these cells. These results agree with previous studies of Clark et al,[23] Macarak and Howard,[24] and Herbst et al.[25] Uninfected endothelial cells also migrate in response to gradients of these three proteins in modified Boyden chambers. As with adhesion and spreading, laminin is ineffective at promoting endothelial cell migration compared to type IV collagen or fibronectin. The adhesion, spreading, and migration of HSV infected endothelial cells is greatly reduced, or entirely eliminated, when low to intermediate coating levels of these three proteins are used. The defect in endothelial cell adhesion to fibronectin is detectable within 4 hours after HSV infection (Figure 1)—many hours before ultrastructural changes can be discerned. Defective adhesion can be partially corrected by using high coating levels of fibronectin, while no such correction is observed when high coating levels of laminin or collagen type IV are used. These results suggest that endothelial cell adhesion to each of these three proteins involves distinct mechanisms, which is consistent with literature reports in other cell types describing distinct surface receptors for these three proteins.[21] Our present studies also demonstrate that the various adhesion mechanisms used by uninfected endothelial cells are differentially affected as a consequence of HSV infection.

Our studies also demonstrate that endothelial cell adhesion to fibronectin involves multiple domains on fibronectin which interact with distinct cell surface receptors. Uninfected endothelial cells adhere on both a 75 kd RGDS containing fragment and a 33 kd heparin binding fragment, which promotes cell adhesion by an RGDS-independent mechanism.[12,13] The presence of multiple ad-

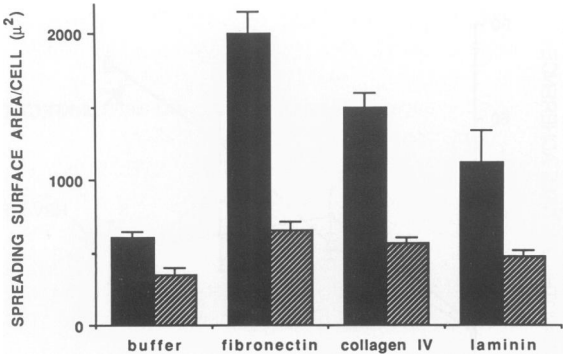

Figure 5. *Decreased HSV-infected endothelial cell spreading on matrix coated substrata. Tissue culture plates were coated with buffer (RPMI/0.5% albumin), fibronectin (200 μg/ml), collagen type IV (200 μg/ml), or laminin (200 μg/ml). Normal (solid bars) or HSV-infected (hatched bars) endothelial cells, (2 × 10⁴/ml) were added for 90 minutes, the cells fixed, stained, and the average area/cell determined as described in the Methods section. The data represent the mean of 30 cell scans in each of duplicate wells ± SE.*

hesion sites for endothelial cells on fibronectin suggests a partial explanation for the relative inability of RGDS to inhibit uninfected endothelial cell adhesion on the intact molecule (Table 1). This has been reported previously for both normal and transformed cells,[26,12] especially when high coating concentrations of fibronectin were employed. In contrast, HSV infected endothelium—capable of adhering to high levels of fibronectin—demonstrates significant sensitivity to the inhibitory effects of soluble RGDS, suggesting that HSV-infected endothelial cells still retain a partially-functional class of integrin-like molecules on their surface. In support of this, the 75 kd RGDS containing fragment of fibronectin still partially promotes the adhesion of infected endothelial cells, whereas adhesion to the 33 kd heparin fragment is virtually eliminated. That adhesion to the 33 kd heparin-binding fragment of fibronectin is eliminated is especially interesting in light of the recent preliminary report that HSV-infected endothelial cells may be deficient in surface-associated heparan sulfate proteoglycan.[27]

The deficient binding of HSV-infected endothelial cells to collagen type IV and laminin is reminiscent of a similar effect seen during incubation of other cultured cells with cycloheximide, a protein synthesis-inhibitor.[28,29] HSV infection both inhibits overall protein synthesis in diverse cells and has been shown to decrease endothelial fibronectin synthesis.[24,30] With the additional abnormality of fibronectin binding sites,[31] it is not surprising that surface fibronectin has been found decreased in virally-infected endothelium. Because cell surface fibronectin can promote cell attachment directly, or by complexing with other matrix proteins such as collagen it remains to be seen whether deficient adhesion of infected cells to collagen reflects loss of hypothetical collagen receptors on the cell surface, or might be due to the loss of surface fibronectin.

We believe these studies may provide insight into the mechanism by which HSV infection engenders endothelial cell detachment from substratum in the presence of granulocytes[5] or activated lymphocytes.[32] The studies not only provide mechanisms by which HSV infection might promote endothelial denudation, but also suggest that such infection might interfere with vascular repair strategies. We find endothelial cells are actively attracted to the extracellular matrix proteins, fibronectin and collagen type IV which might be important in restitution of vascular lining after endothelial cell loss. HSV infection inhibits this migration, and we suggest the loss of adhesogenic protein binding sites may underly this defective motility. That is, cytoskeletal structures containing actin, polymerize and rearrange in association with diverse cell-migration stimuli. Attachment of surface adhesogenic proteins, including fibronectin, have been shown to foster such arrangements of cytoskeletal elements.[33] We suggest the altered binding of HSV-infected endothelial cells to extracellular matrix proteins may affect their cytoskeleton sec-

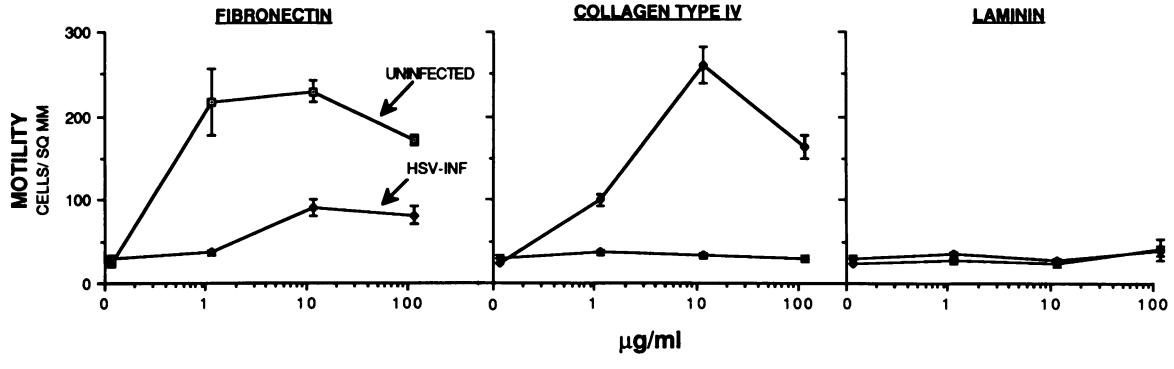

Figure 6. *HSV-infected endothelial cells exhibit defects in migration. The indicated concentrations of fibronectin, collagen type IV, laminin, or buffer (○) were added to the lower wells of modified Boyden chambers and the wells overlain with 8.0 μ polycarbonate filters. Normal (open symbols) or HSV-infected (closed symbols) endothelial cells (2× 10⁴/well) were dispensed into the upper wells and the chambers incubated for 4 hours at 37 C. Filters were fixed, stained, and the number of cells migrating to the lower filter surface quantitated as described. The data are presented as the mean cells/sq mm of triplicate determinations ± SE.*

ondarily and thereby "paralyze" these cells. Indeed, others have shown that disturbances of intracellular cytoskeleton morphology occur during fibroblast infection with HSV,[34] and we suspect a similar disturbance may prove to underlie the defect in migration of HSV-infected endothelium shown herein. In fact, in preliminary studies with fluorescent anti-actin serum, we have reported the loss of parallel actin arrays in HSV-infected endothelium.[11] Alternative processes that are important in cell migration such as endocytosis[35] may be affected by virus infection as well, because HSV induces changes in endocytosis and the membrane bilayer itself.[36,37]

In summary, HSV infection diminishes binding and migration of endothelial cells to extracellular matrix proteins. The binding to different matrix proteins and to their constituent peptides is differentially affected, suggesting that more than one membrane binding site is involved. This model system may prove useful in distinguishing between different adhesion mechanisms that endothelial cells use. Our results may help explain endothelial cell vulnerability to detachment by inflammatory cells, and may provide insight into the vascular damage seen clinically in HSV infections, including perhaps atherosclerosis.

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