Expression of Factor V on Human Umbilical Vein Endothelial Cells Is Modulated by Cell Injury

Anjanayaki E. Annamalai, Gwendolyn J. Stewart, Bruce Hansel, Mark Memoli, Hui-Chong Chiu, Donald W. Manuel, Kundan Doshi, and Robert W. Colman

Since human endothelial cells synthesize Factor V but do not secrete it into the medium, we studied the effects of cell injury on the availability of Factor V at the surface of these cells. Human umbilical vein endothelial cells (HUVEC), grown to confluency and incubated with human $^{125}$I Factor Va, specifically bound 5000 to 7000 molecules per cell. In the absence of added Va, no antigen was detected on adherent HUVEC with either labeled anti-Va monoclonal or polyclonal IgG. However, exogenous Va, not V, prebound to these cells allows binding of labeled $^{125}$I anti-Va. Immunodetectability of bovine Factor V contributed by fetal calf serum in the concentration used in cultures is less than 0.1% of that detected in human plasma. HUVEC, suspended by scraping from dishes, specifically bound 4000 molecules/cell of $^{125}$I monoclonal IgG against V(Va). Although undisturbed cells excluded trypan blue, dye uptake by many of the suspended HUVEC indicated cell injury. Quantitation of injury by $^{51}$Cr release after scraping followed by multiple passages through an 18 g needle showed that $^{51}$Cr release increased with number of manipulations up to 60% and was observed almost immediately after manipulation. We suggest that little Factor V(Va) is present on the surface of intact adherent HUVEC. However, mechanical injury to HUVEC released or exposed endogenous Factor V(Va), resulting in expression of V that might mediate Factor Xa binding as well as activation of protein C by thrombin.

Thus, injured, but not intact, HUVEC could participate in both promoting and limiting blood coagulation. (Arteriosclerosis 6:196–202, March/April 1986)
FACTOR V EXPOSURE ON ENDOTHELIAL CELLS

Annamalai et al.

North Billerica, Massachusetts. \(\text{Na}_2\text{CrO}_4\) in normal saline solution from ICN, Irvine, California. Endothelial cell growth supplement and fibronectin were purchased from Collaborative Research Inc., Lexington, Massachusetts. Fetal calf serum was obtained from Hyclone Sterile Systems, Logan, Utah.

Methods

Factor V

Human plasma Factor V was purified as described by Chiu et al.\textsuperscript{24} It showed a single band with a \(M_n\) of 330,000 on both reduced and nonreduced SDS gel electrophoresis. The specific activity varied from 70 to 100 units per mg and increased 20- to 25-fold on exposure to thrombin.

Preparation of Rabbit Polyclonal Antihuman Factor V(Va) IgG

The antibody was prepared as previously described.\textsuperscript{24} It was monospecific as judged by immunodiffusion and immunoelectrophoresis, and it neutralized plasma and purified Factor V. The crossreaction with bovine plasma was less than 1%.

Preparation of Mouse Monoclonal Antihuman Factor V(Va)

Purified human Factor V (75 \(\mu\)g) was injected intraperitoneally into 3- to 4-month-old BALB/c mice with complete Freund's adjuvant. These mice were boosted with 50 \(\mu\)g of Factor V in saline after 14 days. The spleens were removed from immunized mice 3 days later and used for fusion with mouse myeloma cells.\textsuperscript{25} The cells were cloned by limiting dilution, and the culture media was tested for antifactor V(Va) antibody by use of ELISA or Factor V neutralizing assay.\textsuperscript{24} Cells producing such antibodies were recloned and propagated in the ascites of mice primed with pristane. One clone produced a monoclonal (IgG\(_1\), K) antibody (B.38) that neutralized Factor V at a titer of 1:12,800. This monoclonal antifactor V (B.38) was purified by precipitation with 33% ammonium sulfate and by chromatography of the precipitate (resuspended in 0.02 M Tris-Cl, pH 7.4, containing 0.15M NaCl) on DEAE column.

Assay of Factor V Activity

The coagulant activity of Factor V was assayed by the method of Ware et al.\textsuperscript{26} by using artificially deficient human plasma.\textsuperscript{27} One unit of Factor V is defined as the amount of activity in 1 ml of normal human plasma.

Protein Concentration

Protein was measured either by the Bio Rad protein assay\textsuperscript{28} or by the spectrophotometric method with the following values for A\(_{280}\) IgG, 14.3; Factor V, 9.6.

Radioiodination of Proteins

Human Factor V, mouse monoclonal, and rabbit polyclonal antihuman Factor V IgG were radioiodinated with \(\text{I}^{125}\) by the iodoagen method, and free \(\text{I}^{125}\) was removed rapidly as described by Tuszyński et al.\textsuperscript{9} The specific radioactivity of the labeled Factor V ranged from 7.0 to 9.0 \(\times\) 10\(^6\) cpm per \(\mu\)g, monoclonal antifactor V, 5.0 to 6.0 \(\times\) 10\(^6\) cpm per \(\mu\)g, and polyclonal antifactor V, 4.0 \(\times\) 10\(^5\) cpm per \(\mu\)g. The iodination procedure did not alter either the specific coagulant activity of Factor V or its proteolytic activation by thrombin.

Thrombin Activation of \(\text{I}^{125}\) Factor V

Radiolabeled Factor V (1 to 2 units/ml) was incubated with thrombin (0.05 to 0.1 unit/ml) in buffer (0.02M Tris and 0.15M NaCl, pH 7.4) containing 1% BSA at room temperature for 30 minutes. The coagulant activity increased 15- to 30-fold after incubation. The action of thrombin was stopped by the addition of PPACK, 4-8 \(\times\) 10\(^{-7}\)M.\textsuperscript{30} When the digest was subjected to SDS electrophoresis, four polypeptides, \(M_t = 150,000\) (C1), \(M_t = 105,000\) (D), \(M_t = 74-71000\) (F1F2), and \(M_t = 40,000\) were observed in agreement with Dahlback.\textsuperscript{31}

Specificity of the Monoclonal Antibody B.38

The purified antibody was immobilized to produce an immunoaffinity column as described before.\textsuperscript{24} The column was equilibrated with 0.02 M Tris buffer (pH 7.2) containing 0.15 M NaCl. The radiolabeled thrombin-digested Factor V containing 0.02M EDTA was applied to the column and washed with 0.02M Tris buffer (pH 7.2) containing 0.36M NaCl and again the same buffer containing 0.15M NaCl and 5% glycerol. Specifically bound Factor Va was then eluted with the same buffer containing 25% glycerine and 50% ethylene glycol. The wash contained Cl, D, and \(M_t = 40,000\) polypeptides. The ethylene glycol-glycerine eluate contained the Factor Va light chain, \(M_t = 74-71000\) (F1F2). Thus, the epitope binding specificity for B.38 was found in the light chain.

Human Umbilical Vein Endothelial Cell Culture

Endothelial cells (HUVEC) were harvested and grown essentially by the method of Jaffe et al.\textsuperscript{32} Cells were plated in 35 mm dishes precoated with human fibronectin (50 \(\mu\)g/ml) with Dulbecco's modified Eagles medium (DMEM) plus 20% fetal bovine serum and endothelial cell growth supplement (150 \(\mu\)g/ml final concentration). Cells were subcultured at a 1:2 split ratio by using trypsin and EDTA, and were replated in fibronectin-coated dishes with the same medium. Fetal calf serum in the concentration used gave less than 0.1% crossreactivity in a Factor V ELISA compared to human plasma.

The number of cells per dish was determined by removing and dispersing the layers with 0.1% trypsin plus 0.1% EDTA and by counting aliquots of the cell suspension by phase microscopy. The cell number was determined periodically on three dishes taken at random. Two counts were done on each dish, and the six values were used to give an average cell count. The variability was usually not more than 10%.

Binding of \(\text{I}^{125}\) Factor Va to Adherent HUVEC

Binding of labeled Factor V was carried out by using monolayers of cells in 35 mm dishes (1 \(\times\) 10\(^5\) cells/dish). Serum-containing medium was aspirated, and cultures were washed once with 1 ml of serum-free medium. \(\text{I}^{125}\) Va in 1 ml of serum-free medium containing 1% BSA was added and incubated at 37° C for 30 minutes in a 5% CO\(_2\) atmosphere. At the end of the incubation, medium containing labeled Va (supernatant) was transferred by pipette to a test tube (75 \(\times\) 12 mm) for measurement for radioactivity. The cultures were then washed twice with 1 ml of serum-free medium, which removed 99.7% of the unbound radioactivity as measured with \(\text{I}^{125}\) BSA. The cells were then removed with a rubber policeman and solubilized with 1.5 ml of 0.015M Tris (pH 7.4) containing 0.15M NaCl, 0.01M EDTA, and 0.025% Triton X-100 (wt/vol) (solubilizing buff-
er). The radioactivity of supernatant and cells was measured by Gamma Counter Nuclear Enterprises NE 1600 or LKB 1270 Rack Gamma 11.

Binding of Labeled Mouse Monoclonal Antifactor V(Va) IgG to HUVEC in Suspension

Serum-containing medium was removed from cultures by aspiration. Cells were washed once with 1 ml of serum-free medium. Then 1 ml of medium was added to the cells, and they were scraped off the dishes with a rubber policeman. The suspension was transferred to 75 x 12 mm test tubes. The dishes were rinsed with 2 ml of medium which was added to the cell suspension for a total volume of 3 ml. Cells were centrifuged at 800 rpm for 10 minutes with a table top centrifuge (IEC Centra 7R). About 2.5 ml of the supernatant was carefully pipetted out without disturbing the cells and was discarded. The cells in 0.5 ml of medium were resuspended and dispersed by gently passing them five times through an 18 g needle. To this suspension was added 1 ml of labeled or a mixture of labeled and 50- to 100-fold excess of unlabeled antifactor V IgG (B.38). HUVEC with antifactor Va were returned to the incubator. At the end of the incubation, the cell suspensions were transferred to Eppendorf tubes and centrifuged for 2 minutes; the supernatant was saved for counting. The cells were washed twice with 1 ml of ice-cold medium and were centrifuged as explained above; the supernatant was removed. The radioactivity was counted as described before.

Results

Binding of 125I Human Factor Va to Adherent HUVEC

The binding of radiolabeled exogenous Factor Va to monolayers of adherent cells was rapid and attained equilibrium within 10 minutes. Factor Va (5000-7000 molecules per cell) bound specifically at 37°C (Figure A). The inclusion of 50-fold excess of unlabeled Factor Va resulted in a marked decrease in the binding of 125I Factor Va (0.2-0.6 μg) to about 60% of maximum (Figure 1 A). At higher concentrations of added labeled Factor Va, nonspecific binding became greater than specific binding. A Scatchard plot of binding of Factor Va to HUVEC (Figure 1 B) yielded a binding constant of about 3.73 x 10^-9 M and a total of 10,000 Factor Va molecules/cell at saturation. The number of binding sites obtained here agrees with the data reported by Maruyama et al.34

Binding of 125I Labeled Mouse Monoclonal Antifactor V(Va) IgG to Monolayers of HUVEC

No specific binding to adherent monolayers of the monoclonal antibody to Factor Va could be demonstrated (Figure 2), indicating that no detectable Factor V was expressed on the surface of the endothelial cells. A 50-fold
FACTOR V EXPOSURE ON ENDOTHELIAL CELLS  

Table 1. Binding of Radiolabeled 125I Rabbit Polyclonal Antifactor V(Va) to Monolayers of Human Umbilical Vein Endothelial Cells

<table>
<thead>
<tr>
<th>Labeled antibody added (µg/10^6 cells)</th>
<th>Total binding (ng/10^6 cells)</th>
<th>Nonspecific binding (ng/10^6 cells)</th>
<th>Specific binding (ng/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.133 ± 0.030</td>
<td>0.138 ± 0.026</td>
<td>—*</td>
</tr>
<tr>
<td>1.0</td>
<td>0.194 ± 0.038</td>
<td>0.227 ± 0.016</td>
<td>—*</td>
</tr>
</tbody>
</table>

HUVEC were cultured as described in methods. 1 ml of labeled 125I Antifactor V(Va) in serum-free medium was added to cells in 35 mm dishes and incubated for 30 minutes at 37°C in a 5% CO2 incubator. Total, nonspecific, and specific bindings were measured as described in methods. Each value represents the mean + SD of five determinations.

*The differences between total and nonspecific binding were not significant (p > 0.1).

excess of unlabeled antibody failed to decrease the binding of radiolabeled antifactor V(Va), showing an almost complete absence of specific binding (Figure 2).

**Binding of Labeled 125I Rabbit Polyclonal Antifactor V(Va) IgG to Monolayers of HUVEC**

The failure of the monoclonal antibody to bind to adherent endothelial cells might have been due to the lack of expression of the epitope recognized rather than to the failure of binding. Therefore, rabbit polyclonal antibody was also used in binding studies. Inclusion of a 50-fold excess of unlabeled polyclonal antibody did not decrease the binding of labeled polyclonal antibody as shown on Table 1, thus confirming the virtual absence of Factor V(Va) antigen on the cell surface.

**Binding of 125I Labeled Monoclonal Anti-V(Va) to Monolayers of HUVEC Exposed to Exogenous Factor Va or V**

Since adherent cells did not bind labeled anti-V(Va), as shown in Figure 2, we investigated whether adherent cells to which exogenous Factor Va or V was added could then bind the antibody. At a concentration of 1.6 µg of labeled Va, the cells bound specifically 0.82 ng of Va (Figure 1 A). When the cells were preincubated with 1.6 µg of unlabeled Va, radiolabeled 125I anti-V(Va) was bound specifically. The total binding of anti-V was 12017 ± 740 cpm (mean and SD of triplicate determinations), while the nonspecific binding was 9626 ± 310 cpm (mean and SD of triplicate data). Thus, the specific binding was 2391 cpm, equivalent to 0.85 ng of antibody. No binding of labeled anti-V(Va) was observed when the cells were preincubated with 1.6 µg of unlabeled Factor V.

Figure 1. A. Binding of 125I Factor Va to human umbilical vein endothelial cells (HUVEC). Increasing concentrations of radiolabeled 125I Factor Va were incubated with monolayers of HUVEC in 35 mm dishes (1 × 10^6 cells/dish) in 1 ml of Eagle's modified medium without serum at 37°C in a 5% CO2 atmosphere for 30 minutes. Total binding was measured as described in Methods. Determination of nonspecific binding was carried out in the presence of a 50-fold excess of unlabeled Factor Va. Specific binding was obtained by subtracting the nonspecific binding from the total. Each measurement was a mean of three to five experiments. •—• = total binding; X—X = nonspecific binding; 0—0 = specific binding. B. Scatchard plot of specific binding of Factor Va to HUVEC. Radiolabeled 125I Factor V was added in the concentration range of 0.2 to 2.4 µg to monolayers of HUVEC. The specific binding was measured as described in A.

Figure 2. Binding of labeled 125I monoclonal antifactor V(Va) IgG to monolayers of endothelial cells. Various concentrations of labeled antifactor V(Va) in serum-free medium containing 1% BSA were added to monolayers of HUVEC in 35 mm dishes. •—• = total binding; X—X = nonspecific binding; 0—0 = specific binding. These bindings were determined as described in Methods.
The cells were washed with serum-free medium and subjected to various manipulations as described in Methods. The radioactivity released at 0, 5, 10, 20, and 40 minutes was determined for both the supernates and cells after each manipulation. \( \triangle \Delta \) = adherent; \( \square \square \) = scraped; \( \triangle \square \) = scraped and passaged once through the needle; \( \square \odot \) = scraped and passaged five times; \( \times \times \) = scraped and passaged 10 times.

**Production and Quantification of Cytolytic Cell Injury**

Since the initiation of thrombosis involves damage to blood vessels and detachment of the adherent endothelial cells from the basement membrane, we sought to simulate this process in vitro. The mechanical removal of endothelial cells from their substrate induced considerable cell injury and further cell lysis resulting from the passage of the cells through an 18 g needle. It was immediately obvious that essentially all adherent cells excluded trypan blue. On the other hand, we were unable to obtain an accurate estimate of the percentage of suspended cells that excluded or took up the dye. Even after passage through the needle, a large percentage of the cells remained in sizable, often partially scrolled, clumps making visualization of part of the cells impossible. However, it was obvious that many cells in large clumps excluded dye while most single cells and small clumps took up the dye. It appeared that slow centrifugation reduced but did not eliminate the number of single cells and small clumps in the pellet.

Quantification of cell injury by release of \( ^{51} \)Cr showed that release was only 5% in adherent cells but was about 50% in cells scraped off the dish and about 60% after passage through an 18 g needle (Figure 3).

**Binding of \( ^{125} \)I Mouse Monoclonal Antifactor V(Va) to Detached HUVEC**

Radiolabeled B.38, a Factor V neutralizing antibody, was used to determine the presence of endogenous Factor V on the surface of HUVEC that were injured by removal from adherent monolayers and suspension in serum-free medium. At increasing concentrations of added \( ^{125} \)I antibody 4000, molecules bound specifically per cell and reached apparent saturation (Figure 4). If one assumes an equimolar ratio of 1:1 of antifactor V(Va)/Factor V, then 4000 molecules of Factor V are expressed on the surface of the detached cells. Inclusion of a 50-fold excess of unlabeled antifactor V(Va) IgG reduced the binding of labeled antibody significantly.

**Discussion**

Several recent studies have suggested that the endothelium participates in the regulation of localized coagulation such as might occur in response to vessel wall injury or in atherosclerosis and thrombosis.17-19 Bovine aortic endothelial cells markedly accelerated the conversion of prothrombin to thrombin in the presence of added human Factor Xa and calcium, implying that human Factor Xa binds to these cells.20 Since this activity required no exogenous Factor V and was inhibited by an antifactor V antibody, Rodgers and Shuman20 concluded that endogenous Factor V was available. The synthesis and release of Factor V by bovine aortic cells was shown by Cerveny et al.21 Unlike bovine endothelial cells, human endothelial cells from umbilical veins fail to secrete Factor V into the medium.22, 34 Thus, there is no extracellular source for Factor V. The study of Rodgers and Shuman20 implied that Factor V was available on the cell surface and functioned in the assembly of the prothrombinase complex on bovine aortic cells. Two studies have been concerned directly with the presence and role of Factor V in the regulation of coagulation by HUVEC. Sinha et al.22 from our laboratory recently reported that cultured human umbilical vein endothelial cells contained, but did not secrete, Factor V into the medium. They found that 200 molecules of Factor Xa bound to each endothelial cell in a monolayer resulted in the down-regulation of production of PGI2. This functional effect of
Xa is blocked by anti-V, indicating that endogenous Factor V is required. Maruyama et al.34 reported the binding of radiolabeled antifactor V(Va) to cells that had been removed from the substrate and passed through a 19 g needle. These cells also accelerated the activation of protein C.

While both of these studies suggested that Factor V(Va) was available on the cell surface, we were unable to detect Factor V on the surface of adherent monolayers of HUVEC in extensive studies with radiolabeled mononclonal and polyclonal antibodies (Figure 2 and Table 1). In contrast, removal and suspension of HUVEC resulted in the binding of 4000 molecules per cell of radiolabeled IgG per cell (Figure 2 A), implying an equal number of exposed Factor V(Va) molecules. This is in agreement with the observation of Maruyama et al. In addition, we found that the appearance of Factor V(Va) molecules that were accessible to antifactor V(Va) antibody closely parallel cell injury and death as measured by release of 51Cr. This finding suggests that endogenous Factor V(Va) had been made available possibly through release from damaged cells with consequent binding to intact cells. It is equally possible that Factor V in dead cells is capable of binding the antibody as has been shown by the binding of IgG to dead cells.35 We also showed that binding of 125I-labeled Factor Va to HUVEC was specific and saturable (Figure 1).

The apparent discrepancies between our study and the studies of Rodgers and Shuman,20 Sinha et al., 22 and Maruyama et al.34 necessitate a close examination of all four studies. Indirect evidence for the presence of Factor V on the surface of bovine aortic and human umbilical vein endothelium in the functional studies of Rodgers and Shuman and Sinha et al. and our inability to demonstrate Factor V binding by study can be explained by the greater sensitivity of the functional studies through amplification by product formation. It should be noted that the presence of less than 500 molecules of endogenous Factor V per cell would not have been apparent in our monoclonal antibody binding studies. The observation by Maruyama of less cell damage (70% to 90% of cells excluded trypan blue) than we found (50% to 60% release of 51Cr) apparently depended on differences in technique, since 51Cr is more sensitive than trypan blue. We included all cells in our estimate of damage while they considered only those cells remaining in the pellet after centrifugation at 800 rpm (probably about 150 g). Suspension of endothelial cells apparently leads to cytolytic damage or death which should be considered by investigators using this model to study the interaction of procoagulant proteins with endothelial cells.

Based on our observations, we suggest that human endothelial cells not only bind exogenous Factor V but also express or expose endogenous Factor V activity upon mechanical injury. This observation is of relevance to several clinical situations in which blood vessels are directly injured by mechanical means such as endarterectomy, construction of arterial bypasses, and various forms of catheterization. Other examples of cytotoxic injury occurring in pathologic conditions include the effects of circulating antigen-antibody complexes which activate complement, endotoxin, and cytotoxic viruses. Undisturbed adherent HUVEC express little Factor V on their surface, consistent with their role in maintaining blood fluidity and a nonthrombogenic surface for flowing blood. The exposure of Factor V after endothelial injury or death may play an important role in pathological thrombogenesis.

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