Morphological Alterations in Endothelial Cells Associated With the Release of von Willebrand Factor After Thrombin Generation In Vivo

Mary Richardson, Shawn Tinlin, Marnie De Reske, Sandra Webster, Yotis Senis, Alan R. Giles

Abstract von Willebrand factor (vWF) is synthesized by endothelial cells and stored in endothelium-specific granules, the Weibel-Palade (WP) bodies. The release of vWF from endothelial cells in vitro in response to secretagogues such as thrombin is considered to result in the loss of WP bodies through the fusion of the WP bodies with the plasma membrane. Biochemical and morphological techniques, including transmission (TEM) and scanning (SEM) electron microscopy, were used to examine the plasma profile of vWF in parallel with morphological alterations in endothelial cells associated with the generation of thrombin in vivo. There was a rapid loss of high-molecular-weight multimers of the circulating vWF, with full recovery within 1 hour. Simultaneously, TEM demonstrated that the endothelial cells lost WP bodies and became severely vacuolated; this was associated with the appearance of craters in the endothelial surface on SEM. Release of stored vWF in WP bodies seemed to follow the fusion of multiple rather than individual WP bodies, with the resulting vacuole fusing and rupturing through the plasmatic membrane. Within 1 hour there was increased morphological evidence of metabolic organelle activity associated with replacement of WP bodies, presumably due to de novo synthesis of the basic protomer and its packaging in high-molecular-weight multimeric form in the storage organelles. (Arterioscler Thromb. 1994;14:990-999.)

Key Words • thrombin • Weibel-Palade bodies • von Willebrand factor • endothelial cells
doses, however, there was an apparently paradoxical increase in plasma antigen and functional levels of vWF, associated with the appearance of abnormally high-molecular-weight multimeric forms in the plasma. These observations suggested that thrombin generation in vivo induced consumption of vWF balanced by replacement via the regulated pathway of WP body release.

It appeared that when the intensity of the procoagulant challenge was low, consumption initially exceeded the rate of replacement. When the challenge was substantial, however, not only did release via the regulated pathway exceed consumption, but also abnormally high-molecular-weight vWF was discharged from WP bodies without processing to normal multimeric forms. Consequently, this model offered the opportunity to correlate the changes observed in plasma vWF concentration and multimeric structure after thrombin generation in vivo with morphological changes in endothelial cells in general and WP bodies in particular.

Methods

Reagents

All reagents used were of reagent grade or better. FXa and the PCPS vesicles were prepared and characterized as previously described. Soybean trypsin inhibitor, benazimide hydrochloride, and aprotinin were purchased from Sigma Chemical Co.

Animal Preparation

Male Wistar rats (body weight, 200 to 300 g) were killed at 30 seconds; 2, 15, and 60 minutes; and 24 hours after a single infusion of FXa/PCPS (9.38 x 10^{-14} mol/g and 14.43 x 10^{-14} mol/g, respectively) administered via the penile vein under anesthesia (35 mg/kg IP sodium pentobarbital [Boehringer Ingelheim]). Four animals were infused with saline in place of FXa/PCPS. The animals killed at 30 seconds and 1, 2, 5, 15, and 60 minutes were maintained under anesthesia. Animals in the 24-hour group were allowed to recover consciousness and were reanesthetized before they were killed. The right carotid artery was cannulated with polyethylene tubing (Intramedic, PE 190), and the rats were killed by perfusion with either 2.5% glutaraldehyde or 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer for 24 hours at 4°C. FXa/PCPS was postfixed in phosphate-buffered saline (PBS); changed to 0.1 mol/L glycine for 10 minutes; changed to 0.5% H2O2 in PBS for 10 minutes; washed 3 times (5 minutes each) in PBS; incubated with the primary antibody for 1 hour; washed in tris(hydroxymethyl)-aminoethane (Tris)–buffered saline; blocked with nonimmune rabbit serum in place of the specific antiserum and processed the same way. Tissues for postembedding TEM immunohistochemistry were diced as above but without postfixation, dehydrated in graded ethanol, and embedded in LR white resin. Ultrathin sections were mounted on uncoated copper grids, counterstained with uranyl acetate and lead citrate, and examined in a Philips 301 TEM. Control tissues were exposed to streptavidin–linked horseradish peroxidase for 45 minutes (Vector Stain Kit, Dimension Lab Inc); washed in Tris-buffered saline; postfixed and dehydrated as above, critical-point dried from CO2 in a critical-point dryer (Ladd Res Industries Inc), coated with gold in a sputter coater (Polaron Inc) for 1.5 minutes, and examined in a Philips 301 TEM.

Plasma vWF Levels and Multimeric Analysis

Blood samples, taken before the injection of FXa/PCPS and immediately before the animals were killed, were collected into a buffered citrate anticoagulant (0.06 mol/L sodium citrate, 0.04 mol/L citric acid). Platelet-poor plasma was separated by centrifugation at 11,200g for 1 minute. The plasma was snap-frozen and stored at -70°C until processed.

vWF levels, antigen, and functional activity were determined by enzyme-linked immunosorbent assay, using a goat anti-human vWF antibody (Affinity Biologicals) and ristocetin cofactor assay as previously described. vWF multimers were analyzed by discontinuous sodium dodecyl sulfate–agarose gel electrophoresis performed on 0.8% (wt/vol) agarose stacking gel and 1.6% (wt/vol) running gel as previously described by Toh et al.

Tissue Preparation

The lungs and aorta were prepared for conventional ultrastructural examination using standard procedures. Tissue, fixed initially in paraformaldehyde, was transferred to 2.5% glutaraldehyde after removal from the animal. Full-circumference segments (1 mm in length) of thoracic aorta (between the third and fifth intercostal arteries) and 1-mm cubes of the right lower lobe of the lung were prepared for transmission electron microscopy (TEM). Segments (1 cm long) of the thoracic aorta (between the fifth and seventh intercostal arteries) were processed for scanning electron microscopy (SEM). Tissue prepared for TEM was postfixed in 1% aqueous osmium tetroxide for 1 hour, dehydrated through graded ethanol, and embedded in Spurr resin. Thin sections were mounted on copper grids, stained with lead citrate and uranyl acetate, and examined in a Philips 301 TEM. Samples for SEM were postfixed and dehydrated as above, critical-point dried from CO2 in a critical-point dryer (Ladd Res Industries Inc), coated with gold in a sputter coater (Polaron Inc) for 1.5 minutes, and viewed in a Philips PSEM 501B.

Immunohistochemistry

Samples of lungs and aorta fixed in 4% paraformaldehyde were processed for preembedding and postembedding TEM immunolocalization of vWF using a specific affinity-purified rabbit anti-human vWF (Dako Ltd) and a secondary detection goat anti-rabbit antibody (Janssen Biochemica). For preembedding staining the secondary antibody was biotinylated (Dimension Lab Inc), whereas for postembedding staining the antibody was conjugated with 10-nm colloidal gold (Janssen Biochemica).

Preembedding immunohistochemistry was carried out as described by Reidy et al. After the initial in situ fixation by 4% paraformaldehyde, the tissue was cut up and fixed continued in periodate/lysine/paraformaldehyde (PLP) in 0.04 mol/L phosphate buffer for 24 hours at 4°C. The tissue was treated with 0.01% Triton-X for 15 minutes; washed 3 times (5 minutes each) in PLP and 3 times (5 minutes each) in phosphate-buffered saline (PBS); changed to 0.1 mol/L glycine for 10 minutes; changed to 0.5% H2O2 in PBS for 10 minutes; washed 3 times (5 minutes each) in PBS; incubated with the primary antibody for 1 hour; washed in tris(hydroxymethyl)-aminoethane (Tris)–buffered saline; blocked with nonimmune goat serum; incubated with biotinylated detection antibody for 1 hour; rinsed and then incubated with streptavidin–linked horseradish peroxidase for 45 minutes (Vector Stain Kit, Dimension Lab Inc); washed in Tris-buffered saline; exposed to 0.05% diaminobenzidine in Tris-buffered saline and H2O2 for 15 minutes; washed in Tris and PBS; postfixed in osmium tetroxide; and dehydrated and processed for TEM. Ultrathin sections were mounted on uncoated copper grids, counterstained with uranyl acetate and lead citrate, and examined in a Philips 301 TEM. Control tissues were exposed to nonimmune rabbit serum in place of the specific antisera and processed the same way. Tissues for postembedding TEM immunohistochemistry were diced as above but without postfixation, dehydrated in graded ethanol, and embedded in LR white resin. Ultrathin sections were mounted on Formvar-coated nickel grids and exposed to the primary rabbit anti-human vWF (Dako Ltd) at 1:1000 dilution in 1% bovine serum albumin (BSA) in PBS overnight (16 hours). The sections were rinsed in PBS; blocked with nonimmune goat serum; exposed to goat anti-rabbit serum labeled with 10-nm colloidal gold; diluted 1:50 in 1% BSA in PBS for 1 hour; rinsed in PBS, followed by water; and counterstained with aqueous uranyl acetate and lead citrate. Control sections were incubated with nonimmune rabbit serum in place of the specific antisera and processed in the same way as the test sections. The sections were examined in a Philips 301 TEM.
Fig 1. Transmission electron photomicrograph of a pulmonary arteriole with two layers of smooth muscle cell (SMC), representative of the vessel type used in the quantification of Weibel-Palade (WP) bodies. The endothelial cell contains 29 profiles of WP bodies, which are located at the luminal side of the nucleus. The inset (showing the area between the two small arrowheads) shows WP bodies cut in cross section in which the tubular structure of the organelle is evident. The large arrowhead indicates a single WP body. N indicates nucleus; L, lumen. (Bar=1 µm; inset bar=100 nm.)

Quantitative Morphometric Evaluation of Endothelial Cell Organelles

Sections of aorta and lung were examined by TEM. The number of profiles of WP bodies was counted in all endothelial cells that contained a nuclear profile. In the lung, only endothelial cells in pulmonary arterioles with one to three layers of smooth muscle cells were evaluated. An example of such an arteriole with two layers of smooth muscle cells is illustrated in Fig 1. The presence of Golgi apparatus, dilated rough endoplasmic reticulum, and cytoplasmic vacuoles was recorded and quantified on a 1+ to 4+ basis. In each instance the evaluation was carried out with the operator blinded to the origin of the sample.

Statistical Analysis

The mean value for the number of WP bodies per endothelial cell for aorta and lung from each animal was determined. A one-way ANOVA was carried out among the data from all animals at each time period (0 seconds [control], 30 seconds, 1 to 2 minutes, 5 minutes, 15 minutes, 60 minutes, and 24 hours after infusion of FXa/PCPS). Dunnett’s analysis was carried out using weighted means to determine whether there was a significant difference between the value observed at any one time interval after FXa/PCPS infusion and the value obtained in the control animals receiving saline.

Results

Changes in vWF Levels After FXa/PCPS Infusion

In previous studies of changes in vWF after the infusion of FXa/PCPS, dogs, rabbits, and nonhuman primates had been used. To facilitate the morphological studies proposed, a smaller mammalian species was preferred. Consequently, it was essential to confirm a similar vWF response to FXa/PCPS infusion in the species chosen, ie, the rat.

Dose-escalation studies were performed in pilot studies in which the dose of each component, ie, FXa/PCPS, was increased, but the ratio of the molar concentrations was maintained at 0.65×10⁻⁴. The dose selected, 9.38×10⁻¹⁴ mol (FXa)/14.43×10⁻¹¹ mol (PCPS) per gram body weight, was associated with an immediate and substantial decrease in the concentration of plasma vWF (Table 1) but was well tolerated by the animal. Multimeric vWF analysis confirmed that this was associated with the selective loss of high-molecular-weight forms with recovery to a normal distribution over 30 to 60 minutes (data not shown), as seen previously in other mammalian species.

Morphological Observations

The morphological alterations, rated on a 1+ to 4+ basis, are summarized in Table 2 and illustrated in Figs 1 through 6.

Saline-Treated Control Rats

Examination of the control (saline-treated) animals by TEM demonstrated that the endothelial cells in the

<table>
<thead>
<tr>
<th>Time After Injection of FXa/PCPS</th>
<th>WP Body Content</th>
<th>Plasma vWF Levels, % Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aorta (4)</td>
<td>Lung (4)</td>
</tr>
<tr>
<td>Saline-treated control</td>
<td>9±1 (3)</td>
<td>15±6 (4)</td>
</tr>
<tr>
<td>30 s</td>
<td>4±1 (3)*</td>
<td>6±2 (3)*</td>
</tr>
<tr>
<td>1-2 min</td>
<td>5±1 (4)*</td>
<td>5±1 (4)*</td>
</tr>
<tr>
<td>5 min</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>15 min</td>
<td>6±2 (3)*</td>
<td>6±4 (3)*</td>
</tr>
<tr>
<td>60 min</td>
<td>8±4 (3)</td>
<td>10±6 (3)</td>
</tr>
<tr>
<td>24 h</td>
<td>9±4 (4)</td>
<td>13±5 (4)</td>
</tr>
</tbody>
</table>

FXa/PCPS indicates factor Xa/phosphatidylcholine/phosphatidylserine; WP, Weibel-Palade; vWF, von Willebrand factor; and ND, not done. Values are mean±SD for the average endothelial cell WP content. Number of animals is shown in parentheses.

*P > .05 compared with control values (Dunnett’s analysis) using weighted means.
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TABLE 2. Morphological Features of Endothelium

<table>
<thead>
<tr>
<th>Time After Injection of FXa/PCPS (n)</th>
<th>Vacuoles</th>
<th>SEM Craters</th>
<th>RER</th>
<th>Golgi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline-treated control (4)</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>30 s (3)</td>
<td>+++</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>1 min (2)</td>
<td>+++</td>
<td>+++</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>2 min (2)</td>
<td>+++</td>
<td>+++</td>
<td>±</td>
<td>+</td>
</tr>
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<td>15 min (3)</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>60 min (3)</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>24 h (4)</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

FXa/PCPS indicates factor Xa/phosphatidylcholine/phosphatidylserine; SEM, scanning electron microscopy; and RER, rough endoplasmic reticulum. The frequency of each feature in endothelial cells in both aorta and lung was rated ± to ++++: ±, least frequent; ++++, most frequent.

aorta and pulmonary arterioles of saline-injected rats contained occasional profiles of Golgi apparatus, rough endoplasmic reticulum, and some small vacuoles (Fig 1 and Fig 2a). In the cells that contained a nuclear profile, 98% contained profiles of WP bodies. The WP bodies were similar to earlier descriptions: round or elongated membrane-bound structures, 0.1 μm wide and 0.5 to 1 μm long, containing tubules 10 to 15 nm in diameter, arranged parallel to the long axis of the organelle (Fig 1 [inset]). These were mainly distributed close to the nucleus and were predominantly on the luminal rather than the abluminal side of the cell. Endothelial cells that contained few WP bodies were not vacuolated, and, with the exception of the number of WP bodies, their features were indistinguishable from other endothelial cells in the same vessel.

Immunolocalization of vWF using postembedding staining showed that WP bodies in the pulmonary arterioles and in the aorta were selectively “decorated” with gold particles. There was no evidence of gold label in the vacuoles, the vessel lumen, or the abluminal space (Fig 2b). Tissues exposed to nonimmune rabbit serum and processed for both preembedding and postembedding immunohistochemistry showed no evidence of reaction product or nonspecific staining.

FXa/PCPS-Treated Rats

The number of profiles of WP bodies was clearly reduced in the aorta and pulmonary arterioles 30 seconds after infusion of FXa/PCPS; there was little evidence of further reduction after 2 minutes. Overall, the morphological endothelial cell response was similar at 30 seconds and at 2 minutes. The most marked alteration was a substantial increase in the number and size of cytoplasmic vacuoles (Fig 3a, 3b, and 3c). Some of these contained vWF, visualized by preembedding immunocytochemical staining (Fig 3d), whereas others were apparently rupturing (Fig 3c) and releasing their content of vWF into the vessel lumen (Fig 3d). The SEM of the aortic endothelium was consistent with the increase in the number and size of the cytoplasmic vacuoles seen in TEM, in that it showed large numbers of craters and occasional cells from which the luminal

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Fig 2. Aortic endothelial cells from saline-injected animals. a, Transmission electron photomicrograph of an aortic endothelial cell containing eight profiles of Weibel-Palade (WP) bodies, one small vacuole (V), and a profile of the Golgi apparatus (G). (Bar=1 μm.) b, Transmission electron photomicrograph of an endothelial cell stained after embedding with anti-von Willebrand factor, visualized by colloidal gold. The gold label is present over the WP bodies in both cross and longitudinal section. (Bar=100 nm.) c, Scanning electron photomicrograph of the luminal surface of the aorta. The endothelial cells are regularly arranged; there are microvilli on the surface of some cells and an occasional small crater. (Bar=10 μm.) WP bodies are indicated by arrows. L indicates lumen.
The membrane was apparently lifting (Fig 3e). The metabolic organelles, Golgi apparatus, and rough endoplasmic reticulum were less evident than in endothelial cells of saline-treated control animals.

The WP bodies that remained were frequently less regular in shape and larger than in control vessels. The contents were less electron dense, and a clear halo was distinguished at the margin (Fig 4a). WP bodies were...
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FIG 4. Sections of endothelial cells in the first 2 minutes after the infusion of factor Xa/phosphatidylcholine/phosphatidylserine stained after embedding with anti-von Willebrand factor (vWF) visualized with colloidal gold-conjugated secondary antibody. a, Aortic endothelial cell in which the Weibel-Palade (WP) body, decorated with gold label, is swollen, with a clear halo at the margin. (Bar=1 μm.) b, Transmission electron micrograph of an aortic endothelial cell. Gold label is present over a structure that appears to be formed from three or four coalescing WP bodies. (Bar=500 nm.) c, Arrowhead indicates gold label associated with electron semidense material, which may be the remains of a WP body in a large vacuole in an aortic endothelial cell. (Bar=100 nm.) d, vWF-positive stain in the lumen close to an aortic endothelial cell. This may represent the release of vWF into the circulation. Open arrowheads indicate WP bodies similar to those seen in unstimulated cells. (Bar=500 nm.) Gold-labeled anti-vWF is indicated by arrowheads. L indicates lumen; N, nucleus; and IEL, internal elastic lamina.

seen clustered closely together (Fig 4b), and often it was not possible to define limiting membranes, suggesting fusion. Other WP bodies were closely associated with vacuoles that contained gold particles (Fig 4c). In addition, small foci of gold particles were occasionally observed in the lumen adjacent to the endothelial cell membrane (Fig 4d), but the label was not observed in the subendothelial region. The immunohistochemical localization showed an accumulation of vWF associated with WP bodies but minimal cytoplasmic staining.

By 15 minutes after FXa/PCPS infusion, vacuolization was less marked than at earlier time intervals, but vWF was still present in WP bodies and vacuoles, some of which appeared to be fusing. WP bodies and vacuoles very close to the luminal and abluminal membranes were also observed. Gold label was again observed in small foci in the lumen but not at the abluminal surface of the endothelial cells. SEM of the aorta showed the formation of craters in the endothelial cell surface as before, but no endothelial cell loss was noted. The Golgi apparatus and rough endoplasmic reticulum were more prominent than in the control and the test animals killed at earlier times (Table 2).

By 1 hour after treatment endothelial cell vacuoles were less evident, and WP bodies were present and obviously increasing in number (Fig 5a). An increase in the metabolic organelles, particularly the rough endoplasmic reticulum, was a marked feature at 1 hour and even more evident by 24 hours (Fig 5b and 5c and Table 2). vWF was identified in association with the dilated metabolic organelles by both preembedding and postembedding immunohistochemistry (Fig 5d). Gold label was rarely observed in either the lumen or at the abluminal surface of the cells. By SEM the morphology was indistinguishable from the normal control animals, in that there was little evidence of luminal craters and no evidence of endothelial desquamation.

Despite careful observation, there was minimal evidence of individual WP bodies releasing their contents directly into the circulation. Only six possible examples of this phenomenon were observed, and these were seen at all time intervals. The best examples are presented in Fig 6. Fig 6a shows a WP body stained with anti-vWF in an aorta 2 minutes after stimulation. Fig 6b is from an aorta, 15 minutes after stimulation. One hour
after stimulation (Fig 6c) a structure apparently being discharged into the lumen was observed, and 24 hours after stimulation (Fig 6d) a WP body, seemingly continuous with the luminal membrane, was present in an aortic endothelial cell.

Quantification of Endothelial Cell Weibel-Palade Body Content

The results of the quantitative analysis of WP body content of aortic and pulmonary arteriole endothelial cells before and after FXa/PCPS infusion are shown in Table 1. The data are presented as the mean±SD numbers of WP body profiles in the endothelial cells examined. There was a statistically significant effect of treatment among the groups by one-way ANOVA (P<.05). Further analysis by Dunnett's test with weighted means showed a significant reduction in pulmonary and aortic endothelial cells at 30 seconds (P<.05), and this was maintained through 15 minutes after administration of FXa/PCPS.

Discussion

These data demonstrate that rats challenged with a procoagulant stimulus, which is known to generate thrombin in vivo, respond in a similar way to other species tested with respect to plasma vWF levels. The loss and reappearance of the high-molecular-weight multimers of vWF suggested the discharge of vWF from endothelial cells via the regulated pathway, and the alterations in endothelial cell WP body content are consistent with this. Thus, it has been possible to analyze the order and type of ultrastructural alterations and the associated redistribution of vWF, which occur in endothelial cells during and after the release of vWF in vivo.

After stimulation by FXa/PCPS, there was a significant reduction in the numbers of profiles of WP bodies in aortic and pulmonary artery endothelial cells by 30 seconds, which was maintained for at least 15 minutes, with recovery thereafter to control levels. The loss of WP bodies was coincident with the formation of large cytoplasmic vacuoles, some of which contained vWF and/or opened into the lumen. It is proposed, therefore, that the mechanism by which vWF is discharged from endothelial cells in vivo includes fusion of WP bodies with each other or with other vacuoles to form large vacuoles that encroach on and subsequently fuse with...
the luminal membrane. The possibility that endothelium-specific granules discharge by fusing and forming large vacuoles was first proposed in 1982 by Fujimoto after observations of the ultrastructural alterations that occurred during degradation of endothelium-specific granules in the toad aorta. The appearance of swollen granules in which the contents had decreased electron density is similar to that described in the present study (Fig 4a). Direct fusion of individual WP bodies with the luminal membrane with discharge of the entire contents as described by McNiff and Gill appeared to be a relatively less frequent event and is in agreement with their view that this phenomenon was rare and may represent a chance observation of a spontaneous rather than a stimulus-coupled event. Nonetheless, this direct release mechanism could represent a "basal secretion pathway" of the resting cell described by Matsuuchi and Kelly.

The sequence of events in vivo reported here shows many similarities but also some differences in either the description or interpretation of those that occur during the release of vWF from endothelial cells in culture. Studies of the morphological alterations associated with the loss of vWF from endothelial cells in vitro provide support for the existence of both mechanisms of WP body discharge, although the possibility that regulated release may involve a mechanism other than the direct discharge of individual WP bodies, after the fusion of their limiting membranes with the cell membrane, has not generally been considered. Immunofluorescent staining with anti-vWF antiserum visualized the discrete rodlike WP bodies in cultured endothelial cells. Sporn et al described the formation of larger areas of intense staining, which were interpreted as cytoplasmic patches of vWF remaining after the discharge of the WP bodies. The precise location in relation to the cell membrane could not, however, be determined. It is possible that these "patches" were in fact large vacuoles, either close to or opening onto the surface, as described in the present study. A description of the
ultrastructural alterations in cultured endothelial cells stimulated by PMA to release vWF via the regulated pathway includes a loss of recognizable WP bodies 15 minutes after treatment. The loss of vWF was associated with WP bodies apparently being extruded from the cell and the formation of large vacuoles containing immunocytochemically reactive vWF. These observations are very similar to those reported here and, although not interpreted as such by the authors, are compatible with the suggestion that WP bodies may empty their contents into these vacuoles before their subsequent discharge from the cell.

The timing of morphologically observed events in vivo did not appear always to correspond to those reported in endothelial cells in culture. de Groot and al reported loss of WP bodies at 10 minutes from endothelial cells stimulated by thrombin in vitro, but no observations were made before this. We observed that after the initial loss of WP bodies at 30 seconds, their numbers had returned to near control levels by 1 hour after stimulation. Because the formation of WP bodies is a function of vWF synthesis and subsequent processing to its multimeric forms, it is notable that this event was associated with morphological prominence of both the dilated rough endoplasmic reticulum and Golgi apparatus, organelles known to be involved in the secretion and processing of vWF, including the formation of WP bodies. Therefore, it appeared that in vivo, endothelial cells respond rapidly, apparently within 15 minutes, to the loss of stored vWF, and this includes de novo vWF synthesis and the regeneration of WP bodies. It has been reported, however, that thrombin stimulation of endothelial cells in culture, although effective in inducing the release of vWF from the storage pool, had no immediate effect on the basal levels of vWF-specific mRNA or the subsequent biosynthesis of vWF, suggesting that the replenishment of stored vWF occurred at a steady-state rate similar to that of unstimulated cells. Similarly, replenishment of stored vWF was not evident until 48 hours after PMA stimulation of endothelial cells in culture. To resolve these differences, it will be necessary to correlate the morphological changes noted here with more direct and less subjective evidence of de novo synthesis of vWF. The model described lends itself to approaches such as mRNA quantification and in situ hybridization with species-specific vWF probes. The development of appropriate reagents and protocols for such studies is currently under way.

Little immunohistochemical evidence of abluminal discharge of WP body–stored vWF was observed in these studies in contrast to in vitro observations in which secretion of vWF in both basolateral and apical directions in response to thrombin was reported. There is strong evidence that vWF is essential for the interaction between platelets and exposed extracellular matrix. Based on studies of endothelial cells in culture, however, there are a number of discrepancies concerning the apparent extracellular distribution of vWF after its release. It has been shown to be associated with the extracellular matrix of endothelial cells grown in vitro. Perturbation with thrombin or PMA, however, did not increase the amount in the extracellular matrix, although the ability of the matrix to support platelet adhesion was increased. Extracellular vWF has been identified in human umbilical veins, but none was detected in the subendothelial of human capillary, arteriolar, and venular endothelial cells obtained from human smooth and skeletal muscle, breast, and hemangio mas. Consistent with observations reported here, Reidy and al could not detect vWF in the subendothelial layer at any time where the endothelium was intact. All these observations, including those reported in vivo here, are compatible with the suggestion that in the unstimulated endothelial cell, the contribution to the extracellular matrix is via the constitutive pathway of vWF release. In a situation in which the subendothelial connective tissue is exposed as a result of endothelial cell damage, the source of vWF may be the plasmatic pool, augmented by its release from WP bodies and platelet α-granules. The possibility that the apparent luminal polarity of WP body release, observed in the rat in vivo, is a peculiarity of that species may be addressed by similar studies in other species.

In summary, these observations confirm that, in vivo, in response to the generation of thrombin, the release of vWF by endothelial cells involves the loss of WP bodies. Although the mechanism(s) by which WP bodies release their vWF may include the fusion of the intact organelles with the luminal membrane, this appears to be a rare event but may represent a basal regulated secretion pathway. In stimulated cells, fusion of WP bodies either with other WP bodies or vacuoles occurs more frequently, resulting in the formation of large vacuoles that fuse with the luminal membrane and release vWF into the circulation. These events appear to be followed promptly by de novo synthesis of vWF, and the regulated pathway is reestablished by the regeneration of WP bodies.

Acknowledgments

These studies were performed with grant-in-aid support from the Heart and Stroke Foundation of Ontario (No. B2148). Dr. Giles is a Distinguished Research Professor of the Heart and Stroke Foundation of Ontario. The contribution of Barbara Saunders in the preparation of the manuscript is gratefully acknowledged.

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Arterioscler Thromb Vasc Biol. 1994;14:990-999
doi: 10.1161/01.ATV.14.6.990

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