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Enhanced assay of endothelial exocytosis using extracellular matrix components

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Abstract

Vascular inflammation plays a key role in the pathogenesis of atherosclerosis. The first step in vascular inflammation is endothelial exocytosis, in which endothelial granules fuse with the plasma membrane, releasing pro-thrombotic and pro-inflammatory messenger molecules. The development of cell culture models to study endothelial exocytosis has been challenging because the factors that modulate exocytosis *in vitro* are not well understood. Here we report a method for studying endothelial exocytosis that optimizes extracellular matrix components, cell density, and duration of culture. Human umbilical vein endothelial cells plated on collagen I coated plates and cultured in the confluent state for 7–12 days in low serum media showed robust secretion of von Willebrand Factor when stimulated with various agonists. This exocytosis assay is rapid and applicable to high-throughput screening.

Keywords

endothelial; exocytosis; VWF; extracellular matrix

Introductory Statement

Atherosclerosis is the major cause of morbidity and mortality in the USA and Western world. Atherosclerosis is caused by vascular inflammation, which leads to leukocyte recruitment, followed by development of a fatty streak, a fibrous plaque, and plaque rupture [1–6]. The first step in vascular inflammation is leukocyte recruitment into the vessel wall [7, 8]. Endothelial cells trigger leukocyte rolling by rapidly moving P-selectin from the cell interior to the cell surface [9]. P-selectin is normally stored inside endothelial granules called Weibel-Palade bodies [10, 11]. When the vessel wall is injured, endothelial granules fuse with the plasma membrane, externalizing P-selectin, and releasing VWF along with a

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variety of other pro-inflammatory and pro-thrombotic messengers. This process, which leads to leukocyte rolling, is called endothelial exocytosis.

Exocytosis is a form of vesicle trafficking. In the case of endothelial cell granules, the granules load cargo (P-selectin, von Willebrand Factor (VWF), and other substances) from Golgi stacks, bud off from the Golgi, translocate to the plasma membrane, dock, and then fuse with the plasma membrane, releasing their contents outside the cell and into the blood [12–17]. Several protein families regulate exocytosis and vesicle trafficking [18–23]. NSF (N-ethylmaleimide sensitive factor) is a molecular motor that drives the process of exocytosis. SNAREs (soluble NSF attachment protein receptor) are transmembrane proteins that are inserted into transport vesicles or their target membranes and interact with each other, forming a ternary complex that drives membrane fusion. Complexins act as clamps to prevent SNAREs from fusing membranes; and synaptotagmins are calcium sensors that remove complexins from SNAREs, triggering rapid exocytosis. Rab proteins and their effectors regulate vesicle motility and tethering. The components of the exocytic pathway in endothelial cells include: NSF, VAMP3, syntaxin-4, α 5 and α 6GDS, rab27a, MyRIP, and Munc18c [24–32]. Additional proteins that regulate exocytosis include eNOS, PKC, and Atg7 [24, 33, 34].

Identification of all components of the exocytic machinery is challenging due to the limitations of the current endothelial exocytosis assay used by most investigators. The endothelial cell exocytosis assay was first described over 30 years ago [35]. Analysis of exocytosis depends upon cells cultured in large plates instead of 96-well plates. Detection of secretion can be variable, depending upon poorly defined characteristics of endothelial culture [36–39]. For example, one report notes that endothelial release of VWF following stimulation can vary between 3-fold to 12-fold over baseline, “observed with different HUVEC preparations” [38].

We sought to understand the factors that influence endothelial expression and release of granule contents. We now report 3 key factors that greatly affect endothelial exocytosis: extracellular matrix, cell confluency, and duration of culture. Optimization of these factors produces an exocytosis assay that is robust, reproducible, and suitable for high-throughput screening.

Materials and Methods

Cell Culture

Human umbilical vein cells (HUVEC) from pooled, virus-free cords were purchased from Lifeline Cell Technology and used at passage 1–4. Endothelial cell basal media (EBM, Lifeline Cell Technology) and endothelial growth supplements (EnGS, Lifeline Cell Technology) were used to create endothelial complete media (ECM). Tissue culture treated and matrix coated 6-well plates were from Becton Dickinson and 96-well plates were from Greiner Bio-One. The matrix components coating the 6-well and 96-well plates included lysine, laminin, fibronectin, collagen I and collagen IV. Glass-bottomed 35 mm dishes coated with or without collagen I for microscopy were from MatTek. Cells were seeded at 2000–4000 per well for 96-well plates, or 25,000 per well for 6-well plates. Cells were

maintained at 37°C in humidified air with 5% CO₂ for 3–11 days in ECM and 10 µg/ml gentamicin (Invitrogen). Cells were refed with ECM and EnGS every 2–3 days and were refed with ECM 16–20 hours before exposure to agonists.

Cell Stimulation with Agonists

Histamine (Enzo Life Sciences), calcimycin (Enzo), and ATP (Sigma-Aldrich) were used at 10 µM in EBM without gentamicin pre-warmed to 37°C. Plates were supported by pre-warmed styrofoam to reduce spontaneous VWF exocytosis. Media were removed and replaced with agonists without laminar flow to reduced spontaneous VWF release and immediately returned to the incubator.

Measurement of VWF

Supernatants were collected without touching the HUVEC monolayer in order to reduce spontaneous release of VWF. Debris was removed by centrifugation at 100 × *g* for 6 minutes to reduce background. For preparation of cell lysates, 6-well plates were treated with agonists as described above and decanted by inversion on blotter paper. Cells were lysed with 1% SDS in PBS, collected by scraping and vortexing followed by low speed centrifugation. Lysates were diluted 10:1 and protein was determined by BCA analysis (Pierce). VWF concentration was measured with Sekisui Diagnostics ELISA kits.

Microscopy of Weibel-Palade Bodies

HUVEC were plated on glass coverslips coated with or without collagen I and cultured for 10 days. Media was removed by inversion onto blotter paper and fixed with fresh 1% formalin in PBS for 15 minutes. The fixed monolayers were washed three times with 3 ml PBS and permeabilized with 0.1% Triton X in PBS for 5 minutes. The fixed and permeabilized monolayers were washed three times with 3 ml PBS and blocked overnight at 4°C with goat serum. The blocked monolayers were washed three times with 3 ml PBS. Primary antibody (Abcam) and secondary antibody (Invitrogen) was added. DAPI (Vector) mounting media was used to identify nuclei. Confocal images at 40× were collected and stacked using an Olympus microscope and software. Enumeration of Weibel-Palade bodies and nuclei was performed using ImagePro and ImageJ software.

Statistics

We described the variability of our data using ± S.D. with *P* < 0.05 to indicate significance. The Student's *t*-test was used to compare 2 groups, and ANOVA to compare > 2 groups.

Results

Extracellular matrix affects endothelial content of VWF

We hypothesized that extracellular matrix affects endothelial content of VWF. To test this idea, we plated human umbilical vein endothelial cells (HUVEC) upon non-coated plates or upon plates coated with different extracellular matrix components, including laminin, lysine, fibronectin, collagen I, and collagen IV. We then grew the cells for 4 days until they were confluent, and then cultured the confluent cells for an additional 6 days in the confluent

state. Cells were lysed, lysates were diluted 10 fold, and the concentration of VWF was measured by an ELISA and protein by BCA.

Yield of VWF was unaffected by matrix after 4 days in culture (Fig. 1A, white bars). By day 10 in culture, VWF content increased. Notably, endothelial cells grown on laminin or lysine coated plates had less VWF content than cells grown on non-coated plates (Fig. 1A, black bars). In contrast, plating endothelial cells on collagen I coated plates instead of non-coated plates increased VWF content (Fig. 1A, black bars). Fibronectin or collagen IV coated plates were not statistically different from non-coated plates.

Extracellular matrix affects endothelial exocytosis of VWF

We next explored the influence of extracellular matrix upon endothelial release of VWF. Again we plated HUVEC on plates coated or not with extracellular matrix components, and then cultured the cells. On day 10, the media was aspirated and the cells were refed with endothelial basal media alone or with endothelial basal media and histamine 10 μ M for 1 h. The media was collected and VWF was measured by an ELISA.

Compared to non-coated wells, wells coated with laminin or lysine decreased endothelial exocytosis of VWF (Fig. 1B, black bars). However, wells coated with collagen I or collagen IV increased the ability of endothelial cells to release VWF (Fig. 1B, black bars). Furthermore, basal release of VWF was higher from endothelial cells grown on fibronectin or collagen I or collagen IV compared to cells grown on non-coated wells (Fig. 1B, white bars). Taken together, these data suggest that extracellular matrix regulates endothelial secretion of VWF.

Many investigators culture HUVEC on a gelatin matrix [36–39]. We cultured HUVEC upon wells coated with gelatin, collagen, or upon non-coated wells, in order to compare the effects of these matrices upon endothelial exocytosis. Repeating the VWF secretion assay, we found that gelatin and collagen have similar effects upon endothelial release of VWF in response to histamine (Fig. 1C).

Confluency increases endothelial exocytosis of VWF

We next tested the effect of confluency upon endothelial release of VWF, since we observed that cells grown for 4 days released less VWF than cells grown for 10 days (Fig. 1A). We grew HUVEC on non-coated or collagen I coated 96-well plates for 0 – 12 days. Cells became confluent on day 4, and were cultured in a confluent state between days 5 – 12. Cells were harvested on days 4, 7, or 12, treated with histamine 10 μ M or media alone, and the concentration of VWF released into the media was measured as above.

HUVEC that are confluent for less than 24 h are unable to release significantly more VWF when stimulated than when not stimulated (Fig. 2, day 4). However, HUVEC confluent for at least 3 days respond to histamine by releasing more VWF than non-stimulated cells (Fig. 2, day 7 and day 12). Furthermore, confluent cells grown on a collagen I matrix release more VWF than confluent cells grown on plastic after 7 days and after 12 days (Fig. 2, plastic vs. collagen I). Thus confluency increases the ability of endothelial cells to release VWF following stimulation.

Extracellular matrix affects agonist triggering endothelial exocytosis

A variety of agonists trigger endothelial exocytosis. We tested the ability of extracellular matrix to modulate the endothelial response to ATP and histamine, which trigger exocytosis through purinergic receptors and calcium signaling, and to modulate the endothelial response to ionomycin, a calcium ionophore which directly increases intracellular calcium levels. Each agonist increased the release of VWF by cells grown on uncoated plates (Fig. 3A, white bars). As before, culturing endothelial cells on collagen I coated plates increased basal release of VWF (Fig. 3A, control white and black bars). Culturing cells on collagen I increased endothelial secretion in response to ATP, histamine, or ionomycin (Fig. 3A). Thus the effects of collagen on endothelial exocytosis of VWF are not restricted to histamine signaling.

We next tested the effect of matrices upon endothelial responses to forskolin, a compound that increases intracellular cAMP levels. Cells grown on collagen I or gelatin release more VWF in response to forskolin than cells grown on plastic (Fig. 3B). Interestingly, cells grown on collagen I release more VWF than cells grown on gelatin in response to forskolin (Fig. 3B). In addition, both collagen and gelatin boost the endothelial response to the calcium ionophore A23187 (Fig. 3B).

We also measured the dose-response to histamine. Cells grown on a collagen I matrix release more VWF than cells grown on non-coated plates, throughout a range of histamine concentrations (Fig. 3C).

Extracellular matrix affects the number of endothelial granules

We next explored the effect of extracellular matrix on the number and distribution of endothelial granules, since the factors regulating endothelial granule biogenesis are not completely defined. Endothelial cells were cultured for 10 days on non-coated or collagen I coated glass slides, and imaged by confocal microscopy for DNA and VWF (Fig. 4A). ImageJ and ImagePro software was used to analyze the number of nuclei and granules. The extracellular matrix did not change the number of nuclei per high powered field (Fig. 4). However, collagen I coating tripled the number of granules per field (Fig. 4). Furthermore, the collagen coating increased the fraction of cells with granules: 57% of cells grown on non-coated slides contain granules, but 74% of cells grown on collagen I coated slides contain granules ($P < 0.0001$). Thus collagen I matrix increases the number of granules inside each cell, and the fraction of endothelial cells containing granules.

Discussion

We have identified three factors that together affect endothelial exocytosis *in vitro*: extracellular matrix, confluency, and time in confluent culture. Collagen I extracellular matrix increases the intracellular stores of VWF, the numbers of Weibel-Palade bodies, and the endothelial release of VWF. Confluency increases endothelial secretion. Culture for 4 or more days after confluency increases endothelial exocytosis. The endothelial exocytosis assay is optimized when cells are cultured on a collagen I matrix for 7 days after confluency.

What are the collagen I signaling pathways within endothelial cells?

The extracellular matrix produced by endothelial cells and other vascular cells plays a key role in regulating endothelial cell proliferation, migration, and survival [40, 41]. Our study raises several interesting questions about the interaction of extracellular matrix with endothelial cells, including: what is the endothelial receptor for collagen I? Several families of collagen receptors have been described, including discoidin domain receptors (DDR1 and DDR2), leukocyte-associated immunoglobulin-like receptor-1, glycoprotein VI, and integrins [42]. Endothelial cells express several integrin heterodimers, including integrin $\alpha 1\beta 2$, $\alpha 1\beta 3$, $\alpha 2\beta 1$, $\alpha 1\beta 2$ [43–46]. *In vitro* studies have shown that interrupting integrin signals change endothelial biology [47–49]. *In vivo* studies show that knocking out the gene encoding $\beta 1$ drastically alters endothelial polarity and morphology [50]. Thus it is plausible that an integrin family member or another collagen receptor mediates the effects of collagen I upon endothelial signaling.

Another question raised by our data is: what is the intracellular signal cascade triggered by the collagen receptor that increases VWF secretion? Is this a transcriptional program or a non-genomic pathway? Our data show that collagen I increases VWF content only by a modest amount (Fig. 1A). However, collagen I increases VWF release by a much greater percentage (Fig. 1B). This suggests that collagen I influences the capacity of the cell to release granules. It is possible that collagen signaling increases the expression of components of the exocytic machinery, such as SNAREs or NSF or rab members. Another possibility is that collagen increases the activity of proteins that regulate exocytosis, such as synaptotagmin or complexin family members.

Another possibility is that collagen I signaling increases endothelial exocytosis of VWF by increasing the pool of secretory competent granules. In neurons, a pool of neurovesicles, often referred to as the readily releasable pool, is docked to the presynaptic terminal and is more likely to fuse with the membrane than other pools of neurovesicles [22, 51]. Similarly in endothelial cells, granules containing VWF can be found in secretion competent or secretion incompetent pools [15, 27]. Cutler and colleagues showed that the small GTPase rab27 and its effector forms a complex that regulates the peripheral location and release of endothelial granules [15, 27]. Thus, it is possible that extracellular collagen I modulates the pool of readily releasable VWF granules, perhaps through regulation of rab27 and its effectors. Our data support this conclusion, since the increase in VWF release is greater than the increase in VWF intracellular content (Fig. 1).

How does confluency regulate exocytosis?

Our data show that confluency strongly affects endothelial exocytosis. This result has been shown by others as well [15, 52]. Cells confluent for less than 1 day do not respond to histamine by releasing VWF. There are several possible explanations for this observation. The simplest possibility is that confluent cells express more histamine receptors than non-confluent cells. Another possibility is that cell to cell contact increases the capacity of cells to release granules. Finally, it is possible that the cell cycle affects the ability of cells to secrete VWF. One study shows that subconfluent endothelial cells contain more immature pro-VWF while confluent cells contain more mature, processed VWF [52]. Other studies

have shown that confluency affects endothelial biology. Electron microscopy of confluent “cobblestone” appearing HUVEC cultures revealed myriad hallmarks of endothelial cells [53]. Confluent cells also express more endothelial nitric oxide synthase (eNOS or NOS3) than non-confluent cells [54, 55]. Additionally, confluent endothelial cells express more thrombospondin than non-confluent cells [56]. Proteins in the insulin like growth factor pathway are expressed only in confluent endothelial cells, not proliferating cells [57]. Quiescent endothelial cells express the interleukin-33 receptor, but sub-confluent cells do not [58]. Thus our study adds to the existing literature, demonstrating that confluency affects another aspect of endothelial biology.

Optimization of a high-throughput assay for endothelial exocytosis

The cell culture system we have described is applicable to high-throughput screening to identify genes or small molecules that affect endothelial exocytosis. The collagen I extracellular matrix, confluency, and days in confluent culture together combine to form a stable and reproducible assay. The assay can be performed in a 96-well plate format. Different agonists can be used to trigger exocytosis. Taken together, these characteristics of our assay can be used to study the molecular pathways involved in endothelial exocytosis.

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C **Figure 1.**

Extracellular matrix affects endothelial content of VWF and release of VWF. (A)

Extracellular matrix and VWF content. HUVEC were plated on 6-well plates coated with different extracellular matrix components, cultured for 4 or 10 days, and lysed. An ELISA was used to measure the concentration of VWF in cell lysates ($n = 3 \pm \text{S.D.}$, * $P < 0.03$ ** $P < 0.002$). (B) Extracellular matrix and VWF release. HUVEC were cultured on 6-well plates coated with different extracellular matrix components. On day 8 the HUVEC were aspirated, refed, and cultured overnight. On day 9 HUVEC were aspirated and refed with basal media alone (without serum and without growth factors) or with basal media plus histamine 10 μM for 60 min. Media was collected and analyzed for VWF by an ELISA ($n = 3 \pm \text{S.D.}$, * $P < 0.05$ vs. plastic ** $P < 0.01$ vs. plastic). VWF release is $P < 0.02$ for all control vs. histamine samples. (C) Gelatin and collagen I increase VWF release. HUVEC were cultured on 96-well plates coated with collagen I or gelatin. On day 9 the HUVEC were aspirated, refed, and cultured overnight. On day 10 HUVEC were aspirated and refed with basal media alone (without serum and without growth factors) or with basal media plus histamine 10 μM for 60 min. Media was collected and analyzed for VWF by an ELISA ($n = 6 \pm \text{S.D.}$, * $P < 0.001$ vs. plastic).

Figure 2.

Time at confluency affects endothelial exocytosis. Tissue culture plates were coated with collagen I or no coating. HUVEC were cultured on coated 96-well plates and became confluent on day 4. Cells were studied day 4, 7, or 12 d. HUVEC were refed with media and serum, and cultured 16 h; and the next day cells were aspirated and refed with media alone or with media plus histamine 10 μ M for 60 min. Media was collected and analyzed for VWF by an ELISA ($n = 8 \pm$ S.D. * $P < 0.0001$ vs. control).

Figure 3.

Extracellular matrix affects agonist signaling. (A) Extracellular matrix affects signaling from different agonists. HUVEC were grown on uncoated or collagen coated wells of 96-well plates for 7 days, and then stimulated with different agonists, and the release of VWF after 1 h was measured with an ELISA ($n = 4 \pm \text{S.D.}$, * $P < 0.05$ for agonist vs. control; ** $P < 0.03$ for plastic vs. collagen). Agonists include: Histamine 10 μM , A23187 10 μM , ATP 10 μM . (B) Extracellular matrix affects response to forskolin. HUVEC were grown on plastic or collagen or gelatin coated wells of 96-well plates for 7 days, and then stimulated with

control media or with 0 or forskolin 10 μ M or A23187 10 μ M, and the release of VWF after 1 h was measured with an ELISA ($n = 4 \pm$ S.D. $*P < 0.001$ vs. plastic). (C) Extracellular matrix and a dose-response to histamine. HUVEC were grown on plastic or collagen coated wells of 96-well plates for 7 days, and then stimulated with control media or with 0, 1, 10, or 100 μ M of histamine, and the release of VWF after 1 h was measured with an ELISA ($n = 6 \pm$ S.D. $*P < 0.006$ vs. plastic).

Figure 4.

Extracellular matrix affects number of endothelial granules. (A) Confocal microscopy of endothelial cells grown on non-coated glass (left) or on collagen coated glass (right) for 10 days were stained for VWF (red) and nuclei (blue) and imaged by confocal microscopy. Scale bar: 50 μ m. (B) HUVEC were cultured on collagen coated slides or non-coated slides as above. Computer image analysis was used to count the number of nuclei and VWF

containing granules ($n = 10$ fields $\pm$ S.D. $*P < 0.0001$ vs. glass). (C) Percent of cells containing granules stained for VWF ($n = 10$ fields $\pm$ S.D. $*P = 0.004$ vs. glass).