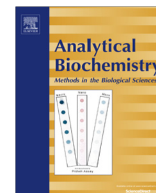
Analytical Biochemistry 452 (2014) 19–24

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio



Enhanced assay of endothelial exocytosis using extracellular matrix components

Michael B. LoMonaco, Charles J. Lowenstein *

Aab Cardiovascular Research Institute, Department of Medicine, University of Rochester Medical Center, Rochester, NY 14642, USA

ARTICLE INFO

Article history:
Received 3 December 2013
Received in revised form 7 February 2014
Accepted 11 February 2014
Available online 20 February 2014

Keywords:
Endothelial
Exocytosis
VWF
Extracellular matrix

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 prothrombotic and proinflammatory 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 medium showed robust secretion of von Willebrand factor when stimulated with various agonists. This exocytosis assay is rapid and applicable to high-throughput screening.

© 2014 Elsevier Inc. All rights reserved.

Atherosclerosis is the major cause of morbidity and mortality in the United States 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 von Willebrand factor (VWF) along with a variety of other proinflammatory and prothrombotic 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, 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 receptors) are transmembrane proteins that are inserted into transport vesicles or their target membranes and interact with one another, 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, RalA and RalGDS, Rab27a, MyRIP, and Munc18c [24–32]. Additional proteins that regulate exocytosis include endothelial nitric oxide synthase (eNOS), PKC, and Atg7 [24,33,34].

Identification of all components of the exocytic machinery is challenging because of 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- and 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 three 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.

* Corresponding author.
E-mail address: charles_lowenstein@urmc.rochester.edu (C.J. Lowenstein).

http://dx.doi.org/10.1016/j.ab.2014.02.015
0003-2697/© 2014 Elsevier Inc. All rights reserved.

Materials and methods

Cell culture

Human umbilical vein cells (HUVECs) from pooled, virus-free cords were purchased from Lifeline Cell Technology and used at passages 1–4. Endothelial cell basal medium (EBM; Lifeline Cell Technology) and endothelial growth supplement (EnGS; Lifeline Cell Technology) were used to create endothelial complete medium (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- 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 for6-well plates. Cells were maintained at 37 °C in humidified air with 5% CO_2 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 h 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 prewarmed to 37 °C. Plates were supported on prewarmed styrofoam to reduce spontaneous VWF exocytosis. Media were removed and replaced with agonists without laminar flow to reduce spontaneous VWF release and immediately returned to the incubator.

Measurement of VWF

Supernatants were collected without touching the HUVEC monolayer to reduce spontaneous release of VWF. Debris was removed by centrifugation at 100*g* for 6 min to reduce background. For preparation of cell lysates, six-well plates were treated with agonists as described above and decanted by inversion on blotter paper. Cells were lysed with 1% SDS in phosphate-buffered saline (PBS) and 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

HUVECs were plated on glass coverslips coated with or without collagen I and cultured for 10 days. Medium was removed by inversion onto blotter paper and fixed with fresh 1% formalin in PBS for 15 min. The fixed monolayers were washed three times with 3 ml PBS and permeabilized with 0.1% Triton X in PBS for 5 min. 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) were added. 4′,6-Diamidino-2-phenylindole (Vector) mounting medium 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 ±SD with $p < 0.05$ to indicate significance. The Student *t* test was used to compare two groups and ANOVA to compare more than two 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 HUVECs on uncoated plates or on plates coated with various 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 ELISA and protein by BCA.

The yield of VWF was unaffected by matrix after 4 days in culture (Fig. 1A). 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 uncoated plates (Fig. 1A). In contrast, plating endothelial cells on collagen I-coated plates instead of uncoated plates increased VWF content (Fig. 1A). Fibronectin- or collagen IV-coated plates were not statistically different from uncoated plates.

Extracellular matrix affects endothelial exocytosis of VWF

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

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

Many investigators culture HUVECs on a gelatin matrix [36–39]. We cultured HUVECs on wells coated with gelatin or collagen or on uncoated wells, 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 HUVECs on uncoated 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 and 12. Cells were harvested on day 4, 7, or 12 and treated with 10 μM histamine or medium alone, and the concentration of VWF released into the medium was measured as above.

HUVECs 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, HUVECs confluent for at least 3 days respond to histamine by releasing more VWF than unstimulated 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.

Download English Version:

https://daneshyari.com/en/article/1173636

Download Persian Version:

https://daneshyari.com/article/1173636

Daneshyari.com