Human Complement Factor H Is a Reductase for Large Soluble von Willebrand Factor Multimers—Brief Report

Leticia Nolasco, Jennifer Nolasco, Shuju Feng, Vahid Afshar-Kharghan, Joel Moake

Objective—Ultrasound von Willebrand factor (vWF) multimers, composed of ≈250 kDa subunits connected by disulfide bonds, are secreted by, and anchored to, human endothelial cells (ECs) stimulated by secretagogues (eg, histamine or cytokines).1-3 Exposed A1 domains in vWF monomeric subunits of EC-anchored ULvWF multimeric strings induce platelet adherence, and exposed A2 domains of ULvWF are cleaved at peptide bonds 1605–1606 (in the presence or absence of flow).4 By a disintegrin and metalloprotease with thrombospondin domains-type 13 (ADAMTS-13) cleaves the ultralarge vWF strings into large soluble vWF multimers. Normal plasma contains a nonproteolytic reducing activity that subsequently rapidly diminishes the size of the large soluble vWF multimers.

Approach and Results—The vWF reductase activity was isolated from normal cryoprecipitate-poor plasma by chromatography and identified as the complement regulatory protein, factor H (FH), by mass spectroscopy, SDS-PAGE, and monospecific anti-FH antibody. Removal of FH from partially purified vWF reductase by immunoabsorption eliminated the reducing activity, and the activity was recovered in the eluates. Recombinant human FH reduced large soluble vWF multimers in a free thiold-dependent reaction that was not inhibited by a variety of protease inhibitors.

Conclusions—FH contributes to the reduction of large soluble vWF multimers. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: complement factor H ■ oxidoreductases ■ von Willebrand factor

Materials and Methods

In contrast, as described for the first time in this report, the complement regulatory protein, factor H (FH),11,12 reduces (in the absence of shear stress) the disulfide bonds that link vWF monomers of various chain lengths into LsvWF.

Results

A partially purified candidate protein with reductase activity for LsvWF, and containing free thiols, was identified as a maleimide–polyethylene glycol (PEG)–biotin–reactive (ie, containing free thiol) band on 7% sodium dodecyl sulfate (SDS) unreduced acrylamide gels (Figure 1). The maleimide–PEG–biotin (and Coomassie stained)–positive band was cut from the gel lanes and identified by mass spectroscopy as compatible with the amino acid composition of FH. The candidate active band reacted with polyclonal antihuman FH antibody (Figure 1).

The LsvWF reducing activity was eliminated from purified preparations by depleting FH, using FH antibody conjugated with protein G or with cyanogen bromide-Sepharose beads (Figure 1). The FH eluted from anti-FH/cyanogen bromide-Sepharose beads was capable of reducing LsvWF (Figure 1).

Control immunoabsorption of C4 and C5, thrombospondin-1, vWF, albumin, or ADAMTS-13 did not remove the
reducing activity for LsvWF from cryosupernatant or from partially purified LsvWF reductase.

Recombinant FH (rFH; Figure 2) reduced LsvWF in the presence or absence of EDTA, but not in the presence of the free thiol-blocking substance, iodoacetic acid (Figure 2). rFH at concentrations as low as 873 ng/mL (final concentration) were capable of reducing LsvWF. As control, there was no reduction of vWF multimers using the conditioned media from HEK 293 cells not transfected with the FH gene. (An example of this latter experiment is shown in Figure 3A; top membrane shown.)

In addition to EDTA, several other protease inhibitors did not inhibit the breakdown of the reducing activity for LsvWF contained in normal cryosupernatant, partially purified vWF reductase, or rFH. These results are demonstrated in Figure 4 and the Table. Furthermore, purification of the LsvWF reducing activity (using normal cryosupernatant as starting material) was done in the presence of EDTA.

In some experiments, the lower molecular weight vWF multimers generated by FH reduction of LsvWF are electrically transferred more rapidly than larger vWF multimeric forms from the thin SDS-1% agarose gel into, and through, the underlying membrane. This can be demonstrated by placing a second transfer membrane beneath the first to capture the rapidly migrating lower molecular weight vWF multimers on the second (bottom-most) membrane (Figure 3B). Reduction by rFH of both soluble ULvWF multimers (from EC supernatant; Figure 3A) and soluble recombinant plasma-type large vWF multimers into smaller vWF multimers is demonstrated in the second (bottom-most) transfer membrane in Figure 3B.

We did not detect any ADAMTS-13–mediated proteolytic products of vWF multimers (including 140–176 kDa heterodimers) using unreduced SDS-1% agarose gels, both first and second (bottom) transfer membranes and anti-vWF antibody. However, more rapidly migrating (smaller) vWF antigenic forms were detected from gel lanes that initially contained a relatively large quantity of vWF antigen in normal plasma. (Figure 3A and 3B).

We cannot rule out the possibility that even smaller vWF forms or fragments move through the second (bottom-most) membrane and remain undetected in our transfer system. However, all of our results demonstrate that the FH reducing activity for LsvWF is thiol dependent and not the result of contamination by ADAMTS-13 or other protease.

In contrast to ADAMTS-13, FH reduces LsvWF, but has no effect on ULvWF multimeric strings anchored to ECs, in our in vitro human umbilical vein endothelial cell/microscopy system (Figure 5).

Partially purified FH was isolated in a form with LsvWF reducing activity, perhaps because it was prepared from cryosupernatant that contains a few mol/L each of cysteine and glutathione. rFH was also released from transfected cells containing cysteine and glutathione. Equivalent plasma cysteine and glutathione concentrations do not reduce ULvWF (data not shown).

In contrast, a commercial, highly purified preparation of FH (CompTech, Tyler, TX) as supplied (in concentrations ≤500 µg/mL) does not have LsvWF reducing activity. This may be because of reversible inactivation of the FH LsWVF reducing

**Figure 1.** Factor H (FH) is the probable reductase for large soluble von Willebrand factor multimers (LsvWF). Partially purified LsvWF reductase (Coomassie-gel band in lane 3 and maleimide–PEG–biotin (MPB)–positive band in lane 4) is indicated by the box and solid arrow, along with adjacent fractions lacking LsvWF reductase activity (lanes 2 and 5). Lane 3, but not lane 2, contained FH by mass spectrometry. Partially purified LsvWF reductase containing FH (lane 6) reduced LsvWF multimers in human umbilical vein endothelial cell supernatant (lane 7) to smaller vWF forms during 15 minutes of mixing at room temperature. Removal of FH by immunoabsorption (using goat antihuman factor H linked to cyanogen bromide [CNBr]-Sepharose beads) eliminated most LsvWF reductase activity (lane 8). The bead eluate contained LsvWF reductase activity (lane 9), as well as 2 bands of ≈150 and 130 kDa identified as FH by immunoblotting (lane 10; dashed arrow; n=4–10). Lanes 1 to 5 and 10, Unreduced sodium dodecyl sulfate (SDS)-7% PAGE. Lanes 6 to 9, Unreduced, SDS-1% agarose gel lanes. Lanes 1 to 3, Coomassie stain. Lanes 4 and 5, Free thiol reactivity using MPB and goat anti–streptavidin horseradish peroxidase. Lanes 6 to 9 identify vWF, and lane 10 identifies FH, by Western blotting/chemiluminescence.
activity during the purification process. Evidence in support of this possibility is provided by the demonstration that commercial FH regains LsvWF reducing activity after incubation at 4°C for 24 hours with either 10 mmol/L EDTA or 1 mol/L urea (Figure 6). These compounds may change the conformation of commercial, purified FH, by altering either the cation environment (EDTA) or the folding pattern (urea) of the highly purified commercial FH molecule, and restore its nascent LsvWF reductase activity. Figure 6 demonstrates that both EDTA-treated and urea-treated commercial, purified FH samples have LsvWF reducing activity associated with a predominant band that is more migrating slowly on unreduced SDS-PAGE than a small quantity of commercial, purified FH.

Discussion

FH family proteins are present in the plasma in several sizes ranging from 24 to 150 kDa and participate in the regulation of the alternative complement pathway. These include FH-like protein (≈40 kDa) and 5 FH-related proteins (≈25–90 kDa), FH monomer (≈150 kDa), and some FH doublets. FH binds to C3b, promotes C3b degradation, and serves as a cofactor for FI-mediated C3b cleavage.

The most abundant FH in normal circulation is the monomeric form. Its estimated size depends on isolation techniques, acrylamide gel/electrophoretic conditions of analysis and, possibly, partial kallikrein proteolysis. The full-length FH monomer comprises 20 short consensus repeat (Sushi) glycosylated subunits of 60 amino acids connected by peptide bonds. Each short consensus repeat domain contains 2 internal disulfide bonds.

We detected free thiols in the FH monomer by maleimide–PEG–biotin, which may have been produced by reduction of ≥1 of the disulfide bonds within short consensus repeat domains. Our experiments using unreduced SDS-1% agarose gel electrophoresis indicate that FH is likely to decrease the size of LsvWF by reducing some of the disulfide bonds that connect chains of vWF monomers into vWF multimers. The precise region of the multidomain FH molecule responsible for the reduction of intermonomeric disulfide bonds in vWF multimers has not yet been identified. The interaction between FH and LsvWF is likely to be evanescent because we could not detect (using SDS-agarose or SDS-acrylamide electrophoresis) the formation of covalent mixed disulfide bonds between FH and vWF.

FH attaches to cell-anchored ULvWF strings and participates in the regulation of the alternative complement pathway. In contrast to its capacity to reduce LsvWF multimers, FH (in the concentrations we have tested) does not reduce EC-secreted/EC-anchored ULvWF multimeric strings. This is the opposite substrate preference from ADAMTS-13 that cleaves EC-anchored ULvWF multimeric strings, but (in the absence of extremely high shear stress) does not cleave either soluble large vWF or soluble vWF multimers.
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ULVWF forms. EC-anchored ULvWF multimeric strings differ considerably in conformation from soluble large vWF (LsvWF). Human umbilical vein endothelial cell supernatant (enriched in ultralarge vWF multimers) was mixed (1:1 vol:vol) with either cryosupernatant (A) or partially purified LsvWF reductase (B), in the presence of the following additions. A, None (lane 1), EDTA (10 mmol/L; lane 2), IAA (1 mmol/L; lane 3), and aprotinin-leupeptin–pepstatin (ALP; 1 μmol/L; lane 4). B, None (lane 1), IAA (1 mmol/L; lane 2), NEM (1 mmol/L; lane 3), and phenylmethylsulfonylfluoride (PMSF; 20 μmol/L; lane 4). Analysis of the mixtures was by unreduced sodium dodecyl sulfate–1% agarose gel electrophoresis and Western blotting using anti-vWF antibody.

ULVWF forms. EC-anchored ULvWF multimeric strings differ considerably in conformation from soluble large vWF and soluble ULvWF forms. These conformational differences are likely to account for the distinct FH (and ADAMTS-13) substrate preferences under our experimental conditions.

The complementary substrate breakdown mechanisms of ADAMTS-13 (for EC-anchored ULvWF multimeric strings) and FH (for soluble large vWF and ULWVF forms) suggest the possibility that ADAMTS-13 and FH may act sequentially to generate the normal vWF multimeric patterns seen in unreduced SDS-agarose gels.

Neither partially purified FH nor rFH at the concentrations tested was capable of reducing EC-secreted/anchored ULvWF multimeric strings in vitro. We do not know, however, whether these results simulate in vivo events because the concentration of FH in normal plasma (≈500 µg/mL) is ≥7-fold greater than we have been able to produce/test on human umbilical vein endothelial cell–secreted/anchored ULvWF strings.

Figure 4. Iodoacetic acid (IAA) and N-ethylmaleimide (NEM), but not protease inhibitors, inhibit von Willebrand factor (vWF) reducing activity in cryosupernatant or in partially purified large soluble vWF (LsvWF). Human umbilical vein endothelial cell supernatant was mixed (1:1 vol:vol) with either cryosupernatant (A) or partially purified LsvWF reductase (B), in the presence of the following additions. A, None (lane 1), EDTA (10 mmol/L; lane 2), IAA (1 mmol/L; lane 3), and aprotinin-leupeptin–pepstatin (ALP; 1 μmol/L; lane 4). B, None (lane 1), IAA (1 mmol/L; lane 2), NEM (1 mmol/L; lane 3), and phenylmethylsulfonylfluoride (PMSF; 20 μmol/L; lane 4). Analysis of the mixtures was by unreduced sodium dodecyl sulfate–1% agarose gel electrophoresis and Western blotting using anti-vWF antibody.

Figure 5. Recombinant factor H (rFH) does not cleave human umbilical vein endothelial cell (HUVEC)–secreted/anchored ultralarge von Willebrand factor (ULvWF) multimeric strings.

A, HUVECs were stimulated with 100 μmol/L histamine for 2 minutes under static conditions, and typhloidized platelets were added to adhere to the cell-anchored ULvWF strings (white arrows). B, rFH (750 ng/mL) was added and the cell-anchored ULvWF strings remained uncleaved during 5 minutes of observation. C, Recombinant a disintegrin and metalloprotease with thrombospondin domains-type 13 (350 ng/mL) cleaved cell-anchored ULvWF strings in <1 minute.

Table. Effect of Protease Inhibitors on FH-Mediated Reduction of Large Soluble von Willebrand Factor

<table>
<thead>
<tr>
<th>Protease Inhibitors</th>
<th>Blockade of FH</th>
<th>Reductase</th>
<th>Final Concentration</th>
</tr>
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<tr>
<td>Phenylmethylsulfonylfluoride (n=7)</td>
<td>No</td>
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<tr>
<td>Aprotinin (n=10)</td>
<td>No</td>
<td>125 kIU</td>
<td></td>
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<tr>
<td>Iodoacetic acid (n=20)</td>
<td>Yes</td>
<td>1 mmol/L</td>
<td></td>
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<tr>
<td>Leupeptin (n=5)</td>
<td>No</td>
<td>1 μmol/L</td>
<td></td>
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<tr>
<td>Pepstatin (n=5)</td>
<td>No</td>
<td>1 μmol/L</td>
<td></td>
</tr>
<tr>
<td>N-Ethylmaleimide (n=10)</td>
<td>Yes</td>
<td>1 mmol/L</td>
<td></td>
</tr>
<tr>
<td>EDTA (n=20)</td>
<td>No</td>
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<tr>
<td>Citrate–hirudin–aprotinin–leupeptin (n=20)</td>
<td>No</td>
<td>10% vol/vol</td>
<td></td>
</tr>
<tr>
<td>Soybean trypsin inhibitor (n=20)</td>
<td>No</td>
<td>10% vol/vol</td>
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</table>

FH indicates factor H.
EDTA and urea, in contrast to the single predominant band of Mr ≈ 150 kDa and a minor band of 130 in lane 6. Commercial, purified FH migrated as a major band of µ concentration were 37.5 (lane 5), or untreated (lane 6), commercial, purified FH. FH final with anti-FH antibody on urea-treated (lane 4), EDTA-treated gel electrophoresis and Western blotting with anti-vWF antibody. fied FH did not possess LsvWF reducing activity (lane 3). Lanes gel lane 1) or 1 mol/L urea (lane 2). Untreated, commercial, puri- soluble large and ultralarge vWF multimers after preincubation for µ factor H (37.5 µ factor H. Commercial, purified factor H (FH) does not have large soluble vWF multimers unless it either type of FH deficiency. If FH deficiency patients have a shift toward larger vWF multimers in plasma, then they might be more susceptible to shear-induced platelet aggregation mediated by soluble large and soluble ULvWF multimers and, possibly, to accentuated microvascular thrombosis.

Acknowledgments
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Disclosures
None.

References


Significance
We demonstrate in this report that factor H, a regulatory protein in the alternative complement pathway, can reduce large soluble von Willebrand factor multimers to smaller von Willebrand factor forms. Defective reduction in the size of large soluble von Willebrand factor multimers may contribute to excessive von Willebrand factor–mediated platelet aggregation in the renal microcirculation, and accentuate renal injury, in patients with factor H deficiency.
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MATERIALS AND METHODS

Human umbilical vein endothelial cells (HUVECs)

Primary endothelial cells were harvested from fresh human umbilical cord veins, plated and grown to confluence as previously described.¹

Preparation of normal human cryoprecipitate-poor plasma (cryosupernatant)

Cryosupernatant² was prepared by 3-4 freeze (-80°C)-thaw (4°C) cycles of acid-citrate-dextrose (ACD), citrated or EDTA-normal human plasma, followed by centrifugation at 10,000 rpm at 4°C for 30 minutes, in order to decrease the quantity of large plasma VWF multimers and other proteins (fibrinogen, fibronectin, IgM) that precipitate at cold temperatures. Some of the purification procedures for VWF reductase using normal cryosupernatant as starting material were done in the presence of EDTA.

Detection of the reducing activity for large soluble VWF multimers (LsVWF)

Normal cryosupernatant (or other test samples) were mixed 1:1 (vol/vol) and incubated for 15 minutes at room temperature with supernatant from HUVECs collected after histamine-stimulation for 10-15 minutes (or after 24 hours in culture in the absence of histamine). HUVEC supernatants were enriched in LsVWF produced by HUVEC-derived ADAMTS-13 cleavage of HUVEC-secreted and anchored ULVWF strings. The mixtures were then denatured using 8 M urea and 1% SDS, heated for 15 minutes at 56°C, and electrophoresed into a SDS 1% ME-agarose gel. The separated proteins were transferred onto a PVDF membrane.

In some experiments, a second (bottom) membrane was placed beneath the first in order to capturing relatively smaller VWF multimers migrating through the first membrane. When using a double membrane electrotransfer, it was important to eliminate air bubbles by careful handling of the 2 membranes.

The transferred proteins were detected using goat polyclonal anti-human VWF antibody (cat. # A80-138), secondary rabbit anti-goat antibody linked to horseradish peroxidase (cat. # A50-100P) (HRP; Bethyl Laboratories, Montgomery, TX) and Chemiluminescent Reagent (Pierce/ Thermo, Rockford, IL). The reducing activity for LsVWF was indicated by the disappearance of large VWF multimeric forms, and was not inhibited by EDTA (indicating that the activity was not dependent on Zn²⁺/Ca²⁺-requiring ADAMTS-13).

Purification of LsVWF reductase from normal human cryosupernatant

A Sephacryl S300-HR column (S-300 cat. # 17-0599-01; GE Health Care, Uppsala, Sweden) was used to separate proteins in cryosupernatant according to their molecular weights. Cryosupernatant (30 ml) was added to 300 ml of S-300 in a pre-
washed column, followed by an inflow of phosphate-buffered saline (PBS), pH 7.4. One ml fractions were collected and screened for LsVWF reducing activity. Active fractions were pooled and concentrated using Centricon concentrator tubes (EMD Millipore Corp.) with 25-50 kDa size exclusion. In the next step, 100 ml of DEAE-Sepharose CL 6B (Sigma Chemicals) were loaded into a column, equilibrated and washed with 10 mM Tris-HCl, pH 8.0. A pooled, concentrated S-300 fraction was applied to the DEAE-Sepharose CL 6B column, allowed to enter completely, and the column was washed with 10 mM Tris-HCl, pH 8.0, in order to remove unbound protein. An increasing concentration (20 mM to 50 mM) of Tris-HCl, pH 8.0, was then added to elute the bound protein. 1.5 ml fractions were collected, and LsVWF reductase was detected as described in the section above.

Protein identification and measurement was based on the absorbance of Coomassie Brilliant Blue (CBB) G25 (Bio Rad Laboratories, Inc., Hercules, CA) at 595 nm. The detection of free thiols in test proteins was done using maleimide-PEG2-biotin (MPB; Pierce/Thermo, Rockford, IL). Partially purified fractions (100 µl) were diluted with an equal volume of phosphate buffer, pH 6.5-7.5, and incubated with 4 mM (final concentration) MPB for 1 hour at room temperature. The MPB-labeled samples were electrophoresed into SDS-7% acrylamide gels, transferred onto PVDF membranes (EMD Millipore Corp.), and detected by chemiluminescence with streptavidin-HRP (GE Health Care, Buckinghamshire, UK).

Identification by mass spectroscopy of the reductase for LsVWF

Gel bands that were CBB positive and either MPB positive (with LsVWF reductase activity) or MPB negative (without LsVWF reductase activity), and at identical locations on SDS-7% acrylamide gels, were processed for protein identification by liquid chromatography/mass spectrometry (LC/MS) analysis and in-gel trypsin digestion following reduction (with dithiothreitol) and alkylation (with iodoacetamide) of potential protein disulfide bonds. LC/MS data acquisition and instrument control was accomplished using MassLynx v4.1 software. Protein identifications were accomplished by searching lock-mass corrected and processed LC/MS spectral data via the ProteinLynx Global Server (PLGS v2.4; Waters Corp.) search engine software. The Uniprot 2011_03 (‘reviewed’) human proteome database (20,227 entries) was used as the target database. The false discovery rate was set at 1%, by using the reversed and randomized human proteome as the decoy target database.

Removal of FH and its effect on LsVWF reductase activity

We used Cyanogen Bromide (CNBR)-activated Sepharose-4B beads (GE Health Care, Buckinghamshire, UK) conjugated with anti-FH antibody in order to deplete FH from partially-purified LsVWF reductase in elutes purified from cryosupernatant or from recombinant FH samples. Briefly, either 300-500 µl of partially purified LsVWF reductase or 500 µl of recombinant FH was mixed with an equal volume of 0.1M NaHCO₃/0.5M NaCl₂ was added to 350 µl of anti-human FH/CNBR-Sepharose, incubated overnight at 4°C, and centrifuged at 5000 rpm for 5 minutes. The supernatant...
was removed and the anti-human FH/CNBR-Sepharose with bound FH was washed with PBS. Bound FH was then eluted using 0.1M glycine-HCl, pH 2.0. Eluted samples were immediately neutralized with 20 µl of 1M Tris-HCl pH 8.0/ 200 µl eluate and concentrated using Micro Centricon Concentrator (100 kDa exclusion) (EMD Millipore Corp.). LsVWF reducing activity was determined in the FH elutes and FH-depleted supernatants as described above.

Factor H was also depleted from partially purified LsVWF reductase or from recombinant FH using polyclonal goat anti-human FH antibody (cat.# A237; Complement Technologies) plus protein G (Sigma Chemicals). Briefly, 200 µl of samples were mixed with 2-5 µg anti-FH antibody at 4°C overnight, and incubated with 50 µl of pre-washed protein G for at least 4 hours at room temperature to overnight at 4°C. After mixing and incubation, the complex was centrifuged and the supernatant (depleted of FH) was aspirated and saved. In some experiments, the depletion procedure was done twice in order to deplete FH completely. Factor H bound to protein G was detected by mixing the beads with non-reducing sample buffer (0.5M Tris-HCl, pH 6.8, and 1% SDS), SDS-7% polyacrylamide gel electrophoresis, transfer to a PVDF membrane, and immunoblotting using anti-FH antibody.

Recombinant human Factor H (rFH)

The production of rFH using baculovirus or yeast systems has been reported. We used human embryonic kidney (HEK) 293 cells (American Type Culture Collection [ATCC], Manassas, VA) to generate FH. Full-length human FH cDNA was generated by polymerase chain reaction (PCR) with 5′ primer (GGACTTTCCAAAATGTCGT) and 3′ primer (ATCCTCGAGCTCTTTTTGCACAAGT), using human FH cDNA (Origene Technologies, Inc., Rockville, MD) as a template. After confirming the sequence, the amplified DNA fragment was digested with Not I (5′) and Xho I (3′) and ligated to pSecTag2B His tag vector (Invitrogen, Grand Island, NY). The plasmid containing full-length FH cDNA were transfected into HEK 293 cells using LipofectamineTM 2000. Stably transfected cells were maintained in Dulbecco’s Modified Eagle media (DMEM) containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 1 mM sodium pyruvate 1% MEM Non-essential Amino Acids and the selection agent, Hygromycin B (250µg/ml).

Recombinant FH in cell culture media was purified using His-select Ni2+-charged affinity gel (Sigma Chemicals) that binds to histidine-tagged rFH. Briefly, 1 ml of cell culture media was mixed with 100 µl of his-select gel, for 15 minutes. His-tagged proteins were then eluted by 200-250 mM imidazole. The purified proteins were electrophoresed into an SDS-7% acrylamide gel, transferred onto a PVDF membrane, and detected with either anti-His-HRP (Sigma Chemicals) or goat anti-human FH antibody and secondary rabbit anti-goat-HRP. Purified rFH was tested for LsVWF reductase activity as described above.
Purified Reductase and Recombinant Factor H Contains Free Thiols

Free thiols in FH were quantified by Ellman’s assay using 5,5’-Dithio-bis-(2-nitrobenzoic acid) (DTNB) that forms a mixed disulfide with free thiols and produces a yellow color measureable at 410 nm. The amount of free thiols was calibrated against a cysteine standard.

Recombinant VWF

The production of rVWF in several cell lines has previously been described in detail.\textsuperscript{17,18}

HUVEC Secreted/ Anchored ULVWF Multimeric Strings

This method is described in detail in Materials & Methods reference #1 and in text references 1,2,4 and 5.

Word count = 1,347 + 18 references

REFERENCES


