Benchmarks

Expression and purification of bioactive, low-endotoxin recombinant human vitronectin

Michael M. Halford¹, Yi-Chao He^{1,2}, and Steven A. Stacker^{1,2}

¹Tumour Angiogenesis Program, The Peter MacCallum Cancer Centre, St Andrews Place, East Melbourne, Victoria, Australia and ²Sir Peter MacCallum Department of Oncology, The University of Melbourne, Parkville, Victoria, Australia.

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The secreted adhesive glycoprotein vitronectin (VTN) is a multifunctional component of plasma and the extracellular matrix. A high-yielding, inexpensive, low endotoxin source of bioactive recombinant human vitronectin (rhVTN) is highly desirable for in vitro use in diverse cell culture systems ranging from basic research settings to clinical-grade production of human cells. We describe modifications to a previously reported heparin-based affinity chromatography procedure that improve yield and achieve efficient removal of endotoxin from washed and urea-solubilized human VTN inclusion bodies following standard autoinduction of expression in *Escherichia coli*. This simple procedure makes accessible the low-cost expression and purification of large quantities of bioactive rhVTN using basic equipment and facilitates its use in a spectrum of endotoxin-sensitive applications.

In developing conditions to support an in vitro genetic screen for modifiers of the human blood endothelial cell (BEC) response to anti-angiogenic agents (1), we sought an inexpensive source of human vitronectin (VTN) for coating large surface areas of polystyrene culture vessels and microcarrier beads. VTN was selected by virtue of its unique ability to engage the $\alpha_v\beta_3$ plus $\alpha_v\beta_5$ integrins, among others, and synergize with multiple growth factor receptors expressed on BECs in the activation of angiogenesis (2). However, commercial

sources of full-length VTN purified from human plasma (hp) or recombinant human VTN (rhVTN) purified from mammalian expression systems were prohibitively expensive for use on a large scale.

Wojciechowski et al. (3) were the first to report expression of rhVTN in *E. coli*, and these authors described its purification based on fusion to a glutathione S-transferase (GST) tag. Chen et al. (4), in establishing chemically defined culture conditions for human embryonic stem (ES) and

induced pluripotent stem cells, modified the procedure to facilitate purification of bioactive untagged rhVTN—and truncated derivatives thereof—by exploiting VTN's heparin binding activity in 8 M urea. Although ES cells are relatively resistant to endotoxin (also known as lipopolysaccharide), most other mammalian cell types are highly sensitive and respond to exposure with rapid modulation of survival and/or proliferation, adhesion, secretion, or specialized tissue-specific functions (5). Thus, the endotoxin that typically contaminates protein purified from *E. coli* can readily generate spurious results in cell-based assays, including those involving ECs (6,7,8).

Here we describe how to effectively deplete endotoxin from rhVTN expressed in and purified from *E. coli*, thus permitting its use with all cell types and/or in chemically defined culture systems. We began by optimizing purification of untagged rhVTN from *E. coli* with a yield ≥ 350 mg/L versus ~ 70 mg/L from the existing method (Figure 1A, Supplementary Protocol) (4). In evaluating methods for subsequent depletion of endotoxin from purified rhVTN, we observed near-complete loss of purified rhVTN on a commercial poly- ϵ -lysine endotoxin-removal column (Pierce High Capacity Endotoxin Removal Spin Column, 0.25 mL, #88273; Thermo Fisher Scientific, Inc., Rockford IL) and failure of a complementary method of endotoxin removal involving extraction of inclusion body preparations with Triton X-114 (data not shown) (9).

The lack of a universally successful method for endotoxin removal from proteins (10) led us to screen for additives to the heparin column wash solution that would dissociate endotoxin from the stationary affinity chromatography phase. In small-scale purification runs, we tested the individual addition of 6 candidates (Isopropanol, 1,2-hexanediol, ASB-14, C7BzO, Igepal CA-630, CHAPS; all purchased from Sigma-Aldrich, Castle Hill, NSW, Australia) to a modified

METHOD SUMMARY

We describe a simple procedure for the low-cost expression and purification of large quantities of bioactive and low-endotoxin recombinant human VTN by modifying the standard heparin-based affinity column chromatography procedure to include the additive Igepal CA-630 in the wash solution in order to remove endotoxin.

Figure 1. Purification of low-endotoxin rhVTN from *E. coli*. (A) Representative Coomassie brilliant blue-stained SDS-PAGE analysis of samples taken during purification of rhVTN from 50 mL of autoinduced culture (as described in the Supplementary Protocol). The molecular weight (in kDa) of size standards is shown at left. At right, the open arrowhead points to full-length rhVTN and the filled arrowhead to a proteolytic derivative; these correspond to V75 and V65, respectively, in mammals. SB+2, SeeBlue Plus 2 Pre-Stained Standard (Life Technologies); T, total lysate; S, soluble fraction; UB, unbound fraction; W, wash fraction; E, eluate fraction. Amounts loaded correspond to 0.005% (T, S, and UB) and 0.025% (W and E) of each fraction. W1 contained 1% Igepal CA-630. (B) Depletion of endotoxin during rhVTN purification. Material purified on a small scale was buffer exchanged into DPBS, and the specific endotoxin content (in EU/mg) was determined (EndoZyme Recombinant Factor C Assay, Hyglos; BCA Protein Assay, Thermo Scientific). Bars depict the mean + standard error of the mean (SEM) from three independent experiments; the corresponding value of the mean is also shown above each bar. Additives used in Wash 1 are shown on the x-axis. EU, endotoxin unit; IPA, isopropanol 1:1 (v/v) mixture with Buffer U; 1,2-HD, 1,2-hexanediol 1:4 (v/v) mixture with Buffer U; IGP, Igepal CA-630 1% (v/v) in Buffer U; ASB-14, 0.5% (w/v) amidosulfobetaine 14 in Buffer U; C7BzO, 0.5% (w/v) 3-(4-heptyl)phenyl-3-hydroxypropyl dimethylammonio propanesulfonate in Buffer U; CHAPS, 1% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate in Buffer U.

Figure 2. Bioactivity of low-endotoxin rhVTN in HUVEC adhesion assays. (A) Promotion of HUVEC adhesion. MaxiSorp 96-well plates (Nunc) were coated with rhVTN at the indicated concentrations (100 μ L/well at 37°C for 1 h; endotoxin content 0.09 EU/mg) then blocked with heat-denatured 2% BSA. HUVECs (Life Technologies) at passage 2 or 3 were detached with Accutase, pelleted and resuspended at 3×10^5 cells/mL in warm EBM-2 (Lonza) containing 20 mM HEPES (pH 7.4 at 37°C), 1 μ g/mL hydrocortisone, 75 μ M (+)-sodium-L-ascorbate, 500 μ M 2-phospho-L-ascorbic acid trisodium salt, 1 μ g/mL gentamicin, and 100 ng/mL heparin. Adhesion of 100 μ L of HUVECs to coated wells was quantified after incubation at 37°C, 5% O₂, 5% CO₂, and 90% N₂ for 90 min in a humidified chamber and is shown relative to that of BSA-blocked wells. Error bars depict the mean $\pm$ SEM from three independent experiments. (B) Comparison of rhVTN with other VTN sources and the effects of antagonists of integrin activity. A function-blocking mouse anti-VTN (LS-C150852, LifeSpan BioSciences, Inc.) or isotype-matched negative control MAb (50 μ g/mL), EDTA (10 mM), RGES or RGDS peptide (10 μ g/mL; Sigma), function-blocking mouse anti- $\alpha_v\beta_3$ (clone LM609, MAB1976Z; Millipore) and/or anti- $\alpha_v\beta_5$ (clone P1F6, MAB1961Z; Millipore) MAb, or a non-function-blocking mouse anti- $\alpha_v\beta_3$ MAb (clone 15F11, MAB2019Z; Millipore) were used to interrogate the mechanism of HUVEC adhesion promoted by rhVTN. Anti-integrin MABs (15) were used at a final concentration of 20 μ g/mL. The adhesion assay was performed as in panel (A) using wells coated with 1 μ M rhVTN, hpVTN, or rhVTN-N (gray bars). Adhesion is expressed relative to that of rhVTN-coated wells (first bar, 100%). Bars depict the mean $\pm$ SEM from three independent experiments. MAb, monoclonal antibody; FB, function-blocking; hp, human plasma.

denaturing wash buffer (Buffer U: 20 mM Tris-HCl, 75 mM NaCl, 8 M urea, 5 mM EDTA, 5 mM DTT, pH 7.6 at 23 °C) to destabilize divalent cation-dependent endotoxin micelles and vesicles (Figure 1B) (5). Isopropanol, 1,2-hexanediol, ASB-14, and C7BzO were effective, but 1% Igepal CA-630 was identified as the most suitable additive by virtue of low cost, low flammability, high compatibility with Buffer U, enhancement of column flow rate, and good efficacy in reducing endotoxin contamination (Figure 1B and data not shown). From larger-scale rhVTN purification runs (*E. coli* pellets from ≥50 mL of autoinduced culture) where washing of the heparin Sepharose column with Buffer U plus 1% Igepal CA-630 (Buffer ER) is more efficient, we achieved endotoxin depletion to <0.1 EU/mg (data not shown). For comparison, Life Technologies' (Mulgrave, VIC, Australia) truncated rhVTN lacking the N-terminal somatomedin B domain (rhVTN-N, Δ20–61, catalog number A14700) (4) is expressed in and purified from *E. coli*, and is supplied with an upper limit of endotoxin contamination of 25 EU/mg.

To evaluate the bioactivity of rhVTN produced using our protocol, we quantified the adhesion of endotoxin-sensitive human umbilical vein endothelial cells (HUVECs) (11) to coated polystyrene wells using an established assay (12). A sigmoidal relationship between the rhVTN concentration used for coating and HUVEC adhesion was observed, with a minimum 4-fold enhancement at ≥0.3 μM (Figure 2A). Comparison with other VTN sources at a coating concentration of 1 μM demonstrated that enhancement of HUVEC adhesion by our rhVTN was equipotent compared to commercial carrier-free hpVTN (2349-VN-100; R&D Systems, Minneapolis, MN) and similar to rhVTN-N (Figure 2B, gray bars). Inclusion of antagonists of integrin activity showed that adhesion promoted by our rhVTN was strongly inhibited by a neutralizing anti-VTN monoclonal antibody and was dependent upon divalent cations, recognition of the RGDS peptide, and the combined activity of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Figure 2B).

In conclusion, we have introduced effective and economical modifications to the established method for rhVTN expression in, and purification from, *E.*

coli. These result in a 5-fold increase in product yield with reduction of endotoxin contamination to a level suitable for use with endotoxin-sensitive cell types and/or in chemically defined culture conditions. The rhVTN and derivatives thereof prepared by this method will be valuable in research settings involving assays (e.g., for cytokine activity), functional screens (e.g., genetic or small molecule driven), and cell biology studies (e.g., of proliferation, differentiation, adhesion, or migration) using phenotypically faithful cell types. Clinical applications that will benefit from this method include the coating of biomaterials to promote colonization with cells following implantation of medical devices (13) and the ex vivo culture, reprogramming, and/or directed differentiation of human cells for use in regenerative therapy (14).

Author contributions

M.M.H. designed experiments; M.M.H. and Y.H. performed experiments; M.M.H., Y.H., and S.A.S. analysed data; M.M.H. prepared the manuscript with assistance from Y.H. and S.A.S.

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Competing interests

The authors declare no competing interests.

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Address correspondence to Michael M. Halford, Tumour Angiogenesis Program, Peter MacCallum Cancer Centre, St Andrews Place, East Melbourne, Victoria, Australia. E-mail: michael.halford@petermac.org

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