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Preparation of human vascular endothelial growth factor-D for structural and preclinical therapeutic studies

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ABSTRACT

Vascular endothelial growth factor-D (VEGF-D), a secreted angiogenic and lymphangiogenic glycoprotein, enhances tumor growth and metastasis in animal models, and its expression correlates with metastasis and poor patient outcome in some cancers – it is therefore considered a target for novel anti-cancer therapeutics. The definition of the structure of the complex of VEGF-D bound to its receptors would be beneficial for design of inhibitors of VEGF-D signaling aimed at restricting the growth and spread of cancer. In addition, there is interest in using VEGF-D protein for therapeutic angiogenesis and lymphangiogenesis in the settings of cardiovascular diseases and lymphedema, respectively. However, VEGF-D has proven difficult to express and purify in a highly bioactive form due to a tendency to exist as monomers rather than bioactive dimers. Here we describe a protocol for expression and purification of mature human VEGF-D, and a mutant thereof with reduced glycosylation, potentially suitable for preclinical therapeutic and structural studies, respectively. The degree of glycosylation in mature VEGF-D was reduced by eliminating one of the two *N*-glycosylation sites, and expressing the protein in Lec3.2.8.1 cells which had reduced glycosylation capacity. Mature VEGF-D and the glycosylation mutant were each enriched for the biologically active dimeric form by optimizing the separation of dimer from monomer via gel filtration, followed by conversion of remaining monomers to dimers via treatment with cysteine. The glycosylation mutant of VEGF-D intended for structural studies preserved all the cysteine residues of mature VEGF-D, in contrast to previous structural studies, exhibited comparable receptor binding to mature VEGF-D and might facilitate structural studies of the VEGF-D/VEGFR-3 complex.

Introduction

The vascular endothelial growth factor (VEGF)[2] family of secreted glycoproteins includes members which induce angiogenesis and/or lymphangiogenesis during embryonic development, and may contribute to maintaining the function of the blood and lymphatic vasculatures during adulthood. Some of the VEGFs are expressed in a range of pathological conditions including cancer [1], and can facilitate the growth and metastatic spread of tumors. An important step in understanding the molecular mechanisms by which VEGFs signal in health and disease is the determination of their tertiary structures in complex with the corresponding receptors. To date, high-resolution structural information from X-ray crystallography on mammalian members of the VEGF family in complex with their receptors is available for VEGF-A with VEGFR-1 domain 2 [2], placenta growth factor (PlGF) with VEGFR-1 domain 2 [3] and a VEGF-C mutant (Cys137Ala) in complex with VEGFR-2 domains 2/3 [4]. The only structure reported for VEGF-D is for the Cys117Ala mutant [5]. There is no structural data available for VEGF-D in complex with either of its receptors (VEGFR-2 or VEGFR-3).

VEGF-D is of interest from a therapeutic perspective given that it can be used to promote angiogenesis or lymphangiogenesis in tissues [6–11], which could be relevant in the clinical settings of cardiovascular diseases and lymphedema, and that it is thought to play a role in vascular homeostasis [12]. Further, VEGF-D promoted the growth and spread of cancer in a variety of xenograft and transgenic animal tumor models [13–16] (for review see [16]) and was reported to be expressed in some prevalent human

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[2] *Abbreviations used:* VEGF, vascular endothelial growth factor; PlGF, placenta growth factor; DMEM, Dulbecco's modified Eagle's medium; MES, 2-(*N*-morpholino)ethanesulfonic acid; HRP, horse radish peroxidase; CD, circular dichroism; Bio21 C[3], Bio21 Collaborative Crystallization Centre.

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cancers (for example see [17]) correlating with lymph node metastasis and patient outcome (for review see [18,19]). It is therefore a potential target for novel anti-cancer therapeutics designed to restrict the growth and spread of cancer.

VEGF-D is initially secreted from the cell as a full-length form consisting of N- and C-terminal propeptides flanking a central VEGF homology domain containing the binding sites for VEGFR-2 and VEGFR-3 [20], receptor tyrosine kinases expressed on the endothelial cells of blood vessels and lymphatics. This form can be proteolytically processed by proprotein convertases to remove the propeptides, giving rise to a mature dimeric form with improved binding affinity for VEGFR-2 and VEGFR-3 [21–24]. However, expression of recombinant mature VEGF-D can give rise to a large proportion of inactive monomer in addition to bioactive dimer [23].

VEGF-D is a member of the cystine knot superfamily of proteins, for which cysteine residues are critical to the core structure of the molecule [25]. The only published structure for VEGF-D [26] is for a mutant in which the cysteine residue Cys117, that is not part of the cystine knot and is located in the dimer interface close to the interchain Cys136–Cys145 disulfide bridge, has been altered to alanine to increase dimer stability [27,28]. Given the potential importance of the mutated cysteine for the structure of the protein, analysis of VEGF-D in which all cysteine residues are preserved would also be beneficial.

Proteins for crystallization have to be prepared in milligram quantities and to be highly homogeneous. Glycoproteins present a special problem for crystallization–glycosylation can be required for correct folding and secretion, however, the chemical and conformational heterogeneity resulting from glycosylation can inhibit crystallization [29]. Here, we report a protocol for expression and purification of partially-glycosylated human mature VEGF-D, with all cysteine residues preserved, that may be suitable for ligand/receptor structural studies. Further, the approaches we describe, particularly conversion of VEGF-D monomers to bioactive dimers, may have utility for preparation of bioactive mature human VEGF-D for preclinical studies of therapeutic angiogenesis and lymphangiogenesis.

Materials and methods

DNA constructs

An expression construct encoding VEGF-DΔNΔC (a form of mature human VEGF-D tagged with FLAG at the N-terminus), based on pEFBOSFLAG, has been described previously [20]. The construct used here was derived by subcloning the expression cassette of the pEFBOSFLAG derivative into the XbaI site of pApex3 [30], and designated pVDApexΔNΔC. To mutate cysteine (C) to alanine (A) and to eliminate N-glycosylation sites by asparagine (N) to glutamine (Q) exchange we used site-directed mutagenesis with overlapping extension PCR [31]. Forward 5′-GAG CTC GGA TCC ***TCT AGA*** CTA GTG CTA GC and reverse 5′-GCC TGC AGG TCG AC***T CTA GA***C TAG TGC flanking primers incorporated an XbaI restriction site (bold, italic). The nucleotide sequences of the mutagenic primer pairs complementary to each other were 5′-C CTT ATC TGT ATG **CAG** ACC AGC ACC TCG, designated Asn1_fw, and 5′-CGA GGT GCT GGT **CTG** CAT ACAGAT AAG G, designated Asn1_rev; 5′-CCT GTT AAA GTT GCC **CAG** CAT ACA GGT TG, designated Asn2_fw and 5′-CA ACC TGT ATG **CTG** GGC AAC TTT AAC AGG, designated Asn2_-rev (bold font indicates nucleotides corresponding to mutated amino acids). Each pair of these primers was designed to produce two mutated DNA fragments on VEGF-D gene sequence using pVDApexΔNΔC as a template for the generation of DNA encoding N155Q (Asn1) and N185Q (Asn2) VEGF-DΔNΔC mutants. Mutated DNA fragments obtained were cloned into the XbaI site of pApex3

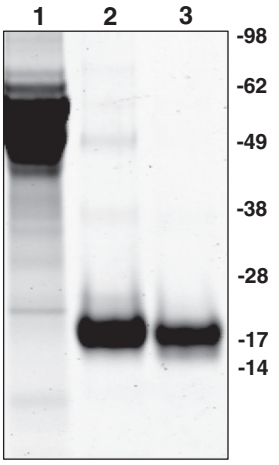

Fig. 1. Purification of the Asn2 mutant protein. Coomassie-stained, reducing SDS gel: lane 1 – conditioned cell culture medium from cells expressing the Asn2 mutant, lane 2 – affinity purified protein, lane 3 – protein after affinity purification and gel filtration. The positions of molecular weight markers (in kDa) are indicated to the right of the panel.

[30]. The desired mutations were confirmed by DNA sequencing. The resulting plasmids were designated pVDApex ΔNΔC/Asn1 and pVDApexΔNΔC/Asn2, correspondingly. For mutation of both glycosylation sites we used Asn2_fw and Asn2_rev primers on pVDApexΔNΔC/Asn1 template and designated this construct as pVDApexΔNΔC/ΔAsn.

VEGF receptor-3 domains 1–3 (VEGFR-3 D1-3) construct was expressed, purified, and tested for functional integrity as described elsewhere (Davydova et al., in preparation).

Cell culture, DNA transfection and analytical protein preparation

293EBNA-1 human embryonic kidney cells (Invitrogen) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Sigma). The Lec3.2.8.1 Chinese Hamster Ovary cell line (obtained from The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia), a derivative of CHO-K1 fibroblasts [32], was maintained in RPMI-1640 supplemented with 5% FBS on tissue culture flasks pre-coated with 1% gelatine in PBS. Both cell lines were transiently transfected with pVDApexΔNΔC and its variants using FuGENE 6 (Roche Molecular

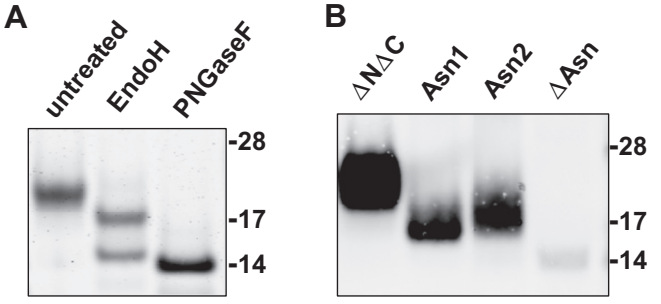

Fig. 2. Deglycosylation of VEGF-DΔNΔC via enzymes or mutagenesis. (A) Coomassie-stained, reducing SDS–PAGE of VEGF-DΔNΔC expressed in 293EBNA-1 cells. Protein was treated with either Endo H or PNGase F, with untreated material as control. (B). Western blotting of conditioned media (CM) from Lec3.2.8.1 cells transiently transfected with VEGF-DΔNΔC and its glycosylation mutants. Transfections were done in parallel under identical conditions on three occasions – the results for one set of transfections are shown, and are representative of results from all transfections. Immuno-detection was performed using anti-FLAG antibody labeled with IRDye 800CW. Equal volumes of CM were loaded in tracks ΔNΔC, Asn1 and Asn2, whereas in track ΔAsn the loading volume was five times greater. The positions of molecular weight markers (in kDa) are shown on the right.