The anti-angiogenic isoforms of VEGF in health and disease

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Abstract
Anti-angiogenic VEGF (vascular endothelial growth factor) isoforms, generated from differential splicing of exon 8, are widely expressed in normal human tissues but down-regulated in cancers and other pathologies associated with abnormal angiogenesis (cancer, diabetic retinopathy, renal vein occlusion, the Denys–Drash syndrome and pre-eclampsia). Administration of recombinant VEGF165b inhibits ocular angiogenesis in mouse models of retinopathy and age-related macular degeneration, and colorectal carcinoma and metastatic melanoma. Splicing factors and their regulatory molecules alter splice site selection, such that cells can switch from the anti-angiogenic VEGFxxxb isoforms to the pro-angiogenic VEGFxxx isoforms, including SRp55 (serine/arginine protein 5S), ASF/SF2 (alternative splicing factor/splicing factor 2) and SRPK (serine arginine domain protein kinase), and inhibitors of these molecules can inhibit angiogenesis in the eye, and splice site selection in cancer cells, opening up the possibility of using splicing factor inhibitors as novel anti-angiogenic therapeutics. Endogenous anti-angiogenic VEGFxxxb isoforms are cytoprotective for endothelial, epithelial and neuronal cells in vitro and in vivo, suggesting both an improved safety profile and an explanation for unpredicted anti-VEGF side effects. In summary, C-terminal distal splicing is a key component of VEGF biology, overlooked by the vast majority of publications in the field, and these findings require a radical revision of our understanding of VEGF biology in normal human physiology.

VEGF (vascular endothelial growth factor)-A alternative splicing

VEGF has become a centre of intense interest due to its essential role in neovascularization (vasculogenesis and angiogenesis) (for reviews see [1,2]) in a variety of physiological and pathological processes, such as the female reproductive cycle [3], wound healing [4], tumours [5], angiogenic eye diseases such as age-related macular degeneration [6] and diabetic retinopathy [7], myocardial ischaemia [8], pre-eclampsia [9] and rheumatoid arthritis [10]. Angiogenesis, the process of new blood vessel formation from pre-existing blood vessels, is important in generating new blood vessels necessary to provide metabolic substrates, such as glucose and oxygen for tissues and transferring substrates for hormone synthesis for endocrine tissues/organisms, as well as efficient removal of waste products and the distribution of hormones synthesized systemically.

The VEGF gene consists of eight exons separated by seven introns and spans approx. 14 kb [11]. The VEGF pre-RNA is differentially spliced to form two families of proteins, each of which contain multiple isoforms of varying amino acid number according to alternative inclusion of exons 6 and 7, which encode heparin-binding domains. The two families are formed by alternative 3′ splice site selection in the terminal exon (exon 8) [12] to give two different C-terminal sequences, and these families are termed VEGFxxx (pro-angiogenic) and VEGFxxxb [13] (anti-angiogenic), where xxx denotes the amino acid number (Figure 1). The most widely studied VEGFxxxb isoform is VEGF165b [14], but VEGF121b [15] and VEGF162b [16] have also been identified at the mRNA and protein levels [13].

The VEGFxxxb family of isoforms is formed by distal splice site selection 66 bp downstream (exon 8b) of the proximal splice site in exon 8 (exon 8a; see Figure 1) [12,14]. This distal splicing event results in an open reading frame of the same number of nucleotides as the proximally spliced variants (pro-angiogenic isoforms); however, the translated amino acid sequence is different (Figure 1), which has implications for the biological properties of the protein. So far, at least 12 isoforms of VEGF-A have been identified: VEGF111, VEGF121, VEGF121b, VEGF143, VEGF148, VEGF162, VEGF164, VEGF165b, VEGF183, VEGF189, VEGF189b and VEGF206 [11,12,16–20]. The present review will focus on the detection, expression and biological functions of VEGFxxxb in human health and disease.

Expression of VEGFxxxb in health and disease in human

VEGF165b mRNA was first isolated in 2002 by RT (reverse transcription)–PCR of tissue of renal cortex [12] using primers that were placed 90 bp 3′ to the exon 8 splice...
Figure 1 | Structure of the VEGF isoforms

(A) mRNAs generated by alternative splicing of the VEGF-A gene. Exon 1 encodes the 5′-UTR and translational start (AUG), and exon 8 the stop codon and 3′-UTR. When pro-angiogenic isoforms are generated the stop codon is 19 nt from the splice site, and the remaining 47 nt of exon 8a are 3′-UTR as is the whole of exon 8b. In the anti-angiogenic isoforms, exon 8a is spliced out and the 3′-UTR is all but the first 19 nt of exon 8b. (B) Sequences of the mRNA and amino acids for the two families of isoforms.

acceptor site, as a shorter PCR product than that predicted from previously identified isoforms. This product was isolated and cloned from seven individuals and then identified in a wide variety of human tissues by RT–PCR. It was subsequently identified in both primary epithelial cells of the glomerulus (podocytes) and in a stable conditionally immortalized podocyte cell line, where it was more highly expressed at the mRNA level in differentiated than in de-differentiated cells [21]. VEGF<sub>165b</sub> mRNA has subsequently been shown to be present in pig [22] and rat [23,24], and dog VEGF<sub>165b</sub> protein has been investigated in [25,26]. Surprisingly, there are no published reports to date of VEGF<sub>165b</sub> mRNA in the mouse. The first indication that this mRNA results in a VEGF protein was provided by Cui et al. [21], where knockdown with siRNA (small interfering RNA) specific for VEGF<sub>165</sub>b resulted in a 66% reduction in VEGF production in differentiated podocytes, as assessed by an ELISA that did not distinguish distal from proximally spliced isoforms. Interestingly, they found very little reduction in de-differentiated podocytes with the same siRNA. In 2004, Woolard et al. [14] showed that a monoclonal antibody raised against the terminal nine-amino-acid sequence of VEGF<sub>165b</sub> detected a protein consistent with VEGF in a variety of tissues. This sequence is unique, with the closest match to the sequence of a protein containing a region of 66% identity (nucleoredoxin, which is not detected by this antibody). Using an antibody that precipitated all VEGF isoforms to capture VEGF<sub>165</sub>b, and the anti-VEGF<sub>xxxb</sub> antibody as a detection antibody, we identified VEGF<sub>xxxb</sub> protein expression in plasma of half of the healthy individuals tested [14], with levels consistent with known circulating levels of VEGF. Subsequently, two more ELISAs have been generated, one using a different, biotinylated VEGF<sub>xxxb</sub>-specific antibody generated by R&D Systems to detect VEGF captured by an anti-pan-VEGF antibody, and a second where the original anti-VEGF<sub>165b</sub> antibody is used to capture and a biotinylated anti-pan-VEGF antibody to detect captured VEGF<sub>xxxb</sub> [27]. A further nine different anti-VEGF<sub>165b</sub> monoclonal antibodies have been generated (by Y. Qiu, R. Mushens, S.J. Harper and D.O. Bates for the 56/1 antibody, and R&D Systems for the others), but are as yet unpublished. All three ELISAs show expression of VEGF<sub>165b</sub> protein in primary human cells, and in human tissue extracts, i.e. retinal pigmented epithelial cells and colonic epithelial cells [21,27], human vitreous fluid [13], lung, bladder, colon, islets, kidney, smooth muscle, circulating plasma, urine and placenta [28]. A direct comparison of two of the ELISAs showed the same expression levels in human colon (129 and 130 pg/ml) [27]. Recently, it was reported that VEGF<sub>165b</sub> is also expressed in normal human breast [29]. Quantification of VEGF<sub>xxxb</sub> has indicated that it may form more than 50% of the total VEGF protein in some of these tissues such as pancreatic islets, colon and vitreous
humour [13,27,30,31], and a significant proportion in kidney, lung and prostate tissue, but a small proportion in placenta (an angiogenic tissue [32]. However, it is difficult to quantify absolutely the amounts of VEGFxxxb and VEGFxxx for the following reasons.

(i) The ELISAs used to detect total VEGF levels use antibodies that recognize the same epitope of the VEGF isoforms. Therefore any inhibitors that may bind may affect the levels, e.g. soluble Flt-1 (Fms-like tyrosine kinase-1) interferes with some of these ELISAs [33,34]. Alternative methods of measurement of VEGF in plasma, for example, give 10–100 times the VEGF levels that ELISAs do [35]. In contrast, the ELISAs used to detect VEGF165b recognize different epitopes on the protein and so are less likely to undergo stoichiometric interference.

(ii) The sensitivity of the ELISAs for the different isoforms (e.g. VEGF121, VEGF121b and VEGF189) and heterodimers of these different isoforms (e.g. VEGF165b–VEGF165 and VEGF165–VEGF121) are not known, but the pan-VEGF ELISA has a lower affinity for VEGF165b than VEGF165, indicating that the difference in affinity needs to be accounted for [27].

(iii) A VEGF165b ELISA that uses an anti-pan-VEGF capture antibody may saturate the antibody with VEGF165 and therefore not bind all the VEGF165b.

(iv) RT–PCR for VEGF isoforms assumes that in the two PCR steps the two products amplify with equal efficiency. However, preliminary results suggest that it is likely that pro-angiogenic isoforms may amplify preferentially, even when mixed at equal amounts or when more VEGF165b cDNA is present.

(v) The secondary structure of the mRNA in the 3′-UTR (3′-untranslated region) is likely to be different. Modelling of the structure using M-fold shows that there is a substantial difference in the melting temperatures of the sequences surrounding the terminal exon splice sites, which would result in differential reverse transcription efficiency and make that efficiency heavily dependent on the conditions of reverse transcription.

(vi) There is no commercially available antibody that specifically detects the VEGFxxx isoforms.

In pathological tissues, VEGF165b has been reported to be expressed, but down-regulated in all cancers investigated so far, including renal-cell (RT–PCR) [12], prostate (RT–PCR and ELISA) [14] and colon carcinomas (RT–PCR, QPCR, ELISA and Western blotting) [27] and malignant melanoma (immunohistochemistry) [36]. It was estimated that over 90% of normal colonic tissue was VEGFxxx, but in colorectal carcinoma samples, the balance switched to favour expression of VEGFxxx, the pro-angiogenic isoforms [27]. In addition to its down-regulation in cancer, VEGFxxx isoform expression is also altered in other diseases characterized by excess neoangiogenesis, including proliferative eye disease [13] and DDS (Denys–Drash syndrome) [37]. In the eye, VEGFxxx is down-regulated in the vitreous fluid of patients with proliferative diabetic retinopathy; in normal vitreous, 65% of VEGF protein is VEGFxxx; however, in diabetes this decreases to 16% [13]. A dysregulation of VEGFxxx expression is observed in the glomeruli of humans with DDS, a disease in which mutations in WT1 (Wilms’ tumour-1) gene affect the transcriptional regulation of VEGF expression, where VEGF165b was down-regulated [37]. Interestingly, WT1 is also expressed in the urogenital bud, where VEGF165b is expressed during development, and inhibition of VEGF165b results in abnormal ovogenesis due to increased angiogenesis [24]. DDS patients also have urogenital disorders, but it is not known whether this is due to altered VEGF splicing.

**VEGF165b in reproductive health**

Angiogenesis is a key process in many aspects of reproduction from development of the reproductive system, where angiogenesis plays a pivotal role in formation of healthy gonads [38], to follicle progression and ovulation [39], corpus luteal development [40], endometrial proliferation [41], oocyte implantation and placentation [42], and of course organogenesis and development [43], but also in secondary reproductive features, such as mammary gland development [44], all of which require blood vessel remodelling and growth. VEGF165b is expressed in many of these tissues, and its expression levels are tightly controlled; for instance, in the ovary it is expressed only in the theca externa, not in the theca interna [29]. To investigate the role of VEGF165b in reproductive health, a transgenic mouse line overexpressing VEGF165b under the control of the mouse mammary tumour virus promoter has been developed. The female mice express VEGF165b during mammary development, in the ovary, adrenal gland, lungs and heart, whereas expression is limited to the salivary gland in males. Overexpression of VEGF165b in the mammary gland during pregnancy results in an inhibition of blood vessel development, impaired alveolar coverage of the fat pad and a significant reduction in milk production [29]. We recently reported that overexpression of VEGF165b in mouse ovary results in reduced litter size, defects in follicle development and lack of cumulus oocyte complex formation [44a,44b], suggesting that the balance between VEGFxxx and VEGFxxx may also be involved in fertility control. Moreover, inhibition of endogenous VEGF165b with a neutralizing antibody in the developing ovary results in follicle progression similar to stimulation with angiogenic isoforms, indicating an endogenous role for anti-angiogenic isoforms in the regulation of follicle development [24]. Additionally, inhibition of VEGFxxx isoforms in developing testes stimulated vascular development and perturbed testicular cord formation, in a similar manner to addition of excess VEGF165 [45]. Overexpression of VEGFxxx in areas where angiogenesis is required therefore appears to be detrimental; however, in normal pregnancy, circulating VEGF165b levels rise rapidly after fertilization, with most patients having levels more than four times the non-pregnant levels within the first few weeks of pregnancy [46].

VEGFxxx expression is at a low level in placenta, but in patients with pre-eclampsia, the VEGFxxx levels are further
down-regulated even though total VEGF levels are increased [32]. Moreover, circulating VEGF<sub>xxx</sub>b appears to be normal in pre-eclamptic patients. Expression of VEGF<sub>xxx</sub>b in placenta has been reported to be altered in Type 1 diabetic patients, but not in gestational diabetes and also by infusion of insulin [47]. VEGF<sub>165</sub>b was also reported to be a survival factor for first trimester trophoblasts, suggesting that the lack of VEGF<sub>165</sub>b may prevent adequate trophoblast invasion into the endometrium, promoting pre-eclampsia development. It therefore appears that VEGF<sub>xxx</sub>b may play a significant role in reproductive biology from gonadal development, through to follicular maturation, implantation and placentation.

**VEGF<sub>165</sub>b in renal health**

VEGF is known to be a critical regulator of renal glomerular function and filtration. However, VEGF<sub>165</sub>b appears to be significantly expressed in the glomeruli of human kidneys [28]. Overexpression of VEGF<sub>165</sub>b in mouse glomeruli leads to proteinuria, glomerular dysfunction and renal failure [48]. In contrast, overexpression of VEGF<sub>165</sub>b does not result in glomerulopathy, and the mice appear healthy and normal. Examination of the permeability characteristics of podocyte-specific VEGF<sub>165</sub>b-expressing glomeruli, however, shows a decreased glomerular permeability that is gene dose-dependent [49]. Moreover, recent studies have reported that VEGF<sub>165</sub>b overexpression can relieve the increased water permeability and proteinuria caused by diabetic nephropathy in STZ (streptozotocin-induced diabetic) mice [50], and an incubation of wild-type mouse glomeruli with recombinant human VEGF<sub>165</sub>b decreases permeability to water [51]. Recombinant VEGF<sub>165</sub>b has also been shown to inhibit glomerular endothelial permeability induced by VEGF<sub>165</sub>b and reduce permeability of glomerular endothelial cells by itself [30]. Electron microscopic examination of glomeruli of podocyte-specific VEGF<sub>165</sub>b-overexpressing mice indicates that alterations in glomerular endothelial fenestrae are apparent, possibly providing an ultrastructural explanation for the decreased permeability (Y. Qiu, J. Ferguson, S. Oltean, C.R. Neal, A. Kaura, E. Wood, L. Sage, S. Lanati, D. Nowak, A.H.J. Salmon, D.O. Bates and S.J. Harper, unpublished work).

**Role of VEGF<sub>165</sub>b in pathology**

**Tumours**

*In vivo* studies have shown that overexpression of VEGF<sub>165</sub>b inhibits tumour growth in colon, prostate and renal cell carcinoma and on the growth of Ewing’s sarcoma cells and metastatic melanoma in xenografted mouse models [14,27,52]. These studies have used overexpression of human VEGF<sub>165</sub>b cDNA in human tumour cells and selection of clones to produce a large excess of VEGF<sub>165</sub>b compared with VEGF<sub>165</sub>b. In human tumours, it appears that, in most of the published studies to date, there is an up-regulation for the VEGF<sub>xxx</sub>b isoforms such that the VEGF<sub>xxx</sub>b levels become less than 50% in most cases, although some human tumours still have an excess of VEGF<sub>165</sub>b [27,36]. Of particular interest is that the balance of anti-angiogenic to pro-angiogenic isoforms seems to affect the sensitivity of tumours to bevacizumab, an anti-VEGF-A antibody [27]. It was demonstrated that VEGF<sub>165</sub>b bound to bevacizumab with similar affinity as VEGF<sub>165</sub>b and inhibits the bevacizumab effect on tumour growth [27]. This is presumably because, in the presence of excess VEGF<sub>165</sub>b, the antibody binds predominantly VEGF<sub>xxx</sub>b so that the concentration of antibody available to bind to VEGF<sub>xxx</sub>b is effectively reduced. Thus, although tumours that have an excess of VEGF<sub>xxx</sub>b grow more slowly than VEGF<sub>xxx</sub>b dominant tumours, they may be resistant to anti-VEGF therapies.

**Potential therapeutic role of VEGF<sub>165</sub>b in eye disease**

Proliferative diabetic retinopathy and wet age-related macular degeneration are both eye diseases associated with neovascularization [52a,52b]. Injection of VEGF<sub>165</sub>b can significantly reduce pre-retinal neovascularization without inhibition of physiological intraretinal angiogenesis in the oxygen-induced retinopathy mouse model of ocular neovascularization [31]. The lack of inhibition of intraretinal revascularization is of particular interest and was shown to be a result of a cytoprotective effect of VEGF<sub>165</sub>b on endothelial cells (A. Magnussen, E.S. Rennel, J. Hua, H.S. Bevan, N. Beazley-Long, C. Lehrling, J. Floege, Harper, S.J. Harper, H.T. Agostini, D.O. Bates and A.J. Churchill, unpublished work). Further study showed that VEGF<sub>165</sub>b was also cytoprotective for epithelial cells of the retina (A. Magnussen, E.S. Rennel, J. Hua, H.S. Bevan, N. Beazley-Long, C. Lehrling, J. Floege, Harper, S.J. Harper, H.T. Agostini, D.O. Bates and A.J. Churchill, unpublished work), indicating a potential role as a therapeutic agent in diabetic retinopathy, where the angiogenesis is often a result of local endothelial cell loss due to the effects of the diabetic milieu on endothelial cell survival. Treatment with agents that inhibit angiogenesis but protect existing endothelial cells from toxicity may be a preferential route to using anti-VEGF agents that cause further cell loss. VEGF<sub>165</sub>b is also shown to be a potent anti-angiogenic agent both through intraocular injection and systemic administration in a mouse model of choroidal angiogenesis (J.J. Hua, C. Spee, S. Kase, E.S. Rennel, A. Magnussen, Y. Qiu, A.H. Varey, S. Dhayade, D.G. Nowak, M. Hagiwara, A.J. Churchill, S.J. Harper, D.O. Bates and D.R. Hinton, unpublished work). Taken together, these results suggest that increasing the ratio of anti- to pro-angiogenic isoforms may be therapeutically effective in diabetic retinopathy and AMD (age-related macular degeneration).

**Signalling pathways**

The mechanisms through which VEGF<sub>165</sub>b signals are still being elucidated. There are a number of amino acid residue changes predicted to significantly alter the configuration of the VEGF<sub>165</sub>b protein compared with its counterpart, VEGF<sub>165</sub>b. The charged C-terminal arginine residues
in VEGF165 are replaced with neutral lysine-aspartic acid residue in VEGF165b [25] and the replacement of a proline residue in VEGF165 with an arginine residue in VEGF165b occurs. In culture, VEGF165b stimulates VEGFR2 (VEGF receptor 2), p42/p44MAPK (p42/p44 mitogen-activated protein kinase) and Akt phosphorylation in human microvascular endothelial cells, but not in umbilical-vein endothelial cells [14]. Results from Kurt Ballmer-Hofer’s laboratory indicate that VEGF165b-mediated signalling through phosphorylation of MAPK is much more transient than VEGF165 [25], and the inhibition of angiogenesis required the C-terminal sequence of VEGF165b, because a mutant form of VEGF lacking the last six amino acids did not inhibit angiogenesis in mouse and chick models. Results from Lena Claesson-Welsh’s laboratory indicate that VEGF165b is a weak agonist of VEGFR2 through phosphorylation not of Tyr1175, Tyr1054 or Tyr1215 in contrast with VEGF165 activation, but of Tyr951 and/or other unidentified tyrosine residues [53]. VEGF165b has been shown to signal through VEGFR1 to transiently increase permeability in frog mesentery blood vessels [54]. However, there is no evidence, from in vivo experiments, on how VEGF165b signals in blood vessels for both angiogenesis and permeability.

**Regulation**

VEGF165b expression is up-regulated in retinal pigmented epithelial cells and podocytes by growth factors such as IGF-1 (insulin-like growth factor 1) and TGFβ (transforming growth factor β) (Figure 2). The mechanism through which TGFβ up-regulates VEGF165b appears to be through activation of p38MAPK, and subsequent downstream activation of the Clk/sty kinases. Inhibition of Clk1 and/or Clk4 results in phosphorylation of the mRNAbinding protein SRp55 (serine/arginine domain protein 55), a key regulatory splice factor, which binds to VEGF pre-RNA in the exon 8b region [55]. This binding appears to favour distal splice site selection and hence VEGF165b expression. In contrast, IGF-mediated VEGF165b down-regulation appears to occur through activation of PKC (protein kinase C) and hence phosphorylation of the RNA-binding protein ASF/SF2 (alternative splicing factor/splicing factor 2) through activation of the SR protein kinase SRPK1 (serine/arginine domain protein kinase 1) [55a]. ASF/SF2 is a splicing factor that acts as a shuttling protein between the cytoplasm and the nucleus [56], and IGF activation results in increased nuclear localization of ASF/SF2. This factor is also able to bind the VEGF pre-RNA, but in the region surrounding the proximal splice site, and binding appears to prevent VEGF165b expression (E.M. Amin, M. Cheung, L. Ni, S.J. Harper, M.A. Saleem, M. Hagiwara, V.A. Schumacher, D.O. Bates and M.R. Ladomery, unpublished work). ASF/SF2 has been identified as an oncogene [57], and this splicing switch may therefore be a key regulator of blood vessel growth in cancer. These findings on ASF/SF2 regulation have recently helped to uncover the mechanism of the down-regulation of VEGF165b splicing in DDS. The WT1 mutant protein that is found in DDS appears to lack suppressor activity of the splice factor kinase SRPK1. Whereas WT1 in wild-type podocytes can bind the SRPK1 promoter at the transcription start site and inhibit its expression, the mutated WT1 from podocytes isolated from a patient with DDS does not bind this site, and SRPK1 expression is 15-fold greater than in wild-type podocytes. Restoration of wild-type WT1 reduces SRPK1 expression and restores binding to the SRPK1 site. In DDS podocytes, excess SRPK1 results in hyperphosphorylation of the splice factor ASF/SF2, which, as in IGF activation, results in its nuclear localization and hence activity. In the absence of SRPK1-mediated phosphorylation, distal splice site selection occurs and VEGF165b is generated, confirmed by knockdown and pharmacological inhibition of SRPK1 [55a], and both result in increased VEGF165b expression in the DDS podocytes. Thus, increasing the ratio of anti-to-pro-angiogenic isoforms by modifying splicing may be therapeutically effective for Wilms’ tumour and DDS.

**Function of other isoforms**

So far, VEGF121b, VEGF165b and VEGF189b have been identified in human tissues. The only other isoform that has been investigated experimentally for its activity is VEGF121b, which has also been found to be anti-angiogenic, both in in vitro migration assay and in vivo tumour development and oxygen-induced neovascularization [15], indicating that the entire family is likely to be anti-angiogenic.

Taken together, the discovery of VEGF165b reveals two families of VEGF, with VEGF165b being angiogenic and permeability-inducing, while VEGF165b is anti-angiogenic and its role in permeability regulation is complex. VEGF165b is endogenously expressed in a variety of tissues. Its expression is down-regulated in the tumours studied so far and neovascularization-associated eye diseases. VEGF165b is also involved in physiological angiogenesis, such as mammary development during pregnancy and lactation, and fertility control. Since there is a switch from anti- to pro-angiogenic isoforms of VEGF in colonic tumours and proliferative diabetic retinopathy, it is believed that
regulation of the ratio of pro- to anti-angiogenic isoforms of VEGF by altering splicing is potentially therapeutic for tumours and other angiogenesis-associated diseases.

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