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Recombinant human $VEGF_{165}b$ protein is an effective anti-cancer agent in mice

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ABSTRACT

Tumour growth is dependent on angiogenesis, the key mediator of which is vascular endothelial growth factor-A (VEGF-A). VEGF-A exists as two families of alternatively spliced isoforms – pro-angiogenic $VEGF_{xxx}$ generated by proximal, and anti-angiogenic $VEGF_{xxx}b$ by distal splicing of exon 8. $VEGF_{165}b$ inhibits angiogenesis and is downregulated in tumours. Here, we show for the first time that administration of recombinant human $VEGF_{165}b$ inhibits colon carcinoma tumour growth and tumour vessel density in nude mice, with a terminal plasma half-life of 6.2 h and directly inhibited angiogenic parameters (endothelial sprouting, orientation and structure formation) *in vitro*. Intravenous injection of ^{125}I-$VEGF_{165}b$ demonstrated significant tumour uptake lasting at least 24 h. No adverse effects on liver function or haemodynamics were observed. These results indicate that injected $VEGF_{165}b$ was taken up into the tumour as an effective anti-angiogenic cancer therapy, and provide proof of principle for the development of this anti-angiogenic growth factor splice isoform as a novel cancer therapy.

1. Introduction

Vascular endothelial growth factor-A (VEGF) is the principal angiogenic promoter in most, if not all, cancers. VEGF is upregulated by hypoxia,[1] and by over-expression of oncogenes in tumours,[2] and stimulates the migration of endothelial cells, sprouting of blood vessels and generation of new vessels from existing vasculature in tumours (reviewed in[3]), resulting in sustained blood flow, oxygen supply and waste removal to the growing tumour. Anti-VEGF therapy has been

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hailed as the fourth line of cancer treatment alongside surgery, chemotherapy and radiotherapy, and antibodies to VEGF have shown clinical benefit in colorectal cancer, renal carcinoma, non-small cell lung cancer, breast cancer[4,5] and are in further phase III clinical trials in other cancers.

VEGF is generated as multiple isoforms by alternative splicing of mRNA from 8 exons. Alternate splicing of exons 6 and 7 results in proteins with differing heparin binding affinity, and numbers of amino acids, such as $VEGF_{165}$, $VEGF_{121}$ and $VEGF_{189}$, termed according to the number of amino acids encoded in the final secreted protein. Alternate splicing into the terminal exon, exon 8, gives rise to two families of isoforms, the $VEGF_{xxx}b$ and $VEGF_{xxx}$ isoforms. The $VEGF_{xxx}b$ family of isoforms, first identified in 2002,[6] are generated by the use of a more distal 3′ splice acceptor site, and result in mRNA species that code for proteins of the same length as the $VEGF_{xxx}$ isoforms, but with different C-terminal six amino acids. Whereas the $VEGF_{xxx}$ isoforms (e.g. $VEGF_{165}$ and $VEGF_{121}$) are pro-angiogenic and are upregulated in tumours, the $VEGF_{xxx}b$ isoforms (e.g. $VEGF_{165}b$ and $VEGF_{121}b$) are anti-angiogenic and downregulated in tumours.[6,7] This anti-angiogenic activity is generated by receptor binding,[7,8] but only weak receptor activation, and inhibition of downstream VEGFR2 signalling.[7] Its activity has led to the hypothesis that $VEGF_{165}b$ may be a useful therapeutic tool in angiogenic conditions such as in tumour growth, or in neovascularisation associated with retinopathy[9] such as in diabetes.

To determine whether $VEGF_{165}b$ has an appropriate pharmacokinetic profile for systemic anti-angiogenic therapy, we have investigated the clearance rates, toxicity, tumour uptake and circulating effects on blood pressure of $VEGF_{165}b$ injection in mice, and whether recombinant human $VEGF_{165}b$ exerts inhibitory effects on tumour growth in tumour-bearing mice.

2. Materials and methods

2.1. Production of recombinant protein

Recombinant human $VEGF_{165}b$ ($rhVEGF_{165}b$), produced in Chinese Hamster Ovary Cells with correct glycosylation, dimerisation and receptor binding, was generated by Cancer Research Technologies, London, United Kingdom or PPS, Israel. Protein was analysed by MALDI-TOF mass spectrometry (Voyager DE-STR, Applied Biosystems, Foster City, CA). Endotoxins were removed by phase separation using Triton X-114 followed by endotoxin detection using Limulus Amebocyte Lysate performed by Cambrex (Cambrex Corporation). All the proteins were considered endotoxin free (endotoxin levels below 5 EU/ml of concentrated stock). Recombinant canine $VEGF_{165}$ ($rcVEGF_{165}$) or $rcVEGF_{165}b$ was produced and assayed for retained activity as previously described.[8]

2.2. Functional effects of recombinant $VEGF_{165}b$

Human umbilical vein endothelial cells (HUVEC) were extracted from umbilical cords from caesarean sections (St. Michael's Hospital, Bristol, UK). HUVECs were maintained in M200 supplemented with low serum growth factor supplements (Cascade Biologics, Portland, OR) in flasks coated with extracellular matrix proteins. Cells were used at passages 3–5. Sub-confluent HUVECs were serum starved for 7 h in M200 medium without supplement, and 100,000 serum starved cells were plated into collagen-coated 8 μm inserts (Millipore, Billerica, MA). Inserts were placed in 24-well plates with 500 μl of chemoattractant in M200 medium with 0.1% v/v FCS and 0.2% w/v BSA and incubated overnight at 37 °C to allow for migration. After incubation, inserts were washed, non-migrated cells were removed and migrating cells were stained in Mayer's haematoxylin. Migrating cells were counted (10 fields per insert) and expressed either as a relative change to basal migration (media without chemoattractant) or% migration and plotted as average ± s.e.m. Experiments were performed in triplicate.

To determine the concentration at which 50% of the migratory response was inhibited, (IC_{50}), increasing amounts of $VEGF_{165}b$ were added (1–100 ng/ml) with or without the optimal concentration of 40 ng/ml of $VEGF_{165}$. For stability tests, recombinant $VEGF_{165}b$ was incubated under sterile conditions at 37 °C for 1 or 2 weeks, whereafter the retained inhibitory migratory response was assayed.

2.3. Angiogenesis assay

Extracellular matrix gel solution was prepared according to manufacturer's instructions (Chemicon International, Temecula CA). Thirty microlitre gel solution was transferred to each well of pre-cooled 8-well culture slides (Falcon, BD, Oxford, Oxfordshire, UK) and incubated at 37 °C to solidify. $VEGF_{165}$, $VEGF_{165}b$ and the combination were placed on one side of the gel to 1 nM final concentration. Human Microvascular Endothelial Cells, HMVEC, were serum starved for 3 h with EBM-2 (Clonetechs, Lonza), and 10,000 cells were seeded onto the gel in 100 μl EBM-2 basal media and incubated at 37 °C for 6 h. Gels were fixed in 4% w/v para-formaldehyde/PBS pH 7.4 for 5 min and washed twice with PBS. F-actin fibres were stained with Alexa 488 phalloidin for 1 h (Molecular Probes, dilution 1:200 in PBS/0.5% v/v Triton) and 10 min with Hoechst 33342 (5 μg/ml PBS/0.5% v/v Triton). Gels were washed twice with PBS/0.5% v/v Triton, twice with PBS and mounted with vectashield (Vector Laboratories Burlingame, CA). Images were taken on a Leica DM RB fluorescence microscope for structure analysis counting branch points, sprouts and closed polygons over the entire area of the well.

2.4. Injections of recombinant human $VEGF_{165}b$ into LS174t tumour-bearing mice

LS174t colon carcinoma cells (2×10^6) in 200 μl sterile PBS were injected into the nape of the neck of nude mice and injected with $rhVEGF_{165}b$ subcutaneously every day or twice weekly (in 200 μl 0.9% w/v NaCl), starting 24 h after the injection of tumour cells (prophylactic) or when tumours reached a diameter of 4–5 mm (therapeutic). Bi-weekly intraperitoneal injection was carried out in mice 4 d after the injection of 2×10^6 LS174t tumour cells as above. Xenotransplanted tumours were measured by calliper every day, and tumour volume was calculated according to (length × width × [length + width]/2). Mice were culled by cervical dislocation and organs and tumours were removed. Tumour vessel