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Regulation of alternative VEGF-A mRNA splicing is a therapeutic target for analgesia☆

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ABSTRACT

Vascular endothelial growth factor-A (VEGF-A) is best known as a key regulator of the formation of new blood vessels. Neutralization of VEGF-A with anti-VEGF therapy e.g. bevacizumab, can be painful, and this is hypothesized to result from a loss of VEGF-A-mediated neuroprotection. The multiple *vegf-a* gene products consist of two alternatively spliced families, typified by VEGF-A_{165}a and VEGF-A_{165}b (both contain 165 amino acids), both of which are neuroprotective. Under pathological conditions, such as in inflammation and cancer, the pro-angiogenic VEGF-A_{165}a is upregulated and predominates over the VEGF-A_{165}b isoform.

We show here that in rats and mice VEGF-A_{165}a and VEGF-A_{165}b have opposing effects on pain, and that blocking the proximal splicing event – leading to the preferential expression of VEGF-A_{165}b over VEGF$_{165}$a – prevents pain in vivo. VEGF-A_{165}a sensitizes peripheral nociceptive neurons through actions on VEGFR2 and a TRPV1-dependent mechanism, thus enhancing nociceptive signaling. VEGF-A_{165}b blocks the effect of VEGF-A_{165}a.

After nerve injury, the endogenous balance of VEGF-A isoforms switches to greater expression of VEGF-A_{xxx}a compared to VEGF-A_{xxx}b, through an SRPK1-dependent pre-mRNA splicing mechanism. Pharmacological inhibition of SRPK1 after traumatic nerve injury selectively reduced VEGF-A_{xxx}a expression and reversed associated neuropathic pain. Exogenous VEGF-A_{165}b also ameliorated neuropathic pain.

We conclude that the relative levels of alternatively spliced VEGF-A isoforms are critical for pain modulation under both normal conditions and in sensory neuropathy. Altering VEGF-A_{xxx}a/VEGF-A_{xxx}b balance by targeting alternative RNA splicing may be a new analgesic strategy.

Introduction

Neutralization of VEGF-A with anti-VEGF-A therapies, such as bevacizumab or VEGF-A receptor inhibitors (e.g., vandetanib) can result in pain, when given alone (Burger et al., 2007; Cohen and Hochster, 2007) or in combination with chemotherapies (Cohen et al., 2007; Garcia et al., 2008; Langenberg et al., 2011; Miller et al., 2007). The clinical findings that VEGF-A contributes to pain are supported by observations that inhibition of VEGF receptor 2 (VEGFR2) exacerbates peripheral neuronal damage, which is often associated with pain (Beazley-Long et al., 2013; Verheyen et al., 2012), and enhances pain behaviors in normal, nerve-injured and diabetic animals (Hulse et al., 2010a; Verheyen et al., 2012).

Abbreviations: VEGF-A, vascular endothelial growth factor-A; SRPK1, serine arginine protein kinase 1; SRSF1, serine arginine splice factor 1; VEGFR2, vascular endothelial growth factor receptor 2; IB4, isolectin B4; TRPV1, transient receptor potential vanilloid 1; CV, conduction velocity; PSNI, partial saphenous nerve ligation injury; DRG, dorsal root ganglia.

☆ Conflict of interest: LFD, DOB, JH, SJH are co-inventors on patents protecting VEGF-A_{165}b and alternative RNA splicing control for therapeutic application in a number of different conditions. LFD, DOB, SJH are founder equity holders in, and Dob and SJH are directors of Exonate Ltd, a new company with a focus on the development of alternative RNA splicing control for therapeutic application in a number of different conditions, including analgesia.

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The *vegf-a* gene encodes two families of isoforms typified by VEGF-A_{165} a and VEGF-A_{165}b (Harper and Bates, 2008). Both families have sister isoforms of the same length so they are referred collectively as VEGF-A_{xxx}a and VEGF-A_{xxx}b where xxx represents the number of amino acids. The isoform families differ only in their six C terminal amino acids (Harper and Bates, 2008), and they are both capable of binding to VEGFR2 with similar affinities, but the functional results of receptor activation are multivariate (Table 1) (Ballmer-Hofer et al., 2011). Control of relative isoform expression occurs by alternative pre-mRNA splicing of either proximal or distal splice sites in exon 8 (Fig. 1).

VEGF-A_{xxx}a is the principal target of anti-VEGF and VEGFR therapies as these isoforms are upregulated and predominate in many pathologies. However, VEGF-A_{xxx}b forms a significant proportion of total (pan-)VEGF-A protein in many normal tissues (Harper and Bates, 2008) so the therapeutic effects of VEGF-A sequestration with many current antibody therapies, or VEGFR2 inhibition are a net result of simultaneous blockade of the actions of *both* families. The impact of the neutralization of the VEGF-A_{xxx}b family on treatment outcomes has only recently been exemplified, in terms of its ability to predict colorectal cancer patients that do not respond to bevacizumab (Bates et al., 2012).

rhVEGF-A_{165}a exacerbated spinal cord contusion-associated pain and damage (Benton and Whittemore, 2003; Herrera et al., 2009; Nesic et al., 2010; Sundberg et al., 2011), and referred mechanical abdominal pain (Malykhina et al., 2012), but local VEGF-A delivery (presumed VEGF-A_{xxx}a) partially reversed diabetic neuropathic mechanical hyperalgesia (Verheyen et al., 2013). Neutralization of all endogenous VEGF-A isoforms or VEGF receptor 2 inhibition increased pain sensitivity in chemotherapy-induced neuropathy (Verheyen et al., 2012), but conversely reversed neuropathic (Lin et al., 2010), and acute inflammatory hyperalgesia (Grosios et al., 2004).

These conflicting observations might be explained by different actions of the distinct isoforms, which have not been studied independently, and their differing actions on VEGFR2 (Ballmer-Hofer et al., 2011). We therefore tested the hypothesis that the alternatively spliced VEGF-A isoform families have different effects on pain. We investigated: a) the effects of specific VEGF-A isoforms on pain/nociception; b) the neuronal mechanisms through which effects on pain might occur; c) whether using control of alternative RNA splicing of VEGF-A could modulate nociception/pain, and d) whether either VEGF-A proteins or alternative splicing control may be potential novel analgesic targets.

Table 1
Overview of the C-terminal sequences, binding domains and interactions with VEGFR2 of the different VEGF-A splice variant isoforms.

	C terminal sequence	Binding domains present	Consequences of receptor binding
VEGF-A_{165}a	CDKPRR	VEGFR1, VEGFR2, NP-1	Full agonist. Binds and stabilizes VEGFR + NP-1 interaction. Complete phosphorylation at Y1175. PIP2 hydrolysis, PKC activation.
VEGF-A_{165}b	SLTRKD	VEGFR1, VEGFR2	Partial VEGFR2 agonist/competitive inhibitor of VEGF-A_{165}a binding. Very weak NP-1 interaction. Weak/incomplete phosphorylation at Y1175 No PIP2 hydrolysis, or PKC activation. Receptor internalization and degradation. (Ballmer-Hofer et al., 2011; Kisko et al., 2011)
VEGF-A_{159}	–	VEGFR1, VEGFR2	Binds VEGFR, no activation. Very weak NP-1 interaction.
VEGF-A_{121}a	CDKPRR	VEGFR1, VEGFR2	Binds VEGFR. Very weak NP-1 interaction. Complete phosphorylation at Y1175

Materials and methods

All procedures using animals were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and with University of Bristol and King's College London Ethical Review Groups approval. Human embryonic and adult tissues were obtained under ethical approval by University of Leiden and adult human DRG under ethical approval by Southmead Hospital Local Research Ethics Committee.

Antibody and pharmacological inhibitors

The following pharmacological interventions were used: pan-VEGF-A neutralization with mouse anti-VEGF-A antibody (Liang et al., 2006), specific VEGF-A_{165}b neutralization using systemic treatment with anti-VEGF-A_{165}b antibody (clone 56/1, (Woolard et al., 2004)) systemic and local VEGF receptor inhibition with selective (PTK787; (Wood et al., 2000)) and/or specific (ZM323881; (Whittles et al., 2002)). VEGFR2 tyrosine kinase inhibitors; systemic or local administration of VEGF-A_{165}a and/or VEGF-A_{165}b; systemic antagonism of TRPV1 with SB366791 (Varga et al., 2005); inhibition of serine-rich protein kinases with SRPIN340 (Fukuhara et al., 2006), and appropriate vehicles.

Measurement of mechanical and thermal nociceptive behaviors

A total of 64 adult male mice (C57Bl6, 25–30 g), 6 TRPV1 congenic knockouts and 6 wild-type strain-matched controls and 24 adult male Wistar rats were used to assess nociceptive behavior. TRPV1 homozygous knockout mice breeding pairs were generated and bred as described at King's College London, (Caterina et al., 2000; Fernandes et al., 2011, 2013) where breeding colonies were regularly backcrossed according to Jackson Laboratory guidelines to avoid sub-strain selection (Lambert, n.d.).

All animals were habituated to testing environments and handling prior to testing, and were allowed to habituate to the environment for at least 15 min at each test session. Nociceptive testing, as previously described (Hulse et al., 2008), consisted of measurement of mechanical allodynia by determination of von Frey hair mechanical withdrawal threshold and thermal hyperalgesia using the Hargreaves test (Hargreaves et al., 1988). Behavioral testing groups were randomized, and all operators were blinded to the drug and surgical treatment (nerve injury/sham) in each animal in all experiments.

Von Frey hair mechanical thresholds — mechanical allodynia

Animals were habituated to chambers with mesh floors. The plantar surface of each foot was stimulated with von Frey hairs (Linton, UK) of increasing gram force breaking points, over a range of 0.07–2 g (mice), or 1–100 g (rats) (Hulse et al., 2008). Each von Frey hair tested was applied a total of 5 times to each hind paw and the number of times an animal removed the paw from each stimulus was counted. The proportion of times that the animal withdrew from each stimulus was plotted against the breaking force, and the withdrawal threshold determined from the resultant stimulus response curve (the gram force at which paw removal occurred at 50% of the stimulations).

Hargreaves test for thermal hyperalgesia

Thermal hyperalgesia was measured using a radiant heat source directed against the plantar surface of the hind paws, through the Perspex floor of the testing chamber (Hargreaves et al., 1988), and the latency to withdrawal was measured. The stimulus intensity was determined at the beginning of each experimental series, to give a control withdrawal latency of ~10 s, and this intensity was subsequently used for each subsequent testing session for that experimental group. A maximum latency duration of 30 s was used to prevent tissue damage/sensitization to