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THERAPEUTIC POTENTIAL OF VEGF AND VEGF-DERIVED PEPTIDE IN PERIPHERAL NEUROPATHIES

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Abstract—Besides its prominent role in angiogenesis, the vascular endothelial growth factor (VEGF) also exerts important protective effects on neurons. In particular, mice expressing reduced levels of VEGF suffer from late-onset motor neuron degeneration, whereas VEGF delivery significantly delays motor neuron death in ALS mouse models, at least partly through neuroprotective effects. Additionally, VEGF protects dorsal root ganglion (DRG) neurons against paclitaxel-induced neurotoxicity. Here, we demonstrate that VEGF also protects DRG neurons against hyperglycemia-induced neuronal stress as a model of diabetes-induced peripheral neuropathy. Specifically, VEGF decreased expression of the stress-related gene activating transcription factor 3 (ATF3) in DRG neurons isolated from streptozotocin-induced diabetic mice (ex vivo) and in isolated DRG neurons exposed to high glucose concentrations (*in vitro*). *In vivo*, local VEGF application also protected against paclitaxel- and diabetes-induced neuropathies without causing side effects. A small synthetic VEGF mimicking pentadecapeptide (QK) exerted similar effects on DRG cultures: the peptide reduced ATF3 expression *in vitro* and ex vivo in paclitaxel- and hyperglycemia-induced models of neuropathy to a similar extent as the full-length recombinant VEGF protein. By using transgenic mice selectively overexpressing the VEGF receptor 2 in postnatal neurons, these neuroprotective effects were shown to be mediated through VEGF receptor 2. Overall, these results underscore the potential of VEGF and VEGF-derived peptides for the treatment of peripheral neuropathies.

Key words: peptide, peripheral neuropathy, VEGF.

INTRODUCTION

The vascular endothelial growth factor (VEGF) was originally discovered as a growth factor capable of increasing vascular permeability and endothelial cell proliferation. After binding to its VEGF receptor 1 (Flt1), VEGF receptor 2 (Flk1) and neuropilin (Nrp-1 and Nrp-2), VEGF promotes endothelial cell proliferation, migration and survival (Leung et al., 1989; Ferrara et al., 2003). VEGF is also a potent endothelial cell survival factor during physiological and tumor angiogenesis (Benjamin and Keshet, 1997; Gerber et al., 1998). Furthermore VEGF is known as a vascular permeability factor, playing a significant role in inflammation and other pathological conditions (Senger et al., 1983; Dvorak et al., 1995). Evidence has been accumulating that VEGF, in addition to well-known effects on angiogenesis, exhibits more pluripotent activities, particularly in the nervous system.

Neurotrophic effects of VEGF were first described by Sondell et al., who reported that VEGF promotes axonal outgrowth of primary dorsal root ganglia (DRG) (Sondell et al., 1999). Whether VEGF also exerts such direct neuroprotective effects on sensory neurons *in vivo* is not known. Although VEGF therapy has already shown therapeutic potential in peripheral neuropathies caused by diabetes or chemotherapy, these effects were mainly attributed to its vessel- and perfusion-promoting effects, rather than to its direct neuroprotective properties (Schratzberger et al., 2001; Kirchmair et al., 2007). The most convincing evidence for *in vivo* neuroprotective effects of VEGF originates from studies in knock-in mice, in which a subtle deletion of the hypoxia-response element reduced expression levels of VEGF and caused adult-onset progressive degeneration of motor neurons, reminiscent of amyotrophic lateral sclerosis (ALS). Moreover, overexpression of VEGF or Flk1 in neurons, substantially delayed disease symptoms in models for ALS (Oosthuyse et al., 2001; Storkebaum et al., 2005).

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Abbreviations: ALS, amyotrophic lateral sclerosis; ANOVA, analysis of variance; ATF3, activating transcription factor 3; AUC, area under the curve; DN, dominant negative; DRG, dorsal root ganglia; GDNF, glial cell-derived neurotrophic factor; PBS, phosphate-buffered saline; SEM, standard error of the mean; STZ, streptozotocin; VEGF, vascular endothelial growth factor; WT, wild-type; YFP, yellow-fluorescent protein.

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Meanwhile, VEGF was also found to be implicated in other neurological diseases, including recovery from stroke (Wang et al., 2009), Alzheimer's disease (Del Bo et al., 2009) and Parkinson's disease (Yasuhara et al., 2005). In most of these studies, however, it was unclear whether VEGF exerted this neuroprotection through direct neurotrophic or perfusion-promoting effects.

Recently, a VEGF-derived peptide was generated with similar biological properties and VEGF receptor-binding capacities as the full-length VEGF protein (D'Andrea et al., 2005; Ziaco et al., 2012). In several later studies, this peptide has been functionally and pharmacologically characterized as pro-angiogenic with positive effects on wound healing (Santulli et al., 2009; Finetti et al., 2012). Whether this VEGF-derived peptide also exerts neuroprotective activities has not been studied before.

In light of the emerging recognition of VEGF as a dual 'neuro-vascular' factor, we investigated whether VEGF, in addition to its well-established vascular effects, exerts direct neuroprotective effects on sensory neurons during diabetic- and chemotherapy-induced neuropathies. Hyperglycemia-induced neuronal stress levels were measured to evaluate *in vitro* and ex vivo protective effects of VEGF. The therapeutic potential of VEGF was tested *in vivo* in streptozotocin (STZ)-induced diabetic and subplantar paclitaxel neuropathy models. Transgenic mice and the anti-Flk1 antibody (DC101) were used to study whether the effects of VEGF were mediated through its Flk1 receptor. Finally, also the therapeutic potential of a recently generated VEGF-derived peptide was evaluated in these different models.

EXPERIMENTAL PROCEDURES

Animals

Male adult Sprague Dawley rats (Harlan, The Netherlands) were used for all rat based experiments. Male C57BL/6JCrl (Charles River, Belgium) or FVB (Harlan, The Netherlands) were used for mouse-based experiments. Transgenic mice: Thy:Flk1^{WT} and Thy:Flk1^{DN} mice expressing a wild-type (WT) or truncated murine *Flk1* transgene in postnatal neurons, were generated by pronuclear injection of a mouse *Thy1.2* expression cassette. These mice were further intercrossed with Thy:YFP mice to obtain Thy:YFP$\times$Thy:Flk1^{WT} mice. Knock-in mice expressing a tyrosine kinase-dead Flt1 receptor (Flt-TK$^{-/-}$ mice) were backcrossed to an FVB background. Before starting the experiment, all animals were habituated to handling and test environments. Rats and mice were housed in conventional facilities with a 12-h light/dark cycle with access to food *ad libitum*. All experiments were approved by the local ethical committee.

Subplantar paclitaxel model

Paclitaxel (Taxol®, Bristol Myers Squibb as 6 mg/ml in Cremaphor EL solution) was formulated in Cremaphor/Ethanol (150 μg per dose, 50 μl) for rats or in saline (75 μg per dose, 25 μl) for mice, and was injected directly into the left hind paw for four consecutive days. The needle was introduced under the skin between the 2nd and 3rd toes and gently slid toward the heel. One hour before each subplantar paclitaxel injection, recombinant rat VEGFA or glial cell-derived neurotrophic factor (GDNF) (50 μg, PeproTech EC, London) diluted in phosphate-buffered saline (PBS), was subplantarily delivered, 1 h before paclitaxel, in the same hind paw in a volume of 10 μl for mice or 50 μl for rats. PBS was used as a control. All animals were pinpricked before subplantar injections. Paw thickness (IITC Inc. Life Science, Woodland Hills, CA, USA) was measured 24 h after each VEGF injection, before paclitaxel injections.

Diabetes rat and mouse models

Seven-week-old male FVB mice (Harlan, The Netherlands) and male Sprague–Dawley rats (Harlan, France) were fed a standard diet and had access to water *ad libitum*. Diabetes was induced by a single intraperitoneal injection of STZ (180 mg/kg for mice and 65 mpk for rats) to animals that were fasted overnight (starvation for 20–24 h). To measure glucose levels, blood samples were taken from the tail vein 7 days after STZ injection. Mice and rats with a blood glucose level above 250 mg/dl were considered diabetic and used for further experiments. Control animals were injected with a buffer solution.

Behavioral analyses

Pinprick test. All animals were allowed to habituate for 30 min in individual testing chambers on a metal mesh floor. A bend 22-gauge needle for rats and a bend 1 ml syringe for mice were gently applied to the plantar surface of the hind paw without breaking the skin. The needle was applied to alternating feet and 30 s was allowed to elapse between applications to the same foot. A response (scored as 0) was defined as lifting, shaking or licking the hind paw. No response was scored as 1. Three scores of each hind paw were taken for each animal.

Von Frey test. The electronic Von Frey test was used to evaluate mechanical (tactile) allodynia. All animals were allowed to habituate for 30 min in individual testing chambers on a metal mesh floor. Gradually increased pressure was applied with a mechanical Von Frey probe (Senselab® Somedic, Hörby, Sweden) positioned perpendicularly into the mid-plantar surface. The stimulus was continued until the hind paw was withdrawn or slowly elevated (defining a withdrawal threshold). Peak forces were recorded in grams. Three measurements were taken for each hind paw and averaged.

Paw pressure test. Mechanical hyperalgesia was quantified with an Analgesy meter (Ugo-Basile, Comerio, Italy). An increasing pressure was applied with a cone-shaped plunger on the dorsal area of the hind