Experimental Neurology 227 (2011) 195–202

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Placental growth factor-2 gene transfer by electroporation restores diabetic sensory neuropathy in mice

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ARTICLE INFO

Article history:
Received 11 August 2010
Revised 23 October 2010
Accepted 26 October 2010
Available online 5 November 2010

Keywords:
Diabetic neuropathy
Electroporation
Gene therapy
Hypoalgesia
Neuropilin-1
Placental growth factor-2

ABSTRACT

Placental growth factor-2 (PlGF-2) exhibits neurotrophic activity in dorsal root ganglion (DRG) neurons through the neuropilin-1 (NP-1) receptor *in vitro*. To examine the potential utility of PlGF-2 therapy for treating diabetic neuropathy, we performed intramuscular PlGF-2 gene transfer by electroporation, and examined its effects on sensory neuropathy in diabetic mice. PlGF-2 was overexpressed in the tibial anterior (TA) muscles of streptozotocin-induced diabetic mice with hypoalgesia using a PlGF-2 plasmid injection with electroporation. The nociceptive threshold was measured using a paw-pressure test. In addition, we overexpressed PlGF-1, an isoform of PlGF that does not bind NP-1. The sciatic nerve and skin were examined 3 weeks after PlGF-2 electro-gene transfer. The overexpression and secretion of PlGF-2 in TA muscles were confirmed by an increase in PlGF levels in TA muscles and plasma, and strongly PlGF positive myofibers in TA muscles. Two weeks after electro-gene transfer into the bilateral TA muscles, the previously elevated nociceptive threshold was found to be significantly decreased in all treated mice. PlGF-1 gene transfer by electroporation did not significantly decrease the nociceptive threshold in diabetic mice. No increase in the number of endoneurial vessels in the sciatic nerve was found in the PlGF-2 plasmid-electroporated mice. A reduction of area of immunoreactivity in epidermal nerves in diabetic mice was restored by PlGF-2 gene transfer. These findings suggest that PlGF-2 electro-gene therapy can significantly ameliorate sensory deficits (*i.e.* hypoalgesia) in diabetic mice through NP-1 in DRG and peripheral nerves.

Introduction

Diabetic neuropathy is a major complication of diabetes and the most frequent cause of peripheral neuropathy (Llewelyn et al., 2005). Polyneuropathy is the most common form of diabetic neuropathy, and symptoms experienced by patients typically include paresthesia, pain or sensory loss in the extremities. In severe cases, diabetic sensory neuropathy can lead to foot ulceration, infection and limb amputation. No specific treatments exist at present, and the only therapeutic strategy is to control blood-sugar levels. There is evidence that diabetic polyneuropathy occurs via a range of pathogenic mechanisms, including enhanced polyol pathway activity, increased nonenzymatic glycation, microvascular insufficiency and reduced availability of neurotrophic factors (Llewelyn et al., 2005).

Vascular endothelial growth factor (VEGF) is a crucial mediator of vasculogenesis and angiogenesis, and has been also demonstrated to have neurotrophic and neuroprotective effects (Sondell et al., 1999; Cheng et al., 2004). Previous studies investigating potential treatments of diabetic neuropathy using animal models reported that both the simple intramuscular injection of a VEGF plasmid, and herpes simplex virus vector-mediated VEGF gene transfer improved nerve function (Schratzberger et al., 2001; Chattopadhyay et al., 2005). In mice, four VEGF isoforms, VEGF 115, 120, 164, and 188, have been generated by alternative splicing. VEGF 120 does not bind to neuropilin-1 (NP-1) receptor, but binds to fms-like tyrosine kinase (VEGFR-1) and fetal liver kinase (VEGFR-2) receptors, whereas VEGF 164 binds to all these receptors. We demonstrated that gene transfer of VEGF 164, but not VEGF 120, by electroporation improves diabetic sensory neuropathy in mice, suggesting that VEGF 164 may improve sensory deficits through NP-1 (Murakami et al., 2006). The findings of a phase 2 randomized trial of intramuscular gene transfer using a VEGF plasmid to treat diabetic polyneuropathy were recently reported (Ropper et al., 2009). Importantly, sensory loss and diabetic neuropathic pain were both found to be improved in this clinical trial.

Placental growth factor (PlGF) is a member of the VEGF family, and its expression is predominantly restricted to the placenta. Four isoforms of human PlGF have been described as a result of alternative splicing (Hauser and Weich, 1993; Maglione et al., 1993; Yang et al., 2003). PlGF-2 has a

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doi:10.1016/j.expneurol.2010.10.015

basic amino-acid rich, heparin-binding domain, and binds VEGFR-1 and NP-1 (Sawano et al., 1996; Migdal et al., 1998). It has been reported that human VEGF 165 corresponding to mouse VEGF 164, and PlGF-2 exert neurotrophic actions through NP-1 in dorsal root ganglion (DRG) neurons *in vitro* (Cheng et al., 2004). Taken together, we hypothesized that PlGF-2 may have neurotrophic properties *in vivo*, and improve diabetic sensory neuropathy through NP-1. We used streptozotocin (STZ)-induced diabetic ddY mice with sensory neuropathy, which have previously been found to exhibit increased nociceptive thresholds, *i.e.* hypoalgesia (Murakami et al., 2006; Christianson et al., 2003a). To examine the potential therapeutic effects of PlGF-2, plasmid DNA expressing PlGF-2 was injected into skeletal muscles of these mice with electroporation, and compared with mice injected with a control plasmid. The findings demonstrated that overexpression of PlGF-2 by electro-gene transfer can improve diabetic sensory deficits in mice.

Material and methods

Plasmid construction

Human PlGF-1 and 2 were synthesized by PCR from human placenta cDNA (Quick-Clone cDNA; BD Biosciences). The cDNA fragments were subcloned into the pCAGGS vector (Tokui et al., 1997) (pCAG-hPlGF1 and 2) and sequenced to confirm insertion of the PlGF-1 and 2 cDNA. The empty pCAGGS plasmid was used as a control. pCAG-hPlGF1, 2 and pCAGGS were purified on Qiagen (Chatsworth, CA) columns, then diluted to 1 mg/ml with 0.9% NaCl.

Animal model

Eight-week-old male ddY mice were used for the induction of diabetes by intraperitoneal injection of STZ (200 mg/kg). The onset of the diabetic state was assessed by the presence of hyperglycemia. Six weeks after the STZ injection, a significant increase in the mechanical nociceptive threshold, *i.e.* hypoalgesia, in the hindpaw was confirmed using a paw-pressure test. All animal experiments were approved by the Animal Research Committee of Kawasaki Medical School and performed according to the protocols of Kawasaki Medical School.

Paw-pressure test

Mechanical nociceptive thresholds were determined using an Ugo Basile analgesymeter (Randall and Selitto, 1957). The nociceptive threshold was defined as the force, in grams, at which a mouse struggled to withdraw its hindpaw. The data are expressed as the percentage of the maximum pressure of 750 g.

Electroporation

Six weeks after the STZ injection, mice with body weight less than 35 g, a blood glucose level greater than 16.7 mmol/l and a nociceptive threshold greater than 80% were used for the gene transfer experiment as the 'diabetic' group. The diabetic mice were anesthetized with sodium pentobarbital. After skin incision, 50 μg of plasmid DNA was injected into the tibial anterior (TA) or quadriceps muscles using an insulin syringe with a 29-gauge needle. A pair of stainless steel electrodes with a 5 mm gap was immediately inserted into the muscles, and a series of six electrical pulses (70 V, 1 Hz, and 50 ms each) were delivered using a standard square-wave electroporator (CUY21; BEX Co. Ltd., Tokyo, Japan).

PlGF ELISA

The TA muscles were removed from the diabetic mice at 2 weeks after plasmid injection. Total protein lysates were obtained from homogenized muscles as previously described (Murakami et al., 2006). Lysates were then assayed using a human PlGF ELISA kit (Quantikine M; R&D Systems), according to the manufacturer's instructions.

Blood samples were taken at 1 and 2 weeks after plasmid injection from the tail of the diabetic mice, using heparinized hematocrit-capillary tubes, and centrifuged at room temperature at 10,000 rpm for 5 min. The plasma PlGF level was then evaluated using the PlGF ELISA kit.

Histology and immunohistochemistry

The TA muscles were isolated at 2 weeks after the plasmid injection. The tissues were immersion-fixed in 10% (vol/vol) buffered formalin, then embedded in paraffin. Paraffin sections from these tissues were stained with hematoxylin and eosin. Paraffin sections of the TA muscles were also stained for PlGF (polyclonal rabbit 10 mg/ml; Abcam), CD3 (polyclonal rabbit 1:200; Calbiochem), CD11b (polyclonal rabbit 1:400; Abcam) and for von Willebrand factor (vWF; polyclonal rabbit 1:200; Chemicon) using the Ventana Discovery automated staining system with the 3,3′-diaminobenzidine (DAB) Map kit (Ventana). The Discovery staining protocol was set to "Research IHC DAB Map XT". The sections were deparaffinized, heated at 100 °C for 60 min for epitope retrieval, and then incubated for 4 h with the primary antibody, followed by 1 h incubation with secondary antibody. All slides were counterstained with hematoxylin, dehydrated, cleared, and mounted.

Segments of mid-thigh sciatic nerves were removed from the diabetic mice at 3 weeks after the plasmid DNA injection, and from 17 week-old non-treated diabetic and healthy ddY mice. Segments were fixed, and then embedded in epoxy resin as previously described (Murakami et al., 2006). One-μm sections were stained with toluidine blue. All blood vessels in the endoneurium were counted by microscopic examination.

The entire plantar surface of the left hind paws was dissected from the diabetic mice at 3 weeks after the plasmid injection, and from non-treated diabetic and healthy ddY mice. The tissues were immersion-fixed in 10% (vol/vol) buffered formalin, and then embedded in paraffin. Cross sections (along the right–left axis) of the anterior plantar skin were cut near volar pads on a microtome. Randomly chosen 5-μm paraffin sections from each mouse were stained for protein gene product 9.5 (PGP9.5; polyclonal rabbit 1:400; UltraClone) using the Ventana Discovery automated staining system with the DAB Map kit as described above except 30 min incubation with the primary antibody. Images were captured using a BZ8100 microscope (Keyence, Osaka, Japan). Twenty Z-stack images were obtained at 0.2-μm intervals through the section and combined. Two fields per section at 400× magnification and four sections per animal underwent quantitative analysis. An area of epidermis and the area of immunoreactive nerve fibers in the epidermis were quantified using software Dynamic cell count BZ-HIC (Keyence), and the ratio was calculated. The epidermal skin area was defined as the basal, spinous and granular layers, but the keratin layer was excluded.

Gene transfer and measurement of the nociceptive threshold

Six weeks after STZ injection, the diabetic mice with hypoalgesia were divided into two groups. In the first group ($n = 11$), pCAG-hPlGF2 was injected into the bilateral TA muscles followed by electroporation. In the second group ($n = 9$), the pCAGGS vector was injected into the bilateral TA muscles followed by electroporation. From 2 weeks after the plasmid DNA injection, the nociceptive threshold was examined weekly using the paw-pressure test.

To examine whether the influence of PlGF was restricted to local effects, pCAG-hPlGF2 or pCAGGS was injected into the left TA and quadriceps muscles of diabetic mice with hypoalgesia ($n = 7$, each) followed by electroporation. From 2 weeks after the plasmid DNA injection, the nociceptive thresholds of both hindpaws were measured weekly using the paw-pressure test.

To examine the effect of PlGF-1, pCAG-hPlGF1 or pCAGGS was injected into the bilateral TA muscles of diabetic mice with hypoalgesia