Original Article

Gene Transfer of an Engineered Transcription Factor Promoting Expression of VEGF-A Protects Against Experimental Diabetic Neuropathy

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Peripheral neuropathy is a common, irreversible complication of diabetes. We investigated whether gene transfer of an engineered zinc finger protein transcription factor (ZFP-TF) designed to upregulate expression of the endogenous vascular endothelial growth factor (VEGF)-A gene could protect against experimental diabetic neuropathy. ZFP-TF–driven activation of the endogenous gene results in expression of all of the VEGF-A isoforms, a fact that may be of significance for recapitulation of the proper biological responses stimulated by this potent neuroprotective growth factor. We show here that this engineered ZFP-TF activates VEGF-A in appropriate cells in culture and that the secreted VEGF-A protein induced by the ZFP protects neuroblastoma cell lines from a serum starvation insult in vitro. Importantly, single and repeat intramuscular injections of formulated plasmid DNA encoding the VEGF-A–activating ZFP-TF resulted in protection of both sensory and motor nerve conduction velocities in a streptozotocin-induced rat model of diabetes. These data suggest that VEGF-A–activating ZFP-TFs may ultimately be of clinical utility in the treatment of this disease. *Diabetes* 55: 1847–1854, 2006

Peripheral neuropathy, characterized by a progressive loss of sensation in the extremities, occurs in ~50% of diabetic individuals after 25 years of disease (1,2). Despite the prevalence of this condition, beyond the careful management of the diabetes itself via glycemic control, no treatment for diabetic neuropathy exists.

In the present study, we examined whether gene transfer of an engineered zinc finger protein transcription factor (ZFP-TF) for the activation of the endogenous vascular endothelial growth factor (VEGF)-A gene could be used to prevent progression of neuropathy in streptozotocin (STZ)-induced diabetic rats. VEGF-A has shown particular promise in this regard. In vitro, the addition of the $VEGF_{165}$-A isoform alone has been shown to have both direct neuroprotective (3–5) as well as neuronal growth-promoting (6–8) effects. In rat and rabbit models of diabetic neuropathy, $VEGF_{165}$-A gene transfer conferred a complete reversal of the deficits in nerve conduction velocities (NCVs) characteristic of diabetes. Preliminary results from clinical studies have indicated improvements in the signs and symptoms of sensory neuropathy in diabetic patients after intramuscular injection of a plasmid DNA encoding $VEGF_{165}$-A (9). Collectively, these studies have provided support for the development of therapies for peripheral neuropathy based on $VEGF_{165}$-A as well as other VEGF gene family members, and human gene therapy clinical trials testing this concept have either been proposed or are underway (10).

Given the potential demonstrated by VEGF-A in experimental as well as clinical diabetic neuropathy, we chose to investigate an alternative strategy for the therapeutic application of this powerful growth factor, namely, the activation of the endogenous VEGF-A gene through the action of an engineered ZFP-TF. ZFP-TFs can be designed to control the expression of any desired gene (rev. in 11). We have previously reported on a ZFP-TF engineered to upregulate VEGF-A mRNA and protein expression in human cells in culture (12) as well as in animal models of disease (13,14). In all cases studied, this regulation results in the expression of all of the different VEGF-A isoforms (12,14), a fact that appears (at least in the context of angiogenesis) to provide for more complete and robust biological function (13–15). Here, we demonstrate that this VEGF-A–activating ZFP-TF is capable of stimulating VEGF-A expression that can protect neuronal-derived SK-N-MC cells from growth arrest in response to serum starvation in vitro. Moreover, in an experimental model of diabetic neuropathy, a single administration by intramuscular injection of a plasmid DNA encoding the VEGF-A–activating ZFP-TF showed significant and dose-related protection of both motor and sensory NCVs. Repeat administration of plasmid DNA encoding the VEGF-A–activating ZFP-TF resulted in sustained robust protection against STZ-induced measurements of diabetic neuropathy. These data suggest that a VEGF-A–activating ZFP-TF may ultimately be of clinical utility in the treatment of diabetic neuropathy.

RESEARCH DESIGN AND METHODS

Plasmid constructs. The VEGF-A–activating ZFP VZ+434 has been previously described (12). In brief, the genetically engineered plasmid, designated

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CMV, cytomegalovirus; DOX, doxycycline; DMEM, Dulbecco's modified Eagle's medium; MNCV, motor NCV; NCV, nerve conduction velocity; NRK, normal rat kidney; SNCV, sensory NCV; STZ, streptozotocin; VEGF, vascular endothelial growth factor; ZFP-TF, zinc finger protein transcription factor.

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VZ+434, encodes the designed three-finger ZFP DNA-binding domain, the nuclear translocation signal from simian virus 40 large T antigen, and the transactivation domain from the p65 subunit of the human nuclear factor-κB, subcloned into pVAX-1 (Invitrogen, San Diego, CA) with expression driven by the human cytomegalovirus (CMV) promoter. Plasmids were formulated at a concentration of 2 mg/ml in 5% poloxamer 188 (BASF, Washington, New Jersey), 150 mmol/l NaCl, and 2 mmol/l Tris (pH 8.0).

Adenoviral vector constructs and production. Recombinant adenoviral vectors, AdEGFPp65-flag, and AdVZ+434-flag were created as follows: the *Mlu* I-*Afl*II fragment of the plasmid pcDNA4/TO (Invitrogen), which consists of the human CMV immediate early promoter/enhancer and two tetracycline operator sequences ($TetO_2$), and the *Afl*II-*Xho*I fragment of the plasmid pcDNA3-EGFPp65-flag or pcDNA3-VZ+434-flag, which contains the ZFP expression cassette, were simultaneously cloned into the *Mlu* I and *Xba*I restriction sites upstream of a bovine growth hormone polyadenylation signal (BGH polyA) in the plasmid pShuttle (Clontech, Palo Alto, CA). The CMV-$TetO_2$-ZFP-BGH polyA cassette was then excised via the unique I-*Ceu* I and PI-*Sce* I restriction sites in the pShuttle and were ligated to the Adeno-X viral DNA previously digested with I-*Ceu* I and PI-*Sce* I (Clontech). All cloned sequences were verified by DNA sequencing.

Recombinant adenoviral vectors were packaged by transfecting T-REx-293 cells (Invitrogen). Adenoviruses were harvested from transfected T-REx-293 cells lysed with three consecutive freeze-thaw cycles and were amplified in T-REx-293 cells before purification by double cesium chloride gradient centrifugation (Qbiogene). Purified recombinant adenoviruses were dialyzed against three changes of 10 mmol/l Tris (pH 8.0), 2 mmol/l $MgCl_2$, and 4% sucrose; aliquoted; and stored at −80°C. Adenoviral particle numbers were determined by absorbance at 260 nm, and infectious titers were determined by using the Adeno-X Rapid Titer kit (Clontech). Differentiated human skeletal myotubes and normal rat kidney (NRK) cells were plated in 24-well cell culture plates and infected with recombinant adenoviruses at 100–250 pfu/cell for 24 h at 37°C. At 48 h postinfection, cells were collected for RNA and protein analyses.

Production and transduction of differentiated skeletal muscle cells. Human cultured skeletal myocytes (Clonetics) were maintained as recommended by the manufacturer and were differentiated via treatment with a low-serum medium for 8 days (16). This procedure results in growth cessation, fusion of many myocytes to form multinucleated fibers, and upregulation of muscle-specific genes such as creatine kinase (data not shown). After differentiation, myotubes were treated with adenovirus expressing either VZ+434-FLAG or EGFP-p65-FLAG or they were left untreated.

Retroviral constructs, virus preparation, and generation of stable lines. A self-inactivating retroviral vector containing a tetracycline-inducible ZFP expression cassette was constructed and used for virus generation. Briefly, the pSIR vector (Clontech) was modified to contain the CMV promoter and the tetracycline operator sequences from pcDNA4-TO (Invitrogen). The coding region of ZFP VZ+434 (12) was inserted downstream of the inducible promoter by cloning into the modified pSIR vector (Clontech). Virus-containing supernatant was generated by transient transfection of the resulting plasmid, pSIR-TO-ZFP VZ+434, into the Phoenix packaging line as previously described (17). For stable cell line generation, HEK293 T-REx cells were transduced with supernatant obtained (described above) containing retrovirus encoding ZFP VZ+434 and were selected in medium containing 800 μg/ml G418 (Invitrogen). Individual clones were isolated and analyzed for doxycycline-dependent expression of ZFP VZ+434 expression and corresponding activation of the endogenous gene target.

Cell culture and viability assays. NRK cells were obtained from the American *Type Culture* Collection, and they were maintained in accordance with their recommendations. SK-N-MC human neuroblastoma cells were obtained from the American *Type Culture* Collection and were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies), supplemented with 10% fetal bovine serum (Invitrogen) and 2 mmol/l L-glutamine (Life Technologies). HEK293 T-REx VZ+434 cells (HEK293 cells stably transformed with the VZ+434 ZFP-TF transgene under the control of a doxycycline-inducible promoter) were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mmol/l L-glutamine, and 200 μg/ml G418.

Cell viability was determined by using the Alamar Blue assay (Biosource) as per the manufacturer's recommendations. Briefly, cells were cultured in test medium in 96-well plates. Alamar Blue reagent was added to a final volume of 10%, and the cells were incubated at 37°C for 3 h. Fluorescence was determined with a Wallac Victor2 plate reader by excitation at 530–560 nm and by measuring emission at 590 nm.

Quantitative RT-PCR analysis of VEGF-A mRNA expression. Total cellular RNA was isolated by using the RNeasy extraction kit (Qiagen) according to the manufacturer's recommendations. RNA (25 ng) was used in RT-PCR analysis with TaqMan chemistry in a 96-well format on an ABI 7700 SDS machine (Applied Biosystems) as previously described (18). Reverse transcription was performed at 48°C for 30 min by using MultiScribe reverse transcriptase. After a 10-min denaturation at 95°C, PCR amplification with AmpliTaqGold DNA polymerase was conducted for 40 cycles at 95°C for 15 s and 60°C for 1 min. The primers and probes used were as previously described (12). Results (relative gene expression) are expressed as a ratio between target gene expression and 18S expression. The results were analyzed with SDS Version 1.6.3 software.

Enzyme-linked immunosorbent assay analysis of VEGF-A protein expression. Secreted VEGF-A protein levels were determined in the culture media after a 24-h accumulation period (i.e., 24 h after fresh media was applied to the cells). Medium was either immediately analyzed upon harvesting or stored at −20°C for analysis at a later date. Aliquots (200 μl) of culture media were assayed, in duplicate, by using the R&D Systems sandwich enzyme-linked immunosorbent assay kit with horseradish peroxidase conjugate detection, according to the manufacturer's recommended conditions.

Preparation of serum-free conditioned medium from HEK293 cells expressing VZ+434. VEGF-A–containing conditioned medium was produced from cultured HEK293 T-REx VZ+434 cells. Cells were plated in complete growth medium (DMEM, 10% fetal bovine serum, and 2 mmol/l L-glutamine), in 15-cm dishes, at a density of 1.3×10^7 cells per dish, resulting in 80–90% confluency 24 h after plating. The cells were induced by the addition of 5 ng/ml doxycycline. Induction was allowed to proceed for 24 h, and then the complete growth medium was removed and replaced with 10 ml of serum-free medium (DMEM, 2 mmol/l L-glutamine) containing 5 ng/ml doxycycline. Twenty-four hours later, the conditioned serum-free medium was harvested and passed through a 0.45-μmol/l filter to remove any cellular material. The concentration of VEGF-A in this medium was determined by an enzyme-linked immunosorbent assay, and the medium was stored at −20°C until ready to be used in neuroprotection experiments. Conditioned medium from noninduced cells (i.e., by omitting doxycycline from all media) served as a control.

Neuroprotection studies. SK-N-MC human neuroblastoma cells (American *Type Culture* Collection) were plated on a 96-well plate at a density of 4,000 cells per well. These cells were allowed to grow for 48 h before the growth medium was removed and replaced with serum-free medium, containing various proportions of the doxycycline-induced serum-free conditioned medium from the HEK293 T-REx VZ+434 cells. Media from HEK293 T-REx VZ+434 cells grown in the absence of doxycyline, complete medium, and serum-free medium alone served as controls. Duplicate samples were run for each set of growth conditions. The treated SK-N-MC cells were then cultured for a additionaln 72 h under these growth conditions, and cell number and metabolic activity were determined by using an Alamar Blue assay (described above).

Induction of diabetes and gene transfer. Diabetes was induced in male Wistar rats (Charles River, U.K.) with an intraperitoneal injection (55 mg/kg) of STZ (Sigma) given after an overnight fast. The drug was freshly dissolved in sterile saline immediately before injection. Three days later, tail vein blood glucose was measured by a strip-operated reflectance photometer (MediSense Optimum; MediSense, Abington, Oxon, U.K.), and STZ-treated rats with blood glucose <15 mmol/l were removed from the study. Rats were group housed and were given free access to food and water in a 12-h light/dark cycle. Age- and weight-matched rats were used as nondiabetic control animals.

Two studies were performed in diabetic rats: *1*) a dose-ranging study and *2*) a study of repeat administration at selected intervals. In the first study, animals received a single intramuscular administration of formulated plasmid DNA encoding VZ+434 (see above) 4 weeks after STZ administration and consequent induction of diabetes. For the injections, animals were anesthetized with isoflurane in oxygen and given a single dose of VZ+434 into muscle groups of the left hindlimb. The single dose was divided into two injections, one into proximal (gastrocnemius) and the other into distal (soleus) muscle groups. Four groups of diabetic rats ($n = 12$ per group) received doses of either 31.25, 65, 125, or 250 μg VZ+434-formulated plasmid DNA (total injection volume of 500 μl). A control diabetic group received 250 μg formulated control vector (pVAX-1) as a sham treatment. The nondiabetic control group received no treatment.

In the second study, diabetic rats received *1*) a single intramuscular injection of formulated plasmid as previously described at 4 weeks post-STZ induction of diabetes or *2*) three injections of 250 μg each of formulated plasmid at 2-week intervals after 2 weeks of STZ treatment (Fig. 4).

Nerve conduction velocity measurements. Rats were anesthetized with isoflurane (2–4% in oxygen), and electromyograms were recorded from plantar foot muscles in response to stimulation at the sciatic notch and then at the Achilles' tendon. Motor NVC (MNCV) was measured from latencies of the compound M waves, and sensory NVC (SNCV) was measured from H reflex latencies in response to stimuli with the same electrodes. Electromyograms were elicited via fine percutaneous electrodes connected to a Powerlab 4 stimulator (1.5–5.0 V, 2-ms pulses) and recorded on a Powerlab 4 with ABI Scope software on a Sony Vaio laptop computer. The H reflexes were

determined to be genuine, as *1*) they are obliterated by dorsal root section (data not shown) and *2*) they appear at a lower stimulus voltage than M waves, a classical feature of H reflexes. Mid-thigh nerve temperature was maintained at 36 ± 0.5°C throughout the procedure. The latency difference between the two sets of M waves was calculated and related to the nerve length separating the two stimulus points (measured ex vivo) to calculate MNCV. H reflex latency differences were used similarly to calculate SNCV. NCVs were measured for both the left (injected) and right (not injected) sides.

Statistical analyses. The MNCV and SNCV are presented as group means ± SD. The critical testing was a comparison of left- and right-side NCVs, which was done by paired *t* tests; there were no multiple comparisons. For completeness, controls and pVAX-1–treated diabetic rats were compared by unpaired *t* tests.

RESULTS

Engineered ZFP-TF VZ+434 induces endogenous VEGF-A gene expression in vitro. The engineered ZFP-TF VZ+434 has been shown to drive activation of the endogenous VEGF-A gene in both cell culture and animal model settings (12–14). To extend these observations to an in vitro model of skeletal muscle, skeletal myocytes (Clonetics) were differentiated by growth in low-serum medium for 8 days. After differentiation, the cells were infected with recombinant adenovirus vectors expressing either VZ+434-Flag (AdVZ+434-Flag) or EGFPp65-Flag (AdEGFPp65-Flag), or cells were left untreated as a control. VEGF-A protein levels determined by enzyme-linked immunosorbent assay and mRNA levels determined by TaqMan real-time PCR analysis are shown in Fig. 1*B*. Infection with AdVZ+434-Flag virus resulted in an ~6- and ~15-fold increase in VEGF-A mRNA and protein, respectively, relative to uninfected cells. Infection with AdEGFPp65-Flag did not result in increased levels of VEGF-A protein or mRNA, demonstrating that the increases in VEGF-A levels were a consequence of VZ+434 rather than adenoviral infection per se.

Since we planned to examine effects of induction of VEGF-A expression in a rat model of diabetes, we next sought to confirm that the VZ+434 ZFP-TF activator of VEGF-A would function robustly in cultured rat cells. As shown in Fig. 1*A*, the binding site of VZ+434 in the VEGF-A locus is conserved across multiple species, including human, mouse, and rat. NRK cells were infected with AdVZ+434-Flag or AdEGFPp65-Flag or they were left untreated. As shown in Fig. 1*C*, infection with AdVZ+434-Flag resulted in an approximately fivefold increase in VEGF-A mRNA and protein expression. As expected, no increase in VEGF-A mRNA or protein expression was seen with AdEGFPp65-Flag (Fig. 1*C*, *left and center panels*), although both vectors drive expression of their respective transgenes (Fig. 1*C*, *right panel*). These data establish that the ZFP-TF (VZ+434) activates VEGF-A expression in both cultured rat cells and in terminally differentiated human skeletal muscle cells in vitro.

VZ+434-induced VEGF-A protects SK-N-MC human neuroblastoma cells from serum starvation-induced growth arrest. Next, we determined whether the ZFP-TF–induced VEGF-A protein was neuroprotective in vitro. Conditioned medium from a HEK293 T-REx cell line stably transformed with a plasmid encoding the VZ+434 zinc finger protein was generated under inducing and noninducing conditions. This line uses a tetracycline-inducible promoter to drive ZFP-TF expression; thus, the addition of doxycycline (DOX) rapidly turns on ZFP-TF expression and results in the activation of the endogenous VEGF-A gene. Pilot experiments indicated that induction with 5 ng/ml of DOX resulted in both the maximal expression of the VZ+434 transcription factor and peak VEGF-A secretion into the culture medium. Assay of several batches of induced conditioned medium were shown to have VEGF-A concentrations of up to 70 ng/ml after a 24-h accumulation period (data not shown).

To evaluate the neuroprotective function of the ZFP-TF–induced VEGF-A protein secreted from these cells, we measured the ability of conditioned medium to promote the survival of cultured neuronal cell lines after a serum starvation insult. Human neuroblastoma cells (SK-N-MC) were grown either in the presence of complete medium, in serum-free conditions, or in the serum-free medium supplemented with increasing amounts of conditioned media (Fig. 2). Conditioned medium was prepared both in the presence and absence of the VEGF-A–activating ZFP (i.e., in the presence or absence of DOX). The media prepared in the absence of DOX serve to control for any potential neuroprotective factor(s) that may be secreted into the media by HEK293 cells alone. The results, shown in Fig. 2, indicate that only conditioned medium prepared from cells induced for expression of the VZ+434 transcription factor provided a dose-dependent protection from serum starvation. Similar data were obtained with ND8 cells, which are a fusion of primary neonatal rat dorsal-root-ganglion neurons with N18Tg2, a mouse neuroblastoma (C1300)-derived azaguanine-resistant line (data not shown). The data described above suggest that ~20–40 ng/ml of ZFP-TF–induced VEGF-A (final concentration) is sufficient to protect cultured human neuroblastoma cells from loss of viability in response to serum starvation (Fig. 2). This result is consistent with previously published studies with recombinant VEGF-A protein, wherein the effective VEGF-A dose was found to be between 10 and 100 ng/ml, with a declining response at higher doses (3,4,19).

VZ+434 protects NCV in STZ-induced diabetic rats. Next, we sought to determine the effect of VZ+434-driven activation of VEGF-A in an established rat model of STZ-induced diabetes (20–25). Eight weeks post-STZ treatment, diabetic rats exhibited characteristic reduction in body weight and increased plasma glucose levels (Table 1). In agreement with our expectations, neither of these indicators of diabetes was altered in any of the treatment groups (Table 1). First, a dose-ranging study using 31.25–250 μg VZ+434-encoding plasmid DNA was performed to establish an efficacious dose. NCV measurements, taken 8 weeks after STZ-treatment and 4 weeks after treatment with the indicated plasmid DNAs, are shown as group mean data in Fig. 3. Treatment with the empty plasmid pVAX-1 was without effect on NCVs (Figs. 3 and 4), since no difference between values was observed between treated and contralateral limbs. Calculation of mean values (treated and untreated) for each animal in control and diabetic pVAX-1–treated groups gave highly significant reductions in both motor (controls = 50.0 ± 5.8 and pVAX-1–treated diabetic rats = 41.7 ± 5.4; $P < 0.003$) and sensory NCV (controls = 54.7 ± 6.7, diabetic rats = 45.5 ± 4.8; $P < 0.002$; Fig. 3). These NCV deficits obtained after 8 weeks of STZ treatment are consistent with, and within, the range reported elsewhere (21,22). Injection of the lowest dose of ZFP-TF expression plasmid (VZ+434 at 31.25 μg) had no effect on MNCVs or SNCVs (Fig. 3). In contrast, diabetic rats treated with all three higher doses of VZ+434 (62.5, 125, and 250 μg) demonstrated a dose-dependent, progressively increas-

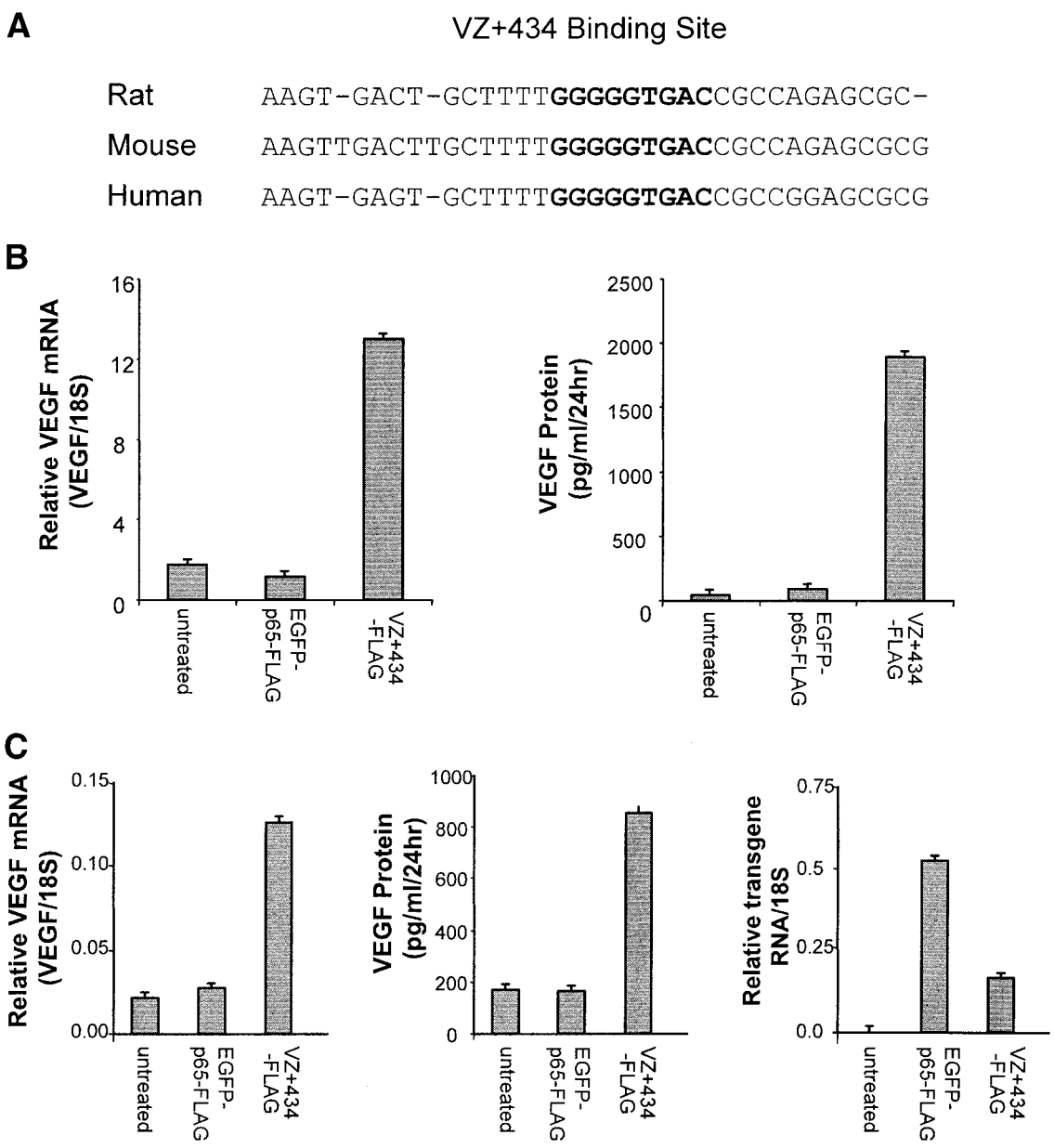

FIG. 1. ZFP VZ+434 activates expression of VEGF-A mRNA and protein in differentiated human skeletal myotubes and rat NRK cells. *A*: The VZ+434 binding site is conserved across multiple species. The human, mouse, and rat VEGF-A sequence in the vicinity of the VZ+434 binding site (indicated in bold) is shown. *B*: Differentiated myotubes were infected with 250 pfu/cell AdVZ+434-Flag or AdEGFPp65-Flag or they were left untreated. Forty-eight hours later, culture medium and cells were harvested for analysis of the levels of VEGF-A mRNA and protein, respectively. *C*: NRK cells were infected with 100 pfu/cell AdVZ+434-Flag or AdEGFPp65-Flag or they were left untreated. Forty-eight hours later, culture medium and cells were harvested for analysis of the levels of VEGF-A protein mRNA, VEGF-A protein, and VZ+34 transgene mRNA, respectively. Results are expressed as a ratio between target gene expression and 18S expression. Results are representative of at least three independent experiments, and the means and SDs are shown.

ing difference between the treated versus untreated limbs with respect to both SNCVs and MNCVs (Fig. 3). In all experiments at these higher doses, the conductance velocity of the nerve on the treated side was always higher than the untreated side, and the differences were all significant ($P < 0.05$; Fig. 3). These data indicate that a single treatment of VZ+434 is capable of neuroprotection in an experimental animal model of early diabetes, as determined by motor and sensory NCV measurements conducted 4 weeks posttreatment.

Having established an effective dose for VZ+434, we next tested the ZFP-TF's ability to function in the context of a repeat administration study, again comparing NCV values from the treated and untreated limbs. The study design (shown in Fig. 4) called for injection of 250 μg VZ+434 plasmid DNA 2, 4, and 6 weeks post-STZ treatment. Interestingly, while a single injection of 250 μg of VZ+434 plasmid DNA at 4 weeks again demonstrated protection of NCVs (data not shown), the repeat intramuscular administration of VZ+434 plasmid DNA at 2, 4, and 6 weeks post-STZ treatment demonstrated a robust and highly significant protection of both MNCV and SNCV (Fig. 4). As expected, no change in NCVs was observed in diabetic animals that received the control vector (pVAX). These repeat injection data may be significant given the chronic nature of the human condition.

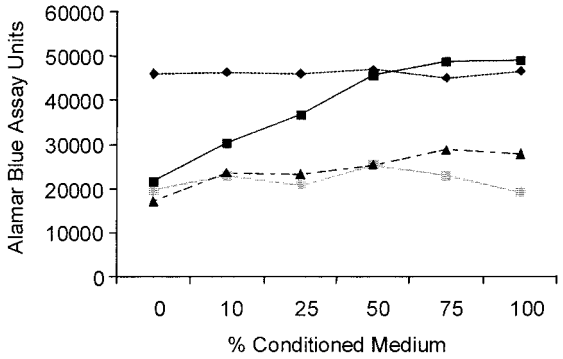

FIG. 2. VZ+434-induced VEGF-A protects SK-N-MC human neuroblastoma cells from serum starvation insult. SK-N-MC cells were cultured for 24 h in complete serum. Culture medium was then removed and replaced with test media: either normal culture medium with 10% fetal bovine serum, serum-free culture medium, conditioned medium from induced HEK293 cells expressing VZ+434 from a doxycycline-inducible promoter (and thus activated VEGF-A levels), or culture medium from the same cells not expressing ZFP VZ+434 (no doxycycline induction, basal VEGF-A levels). Forty-eight hours later, the cells were assayed for viability by using the Alamar Blue assay. Results are shown are means representative of at least three independent experiments. ♦, complete medium; ▨, serum-free medium; ▲, control-conditioned medium; ■, VZ+434-conditioned medium.

DISCUSSION

This study demonstrates a beneficial effect on NCVs after intramuscular injection of a plasmid DNA encoding VZ+434 in an animal model of diabetic neuropathy. Efficacy was observed in two independent animal studies. In the first, a dose-ranging study, the effect on NCV was evident 4 weeks after a single treatment with the engineered ZFP-TF (Fig. 3). The second animal study, which utilized dosing at 2-week intervals, was markedly successful at protecting NCVs in the injected limb.

VZ+434 is an engineered ZFP-TF designed to upregulate the expression of the endogenous VEGF-A gene. Extending previous studies that used this engineered transcription factor in human cell culture (12) and mouse or rabbit (13,14) animal models, we show here that VZ+434 is capable of activating the expression of VEGF-A mRNA and protein both in cultured rat cells and in differentiated human skeletal myocytes (Fig. 1).

Furthermore, we show that conditioned media containing increased VEGF-A levels stimulated by this engineered transcription factor are capable of protecting SK-N-MC cells from serum starvation–induced growth arrest (Fig. 2). An important role for VEGF-A in neuroprotection is becoming increasingly apparent. For example, gene transfer with a plasmid DNA encoding the VEGF-A_{165} isoform improved NCVs in several studies in vivo (20,26). Moreover, in an open-label, dose-escalation trial of the same plasmid DNA encoding the VEGF-A_{165} isoform, four of six patients enrolled who had diabetes showed an improvement in neuropathy in the treated limb (9). Evidence for a direct neuroprotective role for VEGF-A comes from in vitro studies, which have documented VEGF-driven protection of neuronal-derived cell lines (such as HN33 and NSC34) from serum starvation, hypoxic insult, tumor necrosis factor α, and oxidative stress (3–5). Beyond protection, VEGF-A has also been shown to promote neuronal growth with cultures of adult mouse dorsal root ganglion and superior cervical ganglion explants (6,8). In addition, VEGF-A has shown both protective and potent growth factor activity for Schwann cells (6,7), which provide myelination and support functions to neurons, suggesting an additional potential mechanism for the beneficial effects of VEGF-A action in vivo. Note that all of these studies used a single isoform of the VEGF-A growth factor (VEGF-A_{165}). With respect to the angiogenic properties of VEGF-A, the expression of multiple isoforms has been shown to be more effective than any individual isoform alone (13,15). Engineered transcription factors via their action at the promoter of the endogenous gene generate all of the natural splice variants and protein isoforms supported by that cell type (13,14). While it remains to be determined whether the expression of the full complement of protein isoforms will be advantageous in the context of neuropathy, it is known that the isoforms differ in their effects on vascular permeability, tumor progression, receptor binding, and interaction with the extracellular matrix (27–31).

It is remarkable that this positive effect was observed 28 days after a single intramuscular treatment with plasmid DNA encoding VZ+434. This result is perhaps even more surprising given the short (<7 days) duration of transgene expression observed (data not shown). The second study shows that an even more robust effect may be achieved with multiple dosing. This striking result in terms of the potency and duration of effect supports earlier work with a plasmid encoding the VEGF-A_{165} cDNA, demonstrating sustained improvement in NCVs up to 10 weeks after gene transfer (20). While angiogenesis was proposed as a pos-

TABLE 1
Final blood glucose values and starting and ending body weights for all groups

Experimental group	Number of rats	Blood glucose (mmol/l)	Body weight (g)	
			Start	End
Dose-ranging study				
Controls (nondiabetic; not injected)	10	5.2 ± 0.9	306 ± 19	547 ± 60
Diabetic pVAX-1	12	27.9 ± 3.5	297 ± 11	390 ± 22
Diabetic VZ+434 (31.25 μg)	11	28.9 ± 4.0	304 ± 10	385 ± 58
Diabetic VZ+434 (62.5 μg)	13	28.3 ± 3.9	300 ± 13	368 ± 45
Diabetic VZ+434 (125 μg)	10	29.0 ± 3.0	302 ± 15	372 ± 52
Diabetic VZ+434 (250 μg)	14	29.0 ± 2.7	295 ± 10	366 ± 37
Repeat-dosing study				
Control (untreated)	12	7.0 ± 1.0	285 ± 13	534 ± 39
Diabetic pVAX-1 (repeat injections)	13	25.8 ± 2.6	288 ± 15	349 ± 72
Diabetic VZ+434 (repeat injections)	11	28.7 ± 3.9	292 ± 10	354 ± 40

Data are means ± SD from triplicate measurements for blood glucose.

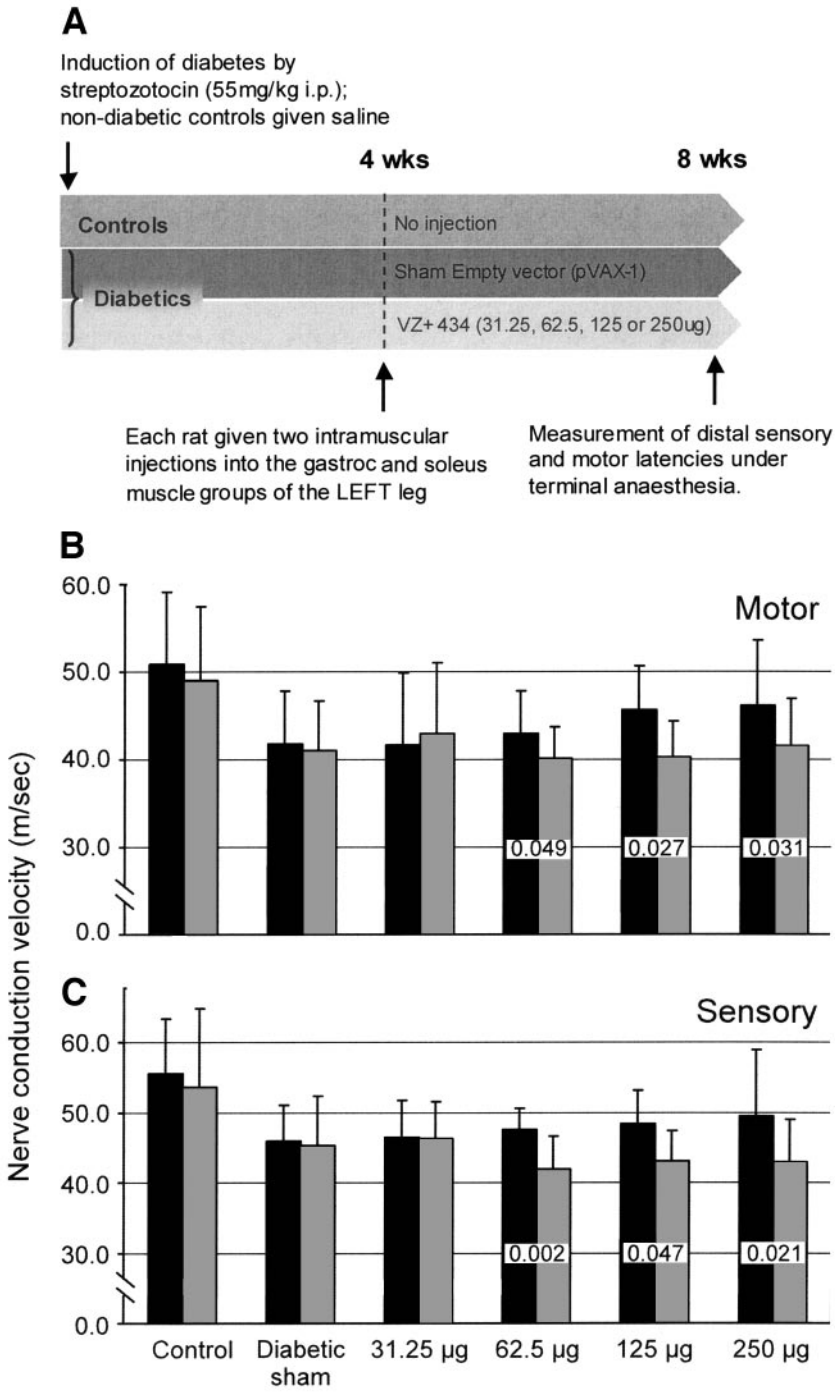

FIG. 3. SNCV and MNCV in STZ-treated diabetic rats 4 weeks after gene transfer of VZ+434. *A*: Experimental study design. MNCV (*B*) and SNCV (*C*) measurements. ■, values (means ± SD) for the injected limb; ▨, values for the uninjected limb. *P* values derived from paired *t* tests between NCV values for injected versus noninjected limbs are shown across each column.

sible mechanism responsible for the therapeutic effect after delivery of the VEGF-A cDNA, the data presented here strongly suggest a direct neuroprotective effect of VEGF-A. This is further supported by work in an ALS mouse model, which showed that VEGF-A, but not the potent angiogenic factor PLGF (placental growth factor), prevented motor neuron degradation (32). Moreover, the latter studies were conducted in animals that were diabetic for 3 months before gene transfer and thus suggest that VEGF-A may be an effective treatment for the long-term effects of DN. In both the present study and the previous studies (20,26), no change in NCVs was observed on the contralateral side (uninjected side), consistent with a local effect of VEGF-A and/or the ZFP-TF treatment. Interestingly, both protection of NCVs and increased percentages of small myelinated nerve fibers were noted following local administration of insulin itself in STZ-induced models of diabetes independent of hyperglycemia (33), data further supporting the importance of direct trophic effects on the treated nerve fibers.

Thus, the current findings indicate a neuroprotective effect for the VEGF-A–activating ZFP-TF VZ+434 both in vitro and in animal models of diabetes in vivo and thus suggest that this engineered transcription factor could represent a novel therapeutic modality for the potential treatment of diabetic neuropathy.

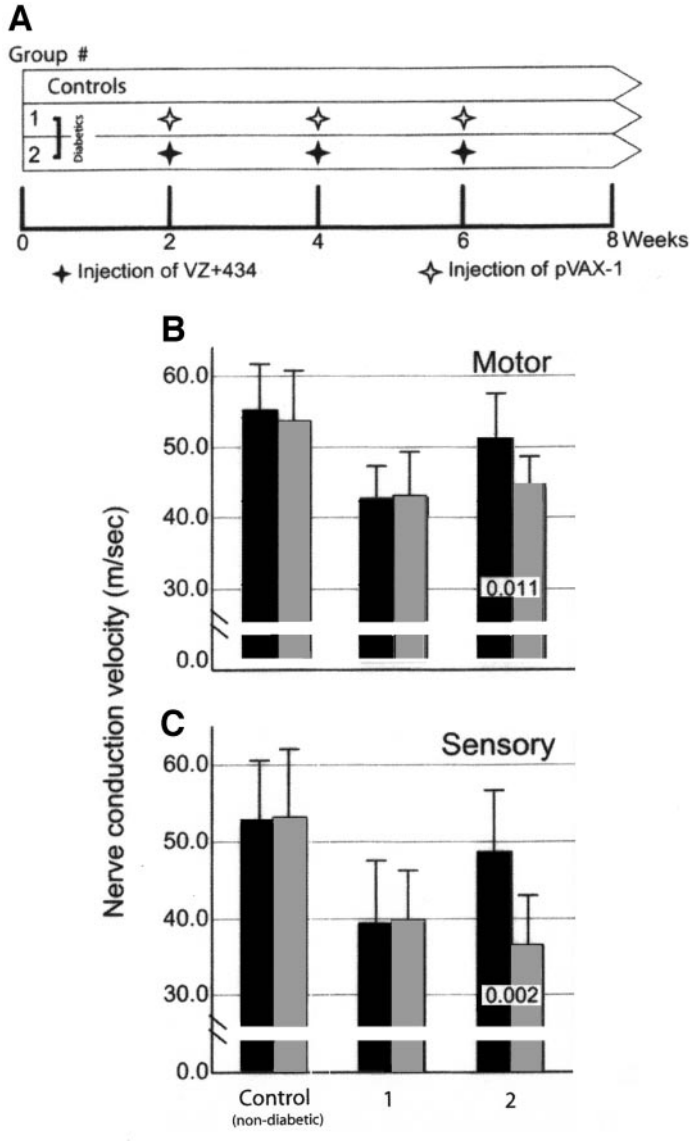

FIG. 4. Effect of VZ+434 repeat administration on SNCVs and MNCVs in STZ-induced diabetic rats. *A*: Experimental study design. MNCV (*B*) and SNCV (*C*) measurements. Two groups of diabetic rats were compared with an untreated, nondiabetic control group. Group 1 received three single injections of 250 μg of the control vector DNA (pVAX-1), given at 2, 4, and 6 weeks post-STZ treatment. Group 2 received a total of three injections of the plasmid expressing VZ+434 (250 μg), one at each of the selected time points as per group 1. The bar charts show NCVs (means ± SD) for injected (■) and uninjected (▨) limbs. *P* values derived from paired *t* tests between NCV values for injected versus noninjected limbs are shown across each column.

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