Protective effect of herpes simplex virus-mediated neurotrophin gene transfer in cisplatin neuropathy

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Summary
Attempts to develop clinical treatments for neuropathy using neurotrophins have not been successful. We tested whether neurotrophin gene delivery to dorsal root ganglia (DRGs) using non-replicating herpes simplex virus (HSV)-based vectors could prevent the development of neuropathy caused by administration of cisplatin. Following subcutaneous inoculation of HSV vectors expressing nerve growth factor (NGF) or neurotrophin-3 (NT-3), neurons in the DRG were transduced to produce NGF or NT-3 in vivo. Inoculation of either the NGF- or the NT-3-expressing vectors 3 days before the start of a 6-week course of cisplatin treatment protected against cisplatin-induced neuropathy assessed by electrophysiological, histological and behavioural measures 2 months later. Iatrogenic neuropathy caused by administration of chemotherapeutic drugs represents an excellent target for a human trial to assess the potential of gene therapy to prevent neuropathy.

Keywords: nerve growth factors; gene therapy; herpes; neuropathy

Abbreviations: CGRP = calcitonin gene-related peptide; DRG = dorsal root ganglion; ELISA = enzyme-linked immunosorbent assay; HCMV IEp = human cytomegalovirus immediate-early promoter; HSV = herpes simplex virus; LAP2 = latency active promoter element; m.o.i. = multiplicity of infection; NGF = nerve growth factor; NT-3 = neurotrophin-3; SP = Substance P; trk = tyrosine receptor kinase


Introduction
During development, neurotrophic factors are produced in close proximity to target neurons where they act at low concentrations through high affinity receptors to prevent the programmed cell death of subpopulations of those neurons (Bothwell, 1995; Kaplan and Miller, 2000). A similarly well-defined role for neurotrophic factor function in the adult nervous system has not been established, but these factors have been shown to provide a protective effect against toxic or traumatic insults to mature neurons in a variety of models (Grill et al., 1997; Zhang et al., 1998; Apfel, 1999). Efforts to extend neurotrophic factor therapy for polyneuropathy or motor neuron disease from animal studies to the treatment of human disease have not met with success (Apfel, 2001, 2002a). The combination of lack of efficacy and/or unacceptable toxicity demonstrated in these human trials in large part results from the failure to achieve adequate neurotrophin levels at the target tissue, and toxicity such as weight loss, injection site inflammation, nausea, stomatitis and fever caused by the systemic administration of these potent and pleiotropic short-lived peptides.

Gene transfer to provide continuous local production of a neurotrophic factor is one approach to achieve a therapeutic effect while avoiding unwanted systemic effects of short-lived peptide factors. We previously have reported that subcutaneous inoculation of a replication-deficient herpes simplex virus (HSV)-based vector encoding either neurotrophin-3 (NT-3) or nerve growth factor (NGF) in vivo prevents the development of neuropathy in the pyridoxine overdose model of subacute sensory neuropathy (Chattopadhyay et al., 2002, 2003) assessed by electrophysiological, morphological and behavioural measures and that subcutaneous inoculation of an NGF-expressing vector protects against the progression of diabetic neuropathy in the mouse (Goss et al., 2002).

Cisplatin is commonly used in the treatment of cancer (Cohen and Lippard, 2001), but causes sensory polyneuropathy after exposure to amounts as low as 200 mg/m2...
Cumulative doses greater than 300 mg/m² uniformly result in a severe sensory neuropathy that limits the maximum chemotherapeutic dose (Cavaletti et al., 1996; Peltier and Russell, 2002). Because cisplatin neuropathy is predictable, begins at a known time and occurs in patients with an underlying systemic malignancy, it represents an ideal condition for phase I/II trials of novel therapeutic approaches to preventing neuropathy. Rodent models of cisplatin treatment faithfully recapitulate the essential aspects of the human disease (Aloe et al., 2000; Shibata et al., 2000), and trophic factors have been demonstrated to be protective in those models (Pradat et al., 2002).

In the current study, we report that transduction of dorsal root ganglion (DRG) neurons by subcutaneous inoculation of HSV-based vectors expressing NT-3 or NGF 3 days before the start of a 6-week course of cisplatin treatment was effective in preventing many of the electrophysiological, histological and behavioural consequences of neuropathy in a rodent model. These results substantially extend the demonstrated time course of the protective effect of vector-mediated gene transfer in neuropathy, and in this animal model relevant to human disease provide pre-clinical data for a human trial of gene transfer in the treatment of neuropathy.

**Methods**

**Vectors**

Four viral vector constructs were employed in this study. SHN contains NGF under the control of the human cytomegalovirus immediate-early promoter (HCMV IEp). SLN contains NGF under the control of the HSV latency active promoter element (LAP2). QL2HNT3 contains NT-3 under the control of a fusion promoter consisting of LAP2 and the HCMV IEp. The control vector QOZHG contains lacZ under the control of the HSV ICP0 promoter and green fluorescent protein under the control of the HCMV IEp.
The construction of vectors has been described previously. Briefly, to construct SHN (Goins et al., 1999), a plasmid containing the coding sequence of murine NGF flanked by the HCMV IEp and SV40 polyadenylation sequences was inserted into the thymidine kinase (TK) locus of an ICP4-deleted HSV 1 recombinant (DeLuca et al., 1985). For vector SLN, HSV LAP2 (Goins et al., 1994) was cloned upstream of the β-NGF sequence in the plasmid in place of the HCMV IEp prior to recombination into the same HSV recombinant. QOZH (Chattopadhyay et al., 2002) was created by a genetic cross of the virus TOZ.1 (ICP4±, ICP27±, ICP22±, UL24± recombinant. QOZH (Chattopadhyay et al., 1985). For vector QOZHG (Chattopadhyay et al., 1999), a plasmid containing the coding sequence of murine NGF flanked by the HCMV IEp and SV40 polyadenylation sequences was inserted into the thymidine kinase (TK) locus of an ICP4-deleted HSV 1 recombinant (DeLuca et al., 1985). For vector SLN, HSV LAP2 (Goins et al., 1994) was cloned upstream of the β-NGF sequence in the plasmid in place of the HCMV IEp prior to recombination into the same HSV recombinant. QOZH (Chattopadhyay et al., 2002) was created by a genetic cross of the virus TOZ.1 (ICP4±, ICP27±, ICP22±, UL24± recombinant. QOZH (Chattopadhyay et al., 1985). For vector SLN, HSV LAP2 (Goins et al., 1994) was cloned upstream of the β-NGF sequence in the plasmid in place of the HCMV IEp prior to recombination into the same HSV recombinant.

Transgene production in vitro and in vivo
Expression in vivo of NGF RNA (Goins et al., 1999; Chattopadhyay et al., 2003) and of NT-3 RNA by vector QL2HNT3 (Chattopadhyay et al., 2002) has been reported previously. NGF and NT-3 protein released by neurons transduced in vitro were measured by enzyme-linked immunosorbent assay (ELISA). Dissociated DRGs from 17-day-old rat embryos were plated on poly-d-lysine-coated coverslips in 500 µl of defined neurobasal medium containing B27, Glutamax I, Albumax II and penicillin/streptomycin (Gibco-BRL, Carlsbad, CA), supplemented with 100 ng/ml of 7.0S NGF/ml (Sigma, St Louis, MO). After 14 days in culture, NGF was removed and 24 h later the cells were infected with either SHN, SLN or QL2HNT3 at a multiplicity of infection (m.o.i.) of 1 for 1 h. The medium was collected 72 h after the infection, and the amount of NGF or NT-3 released was determined using appropriate ELISA kits (Promega, Madison, WI). The amount of NT-3 peptide in sciatic nerve and DRG in vivo 1 week after subcutaneous inoculation of QL2HNT3 was also determined by ELISA.

Cisplatin neuropathy
Male Wistar rats weighing 200–250 g at the start of the study were used in all experiments. Neuropathy was induced by intraperitoneal injection of cisplatin (American Pharmaceutical Partners, Los Angeles, CA) 3 mg/kg twice a week for 6 weeks. Three days prior to the start of cisplatin treatment, 25 µl containing 2.5 × 10⁶ plaque-forming units (p.f.u.) of therapeutic or control vector was inoculated subcutaneously in the plantar surface of both hind feet. Two months after the start of cisplatin treatment (2 weeks after the last dose of cisplatin), electrophysiological and behavioural analyses were performed. At the conclusion of the behavioural analyses, the animals were sacrificed for morphological and molecular biological analyses. Six different groups of 10 animals per group were examined. Control animals received no treatment. Neuropathic animals were inoculated with phosphate-buffered saline (PBS) alone followed by 6 weeks of cisplatin treatment. The remaining four groups were inoculated with vector: (i) QOZH (control vector); (ii) QL2HNT3; (ii) SHN; or (iv) SLN. Ten animals were assigned to each group at the start of the experiment. Animals were injected subcutaneously with 5 ml of normal saline 5 days/week during the 6 weeks of cisplatin treatment. Nonetheless, the animals became ill as a result of the toxic exposure and several animals in each group died; the number was not significantly different between groups (QOZHG, four; QL2HNT3, three; SHN, five; SLN, three; PBS, four).

Electrophysiological measurements
Nerve conduction recordings were made using Nicolet Viking II (Nicolet Biomedical, Madison, WI). Rats were anaesthetized with chloral hydrate (400 mg/kg intraperitoneally), the hind limbs secured at an angle of 30–45° relative to the long axis of the body, and motor nerve conduction velocity and amplitude of the sciatic nerve were determined with a recording electrode inserted in the gastrocnemius muscle. The stimulating electrode pair was placed proximal to the sciatic notch or the knee and a reference recording electrode inserted subcutaneously into the fifth digit of the hind limb. A ground electrode was inserted into the tail and subcutaneous temperature was maintained at 36–37°C. Both latencies and baseline-to-peak amplitudes were determined and conduction velocity was calculated. The H wave was recorded after stimulating at the sciatic notch and recording from a clip electrode placed proximal to the fifth digit. At least eight responses were obtained, and the maximal H wave amplitude determined. For sensory nerve recordings, the electrode placed in the sciatic notch was used as the recording electrode, the stimulating electrode was placed at the ankle digit and the reference electrode was placed at the first digit. The electrophysiological tracings obtained from these types of recordings have been published previously (Chattopadhyay et al., 2002, 2003). The statistical significance of the difference between groups was determined by analysis of variance (ANOVA; Systat 9) using Bonferroni’s correction for the multiple post hoc analyses.

Behavioural evaluation of neuropathy
The severity of neuropathy was assessed by the following tests.

Beam walk. Prior to intoxication, the rats were trained to traverse a 185 cm long dowel, 3 cm in diameter with a pair of black lines 0.6 cm in width painted along the length, 1.05 cm lateral to the midline on each side (Chattopadhyay et al., 2002). Two weeks after the completion of cisplatin treatment, the animals were tested and the placement of the paw (metatarsophalangeal joint) in relation to the score line was evaluated from a videotape recording played at slow speed. The number of slips of the visible hind limb below the painted line on the dowel was recorded.

Rotarod. Prior to cisplatin treatment, the animals were trained on a rotarod device at speeds of 5 and 10 r.p.m. for 5 min. Following the course of cisplatin treatment, on day 15 after the last injection of cisplatin, the animals were retested on the rotating rod and time to fall was recorded. The statistical significance of the difference between groups was determined by ANOVA (Systat 9) using Bonferroni’s correction for the multiple post hoc analyses performed.

Immunocytochemistry
At the completion of testing, animals were perfused transcardially with 0.9% NaCl followed by Zamboni’s fixative (Verdu et al., 1999). The spinal cord and footpads were removed, post-fixed with Zamboni’s fixative for 2 h, and then cryoprotected with 30%
sucrose in PBS overnight. Cryostat sections 10 μm thick were collected on gelatin-coated slides and fixed with 2% paraformaldehyde for 15 min, washed with PBS, incubated with blocking solution (PBS with 1% normal goat serum and 0.3% Triton X-100) for 1 h and washed once. Spinal cord sections were incubated with rabbit anti-calcitonin gene-related peptide (CGRP) or anti-Substance P (SP) antibodies (1 : 1000 dilution; PLI, San Carlos, CA) for 2 h at room temperature; footpads were incubated with mouse anti-β III tubulin (Tuj-1 clone, 1 : 1000 dilution; RDI, Flanders, NJ) overnight at 4°C. All sections were then washed three times and incubated with secondary fluorescent antibody, either goat anti-rabbit IgG for spinal cord or goat anti-mouse IgG for footpad tissue sections (Alexa Fluor 594, 1 : 500 dilution; Molecular Probes, Eugene, OR) for 2 h at room temperature, then washed three times and mounted in water-based Fluoromount G (EMS, Washington, PA).

Morphometric analysis
Immunostained sections were viewed with an Olympus BX61 Fluoview laser-scanning microscope and the digitized images analysed using a PC-based image analysis program (MCID, Imaging Research, Brock, Ontario, Canada) to determine the area occupied by the immunostained sensory nerve terminals in dorsal horn or the proportional area occupied by nerve fibres in the subcutaneous tissue of the footpad. The statistical significance of the difference between groups was determined by ANOVA (Systat 9) using Bonferroni’s correction for the multiple post hoc analyses.

Results
Neurotrophin release in vitro
Each of the vectors produced the appropriate gene product in vitro. Dissociated primary DRG neurons in culture transduced with either QL2HNT3, SHN or SLN at an m.o.i. of 1 resulted in the release of 15.9 pg/10³ cells NT-3, 8.4 pg/10³ cells NGF and 0.37 pg/10³ cells NGF, respectively, into the culture medium over 72 h after infection (Fig. 2).

Neurotrophin production in vivo
We have reported the production of NGF and NT-3 RNA in DRG following subcutaneous inoculation of these vectors (Chattopadhyay et al., 2002, 2003). The production of NT-3 peptide in vivo was confirmed in animals inoculated with QL2HNT3. Five days after subcutaneous inoculation of QL2HNT3 in the foot, a tight ligature was tied around the sciatic nerve, and 24 h later the amount of NT-3 in the DRG and in the sciatic nerve proximal to the ligature was determined by ELISA. Animals transduced with QL2HNT3 had 107.5 ± 1.9 pg of NT3 per mg of protein in the DRG, compared with control DRG that contained 21.4 ± 1.4 pg of NT3 per mg of protein (P < 0.005 by t test). The nerve segment proximal to the ligature in animals inoculated with QL2HNT3 contained 100.7 ± 1.4 pg of NT3 per mg of protein compared with the nerve of control animals which contained 47.4 ± 1.2 pg of NT3 per mg of protein (P < 0.005 by t test). Similar studies could not be performed with NGF because of non-specific cross-reactivity of the secondary antibodies in the commercially available kits (Chattopadhyay et al., 2003).

Vector-mediated trophic factor production preserves sensory nerve function measured electrophysiologically
Animals treated with cisplatin demonstrated a marked decrease in amplitude of the evoked sensory nerve action potential compared with control (control 17.6 ± 0.7 μV, cisplatin 7.2 ± 0.6 μV P < 0.001, Fig. 3). Animals transduced with SHN, SLN or QL2HNT3 3 days prior to cisplatin treatment demonstrated substantial preservation of sensory nerve amplitude (12.1 ± 0.4 μV for SHN, 15.1 ± 2.7 μV for SLN and 13.27 ± 1.2 μV for QL2HNT3, P < 0.005 compared...
with cisplatin treatment, ANOVA, Fig. 3). Animals transduced with control vector QOZHG 3 days prior to cisplatin treatment had a sensory nerve amplitude that was no different from that of cisplatin alone.

The H reflex, a measure of proximal sensory nerve function, was severely attenuated in rats receiving cisplatin compared with control. Animals intoxicated with cisplatin, or intoxicated and treated with QOZHG had essentially no detectable H reflex (control $2.8 \pm 0.2$ mV, cisplatin $0.5 \pm 0.2$ mV, $P < 0.001$, Fig. 4). Animals inoculated with SHN, SLN or QL2HNT3 3 days prior to the start of cisplatin treatment showed preservation of H wave ($2.4 \pm 0.2$ mV for SHN, $3.2 \pm 0.6$ mV for SLN and $2.9 \pm 0.3$ mV for QL2HNT3, $P < 0.005$ compared with cisplatin treatment alone, ANOVA, Fig. 4).

**Functional assessment of nerve function**

In order to test proprioceptive sensory function, rats were trained prior to cisplatin intoxication to walk on a 3.0 cm diameter beam, and tested 2 weeks after completion of cisplatin treatment (2 months after the start of the continuous cisplatin treatment) on the same beam. Control animals had no difficulty traversing the beam, indicated by no slips below the score line. Animals intoxicated with cisplatin experienced substantial difficulty, recording an average of eight slips from the beam during the test period (cisplatin $8.2 \pm 0.9$, control $0.4 \pm 0.2$, $P < 0.001$, Fig. 5). Rats inoculated with SHN, SLN or QL2HNT3 3 days prior to the intoxication performed substantially better both qualitatively and quantitatively than cisplatin-treated animals without vector or inoculated with the control vector (number of slips; $2 \pm 0.3$ for SHN, $1.6 \pm 0.5$ for SLN and $1.8 \pm 0.8$ for QL2HNT3, $P < 0.005$ compared with cisplatin treatment alone, ANOVA, Fig. 5).

Animals were also trained on a rotarod device at speeds of 5 and 10 r.p.m. for 5 min prior to the experiment. As a result of cisplatin-induced sensory nerve damage, treated animals were unable to stay on the rotating rod (control $300$ s; cisplatin $42.04 \pm 17.92$ s, $P < 0.001$, Fig. 6). Neurotrophin vector-inoculated animals tested 2 weeks after the last injection showed a substantial preservation of behavioural function on the rotarod (time to fall $265.46 \pm 35.7$ s for SHN, $286.22 \pm 30.81$ s for SLN and $284 \pm 22.1$ s for QL2HNT3;
$P < 0.001$, compared with cisplatin treatment alone, ANOVA, Fig. 6), while control vector-inoculated animals did not show any improvement ($57.75 \pm 25.91$ s for QOZHG).

**Histology**

After the completion of electrophysiological and behavioural measurements, the animals were sacrificed and morphological and immunohistochemical data assessed in skin and dorsal horn of the spinal cord. Distal innervation of the skin was visualized by immunocytochemical staining using an antibody against β-III tubulin. In control animals, nerve fibres were evenly distributed, while in the cisplatin-treated animals, very few nerve fibres were detectable, and those that were seen were not evenly distributed.

![Image of skin innervation](image-url)

**Fig. 7** Skin innervation was visualized by immunocytochemical staining of nerve branches in subcutaneous tissue using an antibody against β-tubulin. In control animals, nerve fibres were evenly distributed. In the cisplatin-treated animals, few nerve fibres were detectable, and those that were seen were not evenly distributed.
Discussion

In animal models of neuropathy, systemic administration of neurotrophins including NGF, NT-3 and insulin-like growth factor-1 (IGF-1) has been demonstrated to be effective in preventing the progression of neuropathy (Apfel et al., 1994; Fernyhough et al., 1995; Ishii and Lupien, 1995; Zhuang et al., 1996), but side effects following administration to humans have prevented the successful use of neurotrophic factors in the treatment of patients (Apfel, 2002b; Thoenen and Sendtner, 2002). Gene transfer provides the possibility to effect continuous release of short-lived peptide factors that more closely models the natural action of these peptides.

Several different gene therapy approaches to treat neuropathy have been reported. Transduction of muscle either by electroporation-mediated transfer (Pradat et al., 2001a) or by inoculation of an adenoviral vector has been used to produce elevation of systemic neurotrophin levels and has been shown to prevent some aspects of neuropathy caused by diabetes, acrylamide or cisplatin treatment (Pradat et al., 2001b, 2002). However, there are significant limitations to the use of muscle-based gene delivery for the treatment of neuropathy. The amounts of vector required to achieve appropriate circulating levels in a larger animal or human are substantial, as experience with muscle-based gene therapy for haemophilia has demonstrated (High, 2003), and if adequate circulating levels are achieved it may be difficult to avoid the adverse effects resulting from neurotrophin-mediated activation of non-neuronal cells or neuronal populations not intended as targets of that therapy.

Vectors based on HSV possess properties that make them uniquely suited for gene transfer to peripheral nerve. Following primary epithelial infection by HSV (Roizman and Sears, 1996), the viral envelope fuses with the axolemmal membrane of cutaneous nerve terminals, the virion is internalized, and the capsid is carried by retrograde axonal transport to the sensory ganglion where the DNA is injected through a nuclear pore into the nucleus. This process, which depends on both non-specific and selective interactions between envelope glycoproteins and components of the cell membrane (Spear et al., 2000; Shukla and Spear, 2001) as well as specific capsid–transport motor interactions in the axon (Ojala et al., 2000; Ye et al., 2000; Smith et al., 2001), is highly efficient. Intranuclear viral genomes naturally establish a lifelong persistent state as an episomal element (Steiner and Kennedy, 1995). Deletion of one or more essential immediate-early (IE) HSV genes results in recombinants that are severely impaired in their ability to express early (E) and late (L) viral genes, but which can be propagated to high titre on cell lines engineered to provide the IE gene products in trans without rescue of wild-type virus (Ozuer et al., 2002).

In the current study, we have demonstrated that replication-incompetent HSV vectors expressing NGF or NT-3 delivered by subcutaneous inoculation 3 days prior to the onset of cisplatin treatment effectively prevent many of the electrophysiological, histological and behavioural manifestations of cisplatin-treated animals (control 0.097 ± 0.009; cisplatin-treated 0.019 ± 0.01, P < 0.01, Fig. 8). Animals inoculated with SHN, SLN or QL2HNT3 3 days prior to intoxication showed substantial preservation of sensory nerve fibres in the foot (SHN 0.066 ± 0.01; SLN 0.078 ± 0.02; QL2HNT3 0.096 ± 0.01 P < 0.01 compared with cisplatin-treated alone, ANOVA, Fig. 8). Control vector-treated animals showed no preservation of skin innervation (QOZH G 0.028 ± 0.009).

Neuropeptide expression

The amount of immunoreactive CGRP and SP in the dorsal horn was reduced in animals treated with cisplatin (Figs 9 and 10). Animals inoculated with SHN, SLN or QL2HNT3 showed preservation of peptide-immunoreactive fibres in dorsal horn that was substantial and statistically significant for both CGRP and SP. The proportional area occupied by the CGRP nerve terminals in the control was 31.1 ± 4.7 μm² as compared with 14.0 ± 5.3 μm² in cisplatin-treated animals (P < 0.01, ANOVA, Fig. 9). Inoculation with SHN (27.5 ± 2.9 μm²), SLN (21.8 ± 5.3 mm²) or QL2HNT3 (29.9 ± 2.3 μm²) substantially and significantly increased the area of CGRP-immunoreactive fibres in the dorsal horn compared with control QOZH G-inoculated animals (17.3 ± 2.3 μm², P < 0.001 for SHN, QL2HNT3, P < 0.005 for SLN, ANOVA, Fig. 9).

The area occupied by SP-immunoreactive terminals in cisplatin-treated animals compared with normal was also severely reduced (control 28.2 ± 2.5 μm², cisplatin-treated 12.8 ± 2.6 μm², P < 0.001, ANOVA, Fig. 10). Inoculation with SHN (21.3 ± 1.3 μm²), SLN (22.4 ± 1.1 μm²) or QL2HNT3 (27.5 ± 1.2 μm²) 3 days prior to the start of cisplatin treatment substantially preserved SP immunoreactivity as well compared with QOZH G-inoculated cisplatin-treated animals (14.1 ± 1.6 μm², P < 0.001 for QL2HNT3, P < 0.005 for SHN and SLN, ANOVA, Fig. 10).
cisplatin-induced neuropathy. The neurotrophic-expressing HSV vectors efficiently transduced neurons, as demonstrated by the high levels of neurotrophin release from DRG neurons infected in vitro at an m.o.i. of 1. Subcutaneous inoculation of these vectors resulted in delivery of the transgene to the DRG as confirmed by RT–PCR (reverse transcription–PCR) demonstration of transgene RNA in DRGs (Chattopadhyay et al., 2002, 2003). Because IE gene-deleted HSV vectors are incapable of replication in the absence of complementing gene products, the vector genomes in the DRG meet the molecular definition of latency (i.e. they are incapable of reactivation) but fail to meet the full biological definition of latency (which requires the ability to reactivate). Complementing the expression of RNA in the DRG, we found a substantial increase in NT3 peptide by ELISA of DRG and nerve of animals transduced with QL2HNT3. Similar studies of NGF production were not possible because of cross-reactivity of the secondary antibodies in the ELISA kits with rat serum, but it seems reasonable to assume that the NGF RNA similarly results in increased NGF protein.

The mechanisms through which NGF or NT-3 prevents the deleterious effects of cisplatin in vitro or in vivo have not been fully defined. Cisplatin causes platination of DNA in the DRG (Meijer et al., 1999), reduction in nucleolar size in DRG neurons (McKeage et al., 2001), increased expression of gene products associated with re-entry into the cell cycle (Gill and Windebank, 1998) and redistribution of bax and mitochondrial release of cytochrome c (McDonald and Windebank, 2002) leading to cell death by apoptosis. Increased expression of cell cycle markers and redistribution of bax are both prevented by NGF in vitro (Gill and Windebank, 1998; McDonald and Windebank, 2002). In studies in vitro, we have observed that the effects of NGF on signal transduction cascades are reproduced by transduction with vector SHN (data not shown), indicating that the vector-mediated effects of the transgene product activate the same pathways as the peptide alone. Neurotrophins released from transduced neurons may act through receptors on those neurons to protect the transduced cells (autocrine effect) or on neighbouring neurons (paracrine effect). That paracrine
effects occur is suggested by vector-mediated preservation of the H reflex, because the sensory neurons subserving this function project peripherally to muscle spindles and are therefore not directly exposed to the vector injected subcutaneously.

We observed a broad specificity of transgene-mediated neurotrophic effects. The NGF-expressing vectors protected the H wave against cisplatin toxicity, despite the fact that this response reflects activity in large myelinated spindle afferents that express predominantly C high affinity neurotrophin receptors. The NT-3-expressing vector protected SP- and CGRP-containing fibres derived from small DRG neurons that express trkA. The effect of NGF on large myelinated afferents corresponds to a similar result we observed in the pyridoxine model of pure sensory neuropathy (Chattopadhyay et al., 2003), an effect that is probably due to the expression of a relatively low level of functional trkA receptors on large DRG neurons with large diameter myelinated fibres. Regarding the effect of NT-3 on trkA-expressing cells, there is evidence from knockout mice that NT-3 may function through the trkA receptor (Tessarollo et al., 1997), and that in the absence of the low affinity p75NTR, NT-3 may substitute for NGF in the activation of trkA (Francis et al., 1999; Harrison et al., 2000).

In these experiments, the animals were inoculated with the vectors before the onset of cisplatin treatment. There are several reasons for doing this. Cisplatin neuropathy is a predictable disease and one would anticipate inoculating the vectors prior to cisplatin treatment in a human trial. Because most clinically relevant neuropathies are slowly progressive diseases with no currently available treatment, it should be possible even in those situations to treat early in the disease and prevent further progression.

The amount and time course of neurotrophin peptide produced by vector transduction are dependent on the promoter construct employed. In experiments with DRG neurons in vitro, we have found that the HCMV IEp produces high levels of transgene expression that begins within 24 h of

Fig. 10 SP immunoreactivity in dorsal horn assessed by immunocytochemical staining. Cisplatin treatment resulted in a substantial reduction in SP immunoreactivity that was prevented by inoculation with SHN, SLN or QL2HNT3. Mean ± SEM, *P < 0.005, **P < 0.001 ANOVA compared with cisplatin-treated animals. Representative sections of immunostained spinal cord are shown. Control, untreated; the remainder all received 6 weeks of cisplatin treatment and the vector as indicated.
infection, peaks at 48–72 h, and then declines (data not shown). Vectors in which transgene expression was driven by the HCMV IEp (SHN) or the hybrid LAP2–HCMV IEp promoter (QL2HNT3) produced 8–16 pg of neurotrophin peptide per $10^5$ cells over 72 h. In vivo studies using vectors expressing neurotransmitter peptides under the control of the same promoter demonstrate the loss of transgene product bioactivity by 3–4 weeks after inoculation (Goss et al., 2001; Hao et al., 2003). The LAP2 promoter was originally identified on the basis of its ability to drive expression of HSV latency-associated transcripts (LATs) long after the primary infection (Goins et al., 1994) and provide a transportable element to drive prolonged transgene expression from ectopic loci within the HSV genome (Goins et al., 1994). Cells transduced with vector SLN (in which NGF expression is controlled by the LAP2 promoter element alone) produced substantially less NGF ($0.37$ pg/$10^5$ cells/72 h) in vitro than cells transduced with SHN. Nonetheless, we observed that neurotrophin expression driven by any of the promoters was sufficient to protect against sensory neuropathy after 2 months of cisplatin treatment. These results suggest either that transient high level NGF expression driven by the HCMV IEp may be sufficient to establish a state that protects the nerve against subsequent toxic insult over a period of weeks, or that the low level of transgene expression produced by the HCMV IEp at late time points like LAP2-driven transgene expression is sufficient to produce the required therapeutic effect. We are carrying out delayed experiments in an attempt to distinguish these two possibilities.

We previously have demonstrated that neurotrophin-expressing replication-incompetent HSV vectors delivered by subcutaneous inoculation to transduce DRG neurons in vivo effectively prevent the development of neuropathy in models of diabetic (Goss et al., 2002) and pyridoxine-induced neuropathy (Chattopadhyay et al., 2002, 2003). Despite substantial evidence in animal models demonstrating that neurotrophic factors are beneficial in the treatment or prevention of peripheral neuropathy, the treatment of human neuropathies has been limited because of the short half-life of these peptide factors and the side effects engendered by systemic administration. The results of this study demonstrate that gene transfer to the DRG using HSV-based vectors to produce expression of neurotrophins directly in the DRG may circumvent the delivery problem, and thus allow the use of the therapeutic potential of these trophic factors in the treatment of cisplatin neuropathy. Because iatrogenic neuropathies caused by chemotherapeutic drugs occur with a known onset, follow a subacute course and occur in patients with a life-threatening disease, they represent an excellent target for the first human trials of gene transfer to prevent neuropathy.

Acknowledgements
We acknowledge the excellent technical assistance of Vikram Thakur in preparing the vectors and performing the electro-

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