Gene Transfer to the Peripheral Nervous System: Treatments for Polyneuropathy and for Pain

David J. Fink, MD

Department of Neurology
University of Michigan
Ann Arbor VA Healthcare System
Ann Arbor, Michigan

Introduction

Gene transfer to the peripheral nervous system poses special challenges. The target cells—sensory neurons with cell bodies located in the dorsal root ganglia—are widely distributed and relatively inaccessible. In addition, the pseudo-unipolar axons projecting peripherally to the target organ and centrally to the spinal cord are large in comparison with the size of the cell body.

The two categories of disease processes for which gene transfer to the peripheral nervous system would be desirable are sensory polyneuropathy and chronic pain. Polyneuropathy refers broadly to a family of conditions in which peripheral sensory axons degenerate, often in a length-dependent fashion. With the exception of immune-mediated neuropathies that can be treated by immunomodulation, there are no available treatments to effectively prevent the progression of neuropathy resulting from systemic illness (e.g., that caused by diabetes), toxic exposure (e.g., chemotherapy-induced peripheral neuropathy), or genetic defect (e.g., Charcot-Marie-Tooth disease).

Challenges in the Development of Treatments for Pain and Polyneuropathy

Pain is a complex experience comprising sensory discriminative, cognitive, and emotional components. Acute pain is initiated by the activation of a subset of sensory afferents (nociceptors). These nociceptors transmit nociceptive information centrally through a well-characterized ascending pathway that serves to warn the individual of potentially harmful stimuli in the environment, often leading to a reflex withdrawal response. Chronic pain that results from continued activation of nociceptors, or from damage to the neural structures serving pain perception, is equally unpleasant but characteristically results in reduced activity and avoidance of contact. Although the dorsal root ganglia (DRG) are neither necessary nor sufficient for the perception of chronic pain, most of the common forms of chronic pain proceed through the same anatomical pathways as those utilized for acute pain and involve first-order synapses in the dorsal horn of the spinal cord.

For the treatment of polyneuropathy, extensive preclinical animal studies beginning in the 1990s demonstrated that neurotrophic factors delivered by intraperitoneal injection could effectively prevent the progression of polyneuropathy resulting from any of a number of causes, including diabetes, toxic exposures, or genetic defect. Subsequent work extended the range of factors from the classical neurotrophins (e.g., nerve growth factor [NGF], neurotrophin-3) to other peptides with neurotrophic effects, including insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), and erythropoietin (EPO). However, despite the abundant evidence that systemically administering these factors is effective in preventing the progression of neuropathy in animal models, clinical trials have failed to demonstrate a therapeutic effect in patients. Although there are many possible explanations for these discordant results, one obvious problem is that the dose of peptide factor utilized in the animal studies (typically in the range of 5–10 mg/kg) was much higher than the doses tolerated by patients. For example, in the Phase 3 clinical trial of NGF for diabetic neuropathy, a dose of 0.1 μg/kg proved ineffective in preventing the progression of neuropathy in these patients.

A conceptually similar difficulty confronts the treatment of chronic pain. Nociceptive neurotransmission at the first synapse in the dorsal horn between the primary nociceptor and second-order neurons projecting rostrally is subject to complex modulatory influence. This influence is mediated by inhibitory neurotransmitters released from interneurons under the control of descending inputs. Pharmacological activation of these inhibitory neurotransmitter receptors (predominantly but not limited to opioid and GABA), either presynaptically on primary afferents or postsynaptically on second-order neurons, represents one effective means of modulating chronic pain. However, the same receptors are widely distributed throughout the central neuraxis and, in the case of opioids, on nonneural structures as well. Therefore, off-target effects unrelated to analgesia that are elicited by systemic administration of opiates or GABA-active drugs limit their use for pain relief. Alternatively, targeting delivery of these drugs to the spinal level by intrathecal administration allows one to increase the effective dose 10-fold.

Gene transfer offers the possibility of a highly selective, targeted release of bioactive molecules within the nervous system. This method is able to target delivery of neurotrophic factors to the primary sensory afferent for treating polyneuropathy, or of inhibitory neurotransmitters for relieving chronic pain. Through autocrine and paracrine effects, the release of neurotrophic factors from transduced primary sensory afferents could protect sensory neurons from degeneration without requiring high-

dose systemic delivery. Similarly, the release of inhibitory neurotransmitters from primary sensory afferent terminals in the dorsal horn could provide an analgesic effect without engendering side effects by activating these receptors in other sites within the nervous system or other tissues.

Herpes Simplex Virus as a Gene Transfer Vehicle

Among the available gene transfer vectors, herpes simplex virus (HSV) is particularly well suited for the delivery of genes to the DRG. HSV possesses a natural tropism for peripheral sensory neurons of DRG, where the virus establishes a latent state in which viral genomes persist for the life of the host as intranuclear episomal elements. The lifelong persistence of latent genomes in trigeminal ganglion, without the development of sensory loss or histologic damage to the ganglion, attests to the effectiveness of these natural latency mechanisms. Wild-type virus may be reactivated from latency under the influence of a variety of stresses. However, recombinant vectors that are entirely replication-defective retain the ability to establish persistent quiescent genomes in neurons but are unable to replicate (or reactivate) in the nervous system.

Anatomy of HSV and the latent state

The HSV particle consists of a nucleocapsid surrounded by an envelope containing glycoproteins essential for virus attachment and penetration into cells. The HSV genome contains 152 kb of linear, double-stranded DNA encoding more than 80 gene products and consisting of two segments: a unique long (U_L) and unique short (U_S) segment, each of which is flanked by inverted repeats containing important immediate-early (IE) and latency genes. The viral genes are found almost entirely as contiguous transcribable units, making their genetic manipulation relatively straightforward.

In wild-type infection, the virus is transmitted by direct contact, replicating initially in epithelial cells of skin or mucous membranes. Second-generation virions are taken up by sensory nerve terminals and carried by retrograde axonal transport to the neuronal perikaryon in DRG, where viral DNA is injected through a modified capsid penton into the nucleus. In the lytic replication cycle, expression of the viral IE genes (which occurs in the absence of *de novo* protein synthesis) serves to transactivate expression of early (E) genes. Removing essential IE genes from the HSV genome results in the creation of vectors that are unable to enter the lytic cycle

in noncomplementing cells but are nonetheless transported in a normal fashion to the nucleus, where they establish a persistent latent state (Krisky et al., 1998; Wolfe et al., 1999; Fink et al., 2000).

The latent state occurs naturally only in neurons. In this state, following injection of the viral genome into the nucleus, expression of the gene products characteristic of lytic infection is repressed, and the viral genome persists as an intranuclear episomal element. Latent genomes continue to transcribe only one segment of the inverted repeat sequences in UL, just downstream of, and from the strand opposite, the IE *ICP0* gene to produce a family of latency-associated transcripts (LATs). Latent genomes are partially methylated and sequestered as an episomal minichromosome-like structure bound by nucleosomes; in this state, they have no discernible effect on host-cell metabolism or phenotype. Nonreplicating vectors constructed by deleting essential IE genes are forced into a pseudolatent state.

Preclinical studies of HSV-mediated gene transfer in models of polyneuropathy

We have tested nonreplicating HSV vectors in several models of neuropathy. In selective large myelinated fiber degeneration caused by high-dose pyridoxine (PDX), subcutaneous inoculation of a nonreplicating HSV vector coding for neurotrophin-3 (NT-3) resulted in the preservation of sensory nerve amplitude, sensory nerve conduction velocity, and amplitude of the H-wave. Further, it protected large myelinated fiber proprioceptive sensory function and preserved large myelinated fibers in nerve and in the dorsal horn of spinal cord (Chattopadhyay et al., 2002). Similar results were obtained by injecting a nonreplicating HSV vector expressing NGF. In a model of toxin-induced neuropathy caused by cisplatin, subcutaneous inoculation of HSV vectors constructed to express either NGF or NT-3 took place just before a 6-week course of cisplatin. The treatment resulted in significant protection against the development of neuropathy, as assessed by electrophysiological, behavioral, and morphological outcomes (Chattopadhyay et al., 2004).

A model of type 1 diabetes in Swiss Webster mice was created by injecting the animals with streptozotocin (STZ). The subcutaneous inoculation of a nonreplicating HSV vector expressing either NGF (Goss et al., 2002a), VEGF (Chattopadhyay et al., 2005a), or EPO (Chattopadhyay et al., 2009) into both hind feet 2 weeks after inducing diabetes

prevented the loss of sensory nerve action potential amplitude characteristic of neuropathy. Results were measured 4 and 8 weeks after the injection of STZ.

In these initial studies, we employed the human cytomegalovirus immediate early promoter (HCMV IEp) to drive transgene expression and examined the biological effect of vector-mediated transgene expression up to 2 months after inoculation. To achieve prolonged transgene expression, we employed the HSV latency-associated promoter 2 (LAP2) element (nucleotides 118866–119461 of the HSV genome). LAP2 is the sequence responsible for lifelong expression of latency-associated transcripts in neurons infected with wild-type virus. Using a vector with the LAP2 promoter driving expression of NT-3, we found that five and a half months after vector inoculation, animals inoculated with the LAP2-driven NT-3-expressing vector showed preservation of peripheral nerve function in the face of subacute PDX intoxication (Chattopadhyay et al., 2005b). Similarly, in the STZ diabetes model, mice inoculated with the NT-3 expressing vector were protected against the progression of diabetic neuropathy during the course of 6 months (Chattopadhyay et al., 2007).

Because prolonged expression of neurotrophic factors could have unwanted adverse effects, we constructed a nonreplicating HSV vector, vHrtEPO, to express EPO under the control of a tetracycline response element (TRE)–minimal CMV fusion promoter. Primary DRG neurons in culture infected with vHrtEPO express and release EPO in response to exposure to doxycycline (DOX). Animals infected with vHrtEPO by footpad inoculation demonstrated regulated expression of EPO in DRG under the control of DOX administered by gavage. Mice rendered diabetic by injection of STZ, inoculated with vHrtEPO, and treated with DOX 4 days out of 7 each week for 4 weeks were protected against the development of diabetic neuropathy, as assessed by electrophysiological and behavioral measures. These studies indicate that intermittent expression of EPO in DRG, achieved from a regulatable vector, is sufficient to protect against the progression of neuropathy in diabetic animals and provides proof-of-principle preclinical evidence for the development of such vectors for clinical trials.

Preclinical studies of HSV gene transfer for pain

The efficacy of HSV-mediated gene transfer of enkephalin has been tested in several different models of pain in rodents. Pohl and colleagues first showed that a *tk*-defective HSV recombinant, injected subcutaneously in the paw, will transduce DRG neurons that express enkephalin in DRG (Antunes Bras et al., 1998). Wilson and colleagues subsequently demonstrated that a similar tk⁻ HSV–based vector containing the human proenkephalin gene, and injected subcutaneously into the paw, reduces hyperalgesic C-fiber responses ipsilateral to the injection (Wilson et al., 1999). Pohl and colleagues went on to show that subcutaneous inoculation of the vector reduces pain-related behaviors in a rodent model of chronic pain related to polyarthritis induced by injection of complete Freund's adjuvant (CFA) (Braz et al., 2001). Expression of enkephalin from the vector not only reduced pain-related behaviors but also prevented cartilage and bone destruction in the inflamed joints, presumably owing to the release of enkephalin from the peripheral sensory terminals in the joint (Braz et al., 2001). These findings correlated with those demonstrating that axonal transport of the transgene product from transduced cells carries the transgene product toward the periphery (as well as toward the spinal cord), an effect that could be demonstrated by applying a ligature to the nerve (Antunes Bras et al., 2001).

Subcutaneous inoculation of an enkephalin-producing, nonreplicating vector produces an analgesic effect in the delayed phase of the formalin model of inflammatory pain (Goss et al., 2001) in two disease models: the selective spinal nerve ligation model of neuropathic pain (Hao et al., 2003) and the infraorbital nerve constriction model of craniofacial pain (Meunier et al., 2005).

In experiments designed to test the effect of the vector on visceral pain, investigators have injected the vector directly into the end organ rather than the skin. Yoshimura et al. (2001) demonstrated that injecting the nonreplicating enkephalin-expressing HSV vector into the rat bladder wall results in enkephalin expression in relevant DRG. They also demonstrated that vector-mediated enkephalin effectively attenuated capsaicin-induced bladder irritation and resultant bladder hyperactivity (Goins et al., 2001; Yoshimura et al., 2001). Similarly, Westlund and colleagues have shown, in rodent models of acute and chronic pancreatitis, that directly injecting an enkephalin-expressing HSV vector into the pancreas attenuates evoked nocisponsive behaviors (Lu et al., 2007; Yang et al., 2008). In the pancreas, enkephalin expression appeared to reduce the inflammatory response, analogous to the effect reported in polyarthritis (Braz et al., 2001). In a mouse model of bone cancer

pain, subcutaneous inoculation of the HSV vector expressing enkephalin resulted in an attenuation of spontaneous nocisponsive behaviors (Goss et al., 2002b).

Studies of the enkephalin-expressing HSV vector have been extended to primates. Yeomans et al. (2006) demonstrated that peripheral application of the HSV vector expressing enkephalin to the dorsal surface of the foot of macaques reduced A-delta and C-fiber–mediated pain-related responses.

Taken together, these results from several different groups of investigators provide proof-of-principle that HSV vector–mediated delivery of enkephalin can provide an analgesic effect and set the stage for a human trial to treat chronic pain using HSV vector–expressed enkephalin (see below).

Our groups have examined HSV vectors constructed to express other inhibitory neurotransmitters. Endomorphin-2 (EM-2; Tyr-Pro-Phe-Phe-NH2) is an endogenous, highly selective μ-opioid receptor agonist (Zadina et al., 1997), but the gene coding for EM-2 has not yet been identified. We therefore constructed a tripartite synthetic gene cassette with the N-terminal signal sequence of human preproenkephalin (PENK), followed by a pair of EM-2 coding elements, including the addition of a C-terminal glycine residue flanked by dibasic cleavage sites. The gene product is processed by the cellular machinery that processes PENK to enkephalin (Wolfe et al., 2007), and the C-terminal glycine in the cleaved product directs amidation of the cleaved peptide by the widely distributed enzyme peptidylglycine α-amidating mono-oxygenase.

Our findings showed that subcutaneous inoculation of the endomorphin-expressing HSV vector into the footpad of rats with neuropathic pain (induced using selective L5 spinal nerve ligation) resulted in a significant reduction in both mechanical allodynia and thermal hyperalgesia that could be blocked by the highly selective μ-opioid receptor antagonist CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr amide) (Wolfe et al., 2007). It also resulted in a substantial reduction in nocisponsive behaviors in the delayed phase of the formalin test and in the CFA model of inflammatory pain (Hao et al., 2009).

Nocisponsive behaviors can also be attenuated by using HSV vector–mediated gene transfer to modulate the expression of μ-opioid receptors in primary sensory afferents. Consistent with a centrally mediated effect, cutaneous application of an HSV vector (defective in its expression of the viral *thymidine kinase* gene and with the human μ-opioid receptor cDNA in reverse orientation) results in decreased expression of μ-opioid receptors on the central primary sensory afferent terminals in the dorsal horn of the spinal cord and reduced potency of intrathecal [D-Ala^2,N-MePhe^4,Gly-ol^5] enkephalin (DAMGO) on C-fiber nociceptive responses (Jones et al., 2003). Conversely, cutaneous application of an HSV vector expressing the μ-opioid receptor gene in the sense orientation increases μ-opioid receptor immunoreactivity in primary sensory afferents and a leftward shift in the dose response to intraperitoneal lopiramide, indicating an effect at transgene-mediated μ-opioid receptors expressed on the peripheral terminals of the primary sensory neurons (Zhang et al., 2008).

We constructed a replication-incompetent HSV vector encoding the 67 kD isoform of human GAD (Liu et al., 2004). In the selective spinal nerve ligation model of neuropathic pain, inoculation of the GAD-expressing vector resulted in a substantial reduction in mechanical allodynia and thermal hyperalgesia (Hao et al., 2005). In neuropathic pain, the analgesic effect of the GAD-expressing vector is greater in magnitude than the effect produced by either the enkephalin- or endomorphin-expressing vectors. This finding is consistent with the evidence that development of chronic pain after peripheral nerve injury is accompanied by the loss of GABAergic tone in the dorsal horn of the spinal cord (Moore et al., 2002) and the clinical observation that opiate drugs are relatively ineffective in the treatment of neuropathic pain. The GAD-expressing HSV vector also reduces pain-related behaviors in a model of central neuropathic pain created by T13 spinal cord hemisection (Liu et al., 2004).

Phase 1 human trial of a preproenkephalin-expressing HSV vector

Based on the preclinical data, we proceeded to conduct a multicenter, dose-escalation Phase 1 clinical trial of NP2, a replication defective, HSV-based vector expressing human PENK, in subjects with intractable focal pain caused by cancer. NP2 was injected intradermally into the dermatome(s) corresponding to the radicular distribution of pain. The primary outcome was safety. A secondary endpoint, efficacy of pain relief, was assessed using a numeric rating scale (NRS), the Short Form McGill Pain Questionnaire (SF-MPQ), and concurrent opiate usage.

Ten subjects with moderate to severe intractable pain despite treatment with more than 200 mg/day of morphine (or equivalent) were enrolled into the study. Treatment was well tolerated, with no study agent–related serious adverse events observed at any point in the study. Subjects receiving the low dose of NP2 reported no substantive change in pain. Subjects in the middle-dose and high-dose cohorts reported pain relief, as assessed by NRS and SF-MPQ. Treatment of intractable pain with NP2 was well tolerated. There were no placebo controls in this relatively small study, but the dose-responsive analgesic effects were encouraging, and a Phase 2 placebo-controlled trial has been initiated.

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