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Notes:
Pleiotrophin is a neurotrophic factor for spinal motor neurons

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Regeneration in the peripheral nervous system is poor after chronic denervation. Denervated Schwann cells act as a “transient target” by secreting growth factors to promote regeneration of axons but lose this ability with chronic denervation. We discovered that the mRNA for pleiotrophin (PTN) was highly up-regulated in acutely denervated distal sciatic nerves, but high levels of PTN mRNA were not maintained in chronically denervated nerves. PTN protected spinal motor neurons against chronic excitotoxic injury and caused increased outgrowth of motor axons out of the spinal cord explants and formation of “miniventral rootlets.” In neonatal mice, PTN protected the facial motor neurons against cell death induced by deprivation from target-derived growth factors. Similarly, PTN significantly enhanced regeneration of myelinated axons across a graft in the transected sciatic nerve of adult rats. Our findings suggest a neurotrophic role for PTN that may lead to previously unrecognized treatment options for motor neuron disease and motor axonal regeneration.

Peripheral nerve injury leads to Wallerian degeneration of axons and denervation of Schwann cells distal to the site of injury. Denervated Schwann cells secrete a variety of growth factors and assume the role of “transient target” for regenerating axons (1, 2). Among these neurotrophic molecules are well-known ones such as nerve growth factor and glial cell line-derived neurotrophic factor (GDNF). Up-regulation of neurotrophic factors allows regeneration of axons when a repair is made promptly or the gap between the transected ends of the axon is not too long. However, if the duration of denervation is prolonged or when the distances that need to regenerate are very long, as is frequently the case in humans, success of regeneration and functional recovery are suboptimal (3–10).

To identify candidate growth factors underlying neurotrophic support by Schwann cells, we used cDNA microarrays to investigate the gene expression of neurotrophic factors in denervated distal nerve stumps. Pleiotrophin (PTN) gene expression is up-regulated in the distal nerve stump immediately after sciatic nerve transection. PTN (also termed heparin binding growth-associated molecule or heparin binding neurotrophic factor) is a 168-aa, heparin binding secreted protein. PTN, isolated initially from rat uterine as a mitogen for NIH 3T3 cells (11), is a member of the midkine family that has cysteine- and basic amino acid-rich residues distinct from other heparin binding growth factor families (12–14).

In addition to the mitogenic effect on fibroblasts, PTN has activity in a variety of tissues and cell types (14–16). In the nervous system, it has been shown to induce neurite outgrowth from PC-12 rat pheochromocytoma cells (17), and cortical (11) and dopaminergic neurons (18, 19). PTN is also expressed in the developing peripheral nervous system and muscle, and plays a role in postsynaptic clustering of acetylcholine receptors (20). Here, we show that PTN acts as a neurotrophic factor for spinal motor neurons and protects them against chronic excitotoxic cell death in vitro. Furthermore, we show that PTN promotes enhanced regeneration of peripheral nerve axons after sciatic nerve transection and protects neonatal facial motor neurons against cell death induced by deprivation from target-derived neurotrophic support.

Results

PTN Is Up-Regulated in Denervated Schwann Cells and Muscle After Axotomy. To identify candidate neurotrophic factors underlying adaptive responses to chronic nerve degeneration, we used focused cDNA microarrays to investigate the gene expression of neurotrophic factors in denervated Schwann cells. In microarray experiments, 2 and 7 days after the sciatic nerve transection, PTN mRNA was up-regulated in the distal denervated segments compared with the contralateral side (data not shown). To confirm the up-regulation of the PTN mRNA observed in the microarray analysis and further explore the pattern of expression, we performed PTN mRNA measurements in denervated nerves from 2 days to 6 months of denervation. As seen in Fig. 1A, PTN levels were up-regulated in denervated distal sciatic nerves as soon as 2 days after nerve transection and peaked by 7 days. However, the PTN mRNA levels returned back to baseline levels by 3 months. This observation shows that PTN mRNA is up-regulated in acutely denervated Schwann cells, but this up-regulation is not maintained over time, and chronically denervated Schwann cells lose their ability to make PTN. This pattern of mRNA expression mirrors what has been observed with GDNF expression in denervated nerves (21).

As a potential target derived neurotrophic factor, we examined the expression pattern of PTN in muscle during development and after denervation. As seen in Fig. 1B, PTN mRNA is expressed at very high levels in embryonic muscle but is down-regulated to nearly undetectable levels in adult muscle. However, PTN mRNA is rapidly up-regulated in the adult muscle on denervation within days; this mirrors the expression pattern in denervated Schwann cells. In contrast to muscle, there was no significant up-regulation of PTN mRNA in denervated footpad skin (data not shown).

PTN Causes Increased Outgrowth of Motor Axons Out of Spinal Cord Explants. Changes in the pattern of expression of PTN suggested that it might have a neurotrophic role in motor neurons. To examine this potential role for PTN, we used the spinal cord explant culture system. This system has been used extensively to study neuroprotective and trophic properties of growth factors (22–24). In this system, spinal cord slice cultures are prepared on culture inserts with semipermeable membranes and allowed to acclimatize to the
culture conditions for a week. After 1 week, there is a stable population of surviving motor neurons per slice that remain alive in the culture for months. During chronic culture, motor neurons extend axons that can be labeled with anti-neurofilament antibodies, but these axons usually remain in the gray matter and rarely cross the gray–white matter junction and extend out of the explants.

In spinal cord explants where PTN was applied away from the explant in the form of gelfoams soaked with recombinant human PTN (100 ng/ml), there was extensive outgrowth of motor axons out of the explants (Fig. 2). In fact, the motor axons formed “miniventral rootlets.” This pattern of motor axon outgrowth was not seen with gelfoams soaked with the vehicle. In similar experiments, parallel results were observed when the source of PTN was HEK-293PTN cells, but not when the HEK-293vector cells were used (data not shown).

In addition to increased axonal outgrowth induced by gelfoams soaked with PTN, we explored the effect of PTN diffusely available in the culture system. We cultured HEK-293PTN or control HEK-293vector cells in six-well dishes, and then placed filters containing “mature” spinal cord explants (i.e., explants that had been cultured for a week) on top of the HEK-293PTN or control HEK-293vector cells. By using dot blot with appropriate standards, we quantified the amount of PTN in the culture medium when HEK-293PTN cells were used and found it to be ~80–100 ng/ml. After 7 days of coculture, there was a dramatic increase in the number of motor axons traversing the gray–white matter junction and growing into the degenerated white matter tracts (Fig. 2). Similar data were obtained with the recombinant human PTN at a concentration of 100 ng/ml (data not shown).

Because the spinal cord explant culture system is a complex cellular system, it is possible that the effect of PTN is not a direct one, but mediated through the action of PTN on nonneuronal cells in the culture system. To address this possibility, we first examined the effect of PTN on dissociated spinal motor neuron-enriched neuronal cultures and an immortalized murine motor neuronal line MN1 (25, 26). In both experiments, exposure to PTN for 24 h resulted an increase in axonal length [supporting information (SI) Fig. 6]. Second, we measured the levels of other neurotrophic factors including GDNF and did not see any difference. GDNF levels in the spinal cord explant cultures exposed to conditioned medium from HEK-293PTN or HEK-293vector were low and similar to each other (11.2 and 12.2 pg/ml, respectively).

PTN Enhances Peripheral Nerve Regeneration in Vivo. The above observations suggested a potential role for PTN in enhancing peripheral nerve regeneration in vivo. We used a standard sciatic nerve transection and repair paradigm to test this hypothesis. In adult rats, surgical repair with a silicone tube as a graft after transection of the sciatic nerve will result in failure of regeneration...
HEK-293PTN group (33% recovery) but not in the HEK-293vector cells. Furthermore, there was reemergence of compound motor action potentials in the sciatic nerve innervated foot muscles in the nerve). In animals treated with HEK-293PTN cells, there was no regeneration across the gap. In animals treated with HEK-293vector cells compared with HEK-293PTN cells (Left) compared with the animals treated with HEK-293vector cells (Right). There were no regenerated axons in the saline-treated animals.

PTN promotes regeneration of axons after sciatic nerve transection. In adult rats, sciatic nerves were transected at the midtigh level and repaired with silicone tubes filled with HEK-293PTN, HEK-293vector, or saline. Images are from 1-µm transverse sections embedded in plastic at 12 mm distal to the proximal repair site. There were many myelinated axons in the animals treated with HEK-293PTN cells (Left) compared with the animals treated with HEK-293vector cells (Right). There were no regenerated axons in the saline-treated animals. Quantitation of myelinated fibers shows that more axons regenerated into the silicone tubes filled with HEK-293PTN cells compared with HEK-293vector or saline-filled tubes (n = 8–9 per group; *, P < 0.005 compared with the other two groups).

PTN is neuroprotective against chronic excitotoxic glutamate toxicity in vitro and prevents neonatal facial motor neuron death induced by target denervation in vivo. In spinal cord explant cultures, chronic exposure to a glutamate transport inhibitor, threohydroxy-yaspartate (THA), over 4 weeks, results in excitotoxic death of spinal motor neurons (23). We used the same system to examine whether PTN provides neuroprotection against excitotoxic motor neuron death (Fig. 4). In a dose-dependent manner, PTN provided protection and prevented motor neuron death in spinal cord explant cultures. The inverted bell-shaped dose–response curve is similar to what has been observed with other growth factors such as GDNF (22).

To confirm the neuroprotective role of PTN, we used a standard in vivo model of motor neuron death induced by target deprivation. In this model, transection of facial nerve in neonatal mice leads to deprivation of target-derived growth factors and death of facial motor neurons in the brainstem. This model has been used to examine the neuroprotective properties of neurotrophic factors as well as cell replacement strategies (27, 28). We used the same system and transplanted gelfoams loaded with HEK-293PTN or HEK-293vector into the proximal stumps of transected facial nerves in 3-day-old mouse pups (Fig. 4). There was a dramatic rescue of facial motor neurons in animals where the HEK-293 cells secreting PTN were transplanted (63% of the contralateral side), but not with the cells transplanted with the control vector (12% of the contralateral side).

Anaplastic Lymphoma Kinase (ALK) Mediates Trophic Activities of PTN in Motor Neurons. The receptor that mediates neurotrophic activity of PTN is unknown. Four receptors have been proposed to mediate various activities of PTN in different tissues and cell types (reviewed in refs. 14, 15). We hypothesized that in response to axotomy, neurons will up-regulate their receptors for a neurotrophic factor as they prepare to regenerate. We used semiquantitative real-time RT-PCR to measure changes in expression levels of protein tyrosine phosphatase-ζ, ALK, low-density lipoprotein receptor-related protein-5, and syndecan in the ventral spinal cord or dorsal root ganglion (DRG) after sciatic nerve transection in comparison with the contralateral uninjured side (Fig. 5). There was no significant change in any of the receptors postdenervation except ALK, which was up-regulated in the ventral spinal cord 1 week after sciatic nerve transection. This suggested that ALK could be the
ALK mediates neurotrophic activity of PTN. (A) Changes in mRNA levels of PTN receptors in the adult rat ventral spinal cord and DRG (L4 and LS levels) were evaluated by quantitative RT-PCR 1 week after sciatic nerve transection. Values are expressed as percentages of change from ventral spinal cords and DRGs with intact sciatic nerves (*, P < 0.005). (B) Spinal cord explants from postnatal day 8 were double-stained with antineurofilament antibody (NF) and anti-ALK antibody (ALK); a spinal motor neuron is shown. Explants from postnatal day 8 were double-stained with antineurofilament antibody, and the number of axons crossing the gray–white matter junction and the number of axons exiting from the spinal cord explants. By using both measures, the anti-ALK antibody significantly blocked the number of axons exiting from the spinal cord explants. We established spinal cord explant cultures as DRG sensory explant cultures, and although there was an increase in neurite outgrowth, this was minimal compared with the spinal cord explants and required higher doses of PTN (SI Fig. 7).

Discussion

One of the challenges of peripheral nerve regeneration is that, with proximal nerve injury, the distance between the regenerating axon and the target tissue is often too long for the axon to receive target-derived growth factors that may enhance regeneration. Denervated Schwann cells in the distal stumps of injured nerves act as transient target by secreting a variety of growth factors that are normally targeted derived. We exploited this property to identify PTN as a growth factor that was up-regulated in denervated distal nerve segments and showed that PTN is a neurotrophic factor for spinal motor neurons.

The focused microarray we used was designed to detect ~100 known neurotrophic factors and their receptors. It is possible that, with a more comprehensive microarray, we could have detected other molecules whose expression profile mirrored that of neurotrophic factors that are known to be up-regulated [e.g., nerve growth factor (30, 31) and GDNF (21, 32)]. However, the focused microarrays allowed us to do these experiments without any specialized equipment or bioinformatics expertise. We were able to identify PTN as a neurotrophic factor with expression profile similar to that of GDNF. Initial up-regulation and subsequent decline in the expression of GDNF have been proposed to be responsible for failure to regenerate in chronically denervated nerves (21). It is possible that GDNF is not the only growth factor whose increased expression is not maintained with chronic denervation. Lack of up-regulated PTN expression in chronically denervated nerves may contribute to poor regenerative capacity of motor nerves in proximal nerve injuries.

What do we know about the neurotrophic properties of PTN? As reviewed elsewhere (14, 16), PTN is a member of a heparin-binding small cytokine family with close homology to midkine. It was initially isolated as a weak mitogen for fibroblasts and was found to have neurite-promoting properties in neonatal rat brain mixed neuronal and glial cultures (11, 17). More recently, PTN was shown to be a neurotrophic factor for dopaminergic midbrain neurons (19), a feature similar to that of GDNF (33). The dose–response curve in dopaminergic neurons in vitro was similar between PTN and GDNF. In our spinal cord explant cultures, we also observed a similar dose–response curve, where PTN was neurotrophic in the low nanogram per milliliter range. This is a property shared among many neurotrophic factors including nerve growth factor and BDNF.

Although the expression pattern of PTN in denervated nerve and muscle, and the neurotrophic properties of PTN in spinal motor neurons were similar to those of GDNF, there was no up-regulation of PTN in denervated skin or up-regulation of PTN receptors in the DRG. These observations are in clear contrast to other growth factors and suggest that PTN may be a more specific neurotrophic factor for motor neurons. This conclusion, however, needs confirmation by other experimental data. We did test the neurotrophic properties of PTN in DRG sensory explant cultures, and although there was an increase in neurite outgrowth, this was minimal compared with the spinal cord explants and required higher doses of PTN (SI Fig. 7).
PTN knockout mice have been generated, and several studies have been done to elucidate its role in the nervous system (34, 35). So far, these studies have focused on the central nervous system effects of PTN. Evaluation of peripheral nerve regeneration in mice lacking the PTN gene may help answer whether the motor axonal regeneration is differentially more impaired compared with DRG sensory axons. On the other hand, the lack of any obvious phenotype in these mice, despite a wide pattern of expression and multiple actions of PTN in different tissues, suggests that there may be redundancy in the system. For example, expression pattern of midkine, is similar to that of PTN during development (reviewed in ref. 14) and is up-regulated in spinal cord after injury (36). A detailed study of changes in midkine in PTN-deficient mice may help answer the question of whether midkine can substitute for PTN after neural injury.

The receptor biology of PTN is quite complex. Four molecules, protein tyrosine phosphatase-ζ, ALK, syndecan, and low-density lipoprotein receptor-related protein-5, have been shown to bind to PTN and induce intracellular signaling in a variety of cell types (reviewed in ref. 14). Recent data suggest that protein tyrosine phosphatase-ζ regulates Purkinje cell dendritic morphogenesis during cerebellar development (37). It is unclear, however, which receptor mediates neurotrophism in cortical neurons or dopaminergic midbrain neurons. Our observations suggest that ALK may be the receptor responsible for mediating neurotrophic effects of PTN in the motor neuron. This observation is also supported by others who showed that ALK is expressed in spinal motor neurons (38). In contrast, others failed to show phosphorylation of ALK by PTN in neuroblastoma cells (39, 40), but these authors do not reveal the source of PTN. As we have experienced, there are significant differences in neurotrophic properties of PTN from different sources and this largely depends on how the PTN is manufactured. Recombinant PTN may differ from tissue source PTN in terms of its neurotrophic and mitogenic activity (17, 41). Nevertheless, our data will need further confirmation with a conditional knockout animal or other strategies where signaling through the ALK is blocked and motor axonal regeneration is evaluated in vivo. In 2001, Stoica et al. (42) identified PTN as a ligand for orphan receptor, ALK, and showed that binding of PTN to ALK induced phosphorylation of the downstream effecter molecules insulin receptor substrate-1, Shc, phospholipase C-γ, and phosphatidylinositol 3-kinase. Most observations support the notion that PTN mediates its neurotrophic effects through activation of both receptor tyrosine kinases (Trk) and a family of receptor-like molecules (e.g., Ret). In contrast, others showed that ALK is expressed in spinal motor neurons (43), but these authors do not reveal the source of PTN. As we have experienced, there are significant differences in neurotrophic properties of PTN from different sources and this largely depends on how the PTN is manufactured. Recombinant PTN may differ from tissue source PTN in terms of its neurotrophic and mitogenic activity (17, 41). Nevertheless, our data will need further confirmation with a conditional knockout animal or other strategies where signaling through the ALK is blocked and motor axonal regeneration is evaluated in vivo.

In addition to our observations, others have shown that PTN is up-regulated in the rat sciatic nerve after injury (46). However, PTN up-regulation after denervation injury is not unique to the peripheral nervous system. PTN is expressed at high levels in the glial cells of the central nervous system during development and down-regulated in the adult animal (47, 48), but up-regulated in astrocytes in response to ischemic injury in models of stroke (49). Although biological significance of these observations needs elucidation and confirmation, this pattern of expression mirrors what we observed in the peripheral nervous system. Combined, these observations suggest that PTN may play an important role in the response of nervous system to injury. In summary, we identified PTN as a neurotrophic factor that was up-regulated in denervated distal nerve and muscle, and showed that exogenous PTN can enhance axonal regeneration and protect facial motor neurons from trophic factor deprivation-induced cell death in vivo. These observations open up potential avenues of therapeutic research for impairments affecting motor neurons and axons.

Materials and Methods
All animal surgeries were conducted under protocols approved by The Johns Hopkins University Animal Care and Use Committee according to guidelines established by National Institutes of Health and American Association for the Accreditation of Laboratory Animal Care. Routine laboratory methods including real-time RT-PCR, Western blotting, and ELISA are described in detail in SI Methods along with details of the motor neuron-enriched spinal cord neuronal culture and motor neuron-neuroblastoma cell line culture methods used in SI Methods.

Sciotic Nerve Transection Model. Sciatic nerve transection and harvesting of distal denervated sciatic nerves were done as described in ref. 21. Briefly, left sciatic nerve was transected at the upper thigh level under anesthesia in adult female Sprague–Dawley rats and animals were allowed to recover. To investigate the changes in PTN expression in denervated tissues, distal sciatic nerves, and muscles and distal foot skin innervated by sciatic nerve were harvested at 2 and 7 days, and 1, 3, and 6 months after the nerve transection. There were four to six rats per group. In a separate set of animals, the left and right side of the ventral half of the spinal cords and L4 and L5 DRGs were harvested 1 week after sciatic nerve transection as above. These were used to examine the changes in receptor mRNA levels after nerve transection.

Cloning of Rat PTN and Transfection of HEK-293 Cells. Total RNA was extracted from distal segments of 7-day denervated rat sciatic nerve, and full-length PTN was isolated via RT-PCR by using the primers 5′-GCTAGAATTCCAAATTGCTGTTCCAGCATACCAG-3′ (forward) and 5′-ATGCGGATCCATCCAGCATCTTCTCCTGTTC-3′ (reverse). These primers included BamH1 and EcoRI sites facilitating subcloning into pCDNA 3.1(−)-myc-his. The clone was fully sequenced. HEK-293 cells were transfected by using Superfect (Qiagen, Valencia, CA) according to the manufacturer’s specifications. Four micrograms of total plasmid DNA were added to HEK-293 cells grown in six-well dishes. PTN expression in HEK-293 cells and their supernatants were verified by immunostaining and Western blot with anti-myc and anti-PTN antibodies.

Organotypic Spinal Cord Cultures. Organotypic spinal cord cultures were prepared from lumbar spinal cords of 8-day-old rat pups, as described in refs. 22, 24, and 50. Briefly, lumbar spinal cords were collected under sterile conditions and sectioned transversely into 350-μm slices with a McIlwain tissue chopper. Slices were cultured on Millicell CM semipermeable culture inserts at a density of five slices per well. Under these conditions, 95% of cultures retained cellular organization, and a stable population of motor neurons survived for >3 months. Organotypic spinal cord culture media [50% MEM and Heps (25 mM), 25% heat-inactivated horse serum, and 25% Hanks’ balanced salt solution (Invitrogen, Carlsbad, CA) supplemented with d-glucose (25.6 mg/ml) and glutamine (2 mM), at a final pH of 7.2] were changed twice weekly. Drugs and growth factors were added when media were changed. No drugs were added for the first 7 days after culture preparation. In neuroprotection assays, PTN or anti-ALK antibody was added either alone or in combination with the glutamate transport inhibitor THA (100 μM) for 4 weeks (24). In experiments examining the role of ALK, explant cultures were treated with conditioned media from HEK-293 cells expressing vector or PTN with and without a receptor blocking anti-ALK antibody (at a concentration of 50 ng/ml) or a control antibody, anti-GFAP for 3 days. The explants were stained with anti-neurofilament antibody, SMI-32 (see below), and the number of motor axons crossing the gray–white matter junction in the ventral spinal cord and exiting from the explant was counted. The experiments were done in quadruplicate and repeated twice. Statistical analysis was done as above.
HEK-293 Cells and Organotypic Spinal Cord Cultures. HEK-293 cells were transfected with myc-his-PTN or pcDNA 3.1(−)–myc-his in six-well dishes by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. Twenty-four hours after transfection, the culture inserts were transferred to six-well dishes containing HEK-293 cells transfected with PTN or control vector. After 7 days, the culture inserts were transferred to another six-well dish containing HEK-293 cells prepared as above. After an additional 7 days, the organotypic cultures were fixed and stained with SMI-32 antibody as described above.

Sciatric Nerve Regeneration Study. HEK-293 cells were transfected with PTN or control vector as described above. Viability of cells was ascertained and the cells were used within 2 h of harvesting. Under an operating microscope, the sciatic nerves were exposed and a 10-mm segment was resected out at midthigh section. The gap was reconstructed with an operating microscope, the sciatic nerves were exposed and a 10-mm segment was resected out at midthigh section. The gap was bridged with a 15-mm silicone tube (internal diameter, 1.5 mm). HEK-293 cells (5 × 10⁴ cells in 15 μl) containing either the vector with PTN (HEK-293PTN) or vector alone (HEK-293vector) in PBS was infused into the silicone tube. The proximal and distal stumps of the sciatic nerve were inserted 1 mm into the tube and connected with three stitches by using a 10-0 nylon thread. Eight weeks after grafting, animals were perfused transcardially with 4% paraformaldehyde, and the grafted silicone tube and the distal sciatic nerve fibers were further fixed in 4% paraformaldehyde and 3% glutaraldehyde, transferred to Sorenson's buffer, and embedded in plastic according to standard protocols. Toluidine blue-stained 1-μm-thick sections were used to count the number of myelinated fibers per cross section by using stereological methods as described in ref. 51. ANOVA with correction for multiple comparisons was used for statistical analysis.

Facial Axotomy Model. Three-day-old C57BL/6j mice of either sex were anesthetized, and the left facial nerves were transected near the stylomastoid foramen. HEK-293 cells were prepared as described above. Gelfoams measuring 2 × 2 cm were loaded with HEK-293PTN, HEK-293vector, or PBS alone (eight animals per group) and applied to the cut nerve stump (27). The wounds were sutured and the mice were returned to the litter. One week after gelfoam implant, animals were perfused transcardially with 4% paraformaldehyde, and the brainstems were dissected, cryoprotected in sucrose solutions, and serially cut at 40 μm on a cryostat. The serial sections of brainstem were stained with cresyl violet, and six to eight sections were used for facial motor neuron counting on both side of the brainstem (52). Each experiment was done with eight animals per group, and the results were evaluated for statistical significance by using ANOVA with corrections for multiple comparisons.

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