Hypertrophic Neuropathies and Malignant Peripheral Nerve Sheath Tumors in Transgenic Mice Overexpressing Glial Growth Factor β3 in Myelinating Schwann Cells

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The neuregulin-1 (NRG-1) family of growth and differentiation factors exerts a variety of effects on Schwann cells and their precursors during nervous system development; however, NRG-1 effects on adult Schwann cells are poorly defined. Several lines of evidence suggest that NRG-1 actions on adult Schwann cells are distinct from those observed during development. To test this hypothesis, we generated transgenic mice overexpressing the NRG-1 isoform glial growth factor β3 (GGFβ3) in myelinating Schwann cells [protein zero (P0)-GGFβ3 mice]. P0-GGFβ3 mice develop resting tremors, gait abnormalities, decreased hindlimb strength, and paralysis by ~7 months of age. Sciatic nerves from these animals show a hypertrophic neuropathy characterized by demyelination, remyelination, and “onion bulb” formation. Development of this hypertrophic neuropathy is preceded by Schwann cell hyperplasia that is prominent in 1-month-old mice and present but decreased in 2- and 4-month-old animals. P0-GGFβ3 mice also develop peripheral ganglion-associated malignant peripheral nerve sheath tumors. Motor, sensory, and sympathetic ganglia from 1-, 2-, and 4-month-old P0-GGFβ3 mice uniformly contain intraganglionic, likely preneoplastic, Schwann cell proliferations. Examination of bromodeoxyuridine incorporation and caspase-3 activation in sciatic nerves and trigeminal ganglia indicates that Schwann cell hyperplasia in P0-GGFβ3 mice reflects increased proliferation rather than decreased apoptosis. These observations are consistent with the hypothesis that GGFβ3 induces proliferation of adult Schwann cells and demyelination of peripheral nerve axons. Furthermore, overexpression of this NRG-1 isoform frequently induces neoplastic Schwann cell proliferation within PNS ganglia, suggesting that NRG-1 may contribute to human Schwann cell neoplasia.

Key words: Schwann cell; erbB receptor; schwannoma; neuropathy; neuregulin; glial growth factor

Introduction

The phenotype of Schwann cells and their precursors is modulated during development by members of the neuregulin-1 (NRG-1) family of growth and differentiation factors. NRG-1 proteins, which include glial growth factor (GGF), neu differentiation factor, heregulin, and acetylcholine receptor-inducing activity, are alternatively spliced membrane-bound or soluble molecules derived from a single gene (Fischbach and Rosen, 1997). The soluble NRG-1 isoform GGFβ3 promotes glial differentiation of neural crest cells (Shah et al., 1994), the pluripotent precursors from which Schwann cells arise, and prevents these cells from differentiating into neurons. NRG-1 isoforms also regulate the survival, proliferation, and differentiation of Schwann cell precursors, the glial elements derived from neural crest cells (Dong et al., 1995), and prime these cells to express factors necessary for myelination. From late embryogenesis through the neonatal period, axonally derived NRG-1 proteins “match” the number of Schwann cells and axons by promoting the proliferation, migration, and survival of axon-associated committed immature Schwann cells (Topilko et al., 1996; Felts, 1999; Adlkofer and Lai, 2000; Garratt et al., 2000a). It is thus apparent that NRG-1 is critically important throughout Schwann cell development, with the precise responses elicited by these factors depending on the developmental stage of the glia.

Although NRG-1 is also expressed by neurons projecting into the adult PNS (Chen et al., 1994; Bermingham-McDonogh et al., 1997), little is known regarding NRG-1 actions on adult Schwann cells. Several lines of evidence suggest that NRG-1 has in vivo effects on adult Schwann cells differing from those observed during development. Adult Schwann cells, unlike embryonic Schwann cells, depend on autocrine signals for survival (Cheng et al., 1998), having lost a requirement for axonally derived NRG-1 (Meyer et al., 1997; Morris et al., 1999; Wolpowitz et al., 2000); these observations are consistent with an absence of Schwann cell apoptosis in nerves from older rodents, both in the uninjured state and after nerve transection (Grinspan et al., 1996). Furthermore, GGFβ3 induces demyelination in established Schwann cell–neuron cocultures (Zanazzi et al., 2001); this contrasts with the observation that Schwann cell-targeted ablation of the NRG-1 receptor subunit erbB2 inhibits the initial myelination of
axons during embryogenesis and early postnatal life (Garratt et al., 2000b). Additional clues to potential NRG-1 actions on adult Schwann cells are provided by studies in injured peripheral nerves, in which the expression of multiple NRG-1 isoforms has been found to be induced coincident with the onset of Schwann cell proliferation (Carroll et al., 1997). Tyrosine phosphorylation of Schwann cell ErbB receptors is increased with a similar time course (Kwon et al., 1997), suggesting that NRG-1 promotes Schwann cell mitogenesis in the regenerating adult nerve. These observations argue that NRG-1 continues to regulate the phenotype of adult Schwann cells. NRG-1 actions on adult Schwann cells, however, likely differ from those encountered earlier in life. To test this hypothesis, we have generated transgenic mice that overexpress the NRG-1 isoform GGF3 in mature myelinating Schwann cells.

Materials and Methods

Animal care and surgical procedures. Mice were cared for in accordance with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Mice were housed in standard cages with filter lids. Water and food were available ad libitum. Animals were killed by either decapitation or cardiac perfusion after being anesthetized with Metofane (Schering-Plough Research Institute, Union, NJ) or Nembutal (pentobarbital; Abbott Labs, North Chicago, IL).

Construction of a protein zero-GGF3 transgene. A cDNA containing the entire coding sequence for GGF3 was constructed from three distinct clones, an approach that allowed a codon encoding a proline in our sequence and a reverse primer (5′-CTTCTGGCACATCACCAGGTTCCCTCT-3′) corresponds to nucleotides 1164–1174 of the GGF3 rat cdNA sequence (GenBank accession number AF194996). The GGF3 reverse primer (5′-TGACGGGTTTGACAGGTCTCT-3′) corresponds to nucleotides 1414–1393 of the GGF3 rat cdNA sequence. To facilitate normalization of GGF3 mRNA levels, cyclophilin mRNA levels were also assayed. The cyclophilin forward primer (5′-CAAGACTAAGGTGCCTGGATG-3′) corresponds to nucleotides 393–413 in the rat cyclophilin cdNA sequence (Danielson et al., 1988). The cyclophilin reverse primer (5′-TAAATGCAGCCAAGTTCAGAAA-3′) corresponds to nucleotides 561–538 in the rat cyclophilin cdNA sequence. PCR was performed using a PerkinElmer Life Sciences (Emeryville, CA) GeneAmp 2400 thermocycler. PCR conditions and empirical determination of the linear range of the PCR reaction for each primer pair were established as described (Carroll and Frohnert, 1998) using cdNA isolated from SJ1 cells transiently transfected with plasmid pSCL458, which contains the GGF3 construct under the control of the cytomegalovirus immediate early promoter. Cycle parameters, after an initial 3 min melt at 94°C, were 30 sec at 94°C followed by 30 sec at 60°C and 1 min at 72°C. The midpoints of the loglinear range of amplification were determined to be 27 cycles for GGF3 and 28 cycles for cyclophilin; consequently, amplification for assays of GGF3 and cyclophilin mRNA levels in sciatic nerves was performed using 27 and 28 cycles, respectively.

Western blot analyses. Protein from the sciatic nerve was isolated using Trizol (Invitrogen) according to the manufacturer’s protocol. The denatured protein pellet was resuspended in 100 m ς Tris-HCl, pH 6.8, and 1% SDS, heated at 55°C and sonicated twice (30 sec/sonication with a 1 min cooling interval between sonications) in a bath sonicator. Samples were centrifuged at 20,000 χ g for 5 min. The supernatant was removed and assayed for protein content. Twenty micrograms of protein were resolved by SDS-PAGE, immunoblotted onto a polyvinylidene difluoride membrane, and probed with a rabbit polyclonal pan-neuregulin antibody (Carroll et al., 1997) followed by a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA). Immunoreactive species were detected by chemiluminescence (SuperSignal Pico chemiluminescence kit; Pierce, Rockford, IL).

Preparation of plastic sections for light and electron microscopic analyses. Tissues were immersion-fixed in 3% glutaraldehyde in Sorensen’s buffer for 1–2 hr at room temperature and then postfixed in this same fixative overnight at 4°C. Sciatic nerve segments and ganglia were then postfixed in OsO4, dehydrated through graded alcohols, and embedded in Epon-Araldite or Spurr’s medium. Semithin sections of nerves cut perpendicular to the orientation of the axonal fibers were stained with toluidine blue for light microscopic examination. For electron microscopy, thin-layer sections were stained with uranyl acetate and lead citrate. Electron microscopy was performed using a Philips 200 electron microscope.

Preparation of cDNA for Northern analysis. Mice were injected intraperitoneally with bromodeoxyuridine (BrdU) and 5-fluorouracil (5-FU; 60 mg/kg BrdU and 6 mg/kg 5-FU) for 90 min before killing. BrdU-pulsed animals were perfused transcardially with 4% paraformaldehyde in PBS and then postfixed in 4% paraformaldehyde at 4°C. The sciatic nerve, trigeminal nerve, selected PNS ganglia, jejunum (a positive control for BrdU incorporation), and, when applicable, tumor tissue were isolated.

Tissues were embedded in paraffin, and 5–6 ς m sections were prepared and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were deparaffinized in Citrisolve (Fisher Scientific) and rehydrated through isopropanol to water and then PBS. Citrate antigen retrieval was performed by steaming slides for 20 min in a rice steamer and then allowing slides to cool to room temperature for 20 min.

Endogenous peroxidase activity was blocked by treating slides with 3%
H₂O₂ for 5 min, followed by washes performed first with water and then with PBS. Sections were incubated for 15–30 min in tyramide signal amplification blocking buffer (PerkinElmer Life Sciences). After blocking, slides were incubated with goat-anti-BrdU antiserum (1:200,000 dilution in blocking buffer; antiserum kindly provided by Dr. Steven Cohn, Washington University School of Medicine) overnight at 4°C. After three rinses in PBS, sections were incubated at room temperature for 1 hr in blocking buffer containing donkey anti-goat-HRP (1:1000 dilution; Jackson ImmunoResearch). After three washes in PBS, sections were incubated with cyanine 3-tyramide in Plus Amp buffer (PerkinElmer Life Sciences) for 30 min. Sections were then washed two times with PBS (5 min/wash), followed by a wash with PBS containing 0.04 μg/ml bismecamide to label nuclei within the sections. Sections were then washed twice more with PBS and mounted in PBS/glycerol, (1:1 v/v).

Digital images from immunostained preparations were acquired using a Zeiss (Thornwood, NY) Axioskop fluorescence microscope and analyzed using Image-Pro Plus acquisition and analysis software. Nuclei within the endoneurium (excluding those within the endoneurial vasculature) were identified as Schwann cells on the basis of the morphology of their nuclei (oval, blunt-ended nuclei oriented longitudinally relative to the long axis of the nerve); we and others have previously found these criteria to be quite reliable for the identification of Schwann cells in histologic sections (Asbury, 1967; Carroll et al., 1997). Schwann cells actively synthesizing DNA were identified by the presence of BrdU immunoreactivity colocalizing with bismecamide staining.

Immunohistochemistry for activated caspase-3, S100β, and collagen type IV. These immunostains were performed on paraffin sections of nerves. Activated caspase-3 was detected using a rabbit polyclonal antibody specific for this antigen (Cell Signaling, Inc.) with tyramide signal amplification immunohistochemistry performed as described above. S100β immunoreactivity was detected using a rabbit polyclonal anti-S100β primary antibody (Dako, Carpinteria, CA; 1:200 dilution) and a Cy3-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch). Immunostaining for collagen type IV was performed using a mouse monoclonal primary antibody specific for this antigen (clone PHM-12, 1.5 μg/ml; Ventana Medical Systems, Tucson, AZ) followed by a horseradish peroxidase-conjugated secondary antibody, with immunoreactivity detected by diaminobenzidine deposition.

Results

Generation of transgenic mice overexpressing the NRG-1 isoform GGFβ3 in myelinating Schwann cells

To test the hypothesis that NRG-1 is capable of inducing Schwann cell dedifferentiation, proliferation, or both in the non-injured peripheral nerve, we produced transgenic mice constitutively overexpressing NRG-1 in this tissue. We chose to overexpress GGFβ3, an NRG-1 splice variant that is directly secreted without the need for release by a transmembrane domain-specific protease, in the peripheral nerve. The transgene for these experiments (referred to subsequently as the P₀-GGFβ3 transgene) was constructed by inserting sequences encoding GGFβ3 into a genomic clone of the major peripheral myelin P₀ gene, which has been modified so that the cDNA can be inserted at the P₀ initiation codon (Fig. 1A). This vector has been found to reliably produce high-level expression in myelinating Schwann cells, with low-level expression first detectable at postnatal day 5 (P5) and high-level expression evident by P15 and persisting into adulthood (Feltri et al., 1999).

Four P₀-GGFβ3 founders (founders 33–35 and 44) were identified and showed little evidence of neurologic or other abnormalities until they were ~7 months of age (see below). However, two of these founders produced no transgenic progeny, whereas a third produced only a single transgenic pup, which could not be further propagated (Table 1). Transgenic offspring were readily obtained from the fourth founder (founder 33). To assess transgene expression in the progeny of P₀-GGFβ3 founder 33, semi-quantitative RT-PCR analyses were performed on RNA isolated from the sciatic nerves of 4-month-old transgenic animals and their nontransgenic littermates. GGF mRNA levels in the sciatic nerves of nontransgenic mice were barely detectable, with levels much lower than in their nontransgenic littermates. Because of the very low levels of GGF mRNA in the nontransgenic sciatic nerve, we can
only estimate that GGF mRNA levels in the sciatic nerves of 4-month-old P0-GGFβ3 animals are at least 4- to 10-fold higher than in age-matched nontransgenic controls. When probed with an antibody specific for the epidermal growth factor (EGF)-like common domain present in all NRG-1 isoforms (Carroll et al., 1997), sciatic nerve lysates from P0-GGFβ3 mice of line 33 were found to contain increased levels of a 60 kDa NRG-1-like antigen (Fig. 1C). The size of this NRG-1-like antigen is very similar to that previously reported for GGF-II (~59 kDa; Goodearl et al., 1993; Marchionni et al., 1993), indicating that full-length GGFβ3 protein is overexpressed in sciatic nerves from these transgenic animals. Levels of expression of GGFβ3 mRNA and protein showed some variation between individuals of line 33; because GGFβ3 expression in these animals is directed by the regulatory elements of the P0 gene, and GGFβ3 has been found to repress P0 expression (Cheng and Mudge, 1996), variable GGF expression in P0-GGFβ3 mice may reflect cyclic induction and repression of transgene expression.

Mice overexpressing GGFβ3 in myelinating Schwann cells develop hindlimb paralysis associated with hypertrophic neuropathy

At ~7 months of age, founders 33–35 developed gait abnormalities and started dragging their hind legs. When picked up by their tails, these founders clasped their feet tightly to their bellies rather than demonstrating a normal outstretching of the hind legs and splaying of the toes. Hindlimb muscular strength was diminished, as demonstrated by a decreased ability to grasp with their hind feet. As their condition progressed, founders 33–35 developed ptosis, a hunched posture, and a prominent resting tremor. In addition, founder 34 demonstrated severe priapism toward the end of his course. In founder 44, hind leg weakness was the most prominent symptom, which eventually developed into complete hindlimb paralysis (Fig. 2A). No abnormalities of urination or defecation were present in any founder, indicating that spinal cord function was grossly intact. We subsequently observed similar neurologic abnormalities in the progeny of founder 33.

To investigate the anatomic basis of the neurologic abnormalities in these animals, necropsy examinations were performed on founders 44 and 35 and some of the progeny of founder 33. No gross abnormalities were identified in these studies with the exception of atrophy of the hindlimb musculature. One micrometer plastic semithin cross sections of the sciatic nerves were prepared, stained with toluidine blue, and examined by light microscopy. Sciatic nerves from founders 35 (Fig. 2B) and 44 (Fig. 2C) as well as mice from line 33 (see below) showed multiple abnormalities characteristic of a severe hypertrophic neuropathy including numerous well developed “onion bulbs” (e.g., see Fig. 2B, arrowhead). Most of the remaining large axons were thinly myelinated (Fig. 2B, arrows), and occasional structures morphologically consistent with actively degenerating axons were present (Fig. 2C, arrowheads). None of these changes were ever observed in sciatic nerves from age-matched nontransgenic controls (Fig. 2D).

Electron microscopic examination of nerves from founders 35 and 44 confirmed the presence of well developed onion bulbs. Furthermore, these studies demonstrated the presence of endoneurial lipid-laden macrophages (Fig. 2E) and occasional (~0.1%) large unmyelinated (“naked”) axons (Fig. 2F). Sciatic nerves from P0-GGFβ3 transgenic mice therefore showed evidence of both demyelination (lipid-laden macrophages and naked axons) and remyelination (thinnily myelinated large axons). As the same hypertrophic neuropathy occurs in founders 44 and 35 and animals from line 33, we conclude that this neuropathy results from GGFβ3 overexpression rather than line-specific integration site effects.

The hypertrophic neuropathy in P0-GGFβ3 transgenic mice develops in adulthood

The development of neurologic abnormalities at ~7 months of age suggests that the progression of clinical findings in P0-GGFβ3 transgenic mice is paralleled by the development of anatomic changes. To test this hypothesis, sciatic nerves were collected from P0-GGFβ3 transgenic (line 33) and wild-type control mice at 1 (seven transgenic and two wild-type), 2 (eight transgenic and two wild-type), 4 (four transgenic and one wild-type), and 7 (four transgenics and one wild-type) months of age. One micrometer plastic semithin cross sections were prepared from these tissues, stained with toluidine blue, and examined by light microscopy. At 1 month of age, there was no evidence of hypertrophic neuropathy in the sciatic nerves of P0-GGFβ3 mice (Fig. 3A), with myelination in this tissue similar to that seen in age-matched controls (data not shown). By 2 months of age, however, rudimentary onion bulbs were detected in the sciatic nerves of some transgenic animals (Fig. 3B, arrows). By 4 months of age, both onion bulbs and many large, thinly myelinated axons were present, abnormalities that were even more evident in 7-month-old animals (Fig. 3C). Onion bulb formation in particular was very prominent in many 7-month-old transgenic mice of line 33, much as was seen in founders 35 and 44 (see above). None of these pathologic findings were evident in the sciatic nerves of wild-type control animals.
in 4-month-old animals being ~150% of that observed in 1-month-old mice. At all three ages, however, there was no significant difference in the average cross-sectional area of sciatic nerves from transgenic and nontransgenic mice [e.g., nerve area in 4-month-old transgenic mice, 216,814 ± 35531 μm² (mean ± SD of the mean); in 4-month-old nontransgenic mice, 197,226 ± 32658 μm²]. These observations suggest that at least one factor contributing to the age-related decrease in Schwann cell nuclear density in P0-GGFβ3 mice is the endoneurial accumulation of elements (e.g., the Schwann cell processes forming onion bulbs and endoneurial collagen) in the increasingly pathologic nerve, which produces a progressively wider separation of Schwann cell nuclei. A similar age-related decrease in Schwann cell nuclear density occurs in sciatic nerves from nontransgenic animals and probably reflects the endoneurial accumulation of myelin and other molecules associated with maturation of the nerve. Because the average cross-sectional area of sciatic nerves from 4-month-old transgenic and nontransgenic mice is not significantly different, it seems likely that the hypertrophy observed for individual Schwann cell–axon units in P0-GGFβ3 mice is offset by the loss of myelin and axons that occurs as the pathology progresses, resulting in a minimal overall change in nerve diameter.

To verify that the cells producing hyperplasia in nerves from P0-GGFβ3 mice are indeed Schwann cells, sciatic nerve sections from 1-month-old transgenic animals and nontransgenic littermates were stained for the Schwann cell marker S100β and collagen type IV, a protein component of the basal laminae; collagen type IV deposition is found around Schwann cells (Baron-Van Evercooren et al., 1986; Lorimier et al., 1992) and endothelial cells (Lorimier et al., 1992) but not around other cell types within the endoneurium. S100β immunoreactivity was associated with both the normal Schwann cells in nontransgenic nerves (data not shown) and the hyperplastic elements within nerves from P0-GGFβ3 mice (Fig. 5A). Collagen type IV immunoreactivity was evident as faint brown staining outlining Schwann cell–axon units and as stronger endothelial–associated staining in nerves from nontransgenic mice (Fig. 5B). In contrast, collagen type IV immunoreactivity was prominent in nerves from P0-GGFβ3 mice (Fig. 5C) and was frequently observed to invest the hyperplastic cells within this tissue (Fig. 5C, arrows). Because endothelial cells are not S100β-positive, the S100β and collagen type IV immunoreactivity associated with hyperplastic elements in P0-GGFβ3 nerve identifies these cells as Schwann cells.

**Mice overexpressing GGFβ3 in myelinating Schwann cells develop malignant peripheral nerve sheath tumors**

In addition to the neurologic abnormalities described above, founders 33 and 34 demonstrated decreasing responsiveness associated with bulging eyes that first became evident at 6–7 months of age. When autopsied, these mice were found to have large intracranial masses at the base of the skull. The masses were centered on the trigeminal ganglion and displaced the brain upward; both gross and microscopic examinations showed no evidence that these masses invaded the brain. Microscopic examination of the mass from founder 34 (Fig. 6A) showed this tumor to be a hypercellular neoplasm composed of Schwann cell–like spindled cells containing elongated nuclei. Mitoses and areas of
tumor necrosis were common in this neoplasm. Although containing small areas histologically similar to the tumor from founder 34, the majority of the intracranial neoplasm from founder 33 was much more hypercellular and contained even more numerous mitotic figures (Fig. 6B). Ganglionic neurons were found entrapped within both neoplasms, confirming their association with the trigeminal ganglion.

Similar neoplasms were found in three of the older (6- to 10-month-old) progeny of P0-GGFβ3 founder 33. These neoplasms, like the tumor found in founder 33, were markedly hypercellular lesions containing numerous mitotic figures. Two of these tumors were associated with the trigeminal ganglion. The third neoplasm was within the sciatic nerve, producing diffuse enlargement of the nerve that began at the exit of the nerve roots from the vertebral canal and extended along the upper half of the nerve. No such neoplasms were ever observed in the nontransgenic littermates of these mice. Again, because neoplasms were found in founder 34 as well as several mice from line 33, we conclude that the development of these tumors results from transgene-directed GGFβ3 overexpression rather than integration site effects.

The morphologic features of the tumors developing in P0-GGFβ3 mice are highly similar to those of human malignant peripheral nerve sheath tumors (MPNSTs), a type of neoplasm that may occur either sporadically or as part of the hereditary cancer predisposition syndrome neurofibromatosis type 1 (Gutmann et al., 1997; Parada, 2000; Gutmann, 2001). To confirm this impression, immunohistochemical studies and ultrastructural examination of the MPNST-like neoplasms occurring in P0-GGFβ3 mice were performed in accordance with recent consensus recommendations for the evaluation of neoplasms occurring in transgenic mice (Weiss et al., 2002). Extensive areas within the tumors were immunoreactive for the Schwann cell marker S100β (Fig. 6C), indicating that these neoplasms, like human MPNSTs, were Schwann cell tumors. Furthermore, individual tumor cells were surrounded by immunoreactivity for collagen type IV, a basal lamina protein that demonstrates a similar distribution in human MPNSTs (Leong et al., 1997; Vang et al., 2000). Ultrastructural examination demonstrated that the tumor cells had broad, interdigitating processes (Fig. 6E) densely laden with ribosomes and endoplasmic reticulum. Tumor cells were frequently invested by a basal lamina that at times extended away from the cell surface as loops (Fig. 6F, arrowhead). Long-spacing collagen (Luse body) was also identified within the tumor. These ultrastructural features are highly similar to those of human MPNSTs (Taxy et al., 1981; Erlandson and Woodruff, 1982; Arpornchayanon et al., 1984; Herrera and Pinto de Moraes, 1984; Erlandson, 1985; Dickersin, 1987; Takeuchi and Ushigome, 2001). Considered together, the histologic, immunohistochemical, and ultrastructural features of the neoplasms developing in P0-GGFβ3 mice meet the World Health Organization criteria (Woodruff et al., 2000) for classification as MPNSTs. Per consensus recommendations (Weiss et al., 2002), these neoplasms are referred to below as genetically engineered murine (GEM) MPNSTs.
If NRG-1 overexpression promotes the continued growth of the GEM MPNSTs developing in P0-GGFβ3 mice, then expression of the P0-GGFβ3 transgene should be maintained in these neoplasms, and the tumor cells should express the receptors necessary for NRG-1 responsiveness. Examining the expression of the P0-GGFβ3 transgene using transgene-specific primers, we found that GGFβ3 mRNA was readily detectable in trigeminal-associated tumor from transgenic mice but not in trigeminal nerve from nontransgenic littermates (Fig. 6G). Furthermore, the NRG-1 receptor subunits erbB2 and erbB3 were expressed in GEM MPNST (Fig. 6H). A comparison of the levels of erbB2 and erbB3 protein in GEM MPNST and trigeminal nerves from 5- and 7-month-old P0-GGFβ3 mice indicated that both erbB kinases were markedly overexpressed in the tumor relative to the levels evident in nerves not containing grossly evident neoplasms; because it was not possible to detect erbB protein in the trigeminal nerve while simultaneously avoiding saturation of the signals from the tumor, we could not accurately quantify the relative levels of expression in these tissues. Nonetheless, it is apparent that both GGFβ3 and its erbB receptors are expressed in GEM MPNSTs, suggesting that these molecules promote tumor cell proliferation via autocrine or paracrine effects.

Localized Schwann cell hyperplasia is present in multiple peripheral nervous system ganglia in P0-GGFβ3 transgenic mice

The majority of the MPNST-like neoplasms in P0-GGFβ3 mice are associated with the trigeminal ganglion. This observation suggested that preneoplastic lesions might be specifically associated with this structure in P0-GGFβ3 mice. To test this hypothesis, we examined trigeminal nerves and associated ganglia from P0-GGFβ3 transgenic mice and age-matched wild-type controls at 1, 2, 4, and 7 months of age. As in the sciatic nerve, increased numbers of Schwann cell nuclei were uniformly evident within the trigeminal ganglia of P0-GGFβ3 animals at all ages examined (Fig. 7). However, unlike the proliferations in sciatic nerve, Schwann cell hyperplasia was predominantly associated with the ganglia, with much less hypercellularity present in adjacent regions of the trigeminal nerve. Furthermore, hypercellular collections of Schwann cells were frequently seen extending from the areas of hyperplasia within the ganglion along the surface of the trigeminal nerve. As in the sciatic nerve, Schwann cell density appeared to decrease with increasing age within the trigeminal ganglion. Because of the nonuniform distribution of Schwann cells within the trigeminal nerve, however, it was not possible to quantify with accuracy the average Schwann cell density in this structure.

On the basis of these findings, we examined sympathetic (superior cervical, superior mesenteric, and celiac) and sensory (dorsal root) ganglia from P0-GGFβ3 mice for evidence of similar hyperplastic lesions. We found that Schwann cell hyperplasia was evident in sympathetic ganglia, much as seen in the trigeminal ganglia. Such changes were never seen in sympathetic ganglia from age-matched nontransgenic controls. Schwann cell hyperplasia was also present in dorsal root ganglia (DRG) from transgenic animals; furthermore, in some animals, although there was no evidence of massive tumors arising in the DRG, these structures were grossly enlarged and showed histologic findings similar to those of the MPNST-like neoplasms described above (data not shown).

Increased Schwann cell DNA synthesis is evident in sciatic nerves and trigeminal ganglia from P0-GGFβ3 transgenic mice

The increased number of Schwann cell nuclei evident in the sciatic nerves and trigeminal ganglia of P0-GGFβ3 mice could reflect increased proliferation, enhanced survival, or a combination of these two factors. To investigate the possibility that the increase in Schwann cell numbers is attributable to increased mitogenesis, 1-, 2-, and 4-month-old transgenic mice and age-matched nontransgenic controls were injected intraperitoneally with 60 mg/kg BrdU and 6 mg/kg 5-FU (to enhance BrdU incorporation) and killed 90 min later. Sciatic and trigeminal nerves from these animals were fixed, paraffin-embedded, and immunolabeled for incorporated BrdU. Consistent with earlier observations in the sciatic nerves of 2-month-old mice (Asbury, 1967), BrdU-immunoreactive Schwann cell nuclei were rare in sciatic nerves from 1-, 2-, and 4-month-old wild-type mice (Fig. 8A–C). In contrast, multiple BrdU-positive nuclei were seen in sciatic nerve sections from 1-, 2-, and 4-month-old P0-GGFβ3 mice (Fig. 8D–F, arrows). Similar increases in the number of BrdU-immunoreactive Schwann cell nuclei were evident within the trigeminal ganglia of P0-GGFβ3 transgenic mice.

To quantitate these differences, BrdU labeling indices (the number of BrdU-positive Schwann cell nuclei divided by the total number of Schwann cell nuclei) were determined for sciatic nerves and trigeminal ganglia from 1-, 2-, and 4-month-old P0-GGFβ3 transgenic mice and age-matched nontransgenic controls. In the sciatic nerve, BrdU labeling indices were significantly higher in P0-GGFβ3 mice than in wild-type controls at 1 and 2 months of age (Fig. 8G). However, BrdU labeling indices in sciatic nerve from P0-GGFβ3 mice demonstrated an age-related
Discussion

To determine what effects NRG-1 elicits in mature Schwann cells, we generated transgenic mice constitutively overexpressing the NRG-1 isoform GGFβ3 in noninjured peripheral nerves. After an initial period of myelination that occurs coincident with prominent Schwann cell hyperplasia, these mice develop a hypertrophic neuropathy, a condition produced by repetitive rounds of demyelination and remyelination. Many P0-GGFβ3 mice also develop ganglion-associated neoplasms similar to human MPNSTs, a tumor type that occurs sporadically or in association with neurofibromatosis type 1. These MPNST-like neoplasms likely arise from preneoplastic lesions uniformly present in motor, sympathetic, and sensory ganglia from P0-GGFβ3 animals. Considered together, these findings have important implications regarding NRG-1 actions on mature Schwann cells in normal and injured peripheral nerve and the possible involvement of NRG-1 in the pathogenesis of peripheral nerve sheath tumors.

All four P0-GGFβ3 founders and the progeny of founder 33 developed a hypertrophic neuropathy characterized by onion bulb formation with evidence of active demyelination and remyelination. Myelination is relatively normal in 1-month-old P0-GGFβ3 mice, with pathology first becoming evident at ~2 months of age and worsening thereafter. The ability of GGFβ3 to induce demyelination in the noninjured sciatic nerve of P0-GGFβ3 transgenic mice is consistent with a previous report that this NRG-1 isoform promotes demyelination in myelinated co-
cultures of mature Schwann cells and DRG neurons (Zanazzi et al., 2001). GGFβ3-mediated repression of myelin protein expression is also consistent with the development of onion bulbs in the sciatic nerves of P0-GGFβ3 mice; alternative cycles of induction and repression of GGFβ3 expression, which is controlled in these animals by the regulatory elements of the P0 gene, would be expected to produce the repetitive cycles of demyelination and remyelination that produce these pathologic structures. Thus, both in vivo and in vitro studies demonstrate that GGFβ3 induces demyelination in differentiated adult Schwann cells. The NRG-1 effects on myelination in this setting are likely distinct from those reported in the neonatal period, in which ablation of the erbB2 locus inhibits the initial establishment of myelination (Garratt et al., 2000b). The occurrence of demyelination in P0-GGFβ3 mice is thus consistent with the hypothesis that NRG-1 stimulates demyelination when its expression is induced after injury of the adult nerve.

Sciatic nerve pathology in P0-GGFβ3 mice is similar to that of human hypertrophic neuropathies such as hereditary sensory and motor neuropathy I (HMSN I; Charcot–Marie–Tooth disease). Nonetheless, it is unlikely that the NRG-1 locus is mutated in HMSN I. Most cases of HMSN I develop as a consequence of mutations in the peripheral myelin protein 22, connexin 32, P0, and EGR2 genes (Young and Suter, 2001). Furthermore, although rare forms of HMSN I exist for which the gene locus has not yet been identified, none of the mapping studies performed to date have implicated the NRG-1 locus in the pathogenesis of these neuropathies. These observations do not exclude a role for NRG-1 in the development of HMSN I. Indeed, given the effects this growth factor has on myelination, it is quite possible that NRG-1 plays a role in the pathogenesis of human hypertrophic neuropathies.

In addition to demyelination, we found that GGFβ3 stimulates in vivo Schwann cell mitogenesis. Sciatic nerves from P0-GGFβ3 mice as young as 1 month of age show a marked increase in Schwann cell nuclear density relative to nontransgenic controls. Although average Schwann cell densities decreased with age in both transgenic and nontransgenic mice, Schwann cell numbers in P0-GGFβ3 mice were significantly higher than in control mice out to 7 months of age. This GGFβ3-mediated increase in Schwann cell numbers was accompanied by increased bromodeoxyuridine incorporation, indicating that stimulation of Schwann cell proliferation was a major mechanism increasing Schwann cell numbers in P0-GGFβ3 mice. In contrast, there was little evidence of Schwann cells immunoreactive for activated caspase-3, a major mediator of nervous system programmed cell death, in sciatic nerves from 1-, 2-, and 4-month-old wild-type or transgenic mice. We cannot yet rule out the possibility that transgenic overexpression of GGFβ3 diminishes the extent of programmed cell death normally occurring during the first postnatal week (Grinspan et al., 1996), thereby contributing to an initial increase in Schwann cell numbers. Nonetheless, our findings in older mice are consistent with the hypothesis that NRG-1 stimulates Schwann cell mitogenesis in injured adult nerve. It is not yet clear whether the promitogenic action of NRG-1 in this setting is distinct from its demyelinating effects.

Constitutive overexpression of GGFβ3 was also associated with the development of MPNST-like tumors in two P0-GGFβ3 lines. This finding is consistent with several previous observations linking the NRG-1–erbB signaling pathway to Schwann cell neoplasia. First, Brockes et al. (1986) detected high to intermediate levels of a GGF-like activity in a subset of human neurofibro-
mas (the precursor lesion from which many MPNSTs arise) and low levels of this activity in the single MPNST they examined. Furthermore, malignant schwannomas (a MPNST variant) induced by in utero treatment of rats (Perantoni et al., 1987; Nikitin et al., 1991), mice (Buzard et al., 1999b), or hamsters (Buzard et al., 1999a) with the mutagen N-ethyl-N-nitrosourea (EtNU), frequently carry a form of the NRG-1 receptor subunit erbb2 with an activating point mutation. Last, we have found that JS1 cells (Schubert et al., 1974; Kimura et al., 1990), a Schwann cell line derived from an EtNU-induced MPNST, express high levels of several NRG-1 isoforms (including GGF variants), erbb3, and a nonmutated form of erbb2 (Frohnert et al., 2003). JS1 cells demonstrate constitutive tyrosine phosphorylation of these erbb kinases, which can be blocked pharmacologically, resulting in a marked decrease in DNA synthesis. These observations, considered together with our finding that overexpression of GGFβ3 induces MPNST development, are consistent with the hypothesis that constitutive activation of the NRG-1–erbb signaling pathway induces Schwann cell neoplasia in rodents. The role of these signaling events in the pathogenesis of human Schwann cell neoplasms remains to be determined.

Although GGFβ3 overexpression contributes to the development of MPNST-like tumors in P0–GGFβ3 mice, it is unlikely that this is the only molecular event involved in this process. Several factors support this conclusion, including the nonuniform development of these neoplasms in the PNS (see below), the occurrence of MPNSTs in only some animals, and the tendency of these tumors to be found in older animals. At present, it is unclear whether these additional genetic events include those previously identified in human MPNSTs such as inactivation of the NF1 (Perry et al., 2001), Rb (Birindelli et al., 2001), p53 (Menon et al., 1990; Birindelli et al., 2001), and CDKN2A/p16 (Kourea et al., 1999; Nielsen et al., 1999) genes. These additional mutations likely occur in cells within the hyperplastic lesions uniformly present in the motor, sensory, and sympathetic peripheral ganglia of P0–GGFβ3 mice. Several features suggest that the hyperplastic lesions in ganglia are preneoplastic, including their relatively high degree of cellularity, their association with the ganglion (see below), and the tendency of cells in these regions to extend outward along the epineurium.

In humans, MPNSTs are most frequently found in large nerves such as the sciatic nerve. However, human MPNSTs also develop in cranial nerves, most commonly in the trigeminal ganglion or its peripheral branches (Urich and Tien, 1998). Like this minor subset of human MPNSTs, the majority of the MPNSTs developing in P0–GGFβ3 mice arose within trigeminal ganglia. A similar distribution is seen for MPNSTs induced in rats and mice by in utero carcinogenesis with EtNU (Druckrey et al., 1966; Wechsler et al., 1979; Anderson et al., 1989; Buzard et al., 1999b). The preferential association of EtNU-induced tumors with the trigeminal ganglion has been suggested to reflect the developmental state of this structure at embryonic day 15, the time of EtNU exposure. However, because NRG-1 overexpression in P0–GGFβ3 mice likely occurs with a different time course from EtNU exposure, other possibilities must be considered. First, PNS ganglia may contain a distinct Schwann cell subtype particularly sensitive to NRG-1 stimulation. Second, MPNSTs and their precursor lesions may arise from some other NRG-responsive glial lineage specific to ganglia such as satellite cells; alternatively, MPNSTs may develop from immature Schwann cell-like elements or stem cells analogous to those recently identified in the adult gut (Kruger et al., 2002) that are persistently present in perinatal or adult ganglia. Last, other factors unique to the ganglionic microenvironment (e.g., costimulation by growth factors released from ganglionic neurons) may alter Schwann cell responses to NRG-1 stimulation.

In conclusion, overexpression of GGFβ3 in noninjured peripheral nerves induces Schwann cell proliferation and demyelination, ultimately resulting in the development of both a hypertrophic neuropathy similar to Charcot–Marie–Tooth disease and...
neoplasms resembling the human malignant peripheral nerve sheath tumors that occur both sporadically and in association with neurofibromatosis type 1. The effects produced by GGF3 overexpression are therefore consistent with the hypothesis that NRG-1 isoforms mediate both demyelination and Schwann cell proliferation after injury of an adult peripheral nerve. Given its phenotype, the P0-GGF3 transgenic mouse will be highly useful in future studies further examining NRG-1 actions on non-transformed mature Schwann cells in vivo and the role NRG-1 plays in Schwann cell neoplasia.

References


