Tumorigenesis and Neoplastic Progression

Perinatal Epidermal Growth Factor Receptor Blockade Prevents Peripheral Nerve Disruption in a Mouse Model Reminiscent of Benign World Health Organization Grade I Neurofibroma

Jianqiang Wu,* Jason T. Crimmins,*
Kelly R. Monk,* Jon P. Williams,*
Maureen E. Fitzgerald,[†] Susan Tedesco,[‡] and
Nancy Ratner*

From the Division of Experimental Hematology, Department of Pediatrics,* Cincinnati Children's Hospital, and the Departments of Cell Biology, Neurobiology, and Anatomy,[†] and Anesthesia,[‡] University of Cincinnati College of Medicine, Cincinnati, Ohio

Benign peripheral nerve tumors called neurofibromas are a major source of morbidity for patients with neurofibromatosis type 1. Some neurofibroma Schwann cells aberrantly express the epidermal growth factor receptor (EGFR). In a mouse model in which the CNPase promoter drives expression of human EGFR in Schwann cells, nerves develop hypertrophy, mast cell accumulation, collagen deposition, disruption of axon-glia interactions, characteristics of neurofibroma and are hypoalgesic. Administration of the EGFR antagonist cetuximab (IMC-C225) for 2 weeks beginning at birth in CNPase-hEGFR mice normalized all pathologies at 3 months of age as evaluated by hotplate testing or histology and by electron microscopy. Mast cell chemoattractants brain-derived neurotrophic factor, monocyte chemoattractant protein-1, and transforming growth factor- β 1, which may account for mast cell accumulation and fibrosis, were reduced by cetuximab. Later treatment was much less effective. A birth to 2-week pulse of cetuximab blocked hEGFR phosphorylation and Schwann cell proliferation in perinatal mutant nerve, so CNPase-hEGFR Schwann cell numbers correlate with the cetuximab effect. A >250-fold enlarged population of EGFR⁺/p75⁺ cells was detected in newborn *Nf1*^{+/-} mouse nerves. These results suggest the existence of an EGFR⁺ cell enriched in the perinatal period capable of driving complex changes characteristic of neurofibroma formation. (*Am J Pathol* 2006, 168:1686–1696; DOI: 10.2353/ajpath.2006.050859)

Neurofibromatosis type 1 (NF1) is a very common inherited disease, affecting 1:3500 individuals worldwide.^{1,2} Nearly all (95%) NF1 patients develop neurofibromas, benign peripheral nerve sheath tumors that cause disfigurement, nerve compression, and distortion or overgrowth of adjacent structures.³ Cutaneous neurofibromas are associated with small nerve branches, whereas diffuse plexiform neurofibromas develop in childhood and often extend deeply along nerves and involve all levels of skin, fascia, muscle, bone, and even viscera. The lifetime risk for NF1 patients of developing a malignant peripheral nerve sheath tumor (MPNST), mainly from within plexiform neurofibromas, is 8 to 13%.^{4,5}

Currently, the available therapy for neurofibromas is surgical debulking.^{6,7} Total surgical resection is often impossible because neurofibromas are made up of tumor cells within surrounding nerve and adjacent normal tissues. Many patients experience regrowth of the tumor after subtotal resection.⁸ Therapeutic modalities including the use of antihistamines, maturation agents, anti-angiogenic drugs, chemotherapy, and radiotherapy have not shown efficacy in this tumor.⁹ Development of novel, nonsurgical treatments to either prevent tumor formation or inhibit neurofibroma growth is a high priority.

EGFR plays an important role in tumor formation and prognosis in many diseases and is overexpressed or mutated in one-third of human epithelial cancers, including prostate, breast, gastric, colorectal, and ovarian.¹⁰ The activation of HER1/EGFR mediated through ligand

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J.W. is a DOD Neurofibromatosis Research Program fellow.

Address reprint requests to Nancy Ratner, Ph.D., Division of Experimental Hematology, Cincinnati Children's Hospital, 3333 Burnet Ave., Cincinnati, OH 45229-7013. E mail: nancy.ratner@cchmc.org.

binding triggers a network of signaling processes that promote tumor cell proliferation, migration, adhesion, angiogenesis, and decreased apoptosis.^{11,12} The intensive study of EGFR signaling mechanisms has already yielded therapeutic agents in the treatment of cancer.^{13,14} Therapeutic approaches including anti-sense oligonucleotides^{15,16} and small molecule inhibitors of enzymes^{17,18} are being investigated to target members of the EGFR family, particularly HER1/EGFR and HER2. Another therapeutic strategy is the use of monoclonal antibodies (mAbs) against the extracellular domain of the EGFR.^{19,20} The mAb cetuximab (IMC-C225) (ImClone Systems, Inc., New York, NY), a human-specific EGFR monoclonal antibody that binds to the EGF receptor extracellular domain with high affinity, blocks ligand binding and down-regulates receptor expression on the cell surface.^{21–23} Treatment with cetuximab in combination with chemotherapeutic drugs or radiotherapy is effective in eradicating well-established tumors in nude mice.^{20,24,25} Cetuximab is Food and Drug Administration approved in some overexpressing cancers and has clinically significant activity alone or in combination with irinotecan in adults with irinotecan-refractory colorectal cancer.^{20,26}

EGFR expression on neurofibroma Schwann cells implicates EGFR as an attractive target for the treatment of neurofibromas. Schwann cells are believed to be the primary pathogenic cells in neurofibromas because they show biallelic mutation at *Nf1*, dissociation from axons, and angiogenic and invasive properties.²⁷ Normal Schwann cells develop from the neural crest and lack EGFR expression. Expression of EGFR is observed in 1 to 4% of S100⁺ neurofibroma Schwann cells, in some cells from human MPNSTs,^{28,29} and is a common feature of human MPNST cell lines and cell lines derived from compound heterozygous mice bearing loss of function in *Nf1* and *p53*.³⁰ All of the cell lines expressing EGFR respond to EGF by activation of the downstream signaling pathways. Growth of the cell lines is stimulated by EGF *in vitro* and blocked by antagonists of the EGFR.³⁰ Treatment of a human MPNST cell line with cetuximab also significantly inhibits cell growth.²⁸

The objective of this study was to determine the potential therapeutic utility of the mAb cetuximab in a mouse model in which human EGFR is expressed in peripheral nerve Schwann cells.³¹ Treatment with cetuximab only early in postnatal life prevented the development of the neurofibroma-like phenotype in these transgenic mice. We also identified a perinatal cell population expressing EGFR amplified in *Nf1*^{+/-} perinatal nerve and postulate that a critical period exists for neurofibroma formation.

Materials and Methods

Animals

We genotyped CNPase-hEGFR transgenic mice by polymerase chain reaction (PCR) as described.³¹ Mice were on the FVBN strain (*n* = 6 generations backcross). Wild-type and *Nf1* hemizygous mice were on the C57BL/6 background; they were derived and genotyped as described.³² We housed mice in a temperature- and humidity-controlled

vivarium that was kept on a 12-hour dark-light cycle with free access to food and water. The animal care and use committee of the University of Cincinnati or Cincinnati Children's Hospital Medical Center approved all animal use.

Drug Treatment Design

Cetuximab was the kind gift of Dr. Daniel J. Hicklin (ImClone, New York, NY). We dissolved it in phosphate-buffered saline (PBS) at a concentration of 2 mg/ml. It blocks human but not rodent EGFR function with high affinity.^{21,23} We injected cetuximab intraperitoneally twice weekly at a dose of 40 mg/kg according to a previous report.²⁴ Each group contained 12 to 16 mice with approximately half wild-type (WT) and half CNPase-hEGFR. We treated groups of mice with cetuximab at 0 to 2 weeks, 1 to 3 weeks, 2 to 4 weeks, 3 to 5 weeks, birth to 6 weeks, 6 to 8 weeks, or 6 to 12 weeks of age. A control group consisted of littermates injected with the same volume of PBS birth to 6 weeks. We sacrificed mice at 2 weeks or 3 months of age. The dose and time schedule diagram is shown in Figure 1A. EKI-785 was obtained from Wyeth Ayerst Research Laboratory, Pearl River, NY. Intraperitoneal dosing daily at 20 mg/kg/day or with carrier was as recommended.¹⁷ The ages of mice injected is noted in the text.

Hotplate Test (Nerve Sensory Test)

Hotplate procedures were reviewed and approved by the animal care and use committee of the University of Cincinnati before the study was initiated and the study was conducted in compliance with the American Association for Accreditation of Laboratory Animal Care and the ethical guidelines of the International Association for the Study of Pain. The hotplate test was performed in a quiet environment during the day, using an electronically controlled hotplate analgesia meter (Columbus Instruments, Columbus, OH) heated to 50°C (±0.1°C). Mice were housed in a separate room and conditioned for 1 week before the test. The time it took the animals to begin licking their forelegs or hind paws was recorded by two observers blinded to genotype. The cutoff time was set at 30 seconds to minimize tissue damage.^{33,34}

Immunofluorescence

After cetuximab treatment for 2 weeks, mice were fixed by intracardial perfusion with 4% paraformaldehyde in PBS. Sciatic nerves were dissected, cryoprotected, and cut in 10-μm sections for immunofluorescence staining as described.³⁵ Briefly, section slides were fixed in 4% (w/v) paraformaldehyde at room temperature (22 to 25°C) for 10 minutes. After blocking with PBS containing 10% (v/v) donkey serum for 1 hour at room temperature, individual sections were washed in PBS and incubated with anti-phospho-EGFR antibody (SC-12351, 1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C followed by incubation with Alexa 488 rabbit anti-goat IgG and propidium iodine for 1 hour at room temperature. Mouse ear, which has keratinocytes expressing

Figure 1. Hotplate test monitors cetuximab treatment effect. **A:** Diagram of cetuximab injection schedule. Mice were injected intraperitoneally with cetuximab or PBS twice weekly. The **heavy solid line** shows the duration of drug treatment or, for control, of PBS injection. Groups are referred to in the text by the designation at **left** whereby the first digit is the age in weeks that treatment began and the second digit is the duration of treatment in weeks (eg, 0 × 6 = started at birth, treated for 6 weeks). Mice were sacrificed at 3 months of age (S). **B:** Quantification of paw withdrawal latency in hotplate-tested mice. CNPase-hEGFR and WT mice were treated with cetuximab or PBS for designated times (see **A**) and tested for heat sensitivity at 3 months of age. Paw withdrawal latency was significantly increased in untreated CNPase-hEGFR mice (**black bar**) compared to untreated WT mice (**white bar**; $P = 0.009$, $n = 7$). Wild-type thermal sensitivity was restored in CNPase-hEGFR mice treated with cetuximab for 2 or 6 weeks beginning at birth (0 × 2: $P = 0.32$, $n = 5$; 0 × 6: $P = 0.36$, $n = 5$). Sensitivity in the 6 × 6 group was not significantly different from untreated CNPase-hEGFR mice ($P = 0.38$, $n = 6$). Thermal sensitivity was intermediate in CNPase-hEGFR mice treated with cetuximab beginning at 1, 2, or 3 weeks of age.

abundant pEGFR was used as positive control; goat IgG was used as a negative control. Slides were air-dried, coverslipped, and viewed with a laser confocal microscope (Carl Zeiss, Thornwood, NY) equipped with a digital imaging system.

Cell Proliferation

Mice were injected (intraperitoneally) with bromodeoxyuridine (BrdU, Sigma-Aldrich, St. Louis, MO) in PBS (50 mg BrdU per kg of body weight) three times at 2-hour intervals (total 6 hours) before intracardiac perfusion. Sciatic nerves were dissected, paraffin-embedded, and cut into 10- μ m sections. Sections were deparaffinized, washed in PBS, treated with 2 mol/L HCl for 30 minutes at 37°C, washed in PBS, and blocked in PBS containing 0.15% Triton X-100 and 5% donkey serum. We incubated

sections with a rat anti-BrdU antibody (1:200; Zymed Laboratories Inc., South San Francisco, CA) overnight at 4°C. Detection used tetramethyl-rhodamine isothiocyanate-conjugated secondary antibody. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co., St. Louis, MO) for 5 minutes. BrdU-labeled cells and the DAPI nuclei were counted in at least three cross sections made through each sciatic nerve. Data are presented as average numbers of BrdU-labeled cells per section.

Electron Microscopy

Mice were perfused (intracardially) with Karnovsky's fixation solution (3% paraformaldehyde and 3% glutaraldehyde in 0.1 mol/L phosphate buffer, pH 7.4 to 7.6). Saphenous nerves were dissected, embedded, and evaluated as described.³¹

Quantification of Saphenous Nerve Area, Nuclei Counting, and Mast Cell Number

We perfused mice (intracardially) with 4% paraformaldehyde made with 0.1 mol/L PBS. We dissected sciatic and saphenous nerves and immersed them in the same fixative solution overnight at 4°C before paraffin-embedding. Every fifth cross-section (6 μ m) was cut and mounted. Three cross sections through each nerve were analyzed. DAPI, toluidine blue, and Gomori's trichrome stainings were performed for nuclei counting, mast cell counting, and collagen deposition, respectively. MetaMorph image analysis software (Universal Imagine Corp., Downingtown, PA) was used to quantify the nerve area of toluidine blue-stained saphenous sections. Statistical significance was determined by two-tailed Student's *t*-test.

Quantitative Real-Time PCR (QRT-PCR)

mRNA was extracted from WT, CNPase-hEGFR, and cetuximab-treated CNPase-hEGFR mouse sciatic nerve using the Micro-FastTrack kit for isolation of mRNA from small samples (Gibco-Invitrogen, Carlsbad, CA). The mRNA was reverse-transcribed using the Superscript Preamplification System (Gibco-Invitrogen). Superscript II reverse transcriptase was used in the reaction, as per the manufacturer's protocol. Duplicate samples lacking reverse transcriptase were conducted to control for genomic DNA contamination. Mouse GAPDH primers (sense, 5'-ACCCAGAAGACTGTGGATGG-3' and anti-sense, 5'-GGAGACAACCTGGTCCTCAG-3'; expected product size, 300 bp) were included in each reaction as a positive control for cDNA. For QRT-PCR experiments, cDNA was used (as generated above), as were the following primers: brain derived neurotrophic factor (BDNF; sense, 5'-TGGCTGCACTTTTGAGCAC-3' and anti-sense, 5'-GCAGTCTTTTATCTGCCGC-3'; expected product size, 292 bp), monocyte chemoattractant protein 1 (MCP-1; sense, 5'-AGCACCAGCCAACCTCACT-3' and anti-sense, 5'-CGTTAACTGCATCTG-

GCTGA-3'; expected product size, 136 bp), stem cell factor (SCF; sense, 5'-CAGTCTTCAGGAGTGAGCCC-3' and anti-sense, 5'-CAAAGATGCTCCCAAACGCT-3'; expected product size, 254 bp), transforming growth factor- β 1 (TGF- β 1; sense, 5'-TGAGTGGCTGTCTTTTGACG-3' and anti-sense, 5'-TCTCTGTGGAGCTGAAGCAA-3'; expected product size, 293 bp). Triplicate reactions were performed in an ABI Prism 7500 as described.³¹ Briefly, 1 μ l of cDNA or water control was placed into a 50- μ l reaction volume containing 25 μ l of SYBR Green master mix (2 $\times$ concentration; Applied Biosystems, Foster City, CA) and volumes of primers that ranged between 2 μ l and 9 μ l, depending on the optimal conditions for each primer set. The remaining volume was comprised of water. The thermal cycling conditions comprised an initial equilibration step at 60°C (2 minutes), a denaturation step at 95°C (10 minutes), followed by 40 cycles of 95°C (15 seconds), 60°C (1 minute). Cycle threshold (C_t) values were obtained from the point during amplification at which the fluorescent intensity was in the geometric phase, as determined by PE Biosystems analysis software. All PCR products were analyzed on a 2% agarose gel. The ΔC_t values were determined for WT, CNPase-hEGFR, and cetuximab-treated CNPase-hEGFR nerve and relative ligand expression was calibrated to GAPDH expression (primers as above). Fold change of cytokines in CNPase-hEGFR and cetuximab-treated CNPase-hEGFR nerve compared to WT levels were calculated using the established equation (K. Luvak, PE ABI Sequence Detector User Bulletin 2), where C_t is the cycle number at the chosen amplification threshold, $\Delta C_t = C_{t(\text{cytokine})} - C_{t(\text{GAPDH})}$, and $\Delta\Delta C_t = \Delta C_{t(-/-)} - \Delta C_{t(+/-)}$.

Analysis of EGFR⁺p75⁺ Cells from Perinatal Nerves

We dissected sciatic nerves from individual mouse P0 or P1 pups and dissociated nerves in L-15 medium containing 1.25 U/ml Dispase II (Roche, Indianapolis, IN) and 156 U/ml collagen type I (Worthington, Lakewood, NJ) for 30 minutes. We collected cells by centrifugation and plated them onto poly-L-lysine- and laminin-coated eight-well Lab-Tek chamber slides in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% Pen/Strip. Medium was changed the next day to remove debris. Cells were fixed 24 hours later with 4% paraformaldehyde, then double stained with goat anti-EGFR (SC-03-G, 1:20; Santa Cruz Biotechnology) and rabbit anti-p75 (AB1554, 1:200; Chemicon, Temecula, CA) antibodies, followed by secondary antibodies donkey anti-goat Alexa-488 (1:2000 for EGFR; Molecular Probes, Eugene, OR) and mouse anti-rabbit tetramethylrhodamine isothiocyanate (1:200, Molecular Probes). Nuclei were counterstained with DAPI. MPNST cell line 8814 (EGFR-positive) and goat IgG were used as positive and negative controls. Three independent experiments were performed with a total number of nine *Nf1*^{+/-} and six WT pups. For each Lab-Tek well, 10 random fields were chosen for cell counting. The genotype was blind during counting. We used two-tailed Student's *t*-test for data analysis.

Results

Thermal Sensory Decrease in CNPase-hEGFR Mouse

Small caliber axons ensheathed by nonmyelin forming Schwann cells convey most thermal sensory information. Abnormalities in unmyelinated fiber bundle organization in CNPase-hEGFR transgenic mouse³¹ raised the possibility that the sensation is decreased or lost in this mouse model. We used a test of heat sensitivity to assess this possibility. Significantly decreased heat sensitivity (hypoalgesia) was found in 3-month-old transgenic mice as compared to WT littermates ($P = 0.009$, $n = 7$) (Figure 1B).

Based on these results, we tested the thermal sensitivity in cetuximab-treated transgenic or WT mice using the dosing schedule in Figure 1A. Cetuximab treatment of mice for 2 weeks or 6 weeks beginning at birth maintained equivalent thermal sensation to WT levels ($P = 0.32$, $n = 6$ for 0 to 2 weeks group; $P = 0.36$, $n = 5$ for 0 to 6 weeks group) (Figure 1B). However, treatment beginning at 6 weeks of age was ineffective in reversing the hEGFR-driven phenotype ($P = 0.01$, $n = 6$). In mice treated at 1, 2, or 3 weeks of age for 2 weeks, the hypoalgesia was partially maintained. Thus early treatment appeared most effective, while later treatment had lesser effect.

Effects of Cetuximab on CNPase-hEGFR Nerve Histology

To confirm results of hotplate testing we performed detailed histology on nerves from the cohort of WT and mutant mice. We chose saphenous nerve sections from mice for analysis of nerve area, nuclei counts, mast cell number, and collagen deposition because saphenous nerves are highly disrupted in this model by 3 months of age.³¹ As shown in Figure 2, A–C, there are significant differences between transgenic mice and WT mice in nerve area ($P < 0.001$, $n = 6$), nerve nuclei number ($P < 0.001$, $n = 6$), and mast cell number ($P < 0.001$, $n = 6$), and these were maintained with brief exposure to cetuximab beginning at 6 weeks for 2 weeks. When mice were treated by cetuximab for 6 weeks starting at 6 weeks, there was a small significant difference in the nerve area ($P < 0.001$, $n = 6$), nuclei number ($P < 0.001$, $n = 6$), and mast cell number ($P < 0.05$, $n = 6$) compared to untreated CNPase-hEGFR-positive mice. Significant differences remained compared to WT mice in nerve area ($P < 0.001$, $n = 6$), nuclei number ($P < 0.001$, $n = 6$), and mast cell number ($P < 0.05$, $n = 6$). In contrast, when treatment began at birth no significant difference between transgenic mice and WT mice remained, whether we measured nerve area ($P = 0.36$, $n = 5$ for 0 to 2 weeks group; $P = 0.32$, $n = 6$ for 0 to 6 weeks group), nuclei number ($P = 0.10$, $n = 5$ for 0 to 2 weeks group; $P = 0.28$, $n = 6$ for 0 to 6 weeks group), or mast cell number ($P = 0.36$, $n = 5$ for 0 to 2 weeks group; $P = 0.32$, $n = 6$ for 0 to 6 weeks group) (Figure 2, A–C). Counting per high-powered field, normalizing to total area, and counting per

Figure 2. Cetuximab effects depend on treatment starting time. **A:** Quantification of saphenous nerve area in WT (white bars) and CNPase-hEGFR (black bars) mice. Untreated CNPase-hEGFR nerves were significantly larger than WT ($P < 0.001$, $n = 6$). Nerves of CNPase-hEGFR mice treated with cetuximab in the 6×2 group were also significantly larger than WT ($P < 0.01$, $n = 6$). Area of nerves from CNPase-hEGFR mice treated with cetuximab for 2 or 6 weeks beginning at birth did not differ significantly from WT mice (0×2 : $P = 0.36$, $n = 5$; 0×6 : $P = 0.32$, $n = 6$). Nerve area was intermediate in EGFR⁺ mice treated with cetuximab beginning at 1, 2, or 3 weeks of age. **B:** Quantification of nuclear counts in WT (white bars) and CNPase-hEGFR (black bars) nerves. Graph shows average number of nuclei per section. Nuclei counts were significantly higher in untreated CNPase-hEGFR nerves ($P < 0.001$, $n = 6$) and 6×2 CNPase-hEGFR nerves ($P < 0.01$, $n = 6$) compared to WT counterparts. Nerve nuclei counts of CNPase-hEGFR mice treated with cetuximab for 2 or 6 weeks beginning at birth did not differ significantly from WT (0×2 : $P = 0.1$, $n = 5$; 0×6 : $P = 0.28$, $n = 6$). Nerve nuclei counts were intermediate for CNPase-hEGFR mice in the 6×6 , 3×2 , 2×2 , and 1×2 groups. **C:** Quantification of mast cell counts in WT (white bars) and CNPase-hEGFR (black bars) nerves. Graph shows total number of mast cells per nine sections. Mast cell numbers were significantly higher in untreated CNPase-hEGFR nerves ($P < 0.001$, $n = 6$) compared to untreated WT nerves and in all treatment paradigms that did not begin at birth. Mast cell numbers in CNPase-hEGFR nerve from mice treated with cetuximab for 2 or 6 weeks beginning at birth did not differ significantly from WT (0×2 : $P = 0.36$, $n = 5$; 0×6 : $P = 0.32$, $n = 6$). **D–F:** Sciatic nerve tissue sections from 3-month-old mice stained with Gomori's trichrome staining. **D:** WT mouse nerve shows normal staining with little collagen (green). **E:** Untreated CNPase-hEGFR nerve has more collagen than WT mouse. **F:** CNPase-hEGFR nerve treated with cetuximab (0×6) shows collagen similar to WT.

total section gave similar and statistically significant results (data not shown). Early treatment with cetuximab also decreased aberrant collagen matrix staining, assessed without quantification, in the CNPase-hEGFR nerves (Figure 2, D–F). Data were confirmed by treatment of mice for either 8 to 14 weeks or 1 to 4 weeks with the EGFR and ErbB2 tyrosine kinase inhibitor EKI-785 (Supplemental Figure 1, see <http://ajp.amjpathol.org>).

Early Exposure to Cetuximab Reverses Defects in Axon-Glia Interactions

The intimate interactions between axons and Schwann cells are monitored by transmission electron microscopy. In WT mouse nerves, groups of small unmyelinated axons cluster within single non myelin-forming Schwann cells, whereas myelinated Schwann cells show 1:1 interaction with large axons (Figure 3A). We analyzed saphenous nerves of mutant mice before and after drug treatment and considered small axons grouped if three or more were wrapped by a Schwann cell. As shown in Figure 3B, CNPase-hEGFR mouse Schwann cells wrap one or a few small axons rather than the larger numbers wrapped by

WT Schwann cells (Figure 3A). Mice treated beginning at 6 weeks of age for 2 weeks contained axon-Schwann cell units within nerves with ultrastructural characteristics of untreated CNPase-hEGFR mice. Ultrastructure of mice treated beginning at 6 weeks for 6 weeks was intermediate. After treatment with cetuximab for 2 or 6 weeks beginning at birth, the axon-Schwann cell bundles in CNPase-hEGFR saphenous nerve Schwann cells were significantly improved (Figure 3, C–E). This EM analyses confirms that early cetuximab treatment prevents the peripheral nerve dysfunction driven by hEGFR expression in Schwann cells, while later short treatment is ineffective.

Levels of Mast Cell Chemoattractants Decrease after Drug Treatment

Mast cell chemoattractants BDNF, MCP-1, SCF (kit ligand), TGF- β 1, and vascular endothelial growth factor (VEGF) are each up-regulated in CNPase-hEGFR transgenic mouse nerves as determined by QRT-PCR.³¹ We tested if birth to 6-week blockade of EGFR could regulate specific chemoattractants monitored at 3 months. As shown in Table 1, there was no significant change in SCF

Figure 3. Early exposure to cetuximab restores axon-glia interactions. **A–D:** Electron micrographs of saphenous nerves from 3-month-old animals. **A:** WT nerves showed characteristic groups of small unmyelinated axons clustered within single nonmyelin forming Schwann cells. **B:** Untreated CNPase-hEGFR nerves were disrupted; nonmyelinating Schwann cells rarely wrap multiple axons. **C and D:** CNPase-hEGFR nerves treated with cetuximab beginning at birth for 2 weeks (**C**) or 6 weeks (**D**) showed WT levels of nerve organization; nonmyelinating Schwann cells were able to wrap multiple small axons. **E:** Quantification of percentage of grouped axons in nerves from WT, untreated CNPase-hEGFR (EGFR⁺), CNPase-hEGFR mice treated from birth for 2 weeks (0 × 2), birth to 6 weeks (0 × 6), 6 to 8 weeks (6 × 2), or 6 to 12 weeks (6 × 6) of age. Small axons are considered grouped if three or more are wrapped by a single nonmyelinating Schwann cell. Untreated CNPase-hEGFR nerves were significantly more disrupted than WT. CNPase-hEGFR nerves treated with cetuximab beginning at birth did not differ significantly from WT. Original magnifications, ×9375.

(1.19-fold) or VEGF (1.07-fold). TGF- β 1 and MCP-1 were decreased 2.57-fold and 4.14-fold, respectively. The most dramatic change occurred in BDNF, which was no longer detectable in the nerve after cetuximab treatment. These results demonstrate that transient treatment with cetuximab affects Schwann cell expression of mast cell chemoattractants many weeks later.

Cetuximab Inhibits Schwann Cell Proliferation and hEGFR Phosphorylation in CNPase-hEGFR Mouse

To assess the mechanism underlying cetuximab blockade of nerve dysplasia in early postnatal life, we treated

Table 1. Levels of Mast Cell Chemoattractants Decrease after Cetuximab Treatment

Cytokine	WT	EGFR	EGFR + Cetuximab	Fold change: Cetuximab-treated nerve versus EGFR nerve ($2^{-\Delta\Delta C_t}$ method)
BDNF	Present	Present	Absent	n/a
MCP-1	Absent	Present	Present	-4.14 (-4.35 to -3.94)
SCF	Present	Present	Present	-1.19 (-1.47 to -1.04)
TGF- β 1	Absent	Present	Present	-2.57 (-3.23 to -2.04)
VEGF	Present	Present	Present	-1.07 (-1.28 to +1.09)

Mast cell chemoattractants BDNF, MCP-1, SCF, TGF- β 1, and VEGF were measured by quantitative real-time PCR in WT or EGFR mice, or after exposure to Cetuximab (EGFR + Cetuximab) for 6 weeks starting at birth. Fold change was measured as described in Materials and Methods.

newborn mice with cetuximab for 2 weeks and then analyzed EGFR phosphorylation on sciatic nerve sections using an antibody specific for phosphorylated EGFR. As shown in Figure 4A, hEGFR phosphorylation was robust surrounding sciatic nerve nuclei in untreated hEGFR mice. Phosphorylation decreased significantly after a 2-week exposure to cetuximab (Figure 4B) in whole or in part due to direct effects on phosphorylation or to increase turnover and/or degradation of EGFR targeted by cetuximab. Treatment had no effect on WT mice; this result is expected because cetuximab does not inhibit rodent EGFR and because WT mouse Schwann cells do not express EGFR.²⁸

We then tested if increased Schwann cell number might account for effects of cetuximab in perinatal nerve by monitoring cells in the S phase of the cell cycle using BrdU and counting nuclei. WT or transgenic mice were treated with cetuximab or PBS twice a week throughout the first 2 postnatal weeks. As shown in Figure 4D, the number of BrdU-labeled nuclei in the sciatic nerve of hEGFR mice at 2 weeks of age was higher than in WT mouse littermates (Figure 4C). Sciatic nerve nuclei number also increased significantly. After cetuximab treatment, BrdU-labeled nerve nuclei in transgenic mice decreased to WT level ($P = 0.30$, $n = 4$; Figure 4F). Total nuclei number also decreased after cetuximab treatment and become similar to the WT level ($P = 0.34$, $n = 4$; Figure 4G).

Nearly all cells in EGFR⁺ mutant adult nerves express the Schwann cell markers S100 or GFAP.³¹ We found that 90% of cells in perinatal nerves express the transgene and the Schwann cell marker p75 (see below). There are, as in normal nerves, fibroblasts and endothelial elements that could in account for some of the BrdU-positive nuclei. Therefore we specifically excluded perineurial cells and capillary endothelial cells from our BrdU analysis, based on morphology. Taking into account the inhibition of hEGFR phosphorylation by cetuximab, we conclude that the effect of cetuximab in reversing nerve phenotype correlates with hEGFR-driven Schwann cell proliferation.

The enhanced effect of cetuximab on perinatal nerve could result from loss of transgene expression in older mice. To test this, we dissociated Schwann cells from P1 and from P10 CNPase-hEGFR mouse sciatic nerve and stained cells with antibodies recognizing EGFR and the Schwann cell marker p75. There was higher percentage of EGFR/p75 double-positive cells in P1 CNPase-hEGFR mouse Schwann cells ($90 \pm 9.2\%$, $n = 10$) compared to P10 Schwann cells ($42.4 \pm 3.3\%$, $n = 5$). However, both

in newborn nerves and at P10, numerous Schwann cells express detectable levels of the EGFR transgene and might be targeted by cetuximab. The results indicate that an EGFR-expressing cell present around birth might account for much of the abnormality in this EGFR-expressing model. If an EGFR-expressing cell present around birth is related to tumorigenesis in NF1, other *Nf1* model systems might contain such a cell.

Nf1^{+/-} Mouse Nerves Contain a Population of EGFR⁺/p75⁺ Cells

Nf1^{+/-} knockout mice are viable and fertile whereas *Nf1^{-/-}* embryos die *in utero*.³² If *Nf1* mutant cells predispose to EGFR expression within the perinatal period, it should be possible to detect EGFR⁺ cells in *Nf1^{+/-}* mutant peripheral nerve. To test this, we dissociated cells from P0 to P1 mouse sciatic nerves and stained cells with antibodies recognizing mouse EGFR and the Schwann cell marker p75 in three experiments using two to four individual pups/genotype per group. We identified EGFR/p75 double-positive cells among *Nf1^{+/-}* mouse Schwann cells (Figure 5B) but very few in WT cells (Figure 5A). Among 10,000 cells counted in each genotype, only 2 were EGFR⁺ and p75⁺ from WT nerves, these did not show punctuate EGFR staining characteristic of cells in mutant nerves. Quantification revealed >250-fold increase in double-positive cells in *Nf1^{+/-}* mouse nerve cell preparations, compared to cells from WT mice (Figure 5C).

Discussion

We prevented neurofibroma-like peripheral nerve dysfunction driven by human EGFR expression in Schwann cells using the specific human mAb cetuximab. Our results suggest that early, transient blockage of EGFR has prolonged effects on the Schwann cell hyperplasia, mast cell accumulation, fibrosis, and loss of axon-glial interaction characteristic of this model and of human neurofibroma formation. The data indicate that early blockade of EGFR may provide relevant strategies for inhibiting Schwann cell proliferation related to neurofibroma growth. Supporting the relevance of early postnatal glial cells to NF1, we identified a population of EGFR-expressing cells in perinatal *Nf1* mutant nerve.

Figure 4. Cetuximab inhibits Schwann cell EGFR phosphorylation and proliferation. Newborn CNPase-hEGFR mice were treated with cetuximab from birth to 2 weeks of age, and nerves were stained with antibodies specific to phospho-EGFR or BrdU. **A:** Untreated CNPase-hEGFR nerve stained with an antibody specific to phospho-EGFR (green). Nuclei are counterstained with DAPI (red). **B:** CNPase-hEGFR nerve treated with cetuximab (0×2) showed decrease in level of phospho-EGFR (green). **C–G:** BrdU staining of sciatic nerve tissue sections. Nuclei are counterstained with DAPI (blue). **C:** WT nerve contained low levels of BrdU(+) cells (red). **D:** CNPase-hEGFR nerve contained high levels of BrdU(+) cells (red). **E:** CNPase-hEGFR nerve from mice treated with cetuximab (0×2) showed BrdU(+) cell numbers restored to WT levels (red). **F:** Bar graph shows quantification of percentage of cells BrdU(+). Positive nuclei were quantified from control or cetuximab-treated (0×2) CNPase-hEGFR mouse nerves (**black bars**) or WT counterparts (**white bars**). Percentage of BrdU(+) cells was significantly higher in untreated CNPase-hEGFR nerves compared to WT ($P < 0.001$, $n = 4$). Percentage of BrdU(+) cells was not significantly different between WT and cetuximab-treated CNPase-hEGFR nerves ($P = 0.30$, $n = 4$). **G:** Quantification of total nuclei number per high-powered fields from control or cetuximab-treated (0×2) CNPase-hEGFR mouse nerves (**black bars**) or WT counterparts (**white bars**). Nuclei number was significantly higher in untreated CNPase-hEGFR nerves compared to WT ($P < 0.0001$, $n = 4$). Nuclei number was not significantly different between WT and cetuximab-treated CNPase-hEGFR nerves ($P = 0.34$, $n = 4$). Scale bars, 20 μ m.

The ameliorating effect of cetuximab identified by hot-plate sensory testing was confirmed by much more labor-intensive histology and electron microscopy. Hotplate

testing can provide, therefore, preliminary screening of drug therapeutic effects in this model. Reduced thermal transduction in the periphery, decreased initiation and

Figure 5. *Nf1*^{+/-} but not WT mouse nerves contain EGFR⁺/p75⁺ cells. **A** and **B**: Schwann cells were dissociated from P0 to P1 mouse sciatic nerves and stained with anti-EGFR (green) and anti-p75 (red). **A**: Representative WT cell is p75⁺ but EGFR-negative. **B**: *Nf1*^{+/-} cells showing double staining for EGFR and p75. **C**: Graph shows quantification of percentage of EGFR⁺/p75⁺ cells in WT and *Nf1*^{+/-} nerves. The difference between *Nf1*^{+/-} and WT nerve was significant ($P = 0.006$; *Nf1*^{+/-}, $n = 9$; WT, $n = 6$). Scale bar, 10 μ m.

conduction of action potentials, or diminished synaptic transmitter release, can each cause increased time to paw withdrawal. In the CNPase-hEGFR model, the most likely explanation is diminished sensory function because of the impaired axon-glia interaction, although we have not excluded other possibilities.

We found that treatment with cetuximab from birth to 2 weeks of age prevents EGFR nerve pathology; even slightly later treatment had lesser effects. Our data suggest one or more events occurring between birth and 2 weeks of age drives the nerve dysfunction characteristic of this model. Cetuximab inhibited transgenic mouse Schwann cell EGFR phosphorylation and Schwann cell proliferation in 2-week-old sciatic nerves. Thus a candidate mediator of the EGFR phenotype is perinatal Schwann cell proliferation itself, which leads to sustained increases in Schwann cell numbers.

Why is later, short, treatment with cetuximab not effective? Closure of the blood-nerve barrier after the first postnatal weeks or development of immune response against the humanized antibody at this time was each a trivial explanation of the data. We excluded these possibilities by use of the tyrosine kinase inhibitor EKI-785³⁶ (Supplemental Figures 1 and 2, see <http://ajp.amjpathol.org>). As with cetuximab exposure, late treatment had a much lesser effect, with 1 to 3 weeks of treatment intermediate. A likely possibility is that Schwann cells in older animals fail to respond to EGFR signals by cell proliferation. Young and older Schwann cells in peripheral nerve use different mechanisms for proliferation.³⁷ Young nerves express higher levels of Erb2 or Erb3 than do adult nerves. Although EGFR-ErbB2 heterodimers do not occur in adult nerves in the CNPase-hEGFR model,³¹ it is possible that hEGFR heterodimerization with Schwann cell ErbB2 and/or ErbB3 occurs in neonatal mouse, eliciting a signaling program different from that in EGFR-EGFR homodimers. The sets of dimers are known to signal differently³⁷ and signaling cascades downstream of EGFR-ErbB2 dimers may be required for secretion of Schwann cell factors, Schwann cell proliferation, or other changes in nerve function. Indeed proliferation and death are balanced in older CNPase-hEGFR mice.³¹

Nerve area, number of nuclei, and number of mast cells were slightly decreased by 6 weeks of cetuximab treatment starting at 6 weeks. This suggests that long periods of treatment with cetuximab are effective in re-

versing the CNPase-hEGFR mouse phenotype. This is important because use of EGFR antagonists to treat human neurofibromas would necessitate such late treatment.

Increased Schwann cell number and the secretion of factors by an increased number of cells might account for the neurofibroma-like phenotype in the EGFR model. Mast cell accumulation is a characteristic feature of human neurofibromas³⁸ and of peripheral nerves in CNPase-hEGFR mice.³¹ Cetuximab treatment down-regulated only the aberrant expression of TGF- β , MCP-1, VEGF, and BDNF, even though mRNAs encoding mast cell chemoattractants BDNF, MCP-1, SCF, TGF- β 1, and VEGF are each up-regulated in mutant nerves,³¹ and considerable amounts of data support a role for SCF in mast cell recruitment in *Nf1*-deficient mast cells.^{39,40} These results support the hypothesis that one or more of these mast cell chemoattractants secreted by hEGFR Schwann cells contribute to phenotype(s) in this model. *Nf1* mutant Schwann cells also secrete other growth factors that stimulate angiogenesis and attract mast cells.⁴¹ Human neurofibromas contain growth and angiogenesis-promoting factors, including VEGF, HGF, and FGF2, and neurofibroma cells respond by proliferation to bFGF and HGF.⁴²⁻⁴⁷ Our data suggest the possibility that TGF- β , MCP-1, VEGF, and BDNF in addition to SCF may be evaluated for effects in human neurofibromas.

Consistent with a role for EGFR⁺ cells early in postnatal life in *Nf1*, we identified a population of EGFR/p75 double-positive cells in early postnatal *Nf1*^{+/-} mouse nerve. The existence of these cells is consistent with a predisposition of *Nf1* mutant cells to up-regulate EGFR, the mechanism underlying which may include gene amplification.⁴⁸ *Nf1* mutant EGFR⁺ cells are predicted to survive in the nerve environment, as EGFR⁺ cells expressing the SC marker S100 exist in neurofibromas.²⁸

It will be necessary to determine the effect of blocking EGFR in other *Nf1*-driven models of benign peripheral nerve tumorigenesis. Several factors stand in the way of straightforward testing. First, the antibody we used does not block mouse EGFR so that we are unable to test its effect in available mouse *Nf1* models.⁴⁹⁻⁵¹ Second, EGFR antagonists such as tyrosine kinase inhibitors also block ErbB2, a crucial molecule in Schwann cell development and function.⁵² Third, blocking EGFR function early in mouse development can be deleterious in that complete loss of EGFR causes early postnatal lethality.⁵³

The great potential of EGFR-targeted therapies in the treatment of cancer is prompting the continued development of specific agents targeted to the extracellular ligand-binding domain, the intracellular tyrosine kinase domain, the ligand, or to synthesis of the EGFR.^{13,17,18} A SWOG trial of an EGFR tyrosine kinase inhibitor is already ongoing for MPNST (S0330 study, using OSI-774). The data suggest utility of blocking EGFR in neurofibroma as well as MPNST, and the importance of considering timing in neurofibroma prevention and therapy. Although our data do not exclude the possibility that prolonged exposure to EGFR antagonist could prevent enlargement of existing nerve disruption, they support the idea that early exposure of tumor to EGFR antagonist may be most effective.

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