Development/Plasticity/Repair

Inhibiting Epidermal Growth Factor Receptor Improves Structural, Locomotor, Sensory, and Bladder Recovery from Experimental Spinal Cord Injury

Matthias Erschbamer, Karin Pernold, and Lars Olson
Department of Neuroscience, Karolinska Institutet, 171 77 Stockholm, Sweden

Lack of axon regeneration in the adult CNS has been attributed partly to myelin inhibitors and the properties of astrocytes. After spinal cord injury, proliferating astrocytes not only represent a physical barrier to regenerating axons but also express and secrete molecules that inhibit nerve growth, including chondroitin sulfate proteoglycans (CSPGs). Epidermal growth factor receptor (EGFR) activation triggers astrocytes into becoming reactive astrocytes, and EGFR ligands stimulate the secretion of CSPGs as well as the formation of cribriform astrocyte arrangements that contribute to the formation of glial scars. Recently, it was shown that EGFR inhibitors promote nerve regeneration *in vitro* and *in vivo*. Blocking a novel Nogo receptor interacting mechanism and/or effects of EGFR inhibition on astrocytes may underlie these effects. Here we show that rats subjected to weight-drop spinal cord injury can be effectively treated by direct delivery of a potent EGFR inhibitor to the injured area, leading to significantly better functional and structural outcome. Motor and sensory functions are improved and bladder function is restored. The robust effects and the fact that other EGFR inhibitors are in clinical use in cancer treatments make these drugs particularly attractive candidates for clinical trials in spinal cord injury.

Key words: **spinal cord injury; astrocytes; functional recovery; bladder function; plasticity; EGFR**

Introduction

Spinal cord injury (SCI) typically results in neurological dysfunction that cannot be reversed. Injury causes motor and sensory deficits, as well as bladder, bowel, and sexual dysfunction. The lack of regeneration in the CNS has been attributed to a major part to myelin inhibitors and astrocytes (Filbin, 2003; Yiu and He, 2006). After the primary physical damage in SCI, which causes axonal damage and cell death, secondary degenerative events occur over the next several weeks. A dense glial "scar," mainly consisting of reactive astrocytes, not only represent a physical barrier to the regenerating axons but also secrete growth inhibitory molecules, including chondroitin sulfate proteoglycans (CSPGs) (Silver and Miller, 2004; Yiu and He, 2006). Overcoming this inhibitory barrier of reactive astrocytes and inhibitory substances are thought to be crucial for SCI repair (Qiu et al., 2000; Schwab, 2002; Silver and Miller, 2004; Okada, 2006).

Recently, it was shown that the epidermal growth factor receptor (EGFR), in addition to its known ligands (EGF, TGF-α, amphiregulin, heparin binding-EGF, betacellulin, and epiregulin) and its known effects in the CNS (Wong and Guillaud, 2004), may also be involved in nerve growth inhibitory signaling in the CNS via a novel, unknown *trans*-activating mechanism and signaling cascade involving the Nogo receptor (NgR) (Koprivica et al., 2005; Schwab et al., 2006). *In vivo*, PD168393 (4-[3(bromophenyl)-amino]-6-acrylamidoquinazoline), an irreversible EGFR inhibitor, was shown to promote nerve regeneration in the injured optic nerve (Koprivica et al., 2005) and AG1478 [4-(3-chloro-anilino)-6,7-dimethoxyquinazoline], a reversible EGFR inhibitor, to rescue retinal ganglion cells in a chronic glaucoma model (Liu et al., 2006). The latter report found expression of EGFR in astrocytes but not axons, suggesting that beneficial effects of EGFR inhibition might be mediated via astrocytes (Liu et al., 2006).

EGFR activation triggers astrocytes into becoming reactive astrocytes (Liu et al., 2006), and EGFR ligands stimulate the secretion of CSPGs (Smith and Strunz, 2005) as well as the formation of cribriform astrocyte arrangements that might contribute to the formation of glial scars (Liu and Neufeld, 2004). Intrathecal delivery of EGF and FGF-2 has also been shown to stimulate gliogenesis from endogenous stem cells after SCI and results in bigger proliferative lesions (Parr and Tator, 2007). Upregulation of EGFR in astrocytes has been described in different human conditions, including multiple sclerosis (Holley et al., 2003), glaucoma (Liu and Neufeld, 2003), ischemia, stroke, epilepsy, and Alzheimer's disease (Ferrer et al., 1996).

Here we demonstrate robust beneficial effects of local infusion of an irreversible EGFR inhibitor onto the damaged area of the spinal cord on recovery from contusion spinal cord injury in rats. The observed effects and the fact that other EGFR inhibitors are

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Correspondence should be addressed to Matthias Erschbamer, Department of Neuroscience, Karolinska Institutet, Retzius väg 8, 171 77 Stockholm, Sweden. E-mail: matthias.erschbamer@ki.se.

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in clinical use for the treatment of certain lung cancers may help pave the way for clinical trials of EGFR inhibition in spinal cord injury.

Materials and Methods

Animals

A total of 26 adult female Sprague Dawley rats (230–280 g; B&K Universal, Sollentuna, Sweden) were used. Animals were housed under standardized conditions on a 12 h dark/light regimen with food and water *ad libitum*. Two separate experiments were performed to document effects. Twelve animals were used in a first experiment and 16 in a second experiment. Basso, Beattie, and Bresnahan (BBB) scores and subscores were averaged for the two experiments. However, significant effects on BBB scores and subscores remained also when the two experiments were evaluated separately. Histological analysis and 24 h behavior analysis was performed in the first experiment. Residual urine was measured in the second experiment based on observations from the first experiment. Of the 26 operated animals, one animal was excluded from the study because it showed no signs of recovery after 1 week, a behavior not seen in any other animal. For *in situ* hybridization, spared tissue sections from animals ($n = 42$) used in a previous study (Erschbamer et al., 2005) were used. Animal weights were recorded weekly. All tests and measurements were performed in a blinded manner, except urine collection. Experiments were approved by the Stockholm Animal Ethics committee.

Surgical procedures and tissue preparation

Under isoflurane anesthesia, a laminectomy was performed at thoracic level T9. Weight-drop injury was induced using a standard instrument (New York University impactor) (Gruner, 1992) releasing a weight (10 g, rod diameter of 2 mm) from a height of 12.5 mm on the exposed dura of the spinal cord. After injury, animals were assigned to treatment or control groups, and the operation continued. A catheter was inserted through a small hole in the dura rostral to the lesion. The tip of the catheter was positioned subdurally on the dorsal side of the spinal cord over the center of injury. The catheter was secured with several stitches to bone and muscle and connected to a subcutaneous osmotic pump, placed between the shoulder blades. Muscles and skin were sutured. Animals received buprenorphine (Temgesic; Schering-Plough, Kenilworth, NJ) twice a day for 3 d to reduce postoperative pain and antibiotics for 1 week (Borgal; Hoechst, Warren, NJ).

Two weeks after injury, a small skin incision was performed, and the osmotic pump was disconnected and removed. The catheter was examined for patency, injecting 10 μl of solution, then sealed and buried under the skin. Two of 28 catheters, one from each group in the second experiment, were found to be blocked. These two animals were included in the study because the time point of catheter occlusion was unknown and no behavior difference was found for those two animals compared with other animals of their groups. However, all studied effects remained significant even if these two animals were excluded. Animals were killed while under deep anesthesia by transcardial perfusion with 50 ml of Tyrode's solution containing 0.1 ml of heparin, followed by 250 ml of 4% paraformaldehyde with 0.4% picric acid in PBS, pH 7.4. Tissue was kept in the same fixative for 1 h, rinsed for several times, and stored in 10% sucrose PBS at 4°C. Spinal cords were frozen, and 40 μm transverse cryostat sections were collected.

Catheter and PD168393 delivery

A 32 gauge catheter (catalog number CS132G, lot number 20422; ReCathCo, Allison Park, PA) was sealed to a polyethylene (PE) 10 tube, which was inserted into a PE50 tube and connected to the osmotic pump. A small loop was made in the PE50 part, to neutralize any traction forces on the pump and tubing system caused by movements of the animal, thereby securing a locked position of the intrathecally inserted catheter (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). An Alzet (Cupertino, CA) osmotic pump (model 2002; 0.5 μl/h for 2 weeks) was filled with 1 mM PD168393 (resulting in delivery of 4.43 μg per animal per day) (Calbiochem/EMD Biosciences, San Diego, CA) and incubated for 24 h at 37°C before transplantation to provide immediate outflow. PD168393 is a potent, cell-permeable, irreversible, and specific selective EGFR inhibitor (Fry et al., 1998). PD168393 is small molecule (369.2 Da), light sensitive, and with good solubility in DMSO (200 mg/ml). The substance was dissolved in 5% DMSO and HBSS. Control animals were given the same vehicle solution lacking the EGFR inhibitor.

Locomotor score and subscore

Locomotor recovery was scored using the standardized BBB locomotor score (Basso et al., 1995) weekly on a blinded basis by a trained technician. Scores range from 0 (flaccid paralysis) to 21 (normal gait) and involves movement, weight support, and coordination. To obtain a BBB score higher than 13, constant forelimb–hindlimb coordination is required, a major improvement in recovery. To score additional parameters of recovery for animals without constant forelimb–hindlimb coordination, including paw placement, toe clearance, trunk stability, and tail position, the BBB subscore (Lankhorst et al., 1999), with scores from 0 to 13 points, was also used.

Grid walk

Rats had to cross a 1.2 m horizontal grid pathway voluntarily three times, and the number of hindfoot misplacements were counted. This test is an indicator of forelimb–hindlimb coordination: it assesses skilled walking and is assumed to rely on the additional contribution of pathways such as the corticospinal and rubrospinal tracts (Metz and Whishaw, 2002).

Residual urine

After spinal cord injury, rats needed manual assistance to void their bladders. This was done by lifting the rats and applying firm pressure with two fingers on the abdomen above the bladder (Crede's method). Urine was collected twice a day, and volumes were recorded (in the second experiment) until sufficient (autonomic) bladder function recovered but for at least 3 weeks.

Sensory testing

Cold spray. The response to cold was tested with an ethyl chloride spray (Gebauer, Chiroform, Denmark). The spray was applied on the palms of the feet, and response was graded from 1 to 3: 1, no observable response; 2, brief withdrawal and/or licking; and 3, vocalization, repeated withdrawal, and/or aversive reactions.

von Frey hair. The von Frey hair was made to contact the palms of the feet, and pressure was applied. Testing was started with the finest filaments, gradually increasing filament strength until a filament strength was reached that caused withdrawal reactions at least three of five times.

Hotplate. Animals were placed on a preheated plate (53°C), and time until signs of discomfort (paw licking) was measured (Gale et al., 1985). Animals that did not respond within 60 s were removed and assigned the latency score 60 s.

General behavior

General behavior was assessed with an automated behavior analysis and registration system [Laboras (laboratory animal behavior observation registration and analysis system); Metris, Hoofddorp, The Netherlands]. Rats were placed individually in cages on sensing platforms and monitored over 24 h. Animal movement was translated into six behavioral categories (locomotion, grooming, rearing, immobility, eating, and drinking) and automatically registered by a computer (Noldus et al., 2001).

Histology

For indirect immunohistochemistry, we used the following antibodies: GFAP (1:500), 5-HT (1:5000; DiaSorin, Stillwater, MN), tyrosine hydroxylase (TH) (1:300; Dynal Biotech, Oslo, Norway), OX-42 (1:100; Serotec, Raleigh, NC), EGFR [1:100; Santa Cruz Biotechnology (Santa Cruz, CA), Abcam (Cambridge, MA), and Upstate Biotechnology (Lake Placid, NY)], and phosphorylated EGFR (pEGFR) [1:100; Santa Cruz Biotechnology and Cell Signaling Technology (Beverly, MA)]. Histological images were obtained using a confocal microscope (LSM 510 meta; Zeiss, Oberkochen, Germany). Secondary antibodies were conjugated with either cyanine (Cy2) or indocarbocyanine (Cy3) (Jackson ImmunoResearch, West Grove, PA). For myelin quantification, luxol fast blue staining (Sigma, St. Louis, MO) and cresyl violet were used.

5-HT and TH fiber counts

Slides were immunolabeled with 5-HT and TH antibodies, and three sections 7 mm below the lesion were randomly selected from each spinal cord. All immunopositive fibers in the white matter were counted, and the results for the three sections were averaged.

Stereology

Areas consisting of myelin, cyst, spared, and scar tissue were measured in the injured spinal cords from animals of the first experiment. For myelin, spared tissue and cyst quantification luxol fast blue staining was used. Scar tissue was quantified in cresyl violet-stained sections and defined as a cell-dense area, replacing normal SC architecture. A point grid was used, in which each point represented 20,000 μm^2. Nine sections from each animal were analyzed blinded, covering 4.5 mm of the injury center. Section values were averaged to generate animal values.

In situ *hybridization*

We performed in situ hybridization using radiolabeled oligonucleotides as described previously and using spare sections from our previous study (Erschbamer et al., 2005). Two probes complementary to EGFR gave rise to identical *in situ* hybridization patterns. The probe sequences did not match any other sequences in GenBank beside EGFR. Specificity was further confirmed by comparison with previous reports and analysis of known patterns in embryonic tissue.

Statistical analysis

For comparison of groups over time, we used a multiple-measurement ANOVA. For comparison of simple effects, we used a two-tailed Student's *t* test, confidence interval of 95%. Pearson's correlation coefficients were calculated. In all figures, the mean value ± SEM are used to describe the results. Data were analyzed with appropriate software (SPSS 14.0; SPSS, Chicago, IL).

Image processing

Digital images were adjusted for brightness and contrast, and visually verified artifacts were removed (Photoshop and Illustrator CS 2; Adobe Systems, San Jose, CA). Original picture files are available from authors on request.

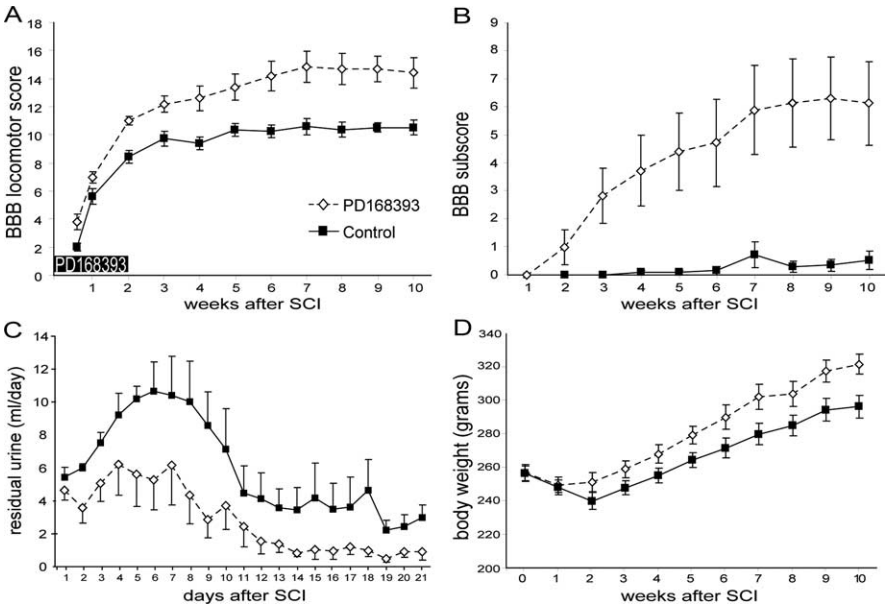

Figure 1. BBB scores and subscores, residual urine, and weight gain. ***A***, PD168393 causes significantly better recovery from SCI. The first BBB score was obtained 4 d after injury, when treated animals ($n = 13$) already showed a higher degree of joint movements. Control animals ($n = 14$) reach a plateau after 3 weeks at a BBB score of ≈10, which corresponds to occasional stepping. Treated animals continue to improve until 7 weeks after injury and reach a score of >14, corresponding to constant stepping with frequent forelimb–hindlimb coordination. Four of the 13 treated animals showed consistent limb coordination. Repeated-measurement ANOVA showed the difference between control and treated animals to be significant ($p = 0.009$). ***B***, The BBB subscore reveals improvement of the treated group during the first 9 weeks, whereas the control group did not improve. ***C***, Residual urine volumes differed dramatically between control and treated groups. As soon as 4 d after injury, this difference became obvious. Residual urine normalized in treated animals after 12 d. Control animals needed longer bladder assistance and did not reach normal residual urine volumes within the 21 d observation period. ***D***, Treated animals ($n = 6$) lost less weight after injury, recovered faster, and gained more weight after injury compared with control animals ($n = 6$).

Results

We induced a standardized 12.5-mm weight-drop injury to the exposed spinal cord at T9 and used implantable osmotic pumps and thin tubing for secure delivery of 0.5 μl/h active drug or vehicle directly to the injured area of the spinal cord for the first 14 d after injury.

PD168393 promotes functional recovery from spinal cord injury

Already 4 d after spinal cord injury, we detected differences in the BBB locomotor scores between treated and control groups (Fig. 1*A*). Recovery in control animals plateaued after 3 weeks at a BBB score of 10, corresponding to occasional stepping, whereas treated animals continued to recover motor function, reaching a plateau at 7 weeks, when they scored higher than 14, which corresponds to constant stepping with frequent forelimb–hindlimb coordination. Four of the 13 treated animals showed consistent limb coordination. Repeated-measurement ANOVA showed the difference between control and treated animals to be significant ($p = 0.009$).

To obtain additional information about hindlimb function, we used the BBB subscore. Treated animals showed improved BBB subscores from week 2, with continued improvement over the next 7 weeks, whereas control animals had minimal subscores throughout the observation period (Fig. 1*B*). Both the BBB and the BBB subscore tests were performed in two separate independent experiments with similar results (supplemental Fig. S2, available at www.jneurosci.org as supplemental material).

We also monitored general behavior (locomotion, grooming, rearing and immobility, eating, and drinking) for a 24 h period 2 weeks after injury. Rearing is a basic rat behavior used to explore the environment. Most animals were able to use hindlimb weight support for rearing, mainly in positions leaning against the cage wall. The duration of rearing was higher in the treated group, reflecting improved hindlimb function (supplemental Fig S2*E*, available at www.jneurosci.org as supplemental material).

To assess supraspinal coordination, we used the grid-walk test and found that treated animals made significantly fewer stepping errors crossing a horizontal grid than control animals (12 vs 20; Student's *t* test, $p < 0.005$). Crossing the grid requires a degree of forelimb–hindlimb coordination that is typically not the case in animals with BBB scores below 11.

Treatment improves sensory function

Our tests using a cold spray, a hotplate, and von Frey hairs (three sensory tests with motor performance readouts) did not reveal any indications of forepaw pain, hypersensitivity, or allodynia with the current treatment protocol. Applying the same sensory

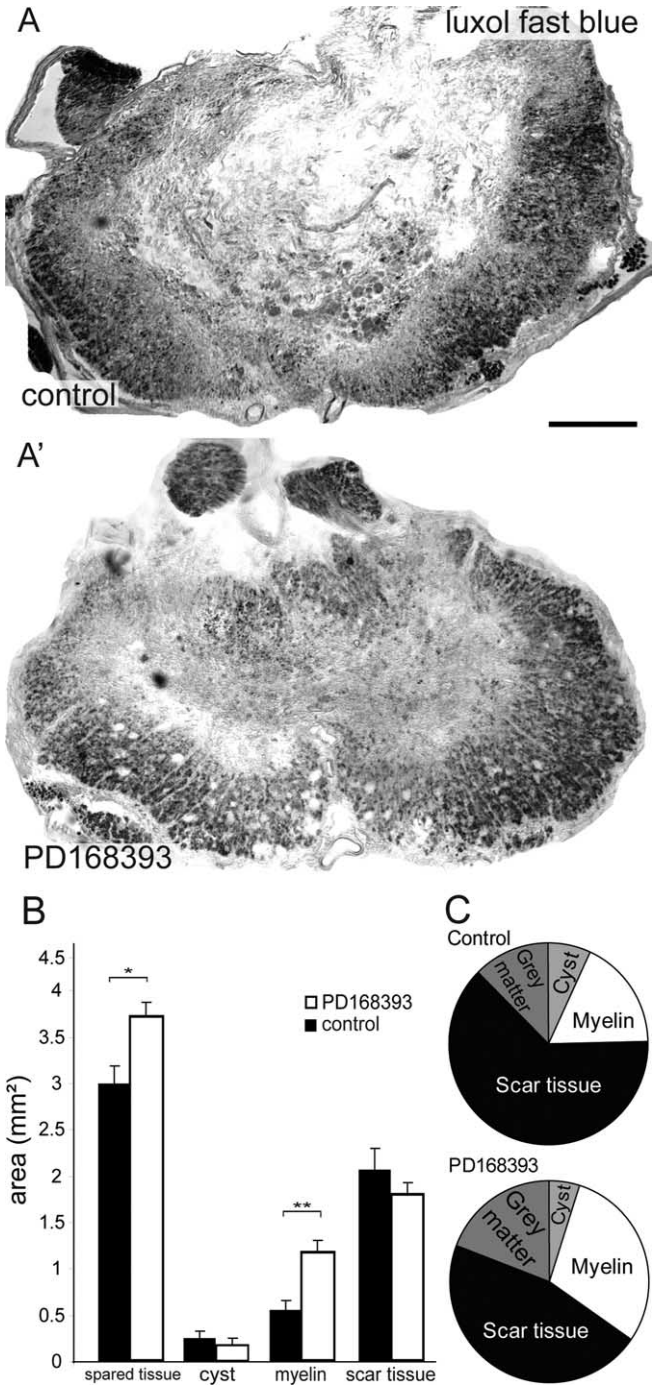

Figure 2. Myelin sparing and tissue analysis. ***A***, ***A′***, After SCI, most dorsal tracts were destroyed and replaced by scar tissue, whereas ventral tracts were spared, visualized here by luxol fast blue to stain myelin. ***B***, Tissue analysis reveals that control spinal cords ($n = 6$) contain half as much myelin in the injury zone compared with treated spinal cords ($n = 6$). Treated cords also show significantly more spared tissue. ***C***, Proportions of different tissue types in the spinal cord. Spinal cords of control animals consisted of a significantly higher degree of scar tissue (63%) compared with control animals (46%). Scale bar, 200 μm.

tests to the hindpaws and combining the two experiments, we found 3 of 12 treated versus 0 of 14 untreated rats to respond to the hot plate by licking their hindpaws within the permitted 60 s test time. Both the cold spray and the von Frey scores were increased in the injured control groups, indicating a degree of hyperreflexia. Both scores decreased, hence were normalized, significantly by EGFR inhibition (cold spray, 1.84 ± 0.02 vs 1.16 ± 0.04, $p = 0.0002$; von Frey hair, 37.7 ± 1.2 vs 51.2 ± 0.98, $p = 0.026$, *t* test, respectively, for the combined experiments).

Treated animals regain bladder control faster and show increased weight gain

After the weight-drop injury, all animals needed assistance with bladder voiding, presumably attributable to an increased urethral sphincter tension and/or lost voluntary control. Measuring manually expelled urine, we found that PD168393 treatment led to significantly less residual urine compared with the control group throughout a 21 d observation period (Fig. 1*C*). Residual urine volumes peaked 7–8 d after injury at ~10.5 ml/d in control animals compared with 6 ml/d in the treated group, peaking 4–7 d after injury. Minimal residual urine volumes were seen from ~12 d after injury in the treated group, whereas untreated injured rats continued to have significant amounts of residual urine also 21 d after injury.

Animals that underwent spinal cord injury typically lost up to 10% of their body weight. PD168393-treated animals recovered faster and gained significantly more weight compared with control animals (repeated-measurement ANOVA, $p = 0.017$) (Fig. 1*D*). Monitoring general behavior 2 weeks after injury showed that treated animals spend twice as much time eating compared with control animals (supplemental Fig. S2*F*, available at www.jneurosci.org as supplemental material).

PD168393-treated animals have more myelin in the lesion center

Stereological quantification of spinal cord tissue in a 4.5-mm-long section centered around the injury center showed that PD168393 treatment led to significantly more spared tissue (Student's *t* test, $p = 0.012$) (Fig. 2*A*,*A′*,*B*,*C*). Detailed analysis revealed that treated animals had twice as much myelin as control animals (5.3 ± 0.6 vs 2.5 ± 0.5 mm^2; Student's *t* test, $p = 0.0039$). Typically, spared myelin tracts were found ventrally and laterally, whereas dorsal aspects of the spinal cord, in which the falling rod had directly impacted the cord, showed little or no evidence of spared tissue and instead more scar tissue. The degree of myelin sparing in individual animals correlated with the BBB scores ($r = 0.66$; $p < 0.02$). Control animals had a significantly higher proportion of scar tissue in the spinal cord compared with PD168393-treated animals (46 vs 63%) and a slightly higher total area of GFAP immunoreactivity 12 weeks after injury (Fig. 2*B*,*C*). Cysts appeared slightly bigger in control animals; however, in this experiment, we observed small cysts or partly no cyst formation, whereas in some animals, cysts, especially close to the surface, seemed collapsed or compressed.

Treated animals have three times more 5-HT- and TH-immunoreactive axons below the lesion

All 5-HT axons in the spinal cord descend from supraspinal nuclei. 5-HT-immunoreactive (IR) fibers caudal to the lesion were therefore selected as one measure of axon sparing and/or regeneration. We found a threefold larger number of 5-HT-IR axons 7 mm caudal to the lesion in white matter of treated animals compared with controls (Fig. 3*A*,*A′*,*C*). These fibers were mostly longitudinally orientated and, in control animals, mainly found in the ventral part of the spinal cord (Fig. 3*B*). Because of their abundance, 5-HT-IR fibers in gray matter were more difficult to count. However, there was an estimated fourfold higher amount of 5-HT-IR fibers in gray matter of treated compared with control animals (1700 vs 400 fibers per section). We found a positive correlation between axon counts in white matter and BBB scores

of individual animals ($r = 0.547$; $p < 0.05$). Amounts of myelin and 5-HT-IR fibers in white matter were highly significantly correlated ($r = 0.876$; $p < 0.001$).

Like 5-HT-IR fibers, TH-IR fibers in spinal neuropil are of supraspinal origin, with the exception of the few scattered sympathetic fibers that can be found accompanying larger intraspinal blood vessels. Amounts of TH-IR axons caudal to injury were affected by injury and by treatment, much like the amounts of 5-HT-IR axons (Fig. 3*C*). In PD168393-treated animals, we counted three times as many TH-IR axons in white matter compared with control animals (average of 778 vs 256; Student's *t* test, $p = 0.001$). Fibers were longitudinally oriented in white matter and branching in gray matter (Fig. 3*C*). A similar pattern of effects on 5-HT and TH fibers was found 14 mm caudal to the lesion.

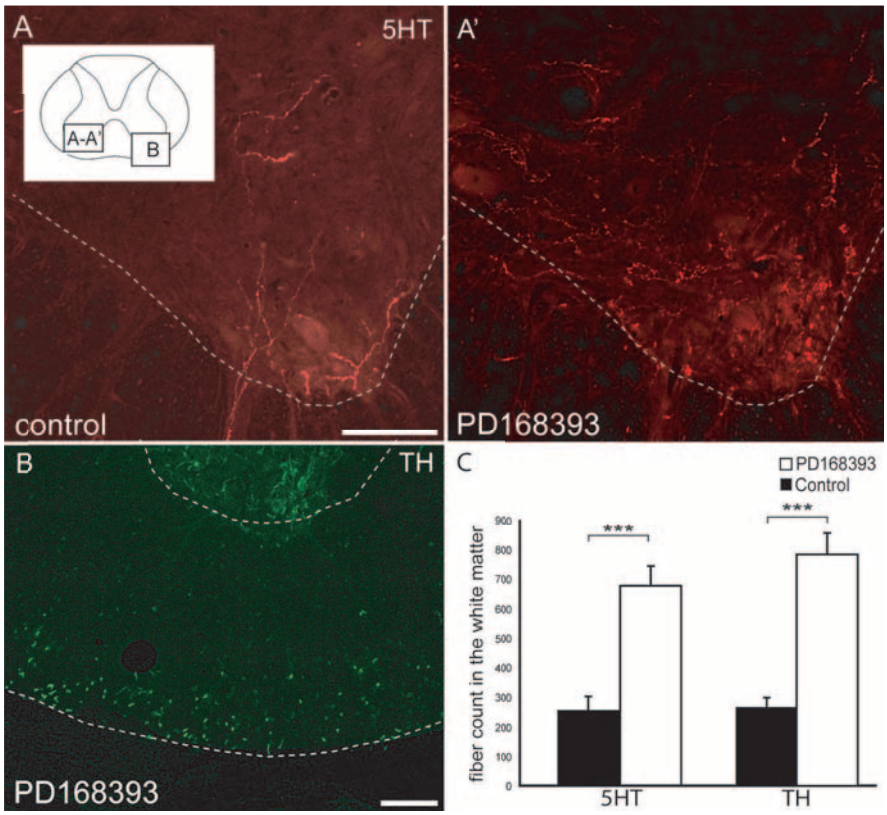

Figure 3. 5-HT- and TH-IR fibers in gray and white matter. ***A***, ***A′***, In treated animals ($n = 6$), significantly larger amounts of 5-HT-IR fibers were found in gray matter compared with control animals ($n = 6$). ***B***, In ventral white matter, fibers run longitudinally. ***C***, Numbers of 5-HT- and TH-IR axons were higher in spinal cords from rats treated with the EGFR inhibitor. Scale bars, 100 μm.

Increased EGFR mRNA and EGFR-like immunoreactivity after SCI

EGFR immunohistochemistry suggested low amounts of EGFR protein in the intact spinal cord, mainly present in a population of astrocytes forming glia limitans and weak signals in gray matter (Fig. 4*A*). In injured spinal cords, astrocytes showed increased EGFR immunoreactivity (Fig. 4*B*), especially in the injury zone. Scattered large neurons in the ventral horn showed modest EGFR immunoreactivity (Fig. 4*A*). Additionally, we found rounded OX-42-IR cells in the lesion center, presumably macrophages, to show EGFR immunoreactivity. DRG neurons expressed EGFR immunoreactivity, with stronger immunoreactivity in small- and medium-sized neurons. Using antibodies against pEGFR, we found a very similar pattern of immunoreactivity as for EGFR, limited to glia limitans in the intact spinal cord (Fig. 4*C*). After injury, astrocytes showed increased pEGFR immunoreactivity, especially surrounding the lesion center (Fig. 4*D*,*E*). *In situ* hybridization to locate EGFR mRNA confirmed immunohistochemical results. EGFR mRNA levels were low in the normal spinal cord, with mRNA mainly found in astrocytes. One day after injury, the expression had increased, mainly cranial to the lesion. After 4 d, increased EGFR mRNA expression was also found in the injury epicenter and caudal to the lesion. These EGFR mRNA increases were long lasting, peaking after 1 month (supplemental Fig. S3*A*, available at www.jneurosci.org as supplemental material). In dorsal root ganglia, we detected EGFR mRNA expression in a subpopulation of the sensory neurons. Glial cells in ganglia also appeared to express moderate levels of EGFR mRNA. After injury, the expression in DRG cells increased and peaked after 2 weeks (supplemental Fig. S3*B*, available at www.jneurosci.org as supplemental material).

Discussion

We show here that intrathecal administration of the potent EGFR inhibitor PD168393 leads to very robust, functionally valuable recovery of hindlimb function and bladder emptying, accompanied by improved sensory function, eating, and weight gain after contusion SCI in rats. These functional improvements are correlated with improvement of several histological parameters, such as tissue sparing, amounts of myelin, and 5HT- and TH-IR fiber tracts.

We observed functional improvement soon after injury, suggesting a protective mechanism of action for PD168393. However, treated animals continued to improve throughout the observation period, whereas controls did not improve further after 3 weeks. Rapid improvements in the first 3 weeks after experimental incomplete spinal cord injury have been observed in response to other treatments of experimental SCI, such as NgR antibodies, C3, or chondroitinase (Bradbury et al., 2002; Dergham et al., 2002; GrandPre et al., 2002; Filbin, 2003). In contrast, improvement as a result of axon regeneration (e.g., graft-assisted regeneration across a total transection of the spinal cord) needs at least 7 weeks to appear (Cheng et al., 1996; Fouad et al., 2005; Tsai et al., 2005; Houle et al., 2006). Several mechanisms for early treatment-induced improvement of recovery have been proposed, including fast synaptic rearrangements and the dampening of secondary damage processes, as well as remyelination (Reier, 2004; Bradbury and McMahon, 2006). We suggest that the effects of EGFR blockade may be attributable to a combination of these possible beneficial effects, because BBB score improvements first appear as early as 4 d after injury but also continue to improve in relation to controls for 7 weeks, BBB subscores as long as 10 weeks. Treatment-induced increased rearing behavior also reflects locomotor improvement. Together with better weight recovery, this suggests increased muscle mass. The improved weight gain is likely attributable to a higher food

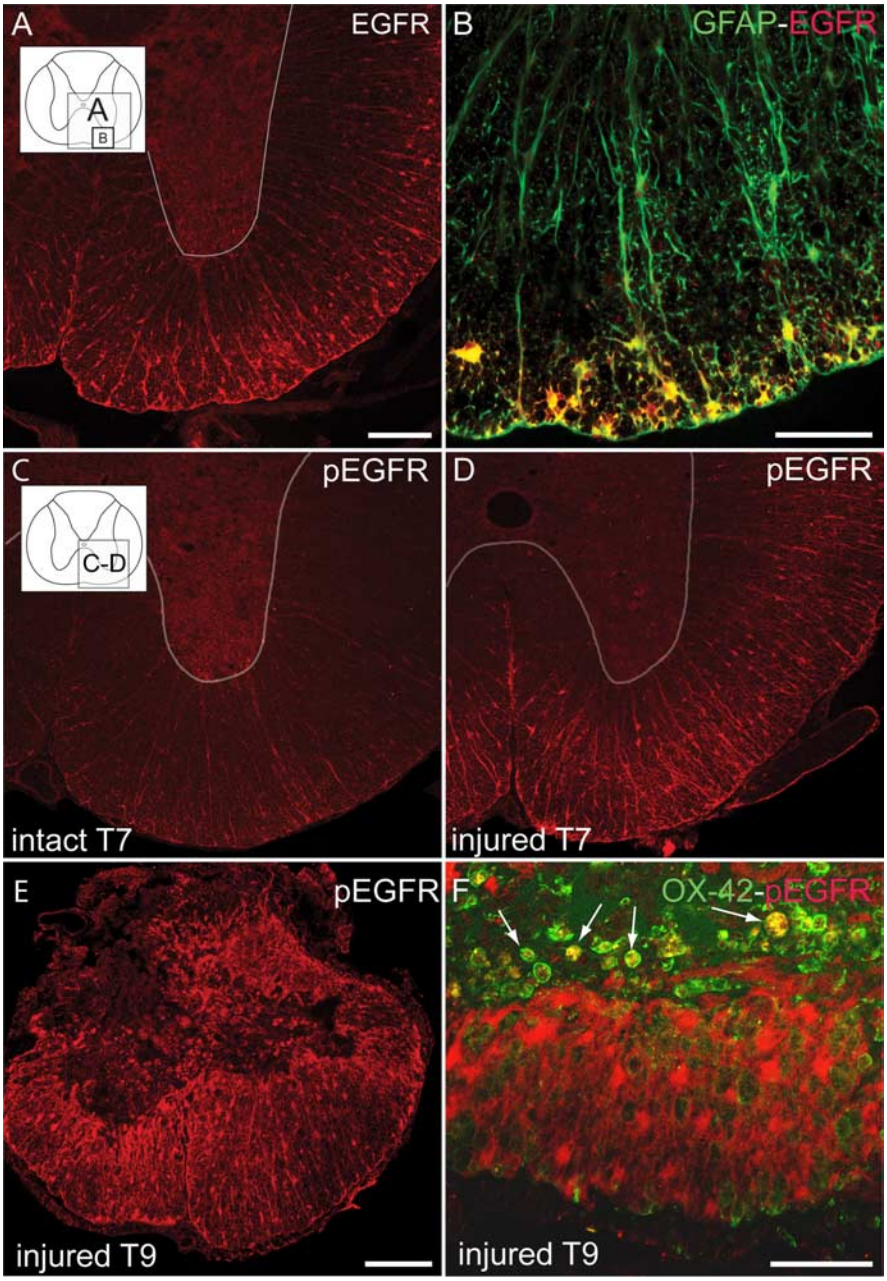

Figure 4. EGFR immunoreactivity and pEGFR expression in the SC. ***A***, EGFR immunoreactivity in the injured spinal cord was mainly located in astrocytes and gray matter. ***B***, Double labeling for GFAP (green) and EGFR (red). ***C***, Peripheral astrocytes are double labeled and appear yellow. pEGFR immunoreactivity in the intact spinal cord is restricted to glia limitans and adjacent astrocytes. ***D***, After injury, a massive upregulation of pEGFR was observed, confined to white matter. ***E***, In the injury zone, a dense network of astrocytes surrounding the lesion is observed. ***F***, Some round-shaped OX-42-positive cells, presumably macrophages, in the lesion center show EGFR-IR colocalization (arrowheads). Scale bars: ***A***, ***B***, ***F***, 100 μm; ***E***, 200 μm.

intake because the treated group spent more time eating than the control group.

Neurogenic pain is a common clinical problem after SCI. It was demonstrated recently that allodynia can be a negative side effect in response to neural stem cell grafting in experimental SCI (Hofstetter et al., 2005). Importantly, therefore, our tests did not reveal signs of pain, hypersensitivity, or allodynia with the current treatment protocol. The amount of spared myelin was compatible with an absence of allodynia (Kloos et al., 2005) and correlated well with BBB scores and subscores. The improved grid-walk performance of treated animals suggests improved sensorimotor coordination. Other tests of sensory function, including cold, heat, and touch, also indicated sensory improvements.

SCI leads not only to sensory and motor malfunction but also to autonomic dysregulation, including bladder dysfunction. Micturition is controlled by a complex network formed by somatic motor, sensory, and autonomic circuitry, dominated by descending pathways and modulated by primary afferent pathways. Incomplete SCI causes initial loss of bladder control with partial recovery, making the model attractive for studies of urinary tract dysfunction (Pikov and Wrathall, 2001). Clinically, bladder dysfunction is a severe urological problem, and autonomic dysreflexia is common in SCI (Santajuliana et al., 1996). Paraplegic and quadriplegic patients prioritize bladder, bowel, and sexual function to increase quality of life (Anderson, 2004). We found rapid reduction of residual urine in treated animals compared with control animals, beginning as soon as 4 d after injury. Twelve days after injury, treated animals showed nearly normal residual urine volumes, whereas residual urine remained present in control animals throughout the 3 week observation period.

After incomplete SCI, the extent of functional recovery depends on the amount of spared fiber tracts, the reorganization of segmental circuitry, and the restoration of supraspinal input. Degenerative events can be detected very early after injury and axon regeneration and reorganization events within 24 h (Kerschensteiner et al., 2005). We attribute at least parts of the early positive effects noted in the present study to protective effects of the local EGFR inhibition. Histological analysis confirmed that the corticospinal tract was mostly replaced by scar tissue, and we therefore suggest that descending tracts other than the dorsomedial corticospinal tract and/or local sprouting may be responsible for the observed improvement of recovery/regeneration. We show that PD168393 treatment is associated with local tissue protection, sparing descending ventral and lateral pathways in the spinal cord. We found that twice as much myelin was spared in the treatment group, mainly in ventral and lateral parts of the cord. Such sparing was correlated to 5-HT-IR and TH-IR fiber densities. This suggests that treatment with the EGFR inhibitor rescues and possibly promotes regeneration and plasticity of both myelinated and nonmyelinated (5-HT and TH) descending pathways after injury. 5-HT and TH fibers were counted 7 mm below the lesions, and comparable amounts of fibers were seen 14 mm below the lesion, suggesting a sparing rather than a regenerative effect.

5-HT and TH fiber alterations have been correlated to sen-

sory, motor, and autonomic disorders (Hains et al., 2002). Initiation of hindlimb movement is influenced by 5-HT activation of motor output via a central pattern generator (Rossignol et al., 1998; Grillner and Wallen, 2002) that organizes automated execution of hindlimb movements. Administration of 5-HT or quipazine has been shown to lead to immediate improvement of locomotion (Antri et al., 2005). 5-HT immunoreactivity has also been associated with bladder recovery (Wrathall and Emch, 2006). Administration of 5-HT agonists facilitates the voiding reflex (Lecci et al., 1992), whereas 5-HT antagonists increase bladder capacity and inhibits the voiding reflex (Testa et al., 1999). The higher numbers of 5-HT-IR fibers in treated rats may thus help explain the drug-induced improvement of bladder function.

To begin understanding the mechanisms by which PD168393 exerts its beneficial effects, we studied the cellular localization of EGFR mRNA and protein in the spinal cord before and after injury. We found a markedly increased expression of EGFR mRNA and EGFR immunoreactivity after injury, mainly confined to astrocytes. Notably, pEGFR immunoreactivity was nearly absent in the uninjured spinal cord. After injury, we found pEGFR mainly in astrocytes with a reactive phenotype, especially surrounding the lesion center. However, a subpopulation of dorsal root ganglion neurons also expressed EGFR immunoreactivity, to a lesser degree pEGFR immunoreactivity, and to a smaller amount also reactive macrophages/microglia.

Astrocytes are thought to play a key role in the pathophysiology of SCI (Okada, 2006) and other CNS diseases (Miller, 2005). Activation of the EGFR triggers transformation of astrocytes into hypertrophic, reactive astrocytes (Liu and Neufeld, 2004). Detailed gene analysis has shown upregulation of several neural disorder-related genes in astrocytes, as regulated by the EGFR pathway (Liu and Neufeld, 2004; Liu et al., 2006). Inhibition of EGFR has been shown to prevent astrocyte activation and found to have neuroprotective effects. Our findings that astrocytes are EGFR-IR and pEGFR-IR, that both forms of EGFR increase markedly after SCI, combined with the marked effects of EGFR inhibition in the present experiments are compatible with previous work on the role of EGFR in astrocytes. Furthermore, it has been observed that intrathecal delivery of both EGF and FGF-2 to healthy and spinal cord injured rats stimulates extensive proliferation of endogenous stem cells from the ependymal region, restricted to a glial lineage (Parr and Tator, 2007). Animals receiving EGF and FGF-2 showed increased gliogenesis and bigger lesions in the spinal cord soon after injury. This might be another mechanism explaining the beneficial effects of EGFR inhibition.

Inflammatory processes are thought to play a crucial role in mediating secondary damage after spinal cord injury (Popovich, 2000; Bareyre and Schwab, 2003; Park et al., 2004). EGFR has been reported to be expressed not only in astrocytes but also in microglia/macrophages in the pathologic rat brain (Ferrer et al., 1996; Planas et al., 1998). We found that part of the OX-42-IR microglia/macrophages showed EGFR immunoreactivity in the spinal cord, mainly in the form of rounded cells in the lesion center, presumably macrophages. Ten weeks after injury, we found no difference in the amount of OX-42-IR or ED-1-IR cells in treated compared with control animals.

In summary, our results show that local blockade of EGFRs in an injured area of the rat spinal cord leads to not only structural but also significantly better functional recovery from contusion SCI both with regard to locomotor behavior and bladder function. EGFR blockers (erlotinib and gefitinib) are already in clinical use for the treatment of specific forms of cancer, and several blockers of different members of the Erb family are in clinical testing. Neurotrophic properties of erlotinib have been reported (Koprivica et al., 2005), and our preliminary experiments suggest a beneficial effect of erlotinib in experimental SCI (M. Erschbamer and L. Olson, unpublished observation). The experience with EGFR inhibitors in clinical use should help in possible future designs of clinical trials with EGFR inhibition in SCI.

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