Nuclear Factor-κB Activation in Axons and Schwann Cells in Experimental Sciatic Nerve Injury and Its Role in Modulating Axon Regeneration: Studies With Etanercept

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Abstract

Early inflammatory events may inhibit functional recovery after injury in both the peripheral and central nervous systems. We investigated the role of the inflammatory tumor necrosis factor/ nuclear factor-κB (NF-κB) axis on events subsequent to sciatic nerve crush injury in adult rats. Electrophoretic mobility shift assays revealed that within 6 hours after crush, NF-κB DNA-binding activity increased significantly in a 1-cm section around the crush site. By immunofluorescence staining, there was increased nuclear localization of the NF-κB subunits p50 but not p65 or c-Rel in Schwann cells but no obvious inflammatory cell infiltration. In rats injected subcutaneously with etanercept, a tumor necrosis factor receptor chimeric that binds free cytokine, the injury-induced rise in NF-κB DNA-binding activity was inhibited, and nuclear localization of p50 in Schwann cells was lowered after the injury. Axonal growth 3 days after nerve crush assessed with immunofluorescence for GAP43 demonstrated that the regeneration distance of leading axons from the site of nerve crush was greater in etanercept-treated animals than in saline-treated controls. These data indicate that tumor necrosis factor mediates rapid activation of injury-induced NF-κB DNA binding in Schwann cells and that these events are associated with inhibition of postinjury axonal sprouting.

Key Words: Axotomy, Dorsal root ganglia, Inflammation, Sensory neuron, TNF-α.

INTRODUCTION

Sensory and motor neurons of the adult peripheral nervous system (PNS) survive and regenerate after injury to a much greater extent than neurons in the central nervous system (CNS). Injury to mature peripheral neurons results in a sequence of molecular and cellular responses that are associated with and may play an important role in axonal regeneration and recovery of function (1). Peripheral nerve transection or crush leads to acute myelin and axonal degeneration in the distal area of the damaged nerve, that is, Wallerian degeneration. This process is associated with macrophage infiltration and Schwann cell proliferation that precede axonal regrowth (2). The proinflammatory cytokine tumor necrosis factor (TNF) and TNF receptor 1 are rapidly upregulated at sites of peripheral nerve injury and mediate many of the events associated with Wallerian degeneration (3–5). In addition, after nerve injury, sensory neuron expression of TNF rises and is associated with increased anterograde axonal transport of this inflammatory mediator to the crush site (6). Through its engagement with TNF receptor 1, TNF activates the transcription factor nuclear factor-κB (NF-κB), which leads to induction of proinflammatory and immunomodulatory genes (4).

Nuclear factor-κB plays a number of critical roles in both developing and postinjury PNS, including initial myelin formation during development and Schwann cell-mediated remyelination after nerve injury (7, 8). Through its intracellular mediation of p75 neurotrophin receptor (p75NTR)–induced antiapoptotic signals, the NF-κB signaling pathway promotes Schwann cell differentiation and myelination after nerve injury (9). After axotomy, sensory neurons of the dorsal root ganglia (DRG) rapidly upregulate endogenous NF-κB DNA-binding activity; this process may be part of the recovery process that protects neurons against cell death and neurodegeneration during axon regeneration in the adult PNS (10). Furthermore, the activation of the NF-κB signaling pathway in CNS neurons has important consequences for neuronal survival and plasticity (11, 12). Sciatic nerve transection leads to an upregulation of NF-κB in spinal cord neurons through a transactivation process that is consistent with NF-κB acting as a neuronal survival signal (13). In addition, NF-κB also mediates numerous inflammatory pathways in multiple cells and organ systems, including the CNS; inflammation is now recognized to exacerbate most if not all neurodegenerative conditions, including Alzheimer disease, Parkinson disease, and infarcts (14, 15). Nerve injury in the PNS has been associated with the production of proinflammatory cytokines in the spinal cord and contributes to nociceptive processing; blocking NF-κB...
activity in spinal glia alleviates pain behaviors in rats with chronic nerve constriction injuries (16). Therefore, although they are necessary for eventual neuronal growth after injury, the actions of TNF and NF-κB may also mediate early detrimental inflammatory events.

We previously assessed the importance of NF-κB within sensory neuron perikarya during axon regeneration (10). In the present study, we focused on the role of NF-κB at the crush site within the sciatic nerve of the adult rat. We analyzed the expression of NF-κB subunits within the multicellular nerve crush environment and determined the effect of blocking TNF signaling and, therefore, NF-κB activation on short-term sciatic nerve regeneration after nerve crush injury.

**MATERIALS AND METHODS**

**Sciatic Nerve Crush**

Adult male Sprague-Dawley rats (250–300 g) underwent unilateral sciatic nerve crush at the midthigh level under isoflurane (Abbott Animal Health, Montreal, Canada)-induced anesthesia. Nerve crush was performed using flat mosquito needle drivers (Fine Science Tools, Heidelberg, Germany) twice from opposite directions for 30 seconds in each direction to ensure a uniform crush across the nerve. The contralateral side was also opened, but no crush procedure was performed. Before surgery, the animals were randomly assigned to one of 3 treatment groups: 1) saline (Baxter, Mississauga, Canada)-injected controls; 2) 100 μg etanercept (Enbrel; Amgen, Mississauga, Canada); or 3) 500 μg etanercept. All injections were subcutaneous and performed at the time of the surgery. The animals were allowed to recover and underwent perfusion fixation or were humanely killed at various time points; the required tissues were quickly removed and snap-frozen on dry ice. All animal procedures were strictly performed to standards set forth by the Canadian Council on Animal Care and the University of Manitoba Ethics Committee.

**Sensory Neuron Cultures**

Primary cultures of DRG sensory neurons were obtained from adult male Sprague-Dawley rats weighing between 250 and 300 g, as previously described (17–19). Ham F-12 medium was used during dissociation of the cells with Bottenstein N2 additives and 0.01 mol/L cytosine arabinoside to prevent nonneuronal cell proliferation. The cells were plated onto polyornithine-laminin–coated no. 1 thickness 22-mm-diameter glass coverslips (Cedar Lane, Burlington, Canada) and cultured for 40 hours in F12 with N2 and cytosine arabinoside until staining. The sciatic nerve tissues were embedded in optimum cutting temperature (Sakura Finetek, Torrance, CA) media and cryoprotectant, PFA, and optimum cutting temperature. For immunofluorescence, cultured cells were fixed using 2% PFA in 0.05 mol/L phosphate buffer. The tissues, 10 mm of sciatic nerve on either side of the crush site, were removed and placed in PFA in 0.1 mol/L phosphate buffer (Fisher). The tissues, which were from Sigma. Protein levels were determined by the Bradford method (Bio-Rad, Hercules, CA), and samples were stored at −80°C. Equal amounts of protein were incubated in a 20-μL reaction mixture containing 20 μg of bovine serum albumin (Sigma), 1 μg of poly(dI-dC) (Amersham, Baie d’Urfé, Canada), and 2 μL of buffer containing 20% glycerol (Fisher), 100 mmol/L KCl (Fisher), 0.5 mmol/L EDTA (Fisher), 0.25% Nonidet P-40 (Calbiochem, Gibbstown, NJ), 2 mmol/L dithiothreitol, 0.1% PMSF, and 20 mmol/L HEPES (pH 7.9); 4 μL of buffer containing 20% Ficoll 400 (Fisher), 300 mmol/L KCl, 10 mmol/L dithiothreitol, 0.1% PMSF, and 100 mmol/L HEPES (pH 7.9); and 20,000 to 50,000 CPM of 32P-labeled oligonucleotide (S) corresponding to an NF-κB binding site (5′-AGTGTAGGGGACTTTCCCAGGC-3′) (Santa Cruz Biotechnology, Santa Cruz, CA). After 20 minutes at room temperature, reaction products were separated on a 12% nondenaturing polyacrylamide gel. Radioactivity of dried gels was detected by exposure to Kodak X-Omat film (VWR, Mississauga, Canada); images on the developed film were scanned into a computer using a UMAX 1200s scanner (Techville, Inc, Dallas, TX). Densitometry was performed using Image software (Scion Corp, Frederick, MD). Paint Shop Pro software (Jasc Software, Minneapolis, MN) was used for preparation of the final figures.

**Fixation of Tissues and Cultured Cells**

Animals to be perfused were anesthetized with 90 mg/kg ketamine and 10 mg/kg xylazine (both from MVDC, Winnipeg, Canada). The animals were fixed using 1% heparinized saline (Baxter) and 4% paraformaldehyde (PFA) (Sigma) in 0.1 mol/L phosphate buffer (Fisher). The tissues, 10 mm of sciatic nerve on either side of the crush site, were removed and placed in PFA in 0.1 mol/L phosphate buffer for 3 hours to postfix. The nerve tissues were then washed 3 times and stored in 20% sucrose (Fisher) and PBS (Fisher) solution at 4°C. For immunofluorescence, cultured cells were fixed using 2% PFA in 0.05 mol/L phosphate buffer. The cells were incubated at room temperature for 15 minutes, and the fixative was removed. Slides were stored in 0.01 mol/L PBS until staining.

**Immunofluorescent Staining for NF-κB Subunits**

The sciatic nerve tissues were embedded in optimum cutting temperature (Sakura Finetek, Torrance, CA) media and stored at −80°C. Embedded tissue samples were sectioned using an HM500 OM cryostat (Microm, Walldorf, Germany). All nerve sections were cut to a thickness of 10 μm. Sections were air-dried overnight at 4°C then used the following day or stored at −20°C in cryoprotectant (30% ethylene glycol [Sigma], 30% glycerol in 0.01 mol/L PBS). Slides underwent washes in 0.01 mol/L PBS to remove cryoprotectant, PFA, and optimum cutting temperature. For cultured cells, after PBS wash, slides were placed in ice-cold
methanol for 3 minutes to permeate the membrane and allow intracellular antibody penetration. A volume of 0.2% Triton X-100 (Sigma) in 0.01 mol/L PBS was used to permeate tissue sections and further permeate cultured cells. Samples, tissue sections and fixed cultured cells, were washed in 0.01 mol/L PBS. Tissue sections to be treated with the same antibody were dried, and a hydrophobic immunopen (Chemicon, Temecula, CA) was used to draw around the samples to separate antibody types and keep solutions on the slide. Slides were blocked using 10% donkey serum (Jackson ImmunoResearch, West Grove, PA) in 0.2% Triton X-100 in 0.01 mol/L PBS for 1 to 2 hours at room temperature in a humidified chamber. Slides were incubated with the primary antibody (or mixture of primary antibodies for double staining) in 0.2% Triton X-100 in 0.01 mol/L PBS overnight at 4°C. The primary antibodies used were anti-GAP43 (1:1000), anti-c-Rel (1:200), anti-p50 (1:200), anti-p65 (1:200), and anti-S100 (1:4000). Primary antibodies were all polyclonal from rabbit and were purchased from Santa Cruz Biotechnology except the GAP43 antibody, which was purchased from Chemicon, and the S100 antibody, which was monoclonal from mouse and was purchased from Sigma. The secondary antibody was fluorescein isothiocyanate–conjugated donkey anti-rabbit (1:200 dilutions, Jackson ImmunoResearch), except for detection of S-100 primary antibody that was Cy3-conjugated donkey anti-mouse (1:400 dilution, also from Jackson ImmunoResearch). Control slide tissues were incubated with 0.2% Triton X-100 in 0.01 mol/L PBS without antibody. After incubation, the slides underwent washes with

FIGURE 1. Sciatic nerve crush induces activation of nuclear factor-κB (NF-κB) at the crush site. Adult Sprague-Dawley rats underwent unilateral sciatic nerve crush injury. The crush site (5 mm proximal and distal) was removed at the indicated time points and NF-κB binding activity determined. Autoradiogram (upper panel) and density analysis (graph) of the main NF-κB binding complex. By 14 hours after crush, there was significant NF-κB activation at the crush site; at 24 hours, there was NF-κB activation on the contralateral side. Values are means ± SEM (n = 3); * p < 0.05 versus contralateral side. C, naive; L, ipsilateral to nerve crush; R, contralateral.

FIGURE 2. Nuclear factor-κB (NF-κB) subunit expression and effect of nerve crush on expression levels. (A) Representative Western blots demonstrate expression of 3 major NF-κB subunit groups, p50, p65, and c-Rel, in various neural tissue samples. (B) Western blots of samples collected from a 12-hour double crush injury (1 cm apart) of the sciatic nerve. (A) Image shows the most proximal or anterograde segment (a measure of accumulation by axonal transport combined with local synthesis in response to nerve damage). L2 is the segment proximal to the second more distal crush (primarily, a measure of local synthesis). (C) Contralateral nerve. Bar graph of NF-κB subunit expression in the blots shown in (B) in which the signal in (A) is compared with the signal in (C). Blots were also probed for β-actin to demonstrate even loading of samples. Means ± SEM (n = 4). * p < 0.05 versus contralateral. DRG, dorsal root ganglion; D. root, dorsal root; V. root, ventral root.
0.01 mol/L PBS. The light-sensitive fluorescing secondary antibody (or mixture of secondary antibodies for double fluorescence), diluted in 0.01 mol/L PBS only, was then applied, and slides were incubated for 1 hour at room temperature in a humidified chamber. The chamber was wrapped in black plastic to prevent light reaching the antibody and quenching the fluorescence. Slides were washed with 0.01 mol/L PBS. Sections were viewed by light microscopy (Axioskop 2 mot, Zeiss, Thornwood, NY) using the appropriate wavelength filter and exposures taken by a camera (Axiocam, Zeiss) using Axiovision Ver.4.3 software (Zeiss).

**Data Analysis**

Student *t*-test was used for all statistical analyses.

**RESULTS**

Adult rats underwent a unilateral nerve crush of the sciatic nerve at midthigh. By 2 hours after nerve crush, there was a nonsignificant increase in the NF-κB DNA-binding levels in the nerve on the side of the nerve crush (Fig. 1). At 14 hours after injury, NF-κB binding activity was significantly increased compared with the contralateral side; both sides remained significantly elevated compared with control nerves. Supershift analysis revealed p50 and p65 subunit components (data not shown).

Expression levels of the NF-κB subunits were measured in the sciatic nerve by semiquantitative Western blotting. Representative Western blots demonstrating p50, p65, and c-Rel expression in various neural tissue samples are shown in Figure 2A. Blots of samples collected from sciatic nerve 12 hours after a double crush (1 cm apart) demonstrate p50, p65, and c-Rel expression (Fig. 2B). All 3 subunits showed significant increases in expression in tissues proximal to the crush site compared with tissue from the contralateral side at 12 hours after crush (Fig. 2C).

Immunofluorescent staining was performed to examine the localization of the NF-κB subunits within the sciatic nerve. Within the 5-mm proximal nerve segment at 12 hours after sciatic nerve crush, p50 levels appeared greater only in the Schwann cells at the crush site compared with naive (precrush) level. The NF-κB DNA-binding activity was further increased on the ipsilateral side at 24 hours after nerve crush and remained significantly increased compared with the contralateral side. Both sides remained significantly elevated compared with control nerves. Supershift analysis revealed p50 and p65 subunit components (data not shown).

FIGURE 3. Localization and expression of the p50 subunit of nuclear factor-κB (NF-κB) in transverse sections of naive (A, C, E) and 12 hours after crush (B, D, F) sciatic nerves. (A, B) p50 staining; (C, D) S-100 (Schwann cell) staining; (E, F) merged images. There is greater p50 staining in the Schwann cells (blue arrows) in the crushed sciatic nerve; staining is absent in endoneurial blood vessels (orange arrows) and axons in both. Scale bar = 100 μm.

FIGURE 4. Localization and expression of the p65 subunit of nuclear factor-κB (NF-κB) in transverse sections of naive (no surgery performed) (A, C, E) and 12 hours after crush (B, D, F) sciatic nerves. (A, B) p65 staining; (C, D) S-100 (Schwann cell) staining; (E, F) merged images. There appears to be greater p65 staining in the Schwann cells (blue arrows) in the crushed sciatic nerve; staining is unchanged in endoneurial blood vessels (orange arrows) and axons in both. Orange arrows, blood vessels; red arrows, axons; blue arrows, Schwann cells. Scale bar = 100 μm.
tissue; the levels in the blood vessels appeared the same between crushed and naive nerve for both subunits (Fig. 3). In tissues stained for p65 (Fig. 4), staining appeared greater in the Schwann cells of the 12-hour crushed nerve, with no change in the amount of staining seen in the blood vessels. Staining for p65 was also present in the axons, but only in the crushed nerve. Figure 5B shows high-power images of the p65 expression and axonal localization in the sciatic nerve at the crush site after 12-hour sciatic nerve crush. The transverse sciatic nerve sections show positive staining for the p65 subunit in Schwann cells, blood vessels, and within the axons. The levels of c-Rel also appeared greater in the Schwann cells at the crush site compared with those in control nerves (Fig. 6).

In addition to observing the localization of p50 and p65 in the sciatic nerve after a nerve crush, DRG neurons were axotomized and cultured for 40 hours and stained for p50, p65, and β-tubulin (III). β-tubulin (III) is a neuron-specific axonal cytoskeletal component and, therefore, is an effective marker of neurites that extend from the cell body of the dissociated DRG neurons (Figs. 5E, F). When NF-κB subunit staining of the neurites was compared with secondary antibody background controls, only the p65 subunit appeared to be positively expressed in the neurites, coexpressing with the β-tubulin (III) (Figs. 5D, H). The p50 subunit remained within the cell body (Fig. 5C) and without any apparent neurite staining (Fig. 5G). The p65 subunit expression in the neurite appeared to be uniform, with no accumulation at the growth cones.

FIGURE 5. Nuclear factor-κB (NF-κB) subunit localization in transverse sections of crushed sciatic nerves and cultured dorsal root ganglion (DRG) neurons. (A, B) Sciatic nerve 12 hours after crush injury. (A) S-100-positive Schwann cells in crushed sciatic nerve. (B) S-100 and p65 image overlay in same field as in (A). Blue arrows indicate positive Schwann cell staining for S-100 and p65. Orange arrows indicate positive blood vessel staining for p65. Red arrows indicate positive staining within axons for p65. Original magnification: 40×. Scale bar = 50 μm. (C–H) Neurite expression of NF-κB subunits p50 and p65 in cultured DRG neurons. (C) p50 staining; (D) p65 staining; (E) β-tubulin (III) (β-tub III) staining in the same sample as C. (F) β-tub (III) staining in the same sample as (D). (G) p50 and β-tubulin (III) overlay. (H) p65 and β-tubulin (III) overlay. (C–H) The p65 subunit was expressed within the neurites of neurotrophin-treated dissociated DRG neurons in vitro (H), whereas p50 was not (G). Scale bar = 30 μm.

FIGURE 6. Localization and expression of the c-Rel subunit of NF-κB in transverse sections of normal (naive) (A, C, E) and 12 hours after crush (B, D, F) sciatic nerves. (A, B) c-Rel staining; (C, D) S-100 (Schwann cell) staining; (E, F) S-100 and c-Rel image overlay. Blue arrows indicate S-100-positive Schwann cells. Staining for c-Rel appeared to be increased in Schwann cells in the injured nerve. Scale bar = 100 μm.
The figures show that NF-κB expression and DNA-binding activity were upregulated in the sciatic nerve after a crush injury, similar to the enhancement in TNF expression seen in previous studies (4, 6). Because TNF increases the NF-κB binding activity in DRG neurons in vivo (20) and in vitro (10) (and unpublished data, the Glazner Laboratory, St. Boniface Hospital Research Center, 2005), we examined the relationship of TNF and NF-κB in vivo after nerve injury. Adult rats were administered vehicle or 100 or 500 μg of etanercept by subcutaneous injection at the time of nerve crush. After 3 days, the animals were killed and tissues were harvested (Figs. 7Y12). Etanercept injection blocked injury-induced enhancement of NF-κB DNA-binding activity in sciatic nerve and reduced to a lesser degree injury-induced enhancement in the DRG compared with the saline-injected controls (Fig. 7).

Immunostaining of longitudinal sections of naive (i.e. no crush injury) sciatic nerves demonstrated faint cytoplasmic and nuclear expression of NF-κB subunits p50, p65, and c-Rel in presumably mostly Schwann cells (Figs. 8A, 9A, and 10A, respectively). In vehicle saline-treated animals 3 days after nerve crush, the cytoplasmic content of all 3 NF-κB subunits was increased (Figs. 8C, 9C, and 10C). Inspection of levels of nuclear staining, however, showed that p50 was increased in nearly all nuclei (Fig. 8D), whereas c-Rel was only increased in some nuclei (Fig. 10D); p65 levels did not appear to be increased in nuclei (Fig. 9D). Increases in cytoplasmic expression of the NF-κB subunits similar to those in the vehicle-treated animals were seen in animals that had been treated with 500 μg of etanercept (Figs. 8E, 9E, and 10E). The nuclear staining of p50 and c-Rel, however, appeared to be less than in the saline-injected animals, whereas p65 expression appeared relatively unchanged. Figure 11 shows that the decreased levels of p50 and c-Rel staining in the nucleus of etanercept-treated animals were, indeed, significantly lower than those seen in the saline-treated animals; there was no difference in p65.

Axon regeneration was assessed by measuring immunofluorescent staining for the neuron-specific growth-associated

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**FIGURE 7.** Inhibition of tumor necrosis factor by etanercept inhibits nerve crush injury-induced elevation in nuclear factor-κB (NF-κB) activity in sciatic nerve. Adult male Sprague-Dawley rats were injected subcutaneously with either 500 μg etanercept in 1 mL saline or saline alone and subjected to sciatic nerve crush injury. After 3 days, the animals were killed, and whole L4–L6 dorsal root ganglia (D or DRG), a 1-cm section of sciatic nerve centered on the crush site (N), and L4–L6 dorsal root (Dr or D. root) and ventral root (Vr or V. root) were removed and processed for NF-κB electrophoretic mobility shift assay. (A) A representative autoradiograph exposure demonstrating 2 major NF-κB–binding bands. (B) Quantification of band density. Data are means ± SEM (n = 3); *p < 0.01.

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**FIGURE 8.** Localization and expression of the p50 subunit of nuclear factor-κB in longitudinal sciatic nerve sections in an uninjured animal (A, B) and in nerves 3 days after crush (C–F). Images are from just proximal to the site of the crush. Original magnification: 63×. (A) Naive animal (no surgery performed) stained for p50. (B) Naive animal with p50 and 4,6-diamidino-2-phenylindole (DAPI) image overlay. (C) Vehicle (saline)-treated injured nerve stained for p50. (D) Vehicle-treated injured nerve with p50 staining and DAPI overlay. (E) Nerve from animal treated with 500 μg etanercept stained for p50. (F) Overlay of (E) with DAPI. Arrows indicate positive p50 nuclear staining; arrowheads indicate unusual DAPI staining and low nuclear p50 staining. Scale bar = 30 μm. The nuclear expression of p50 was increased at the crush site after 3 days in the vehicle-treated animal compared with the uninjured control. Treatment with 500 μg of the TNF blocker reduced the crush-induced increase in nuclear expression of p50.
protein GAP-43 from the center of the crush site to the farthest point that the longest regenerating axon had extended into the nerve trunk 3 days after nerve crush injury (Fig. 12, top panel). The mean regeneration distance was significantly increased in animals treated with 500 μg etanercept compared with saline-treated controls (Fig. 12, bottom panel).

**DISCUSSION**

After peripheral nerve damage, well-described molecular and anatomical events, including Wallerian degeneration of the distal nerve stump and regrowth of axons, can lead to whole or partial recovery of nerve function. Among the early nerve-centered inflammatory events are increased local levels of TNF and activated NF-κB (3, 4, 6, 21, 22). We previously reported that NF-κB DNA-binding activity increases in lumbar DRG within 2 hours of sciatic nerve crush that lasts at least 5 days (10). Here we demonstrate, however, that NF-κB DNA binding in the nerve itself is not significantly increased until approximately 14 hours after crush and that it remains elevated for at least 24 hours as measured by electrophoretic mobility shift assay. Western blot analysis shows that at 12 hours, there is a significant increase in expression of all 3 of the NF-κB subunits in the tissue proximal to the crush site at 12 hours after crush. At 12 hours after sciatic nerve crush, the expression of p50, p65, and c-Rel was greatly increased in cells of the sciatic nerve, as assessed using immunofluorescent staining. Much of the upregulated p65 is likely to be a homodimer, but because of the increases in expression seen in both p50 and c-Rel at 12 hours after nerve crush, there are also likely to be p65/c-rel and p50/p65 heterodimers. Because hematogenous macrophages do not...
infiltrate damaged sciatic nerve until 3 or 4 days after injury (23, 24), this upregulation in NF-\(\kappa\)B must be caused by activation of Schwann cells or endogenous endoneurial macrophages (25). This result is consistent with a previous work by Ma and Bisby (26), who found that NF-\(\kappa\)B increased within Schwann cells close to the site of injury.

Although the levels of p65 in the blood microvessels of the sciatic nerve did not appear to be altered after sciatic nerve crush injury, the positive staining for p65 in apparent microvascular endothelial cells suggests a possible role for p65 in blood vessel function. Similarly, previously published data found that p65, but not p50, was positively expressed and found to increase expression in vessel endothelial cells in human brain tissues after contusion (27). Tumor necrosis factor is also known to be expressed in blood vessel endothelial cells in the sciatic nerve after injury; it is thought to increase the permeability of the blood vessels by activating gelatinases (e.g. the matrix metalloproteinases MMP-2 and MMP-9), thus allowing greater macrophage infiltration (4). In the present study, there was no change detected in the levels of p65 capillary endothelial cell staining in the sciatic nerve after crush injury, therefore, it seems unlikely that there is a link between TNF and p65 in this tissue; the mechanisms may be different from those in brain vessel endothelial cells.

The p65 subunit was also present in axons at the crush site 12 hours after injury, suggesting that p65 was either axonally transported to the axon or secreted into the axon from the surrounding Schwann cells. In dissociated DRG neurons cultured for 40 hours, we found that p65 was expressed throughout the neurite, whereas p50 was found only in the cell body with no neurite staining (Fig. 5C–H). Further work is necessary to determine the exact cellular source of the NF-\(\kappa\)B p65 subunit, but our in vitro observations suggest that p65 may be anterogradely transported in the nerve. Indeed, we have preliminary data showing anterograde accumulation of p65 at the site of a nerve crush using a 12-hour double-ligature paradigm (28, 29). Moreover, recent studies show that important components of the NF-\(\kappa\)B signal transduction pathway (IKB\(\alpha\) and IKK) were present in axonal compartments at nodes of Ranvier in central and peripheral nerves (30).

The physiological effect of TNF on NF-\(\kappa\)B activation and nerve regeneration after nerve crush was determined by using the blocker etanercept, which binds to and sequesters soluble TNF (31). Animals that had been given a single 500-\(\mu\)g subcutaneous injection of etanercept immediately before sciatic nerve crush had significantly reduced crush-induced elevation in NF-\(\kappa\)B 3 days later. Thus, TNF had a direct and robust control over NF-\(\kappa\)B in the sciatic nerve at the site of injury after sciatic nerve crush. Inhibition of TNF also increased the initial rate of axon regeneration (Fig. 12). Because TNF is systemically blocked by this treatment, it is not clear whether this is caused by inhibition of TNF locally at...
the crush site or at the DRG because there can be significant anterograde transport of TNF in the sciatic nerve (6).

NF-kB subunit staining of sciatic nerve sections 3 days after sciatic nerve crush showed an increase in the expression of p50, p65, and c-Rel compared with naive (noncrush) tissue. The increase in expression of NF-kB subunits at 3 days after crush may be caused by invading macrophages, which deliver TNF to the site of injury, in addition to endogenous factors causing an upregulation of all 3 NF-kB subunits. Tumor necrosis factor is a key modulator in Wallerian degeneration and is required to prepare the nerve for successful axon regeneration (6, 21, 32, 33). The inflammatory response at the site of injury seems either to delay the onset of axonal sprouting after injury (with the initial fibroblast and Schwann cell production of TNF) or to slow the rate of axon elongation through the second TNF peak generated by invading blood-borne macrophages (2–3 days after axotomy) or by other mechanisms (3, 34, 35). Because we have shown that NF-kB activation is mediated by TNF at the site of injury after a sciatic nerve crush, the likely mechanism by which TNF slows regeneration is downstream activation of NF-kB, in particular, transcription via the p65, p50, and/or c-Rel subunits.

Reestablishment of neuronal function after damage involves both tissue repair and neuronal regeneration. We describe early inflammation-induced expression, localization, and activation of NF-kB in the sciatic nerve after crush injury and found that this is under the regulation of TNF. As in other neuropathologic processes, inflammation seems to exert an inhibitory effect on axonal regeneration after nerve damage (36–38), and recent studies have shown the use of anti-inflammatory treatments for neuropathy and neuropathic pain (39–42). Our current results provide experimental evidence that support this therapeutic approach and elucidate the role of TNF in these early inflammatory events.

REFERENCES