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Minocycline Reduces Cell Death and Improves Functional Recovery after Traumatic Spinal Cord Injury in the Rat

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ABSTRACT

We examined the effects of minocycline, an anti-inflammatory drug, on functional recovery following spinal cord injury (SCI). Rats received a mild, weight-drop contusion injury to the spinal cord and were treated with the vehicle or minocycline at a dose of 90 mg/kg immediately after SCI and then twice at a dose of 45 mg/kg every 12 h. Injecting minocycline after SCI improved hind limb motor function as determined by the Basso-Beattie-Bresnahan (BBB) locomotor open field behavioral rating test. Twenty four to 38 days after SCI, BBB scores were significantly higher in minocycline-treated rats as compared with those in vehicle-treated rats. Morphological analysis showed that lesion size increased progressively in both vehicle-treated and minocycline-treated spinal cords. However, in response to treatment with minocycline, the lesion size was significantly reduced at 21–38 days after SCI when compared to the vehicle control. Minocycline treatment significantly reduced the number of terminal deoxynucleotidyl transferase (TdT)–mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL)–positive cells 24 h after SCI as compared to that of the vehicle control. DNA gel electrophoresis also revealed a marked decrease in DNA laddering in response to treatment with minocycline. In addition, minocycline treatment significantly reduced the specific caspase-3 activity after SCI as compared to that of vehicle control. Furthermore, RT-PCR analyses revealed that minocycline treatment increased expression of interleukin-10 mRNA but decreased tumor necrosis factor–α expression. These data suggest that, after SCI, minocycline treatment modulated expression of cytokines, attenuated cell death and the size of lesions, and improved functional recovery in the injured rat. This approach may provide a therapeutic intervention enabling us to reduce cell death and improve functional recovery after SCI.

Key words: apoptosis; BBB score; caspase-3; DNA laddering; functional recovery; IL-10; inflammation; lesion area; minocycline; spinal cord injury; TUNEL

INTRODUCTION

TRAUMATIC INJURY to the spinal cord induces immediate mechanical damage followed by a secondary cascade of degenerative processes that leads to a progressive loss of tissues (Anderson and Hall, 1993; Blight, 1992; Lu et al., 2000; Schwab and Bartholdi, 1996; Young, 1993). Inflammatory reactions play an important

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role in the secondary injury cascade and contribute largely to the neuropathology associated with chronic SCI (Blight, 1992; Blight et al., 1997; Dusart and Subwab, 1994; Popovic et al., 1994; Zhang et al., 1995). Although many inflammatory responses are mediated by pro-inflammatory cytokines in spinal cord injury (Klusma and Schwab, 1997; Streit et al., 1998; Wang et al., 1996; Zhang et al., 1997), the effects and functions of the cytokines on progressive necrosis need to be characterized. Furthermore, the beneficial effects of anti-inflammatory agents on the recovery cascade following traumatic SCI largely remain to be explored.

Minocycline is a tetracycline derivative that has both anti-microbial effects and anti-inflammatory effects (Smilack, 1999). This drug is well tolerated clinically and currently used for treatment of rheumatoid arthritis, a chronic inflammatory disease (Mantovani et al., 1997; Tilley et al., 1995). Minocycline is also a highly lipophilic compound and penetrates the brain–blood barrier easily (Klein and Cunha, 1995). Recent reports show that minocycline exerts neuroprotective effects on a variety of CNS neurons *in vivo* and *in vitro*. For example, minocycline reduces expression of caspases and inducible nitric oxide synthetase (iNOS), and delays progression in a mouse model of Hungtington's disease (Chen et al., 2000). Minocycline treatment also reduces the loss of hippocampal pyramidal neurons in a gerbil model of ischemia (Yrjanheikki et al., 1998). Furthermore, minocycline inhibits microglial activation and protects CNS neurons against 6-hydroxydopamine- or glutamate-induced toxicity *in vivo* and *in vitro* (He et al., 2001; Tikka and Koistinaho, 2001). These observations indicate that minocycline provides neuroprotection from insults via an anti-inflammatory mechanism.

The present experiments were undertaken in order to examine whether compounds with anti-inflammatory properties can reduce progressive necrosis that occurs after SCI. For this purpose, we assessed the effects of minocycline on the functional behavior and histopathology of SCI. We report that minocycline treatment reduced apoptotic cell death and progressive necrosis of tissues, increased expression of interleukin (IL-10), an anti-inflammatory cytokine, and improved functional behavior after SCI. Our results suggest that minocycline may represent a potential therapeutic agent for treatment of SCI.

MATERIALS AND METHODS

Materials

Minocycline hydrocloride was purchased from Sigma (Sigma, St. Louis, MO). An Apoptag peroxidase kit for TUNEL-staining was purchased from Oncor (Gaithersburg, MD). Z-DEVD-AFC, a caspase-3 enzyme substrate was purchased from Enzyme Systems (Enzyme Systems Products, Livermore, CA). TRIZOL Reagent and MMLV Reverse transcriptase were purchased from GibcoBRL (Gaithersburg, MD).

Spinal Cord Injury

Traumatic injury was induced using the force-calibrated weight-drop device developed at New York University (Gruner, 1992). Sprague-Dawley rats [male; 230–250 g rats; Sam:TacN (SD) BR, Korea] were anesthetized with chloral hydrate (50 mg/kg, i.p.) During surgery, rectal temperature was maintained at 37°C by a thermostatically regulated heating pad. A laminectomy was performed at vertebral level T9–T10, exposing the underlying cord without disrupting the dura. After the spinous processes of T8 and T11 were clamped to stabilize the spine, the exposed dorsal surface of the cord was subjected to a weight drop impact using a 10-g rod (2.5 mm in diameter) dropped from a height of 12.5 mm following the procedural guidelines established by a multicenter consortium (Multicenter Animal Spinal Cord Injury Study; Basso et al., 1995, 1996a,b). After the injury, the muscles and skin were closed in layers, and the rats were placed in a temperature and humidity-controlled chamber overnight. Manual expression of the urinary bladder was performed three times per day until reflexive bladder emptying was established. The Animal Studies Committee of Korea Institute Science and Technology (KIST) maintained oversight on all of the surgical interventions and animal care in accordance with the Guidelines and Policies for Rodent Survival Surgery Provided.

Minocycline Administration

Minocycline was dissolved in sterile PBS and administered intraperitoneally after injury in animals randomly assigned to control or to a treatment group (for lesion area analysis, $n = 3$ for vehicle or minocycline, total 24 rats; for TUNEL staining, $n = 3$ for vehicle or minocycline, total 6 rats; for caspase-3 activity, $n = 3$ for sham-operated, vehicle or minocycline, total 9 rats; for cytokine expression, $n = 3$ for normal, sham-operated, vehicle or minocycline, total 30 rats; for behavioral testing, $n = 13$ for each vehicle or minocycline, total 26 rats). Rats receiving the 12.5-mm insult received intraperitoneal injections of minocycline at a dose of 90 mg/kg immediately after SCI and then twice at a dose of 45 mg/kg every 12 h (Sanchez Mejia et al., 2001; Yrjanheikki et al., 1998); the vehicle control group received injections of sterile PBS. For the sham-operated controls, the animals underwent a T9–T10 laminectomy without contusion injury, received no pharmacological treatment, and were

sacrificed at the same time intervals as the treatment groups.

Behavioral Testing

The Basso-Beattie-Bresnahan (BBB) locomotor rating scale, as described previously (Basso et al., 1995), was used to evaluate the functional consequences of SCI and to determine the effects of minocycline on recovery (n = 13). Testing was performed by trained investigators who were blind as to the experimental conditions, and who began testing 1 day after the 12.5-mm weight-drop injury and then continued to test twice weekly for up to 38 days.

Lesion Area Analysis

Rats treated with vehicle or minocycline at 14, 21, 28, and 38 days after injury (n = 3 for each time point) were anesthetized and perfused intracardially with 0.1 M PBS, pH 7.4 followed by 4% paraformaldehyde in PBS, pH 7.4. For histological evaluation, a 2-cm cord segment centered at the injury site was removed from the vertebral column, placed in the same fixative overnight, placed in 30% sucrose in 0.1 M PBS, pH 7.4, and embedded in OCT compound. Serial longitudinal sections (8 μm) through the central canal from vehicle- and minocycline-treated rat spinal cords were cut and stained with Cresyl violet. The total areas of lesions (four serial sections per spinal cord) were determined by measuring the area of cavitation at the epicenter of the injury using a 1.25× objective and then calculated by means of a Metamorphor imaging program (Universal Imaging Corp., West Chester, PA). In some experiments, cross sections (10 μm) through the lesion center from vehicle- and minocycline-treated spinal cords were cut and stained with Cresyl violet.

TUNEL Staining

Twenty hours after SCI, rats were anesthetized and perfused via cardiac puncture with saline and subsequently with 4% paraformaldehyde in 0.1 M PBS. A 20-mm section of the spinal cord, centered at the lesion site, was dissected out and post-fixed by immersion in 4% paraformaldehyde overnight. The segment was embed-

Table 1. PCR Primers, Product Size, and PCR Reaction

	Sequence[a]	*Product size*	*PCR parameter*	*Number of cycles*
β-actin	*S*: 5′-CTTCTGCATCCTG TCAGCGATGC-3′	241	94°C 30 s, 58°C 30 s, 72°C 30 s,	25
	A: 5′-AGAAGAGCTATG AGCTGCCTGACG-3′			
TNF-α	*S*: 5′-CCCAGACCCTCA CACTCAGAT-3′	215	94°C 30 s, 55°C 30 s, 72°C 30 s,	30
	A: 5′-TTGTCCCTTGAA GAGAACCTG-3′			
TGF-β	*S*: 5′-GAGAGCCCTGGA TACCAACTACTG-3′	278	94°C 30 s, 60°C 30 s, 72°C 30 s,	30
	A: 5′-CTCCACCTTGGG CTTGCGACCCAC-3′			
IL-10	*S*: 5′-GAGTGTTCAAAG GGAAATTATAT-3′	201	94°C 30 s, 55°C 30 s, 72°C 30 s,	30
	A: 5′-CTGGTTTCTCTTC CCAAGAC-3′			
	S: 5′-AAGTTTCTCTCC GCAAGATACTTCCAG CCA-3′	327	94°C 30 s, 55°C 30 s, 72°C 30 s,	30
	A: 5′-AGGCAAATTTCCT GGTTATATCCAGTTT-3′			
IL-1β	*S*: 5′-GCAGCTACCTATG TCTTGCCCGTG-3′	290	94°C 30 s, 50°C 30 s, 72°C 30 s,	30
	A: 5′-GTCGTTGCTTGTC TCTCCTTGTA-3′			

[a]*S*, sense; *A*, antisense.

ded in paraffin. Longitudinal sections (8 μm) through the anterior horn from vehicle controls and minocycline-treated spinal cords ($n = 3$ for each group) were used for TUNEL staining using an Apoptag peroxidase kit; the sections were counterstained with methyl green. Diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA) was used as a substrate for peroxidase. All TUNEL analyses were carried out by investigators who were blind as to the experimental conditions. Briefly, quantitation was accomplished by counting the number of cells labeled positively using a 20× objective. Only those TUNEL-labeled cells showing morphological features of nuclear condensation and/or compartmentation were counted as TUNEL-positive. All cells stained positively within an area extending from 2 mm rostral to 2 mm caudal to the lesion center were counted from each section (two sections from each spinal cord), without discriminating between gray and white matter or differentiating between neural and non-neural cells.

DNA Laddering

Spinal cord DNA was isolated with DNA binding resin according to the manufacturer's protocol with a few modifications (Omega Biotek, New Orleans, LA). Briefly, rats received injections of vehicle or minocycline after injury ($n = 3$) and were then euthanized at 24 h after SCI. Segments of spinal cord (1 cm) were then isolated using the lesion site as the epicenter. The segments were homogenized in a lysis buffer (7 M guanidinium hydrochloride, 800 mM guanidine HCl, 30 mM Tris-HCl [pH 8.0], 30 mM EDTA, 5% Tween-20, 0.5% Triton X-100). Mixing proceeded on a rocker platform for 20 min, and the contents were then centrifuged at 10,000 × g for 15 min. DNA binding resin was added to the extraction mix, this was incubated for 5 min, and then centrifuged at 10,000 × g for 5 min. The supernatant was discarded and the DNA binding resin was washed with a washing solution (90 mM sodium chloride, 9 mM Tris-HCl, pH 7.4, 2.25 mM EDTA, 55% ethanol). The DNA was then treated with DNase-free RNase (10 mg/mL; Sigma, St. Louis, MO) for 1 h at 37°C and assayed by optical absorption at 260 nm (Ultraspec 1000, Amersham Pharmacia Biotech, Uppsala, Sweden). One μg of DNA was labeled with ^{32}P-dCTP (Amersham Pharmacia Biotech, Uppsala, Sweden) using DNA polymerase (PerkinElmer, Madison, WI). After quantification of DNA by spectrophotometer (PerkinElmer, Emeryville, CA), 1 μg of DNA was incubated for labeling in 20 μL of labeling mixture [10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 10 mM $MgCl_2$] containing 2 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol, Amersham Biosciences, Arlington Heights, IL) and 2.5 U of Taq DNA polymerase (Perkin-Elmer, Emeryville, CA) at room temperature for 20 min. The reaction was stopped by the addition of 2.5 μL of 0.5 M EDTA (pH 8.0). Radiolabeled DNA was loaded onto a 1.5% agarose gel, separated by electrophoresis at 50 V for 2.5 h in a 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA). After the gel was dried, radiolabeled DNA in the dried gel was detected by exposure to x-ray film (Medical X-ray film blue, Agfa-Gevaert NV). Experiments were repeated three times to ensure reproducibility.

Caspase-3 Activity

The caspase-3 enzyme activity was assayed using a procedure described by Springer et al. (1999). Briefly, segments of spinal cord (1 cm) from sham-operated, vehicle- or minocycline-treated rats ($n = 3$) were isolated using the lesion site as the epicenter and homogenized in 0.5 mL of homogenization buffer (pH 7.4) containing 10 mM HEPES, 250 mM sucrose, 1 mM EDTA, 0.1% CHAPS, 5 mM DTT, and 10 μl each of 1 mg/mL pepstatin, 2.5 mg/mL leupeptin, 2 mg/mL aprotinin, and 0.2 M PMSF. The samples were centrifuged at 40,000 × g for 30 min, and the protein levels of the supernatant were determined using the BCA assay (Pierce, Rockford, IL). One hundred micrograms of cytosolic fraction of spinal cord protein was added to 1 mL of caspase homoge-

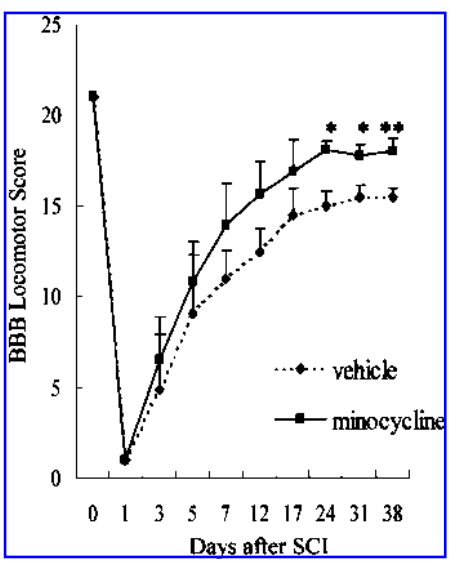

FIG. 1. Effect of minocycline on functional recovery after spinal cord injury. Functional recovery was assessed by BBB behavioral testing once a week for 38 days as described in Materials and Methods. Note that the BBB scores were significantly higher in minocycline-treated rats 24 to 38 days after SCI as compared to those in vehicle-treatedrats. Data represent mean ± SD ($n = 13$ per group). *$p < 0.05$, **$p < 0.005$ vs. vehicle.

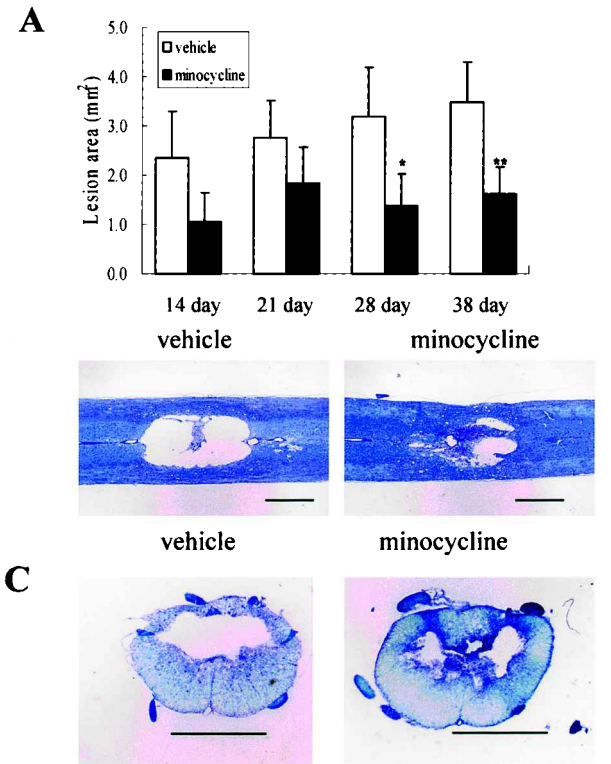

FIG. 2. Effect of minocycline on the lesion area after spinal cord injury. Total lesion areas were determined by calculating the area of cavitation at the epicenter of the injury as described in Materials and Methods. **(A)** Lesion areas 14, 21, 28, 38 days after SCI. Data represent mean ± SD ($n = 3$ for each time point). Note that minocycline treatment significantly reduced the lesion size between 28 and 38 days after SCI as compared to the vehicle control. $*p < 0.05$, $**p < 0.005$ vs. vehicle. Representative photomicrographs of spinal cord lesion areas in longitudinal section **(B)** and cross section **(C)** through the lesion epicenter 38 days after SCI of vehicle- and minocycline-treated spinal cords. Bar = 1 mm.

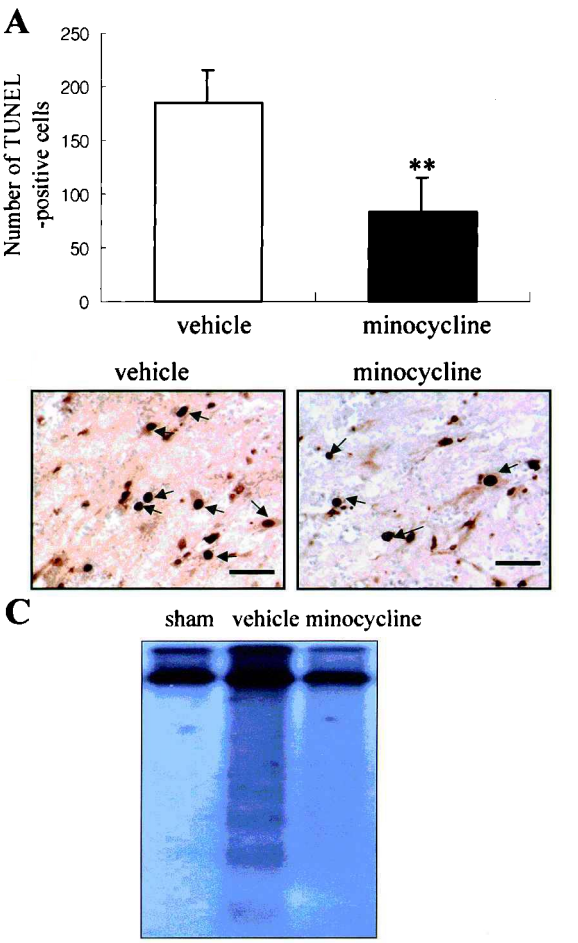

FIG. 3. Effect of minocycline on apoptotic cell death after spinal cord injury. Rats were treated with vehicle or minocycline after injury, and spinal cord tissues were then collected 24 h post-injury. Longitudinal sections were processed for TUNEL staining, and TUNEL-positive cells were counted as described in Materials and Methods. **(A)** TUNEL-positive cells in injured spinal cords. Data represent mean ± SD ($n = 3$). Note that treatment with minocycline after SCI significantly reduced the number of TUNEL-positive cells as compared to the positive cells seen in the vehicle control. $**p < 0.001$. **(B)** Representative photomicrographs of TUNEL-positive cells (arrows) 24 h after spinal cord injury treated with vehicle or minocycline. Bar = 50 μm. **(C)**. DNA gel electrophoresis 24 h after SCI. DNA was isolated from the spinal cord tissues, labeled at the 3′ end with [^{32}P]deoxycytidinetriphosphate (dCTP) and separated by gel electrophoresis as described in Materials and Methods. *Lane 1*, untreated, sham-operated control; *lane 2*, treated with vehicle; *lane 3*, treated with minocycline. Note that DNA laddering decreased following treatment with minocycline after SCI when compared to the laddering seen in the vehicle control. The gel presented is a representative of results from three separate experiments.

nization buffer containing 15.0 μM of Z-DEVD-AFC (Enzyme Systems Products, Livermore, CA). Samples were incubated at room temperature for 5 min, and relative fluorescence (excitation at 400 nm and emission at 505 nm) was measured for 1 h using a K2 multi-frequency phase fluorometer (ISS Inc., Champaign, IL). The specific activity of the samples was calculated relative to a standard curve using recombinant caspase-3 (Upstate Biotechnology, Lake Placid, NY).

RNA Purification and RT-PCR

The spinal cord tissues from normal, sham-operated, vehicle- or minocycline-treated rats ($n = 3$ for each group at 0, 1, 6, 12, and 24 h after SCI) were isolated and processed as described above. RNA was purified using

TRIZOL Reagent according to the manufacturer's instructions. To ascertain that all RNA samples would be DNA-free, samples were treated with RNase-free DNase I (Sigma, St. Louis, MO). After spectrophotometric quantification, the purified RNA was separated on a formaldehyde-agarose gel to check the extent of degradation. One microgram of total RNA was reverse-transcribed into first strand cDNA in each 20-μL reaction mixture, using MMLV according to the manufacturer's instructions (Invitrogen, Groningen, Netherlands). A 20-μL PCR reaction contained 2 μL of first-strand cDNA, 0.6 U of Amplitaq polymerase (Perkin-Elmer, Branchburg, NJ), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 250 μM dNTP, and 10 pmol of each specific primer. Primers for tumor necrosis factor–α (TNF-α) (Estler et al., 1992), transforming growth factor–β (TGF-β) (Streit et al., 1998), interleukin-10 (IL-10) (GeneBank accession no. NM_012854), interleukin-6 (IL-6) (Streit et al., 1998), interleukin-1β (IL-1β) (GeneBank accession no. EO1884), and β-actin (Nudel et al., 1983) were subjected to PCR reactions on a thermocycler (Perkin-Elmer, Emeryville, CA). Primers with the target gene sequences were synthesized by the Genetech Corp (Daejeon, Korea). Primers, PCR product sizes and reaction conditions for target genes are summarized in Table 1. Negative controls consisted of PCR reactions lacking primers or reverse transcriptase. After amplification, PCR products were subjected to a 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The relative density of bands was analyzed by the ChemiImager™ 4400 (Alpha Innotech Corporation, San Leandro, CA). Quantification of PCR bands was performed as described previously (Murphy et al., 1993; McCombe et al., 1998). Experiments were repeated three times, and the values obtained for the relative intensity were subjected to statistical analysis.

Statistical Analysis

Data are presented as mean $\pm$ SD values. Quantitative data from open field locomotor scores and lesion areas were evaluated for statistical significance using Student's paired *t*-test; data from TUNEL-positive cells, caspase-3 activities and PCR analyses were evaluated for statistical significance using two-way ANOVA with a *post hoc* Tukey test. In all analyses, a p value of <0.05 was considered statistically significant.

RESULTS

Minocycline Improves Functional Recovery after SCI

The effect of minocycline on locomotor function was determined to see whether the drug has beneficial effects on functional recovery after SCI. Immediately after SCI, all injured rats were paralyzed in their hind limbs. The functional recovery was assessed by the BBB test, a standard method for assessing hind limb motor function after SCI in rats (Basso et al., 1996a,b). As shown in Figure 1, the BBB scores were significantly higher in minocycline-treated rats 24–38 days after SCI as compared to those in vehicle-treated rats. At 38 days after injury, BBB scores of minocycline-treated and vehicle-treated rats were 18 $\pm$ 0.7 ($p < 0.01$) and 15 $\pm$ 0.5, respectively.

Minocycline Reduces Tissue Necrosis after SCI

The lesion size increased progressively in both vehicle-treated and minocycline-treated spinal cords (Fig. 2). Fourteen days after injury, however, a marked decrease in the lesion size was observed in the minocycline-treated cords when compared to that of vehicle (Fig. 2). The lesion size, when compared to that of vehicle control, was significantly reduced at both 28–38 days after SCI by treatment with minocycline (Fig. 2). At 38 days after injury, for example, the lesion areas of vehicle-treated and minocycline-treated spinal cords were 3.5 $\pm$ 0.8 mm^2 and 1.6 $\pm$ 0.5 mm^2 ($p < 0.01$), respectively.

Minocycline Treatment Reduced Cell Death

Trauma to the spinal cord results in extensive apoptotic cell death of neurons and glial cells (Beattie et al., 2000; Crowe et al., 1997; Lee et al., 2000, Li et al., 1996; Li and Wong, 2000; Liu et al., 1997). Since minocycline exerts protective effects on a variety of CNS neurons against insults (He et al., 2001; Tikka and Koistinaho, 2001; Yrjanheikki et al., 1998), we examined whether the drug alleviates apoptotic cell death after SCI. Minocycline treatment significantly reduced the number of TUNEL-positive cells 24 h after SCI as compared to the vehicle control (Fig. 3A and B). For example, the number of TUNEL-positive cells in vehicle-treated and minocycline-treated spinal cords was 185 $\pm$ 30 and 84 $\pm$ 29 ($p < 0.001$), respectively (Fig. 3A). Also, DNA gel electrophoresis revealed a marked decrease in DNA laddering following minocycline treatment (Fig. 3C).

Minocycline Reduces Caspase-3 Activity

Evidence indicates that activation of caspase-3 is involved in apoptotic cell death after SCI (Emery et al., 1998; Matsushita et al., 2000; Springer et al., 1999). Since minocycline treatment reduced apoptosis after SCI as revealed by TUNEL staining and DNA gel electrophoresis (Fig. 3), we examined whether the drug exerts effects on caspase-3 activity. As shown in Figure 4, minocycline treatment significantly reduced specific caspase-3 activity 4 h after SCI as compared to that of vehicle control.

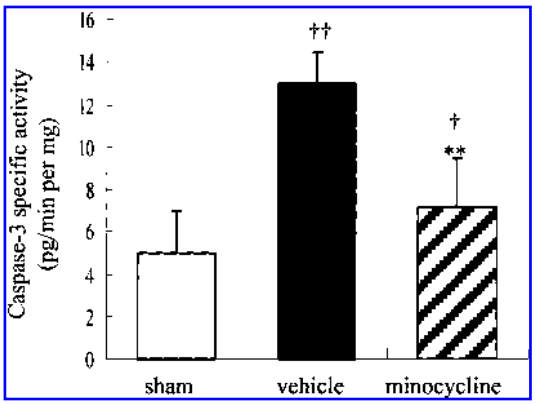

FIG. 4. Effect of minocycline on caspase-3 activity after spinal cord injury. Fluorogenic caspase activity was measured as described in Materials and Methods. Note that caspase-3 specific activity was reduced in minocycline-treatment as compared to that of vehicle control. Data represent mean ± SD (n = 3 per group). $^{**}p < 0.005$ vs. vehicle, $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.005$ vs. sham.

Minocycline Increases IL-10 Expression and Decreases TNF-α Expression

Since minocycline has anti-inflammatory properties *in vivo* (Mantovani et al., 1997; Smilack, 1999; Tilley et al., 1995), we examined the effect of minocycline on expression of several cytokines after SCI. As shown in Figure 5, RT-PCR analyses revealed that, by 6 h after SCI, minocycline treatment significantly increased the mRNA level of IL-10, a potent anti-inflammatory cytokine, as compared to that of vehicle control. By contrast, drug treatment significantly decreased the mRNA level of TNF-α, a pro-inflammatory cytokine. However, after injury, minocycline had no significant effect on the mRNA levels of Il-1β, TGF-β, and IL-6 as compared to those of vehicle control (Fig. 5).

DISCUSSION

The main objective of this study was to assess the long-term effect of minocycline, an anti-inflammtory agent, on locomotor function after SCI. Two essential findings of these experiments are (i) that treatment with minocycline improves functional recovery after SCI; (ii) that the drug treatment attenuates the size of lesions after SCI. In addition, minocycline reduces apoptotic cell death and caspase-3 activity after SCI. Furthermore, minocycline treatment increases IL-10 expression but decreases TNF-α expression. These results indicate that the beneficial effect of minocycline treatment may be attributable primarily to its neuroprotective activity against injury-induced apoptosis thereby reducing the size of lesions after SCI.

In the present study, rats received injections of minocycline at a dose of 90 mg/kg immediately after SCI; they then received two doses of 45 mg/kg every 12 h. At 24–38 days after SCI, these animals showed a significant improvement of hind limb motor function. A similar treatment regimen also showed a significant neuroprotective activity against focal ischemia or traumatic brain injury (Sanchez Mejia et al., 2001; Yrjanheikki et al., 1998). Yrjanheikki et al. (1998) reported that following ischemic insults, injections of minocycline at a dose of 180 mg/kg for the first day after ischemia followed by 90 mg/kg daily resulted in neither significant side effects nor an increase in mortality. In our preliminary studies, by contrast, rats which received injections of minocycline at a dose of 90 mg/kg immediately after SCI followed by 45 mg/kg daily for 10 days showed such side effects as weight loss during treatment for a period of up to 38 days. Also, no improvement of locomotor function was observed in those rats receiving such a long-term treatment with the drug (data not shown). Furthermore, injections of minocycline prior to SCI (pre-treatment: 45 mg/kg 12 h prior to SCI, 90 mg/kg immediately after SCI, and then twice at a dose of 45 mg/kg every 12 h) showed a less beneficial effect on functional recovery and neuroprotective activity as compared to those treated "post-injury" (data not shown). These observations thus suggest that the timing of minocycline injection as well as its dosage could be important factors for determining the outcome of functional recovery after SCI.

Caspases are known to be involved in neuronal apoptosis in CNS injury and neurodegenerative diseases (Chen et al., 2000; Friedlander and Yuan, 1998; Ona et al., 1999; Li and Wong, 2000; Schielke et al., 1998; Yuan and Yankner, 2000). Recent evidence indicates that minocycline has neuroprotective properties expressed by inhibiting several macromolecules involved in apoptosis. In animal models of brain ischemia, for example, minocycline treatment attenuates expression of such macromolecules as caspase, iNOS, and cyclooxygenease (COX)–2, prevents activation of microglia, and reduces infarction volume (Yrjanheikki et al., 1998; 1999). Minocycline also reduces caspase activity and lesion volume, and improves neurological function after traumatic brain injury (Sanchez Mejia et al., 2001). Recently, a preliminary report by Arnold et al. (2001) showed that minocycline reduces apoptosis and caspase-3 activity, and improves functional recovery after SCI. Furthermore, our results demonstrated that minocycline treatment reduced capase activity, lesion volume and the number of TUNEL-positive cells, and improved functional recovery after SCI. Thus, it is clear that minocycline provides neuroprotec-

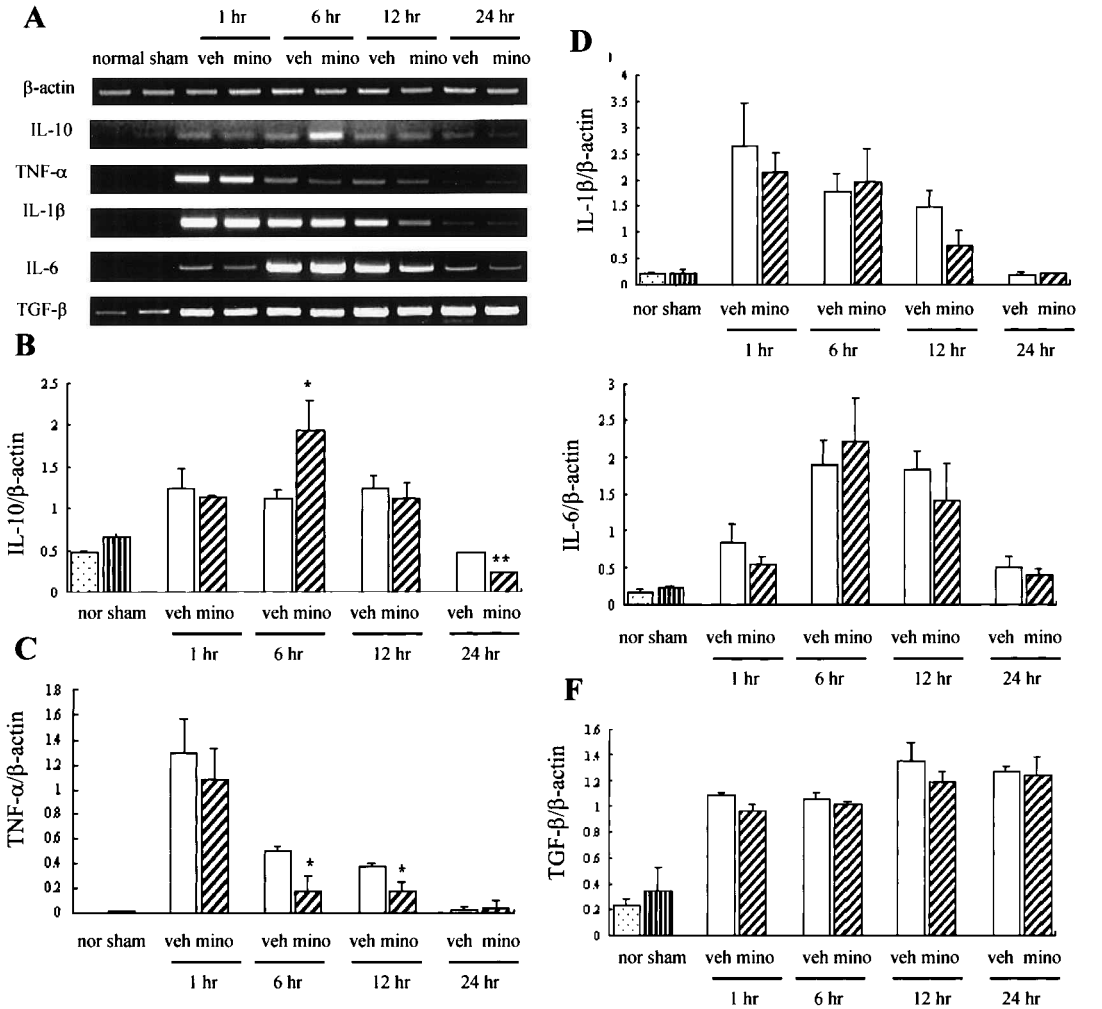

FIG. 5. Effect of minocycline on expression of cytokines after spinal cord injury. Vehicle and minocycline (90 mg/kg) were injected immediately after injury and spinal cord tissues were collected 1, 6, 12, and 24 h post-injury. RT-PCR was performed as described in the Materials and Methods. **(A)** RT-PCR analyses of IL-10, TNF-α, IL-1β, IL-6, and TGF-β cytokine expressions after minocycline treatment. The gels presented are representative of results from three separate experiments. **(B–F)** Densitometry readings of gel bands were expressed as arbitrary units and the data was expressed as a ratio to β-actin mRNA standard. Note that minocycline treatment increased IL-10 expression but decreased TNF-α expression after injury. Values are mean ± SD of three separate experiments. $*p < 0.05$, $**p < 0.005$, vs. vehicle.

tion against CNS injury, possibly through a caspase-dependent mechanism. Because minocycline inhibits neuronal apoptosis, the drug represents a potential therapeutic agent for treatment of CNS injury or diseases.

Inflammation has been implicated in a number of neurodegenerative conditions such as Alzheimer's disease (Hull et al., 2002), AIDS-related dementia (Dalgleish and O'Byrne, 2002; Gentleman et al., 1994; Gentleman and Tardieu, 1994), Parkinson's disease (McGeer et al., 2001), ischemic insult (Liao et al., 2001), and acute CNS trauma (Chrisstensen, 2001; Clark et al., 1994; Dusart and Subwab, 1994). Following SCI, acute inflammatory responses involve the recruitment of neutrophils and macrophages to the site of injury and activate resident microglia (Taoka and Okajima, 1998). Injury to the spinal cord also triggers a rapid and robust upregulation of proinflammatory cytokines that include IL-1β, TNF-α, and IL-6 (Hayashi et al., 2000; Pan et al., 2002; Streit et al., 1998; Wang et al., 1996, 2002). Administration of IL-1 receptor antagonists significantly inhibits apoptotic cell death and caspase activation after SCI (Nesic et al., 2001). Injection of a COX-2 inhibitor, NS-398, also im-

proved hind limb motor function and reduced lesion volume after SCI (Hains et al., 2001). Furthermore, injection of interleukin-10, a potent anti-inflammatory cytokine, reduces TNF-α production in the spinal cord thereby promoting functional recovery following SCI (Bethea et al., 1999). By contrast, Takami et al. (2002) reported that administration of IL-10 after SCI failed to improve functional recovery. We recently demonstrated that injecting a neutralizing antibody to TNF-α reduced apoptosis after SCI (Lee et al., 2000; Yune et al., 2003). The present study also demonstrated that after SCI, minocycline treatment increased IL-10 expression but decreased TNF-α expression. These results indicate that the increased inflammatory response after SCI might stimulate apoptosis and be detrimental to recovery of functions, and that the beneficial effect of minocycline treatment may be attributable to its anti-inflammatory activity. Nevertheless, the answer to improved drug treatment of SCI is to reduce inflammatory responses by administering anti-inflammatory agents that mitigate apoptosis and thereby alleviate functional deficits. The present study corroborates a view that after SCI, treatment with minocycline, an anti-inflammatory drug, improves functional recovery.

The findings reported in the present study indicate that minocycline provides neuroprotection against SCI. Methylprednisolone is currently being applied clinically to treat SCI (Bracken et al., 1997; Constantini and Young, 1994). Although the effect of methylprednisolone is promising, other therapeutic agents designed to reduce cell death and to recover functional deficits after SCI must be investigated. Because minocycline, a derivative of the tetracyclines, has been used clinically, the drug might provide a new approach for neuroprotection in human SCI. Further studies are necessary to determine the precise mechanisms by which minocycline provides neuroprotection and the optimal dose for treating SCI. These studies are now under way.

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