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Increased Production of Tumor Necrosis Factor–α Induces Apoptosis after Traumatic Spinal Cord Injury in Rats

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ABSTRACT

We showed previously that, after spinal cord injury (SCI), tumor necrosis factor–α (TNF-α) may serve as an external signal, initiating apoptosis in neurons and oligodendrocytes. To further characterize the apoptotic cascade initiated by TNF-α after SCI, we examined the expression of TNF-α, inducible nitric oxide (NO) synthase (iNOS), and the level of NO after SCI. Western blots and reverse transcription polymerase chain reactions showed an early upregulation of TNF-α after injury. A peak TNF-α expression was observed within 1 h of injury. By 4 h after injury, the expression of iNOS and the level of NO were markedly increased in the injured spinal cord. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL)–positive cells were also first observed in the lesioned area 4 h after SCI. The largest number of TUNEL-positive cells was observed between 24–48 h after SCI. Injecting a neutralizing antibody against TNF-α into the lesion site after injury significantly reduced the expression of iNOS, the level of NO and the number of TUNEL-positive cells in the injured spinal cord. Injecting the NOS inhibitors, N^G-monomethyl-L-arginine monoacetate and *S*-methylisothiourea sulfate, or an NO scavenger, carboxy-PTIO, into the lesion site also significantly reduced the level of NO and the degree of DNA laddering in the injured spinal cord. These data suggest that after SCI, apoptosis induced by TNF-α may be mediated in part by NO via upregulation of iNOS, induced in response to TNF-α.

Key words: apoptosis; iNOS; NO; NOS inhibitors; spinal cord injury; TNF-α; TUNEL

INTRODUCTION

TRAUMATIC SPINAL CORD INJURY (SCI) initiates a complex series of cellular and molecular events that induce massive cell death leading to permanent neurological deficits. Aside from the immediate cell death caused by necrosis (Balentine, 1978; Selina et al., 1989), apoptosis contributes to a prolonged death of neurons and oligodendrocytes occurring after traumatic injury (Crowe et al., 1997; Emery et al., 1998; Li et al., 1996; Liu et al., 1997; Shuman et al., 1997; Yong et al., 1998). The external apoptotic death signals generated after SCI have

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not been identified, however. It is known that expression of TNF-α is upregulated rapidly at the lesion site after SCI (Hayashi et al., 2000; Streit et al., 1998; Wang et al., 1996; Wang et al., 2002; Yan et al., 2001). TNF-α is known to induce apoptosis of neurons *in vitro* (D'Souza et al., 1995; Sipe et al., 1996) and of oligodendrocytes *in vivo* (Akassoglou et al., 1998). 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). Therefore, a rapid accumulation of TNF-α following SCI may initiate apoptosis in neurons and glial cells (Shuman et al., 1997). We recently demonstrated that TNF-α may act as an external signal initiating apoptosis after SCI (Lee et al., 2000). Despite this finding, the intracellular signaling events initiated by TNF-α that lead to apoptotic cell death are largely unknown.

Nitric oxide (NO) is an important regulatory molecule, which elicits such diverse biological actions as vasodilation and neurotransmission (Lowenstein and Snyder, 1992). NO, a highly reactive free radical, has also been implicated in neuronal injury and in neurodegenerative disease (Beckman and Koppenol, 1996; Brosnan et al., 1998; Coyle and Puttfarken, 1993; Dawson, 1995; Lipton et al., 1994; Parkinson et al., 1997). In particular, high levels of NO produced by inducible NOS (iNOS) are known to elicit neurotoxicity. In models of CNS inflammation and injury, iNOS is expressed in neurons, glial cells and macrophages (Minc-Golomb et al., 1996; Sato et al., 1996; Wu, 1993). Since expression of iNOS can be induced by such proinflammatory cytokines as TNF-α, interleukin-1 (IL-1), and interferon-γ (IFNγ) (Bhat et al., 1999; Coyle, 1996) and TNF-α is rapidly expressed in neurons and glial cells after injury (Lee et al., 2000, Streit et al., 1998), it is likely that after SCI, TNF-α might mediate the apoptotic cell death, in part, via increased NO through the prior induction of NOS. However, the exact mechanism of iNOS regulation at the transcriptional level is not known.

In the present report, we investigated expression TNF-α and its potential downstream target molecules such as iNOS and NO after SCI. Our data show that the temporal expression of TNF-α and iNOS, and the production of NO were up-regulated after SCI. After SCI, furthermore, treatments with a neutralizing antibody against TNF-α significantly reduced the gene expression of iNOS, the level of NO and the number of TUNEL-positive cells. Treatments with NOS inhibitors and with an NO scavenger also significantly reduced the level of NO and the degree of DNA laddering after SCI. These data suggest that apoptosis induced by TNF-α after SCI may be mediated in part via NO produced in response to enzymatic synthesis by NOS which enzyme is itself induced by TNF-α.

MATERIALS AND METHODS

Materials

Polyclonal antibodies directed towards TNF-α, and iNOS were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). A neutralizing antibody against TNF-α was purchased from R&D (Minneapolis, MN) and from Genzyme (Cambridge, MA); normal rabbit serum was purchased from Dako (Carpinteria, CA). N^G-monomethyl-L-arginine monoacetate (L-NMMA), *S*-methylisothiourea sulfate (SMT), carboxy-PTIO were purchased from Alexis Biochemical (San Diego, CA).

Spinal Cord Injury and Pharmacological Treatment

Traumatic injury was induced by crushing the spinal cord of adult rats (male; Sprague-Dawley; 300–350 g) extradurally with no. 5 Dumont tweezers at the level of T-5 as described previously (Du et al., 1999; Lee et al., 2000). All animal care and surgical procedures were approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore and were in strict concordance with NIH-approved guidelines for humane animal housing, care, surgery and treatment. Immediately after crush, 2–4 μL of various pharmacological reagents were injected directly into the spinal cord at the lesion's epicenter. Rats were assigned randomly to one of five experimental groups according to which reagents were to be injected into the spinal cords. The groups ($n = 8$ for each group) were as follows: vehicle control (normal rabbit serum or normal saline); rabbit neutralizing antibody against TNF-α; L-NMMA (25 mg/mL in saline); SMT (50 mg/mL in saline); carboxy-PTIO (25 mg/ml in saline). For the sham-operated controls, the animals underwent a T5 laminectomy without crush injury, received no pharmacological treatment, and were sacrificed at the same time intervals as the treatment groups.

Tissue Preparation

At timed intervals after SCI (30 min, 1 h, 4 h, and 24 h), animals were deeply 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 embedded in paraffin or OCT for frozen sections as described (Du et al., 1999; Lee et al., 2000). Longitudinal sections were then cut at either 10 μm for paraffin-embedded tissues or at 8 μm for frozen tissues.

Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate-biotin Nick End Labeling (TUNEL) Staining

Longitudinal, paraffin sections through the anterior horn taken from vehicle controls and from animals treated with neutralizing antibody against TNF-α ($n = 8$ for each group) were used for TUNEL staining using an Apoptag peroxidase kit (Oncor, Gaithersburg, MD); 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 the cells stained positively within an area extending 2 mm rostral to 2 mm caudal to the lesion site were counted from each section.

Immunostaining

The frozen sections were processed for immunocytochemistry with a polyclonal antibody against TNF-α (1:100 dilution), and iNOS (1:100 dilution). Sections were blocked in 5% normal serum and 0.1% triton X-100 in TBS for 1 h at RT and then incubated with primary antibodies overnight at 4°C, followed by HRP-conjugated secondary antibodies (Dako, Carpinteria, CA). The ABC method was used to detect labeled cells using a Vectastain kit (Vector Laboratories, Burlingame, CA). DAB or tetramethylbenzidine (TMB) served as the substrate for peroxidase. Some iNOS and TUNEL stainings were double-labeled using antibodies to either neuron specific nuclear protein (NeuN; 1:200 dilution; Chemicon, Temecula, CA), a monoclonal antibody to the oligodendrocyte-specific antigen, APC (1:200 dilution; Oncogene, Cambridge, MA), or an antibody specific for microglia, OX-42 (1:200 dilution; Chemicon, Temecula, CA). For double labeling, FITC (Amersham Biosciences, Arlington Heights, IL) or TRITC-conjugated secondary antibodies (Dako, Carpinteria, CA) were used. In some experiments, nuclei were labeled with DAPI according to the protocol of the manufacturer (Molecular Probes, Eugene, OR). Images were collected using an Olympus microscope and SPOT™ (Diagnostic Instrument Inc). In all immunocytochemistry controls, reaction to the substrate was absent if the primary antibody was omitted or if the primary antibody was replaced by a non-immune, control antibody. Serial paraffin or frozen sections were also stained with cresyl violet acetate for histological analysis.

Western Blot Analysis

At appropriate times after treatment, segments of spinal cord (5 mm) were isolated using the lesion site as the epicenter. The experiments described were all performed at 4°C. The tissues were resuspended in a lysis buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 100 mM EDTA, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL leupeptin and aprotinin] and homogenized in a Dounce homogenizer. Tissue homogenate was centrifuged at $14{,}000 \times g$ for 30 min at 4°C, and the supernatant was centrifuged further at $70{,}000 \times$ g for 1 h at 4°C. Protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, IL). For analyses by Western blot, 100 μg of protein were resolved in 10% or 12.5% SDS-PAGE, and the proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Blots were blocked with 5% nonfat dry milk in TBS for 1 h at room temperature and then the membranes were incubated with antibodies against TNF-α (1:100) or iNOS (1:100) overnight at 4°C. The membranes were then processed with HRP-conjugated anti-rabbit secondary antibody (Pierce, Rockford, IL). Immunoreactive bands were visualized by chemiluminescence using Supersignal (Pierce, Rockford, IL).

NO Measurement

The spinal cord tissues were isolated and processed as described above. The tissues were resuspended in homogenizing buffer (10 mM PBS, pH 7.5, 250 mM sucrose, 0.5 mM PMSF, 10 μg/mL leupeptin and aprotinin) and homogenized in a Dounce homogenizer. The homogenate was centrifuged at $14{,}000 \times g$ for 10 min and the supernatant was used for nitrate assay. For measurement of NO (defined as $NO_2^- + NO_3^-$ within equal amounts of spinal cord extracts), we used the NO-specific NO/ozone chemiluminescence technique with an Antek nitrate/nitrite reduction assembly and NO analyzer (models 745 and 7020, respectively; Antek Instruments, Inc., Houston, TX). This method was previously described in detail (Kim et al., 1995). In brief, NO is a very unstable compound in a biological system and is readily oxidized to nitrite within a short time. To overcome this difficulty, the NO analyzer detected the concentration of nitrite/nitrate compounds in the homogenate using V^{3+} to chemically reduce the NO_x, the stable end-product of NO. Free NO was reacted with ozone ($NO + O_3 \rightarrow NO^{\cdot}{}_2 + O_2$; $NO^{\cdot}{}_2 \rightarrow NO_2 + h\nu$) and then detected in a chemiluminescence spectrometer with $NaNO_2$ used as the reference standard. The measurement of NO was performed in three independent experiments and expressed as mean ± SD.

DNA Laddering

The spinal cord tissues were isolated and processed as described above. Genomic DNA was isolated with a Quiagen genomic DNA kit (Quiagen, Germany) according to the manufacturer's instructions. In brief, the tissues were homogenized and incubated for 4 h in 10 mL of lysis buffer [800 mM guanidine HCl, 30 mM Tris-HCl (pH 8.0), 30 mM EDTA, 5% Tween-20, 0.5% Triton X-100] with 5 mg of proteinase K (Sigma, St. Louis, MO) at 50°C. After incubation, genomic DNA was eluted with Quiagen genomic-tip 100/G and precipitated by adding 0.7 volumes of isopropanol at room temperature. The DNA was recovered by centrifugation at 7,000 × *g*

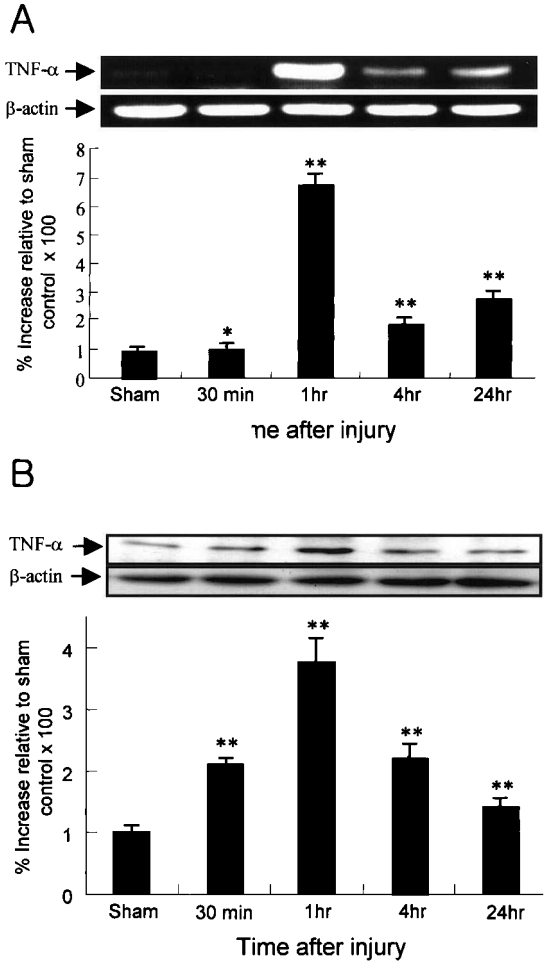

FIG. 1. Time-dependent changes of TNF-α in the spinal cord after injury. (**A**) RT-PCR analysis of TNF-α mRNA after SCI. Note that TNF-α mRNA peaked 1 h after injury and decreased thereafter. (**B**) Western blot analysis of TNF-α protein after SCI. Note that TNF-α protein (17 kDa) reached its peak 1 h after injury and declined thereafter. (**Top panels**) Samples from sham control and injured spinal cords were prepared for RT-PCR and Western blotting at 30 min, 1 h, 4 h, and 24 h after SCI. The gels presented are representative of results from three separate experiments. (**Bottom panels**) Densitometry readings of gel bands were expressed as arbitrary units; data were converted to percentages as compared to the arbitrary densitometric units of the sham control. Values are mean ± SD of three separate experiments. $*p < 0.005$, $**p < 0.001$.

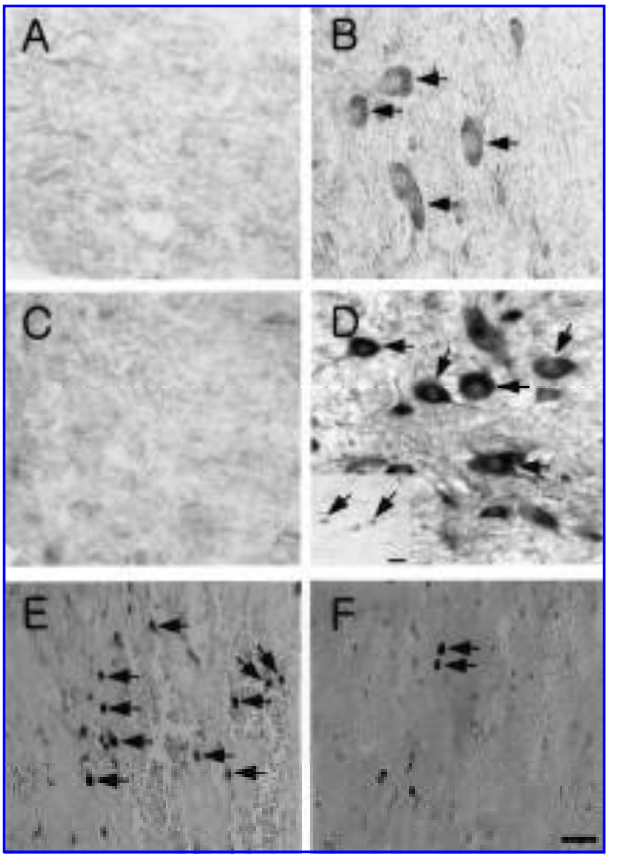

FIG. 2. Immunocytochemical detection of TNF-α, iNOS, and TUNEL labeling after spinal cord injury. (**A**) TNF-α immunoreactivity in the sham-operated spinal cord. Note that TNF-α immunoreactivity was not detected in sham-operated spinal cords. (**B**) TNF-α immunoreactivity 1 h after spinal cord injury. Note that TNF-α immunoreactivity was detected in neurons (arrows) in the gray matter. (**C**) Inducible NOS immunoreactivity in the sham-operated spinal cord. Note that no iNOS immunoreactivity was observed in sham-operated spinal cords. (**D**) Inducible NOS immunoreactivity 4 h after spinal cord injury. Note that intense iNOS immunoreactivity was observed in neurons (arrows) in the gray matter. (**Insert**) Inducible NOS-positive presumptive glial cells (arrows) in the white matter. (**E**) TUNEL-positive cells (arrows) 24 h after spinal cord injury treated with non-immune rabbit serum (2 μL, vehicle control). (**F**) TUNEL-positive cells (arrows) 24 h after spinal cord injury treated with antibodies neutralizing TNF-α (2 μL). Note the significant reduction in the number of TUNEL-positive cells as compared to Figure 1E. Bar = 20 μm.

for 30 min at 4°C and washed twice with 75% EtOH. The DNA pellet was dried and resuspended in 50 μL of 10 mM Tris-HCl (pH 8.5). Genomic DNA was labeled as described by Yakovlev et al. (1997), with slight modifications. After quantification of DNA by spectrophotometer (Perkin-Elmer, 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 drying the gel, radiolabeled DNA in the dried gel was detected by exposure to x-ray film. Experiments were repeated three times to ensure reproducibility.

RNA Purification and RT-PCR

The spinal cord tissues were isolated and processed as described above. RNA was purified using RNA STAT-60 Reagent (TEL-TEST, Inc., Friendswood, TX) 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 SuperScript II RNase H-reverse transcriptase (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. A 20-μL PCR reaction contained 2 μL first strand cDNA, 0.6 U 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. Samples were subjected to 25–30 cycles of 94°C for 30 sec, 52–57°C for 30 sec, and 72°C for 30 sec on a thermocycler (Perkin-Elmer, Emeryville, CA). The primers used for this experiment were designed according to the sequences previously reported for TNF-α (Estler et al., 1992), iNOS (Galea et al., 1994), and β-actin (Nudel et al., 1983) cDNA. The Primer sequences were as follows: TNF-α forward, CCCAGACCCTCACACTCAGAT; TNF-α reverse, TTGTCCCTTGAAGAGAACCTG; iNOS forward, CTCCATGACTCTCAGCACAGAG; iNOS reverse GCACCGAAGATATCCTCATGAT; β-actin forward, ATTTGGCACCACACTTTCTACA; and β-actin reverse, TCACGCACGATTTCCCTCTCAG. Negative controls included PCR reactions lacking primers or reverse transcriptase. After amplification, RT-PCR products were separated on a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide. The amplified cDNA fragments were visualized under ultraviolet light. Densitometry readings of gel bands (as well as those of Western blots) were performed using a Chemiimager™4400 (Alpha Innotech Co, San Leandro, CA). Experiments were repeated three times and the values obtained for the relative intensity were subjected to statistical analysis.

Statistical Analysis

Data were evaluated for statistical significance using analysis of variance (ANOVA) with a computerized statistical package. The confidence level for statistical significance was set at a probability value of 0.05.

RESULTS

Morphological Features after Crush Injury

Within hours of an acute SCI, a hemorrhagic zone appeared within the gray matter of the injured spinal cord. Cell loss, resulting from necrosis and caused primarily by the initial mechanical insult, formed a lesion whose center had characteristic features including infiltration with RBCs and blood-borne mononuclear phagocytes. Histological analysis with cresyl violet revealed that the loss of neuronal Nissl staining was evident within the epicenter of the lesion site with a breakdown of axonal segments in white matter. A well-defined area of injury increased with time; the size of this lesioned area was reproducible after crush injury For example, by 2 days after crush, the injured region extended longitudinally an average of 3.1 ± 0.1 mm, and extended transversely an average of 1.7 ± 0.1 mm (Du et al., 1999; Lee et al., 2000).

Rapid Expression of TNF-α after Injury

To investigate the expression of TNF-α after crush injury, we examined the temporal expression of TNF-α mRNA and protein at pre-defined time intervals (30 min, 1 h, 4 h, and 24 h after SCI; $n = 8$ for each time point). RT-PCR analysis revealed that TNF-α mRNA peaked 1 h after injury and decreased rapidly thereafter (Fig. 1A). Western blot analysis also showed an increase in TNF-α after SCI (Fig. 1B). TNF-α protein reached its peak at 1 h after injury and declined thereafter. Low levels of TNF-α mRNA and protein were detected in the unoperated, normal or sham controls (Fig. 1A,B). Also, immunostaining revealed that as early 1–2 hr after injury, TNF-α immunoreactivity was observed in neurons within the gray matter in the lesion area—even in those cells located several mm from the lesion both rostrally and caudally (Fig.

2B). Four to eight hr after injury, TNF-α-positive presumptive glial cells were also seen in the white matter in the lesion area (data not shown). Little to no TNF-α immunoreactivity was seen in the uninjured sham-operated spinal cords (Fig. 2A). To authenticate that the type of TNF-α-positive cells in the gray matter were neurons, double staining was performed. As shown in Figure 3, TNF-α-positive cells in the gray matter were co-localized with NeuN, a marker for neurons (Fig. 3A–D).

Induction of iNOS after Injury

To investigate the expression of iNOS after injury, we examined the temporal profiles of expression for iNOS by immunocytochemistry, Western blot and RT-PCR analyses. Both injured and sham-operated spinal cords were evaluated for iNOS expression at four pre-defined time points (30 min, 1 h, 4 h, and 24 h after SCI; $n = 8$ for each time point). One to two hr after injury, weak iNOS immunoreactivity was first detected in a few neurons within the gray matter of the lesioned area. Intense immunoreactivity to iNOS was seen in neurons in the gray matter by 4 h after injury (Fig. 2D); this immunoreactivity decreased thereafter. As expected, no iNOS immunoreactivity was observed in sham-operated spinal cords (Fig. 2C). Double staining also revealed that iNOS-positive cells in the gray matter were co-localized either with a neuronal marker, NeuN (Fig. 3E–H) or OX-42, a marker for microglia (Fig. 4D–F). Inducible NOS-positive cells in the white matter were co-localized with APC, a marker for oligodendrocytes (Fig. 4A–C). In addition some iNOS-positive cells in the gray matter were co-localized with TUNEL staining (Fig. 4G–I). As shown in Figure 5, Western blot and RT-PCR analyses also showed that iNOS expression was up-regulated after SCI. Inducible NOS mRNA and protein were detectable by 1 h and peaked 4 h post-injury. The levels of iNOS mRNA and protein decreased thereafter.

Production of NO after Injury

To examine the production of NO after SCI, we quantitated the levels of NO in the injured spinal cords at predetermined times (30 min, 1 h, 4 h, and 24 h after injury; $n = 6$ for each time point). As shown in Figure 6A, the level of NO increased gradually after injury and peaked at 4 h; there was a threefold increase over the level in sham controls. NO levels decreased thereafter and reached the level of sham-operated controls within 24 h after injury.

Inhibition of iNOS Expression and NO Production by Neutralizing Antibodies to TNF-α

To demonstrate the TNF-α-induced expression of iNOS and the subsequent increased production of NO after crush injury, we injected neutralizing antibodies to TNF-α into the spinal cords after SCI ($n = 6$). As shown in Figure 7, the induction of iNOS mRNA and protein were inhibited significantly by treatment with neutralizing antibodies to TNF-α. NO production also decreased significantly in response to treatment with neutralizing antibodies to TNF-α as compared with the production in the vehicle control (Fig. 6B). Neutralizing antibodies to TNF-α from either R&D or Genzyme produced the same relative effects on iNOS expression and NO production. Furthermore, treatment with such inhibitors of NOS as L-NMMA and SMT, and with the NO scavenger, carboxy-PTIO, also reduced NO production significantly as compared to the vehicle control (Fig. 6B).

Inhibition of Apoptosis by Neutralizing Antibodies to TNF-α after Injury

A few TUNEL-positive cells were initially present in the lesion area of SCI within 4 h after injury, with the largest number being present 24–48 h after injury (Fig. 2E). To demonstrate apoptosis induced by TNF-α after SCI, we injected neutralizing antibodies to TNF-α into the spinal cords after injury and assessed apoptotic cell death by TUNEL staining and DNA gel analyses 24 h later. By treating the injured spinal cord with an antibody that neutralized TNF-α, we observed a marked reduction in the number of TUNEL-positive cells in the cord as compared to those found in vehicle controls treated only with non-immune serum (Fig. 2E,F). For example, when counted the number of TUNEL-positive cells, treatment with neutralizing antibodies to TNF-α significantly reduced the positively labeled cells 24 h after injury by 42% (Fig. 8A). Also, DNA gel electrophoresis revealed a marked decrease in DNA laddering by treatment with neutralizing antibodies to TNF-α (Fig. 8B). In addition, treatment with either of the NOS inhibitor, SMT or the NO scavenger, carboxy-PTIO after SCI also reduced DNA laddering (Fig. 8B).

DISCUSSION

This report provides evidence that TNF-α serves as an external signal triggering apoptosis in spinal cords after SCI. Our results suggest, furthermore, that the apoptotic cell death induced by TNF-α is mediated, in part, by the subsequent increased NO production. Furthermore, this moiety appears to be produced enzymatically by iNOS, which itself is likely expressed in response to TNF-α. Expression of TNF-α was upregulated rapidly in the spinal cord as early as 1 h after injury. Expression of iNOS and an increase in the level of NO in the spinal cord both

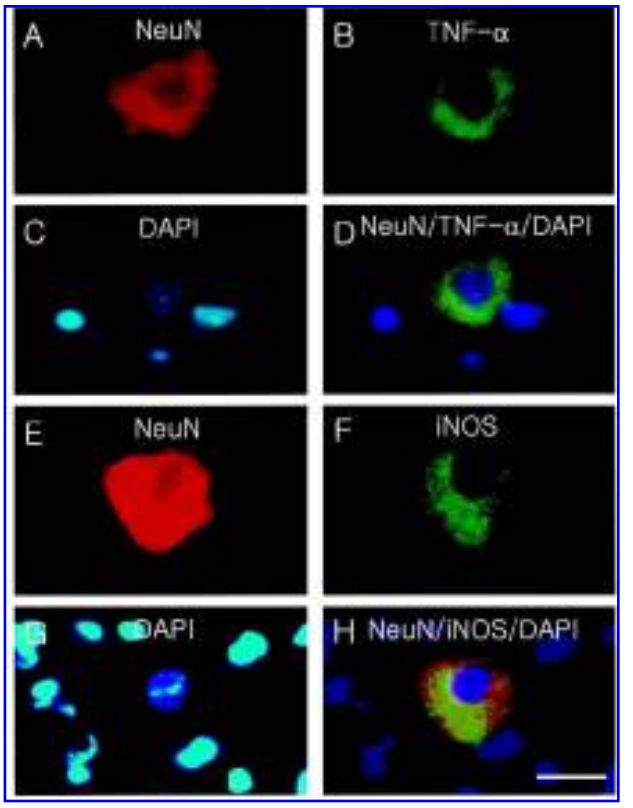

FIG. 3. Double immunostaining for NeuN with TNF-α or iNOS. A NeuN-positive neuron (**A**) in the gray matter was co-localized with TNF-α (**B**) and labeled its nucleus with DAPI (**C**) 1 h after SCI (**D,** merged image). A NeuN-positive neuron (**E**) in the gray matter also was co-localized with iNOS (**F**) and labeled its nucleus with DAPI (**G**) 4 h after SCI (**H,** merged image). No TNF-α or iNOS immunoreactivity was observed NeuN-positive neurons in sham-operated spinal cords. Bar = 20 μm.

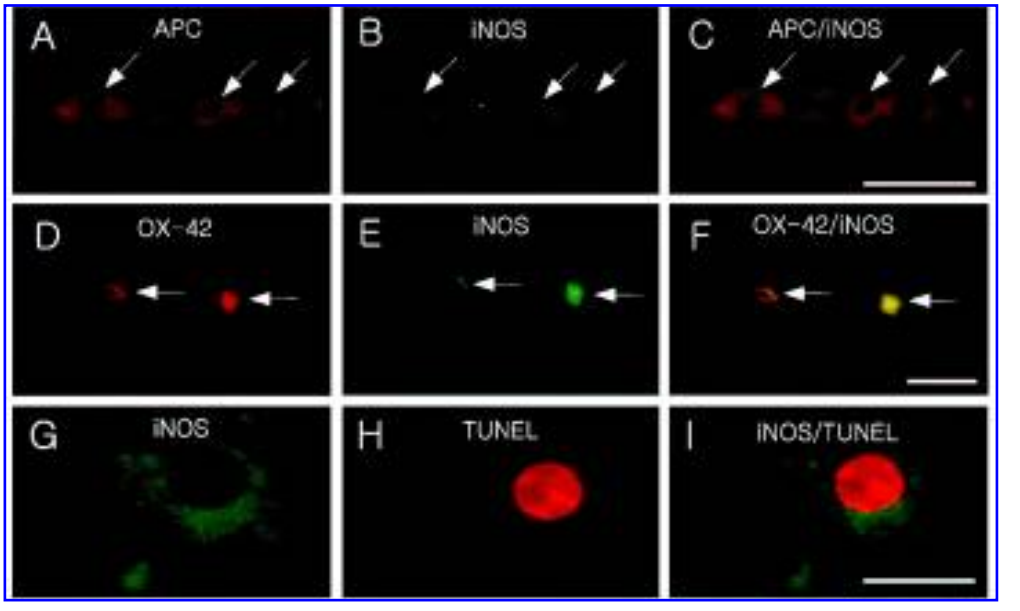

FIG. 4. Double immunostaining for iNOS with specific glial markers or TUNEL. APC-positive oligodentrocytes (**A,** arrows) in the white matter were co-localized with iNOS (**B,** arrows) 4 h after SCI (C, merged image). Also, OX-42–positive microglia (**D,** arrows) in the gray matter were co-localized with iNOS (**E,** arrows) 4 h after SCI (**F,** merged image). An iNOS-positive presumptive neuron (**G**) in the gray matter was co-localized with TUNEL (**H**) 24 h after SCI (**I,** merged image). No iNOS immunoreactivity was observed in glial cells in sham-operated spinal cords. Bar = 20 μm.

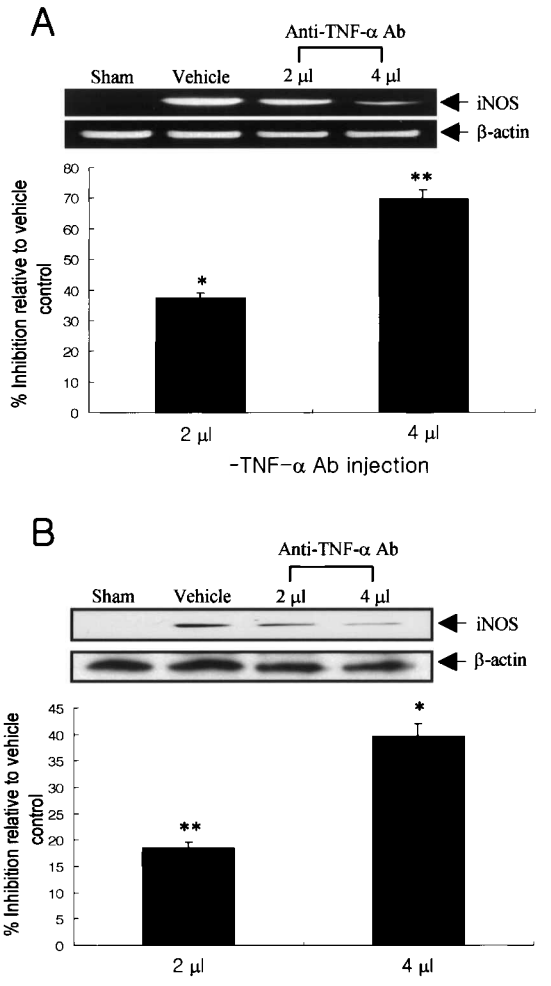

FIG. 5. Time-dependent changes of iNOS after spinal cord injury. (**A**) RT-PCR analysis of iNOS mRNA after SCI. (**B**) Western blot analysis of iNOS protein after SCI. Note that both iNOS mRNA and protein peaked 4 hr after SCI. (**Top panels**) Samples from sham control and injured spinal cords were prepared for RT-PCR and Western blotting at 30 min, 1 h, 4 h, and 24 h after SCI. The gels presented are representative of results from three separate experiments. (**Bottom panels**) Densitometry readings of gel bands were expressed as arbitrary units; data were converted to percentages as compared to the arbitrary densitometric units of the sham control. Values are mean ±SD of three separate experiments. $*p < 0.005$, $**p < 0.001$.

peaked 4 h after injury at a time when a few TUNEL-positive apoptotic cells were first being observed in the lesion area. The peak of apoptotic cells was observed 24–48 h after crush injury. Neutralizing TNF-α by means of specific antibodies significantly reduced the expression of iNOS and the level of NO, the number of apoptotic cells and the degree of DNA laddering after injury. Our results thus suggest an apoptotic signaling pathway after SCI mediated, in part, by TNF-α. However, the intracellular signaling events initiated by TNF-α that lead to apoptotic cell death are largely unknown. In this regard, Springer el al. (1999) showed that upstream and

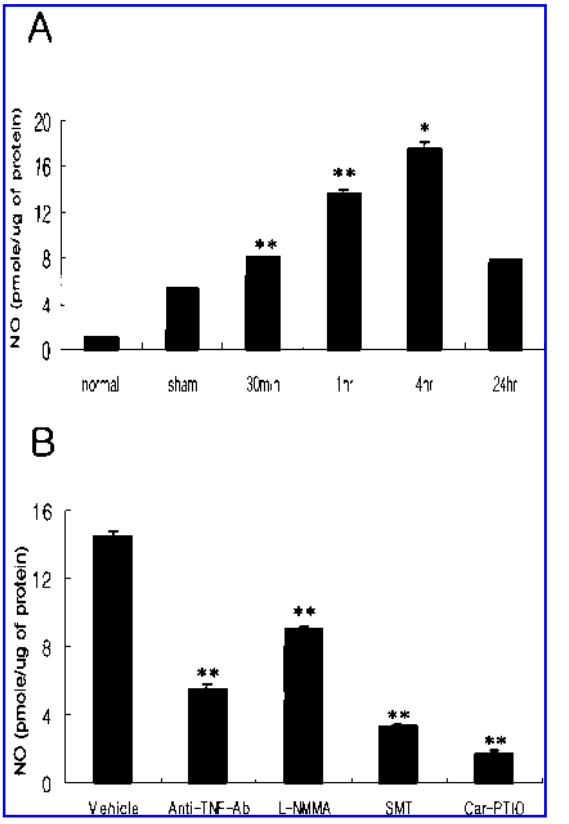

FIG. 6. Production of NO and effects of pharmacological treatments on the level of NO after spinal cord injury. (**A**) Time-dependent changes of NO levels after SCI. Samples from sham control and injured spinal cords were prepared for NO measurement at 30 min, 1 h, 4 h, and 24 h after SCI. Note that the level of NO increased gradually, peaked 4 h after injury and decreased thereafter. Data are the means ± SD of three separate experiments. $*p < 0.005$, $**p < 0.001$. (**B**) Effects of pharmacological agents on the level of NO after spinal cord injury. Immediately after crush, 2 μL of various pharmacological reagents were injected directly into the spinal cord at the lesion's epicenter as described in the Materials and Methods. Each group ($n = 8$) was injected with vehicle solution (normal saline or normal rabbit serum), rabbit neutralizing antibody against TNF-α, L-NMMA (25 mg/mL in saline), SMT (50 mg/mL in saline), or carboxy-PTIO (25 mg/mL in saline). Samples from vehicle or from spinal cords treated with pharmacological agents were prepared for the determination of NO levels 4 h after SCI. Equal amounts of spinal cord extracts were used to determine the production of NO. Note that the level of NO production decreased significantly in response to treatment with various pharmacological agents as compared to that of the vehicle control. Data are the means ± SD of three separate experiments. $**p < 0.001$.

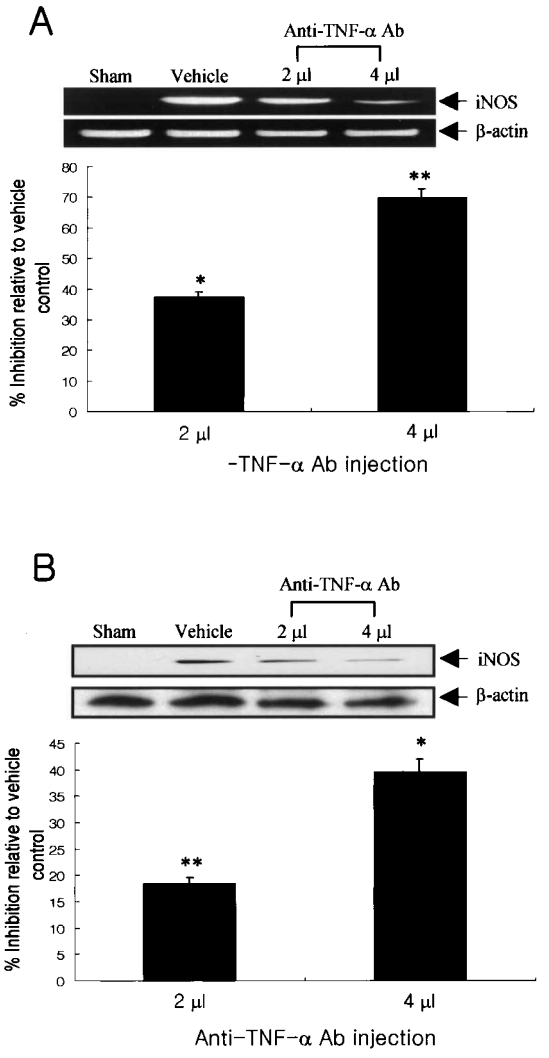

FIG. 7. Effects of neutralizing antibodies against TNF-α on expressions of iNOS mRNA and protein after spinal cord injury. Vehicle or antibodies to TNF-α were injected into the lesion sites immediately after crush and spinal cord tissues were then collected 4 h post-injury. RT-PCR and Western blot analyses were performed as described in the Materials and Methods. (**A**) RT-PCR analysis of iNOS mRNA after SCI. (**B**) Western blot analysis of iNOS protein after SCI. (**Top panels**) *Sham*, Sham control. *Vehicle*, vehicle control that had received a spinal cord crush and was treated with 2 μL of a non-immune rabbit serum; *Anti-TNF-α Ab*, neutralized TNF-α that had received a spinal crush and were treated with 2 μL or 4 μL of a rabbit antibody that neutralized TNF-α. The gels presented are representative of results from three separate experiments. (**Bottom panels**) Densitometry readings of gel bands were expressed as arbitrary units; data were converted to percentages as compared to the arbitrary densitometric units of the vehicle control. Note that treatment with antibodies neutralizing TNF-α after SCI reduced significantly the expression of iNOS mRNA and protein. Values are mean ± SD of three separate experiments. $*p < 0.005$, $**p < 0.001$.

downstream components of the caspase-3 apoptotic pathway are activated after SCI in the rat. Furthermore, a recent study by Zhao et al. (2001) suggested that TNF-α–induced apoptosis may be mediated in part by caspase-3 activation in primary septo-hippocampal cultures.

It is well known that TNF-α expression is upregulated after traumatic brain and spinal cord injury (Hayashi et al., 2000; Shohami et al., 1994; Streit et al., 1998; Taupin et al., 1993; Wang et al., 1996; Wang et al., 2002; Yan et al., 2001). The source of this TNF-α in injured spinal cord was not clear, however. It is likely that microglia, reactive astrocytes, neurons, endothelial cells, and infiltrating macrophages may all produce such inflammatory cytokines as TNF-α after injury (Benveniste, 1992; Eng et al., 1992; Knoblach et al., 1999; Lieberman et al., 1989; Morioka et al., 1991; Tchelingerian et al., 1994; Yan et al., 2001). Our results also demonstrated TNF-α immunoreactivity in neurons and presumptive glial cells after SCI. Although the function of TNF-α remains unclear, several studies of multiple sclerosis (MS) and its animal model, experimental allergic encephalitis (EAE), indicate that TNF-α may be involved in inflammatory CNS demyelination (Sharief and Hentges, 1991; Ruddle et al., 1990; Selmaj et al., 1991). Although we have not assessed the role of TNF-α in the development of demyelination in the late phase of SCI, TNF-α is known to induce apoptosis of neurons and oligodendrocytes (Akassoglou et al., 1998; D'Souza et al., 1995; Sipe et al., 1996). The present experiments also demonstrate an important role for TNF-α in apoptosis in the early phase of SCI. Furthermore, based on the observation of Figures 3 and 4, this is likely to be mediated in part by NO via upregulation of iNOS induced in response to TNF-α. As such, an early and rapid production of TNF-α after injury appears to be deleterious to the CNS by triggering apoptosis. Although the present study did not determine neuonal NOS (nNOS) expression after SCI, we cannot completely rule out a potential role of nNOS in apoptosis since it is a well-known mediator of cell death after CNS injury (Farooque et al., 2001; Sasaki et al., 2002). On the other hand, TNF-α may also exert beneficial effects on the CNS after injury. For example, TNF-α protects against amyloid-β peptide-induced neurotoxicity and induces synthesis of neuronal growth factors *in vitro* (Barger et al., 1995; Gadient et al., 1990). TNF-α also upregulates calbindin and manganese superoxide dismutase (MnSOD) (Bruce et al., 1996; Matton et al., 1995) and promotes regeneration of injured CNS axons *in vivo* (Schwartz et al., 1991). Furthermore, Kim et al. (2001) showed that deletion of TNF-α receptors in mice reduces NF-κB activation and cellular inhibitor of apoptosis protein 2 expression, and increases the active form of caspase-3 protein and the numbers of apoptotic cells after SCI. Therefore, it seems

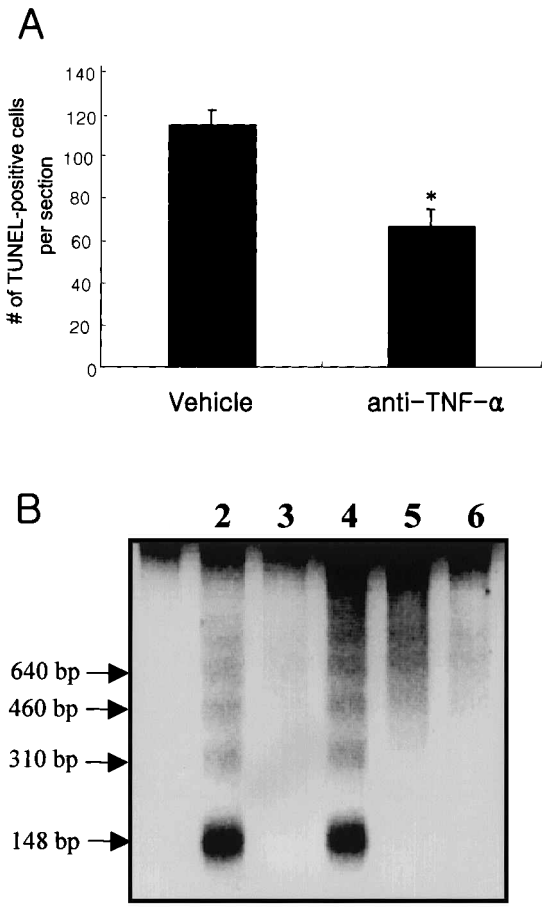

FIG. 8. Effect of neutralizing antibodies against TNF-α on apoptotic cell death after spinal cord injury. Vehicle or TNF-α antibodies (2 μL) were injected into the lesion sites immediately after crush and spinal cord tissues were then collected 24 h post-injury. Longitudinal sections were processed for TUNEL staining, and TUNEL-positive cells were counted as described in the Materials and Methods. (**A**) TUNEL-positive cells in injured spinal cords. Note that treatment with antibodies neutralizing TNF-α after SCI significantly reduced the number of TNUEL-positive cells as compared to the positive cells seen in the vehicle control. $*p < 0.001$. (**B**) DNA gel electrophoresis after SCI. DNA was isolated from the tissues, labeled at the 3′ end with [^{32}P]deoxycytidine triphosphate (dCTP) and separated by gel electrophoresis as described in the Materials and Methods. *Lane 1*, untreated, sham-operated control; *lane 2*, treated with a non-immune rabbit serum (*vehicle control*); *lane 3*, treated with antibodies neutralizing TNF-α; *lane 4*, treated with saline (*vehicle control*); *lane 5*, treated with the NOS inhibitor, SMT; lane 6, treated with the NO scavenger, carboxy-PTIO. Note that DNA laddering decreased following treatment with antibodies neutralizing TNF-α, SMT or carboxy-PTIO after SCI when compared to the laddering seen in the vehicle control. The gel presented is representative of results from three separate experiments.

likely that overproduction of TNF-α in the initial phase of injury may be detrimental to the CNS by activating apoptotic death signals whereas in the later stage of injury, TNF-α may activate components of cell survival pathways.

Liu et al. (1997) first provided the direct evidence for neuronal and glial apoptosis after SCI. In their study, multiple criteria for apoptosis after SCI were used: morphology under both light and electron microscopic examination, nuclear chromatin condensation authenticated by Hoechst 33342 and by TUNEL, histone-associated DNA fragmentation, and DNA laddering on gel electrophoresis. TUNEL-positive cells after SCI were identified as neurons and oligodendrocytes by double staining (Liu et al., 1997). In the present study, we assessed apoptosis after SCI by TUNEL staining and DNA laddering on gel electrophoresis. Four hr after injury, TUNEL-positive apoptotic cells were first observed in the lesion area after SCI. Thereafter, the number of TUNEL-positive cells in the gray matter peaked at 24–48 h after injury. Although the present experiments have not employed double labeling using TUNEL and cell type-specific markers, our previous study demonstrated that many TUNEL-positive cells were neurons and oligodendrocytes (Lee et al., 2000). In addition neurons, glial cells, endothelial cells and inflammatory cells are also known to undergo apoptosis after SCI (Liu et al., 1997; Satake et al., 2000; Yan et al., 2001). Furthermore, treatments with a neutralizing antibody against TNF-α after SCI significantly reduced the gene expression of iNOS, the level of NO and the number of TUNEL-positive cells, furthermore. DNA gel electrophoresis also revealed a decrease in DNA laddering by treatment with neutralizing antibodies to TNF-α, NOS inhibitors, or the NO scavenger after SCI (Fig. 8). These results suggest that apoptosis is induced by TNF-α via induction of iNOS expression following NO production after SCI. However, Cohen et al. (1996) reported that infusion of L-NMMA, an inhibitor of NOS, after SCI shows no beneficial effects on the degree or rate of secondary loss of spinal cord function and on the cross-sectional area of surviving white matter. Nevertheless, Bethea et al. (1998) showed that iNOS immunoreactivity is co-localized with activated NF-κB in neurons after traumatic SCI. This further suggests that the activation of this transcription factor may play an important role in regulating apoptotic programs in neurons.

While our results shed further light on the pathways involved in apoptosis after SCI, it is now evident that both necrosis and apoptosis contribute to the loss of neurons and glial cells occurring after this form of trauma. Although the molecular mechanisms underlying cell

death following injury is fairly well known (Ashkenazi and Dixit, 1998), many important questions related to cell death after SCI still remain to be answered. For example, the intracellular signaling events initiated by external apoptotic signals such as TNF-α are largely unknown. Even the criteria for definitively distinguishing apoptosis from necrosis after SCI are not fully established. In this regard, recent evidence indicates that apoptosis and necrosis may not be mutually exclusive forms of cell death, as once thought but may occur in parallel within a single cell (Ankarcrona, 1998; Nakajima et al., 2000; Zipfel et al., 2000). It is well known that apoptosis during neuronal development and neurodegeneration requires *de novo* RNA synthesis. However, the time course and the regulation of arrays of target genes after SCI are not well characterized. By using a cDNA array analysis, Chiang et al. (2001) have demonstrated that many known pro- and anti-apoptotic proteins are coordinately regulated, including many transcription factors, receptors, Bcl-2 family members, and caspases. Therefore, it might be that in order to fully comprehend cell death after SCI, it is necessary to identify the genes controlling cell death (as for example, Chiang et al., 2001) and to understand the mechanisms by which these genetic interactions produce their effects. This approach may best lead to therapeutic intervention(s) designed to reduce cell death and functional deficits recovery after SCI in the adult.

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