Experimental Neurology **166,** 190–195 (2000)
doi:10.1006/exnr.2000.7494, available online at http://www.idealibrary.com on IDEAL®

BRIEF COMMUNICATION

Role of Tumor Necrosis Factor-α in Neuronal and Glial Apoptosis after Spinal Cord Injury

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Received October 18, 1999; accepted May 24, 2000

We investigated the role of tumor necrosis factor (TNF)-α in the onset of neuronal and glial apoptosis after traumatic spinal cord crush injury in rats. A few TUNEL-positive cells were first observed within and surrounding the lesion area 4 h after injury, with the largest number observed 24–48 h after injury. Double-labeling of cells using cell type-specific markers revealed that TUNEL-positive cells were either neurons or oligodendrocytes. One hour after injury, an intense immunoreactivity to TNF-α was observed in neurons and glial cells in the lesion area, but also seen in cells several mm from the lesion site rostrally and caudally. The level of nitric oxide (NO) also significantly increased in the spinal cord 4 h after injury. The injection of a neutralizing antibody against TNF-α into the lesion site several min after injury significantly reduced both the level of NO observed 4 h thereafter as well as the number of apoptotic cells observed 24 h after spinal cord trauma. An inhibitor of nitric oxide synthase (NOS), N^G-monomethyl-L-arginine acetate (L-NMMA), also reduced the number of apoptotic cells. This reduction of apoptotic cells was associated with a decrease in DNA laddering on agarose gel electrophoresis. These results suggest that: (i) TNF-α may function as an external signal initiating apoptosis in neurons and oligodendrocytes after spinal cord injury; and (ii) TNF-α-initiated apoptosis may be mediated in part by NO as produced by a NOS expressed in response to TNF-α. © 2000 Academic Press

Key Words: **TNF-α neutralizing antibody; TUNEL; NO; NOS; L-NMMA; DNA laddering.**

Trauma to the spinal cord induces immediate and massive *necrosis* of neurons and glial cells. Recent studies show that *apoptosis* plays an important role in the prolonged loss of cells within the spinal cord subsequent to the injury (1–4). The external factors initiating apoptosis in the injured spinal cord have not been identified, however. The intracellular signaling events leading to the apoptotic cell death are also unknown. It is known, however, that TNF-α accumulates rapidly at a lesion site within the CNS, and this accumulation is likely to be produced by microglia, astrocytes, neurons, and infiltrating macrophages (5–9). TNF-α is a pleiotrophic proinflammatory cytokine that elicits diverse biological actions, including the induction of apoptosis (10). TNF-α can induce apoptosis of oligodendrocytes and in a neuronal cell line *in vitro* (11, 12); apoptosis of oligodendrocytes *in vivo* was induced by the overexpression of TNF-α receptor (TNFR1) (13). The discovery and studies of a "death domain" in the TNFR1 and in other related receptors has revealed information on the signaling pathways leading to the activation of caspase-8 and caspase-3, prior to apoptosis (14). It seems reasonable to suggest, therefore, that following a traumatic insult to the spinal cord, an accumulation of TNF-α may act to initiate an apoptotic cascade via receptor-mediated signaling. In fact, TNF-α has been proposed as a hypothetical mediator of oligodendrocyte apoptosis (3). Furthermore, a recent report shows that administration of interleukin-10, a potent anti-inflammatory cytokine, reduces TNF-α production in the spinal cord thereby promoting functional recovery following spinal cord injury (15). In the present report, we used an acute *in vivo* crush model of spinal cord injury and examined the relationship between expression of TNF-α and the appearance of apoptosis in cells of the injured cord. Our results demonstrate that after spinal cord injury in rats, TNF-α may mediate apoptotic cell death of neurons and oligodendrocytes in part by NO through the induction of NOS.

Traumatic injury was induced by crushing the spinal cord of adult rats (male; Sprague–Dawley; 300–350 g) extradurally with a No. 5 Dumont tweezer at the level of T5 as described previously (16). All animal proce-

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dures were approved by the Institutional Animal Care and Use Committee of the University of Maryland at Baltimore. Randomized groups of 4 rats (48 rats, total) were killed 5 min, 30 min, 1, 2, 4, 8, 12, 16, 24, 48 h, 1 week, and 2 weeks after the crush injury and processed for perfusion with 4% paraformaldehyde. Uninjured, sham-operated rats ($n = 4$) served as controls. A 20-mm section of the spinal cord, centered at the lesion site, was dissected out and postfixed by immersion in 4% paraformaldehyde overnight and embedded in paraffin as described (16). Longitudinal sections (10 μm) were used for TUNEL staining using an Apoptag peroxidase kit (Oncor, Gaithersburg, MD) and counterstained with methyl green. Diaminobenzidine (DAB) served as the substrate for peroxidase. For double-labeling, serial paraffin sections were first subjected to TUNEL labeling using DAB to visualize TUNEL-positive nuclei. The sections were then processed for immunocytochemistry with: (i) a polyclonal antibody directed against neuron-specific enolase (NSE; Dako, Carpinteria, CA); (ii) a monoclonal antibody directed against the oligodendrocyte-specific antigen, APC (17; Oncogene, Cambridge, MA); or (iii) a polyclonal antibody directed against bovine glial fibrillary acidic protein (GFAP; Dako, Carpinteria, CA), which is specific for astrocytes. The ABC method was used to detect cells labeled specifically using a Vectastain kit (Vector Laboratories, Burlingame, CA). To visualize the various cell markers, a tetramethylbenzidine (TMB) substrate kit (Vector Laboratories, Burlingame, CA) was used as a substrate for peroxidase. Serial paraffin sections were processed for immunocytochemistry for TNF-α using a rabbit antiserum to mouse TNF-α (Genzyme, Cambridge, MA), and were also stained with cresyl violet acetate for histological analysis.

To assess pharmacological treatments, 24 rats were assigned to one of 4 experimental groups: untreated, sham-operated controls ($n = 6$) that received neither spinal cord injury nor any pharmacological treatment; vehicle controls that had a spinal cord crush and received 2 μl of a non-immune rabbit serum ($n = 6$); TNF-α-neutralized that had a spinal cord crush and received 2 μl of a rabbit neutralizing antiserum to mouse TNF-α (Genzyme, Cambridge, MA; $n = 6$); and NOS inhibitor-treated that had a spinal cord crush and received 2 μl of the NOS inhibitor, L-NMMA (25 mg/ml in saline; RBI, Natick, MA; $n = 6$). Treatment was given by injecting the agents directly into the center of the spinal cord lesion several min after the crush. In pilot experiments, we found that it was necessary to inject the minimal volume (2 μl) of vehicle or inhibitors to obtain consistent positive results. Rats were sacrificed 24 h after the treatments, and the tissue was processed for TUNEL labeling as described above. In a separate experiment, 40 rats were assigned to one of five experimental groups: four of the groups were identical to those used above for TUNEL studies; an additional group, sham-operated controls that received no spinal cord crush but received 2 μl of a nonimmune rabbit serum, was also added. A 5-mm segment of the spinal cord centered at the injury site was dissected following intracardiac perfusion under anesthesia with 200 ml cold saline 24 h after treatment. For each group, three spinal cords (for Western blot analysis of TNF-α; 18) and five spinal cords (for DNA gel electrophoresis; 19, 20) were pooled and processed, respectively. Polyclonal goat anti-mouse TNF-α antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a primary antibody for Western blots.

In another experiment, the spinal cords ($n = 3$) were isolated 30 min, 1, 4, and 24 h after injury and dissected as described above. Sham-operated animals ($n = 3$) with no spinal cord injury served as controls. The level of NO in spinal cord extracts was determined by NO-specific chemiluminescence as measured using an Antek nitrate/nitrite reduction assembly and NO analyzer (models 745 and 7020, respectively; Antek Instruments, Houston, TX) as described (21). To assess the effect of neutralizing antibodies to TNF-α on the level of NO, six rats were assigned to one of two experimental groups: vehicle controls that had a spinal cord crush + 2 μl of a nonimmune rabbit serum ($n = 3$); and TNF-α-neutralized that had a spinal cord crush + 2 μl of a rabbit neutralizing antiserum to mouse TNF-α (Genzyme, Cambridge, MA; $n = 3$). Rats were sacrificed 4 h after the treatments, and the tissue was processed for NO assay as described above.

TUNEL labeling and immunocytochemistry were assessed by a single investigator (THO) who was blind as to the experimental conditions; the quantitation of all data was accomplished by a second investigator (YBL). 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. Quantified data are expressed as mean ± SD. Student's t test was used for statistical analysis with a probability value of $P < 0.05$ considered to be significant. In all immunocytochemistry controls, reaction to DAB or TMB were absent if the primary antibody was omitted or if the primary antibody was replaced by a nonimmune, control antibody.

Acute spinal cord injury resulted in a massive loss of neurons and disruption of axons as previously revealed by histological analysis with cresyl violet (16). Cell loss, caused primarily by the initial mechanical insult and resulting *necrosis,* formed a lesion center with characteristic features including: absence of Nissl bodies, a hemorrhagic clot, infiltration with blood-borne phagocytes, and the disruption of ascending and descending axonal tracts (16). A well-defined area of the injured

region increased over time, and the size of this lesion 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 (mean ± SD, $n = 10$) (16). No TUNEL-positive cells were observed in uninjured sham-operated spinal cords (data not shown). Four hours after injury, a few TUNEL-positive cells were first observed in the lesion area (data not shown). By 16 h after injury, many TUNEL-positive cells were observed within the gray matter, but were restricted to the lesion area. The number of TUNEL-positive cells in the gray matter peaked at 24–48 h after injury (Fig. 1A). Most of these cells showed such morphological features as nuclear condensation/compartmentalization and shrinkage of the cytoplasm; both of these are recognized hallmarks of apoptosis. Double-labeling for a neuron-specific marker (NSE) indicated that many of these cells in the gray matter were neurons (Fig. 1D). A few TUNEL-positive presumptive glial cells were also observed beginning 4 h after injury. By 24 to 48 h after injury, several TUNEL-positive glial cells were observed within the lesion area and in the adjacent white matter (Fig. 1B). Double-labeling for glial cell-specific markers showed that TUNEL-positive cells in the white matter were oligodendrocytes (Fig. 1E); we were unable to identify TUNEL-positive astrocytes (data not shown). By 4 days after injury, TUNEL-positive cells within and surrounding the lesion area had almost disappeared, but reappeared again 3 days thereafter (data not shown). Agarose gel electrophoresis also showed an increase in DNA laddering 24 h after spinal cord injury (Fig. 2A).

Weak immunoreactivity to TNF-α was first seen in a few neurons within the gray matter of the lesion area as early as 5 and 30 min after the initial insult, however, little to no immunoreactivity was seen in the uninjured sham-operated spinal cords (data not shown). One hour after injury, intense immunoreactivity to TNF-α was observed in neurons and glial cells in the lesion area, even in those cells located several mm from the lesion area both rostrally and caudally (Fig. 1G). Immunoreactivity to TNF-α decreased gradually thereafter, but was still detectable for up to 1 week after injury (data not shown). Western blot analysis also showed an increase in TNF-α 24 h after spinal cord injury (Fig. 2B). The level of NO was also significantly increased in the spinal cord 4 h after injury (mean ± SD; *sham-operated* controls, 5.4 ± 0.1 pmol/μg protein, $n = 3$; *operated,* 17.5 ± 1.1 pmol/μg protein, $n = 3$, $P < 0.005$). Thereafter, the level of NO decreased to that of controls within 24 h after injury.

By treating the injured spinal cord with an antibody that neutralized TNF-α, we observed a significant reduction in the number of TUNEL-positive cells in both gray and white matter as compared to those found in *vehicle controls* treated only with nonimmune serum (Fig. 1C). For example, when expressed as a percentage of the vehicle control, treatment with antibody to TNF-α reduced TUNEL-positive cells 24 h after injury by about 89% (TUNEL-positive cells per slide, mean ± SD; *vehicle controls,* 42.2 ± 10.8, $n = 5$; *TNF-α-neutralized,* 4.8 ± 1.2, $n = 7$; $P < 0.01$). Treatment with antibodies to TNF-α also significantly reduced the level of NO in the spinal cord 4 h after injury (mean ± SD; *vehicle controls,* 14.2 ± 0.2 pmol/μg protein, $n = 3$; *TNF-α-neutralized,* 4.7 ± 0.2 pmol/μg protein, $n = 3$; $P < 0.01$). Treatment with a broad spectrum inhibitor of NOS, L-NMMA, also significantly reduced the number of apoptotic cells as compared to the *vehicle controls* by 42% 24 h after injury (TUNEL-positive cells per slide, mean ± SD; *NOS inhibitor-treated,* 24.3 ± 5.8, $n = 3$; $P < 0.005$) (see also Fig. 1F). Furthermore, agarose gel electrophoresis showed a decrease in DNA laddering following treatment with TNF-α neutralizing antibody or L-NMMA (Fig. 2A).

Our results appear to provide evidence that TNF-α serves as an external signal triggering apoptosis in neurons and oligodendrocytes after spinal cord injury. This peak of apoptosis was observed 24–48 h after injury. TNF-α appeared to lead to cellular apoptosis in part via NO produced by the induction of NOS after injury. Intense immunoreactivity to TNF-α was observed in cells as soon as 1 h after injury. The level of NO in the spinal cord peaked at 4 h after injury at the time a few apoptotic cells were first observed in the lesion area. Neutralization of TNF-α by means of selective antibodies reduced significantly both the number of apoptotic cells and the level of NO. Inhibition of NOS by means of L-NMMA also reduced the number of apoptotic cells. Furthermore, agarose gel electrophoresis demonstrated an increase in the degree of DNA laddering after injury, but a decrease in this laddering following treatment with a neutralizing antibody to TNF-α or L-NMMA.

It is known that TNF-α is upregulated after spinal cord injury (22, 23). The source of this TNF-α in injured spinal cord is not clear. It is clear that local cells residing in the injured cord—which include microglia, reactive astrocytes and neurons—may all produce such inflammatory cytokines as TNF-α (5–9). The inflammatory response in traumatic injury is further accelerated by the rapidly spreading necrosis of neurons and glial cells. While the function of TNF-α remains controversial, recent studies of multiple sclerosis (MS) and its animal model, experimental allergic encephalitis (EAE), suggest an important role for TNF-α in inflammatory CNS demyelination. For example, systemic treatment by neutralizing TNF-α antibodies prevented the progression of EAE (24, 25). Also, the level of TNF-α in cerebrospinal fluid correlates positively with MS severity (26). However, we have not evaluated the role of TNF-α and other proinflammatory cytokines

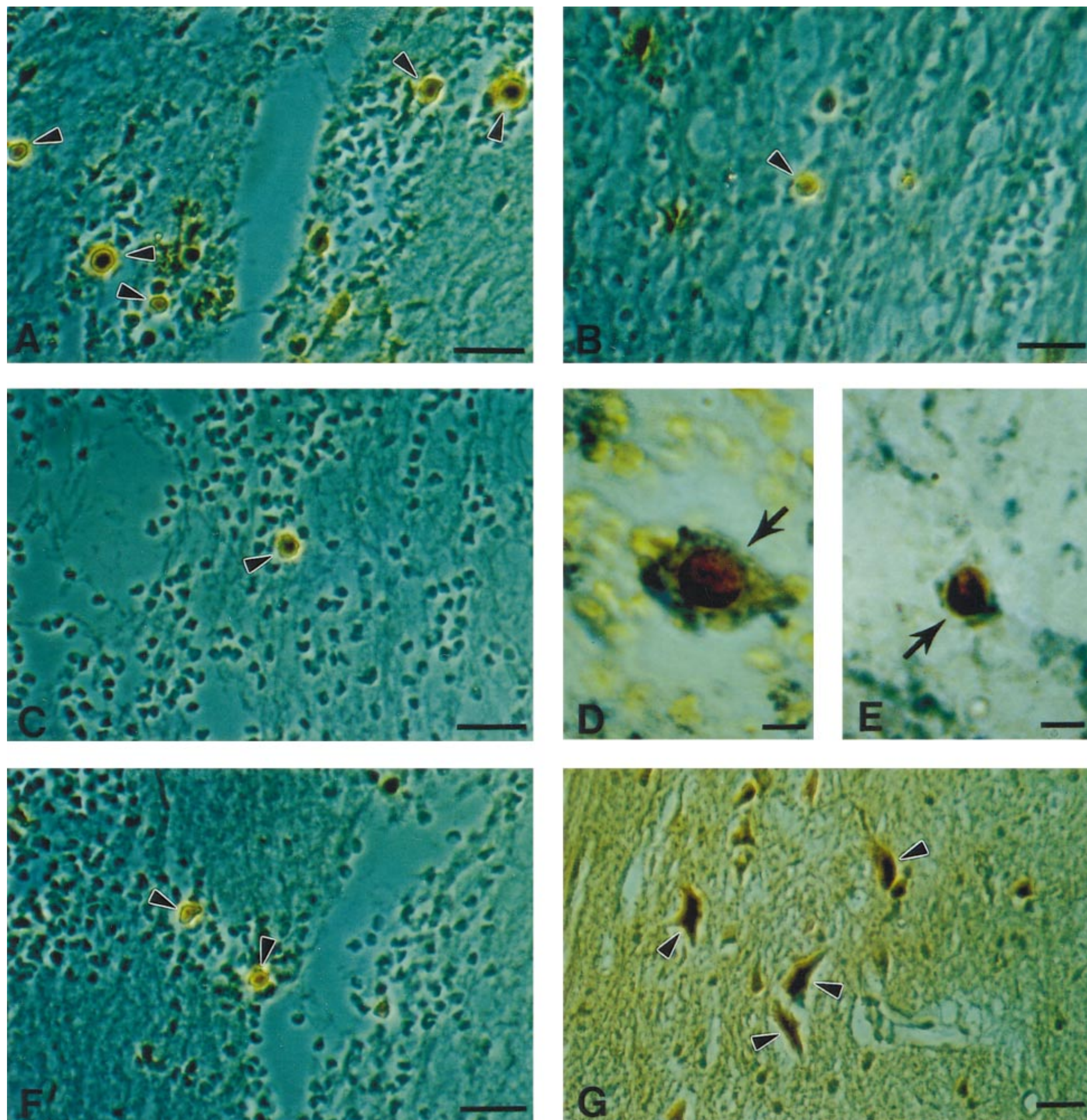

FIG. 1. TUNEL labeling and TNF-α immunoreactivity in the rat spinal cord after injury. For TUNEL labeling, phase-contrast micrographs were taken 24 h after crush injury from a region 1 mm caudal to the lesion site. (A) TUNEL-positive cells (arrowheads) in the gray matter of an injured spinal cord. Bar, 20 μm. (B) A TUNEL-positive glial cell (arrowhead) present in the white matter 24 h after injury. Bar, 20 μm. (C) A TUNEL-positive cell (arrowhead) 24 h after spinal cord injury treated with an antibody that neutralizes TNF-α. Note the significant reduction in the number of TUNEL-positive cells as compared to A. Bar, 20 μm. (D) Double-labeling of a TUNEL-positive cell (brown) with NSE, a specific neuronal marker (blue) (arrow). Bar, 5 μm. (E) Double-labeling of a TUNEL-positive cell (brown) with APC, a specific oligodendrocyte marker (blue) (arrow). Bar, 5 μm. (F) TUNEL-positive cells (arrowheads) 24 h after spinal cord injury treated with the inhibitor to NOS, L-NMMA. Note the significant reduction in the number of TUNEL-positive cells as compared to A. Bar, 20 μm. (G) TNF-α immunoreactivity 1 h after spinal cord injury. A phase-contrast photograph was taken from within an area 2 mm caudal to the lesion site. Note the intense immunoreactivity to TNF-α in neurons (arrowheads) in the injured spinal cord. Bar, 30 μm.

in the development of Wällerian demyelination in the latter phase of traumatic spinal cord injury. Nonetheless, our study demonstrates a potential role for TNF-α in apoptosis in the early phase of spinal cord injury; this appears to be mediated in part by NO, as induced by TNF-α.

A recent study by Bethea *et al.* (27) showed the activation of NF-κB following spinal cord injury. Acti-

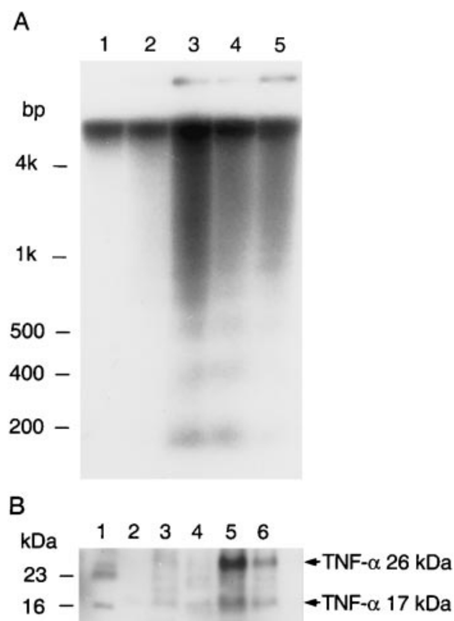

FIG. 2. Agarose gel electrophoresis of DNA laddering (A) and Western blot of TNF-α (B) 24 h after spinal cord injury. DNA was isolated from rat spinal cords, labeled at the 3′ ends with [^{32}P]deoxycytidine triphosphate (dCTP), and analyzed by autoradiography (19, 20). In (A), equal amounts of DNA (500 ng) were subjected to 2% agarose gel electrophoresis. In (B), equal amounts of protein (10 μg) were subjected to 10% tricine polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes as described (18). Polyclonal goat anti-mouse TNF-α antibody (1:500 dilution) was used as a primary antibody. The ABC method was employed using a Vectastain kit (Vector Labs, Burlingame, CA) and immunoreactivity was detected by enhanced chemiluminescence (ECL) autoradiography (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. The gel presented shows a representative of two separate experiments. (A) Lane 1, untreated, sham-operated control that received neither spinal cord injury nor any pharmacological treatment; Lane 2, sham-operated control that had no spinal cord crush but received 2 μl of a nonimmune rabbit serum; Lane 3, vehicle control that had a spinal cord crush and received 2 μl of a nonimmune rabbit serum; Lane 4, TNF-α-neutralized that had a spinal cord crush and received 2 μl of a rabbit neutralizing antibody to TNF-α; Lane 5, NOS inhibitor-treated that had a spinal cord crush and received 2 μl of the NOS inhibitor, L-NMMA. Note an increase in DNA laddering after spinal cord injury (vehicle control). Also, note a decrease in DNA laddering following treatment with TNF-α antibody or L-NMMA after spinal cord injury when compared to the laddering of vehicle control. (B) Lane 1, SeeBlue prestained molecular weight standards (Novex, San Diego, CA); Lane 2, mouse TNF-α standard (17 kDa) (Santa Cruz Biotech., Santa Cruz, CA); Lane 3, untreated, sham-operated that received neither spinal cord injury nor any pharmacological treatment; Lane 4, sham-operated control that had no spinal cord crush but received 2 μl of a nonimmune rabbit serum; Lane 5, vehicle control that had a spinal cord crush and received 2 μl of a nonimmune rabbit serum; Lane 6, NOS inhibitor-treated that had a spinal cord crush and received 2 μl of the NOS inhibitor, L-NMMA. Note the increases in TNF-α (17 kDa) and proTNF-α (30; 26 kDa) after spinal cord injury and the decreases in TNF-α and proTNF-α after spinal cord injury treated with L-NMMA.

vation of NF-κB by TNF-α may also induce transcriptional activation of proapoptotic genes such as NOS; at least one κB site was reported to be essential for its promoter activity. As an inducible isoform of the NOS family, iNOS, once expressed, produces excessive amounts of NO, a deleterious reactive oxygen species (28). However, the exact mechanism of iNOS regulation at the transcriptional level is not known. In a pilot study, we observed a transient activation of NF-κB in neurons and glial cells shortly after the appearance of TNF-α in injured spinal cord (data not shown). We are currently studying the expression of iNOS, a target gene of NF-κB, after spinal cord injury.

The present study shows that treatment with antibodies to TNF-α significantly reduced both the number of TUNEL-positive cells and the level of NO after spinal cord injury. L-NMMA, a NOS inhibitor, also reduced apoptosis after injury. However, a reduction in apoptosis following treatment with L-NMMA (42% decrease) was less than half of that produced by treatment with TNF-α antibody (an 89% decrease). This result leads us to postulate that TNF-α-initiated apoptosis after spinal cord injury may also be mediated in part by a signaling transduction pathway(s) other than NO through the induction of NOS. In fact, a recent report shows that upstream and downstream components of the caspase-3 apoptotic pathway are activated after traumatic spinal cord injury in rats (29). Nevertheless, the present study suggests that TNF-α-initiated apoptosis after spinal cord injury might be mediated in part by NO as produced by NOS, which itself is expressed in response to TNF-α. TNF-α, through its membrane receptors, may activate multiple signaling pathways that result in many cellular functions. The exact pathway(s) leading to apoptosis still need further study. Finally, the functional significance, if any, of ameliorating apoptosis in the recovery of the spinal cord from injury requires further assessment.

ACKNOWLEDGMENTS

The authors extend their appreciation to Drs. Chong-Heon Lee (NIH) and Inho Jo (KNIH) for their helpful input relating to DNA gel electrophoresis and NO assays, respectively. This research was supported in part by grants from the NIH, the Brain Science Program (MOST), and the KIST, Korea.

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