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Expression of the type 1 and type 2 receptors for tumor necrosis factor after traumatic spinal cord injury in adult rats

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Abstract

Posttraumatic inflammation has been implicated in secondary tissue damage after spinal cord injury (SCI). Tumor necrosis factor-α (TNF-α) is a key inflammatory mediator that is increasingly expressed after SCI. The effect of TNF-α is mediated through its receptors TNFR1 (p55) and TNFR2 (p75). However, whether these two receptors are expressed after SCI has not been demonstrated. In the present study, the temporo-spatial expression of TNFR1 and TNFR2 was examined in rats that had received a 10 g impact injury dropped at a height of 12.5 mm using the New York University impact device. In sham operates, no detectable TNFR1 or TNFR2 immunoreactivity (IR) was observed. In contused spinal cord, TNFR1 protein expression and immunoreactivity (IR) were detected as early as 15 min postinjury, reached its peak at 8 h, and declined markedly after 1 and 3 days postinjury. The temporal pattern of TNFR2 expression was similar to that of TNFR1 but its expression peaked at 4 h postinjury. During peak expression, TNFR1- and TNFR2-IR were most intense at the site of injury and decreased gradually from the injury epicenter. TNFR1- and TNFR2-positive cells included neurons, astrocytes, and oligodendrocytes. Methylprednisolone (MP), a synthetic glucocorticoid, partially inhibited the injury-induced expression of TNFR1 and TNFR2, an effect which could be reversed by RU486, an antagonist of glucocorticoid receptors. We suggest that the expression of TNFR1 and TNFR2 after SCI may contribute to posttraumatic inflammatory responses of TNF-α.

Keywords: Glucocorticoids; Inflammation; Rat; Spinal cord injury; TNF-α; TNF receptor

Introduction

Tumor necrosis factor-α (TNF-α) is a key inflammatory mediator that plays a critical role in the initiation and maintenance of inflammatory reaction in diseases or trauma affecting the central nervous system (CNS) (see Arvin et al., 1996; Feuerstein et al., 1994). TNF-α acts on cells through binding to specific surface receptors; two distinct TNF receptors of approximately 55 kDa (TNFR1 or p55) and 75 kDa (TNFR2 or p75) have been identified (Goodwin et al., 1991; Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990). TNF-α binds with high affinity to both receptors, which mediate a large number of biological effects including inflammatory and immunoregulatory responses (Armitage, 1994; Bethea et al., 1998; Beutler and van Huffel, 1994a; Vandenabeele et al., 1995). The biological responses to TNF-α are thought to reflect the balance of multiple signals delivered via both TNFR1 and TNFR2. Thus, mechanisms modulating the expression of the two receptors may affect responsiveness to TNF-α and therefore the inflammatory activity (Bethea et al., 1998; Beutler and van Huffel, 1994a).

Recently, spinal cord injury- (SCI) induced TNF-α expression has been demonstrated at both mRNA and protein levels (see Bartholdi and Schwab, 1997; Wang et al., 1996;

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Table 1
Rats used for the time course and cellular localization of TNF receptors

Group	Sham	15 min	1 h	4 h	8 h	1 day	3 days	7 days
Immunohistochemistry	4	4	—	4	4	4	4	4
Western blot	4	—	4	4	4	4	4	—
Imm-DB-label.	3	—	—	3	—	—	—	—
Imm-EM	2	—	—	3	—	—	—	—

Yakolev and Faden, 1994). We have also confirmed an increase in TNF-α expression after SCI that was preceded by an increase in nuclear factor kappa B (NF-κB) binding activity in nuclear proteins isolated from the injured spinal cord (Xu et al., 1998). Importantly, high doses of methylprednisolone (MP) significantly reduced NF-κB binding activity and TNF-α expression (Xu et al., 1998). Moreover, colocalization of TNF-α and glucocorticoid receptor (GR) in neurons and glial cells was found, suggesting that MP effects on TNF-α expression may be mediated at the cellular level (Yan et al., 1999). These findings, along with others (Bartholdi and Schwab, 1995, 1997; Bethea et al., 1998; Wang et al., 1996; Yakovlev and Fadden, 1994), suggest that posttraumatic inflammation may involve TNF-α as a key mediator that can be suppressed by MP.

In the present study, we sought to determine (1) the temporo-spatial patterns of TNFR1 and TNFR2 expression after SCI, (2) the specific cell types that express the two receptors, and (3) the effects of MP and RU486 on their expression. Our results demonstrate that both TNFR1 and TNFR2 are increasingly expressed following SCI in a time- and site-specific manner and their expression can be partially suppressed by MP through a glucocorticoid receptor mechanism. The inhibitory effect of MP on the expression of these receptors supports the notion that MP mediates posttraumatic inflammatory responses of TNF-α (Hsu and Dimitrijevic, 1990).

Material and methods

A total of 79 adult Long Evans female rats (Simonsen Lab, Gilroy, CA), weighing 200–250 g, were used for this study (Tables 1 and 2). These included animals used for immunohistochemistry, Western blot, immunofluorescence double labeling (imm-DB-label), and immunoperoxidase preembedding EM.

Table 2
Rats used for studying effects of MP and RU486 on TNF receptor expression

Group	Sham	SCI	SCI+MP	SCI+MP+RU
Western blot	4	4	4	4

Spinal cord injury and drug administration

Impact injury was induced using a weight drop device developed at New York University (Gruner, 1992). The method for producing spinal cord injury has been described in detail by a multicenter consortium (Basso et al., 1996) and has been reported previously (Liu et al., 1997; Xu et al., 1998). Briefly, rats were anesthetized with an intraperitoneal injection (ip) of pentobarbital (50 mg/kg) and a laminectomy was performed at the T9-T10 level. 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 injury using a 10-g rod (2.5 mm in diameter) dropped at a height of 12.5 mm. After the injury, the muscles and skin were closed in layers, and rats were placed in a temperature- and humidity-controlled chamber overnight. Manual bladder expression was performed three times per day until reflex bladder emptying was established. For sham-operated rats, a T10 laminectomy without injury was performed. A subpopulation of rats received intravenous MP treatment (30 mg/kg; Upjohn Co., Kalamazoo, MI) at 15 min postinjury and/or intraperitoneal RU486 administration (15 mg/kg; Sigma Chemical Co., St. Louis, MO) at 30 min prior to the injury. After appropriate survival, rats were sacrificed and the cord segments containing the injury epicenter were collected for either Western blot or immunohistochemical preparations. All surgical interventions and postoperative animal care were provided in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and the Guidelines and Policies for Rodent Survival Surgery provided by the Animal Care and Use Committees of Saint Louis University and Washington University.

Western blotting

The Western blotting assay has been described previously (Xu et al., 1998; Yan et al., 1999). Briefly, control rats and rats at varying postoperative survival times or treatments ($n = 4$ per time point; Tables 1 and 2) were given a lethal dose of pentobarbital (80 mg/kg, ip) and perfused intracardially with normal saline. A 8-mm cord segment containing the injury epicenter was removed and homogenized by sonication in a lysis buffer. Twenty micrograms of protein, measured using Lowry's method (Lowry et al., 1951), from the supernatant of each sample and Full Range

Rainbow marker were loaded onto 10% polyacrylamide gel, separated by SDS/PAGE, and transferred to PVDF membranes by electrophoresis. The membranes were blocked in 5% milk in TBST (Tris buffered saline + Tween 20) for 1 h at room temperature (RT). Mouse anti-TNFR1 (1:500; Biosource Int., Camarillo, CA) or mouse anti-TNFR2 antibody (1:500; Biosource) was added to the membrane and incubated at 4°C overnight. The membrane was washed with TBST 3 times at 10-min intervals and incubated with the secondary antibody, sheep anti-mouse IgG conjugated with horseradish peroxidase (1:2000; Amersham, Arlington Heights, IL) at RT for 2 h. The membrane was then washed 3 times with TBST at 10-min intervals and 2 times with TBS (TBST without Tween 20) at 2-min intervals and the proteins were detected by enhanced chemiluminescence (Amersham, Piscataway, NJ). Data were expressed as mean ± standard error of the mean (SEM) and one-way ANOVA was used for statistical comparison of the means with the mean values of sham-operated controls as a baseline control (100%; Arbitrary Unite). Significant results ($P < 0.05$) were followed by Tukey's post hoc tests (GraphPad Prizm 3.0, San Diego, CA). Control blots using no primary antibody showed no band suggesting the two primary antibodies were specific.

Immunohistochemistry

Rats were sacrificed at 15 min, 4 h, 8 h, 1 day, 3 days, and 7 days postinjury (Table 1). In addition, rats that received a T10 laminectomy and were perfused 4 h later served as sham-operated controls. After appropriate survival, animals were deeply anesthetized with pentobarbital (80 mg/kg, ip) and perfused transcardialy with 100 ml of 0.9% saline followed by 500 ml of modified Zamboni fixative (Holets et al., 1987). After perfusion, the spinal cord was carefully removed and a 14-mm segment containing the injury epicenter was blocked and post fixed for an additional 2 h in the same fixation solution. The specimen was transferred to a solution containing 30% sucrose in 0.1 M phosphate buffer (PB, pH 7.4) overnight at 4°C. The specimen was then blocked, from the center of the injury to both rostral and caudal directions, into 2 segments: the first segment 1–5 mm away from the injury epicenter for horizontal sections and the second segment 5–7 mm away for transverse sections. Horizontal sections of the 1–5 mm segment were used to detect the extent of TNFR1- and TNFR2-IR proximodistally while transverse sections of the 5–7 mm segment were used to visualize the lamina distribution of the labeling at varying time points. All sections were cut at 40 μm on a cryostat. The free-floating sections were processed for TNFR1- and TNFR2-IR using the avidin-biotinylated peroxidase complex (ABC) method (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's recommendation. Briefly, sections were incubated with goat anti-TNFR1 (1:1000, R&D System Inc., Minneapolis, MN) and hamster anti-TNFR2 antibody (1:1000; Genzyme, Cambridge, MA) containing 0.3% Triton X-100 and 1% normal goat serum for 24 h at 4°C. After several rinses in 0.01 M PBS, the sections were reacted with biotinylated rabbit anti-goat IgG (1:400; Vector Laboratories, Inc.,) and goat anti-hamster IgG (1:500; Vector Laboratories), respectively, for 1 h and subsequently with Vector avidin-biotin-peroxidase complex (1:500; Vector Laboratories) for 1 h at room temperature. The reaction product was revealed by incubation for 5 min with 0.02% diaminobenzidine tetrahydrochloride (DAB) and 0.003% H_2O_2 in 0.05 M Tris-HCl (pH 7.6). After reaction, the sections were mounted on slides, dehydrated, cleared, and coverslipped. Slides were examined using an Olympus BX60 light microscope. Primary antiserum omission controls and normal goat and hamster serum controls were used to further confirm the specificity of the immunohistochemical labeling.

Immunofluorescence double labeling

The immunofluorescence double-labeling method was described previously (Yan et al., 1999). Briefly, spinal cord segments from either sham-operated or injured animals were embedded in tissue freezing medium, cut longitudinally or horizontally at 12 μm on a cryostat, and mounted on gelatin-coated slides. Before primary antibody incubation, the sections were permeabilized and blocked with 0.3% Triton X-100/3% normal goat serum in 0.01 M phosphate buffered saline (PBS) for 15 min at 4°C. A mixture of goat anti-TNFR1 (1:10, R&D System Inc.) or goat anti-TNFR2 antibody (1:10 Santa Cruz Biotechnology, Inc. Santa Cruz, CA) and a cell-specific monoclonal antibody was applied to the sections overnight at 4°C. The cell-specific monoclonal antibodies included mouse anti-MAP-2 antibody (1:100; Sigma Chemical Co., St. Louis, MO) to identify neurons, mouse anti-glial fibrillary acidic protein antibody (GFAP; 1:100, Sigma) to identify astrocytes, mouse anti-RIP antibody (1:20, a gift from Dr. Scott R. Whittemore, University of Louisville) to identify oligodendrocytes, and mouse anti-OX42 antibody (1:20; Harlan Sera-lab Ltd., Sussex, England) to recognize microglial cells. On the following day, the sections were incubated with fluorescein-conjugated donkey anti-goat (FITC; 1:100; Jackson ImmunoResearch Lab, Inc., West Grove, PA) and rhodamine-conjugated donkey anti-mouse (RITC; 1:100; Jackson ImmunoResearch Lab) antibodies. Sections were washed, mounted, and examined using an Olympus BX60 microscope. Primary antiserum omission controls and normal mouse and goat serum controls were used to further confirm the specificity of the immunofluorescence labeling.

Immunohistochemistry for electron microscopy

For immuno-EM, rats were perfused with the same perfusion fixative as described above for immunohistochemis-

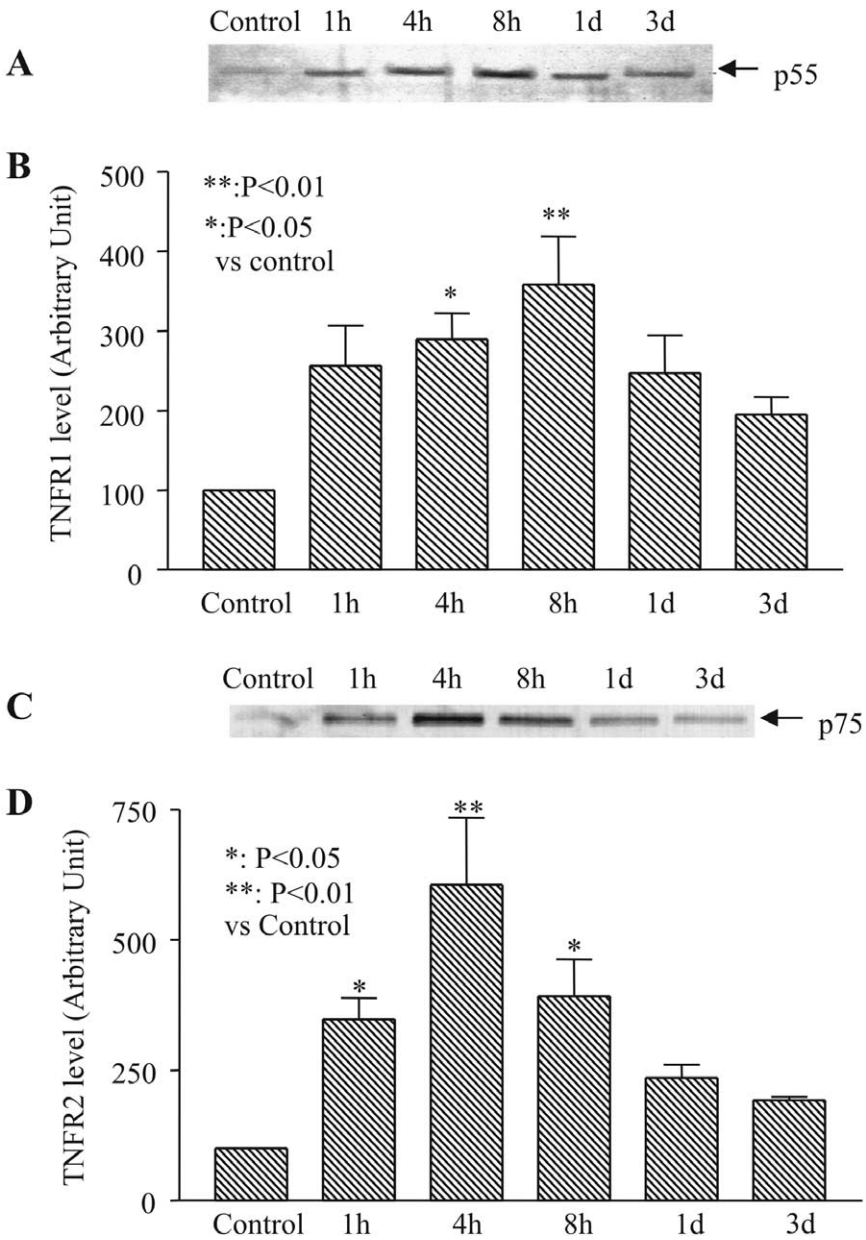
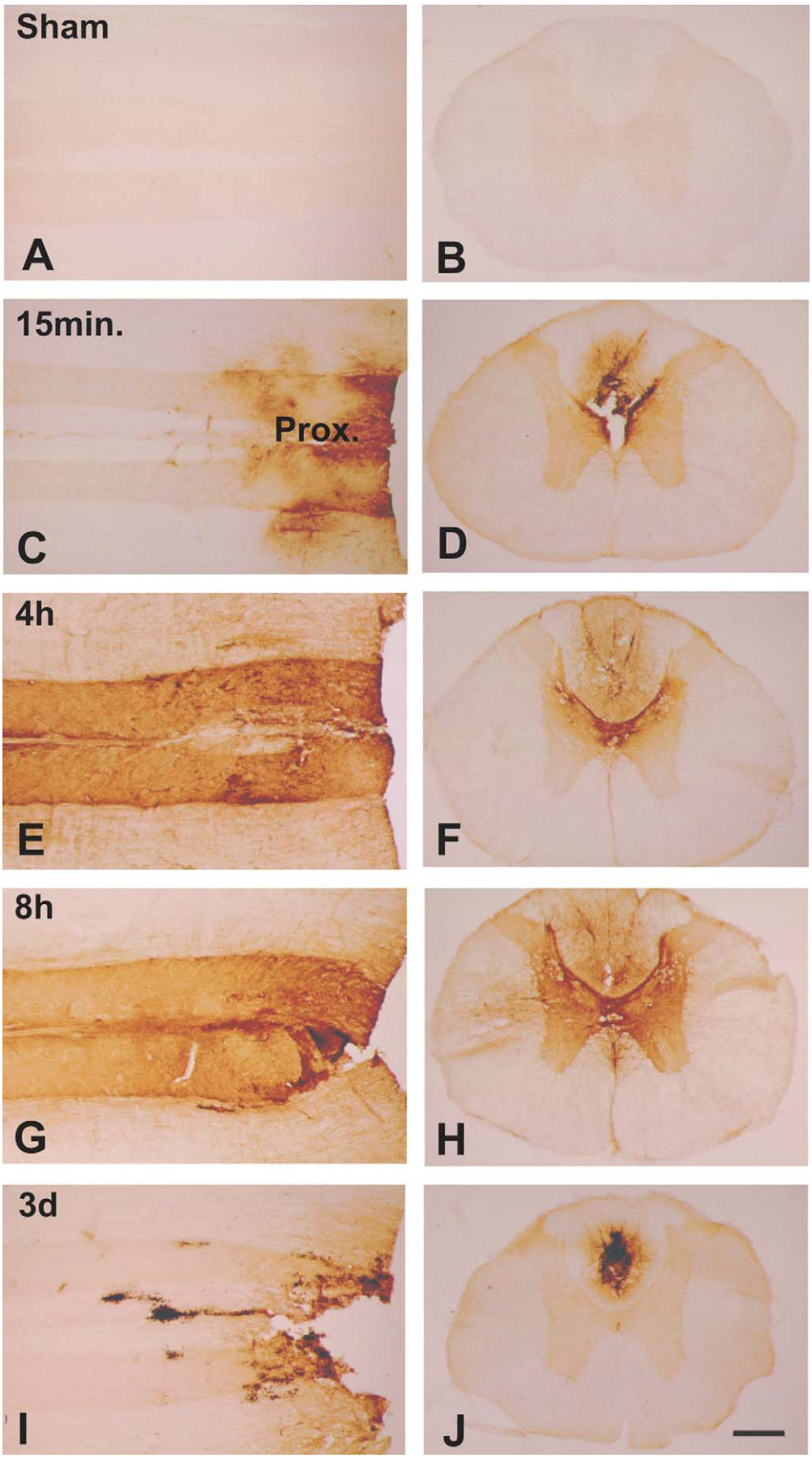


Fig. 1. Western blot analysis showing TNFR1 (p55; A, B) and TNFR2 (p75; C, D) expression at varying time points after SCI. Note that the expression of TNFR1 peaked at 8 h postinjury (3.58-fold) and that of TNFR2 peaked at 4 h post-injury (6.06-fold). Each bar in B and D represents the mean ± SEM (n = 4 rats/group). *$P < 0.05$; **$P < 0.01$.

try but with the addition of 0.1% glutaraldehyde in the fixative. After perfusion, the spinal cord was carefully removed and blocked into a proximal segment (1–5 mm away from the injury epicenter) and a distal segment (5–7 mm away from the epicenter) in both rostral and caudal directions. Horizontal sections of the proximal segment and transverse sections of the distal segment were cut at 50 μm on a vibratome. The sections were subjected to immunohis-

Fig. 2. Temporo-spatial distribution of TNFR2 immunoreactivity (IR) in the spinal cords of sham-operated or injured rats. Left column, TNFR2-IR in horizontal sections of the spinal cord, 1–5 mm from the injury center. The area proximal (prox) to the injury center is indicated in C. Right column, TNFR2-IR in transverse sections of the cord, 5–7 mm distal to the injury center. (A, B) TNFR2-IR was not detectable in a sham-operated control. (C, D) At 15 min after SCI, there was a slight increase in TNFR2-IR, mainly confined within areas close to the injury center (C) and in the dorsal funiculus (D). The ventral portion of the cord contained a negligible amount of TNFR2-IR as is shown in both horizontal (C, left side) and transverse (D) sections. (E, F) TNFR2-IR peaked at 4 h postinjury and extended throughout the entire length of the specimen. (G, H) TNFR2-IR declined at 8 h. (I, J) TNFR2-IR confined to areas adjacent to the lesion site at 3 days postinjury. Scale bars: A–J, 500 μm.


Sham
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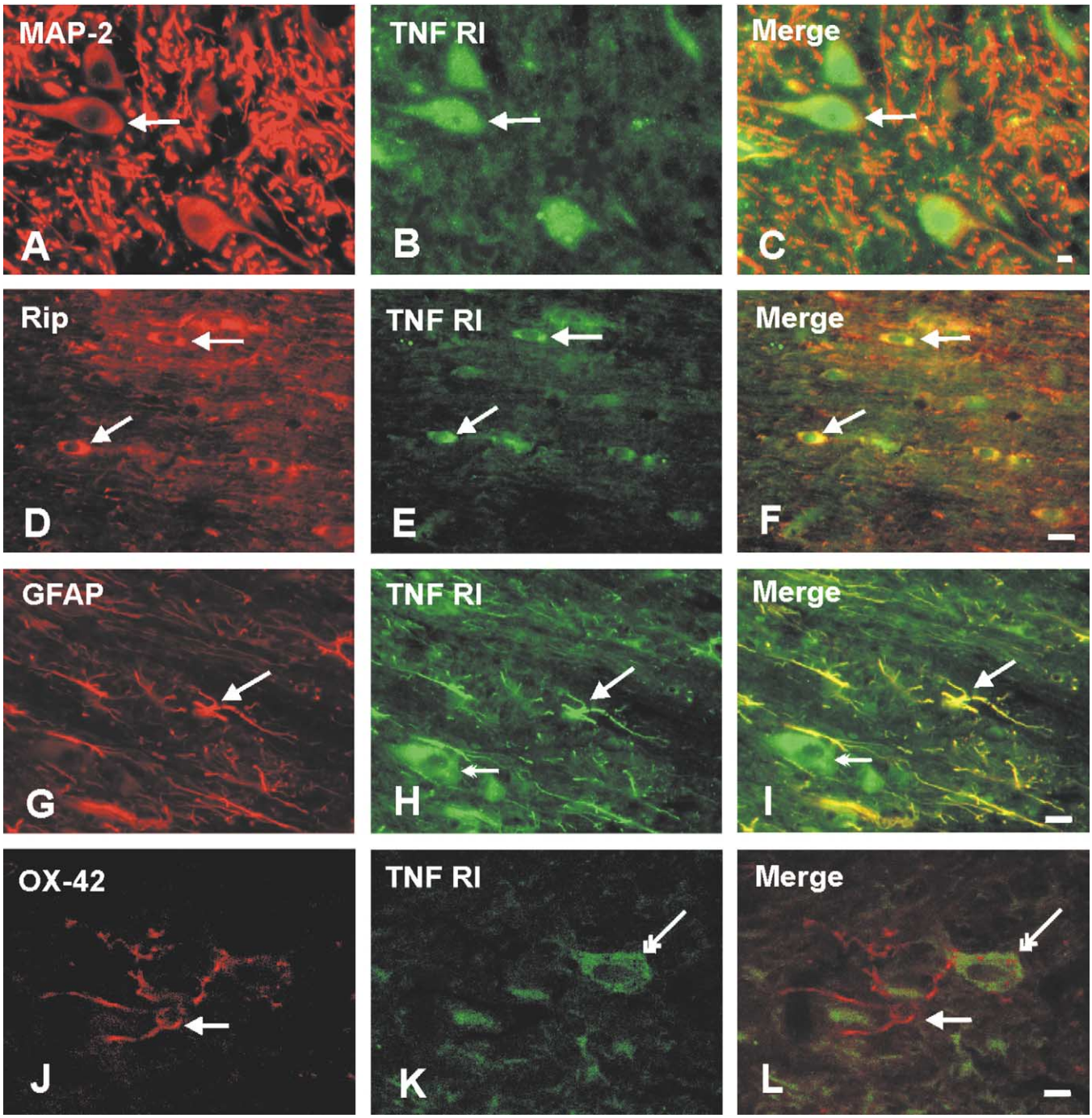

Fig. 3. Colocalization of cell specific markers (red, left column) and TNFR1 (green, middle column) in the adult rat spinal cord 2–4 mm distal to the injury epicenter at 4 h postinjury. Note that TNFR1 was colocalized in neurons (MAP-2-IR, A), oligodendrocytes (Rip-IR, D), and astrocytes (GFAP-IR, G), which can be appreciated in the merge of double exposures for each (C, F, I). Arrows in A–I indicate colocalization of TNFR1 and each cell specific marker. Arrows in J–L indicate that a microglia, identified by OX-42-IR, was negative for TNFR1 (K). Note that the ramified microglia was not morphologically activated. Double arrows in K and L indicate an adjacent cell that is positive for TNFR1 but negative for OX-42, suggesting the specificity of the double-labeling method. Scale bars: A–L, 10 μm.

tochemical processing for TNFR2 according to the method described above for light microscopy with modifications. Specifically, a freeze-thaw method was used to increase the antibody penetration (Eldred et al., 1983). After DAB reaction, the sections were postfixed in 1% osmium tetroxide in 0.1 M PB, pH 7.4, for 50 min at room temperature and dehydrated in graded ethanol and propylene oxide. The sections were then flat-embedded in Spurr on slides. After curing, sections were examined, and areas of interests were cut out and glued to Spurr cylinders for ultrathin sectioning.

The ultrathin sections were mounted on grids, examined, and photographed using a Zeiss 109 electron microscope (Zeiss, Thornwood, NY).

Results

Western blot analysis of TNFR1 and TNFR2 expression after SCI

Western blot analysis for TNFR1 and TNFR2 protein expression after SCI showed an apparent single band labeling with a molecular weight of either 55 kDa (Figs. 1A and B) or 75 kDa (Figs. 1C and D), respectively. In sham-operated controls, there were only light bands of baseline labeling. TNFR1 was increasingly expressed at 1 h postinjury, significantly increased at 4 h ($P < 0.05$), and peaked at 8 h (3.58-fold, $P < 0.01$) postinjury. Increase in TNFR1 expression was still evident at 1 day (2.47-fold) or 3 days (1.94-fold) postinjury although the increase was not statistically significant ($P > 0.05$). The expression of TNFR2 was significantly increased at 1 h (3.1-fold, $P < 0.05$), peaked at 4 h (6.06-fold, $P < 0.01$), and remained at significantly higher level at 8 h (3.27-fold, $P < 0.05$) postinjury. Increases in TNFR2 expression were still evident at 1 day (2.36-fold) or 3 days (1.93-fold) postinjury, although the increases were not statistically significant ($P > 0.05$).

Temporo-spatial distribution of TNFR1 and TNFR2 immunoreactivity after SCI

To confirm the Western blot analysis of TNFR1 and TNFR2 expression and to determine the temporo-spatial distributions of the two receptors after SCI, immunohistochemistry for TNFR1 and TNFR2 was performed. Since similar patterns of TNFR1- and TNFR2-IR were observed, TNFR2-IR was used to demonstrate its distribution after SCI (Fig. 2). In sham-operated controls, TNFR2-IR was not detectable at low magnification in either the gray or white matter (Figs. 2A and B). At 15 min postinjury, TNFR2-IR was increased in the gray matter (Fig. 2C) and in the dorsal funiculus (Fig. 2D), proximal to the injury site. TNFR2-IR reached its peak at 4 h postinjury, during which time intense labeling was observed throughout the entire length of the specimen that was examined (Figs. 2E and F). However, labeling was much stronger in the gray matter than in the white matter. At 8 h postinjury, TNFR2-IR remained strong and extended from the injury epicenter to more distal areas (Figs. 2G and H). TNFR2-IR was noticeably reduced at 1 day postinjury (data not shown) and, at 3 days, it was confined mainly to areas adjacent to the lesion site (Figs. 2I and J). The spatial distribution of TNFR1-IR was similar to that of TNFR2-IR with the exception that the former peaked at 8 h postinjury (data not shown). The time course of TNFR1 and TNFR2 immunoreactivity paralleled the protein expression levels described above using Western blot analysis (Fig. 1).

Cellular localization of TNFR1 and TNFR2 following SCI

Immunofluorescence double labeling or immunoperoxidase preembedding EM experiments were performed to study cellular sources of TNFR1 or TNFR2, respectively, at 4 h postinjury when the two receptors were either highly expressed (TNFR1) or peaked (TNFR2). Using the immunofluorescence double-labeling method, coexpression of TNFR1 and MAP-2 was observed in many neurons, mainly in the intermediate gray of spinal cord injured rats (Figs. 3A–C). Although TNFR1-IR was strongest at sites adjacent to the injury epicenter, TNFR1 and MAP-2 double-labeled neurons were found throughout the entire length of the specimens that were examined (up to 7 mm away from the injury epicenter). In the white matter, colocalization of TNFR1 and RIP (Figs. 3D–F) or GFAP (Figs. 3G–I) was found, indicating its expression in both oligodendrocytes and astrocytes, respectively. TNFR1 was not colocalized in resting microglia that remained ramified several millimeters away from the injury (OX-42-IR; Figs. 3J–L). TNFR1-expressing neurons and glial cells were recognized not only by their cell-specific markers but also by their morphological characteristics. At the EM level, neurons (Fig. 4A; Neu), astrocytes (Fig. 4D; Astro), and oligodendrocytes (Fig. 4C; Oligo) all showed positive TNFR2-IR. Although some axons (Fig. 4B; Ax1) were positive for TNFR2, many others were negative (Fig. 4B; Ax2). At the synaptic terminal level, a postsynaptic element could be positive for TNFR2 whereas the presynaptic component could be negative (Fig. 4 A; inset).

Effects of MP and RU 486 on TNFR1 and TNFR2 expression after SCI

In sham-operated controls, there was only a light band of baseline labeling for both TNFR1 and TNFR2 (Fig. 5). SCI significantly increased the expressions of TNFR1 ($P < 0.001$) and TNFR2 ($P < 0.001$) at 4 h postinjury as compared to controls. MP markedly suppressed the expression of TNFR1 by 55% ($P < 0.01$) and TNFR2 by 65% ($P < 0.001$) at 4 h postinjury. RU486 partially, but significantly, reversed the MP effect on TNFR1 ($P < 0.05$) and almost completely reversed the MP effect on TNFR2 ($P < 0.001$).

Discussion

In the present study, temporo-spatial patterns of TNFR1 and TNFR2 expression and their cellular localization were studied in normal and spinal cord injured adult rats. We demonstrated that the expression of TNFR1 and TNFR2 was slightly increased at 15 min postinjury, reached the peak at 4 h for TNFR2 (6.06-fold, $P < 0.01$) and 8 h for

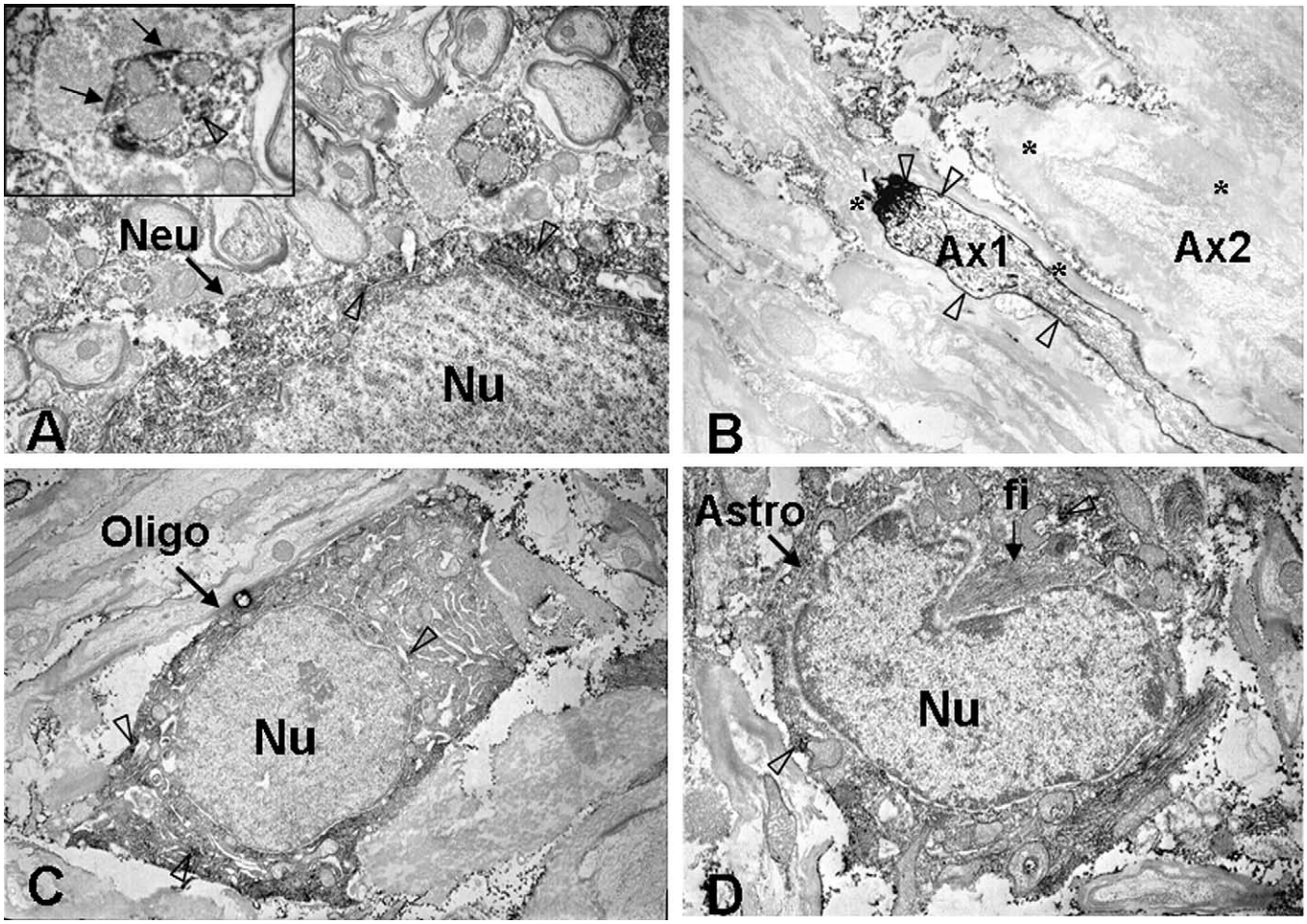

Fig. 4. Immunoelectron microscopy demonstrating cellular and subcellular distribution of TNFR2 in an adult rat spinal cord perfused at 4 h postinjury. TNFR2-IR was observed in neurons (Neu; A), axons (Ax1; B), oligodendrocytes (Oligo; C), and astrocytes (Astro; D). All open arrowheads point to the sites of TNFR2 expression. While a postsynaptic element of a synapse showed TNFR2-IR (arrows; inset in A), its presynaptic component was not. Some axons were positive for TNFR1 (Ax1, B) whereas others were not (Ax2; B). Note the cell in D contains bundles of intermediate filaments (fi), a unique characteristic of the astrocytes. Asterisks in B indicate unstained myelin. Astro, astrocytes; Ax1, a TNFR2-positive axon; Ax2, a TNFR2-negative axon; fi, intermediate filaments; Neu, neuron; Nu, Nucleus; Oligo, oligodendrocytes. Scale bars: A–D, 1 μm.

TNFR1 (3.58-fold, $P < 0.01$), and declined markedly after 1 and 3 days. Spatially, TNFR1- and TNFR2-IR was initially observed at the impact site, spread to the distant areas during the peak expression, and confined to the lesion area at later time points. Colocalization of TNFR1 and TNFR2 was found in neurons, oligodendrocytes, and astrocytes. Importantly, injury-induced expression of TNFR1 or TNFR2 was significantly inhibited by MP, an effect which could be partially (for TNFR1) or completely (for TNFR2) reversed by RU486. The MP inhibition on TNFR1 or TNFR2 expression and its reversion by RU486 indicates that MP mediates posttraumatic inflammatory responses of TNF-α.

Cellular localization of TNFR1 and TNFR2

Both TNFR1 and TNFR2 were expressed in neurons, oligodendrocytes, and astrocytes following SCI. At the EM level, TNFR2 was compartmentally localized in neuronal cell bodies, postsynaptic dendritic elements, and axons, but not in presynaptic elements of axonal terminals (Fig. 4). In addition, only a subpopulation of axons was positive for TNFR2. It is possible that the TNFR2-positive axons may represent those that were traumatized by the injury. The inconsistent labeling of TNFR2 in axons may indicate a dynamic responses of axons to the timing of and proximity to the injury. In addition, the lack of TNFR2 labeling in presynaptic terminals suggests that the receptor can be heterogeneously distributed in specific compartments of a single neuron. Interestingly, TNFR1- and TNFR2-IR were not found in ramified microglia, indicating that these inactivated microglia did not express appreciable levels of the two receptors at 4 h postinjury. TNF receptors, however, have been found in microglia (Dopp et al., 1997), macrophages (Miller-Graziano et al., 1994), and endothelial cells (Okuyana et al., 2000) in vitro in various experimental

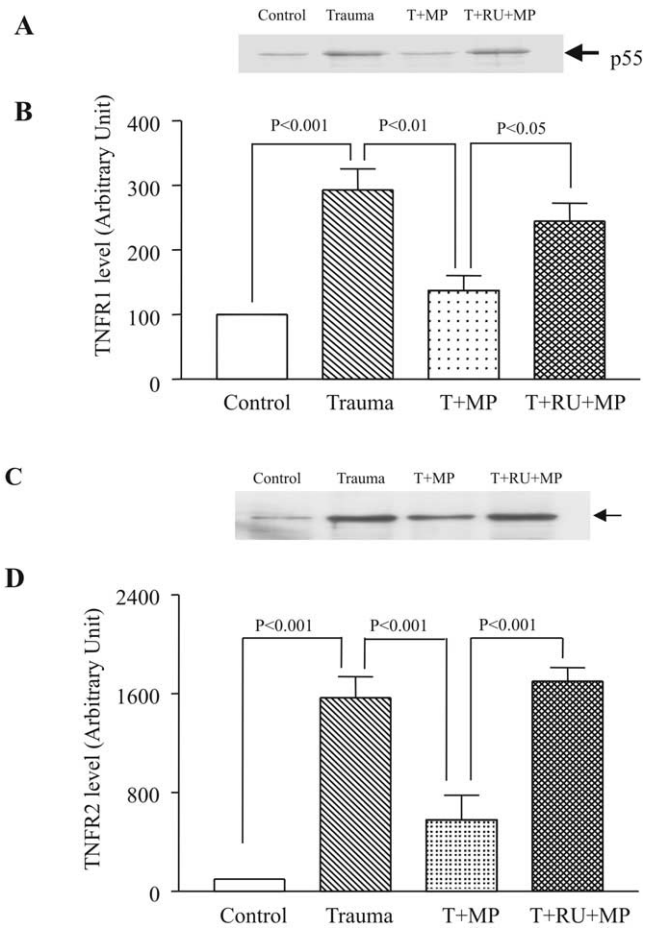

Fig. 5. Effects of MP and RU486 on the expression of TNFR1 (p55; A, B) and TNFR2 (p75; C, D) in the injured spinal cord at 4 h postinjury. (A, B) Expression of TNFR1 significantly increased ($P < 0.001$) above the control level at 4 h postinjury. MP markedly inhibited posttraumatic expression of TNFR1 ($P < 0.01$), an effect that was partially reversed by RU486 ($P < 0.05$). Each bar in B and D represents the mean ± SEM ($n = 4$ rats/group). (C, D) Expression of TNFR2 significantly increased ($P < 0.001$) above the control level at 4 h postinjury. MP markedly inhibited posttraumatic expression of TNFR2 ($P < 0.001$), an effect that was almost completely reversed by RU486 ($P < 0.001$). Each bar in B and D represents the mean ± SEM ($n = 4$ rats/group).

paradigms, as well as in microglia in vivo in the experimental allergic encephalomyelitis spinal cord (Kahn et al., 1999). The lack of TNF receptors expression in ramified microglia in the current study may indicate that those microglia located distant from the injury may not be activated and express detectable levels of TNF receptors at 4 h postinjury.

TNF-α and TNF receptors

Inflammatory cytokines, particularly TNF-α, can signal a large number of cellular responses including cytotoxicity, inflammation, immunoregulatory activities, and transcriptional regulation of many genes (Hsu et al., 2001; Tartaglia et al., 1993a). TNF-α expression increases following SCI (Bartholdi and Schwab, 1997; Wang et al., 1996; Xu et al., 1998; Yakovlev and Fadden, 1994), and resident neurons, glial cells, and vascular endothelial cells all contribute to the TNF-α production in response to acute SCI (Yan et al., 2001). Since both TNFR1 and TNFR2 are increasingly expressed in neurons, astrocytes, and oligodendrocytes following SCI and since the time course of TNF-α and TNFRs expression is closely related, it is possible that an "autocrine" mechanism is involved in the expression of TNF-α to exert its biological function at the cellular level. Further, concerning the fact that TNF-α and glucocorticoid receptor were colocalized in neurons and glial cells, the effect of MP on the suppression of TNF-α and TNF receptors could also occur at the cellular level (Yan et al., 1999, 2001). Although we demonstrated that MP suppresses the expression of both TNF-α (Xu et al., 1998) and TNFRs (the present study), the long-term significance of such an effect on tissue sparing or neurological recovery remains to be elucidated.

Possible roles of TNFR1 and TNFR2 in SCI

The multiple activities of TNFα are mediated through two distinct but structurally homologous receptors TNFR1 and TNFR2 (Vandenabeele et al., 1995). The mechanism of interaction between TNF-α production and elevated expression of TNF receptors after SCI remains to be elucidated. Overproduction or shedding of TNF receptors are capable of autoregulating TNF-α activity (Armitage, 1994; Miller-Graziano et al., 1994) and SCI leads to an increase in the production of TNF-α, IL-β, and iNOS (Wang et al., 1996, 1997; Xu et al., 2001b), which may be responsible for the increase in the expression of TNF receptors. Further, NF-κB is activated by TNF-α-TNFR binding, which leads to transactivation of a range of proinflammatory genes (Bethea et al., 1998).

Both TNFR1 and TNFR2 can bind to TNF-α with high affinity, but the exact role of the two receptors in mediating the effects of TNF-α is still controversial. It has been suggested that TNFR1 is responsible for the majority of biological activities of TNF-α including its effects on apoptosis or programmed cell death, and cytotoxicity (Tartaglia et al. 1993a,c; Weigmann et al., 1992). TNFR2, on the other hand, may play a passive role in mediating TNF-α responses due to the lack of an intracellular death domain (Tartaglia, 1993b), However, recent data demonstrate that TNFR2 is able to independently mediate TNF-α biological activity, such as inducing apoptosis (Grell et al., 1998; Haridas et al., 1998). TNFR2-mediated apoptosis is associated with the down-regulation of antiapoptotic proteins Bcl-xL and/or Bcl-2 (Boise et al., 1993; Lin et al., 1997). The temporo-spatial expression of both TNFR1 and TNFR2 in the present study may suggest that the two receptors may work individually or synergistically on mediating TNF-α biological activities.

Glucocorticoid receptor and TNF receptor

It is known that glucocorticoids (GCs) inhibit the expression of cytokines such as TNF-α and iNOS (Arzt et al., 1994; Brenner et al., 1994; Hayashi et al., 2000; Xu et al., 1998) and block trafficking of inflammatory cells to reducing swelling, inflammation, glutamate release, and free radical accumulation (Heyderman et al., 1995; Perretti and Flower, 1994; Steer et al., 1998; van Furth et al., 1995). MP, a synthetic GC, reduced the expression of TNF-α and the activation of NF-κB and AP-1, two proinflammatory transcription factors in rodent SCI models (Xu et al., 1998, 2001). It has been suggested that biological activities of GCs are largely mediated by glucocorticoid receptors (GRs: Tsai et al., 1988; Yamamoto, 1985). GR also serves as an anti-inflammatory transcription factor that transrepresses pro-inflammatory genes and transactivates anti-inflammatory genes by binding to the glucocorticoid responsive element (GRE) (Hsu et al., 2001b). GR protein expression increased within hours after SCI and the temporal expression of GR (Yan et al., 1999) was similar to that of TNFRs (Fig. 1). Additionally, GRE binding activity was also increased. The posttraumatic increase in the expression of GR and GRE-binding activity suggests the possible existence of endogenous anti-inflammatory activity (Xu et al., 1998, 2001a,b; Yan et al., 1999). The effect of MP on the two receptors in this study is consistent with the observation that MP's transrepressing inflammatory gene is mediated by a GR mechanism (Xu et al., 2001a). As MP partially inhibited the expression of both TNFR subtypes after SCI, which could be reversed by RU486, the beneficial mechanism of MP treatment after SCI may involve its modulation of TNF-α-TNFR-mediated inflammatory responses.

Beneficial or deleterious effects of TNF-α

Conflicting evidence on the role of increased TNF-α production after different CNS insults suggests that this pathway may be involved in both processes of damage and repair (Morganti-Kossman et al., 1997). For example, exogenously administrated TNF-α exacerbates focal ischemic injury (Barone et al., 1997), and TNF-α inhibition by pharmacologic agents, neturalizing antibodies, or soluble receptors has protective effects (Barone et al., 1997; Lavine et al., 1998; Nawashiro et al., 1997a,b; Yang et al., 1998). In the spinal cord, nanoinjections of either TNF-α or kainate alone into the thoracic gray resulted in almost no tissue damage or cell death 90 min after injection. However, the combination of TNF-α and kainate at these same doses produced a large area of tissue necrosis and neuronal cell death, an effect that can be blocked by the AMPA receptor antagonist CNQX (Hermann et al., 2001). The deleterious effect of TNF-α is supported by the observation that IL-10, which blocks the injury-induced increase in TNF-α, can protect the spinal cord from secondary injury (Bethea et al., 1999).

In contrast, a protective role of TNF-α after CNS injury has been recently emphasized in cerebral ischemia, excitotoxic neuronal injury, autoimmune-mediated demyelination, and traumatic brain injury (Brenner et al., 1994; Liu et al., 1998; Scherebel et al., 1999; Sullivan et al., 1999). It has been suggested that TNFRs are involved in antiapoptotic activities through the TNFR-NF-κB signal transduction pathway, which activates a recently identified endogenous caspase inhibitory system mediated by cellular inhibitor of apoptosis protein 2 (c-IAP2). NF-κB transactivates c-IAP2, which in turn inhibits caspase-3 activation. TNFR1 $-/-$ mice exhibited reduced NF-κB activation, decreased c-IAP2 expression, and poor functional recovery after SCI, as compared to their wild-type counterparts (Kim et al., 2001). Consistent with reduced c-IAP2 expression, expression of the active caspase-3 and the number of TUNEL-positive cells were increased in the injured cord in TNFR1$-/-$ mice. Moreover, others have reported that when a mixture of murine recombinant IL-1β, IL-6, and TNF-α is administered to the lesioned spinal cord of adult mice, the amount of tissue sparing 7 days after trauma is increased in animals receiving cytokine treatments compared to controls (Klusman and Schwab, 1997). These findings collectively suggest that the TNFR-NF-κB cascade may play an important role in initiating the endogenous caspase inhibitory system after SCI.

Thus, the role of various inflammation responses after SCI is complex. Whether the activation of TNF-α-TNFR-NF-κB cascade plays a beneficial or deleterious role after injury may be dependent on multiple factors including the nature of injury, the timing and levels of cytokine and receptor expression, and the responses of neurons and glial cells to a specific insult. The ability to specifically activate the beneficial TNF-α-TNFR-NF-κB pathway, without affecting its deleterious pathway, may provide new therapeutic approaches to the treatment of secondary spinal injury.

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