Immunomodulatory Effects of Etanercept in an Experimental Model of Spinal Cord Injury

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ABSTRACT

Etanercept is a tumor necrosis factor antagonist with anti-inflammatory effects. The aim of our study was to evaluate for the first time the therapeutic efficacy of in vivo inhibition of tumor necrosis factor-α (TNF-α) in experimental model of spinal cord trauma, which was induced by the application of vascular clips (force of 24 g) to the dura via a four-level T5–T8 laminectomy. Spinal cord injury in mice resulted in severe trauma characterized by edema, neutrophil infiltration, and cytokine production that it is followed by recruitment of other inflammatory cells, such as production of a range of inflammation mediators, tissue damage, apoptosis, and disease. Treatment of the mice with etanercept significantly reduced the degree of 1) spinal cord inflammation and tissue injury (histological score); 2) neutrophil infiltration (myeloperoxidase evaluation); 3) inducible nitric-oxide synthase, nitrotyrosine, cyclooxygenase-2, and cytokines expression (TNF-α and interleukin-1β); and 4) apoptosis (terminal deoxynucleotidyl transferase dUTP nick-end labeling staining and Bax and Bcl-2 expression). In a separate set of experiment, we have also clearly demonstrated that TNF-α inhibitor significantly ameliorated the recovery of limb function (evaluated by motor recovery score). Taken together, our results clearly demonstrate that treatment with etanercept reduces the development of inflammation and tissue injury events associated with spinal cord trauma.

Post-traumatic inflammatory reactions may play an important role in the secondary injury processes after spinal cord injury (SCI) (Bartholdi and Schwab, 1995). In fact, the primary traumatic mechanical injury to the spinal cord causes the death of a number of neurons that cannot be recovered or regenerated to date. However, neurons continue to die for hours after SCI and this represents a potentially avoidable event. This secondary neuronal death is determined by a large number of cellular, molecular, and biochemical cascades. One such cascade that has been touted to contribute importantly to the evolution of this secondary damage is the local inflammatory response in the injured spinal cord.

The inflammatory response in the CNS involves the participation of different cellular types of the immune system and resident cells of the CNS, adhesion molecules, cytokines, and chemokines among other proteic components. During neuroinflammation, chemotaxis is an important event in the recruitment of cells to the CNS. If this process is not controlled but is prolonged, inflammation loses its repairing function and can be the cause of damage.

Both necrotic and apoptotic mechanisms of cell death after SCI have been well and extensively characterized in animal SCI models. It is has been thought that microglial cells might be the source of cytotoxic cytokines, such as tumor necrosis factor-α (TNF-α), that kill oligodendrocytes. Within 1 h after spinal cord injury, increased synthesis and/or secretion of TNF-α is detectable at the injury site. Harrington et al. (2005) demonstrated that 6 h postacute SCI, there were an increased neuronal expression of TNF-α and its receptors. They also demonstrated that, at 1 h after acute SCI, TNF-α levels in the cerebrospinal fluid were significantly higher than those found in the sham-injured animals, indicating the release of this cytokine into the interstitial fluid. Likewise, other authors have shown that, after experimental SCI, the levels of TNF-α and interleukin 1β (IL-1β) are significantly increased within the first few hours (Hayashi et al., 2000).

TNF-α has an important role in several pathologies, such as rheumatoid arthritis (Bazzoni and Beutler, 1996). TNF-α binds to two receptors, the type 1 TNF receptor (p55) and the type 2 TNF receptor (p75). TNF-α receptor 1 (p55) binds to TNF-α with higher affinity than TNF-α receptor 2 (p75), and binding to receptor 1 causes the activation of a wide range of intracellular signaling cascades, including mitogen-activated protein kinase (MAPK) and nuclear factor κB (NFκB) signaling pathways, which are associated with cell proliferation, survival, and death. TNF-α receptor 2 (p75) binds to TNF-α with lower affinity than receptor 1, and binding to receptor 2 leads to the cleavage of TNF-α receptor 1 (p55) and subsequent shedding of the ligand.

ABBREVIATIONS: SCI, spinal cord injury; CNS, central nervous system; TNF, tumor necrosis factor-α; iNOS, inducible nitric-oxide synthase; PBS, phosphate-buffered saline; COX-2, cyclooxygenase-2; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling staining; IL-1β, interleukin 1β; BBB, Basso, Beattie, and Bresnahan (score); TdT, terminal deoxynucleotidyl transferase; MPO, myeloperoxidase.
type 2 TNF receptor (p75), that are expressed on many types of the cells. The biologic activity of TNF-α can be attenuated by soluble TNF receptors. Studies in animals have provided key evidence that antagonizing TNF-α is a viable therapeutic strategy (Kapadia et al., 1995). Subsequent studies in patients with rheumatoid arthritis indicated that blocking TNF improved symptoms. Biological agents that are currently available include three agents that decrease the activity of tumor necrosis factor-α (infliximab, adalimumab, and etanercept). The major therapeutic goal when administering TNF antagonists is to eliminate the surplus of TNF from the circulation and from sites of inflammation.

Etanercept inhibits TNF activity by competitively binding to it and preventing interactions with its cell-surface receptors. Etanercept is a recombinant dimer of human TNF receptor proteins fused and bound to human IgG1. It binds TNF-α and TNF-β, the binding is reversible, and when it is dissociated, TNF remains bioactive. Etanercept, although originally developed for rheumatoid arthritis and Crohn disease, has also demonstrated beneficial activity in other inflammatory diseases. This article investigated the role of TNF in the pathogenesis of SCI and evaluates the therapeutic effects of etanercept.

In particular, we have determined the following endpoints of the inflammatory response: 1) histological damage; 2) motor recovery; 3) neutrophil infiltration; 4) cytokine expression (TNF-α and IL-1β); 5) nitrotyrosine, inducible nitric-oxide synthase (iNOS), and COX-2 expression; 6) apoptosis (TUNEL staining); and 7) Bax and Bcl-2 expression.

Materials and Methods

Animals

Male adult CD1 mice (25–30 g, Harlan Nossan, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (Ministerial Decree 116192) as well as with the European Economic Community regulations (Official Journal of E.C. L 358/1 12/18/1986).

Spinal Cord Injury

Mice were anesthetized using chloral hydrate (40 µg/kg body weight). A longitudinal incision was made on the midline of the back, exposing the paravertebral muscles. These muscles were dissected away, exposing T6–T7 vertebrae and laminae of the spinal cord. The spinal cord was exposed via a four-level T6–T7 laminectomy, and SCI was produced by extradural compression of the spinal cord using an aneurysm clip with a closing force of 24 g. After surgery, 1.0 cc of saline was administered subcutaneously to replace the blood volume lost during the surgery. During recovery from anesthesia, the mice were placed on a warm heating pad and covered with a warm towel. The mice were singly housed in a temperature-controlled room at 27°C for a survival period of 10 days. Food and water were provided to the mice ad libitum. During this time period, the bladders of the animals were manually voided twice a day until the mice were able to regain normal bladder function. In all of the injured groups, the spinal cord was compressed for 1 min. Sham-injured animals were only subjected to laminectomy.

Experimental Groups

Mice were randomly allocated into the following groups.

1) SCI + Saline Group. Mice were subjected to SCI plus administration of saline (n = 30).

2) Etanercept Group. This group was the same as the SCI + saline group, with the exception that etanercept at the dose of 5 mg/kg was administered 1 h before and 6 h after SCI i.p. (n = 30).

3) Sham + Saline Group. Mice were subjected to the surgical procedures as the above groups, with the exception that the aneurysm clip was not applied (n = 30).

4) Sham + Etanercept Group. This group was identical to sham + saline group, with the exception that the administration of etanercept was administered i.p. 1 h before and 6 h after SCI (n = 30).

In the experiments regarding the motor score, the animals were treated with etanercept 1 h before and 6 h after SCI daily until day 9. At different time points (see Fig. 1), the animals (n = 10 mice from each group for each time point) were sacrificed in order to evaluate the various parameter as described below.

Myeloperoxidase Activity

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was determined in the spinal cord tissues as described previously (Mullane et al., 1985) at 4 h after SCI. The time of 4 h after SCI was chosen in agreement with other studies (Hamada et al., 1996). MPO activity was defined as the quantity of enzyme degrading 1 µmol of peroxide min⁻¹ at 37°C and was expressed in milliunits grams⁻¹ of wet tissue.

Immunohistochemical Localization of TNF-α, IL-1β, Nitrotyrosine, iNOS, COX-2, Bax, and Bcl-2

At 24 h after SCI, the tissues were fixed in 10% (v/v) PBS-buffered formaldehyde and 8-mm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Nonspecific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA srl, Milan, Italy), respectively. Sections were incubated overnight with anti-TNF-α (1:500 in PBS, v/v), anti-IL-1β polyclonal antibody (1:500 in PBS, v/v), antinitrotyrosine rabbit polyclonal antibody (1:500 in PBS, v/v), anti-iNOS polyclonal antibody rat (1:500 in PBS, v/v), anti-COX-2 monoclonal antibody (1:500 in PBS, v/v), anti-Bax rabbit polyclonal antibody (1:500 in PBS, v/v), or anti-Bcl-2 polyclonal antibody rat. Sections were washed with PBS and incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex. To verify the binding specificity for nitrotyrosine, iNOS, TNF-α, IL-1β, COX-2, Bax, and Bcl-2, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations, no positive staining was found in the sections, indicating that the immunoreac-

Fig. 1. Mice were sacrificed at different time points to evaluate the various parameters; n = 10 mice from each group for each time point. See Materials and Methods for further explanations.
tions were positive in all of the experiments carried out. Immuno-
histochemical photographs (n = 5 photos from each samples collected from all mice in each experimental group) were assessed by densi-
tometry as described previously (Shea, 1994; Cuzzocrea et al., 2001) by using Optilab Graftek software on a Macintosh personal com-
puter.

Terminal Deoxynucleotidyltransferase-Mediated UTP End
Labeling Assay

TUNEL assay was conducted by using a TUNEL detection kit according to the manufacturer’s instruction (Apotag, HRP kit; DBA sr1). In brief, sections were incubated with 15 μg/ml proteinase K for 15 min at room temperature and then washed with PBS. Sections were immersed in TdT buffer containing deoxynucleotidyl transferase and biotinylated dUTP in TdT buffer, incubated in a humid atmosphere at 37°C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with anti-horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminoben-
zidine.

Total Protein Extraction and Western Blot Analysis for
Bax and Bcl-2

Tissue samples from SCI-injured animals were homogenized with a Ultra-turrax T, homogenizer (UNILAB CELBIO, Catania, Italy) in a buffer containing 20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM

Fig. 2. Effect of etanercept on histological alterations of the spinal cord tissue 24 h after injury. No histological alteration (A) and no modification of the myelin structure (A1) were observed in the spinal cord tissues from sham-operated mice. B, twenty-four hours after trauma, a significant damage to the spinal cord from no-treated SCI-operated mice at the perilesional area was assessed by the presence of edema and alteration of the white matter. C, notably, a significant protection from the SCI was observed in the tissue collected from etanercept SCI-treated mice. Likewise, at 24 h after the injury in no-treated SCI-operated mice (B1), a significant loss of myelin was observed. In contrast, in etanercept SCI-treated mice, myelin degradation was attenuated (C1). This figure is representative of at least three experiments performed on different experimental days. wm, white matter; gm, gray matter.
phenylmethylsulfonyl fluoride, 1.5 μg/ml trypsin inhibitor, 3 μg/ml peptatin, 2 μg/ml leupeptin, 40 μM benzamidine, 1% Nonidet P-40, and 20% glycerol. The homogenates were centrifuged at 13,000 rpm for 15 min and at 4°C, and the supernatant was collected to evaluate contents of Bax and Bcl-2. Protein concentration was determined with the Bio-Rad protein assay kit (Hercules, CA). Proteins were mixed with gel-loading buffer [50 mM Tris, 10% (w/v) SDS, 10% (w/v) glycerol, 10% (v/v) 2-mercaptoethanol, and 2 mg ml⁻¹ bромphenol blue], boiled for 3 min, and centrifuged at 10,000 rpm for a few seconds. Protein concentration was determined in equivalent amounts (75 μg) of each sample electrophoreses in a 12% (w/v) discontinuous polyacrylamide minigel (BIORAD Laboratories srl, Milan, Italy). Proteins were separated electrophoretically and transferred to nitrocellulose membranes. For immunoblotting, membranes were blocked with 10% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 and incubated with AffiniPure Goat Anti-Rabbit IgG coupled to peroxidase (1:2000; DBA srl). The immune complexes were visualized using the SuperSignal West Pico chemiluminescence substrate (Pierce, Milan, Italy).

Light Microscopy
Spinal cord biopsies were taken at 24 h after trauma. The biopsies were fixed for 24 h in paraformaldehyde solution (4% in 0.1 M PBS) at room temperature, dehydrated by graded ethanol, and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Tissue sections (thickness 5 μm) were deparaffinized with xylene, stained with H&E and Luxol Fast Blue staining (used to assess demyelination; DBA level of the perilesional area, assessed by the presence of after injury. A significant damage to the spinal cord at the level of the perilesional area, assessed by the presence of edema as well as alteration of the white matter (Fig. 2B), was observed in SCI + saline-operated mice compared with spinal cord tissue collected from sham-operated mice (Fig. 2A). Notably, a significant protection of the spinal cord injury was observed in the tissue collected from etanercept-treated mice (Fig. 2C).

Myelin structure was observed by Luxol Fast Blue staining. In sham animals (Fig. 2A1), myelin structure was clearly stained by Luxol Fast Blue in both lateral and dorsal funiculi of the spinal cord. At 24 h after the injury in SCI + saline-operated mice (Fig. 2B1), a significant loss of myelin in lateral and dorsal funiculi was observed. In contrast, in etanercept-treated mice, myelin degradation was attenuated in the central part of lateral (Fig. 2C1) and dorsal funiculi.

To evaluate whether histological damage to the spinal cord was associated with a loss of motor function, the modified BBB hind limb locomotor rating scale score was evaluated. Although motor function was only slightly impaired in sham mice groups (Fig. 3A), operated mice undergoing SCI had significant deficits in hind limb movement (Fig. 3B). In contrast, a significant amelioration of hind limb motor disturbances was observed in etanercept-treated mice (Fig. 3B).

Effects of Etanercept on Neutrophil Infiltration. The above-mentioned histological pattern of spinal cord injury seemed to be correlated with the influx of leukocytes into the spinal cord. Therefore, we investigated the role of inhibition

Statistical Evaluation
All values in the figures and text are expressed as mean ± S.E.M. of n observations. For the in vivo studies, n represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. The results were analyzed by one-way ANOVA followed by a Bonferroni’s post hoc test for multiple comparisons. A p value of less than 0.05 was considered significant. BBB scale data were analyzed by the Mann-Whitney test and considered significant when p value was < 0.05.

Results
Etanercept Reduces the Severity of Spinal Cord Trauma. The severity of the trauma was observed at 24 h after injury. A significant damage to the spinal cord at the level of the perilesional area, assessed by the presence of

![Fig. 3](image) Effect of etanercept on hind limb motor disturbance after spinal cord injury. The degree of motor disturbance was assessed every day until 10 days after SCI by Basso, Beattie, and Bresnahan criteria. Whereas motor function was only slightly impaired in sham mice groups (A), operated mice undergoing SCI had significant deficits in hind limb movement (B). Treatment with etanercept reduces the motor disturbance after SCI (B). Values shown are mean ± S.E.M of 10 mice for each group. *, p < 0.01 versus sham; †, p < 0.01 versus SCI.

![Fig. 4](image) Effects of etanercept on MPO activity. After the injury, MPO activity in spinal cord of no-treated SCI-operated mice was significantly increased at 4 h after the damage compared with sham mice. Treatment with etanercept significantly reduced the SCI-induced increase in MPO activity. Data are means ± S.E.M of 10 mice for each group. *, p < 0.05 versus sham; †, p < 0.01 versus SCI.
of TNF-α on the neutrophil infiltration by measurement of the activity of MPO. MPO activity was significantly elevated at 4 h after SCI in SCI + saline-operated mice compared with spinal cord tissue collected from sham-operated mice (Fig. 4). In etanercept-treated mice, spinal cord MPO activity, at 4 h, was significantly attenuated compared with those of SCI + saline-operated animals.

**Etanercept Modulates Expression of TNF-α and IL-1β after SCI.** Sections of the spinal cord were taken at 24 h after SCI to also determine the immunohistological staining for TNF-α and IL-1β expression to test whether administration of etanercept may modulate the secondary inflammatory reaction also through the regulation of the secretion of cytokines. A substantial increase of IL-1β (Fig. 5B) and TNF-α (Fig. 5B1) formation was found in various cells in the white and gray matter of the spinal cord tissues collected from SCI + saline-treated mice at the 24th hour after SCI (see densitometry analysis in Fig. 6). Spinal cord levels of IL-1β (Fig. 5C) and TNF-α (Fig. 5C1) were significantly attenuated in etanercept SCI-treated mice compared with those of SCI + saline-treated animals (see densitometry analysis in Fig. 6). There was no staining for either IL-1β or TNF-α in spinal cord obtained from the sham group of mice (Fig. 5, A and A1, respectively).

**Etanercept Modulates Expression of Nitrotyrosine, iNOS, and COX-2 after SCI.** To determine the localization of "peroxynitrite formation" and/or other nitrogen derivatives produced during SCI, nitrotyrosine, a specific marker of nitrosative stress, was measured by immunohistochemical analysis in the spinal cord sections of 10 μm at 24 h after SCI.

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**Fig. 5.** Immunohistochemical localization of TNF-α and IL-1β. No positive staining for both IL-1β (A) and TNF-α (A1) was observed in spinal cord tissues obtained from the sham group of mice. SCI caused, at 24 h, an increase in the release of the cytokines IL-1β (B) and TNF-α (B1). Treatment with etanercept significantly inhibited the SCI-induced increase in IL-1β (C) and TNF-α (C1). Figure is representative of at least three experiments performed on different experimental days. wm, white matter; gm, gray matter.
Sections of the spinal cord were taken at the same hour after SCI to also determine the immunohistological staining for iNOS and COX-2. Sections of spinal cord from sham-operated mice did not stain for nitrotyrosine (Fig. 7A) or iNOS (Fig. 8A) or COX-2 (Fig. 8A1). Spinal cord sections obtained from SCI-saline-operated mice exhibited positive staining for nitrotyrosine (Figs. 6 and 7B), iNOS (Figs. 6 and 8B), and COX-2 (Figs. 6 and 8B1) localized in various cells in the white and gray matter of the spinal cord tissues collected from SCI-saline-treated mice. Etanercept (1 h before and 6 h after SCI induction) reduced the degree of positive staining for nitrotyrosine (Figs. 6 and 7C), iNOS (Figs. 6 and 8C), and COX-2 (Figs. 6 and 8C1) in the spinal cord section of SCI-treated mice.

Effects of Etanercept on Apoptosis in Spinal Cord Tissue after Injury. To test whether tissue damage was

Fig. 6. Typical densitometry evaluation. Densitometry analysis of immunocytochemistry photographs (n = 5 photos from each sample collected from all mice in each experimental group) for TNF-α, IL-1β, iNOS, nitrotyrosine, COX-2, Bax, and Bcl-2 from spinal cord tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as the percentage of total tissue area. *, p < 0.01 versus sham; ○, p < 0.01 versus SCI. ND, not detectable.

Fig. 7. Immunohistochemical localization of nitrotyrosine. No positive staining for nitrotyrosine (A) was observed in spinal cord tissues obtained from the sham group of mice. SCI caused, at 24 h, an increase in the nitrotyrosine formation (B). Treatment with etanercept significantly inhibited the SCI-induced nitrotyrosine formation (C). Figure is representative of at least three experiments performed on different experimental days. wm, white matter; gm, gray matter.
associated with cell death by apoptosis, we measured TUNEL-like staining in the perilesional spinal cord tissue. Almost no apoptotic cells were detectable in the spinal cord tissue of sham-operated mice (Fig. 9A). After 24 h, the tissues obtained from SCI/saline-operated mice demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments (Fig. 9, B and B1) in the gray matter of the spinal cord tissues. In contrast, tissues obtained from etanercept-treated mice (Fig. 9C) demonstrated a small number of apoptotic cells or fragments.

**Western Blot Analysis and Immunohistochemistry for Bax and Bcl-2.** The appearance of Bax in homogenates of spinal cord tissues was investigated by Western blot at 24 h after SCI. A basal level of Bax was detectable in the homogenized spinal cord tissues from sham-operated animals (Fig. 10, A and A1). Bax levels were substantially increased in the spinal cord tissues of saline-treated mice (Fig. 10, A and A1). On the contrary, etanercept treatment (1 h before and 6 h after SCI induction) prevented the SCI-mediated Bax expression (Fig. 10, A and A1).

To detect Bcl-2 expression, whole extracts from spinal cord tissue of each rat were analyzed also by Western blot analysis. A low basal level of Bcl-2 expression was detected in spinal cord homogenates from the tissue of sham-operated mice (Fig. 10, B and B1). The Bcl-2 expression significantly was diminished in whole extracts obtained from spinal cord tissues of vehicle-treated mice 24 h after SCI (Fig. 10, B and B1). Treatment of mice with etanercept (1 h before and 6 h...
after SCI induction) significantly reduced the SCI-induced inhibition of Bcl-2 expression (Fig. 10, B and B1).

Moreover, spinal cord tissues were taken at 24 h after SCI to determine the immunohistological staining for Bax and Bcl-2. Sections of spinal cord from sham-operated mice did not stain for Bax (Fig. 11A). Spinal cord sections obtained from SCI/H11001 saline-operated mice exhibited positive staining for Bax (Fig. 11B). Etanercept treatment (1 h before and 6 h after SCI induction) reduced the degree of positive staining for Bax in the spinal cord section of SCI-treated mice (Fig. 11C).

In addition, sections of spinal cord from sham-operated mice demonstrated positive staining for Bcl-2 (Fig. 11A1). Spinal cord sections obtained from SCI + saline-operated mice exhibited significantly less staining for Bcl-2 (Fig. 11B1). Etanercept treatment (1 h before and 6 h after SCI induction) reduced the loss of positive staining for Bcl-2 in the spinal cord section of SCI-treated mice (Fig. 11C1).

**Discussion**

In this report, we demonstrate that a tumor necrosis factor antagonist, etanercept, which inhibits TNF activity by competitively binding to it and preventing interactions with its cell-surface receptors, exerts beneficial effects in a mouse model of SCI. The main findings of the current study are that treatment with etanercept attenuates: 1) the degree of TNF-α and IL-1β in the injured spinal cord, 2) the infiltration of the injured spinal cord with neutrophils, 3) cell apoptosis, 4) the iNOS and COX-2 expression, 5) the nitrotyrosine for-
been well demonstrated that, in SCI, the expression of proinflammatory cytokines, including TNF-α and IL-1β, at the site of injury regulates the precise cellular events after spinal cord injury (Streit et al., 1998). In the present study, we have clearly demonstrated, by immunohistochemistry, a significant increase of positive staining for TNF-α and IL-1β in SCI compared with sham-control animals. On the contrary, no significant expression of TNF-α and IL-1β was observed in the spinal cord sections obtained from etanercept-treated mice. There is a large amount of evidence that TNFα and IL-1β also play an important role in the induction of iNOS, which is known to play an important role in the development of SCI (Matsuyama et al., 1998). In this study, we observe that etanercept treatment reduces the expression of iNOS in SCI-operated mice and we propose that the attenuation of the induction of iNOS expression observed in SCI-operated mice, which are treated with etanercept, is secondary to a reduced formation of endogenous TNFα and IL-1β. This observation is in agreement with other studies that have clearly demonstrated that iNOS expression is reduced by TNF-α antagonists (Perkins et al., 1998). Similar to iNOS, the expression of COX-2 is also mediated by TNFα and IL-1 (Tonai et al., 1999). Recently, Adachi et al. (2005) have point out COX-2 gene induction after traumatic SCI seems not to require any new protein synthesis and that COX-2 protein accumulated in endothelial cells may be a component of the successive inflammatory processes in the damaged area. In this study, we demonstrated that, in sections of spinal cord of etanercept-treated mice, there is a much less positive staining for COX-2 compared with that of SCI + saline-operated mice. Our results, in agreement with other observation (Sairanen et al., 1998), demonstrated a relationship between TNF-α production and the COX-2 expression. In addition to prostaglandins and nitric oxide, several studies have implicated the formation of reactive species of oxygen and reactive species of nitrogen in the secondary neuronal damage of SCI (Xu et al., 2001). In particular, it has been demonstrated that peroxynitrite probably contributes to secondary neuronal damage through pathways resulting from the chemical modification of cellular proteins and lipids (Xu et al., 2001). To confirm the pathological contributions of peroxynitrite to secondary damage after SCI, we have evaluated the nitrotyrosine formation in the injured tissue. We have observed in this study that the immunostaining for nitrotyrosine is reduced in SCI-operated mice treated with etanercept. Nitrotyrosine formation, along with its detection by immunostaining, was initially proposed as a relatively specific marker for the detection of the endogenous formation “footprint” of peroxynitrite (Beckman, 1996). However, there is recent evidence that certain other reactions can also induce tyrosine nitration; e.g., the reaction of nitrite with hypochlorous acid and the reaction of myeloperoxidase with hydrogen peroxide can lead to the formation of nitrotyrosine (Endoh et al., 1994). Therefore, increased nitrotyrosine staining is considered as an indication of “increased nitrosative stress” rather than a specific marker of the peroxynitrite generation. Recent studies have demonstrated the induction of apoptosis in a different cell line in response to reactive oxygen species, peroxynitrite, and nitric oxide (Merrill et al., 1993). One of the most important biological triggers of oligodendrocyte apoptosis in spinal cord injury is TNF-α. In particular, it has been shown that TNF-α induces apoptosis in oligodendrocytes both in vitro and in vivo (Muzio et al., 1997) by the...
activation of caspase-3 and caspase-8 (Hisahara et al., 1997). In the present study, using the tunnel coloration, we have clearly confirmed that TNF-\( \alpha \) plays an important role in the induction of apoptosis during SCI and that the treatment with etanercept attenuates the degree of apoptosis. Moreover, it is well known that Bax, a proapoptotic gene, plays an important role in developmental cell death (Chittenden et al., 1995) and in CNS injury (Bar-Peled et al., 1999). Likewise, it has been shown that the administration of Bcl-xL fusion protein (Bcl-2 is the most expressed antiapoptotic molecule in adult central nervous system) into injured spinal cords significantly increased neuronal survival, suggesting that SCI-induced changes in Bcl-xL contribute considerably to neuronal death (Nesic-Taylor et al., 2005). Based on these evidences, we have identified proapoptotic transcriptional changes, including up-regulation of proapoptotic Bax and down-regulation of antiapoptotic Bcl-2, using Western blot assay and by immunohistochemical staining. We report in the present study for the first time that the treatment with etanercept by inhibiting TNF-\( \alpha \) in SCI prevents the loss of apoptosis induced by SCI.

Fig. 11. Immunohistochemical expression of Bax and Bcl-2. No positive staining for Bax was observed in the tissue section from sham-operated mice (A). SCI caused, at 24 h, an increase in the release of Bax expression (B). Treatment with etanercept significantly inhibited the SCI-induced increase in Bax expression (C). On the contrary, positive staining for Bcl-2 was observed in the spinal cord tissues of sham-operated mice (A1). At 24 h after SCI, significantly less staining for Bcl-2 was observed (B1). The etanercept treatment significantly prevents the loss of Bcl-2 expression induced by SCI (C1). Figure is representative of at least three experiments performed on different experimental days.
the antiapoptotic way and reduces the proapoptotic pathway activation with a mechanism still to discover. Finally, in this study, we demonstrate that etanercept treatment significantly reduced the SCI-induced spinal cord tissue alteration and improve the motor function. The purpose of this article was to highlight our current knowledge on the interaction of post-traumatic immune reactivity and the development of complications. A better understanding of these mechanisms might lead to the introduction of preventive and therapeutic strategies into clinical practice.

References


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