Upregulation of Tumor Necrosis Factor α Transport across the Blood–Brain Barrier after Acute Compressive Spinal Cord Injury

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Tumor necrosis factor α (TNF) is a cytokine that is involved in the inflammatory process after CNS injury and is implicated in neuroregeneration. A saturable transport system for TNF located at the blood–brain barrier (BBB) is responsible for the limited entry of TNF from blood to the CNS in normal mice.

After partial disruption of the BBB by compression of the lumbar spinal cord, permeability to TNF was increased not only in the lumbar spinal cord but also in brain and distal spinal cord segments, where the BBB remained intact. The increase in the entry of TNF to the CNS followed a biphasic temporal pattern, with a first peak immediately after injury and a second peak starting on day 3; these changes lasted longer than the mere disruption of the BBB. The increased entry of TNF was abolished by addition of excess unlabeled TNF, showing that the transport system for TNF remained saturable after spinal cord injury (SCI) and providing evidence that the enhanced entry of TNF could not be explained by diffusion or leakage.

This study adds strong support for our concept that the saturable transport system for TNF across the BBB can be upregulated in the diseased state, and it suggests that the BBB is actively involved in the modulation of the processes of degeneration and regeneration after SCI.

Key words: TNFα; SCI; BBB; transport system; CNS; neuroregeneration; upregulation

Spinal cord injury (SCI) in mammals is associated with disruption of the blood–brain barrier (BBB), increased cytokine production, and degeneration of CNS tissue with poor functional recovery (McKenzie et al., 1995; Popovich et al., 1996; Jaeger and Blight, 1997; Pan et al., 1997b). Production of tumor necrosis factor α (TNF) is increased after SCI (Wang et al., 1996). The CNS effects of this cytokine can be either detrimental or beneficial. Detrimental effects include apoptosis and direct cell toxicity (Gelbard et al., 1993; Chao and Hu, 1994; Miura et al., 1995; Vandenabeele et al., 1995). Beneficial effects include stimulation of the synthesis of neurotrophic proteins and facilitation of the permissiveness of the substrate for neurite outgrowth after CNS injury (Schwartz et al., 1991; Genestier et al., 1995; Mattson, 1997). Thus, TNF can be involved in neuroregeneration.

TNF also can exert different effects on the BBB, including changes in permeability (Camussi et al., 1991; Kim et al., 1992; Duchini et al., 1996; Descamps et al., 1997; Pan et al., 1997c). It is not known whether TNF is involved in the biphasic opening of the BBB that has been observed in some pathological states, including cortical contusion (at 4–6 hr and again 3 d after injury) (Baskaya et al., 1997), transient unilateral middle cerebral artery occlusion (immediately after recirculation and again 5–72 hr later) (Kuroiwa et al., 1985), and lumbar SCI by transection (an immediate increase in adjacent areas and a latent increase in more distal regions) (Pan et al., 1997b). The production of TNF also may be biphasic, such as in traumatic brain injury (Fan et al.,

1994). Whether the increase in TNF after trauma is related to disruption of the BBB, however, is not clear.

The interaction between TNF and the BBB in normal animals involves a saturable transport system at the BBB, which regulates the entry of TNF in peripheral blood to the brain and spinal cord unidirectionally (Gutierrez et al., 1993; Pan et al., 1997a). In animals with SCI, one possibility is that the resulting disruption of the BBB might cause greater entry of TNF from the periphery to the CNS secondary to leakage. However, after acute lumbar SCI by transection in mice, we have found that an enhanced transport system for TNF rather than leakage is mainly responsible for the increased entry of TNF (Pan et al., 1997b). Upregulation of the TNF transport system also occurs in experimental autoimmune encephalomyelitis (EAE), a nontraumatic model in which the autoimmune attack is directed primarily against central myelin components, resulting in an ascending but generalized disruption of the BBB that correlates with the functional deficit (Pan et al., 1996). Because TNF is produced in the CNS by both neurons and astrocytes (Pan et al., 1997c), the increased entry of TNF from the periphery may be indicative of an autoregulatory function restricting further excessive production in the CNS by a negative feedback mechanism.

Having found that the TNF transport system across the BBB is increased in two different pathological states (SCI by complete transection and EAE), we investigated the possibility that this upregulation is a more generalized phenomenon. In the study reported here, we applied a compression model of SCI to further study the alterations of the saturable transport system for TNF. The time course and spatial pattern of TNF entry at various times after SCI were measured and compared with entry of radiolabeled albumin, which was indicative of disruption of the BBB. The results were designed to answer the following questions. (1) How does the disruption of the BBB differ in the compressive

model of SCI? (2) Does TNF enter the injured CNS by leakage only? (3) If not, where is the transport system for TNF preserved and how does it function? In general, knowledge about the regulation of the availability of TNF to the injured CNS should provide a novel approach, with possible therapeutic implications to understanding how inflammatory processes limit degeneration in the CNS.

MATERIALS AND METHODS

Spinal cord compression. Adult male ICR mice received deep anesthesia by intramuscular ketamine and xylazine (Sigma, St. Louis, MO). The skin was incised longitudinally on the lower back, the supraspinal muscles were separated, and laminectomy was performed on L1 and L2 vertebral bones with a pair of iridectomy scissors (Fine Science Tools, Foster City, CA). The spinal cord at this level was compressed by initially flanking the spinal cord with a pair of modified cover glass forceps at a length of 6 mm (Fine Science Tools). SCI involved steady compression of the 3 mm spinal cord to a width of 1 mm for 5 sec. After the injury, the subcutaneous tissue and skin were sutured in layers. The mice were placed on a heating pad until recovery from anesthesia within 1 hr and then were returned to their cages containing soft bedding and easy access to food and water. Manual compression of the bladder was performed for those with bladder distension. The extent of injury was quantified daily with the locomotor rating scale shown in Table 1 ($n = 14$–18 for daily scoring).

Measurement of BBB permeability. Twelve groups of mice were studied ($n = 8$–12 per group). Experimental groups were tested immediately after SCI (5 min) at 0.5, 1, 2, 4, and 12 hr and then 1, 2, 3, 4, and 5 d after SCI. A nonsurgical control group was included in each experiment. The mice were anesthetized by intraperitoneal injection of 40% urethane (Sigma). The neck of the mouse was exposed, and the jugular veins and the right carotid artery were separated. Approximately 0.9 μCi/mouse ^{125}I-TNF (R & D Systems, Minneapolis, MN), together with 1.8 μCi/mouse ^{99m}Tc-albumin (Amersham Health Care, San Antonio, TX), was injected in a total volume of 200 μl into the left jugular vein. The radioactive solution was mixed in lactated Ringer's solution containing 1% bovine serum albumin. At 10 min after intravenous injection, blood was collected from a cut in the carotid artery, and the mouse was decapitated immediately. Samples of brain and spinal cord (cervical, thoracic, and lumbar regions) were collected and weighed. The tissue sample and 50 μl of serum were counted in a dual channel γ-counter (Wallac, Gaithersburg, MD). The tissue/serum ratio was calculated and expressed as microliters per grams. The entry of ^{99m}Tc-albumin was used as a measurement of BBB permeability in general, whereas the entry of ^{125}I-TNF may be a combination of leakage and transport across the BBB.

Inhibition study to test saturability. Four groups of mice were studied ($n = 8$–9 per group): (1) control (non-SCI) injected with ^{125}I-TNF and ^{99m}Tc-albumin only; (2) control with addition of 1 μg/mouse (~40 μg/kg body weight) unlabeled TNF in the injection solution of ^{125}I-TNF and ^{99m}Tc-albumin; (3) mice at 3 d after SCI injected with ^{125}I-TNF and ^{99m}Tc-albumin only; and (4) mice at 3 d after SCI with addition of 1 μg/mouse unlabeled TNF in the injection solution with the radiolabeled compounds. BBB permeability was studied as mentioned above.

Statistical analysis. Statistical Program for the Social Sciences software was used. Means were expressed with their SEs, ANOVA was performed for all groups, and, subsequently, Tukey's range test was performed if ANOVA showed statistical significance ($p < 0.05$). Sigma Plot was used to generate the figures.

RESULTS
Functional deficit after SCI

Figure 1 shows that SCI produced a deficit of locomotor activity in the mice that began immediately after their awakening from anesthesia ($n = 14$–18). Eighteen mice underwent SCI on the same day and were evaluated subsequently for 23 d; four of them died of complications during the first week. The reduction in the combined scores was statistically significant during the first 8 d after injury ($p < 0.01$ for days 3, 4, 5, and 6; $p < 0.05$ for the rest of the 8 d).

Table 1. Scoring scales for mice after mild to moderate compressive spinal cord injury

Locomotor activity
0   No movement in hind limbs, extended paralysis bilaterally, limp tail
1   One hind limb paralyzed and extended, one hind limb paretic and flexed. Tail partially weak
2   One hind limb paralyzed and extended, one hind limb has weight bearing, or bilateral hind limbs have flexed paresis
3   Bilateral hind limbs flexed with minimal weight bearing, or one hind limb relatively normal with weight bearing, one hind limb has flexed paresis
4   Walks with mild deficit, bears weight in bilateral hind limbs
5   Normal walking

Extension withdrawal (reflexion of the hind limb when manually extended)
0   No withdrawal
1   Weak, slow withdrawal on one side, or with normal withdrawal on one side, no withdrawal on the other
2   Weak, slow withdrawal on both sides
3   Normal withdrawal bilaterally

Parachuting reflex (posturing of the body and limbs when held by tail with head downwards)
0   Total body flexed with tremor, no toe spread
1   Moderately impaired body and hind limb positioning, limited toe spread on either side
2   Mildly impaired body and hind limb positioning, nearly full toe spread
3   Normal

Righting reflex (return to prone position after being placed supine on a smooth surface)
0   No righting
1   Prolonged return to prone position
3   Normal righting

Inclined plane (the angle of the test plane to the horizontal plane when the mouse is able to hold for >5 sec while facing downward)
0   No grabbing on hind limbs
1   Angle <30°
2   Angle ≥30°, but <45°
3   Angle ≥45°, but 60°
4   Angle ≥60°

Time course of BBB disruption after SCI as measured by ^{99m}Tc-albumin

The extent of BBB disruption at different time intervals after SCI was reflected in the tissue/serum ratios of ^{99m}Tc-albumin injected intravenously 10 min before each determination. In the baseline (time 0) control group (no SCI), the cervical area had the greatest permeability among the four regions. After SCI, statistically significant changes occurred in the thoracic and especially the lumbar spinal cord but not in the brain or the cervical spinal cord. The significant change in the thoracic spinal cord consisted of decreased permeability at day 5 after injury ($p < 0.05$).

In the lumbar spinal cord, where most of the disruption was evident, a brief increase in entry occurred immediately (5 min after injury) ($p < 0.01$) after SCI and remained elevated 0.5 hr later ($p < 0.01$). Permeability to ^{99m}Tc-albumin in the lumbar area returned to basal levels 1 hr after SCI and remained there, except for a smaller second increase ($p < 0.05$) on day 3 (Fig. 2).
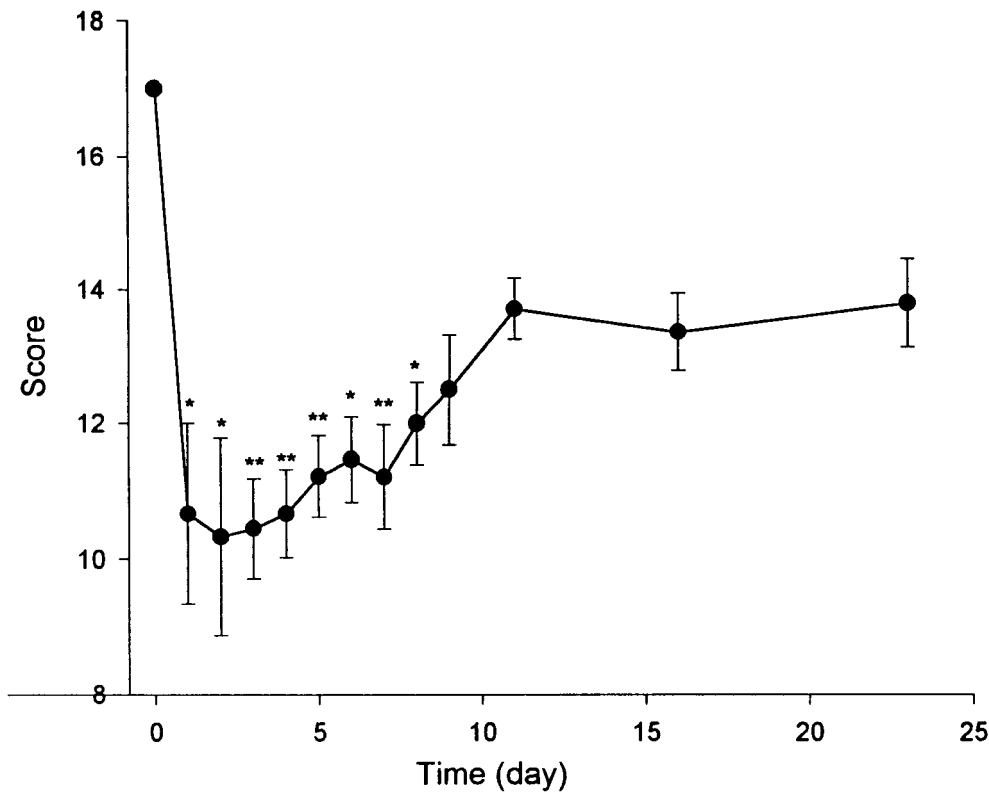
Figure 1. Functional deficit after acute compression of the lumbar spinal cord on day 1. Time 0, Control without SCI with a full score of 17. The decrease in scores was statistically significant from day 1 to day 8. $*p < 0.05$; $**p < 0.01$ versus control ($n = 14$–18 per group).
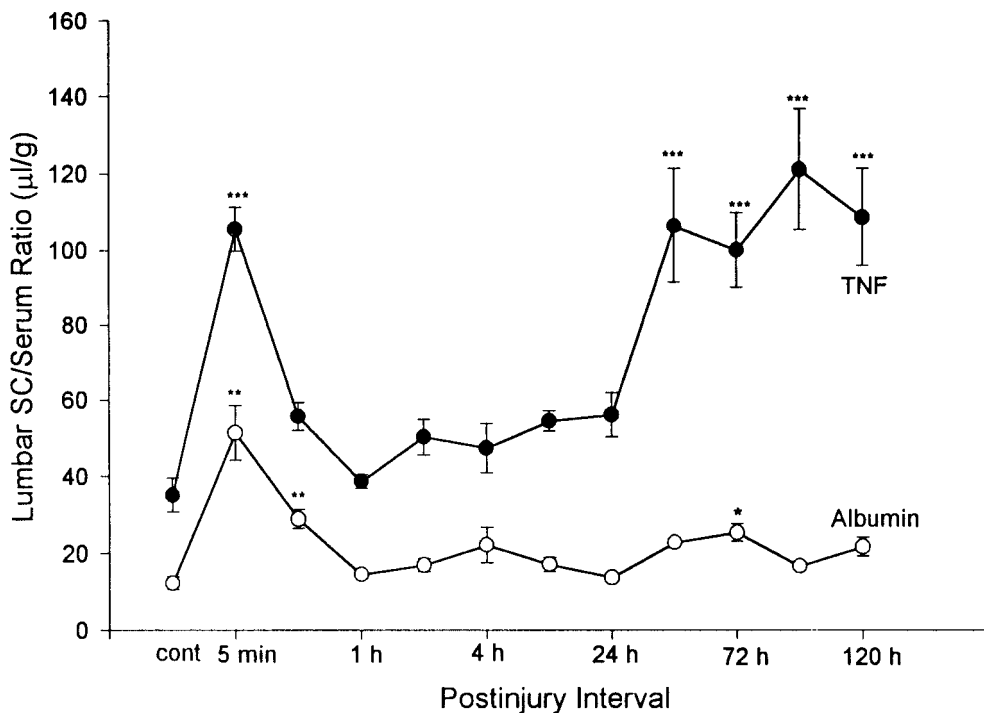

Figure 2. BBB permeation of ^{99m}Tc-albumin and ^{125}I-TNF in the lumbar spinal cord after acute lumbar SCI. Postinjury intervals signify the different time points after injury when the entry of the two compounds was studied. The intervals are as follows: (1) control without injury; (2) immediately (5 min) after injury; (3) 0.5 hr; (4) 1 hr; (5) 2 hr; (6) 4 hr; (7) 12 hr; (8) 24 hr; (9) 48 hr; (10) 72 hr; (11) 96 hr; and (12) 120 hr after injury. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ versus control group. *SC*, Spinal cord.

Increased permeability to ^{125}I-TNF in comparison with BBB disruption

The BBB permeability to ^{125}I-TNF, also measured 10 min after intravenous injection, was higher than that to ^{99m}Tc-albumin in each CNS region in both control and SCI mice. In comparison with the control group, entry of ^{125}I-TNF was significantly increased in the brain on days 3 ($p < 0.01$), 4 ($p < 0.001$), and 5

($p < 0.001$). The spinal cord had a greater increase in the entry of ^{125}I-TNF than the brain. The cervical spinal cord showed increases at 12 hr ($p < 0.05$) and day 5 ($p < 0.001$), whereas the thoracic spinal cord showed an increase on day 2 ($p < 0.001$). In the lumbar spinal cord, there was a transient peak immediately (5 min) after SCI ($p < 0.001$), which paralleled the increase of ^{99m}Tc-albumin temporally as shown in Figure 2; permeability
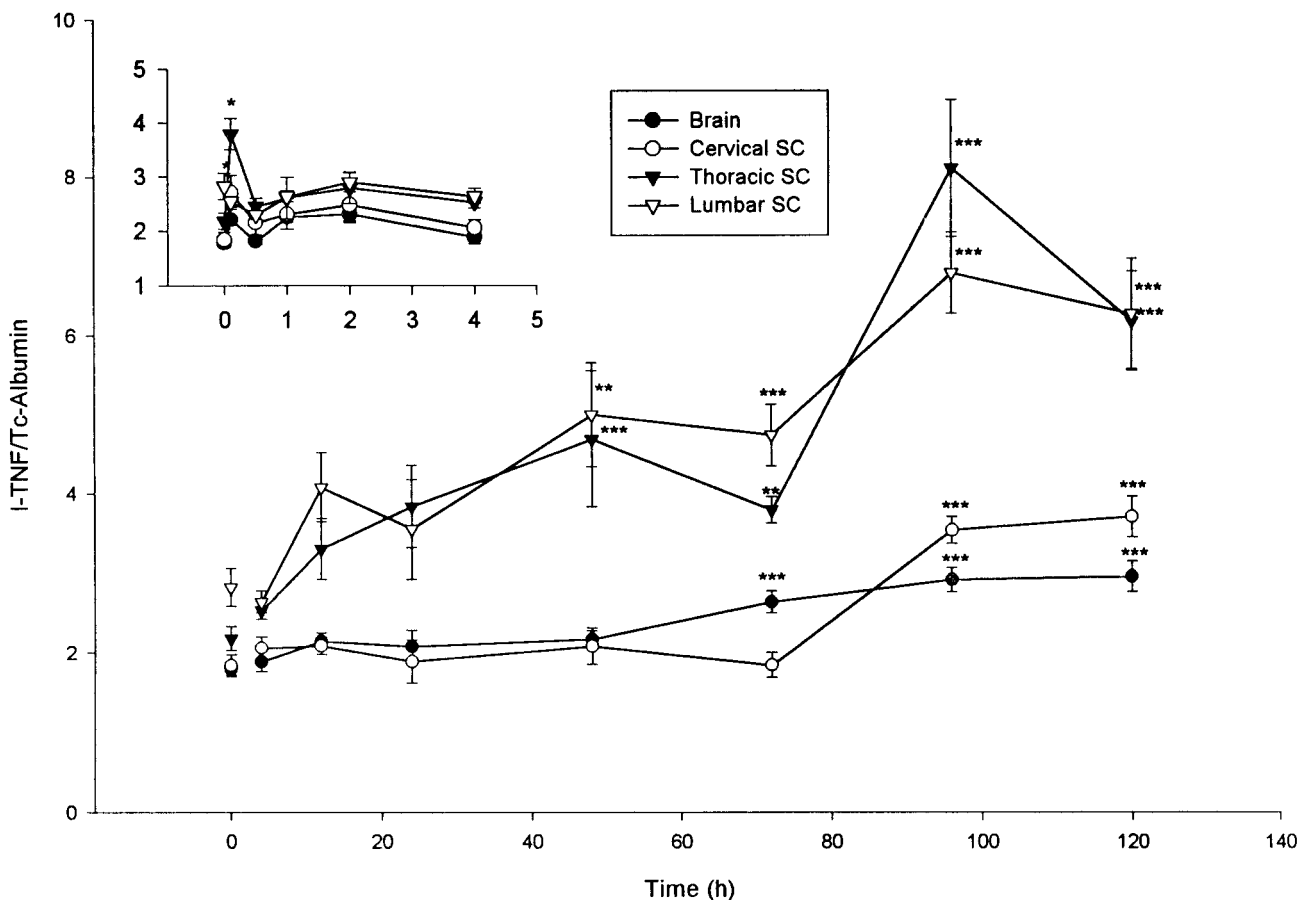
Figure 3. Differential permeability of the BBB after acute compression of the lumbar spinal cord. The ratio of the entry of ^{125}I-TNF to ^{99m}Tc-albumin is plotted against time after SCI performed at time 0. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control. The *inset* shows the earlier times (in hours). *SC*, Spinal cord.

returned to almost normal between 0.5 and 24 hr after injury. A second, more prolonged, peak of increased permeability to ^{125}I-TNF in the lumbar spinal cord occurred at 48 hr (day 2, $p < 0.001$), earlier than the second increase in ^{99m}Tc-albumin, and remained elevated ($p < 0.001$) up to the end of the study at 120 hr (day 5 after injury).

When the ratio of the entry of ^{125}I-TNF to that of the entry of ^{99m}Tc-albumin was plotted against time to clearly illustrate differential permeability (Fig. 3), it was seen that the immediate increase was significant ($p < 0.05$) in the cervical and thoracic areas, as well as the lumbar spinal cord. Furthermore, the prolonged second increase was significant in the thoracic ($p < 0.001$) and lumbar spinal cord ($p < 0.005$) from day 2 after injury, remaining elevated ($p < 0.001$) for the 5 d of the study. Increases also were present in brain (day 3–5, $p < 0.001$) and cervical spinal cord (day 4–5, $p < 0.001$).

When the entry of ^{99m}Tc-albumin was subtracted from the entry of ^{125}I-TNF as a correction for vascular volume and leakage secondary to BBB disruption, the pattern was somewhat different from that of the differential permeability. The initial increase was transient and appeared only in the lumbar spinal cord ($p < 0.005$). For brain and lumbar spinal cord, the second peak started on day 2 after injury ($p < 0.001$) and lasted until day 5. For the thoracic spinal cord, the elevation was significant on day 2–4 ($p < 0.05$). The cervical spinal cord showed a significant increase at day 5 ($p < 0.005$).

Inhibition of increased permeability to ^{125}I-TNF

Figure 4 shows the effects of unlabeled TNF on the differential permeability of TNF/albumin 3 d after SCI. After SCI, the increase in the differential permeability was statistically significant ($p < 0.01$) in the brain, thoracic, and lumbar spinal cord but not in the cervical region. Just as saturability in the control groups was demonstrated by the self-inhibition seen after addition of unlabeled TNF ($p < 0.001$), a similar pattern of inhibition of ^{125}I-TNF entry also was found in the SCI groups. Not only was inhibition of entry of ^{125}I-TNF found with unlabeled TNF in the SCI groups in which entry was already increased by the injury, but saturability occurred in all four of the CNS regions ($p < 0.001$).

DISCUSSION

In the acute stage after lumbar SCI, disruption of the BBB (indicated by dynamic changes in the entry of albumin) was biphasic and restricted to the lumbar spinal cord. In contrast, the entry of TNF was more pronounced in several ways: its magnitude was greater; it occurred earlier; it lasted for a longer time; it involved distal regions, as well as the lumbar spinal cord; and it retained the self-inhibition characteristic of a saturable transport system, which would not be seen with diffusion or leakage.

SCI by compression to a set thickness provides a reproducible model of functional deficit with correlated histological findings that have been well characterized in guinea pigs (Blight, 1991). In our study, the deficit was mild but significant, and the mice
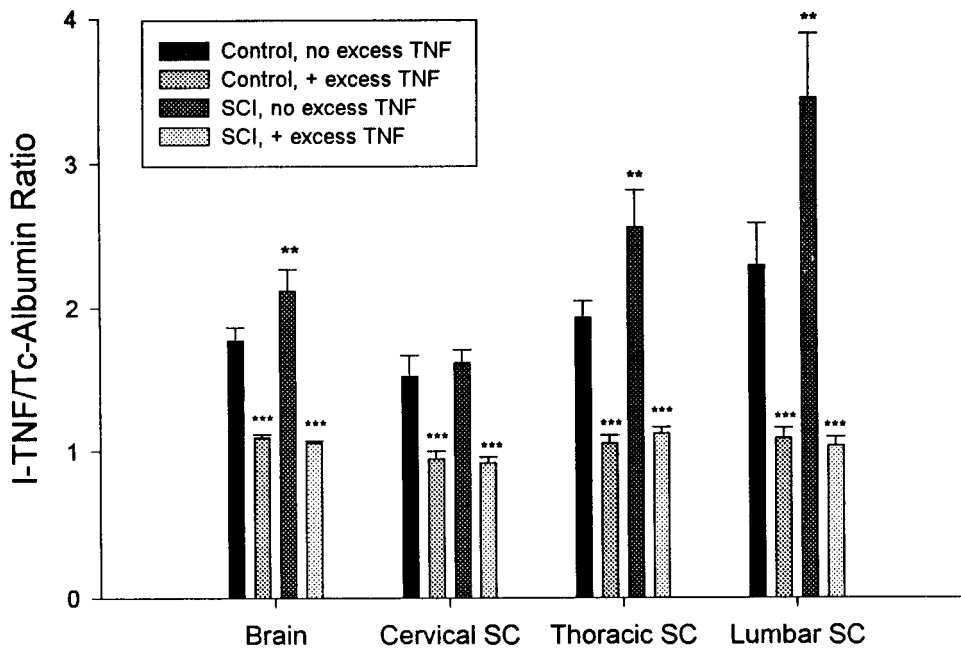
Figure 4. Inhibition of the differential permeability of the BBB after acute compression of the lumbar spinal cord. Groups are as follows: (1) control with ^{125}I-TNF and ^{99m}Tc-albumin; (2) control with ^{125}I-TNF and ^{99m}Tc-albumin and 1 μg/mouse unlabeled TNF; (3) 72 hr after injury with ^{125}I-TNF and ^{99m}Tc-albumin; and (4) 72 hr after injury with ^{125}I-TNF and ^{99m}Tc-albumin and 1 μg/mouse unlabeled TNF. SCI significantly (**$p < 0.01$) increased the TNF/albumin ratio in brain and in thoracic and lumbar spinal cord (group 3 vs group 1). Unlabeled TNF significantly (***$p < 0.001$) decreased the TNF/albumin ratio compared with both control group 1 and SCI group 3. *SC*, Spinal cord.

showed functional improvement within 10 d. Meanwhile, leakage of the BBB, measured by the entry of radiolabeled albumin (which essentially does not cross the intact BBB and reflects the vascular space), showed an increase confined to the lumbar spinal cord. This disruption of the BBB appeared to be biphasic, peaking immediately after injury and again at day 3 after SCI, although both peaks were transient. This is different from the results of previous subacute or chronic studies (Jaeger and Blight, 1997; Banks et al., 1998). In general, disruption of the BBB in the acute stage of SCI did not correlate with the observed functional deficit. Because BBB disruption is related to the activation and invasion of macrophages from the periphery (Fitch and Silver, 1997), a major cytokine product of activated macrophages (TNF) may play an important role in the recurrence of BBB opening.

The entry of TNF into the CNS, in comparison with albumin, initially followed a similar pattern in that there was an early peak of BBB opening in the lumbar spinal cord. However, the second increase in the entry of TNF preceded that of albumin, was much greater, and lasted a longer time. In addition, in regions that are distal to the site of injury and that did not show the disruption of the BBB characterized by albumin, the entry of TNF also was increased. Because TNF circulates in the blood as a trimer (Smith and Baglioni, 1987) and is comparable in molecular size with albumin (60 kDa), the sensitivity of TNF as a detector of BBB leakage should be similar to that of albumin. Because its entry was much greater than that of albumin and was saturable, the increased entry of TNF after SCI was not solely attributable to leakage of the BBB. Moreover, if TNF caused further disruption of the BBB and thereby facilitated its own penetration into the CNS, the coadministered albumin should have shown similar changes. In our studies, disruption of the BBB is not observed after intravenous bolus administration of up to 100 μg/kg TNF or other cytokines (Gutierrez et al., 1993; Pan et al., 1997a).

Thus, the more widespread and persistent increase in the entry of TNF from the periphery is not related to disruption of the BBB but rather to alternative mechanisms of BBB transport. To illustrate this point further, we performed two conversions. The first showed the differential permeability of TNF and albumin by

expression of the results as the ratio of ^{125}I-TNF to ^{99m}Tc-albumin. The differential permeability best reflects dynamic changes during partial disruption of the BBB in which molecules with different sizes cross at different rates (Ziylan et al., 1983), and it also has been shown to be a good indicator when two substances use different mechanisms for passage (Pan et al., 1996, 1997b). The second conversion corrected for nonspecific leakage by subtraction of albumin from the entry of TNF (^{125}I-TNF − ^{99m}Tc-albumin). Both results clearly demonstrate that the entry of TNF differs from that caused by disruption of the BBB.

In normal mice, TNF has a unidirectional transport across the BBB from blood to the CNS, with an influx rate of 0.622 ± 0.092 $\mu l \cdot g^{-1} \cdot min^{-1}$ in the lumbar region and a very low initial volume of distribution that indicates little nonspecific association with the cerebral vasculature (Pan et al., 1997a). Most ^{125}I-TNF in the brain is present in parenchyma as demonstrated by capillary depletion studies. In addition, the radioactivity represents intact TNF rather than a degradation product as shown by reversed-phase HPLC (Gutierrez et al., 1993). The self-inhibition of ^{125}I-TNF entry by unlabeled TNF proves the saturability of the transport system in all regions of spinal cord, as well as in the brain (Pan et al., 1997a). Because the increased entry of TNF after SCI involved regions and time intervals for which BBB disruption was not detected, the evidence indicates that an enhanced transport system was responsible for the increased entry of TNF. As would be expected for any transport system across the BBB, even an enhanced one such as seen with TNF in this study, there was significant self-inhibition. Together, the results indicate that upregulation of the saturable transport system for TNF is responsible for the increased entry of TNF after SCI. This transport system is likely to be a carrier protein at the BBB that is specific for TNF without interaction with the transport systems of other cytokines, such as interleukins (Gutierrez et al., 1993).

There are pathophysiological considerations for the upregulation of TNF transport system at the BBB. On the one hand, adequate TNF in the damaged CNS is related to increased neurite outgrowth by recruitment and activation of macrophages, counteracting myelin-associated inhibitory molecules so as to

provide a permissive substrate for axonal regrowth (Lotan and Schwartz, 1994; Schwartz et al., 1994) and facilitate neuroregeneration (Schwartz et al., 1991; Klusman and Schwab, 1997). After compressive SCI in the rat, axonal regeneration is facilitated by transplantation of macrophages, which secrete TNF but not neurotrophins (Franzen et al., 1998). On the other hand, TNF overproduction may lead to apoptosis after SCI (Li et al., 1996) or result in direct toxicity, as well as hyperactive inflammatory and immune responses (Probert and Selmaj, 1997; Probert et al., 1997; Shohami et al., 1997).

Production of TNF within the CNS is induced after injury (Fan et al., 1994, 1996). The TNF could arise from several CNS cells, such as microglia (Boddeke et al., 1995) and neurons (Liu et al., 1994) after focal ischemia, or astrocytes in various pathological conditions (Pan et al., 1997c). Yet a peripheral source still seems to be prominent, particularly because CNS trauma is usually accompanied by peripheral tissue injury. The second peak of TNF entry preceded the general disruption of the BBB measured by albumin entry and reflected upregulation of TNF transport system in this study. This peak corresponds with the marked increase in macrophage recruitment observed in a mouse model of compressive SCI (Fujiki et al., 1996) and also is similar in time course to infiltration of inflammatory leukocytes after traumatic brain injury (Soares et al., 1995). Thus, the second peak of TNF entry into the CNS provides a direct source of TNF that may be accompanied by an extra indirect source from infiltrating macrophages. The limited entry of TNF from the periphery to regions distant from the site of injury might not only provide a feedback signal to restrict local production but may also serve as a mediator for neuroregeneration. Above all, an adequate concentration of TNF seems to be essential for homeostasis in the CNS, and a saturable transport system regulated by pathological changes may help to control the entry of additional TNF from the periphery and restrict TNF production at the site of SCI.

In summary, although lumbar spinal cord compressive injury caused a restricted biphasic opening of the BBB, it caused a much greater increase in the permeation of TNF from blood to all CNS regions. The prolonged increased entry of TNF during the second phase was related to the upregulation of a saturable transport system rather than to disruption of the BBB. This upregulation showed active involvement of the BBB during SCI in controlling the availability of the blood-borne cytokine. The increased, but still limited, entry of TNF from blood to the CNS may be beneficial in restricting secondary tissue damage and in aiding regeneration of the CNS.

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