EFFECTS OF ANTITHROMBIN III ON MYELOPEROXIDASE ACTIVITY, SUPEROXIDE DISMUTASE ACTIVITY, AND MALONDIALDEHYDE LEVELS AND HISTOPATHOLOGICAL FINDINGS AFTER SPINAL CORD INJURY IN THE RAT

Tahsin Erman, M.D.
Department of Neurosurgery, Çukurova University School of Medicine, Adana, Turkey

M. Şule Yıldız, Ph.D.
Department of Biochemistry, Çukurova University School of Medicine, Adana, Turkey

A. İskender Göçer, M.D.
Department of Neurosurgery, Çukurova University School of Medicine, Adana, Turkey

Suzan Zorludemir, M.D.
Department of Pathology, Çukurova University, School of Medicine, Adana, Turkey

Hakan Demirhindi, M.D.
Department of Public Health, Çukurova University, School of Medicine, Adana, Turkey

Metin Tuna, M.D.
Department of Neurosurgery, Çukurova University, School of Medicine, Adana, Turkey

Reprint requests:
Tahsin Erman, M.D., Department of Neurosurgery, Çukurova University School of Medicine, Balcalı-Adana 01330, Turkey.
Email: ermant@cu.edu.tr

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OBJECTIVE: Among the many possible mechanisms of the secondary spinal cord injury (SCI), microcirculatory disturbances as a result of activated leukocyte-induced endothelial cell injury is important because it is potentially treatable and reversible. Currently, clinically available pharmacological agents for treatment of acute SCI do not inhibit neutrophil activation. The effect of antithrombin III (AT-III) on neutrophil activation was studied in rats with SCI produced with an aneurysm clip on the T2–T7 segments.

METHODS: Forty rats were randomly allocated to four groups. Group I (10 rats) was killed to provide normal spinal cord tissue for testing. Group II (10 rats) underwent a six-segment laminectomy for the effects of total laminectomy to be determined. In Group III, 10 rats underwent a six-segment laminectomy and SCI was produced by extradural compression of the exposed cord. The same procedures were performed in 10 rats in Group IV, but they also received one (250 IU/kg) intraperitoneal injection of AT-III immediately after the injury and a second dose 24 hours later. The animals from Groups II through IV were killed 48 hours after the trauma. The effect of AT-III on the myeloperoxidase activity, superoxide dismutase activity, and malondialdehyde levels and histopathological findings were studied.

RESULTS: Myeloperoxidase activity, superoxide dismutase activity, and malondialdehyde levels were significantly lower and there was less histopathological damage in the AT-III treatment group than in the trauma group.

CONCLUSION: The results demonstrate that AT-III treatment may reduce secondary structural changes in damaged rat spinal cord tissue by inhibiting leukocyte activation.

KEY WORDS: Activated leukocyte, Antithrombin III, Malondialdehyde, Myeloperoxidase, Rat, Spinal cord injury, Superoxide dismutase

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Injury to the spinal cord often results in irreversible functional loss. In addition to the immediate neuronal loss occurring after trauma in the spinal cord, secondary degeneration is now recognized as a major contributor to the severity of the damage (40). Secondary degeneration results from autodestructive events that affect uninjured surrounding neurons and lead to their degeneration and death (8, 40). The extent and severity of the secondary injuries depend on the magnitude of the initial insult as well as on contributing factors, including substances released into the microenvironment, cell reactions in the cord tissue, reduction in blood flow to the site, changes in the use of O_2 and glucose by the damaged tissue, reduction of extracellular Ca^{2+}, release of free radicals, lipid peroxidation of cell membranes, changes in levels of neuropeptides and monoamines, production of arachidonic acid metabolites, and overproduction of thrombin (5, 10, 37, 38, 40).

Activated neutrophils have been demonstrated to be implicated in the secondary degeneration of spinal cord injury (SCI) in rats (14, 31, 42, 43). However, little is known about the role of neutrophils in the pathogenesis of SCI. Activated neutrophils damage the endothelial cells by releasing inflammatory media-

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tors (14, 31, 42, 43). This endothelial cell injury could, in turn, induce microcirculatory disturbances leading to spinal cord ischemia (26, 39–42). Recently, Xu et al. (51) demonstrated that neutrophil infiltration as determined by myeloperoxidase (MPO) activity is proportional to the magnitude of spinal trauma.

Among the many possible mechanisms of secondary injury, microcirculatory disturbance as a result of activated leukocyte-induced endothelial cell injury is important because it is potentially treatable and reversible. Currently, clinically available pharmacological agents for treatment of acute SCI do not inhibit neutrophil activation (40, 42). Taken together, these observations raise a possibility that pharmacological agents inhibiting neutrophil activation may have a beneficial effect in the treatment of traumatic SCI.

Antithrombin III (AT-III), an α_2-globulin, is the physiological inhibitor of thrombin and other serine proteases (6, 18, 19, 22, 31, 45). AT-III may exert a therapeutic effect independent of its anticoagulant effect. It has been demonstrated to promote the endothelial release of prostacyclin (PGI_2). PGI_2 is a potent inhibitor of platelet aggregation and leukocyte activation, it has vasodilatory action, and it may contribute to the maintenance of normal microcirculation (29, 31, 46). In clinical and experimental studies, AT-III is known to attenuate acute inflammation and disseminated intravascular coagulation caused by sepsis, trauma, and endotoxin (7, 15, 18, 19, 31). Additionally, the protective effect of AT-III on ischemia-reperfusion–induced organ injury, such as that to the liver, lung, intestine, kidney, and optic nerve, has been described (31, 34, 45). It is well known that ischemia and reperfusion can induce inflammatory reactions within the microcirculation similar to traumatic SCI.

AT-III exists physiologically in plasma and is widely used in human patients without any reported serious side effects. We did not find any information about the effect of AT-III on SCI, however. We hypothesized that AT-III might be effective in the treatment of SCI. Therefore, in the present study, we examined the effect of AT-III on SCI and the probable effect mechanisms of AT-III in an experimental SCI model. We evaluated the results of this treatment by determining MPO activity, superoxide dismutase (SOD) activity, and malondialdehyde (MDA) levels and examining histopathological findings.

MATERIALS AND METHODS

Seventy adult male Wistar rats, each weighing between 270 and 300 g, were used in the study. All animals received an anesthetic dose of ketamine (50 mg/kg) and xylazine (10 mg/kg). We used the clip compression model described by Rivlin and Tator (33) and produced SCI by extradural compression of a section of cord exposed by a four-segment T2–T7 laminectomy, in which the prominent spinous process of T2 was used as a surgical guide. A six-segment laminectomy was chosen to expedite timely harvest and to obtain enough spinal cord tissue for biochemical examination. With the aneurysm clip applicator oriented in the bilateral direction, an aneurysm clip (Yasargil, FE710K; Aesculap AG, Tuttlingen, Germany) with a closing force of 50 g was abruptly applied externally to the dura. The clip was then rapidly released from the clip applicator, which caused spinal cord compression. In the injured groups, the cord was compressed for 1 minute. The same clip was used in all animals in which SCI was induced.

To assess for possible variation in lesion severity, we conducted a preliminary study and applied this method in a separate group of 20 rats. Ten weeks after clip compression injury, the rats' spinal cords were removed, fixed in buffered neutral formalin, embedded in paraffin, cut in 8-μm-thick serial sections, and stained by the Klüver-Barrera Luxol fast blue method. Six rats died during the 10-week period after injury, and these animals were excluded. All specimens were examined under light microscopy, according to the degree of cavitation over the entire surface of the cord and in the corticospinal tract. The results were all in the range of 75 to 85%, which indicated that there was no significant animal-to-animal difference in lesion severity using this method.

The 40 rats in the experiment were randomly allocated to the following groups: a group of 10 uninjured controls (Group I), a group of 10 rats that underwent a six-segment laminectomy only (Group II, sham-operated), and a group of 20 rats in which SCI was induced. Immediately after SCI was produced, each of these 20 rats was randomly assigned to the trauma (Group III, 10 rats) or AT-III–treated group (Group IV, 10 rats). All evaluations were performed in a blind fashion, and all surgical procedures were conducted with the aid of an operating microscope.

Group I (controls) was used to determine normal SOD activity, MPO activity, and MDA levels as well as spinal cord histological findings in the absence of surgery or medication. Group II (sham-operated) underwent a six-segment laminectomy and received an intraperitoneal injection of saline equal in volume to that received by AT-III–treated rats in Group IV. The aim was to determine the impact of laminectomy alone on the biochemical factors measured and on the cord histopathological findings. Group III (trauma-only) rats received an intraperitoneal saline injection identical to that used in Group II rats. Group IV (AT-III–treated) received one intraperitoneal injection of AT-III (250 IU/kg; Kybernin P 500 [AT-III, pasteurized]; Aventis Behring, Marburg, Germany) immediately after SCI and received a second dose 24 hours later.

Experimental and clinical studies have revealed that high plasma levels of AT are required to obtain beneficial anti-inflammatory effects (9, 19, 50). In addition, animal experiments and clinical studies strongly suggest that AT-III should be infused in large doses and as soon as possible after the injury (32). The AT-III dose and regimen were determined from these previous studies, which indicated that a high intraperitoneal dose is more effective than other regimens and is not toxic (15, 19, 31, 32, 47, 50).

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Sample Collection

Immediately after Group I animals were killed, their spinal cord tissue was prepared for assays of SOD, MDA, and MPO as well as histopathological analysis. In Groups II through IV, 10 animals from each group were killed at 48 hours after surgery alone (Group II) or trauma. Each rat was anesthetized and underwent transcardial perfusion, first with approximately 100 ml of 0.1-mol/L phosphate-buffered saline (pH 7.3) and then with 150 ml of 4% paraformaldehyde under constant pressure. Each animal's exposed cord segment was removed immediately after perfusion. The sample obtained from the injury site was divided transversely into four blocks from a cranial to caudal direction. The first, second, and fourth blocks were stored in a freezer at −80°C for assays of SOD, MDA, and MPO, respectively. The third block was used for histopathological analysis.

Spinal cord tissue samples for biochemical analysis were homogenized in 0.01 mol/L of potassium phosphate buffer (pH 7.0). Homogenates were centrifuged for 30 minutes at 12,500 × *g* at 4°C. The supernatants were used for MPO, SOD, and MDA assays.

Determination of SOD Activity

The SOD enzyme analysis was performed using the Randox/Ransod kit technique (Product No. ORD04, Lot No. 108RD; Randox Laboratories, Ltd., Ardmore, Crumlin, Scotland). Specific activities were calculated as units per gram of protein.

Determination of MPO Activity

Spinal cord tissue MPO activity was determined by the method of Glowiek and Kaplan (12). The MPO activity was assessed by measuring the H_2O_2-dependent oxidation of *O*-dianisidin. One unit of enzyme activity was defined as the amount of MPO that caused an absorbance of 1.0 per minute at 410 nm and 37°C (12).

Determination of MDA Levels

MDA was measured as thiobarbituric acid–reactive substances according to the method of Ohkawa et al. (27). The reaction mixture contained 0.1 ml of supernatant, 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid solutions of various pHs, and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid. The mixture was finally made up to 4.0 ml with distilled water and heated at 95°C for 30 minutes. After cooling with tap water, 5.0 ml of a mixture of *n*-butanol and pyridine (15:1 vol/vol) was added, and the mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 minutes, the organic layer (upper layer) was measured at 532 nm and expressed as nanomoles per milliliter of supernatant (27).

Histological Examination of the Spinal Cord

The spinal cord tissue samples were immediately fixed in Karnovsky's fixative for 1 hour and were then postfixed in 1% osmium tetroxide, dehydrated by a graded series of ethanol, and finally embedded in Epon. Transverse semithick sections 1 μm in thickness were prepared. These sections were stained with toluidine blue special stain and examined under the light microscope.

Statistical Analysis

The difference between groups was detected by Kruskal-Wallis variance analysis, and the means were then compared by the Mann-Whitney *U* test with Bonferroni's correction.

RESULTS

The SOD activity, MPO activity, and MDA levels are presented in *Table 1* with statistical evaluations.

TABLE 1. Distribution of superoxide dismutase, malondialdehyde, and myeloperoxidase values between groups (mean ± standard deviation) with statistical values[a]

Groups	SOD (U/g protein)	MDA (nmol/ml)	MPO (U/g)
Group I	3304.50 ± 469.89	12.88 ± 0.18	0.24 ± 0.04
P [b] (Group I versus II)	0.631	0.000	0.000
Group II	3415.70 ± 357.94	14.41 ± 0.92	0.29 ± 0.03
P [b] (Group II versus III)	0.000	0.000	0.000
Group III	9966.60 ± 1166.87	18.44 ± 1.66	0.75 ± 0.09
P [b] (Group III versus IV)	0.000	0.000	0.000
Group IV	7733.70 ± 564.20	14.70 ± 0.79	0.47 ± 0.03

[a] SOD, superoxide dismutase; MDA, malondialdehyde; MPO, myeloperoxidase.
[b] *P* values according to Mann-Whitney *U* test. *P* = 0.000 (Kruskal-Wallis variance analysis).

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Activity of SOD

Figure 1 demonstrates the effect of injury with and without AT-III treatment on SOD activity in the spinal cord homogenates 48 hours after trauma. Although SOD activity was similar between the sham-operated animals and control rats ($P > 0.05$), the activity was significantly greater in the traumatized rats than in those in the sham-operated and control groups ($P < 0.05$), and AT-III treatment significantly decreased SOD activity compared with the values in the trauma-only group ($P < 0.05$).

Level of MDA

Figure 2 demonstrates the effect of injury with and without AT-III treatment on MDA levels in the spinal cord homogenates 48 hours after trauma. MDA levels were higher in the sham-operated animals than in control rats ($P < 0.05$). The activity was significantly greater in the traumatized rats than in those in the sham-operated and control groups ($P < 0.05$), and AT-III treatment significantly decreased MDA levels compared with the values in the trauma-only group ($P < 0.05$).

Activity of MPO

Figure 3 demonstrates the effect of injury with and without AT-III treatment on MPO activity in the spinal cord homogenates 48 hours after trauma. MPO activity was greater in the sham-operated animals than in control rats ($P < 0.05$). The activity was significantly greater in the traumatized rats than in those in the sham-operated and control groups ($P < 0.05$), and AT-III treatment significantly decreased MPO activity compared with the values in the trauma-only group ($P < 0.05$).

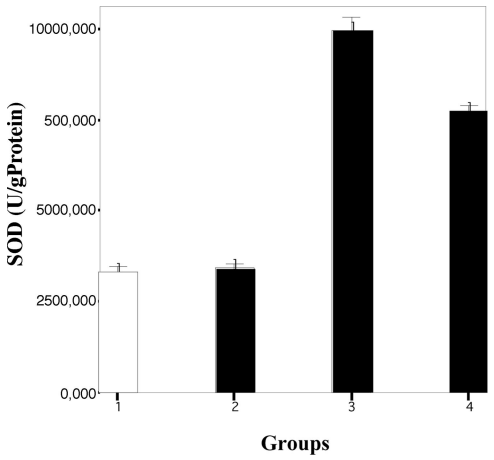


FIGURE 1. *Graph showing the effect of injury on SOD activity in the spinal cord with and without AT-III treatment. Values are expressed as means;* vertical bars *indicate standard errors of the means.*

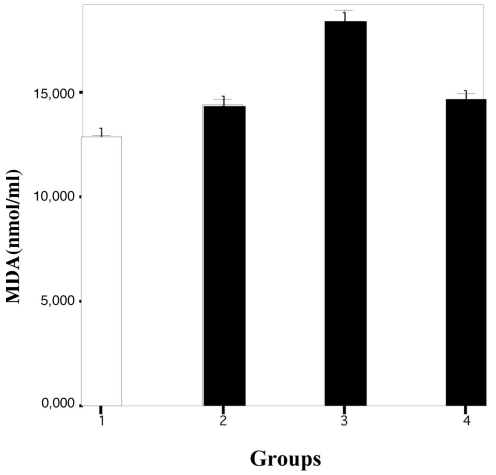


FIGURE 2. *Graph showing the effect of injury on the MDA levels in the spinal cord with and without AT-III treatment. Values are expressed as means;* vertical bars *indicate standard errors of the means.*

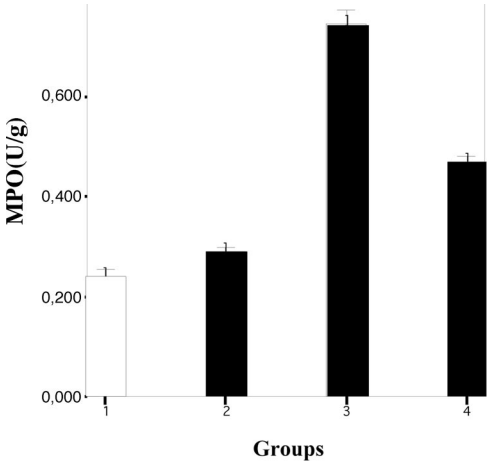


FIGURE 3. *Graph showing the effect of injury on the MPO activity in the spinal cord with and without AT-III treatment. Values are expressed as means;* vertical bars *indicate standard errors of the means.*

Histological Findings

Group I

In the gray and white matter of the spinal cord, the nerve cells, glial cells, and nerve fibers demonstrated a normal structure (*Fig. 4A*).

Group II

Although some of the myelinated nerve fibers revealed slight myelin changes in the white matter, the histological observations were similar to those in Group I in the white and gray matter.

Group III

Extensive hemorrhage, edema, and severe necrosis were observed in the gray and white matter. These finding were

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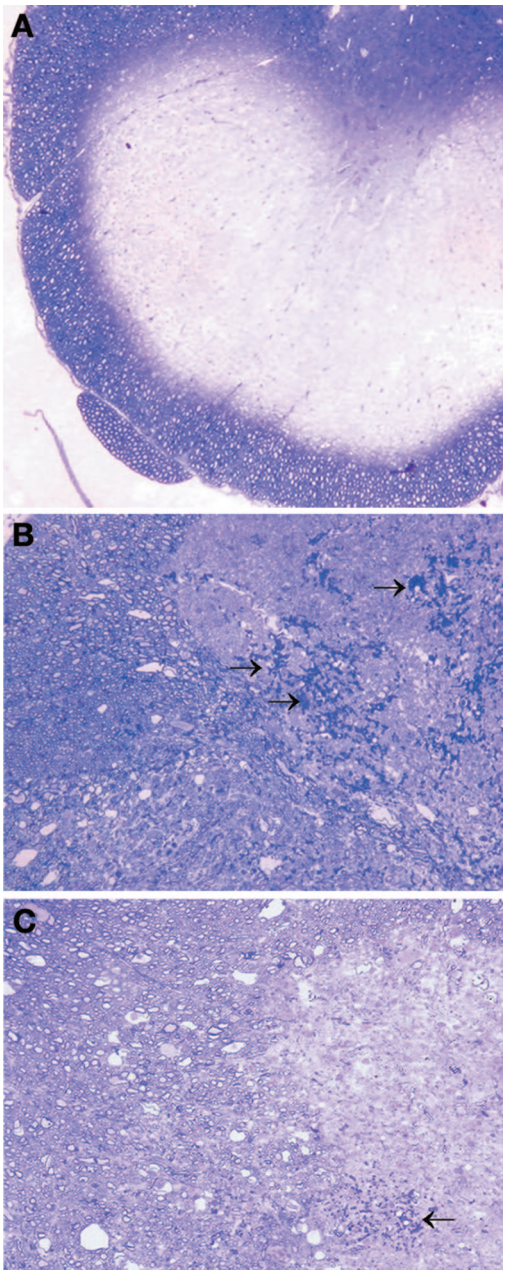


FIGURE 4. *Light microscopy studies.* A, *microphotograph showing normal structure of the spinal cord (toluidine blue; original magnification, ×100).* B, *Group III. Microphotograph showing extensive hemorrhage* (arrows)*, edema, and severe necrosis in the gray and white matter (toluidine blue; original magnification, ×200).* C, *Group IV. Microphotograph showing focal hemorrhagic* (arrow)*, necrotic, and edematous areas in the gray and white matter (toluidine blue; original magnification ×200).*

observed more frequently in the gray matter than in the white matter. Furthermore, there was neuronal necrosis, disrupted myelin sheaths, and axonal degeneration in the gray matter (*Fig. 4B*).

Group IV

The focal hemorrhagic, necrotic, and edematous areas were also observed in this group, but they were less extensive (*Fig. 4C*) than in Group III. The myelinated nerve fibers exhibited slight to moderate myelin sheath degeneration. Although there was some axonal degeneration, most of the fibers demonstrated a normal structure in general.

DISCUSSION

Activated neutrophils have been reported to be implicated as pathophysiological factors in central nervous system injury since 1966 (11). The presence of leukocytes in brain and spinal ischemic tissue has been considered to represent a pathophysiological response to existing injury (42). Although several lines of evidence have suggested a potential relationship between neutrophil accumulation and SCI, a causal relationship between these two entities has not been elucidated. Recently, Xu et al. (51) demonstrated that neutrophil infiltration was proportional to the magnitude of trauma and amount of leukotriene B_4, which is a major eicosanoid synthesized by neutrophils in SCI, induced by a weight-dropping technique in rats (40). More recently, Taoka et al. (43) also demonstrated that activated neutrophils were involved in compression trauma–induced SCI in rats. Activated neutrophils have also been demonstrated to release reactive oxygen species capable of damaging endothelial cells (41). Such oxygen species have been implicated in the progressive injury process in traumatic SCI (23, 41).

AT-III has been demonstrated to promote endothelial release of PGI_2 by interacting with the endothelial surface glycosaminoglycans in vitro (16, 52) and in vivo (48). PGI_2 is a well-known cytoprotective agent that is synthesized in endothelial cells (13, 16, 20, 34, 47, 52). PGI_2 potently inhibits platelet aggregation (44) and induces vasodilation (13, 24), thereby maintaining proper organ microcirculation. PGI_2 has also been demonstrated to inhibit leukocyte activation by inhibiting tumor necrosis factor-α production by monocytes, neutrophil activation, and neutrophil adhesion to endothelial cells (7, 16, 28). AT-III also inhibits proinflammatory mediators such as tumor necrosis factor-α, interleukin-1, interleukin-6, intercellular adhesion molecules, and E-selectin (18, 19, 26, 28, 29, 31). In addition, it acts as an anti-inflammatory mediator by inhibiting leukocyte activation (7, 13, 19, 26, 30, 31, 34, 46, 47, 49).

We used MPO activity as an index of neutrophil accumulation in spinal cord tissue. This activity has been accepted as a reproducible and objective standard for reliably estimating neutrophil infiltration into inflamed tissues and traumatized spinal cord segments (21, 31). Taoka and Okajima (41) reported that increased MPO activity revealing neutrophil infiltration was observed 3 hours after the spinal cord trauma. In this study, MPO activity in spinal cord tissue was greater in the trauma group, reflecting neutrophil infiltration. The decrease in MPO activity in the AT-III treatment group demon-

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strated that this agent inhibited the activation of neutrophils in spinal cord tissue. Inhibition of neutrophil activation in the endothelial cells by AT-III may contribute to prevention of endothelial cell damage that would have been induced by inflammatory mediators released from the activated neutrophils.

After trauma, eicosanoids, leukotrienes, and superoxide anions accumulate in the intra- and extracellular spaces (3, 5). These substances exert a toxic effect on the cells that respire. It is well known that neutrophils may accumulate at the endothelial cell surface, where they may damage the endothelial cells by releasing a wide variety of inflammatory mediators such as neutrophil elastase and oxygen free radicals (14, 31, 43). The cellular endogenous antioxidant defense system against the excessive production of free radicals includes three concerted action enzymes, SOD, glutathione peroxidase, and catalase (2, 4, 5, 23). SOD is a specific free-radical scavenger (4). Its activity in the rats' spinal cords reflected the impact of AT-III treatment on free-radical production. Nakauchi et al. (25) demonstrated that lecithinized SOD, a long-acting SOD, markedly attenuated the severity of motor functions after compression trauma to the spinal cord in rats and significantly suppressed MPO activity in the injured spinal cord and spinal cord edema. We found that the SOD activity in the AT-III-treated rats was significantly lower than that in the trauma-only group. It is logical that the neutrophil activation during secondary injury causes free-radical production and that, with time, SOD would rise in parallel with the increasing levels of free radicals. AT-III treatment attenuates neutrophil activation, which, in turn, decreases the level of free-radical production in the spinal cord tissue. Thus, secondary neurological damage is reduced.

Demopoulos et al. (5) have also suggested that lipid peroxidation is a major factor in the postinjury process after SCI. Free radicals formed at the time of SCI are also able to attach to polyunsaturated fatty acids in membrane, thus initiating lipid peroxidation. MDA is an essential breakdown product of peroxidized polyunsaturated fatty acids in spinal myelin neuronal and glial membranes and other cellulose elements (36). A significant increase in MDA levels as an index of lipid peroxidation has been observed in the central nervous system during injury (5, 17, 40). The neutrophil activation during the secondary SCI causes free-radical production; with time, lipid peroxidation would rise in parallel with the increasing levels of free radicals (17, 40). In our study, the elevated MDA levels in the trauma group support the proposition that lipid peroxidation processes occur during SCI. A decrease in MDA levels was detected in rats treated with AT-III, which implicated lipid peroxidation inhibition by this agent. AT-III treatment attenuates neutrophil activation, which, in turn, decreases lipid peroxidation and MDA levels in the spinal cord tissue. The beneficial effect of AT-III on SCI may be the result of attenuation of the lipid peroxidation.

Recent evidence also suggests that platelets play a major role in the pathogenesis of SCI (26, 40). It is well known that platelets cause postischemic tissue damage through formation of thrombi (35). In addition, other investigators have reported platelets that accumulate in ischemic regions to release oxidants and inflammatory mediators, such as serotonin, leukotrienes, thromboxane-A_2, and platelet-derived growth factor, all of which cause tissue damage (1). Platelets have also been reported to recruit leukocytes to ischemic sites through the expression of adhesion molecules on their surface or by the release of cytokines (26, 30). Administration of AT-III just after the induction of trauma significantly inhibits the recruitment of platelets and may reduce secondary neurological damage.

AT-III is also well known as an anticoagulant agent. AT activities are substantially decreased in patients with trauma, shock, and sepsis (15). The acute consumption of AT-III during the process of coagulation, its increased leakage from the intravascular compartment, and the proteolytic inactivation by elastase are the most likely pathomechanisms responsible for the acquired AT-III deficiency in these patients.

After acute traumatic injury, the spinal cord undergoes sequential pathological changes, including hemorrhage, edema, axonal and neuronal necrosis, and demyelination, after which cyst formation and infarction occur (40). In our study, histological examination revealed marked histopathological changes in the gray and white matter of the spinal cord obtained in the trauma-only rats. In contrast, the spinal cords obtained in the AT-III–treated group exhibited milder hemorrhage and less edema. These findings demonstrate that AT-III treatment partially blocks the progression of edematous and histopathological changes in the rat spinal cord after clip compression injury. On this basis, it seems that there may be therapeutic value in administering AT-III to reduce secondary spinal cord damage.

CONCLUSIONS

During the past 2 decades, considerable research has been conducted to determine the pathological findings, pathophysiology, and treatment of SCI. To the best of our knowledge, this is the first study to demonstrate the role of AT-III in the treatment for SCI. AT-III may prevent secondary structural changes in damaged rat spinal cord tissue by inhibition of leukocyte activation. This beneficial effect of AT-III is explained, at least in part, by the inhibition of MPO activity, SOD activity, and MDA levels. Although the results of the present study have provided some interesting data confirming the useful effect of AT-III after SCI, further studies are under way to verify this mechanism in detail and to clarify the therapeutic window, dosage, and duration of treatment.

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COMMENTS

Erman et al. present a straightforward study of the effects of antithrombin III on histological findings and markers of leukocyte activation in an experimental model of acute spinal cord injury. Their data indicate that antithrombin III may be a promising agent for mitigating some of the endogenous inflammatory response to spinal cord injury. However, this study addresses only histological and biochemical measurements 48 hours after injury. No functional or behavioral endpoints are assessed, and no markers of neuronal regeneration or repair are assayed. Longer-term assessments are not carried out.

The authors seem to be aware of the limits of this study. To their credit, they do not attempt to overstate the significance of these results. They appropriately conclude that further studies are needed to investigate the potential benefits of antithrombin III , as well as to determine such parameters as dosage and duration of treatment and the potential therapeutic window. A special concern would be whether administration of this drug is associated with an increase in bleeding complications, especially in and around the damaged spinal cord.

Alex B. Valadka
Houston, Texas

This study provides important insight into some of the mechanisms through which antithrombin III may reduce secondary injury following spinal cord injury. The authors have clearly demonstrated a reduction in the cellular inflammatory response after treatment. It is not entirely clear, however, if this is a primary effect of treatment or secondary to enhanced perfusion in the microcirculation. Antithrombin III inhibits platelet aggregation and, in some studies at least, has vasodilatory effects. It is possible that its primary effect is to improve blood flow in ischemic tissue early after injury, and that the reduced inflammation observed in this study is a result of a reduction in the volume of ischemic tissue. The results of this study are certainly compelling and will hopefully lead to further studies of changes in local blood flow, and of functional outcomes. Finally, it is important to note that the administration of relatively high doses of this anticoagulant early after spinal cord injury did not result in worsened focal hemorrhage in the lesion site. This supports the argument that the risk of exacerbating posttraumatic hemorrhage is outweighed by the benefit of improving perfusion to ischemic tissue with the use of this and other anticoagulants.

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