Effect of Thyrotropin-Releasing Hormone (TRH) in Experimental Spinal Cord Injury: A Quantitative Histopathologic Study

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ABSTRACT — Spinal cord injuries in rats were experimentally produced by compressing the cord (T11 vertebra level) for 60 min with stainless steel screws. Morphometric analysis of the injured cord revealed that at 14 days post-injury, there were significant correlations between the neurologic score (NS) and all morphometric parameters, including total cross-sectional area ($r_s = 0.438$), lesioned area ($r_s = -0.421$) and area of the gray ($r_s = 0.377$) and white matter ($r_s = 0.704$). Although rats treated with thyrotropin-releasing hormone (TRH; 22.5 mg/kg, s.c., twice daily for 7 days starting 24 hr post-injury) showed significant improvement in NS 14 days post-injury, there were no significant differences in morphometric parameters between saline- and TRH-treated rats. In addition, no significant correlation was observed between NS and any of the morphometric parameters in TRH-treated rats, even though there was a significant correlation between the area of white matter and NS in saline-treated rats. These results suggest that neurologic recovery closely reflects the histopathological changes evident at the injury site in the present model, and that the improvement of neurologic status seen in rats with cord injury given TRH starting 24 hr post-injury is not due to protection against progression of neural damage at the injury site.

Thyrotropin-releasing hormone (TRH) was first reported by Faden et al. (1) to be effective in improving the neurologic outcome in an experimental spinal cord injury model in cats. Recently, an ameliorating effect of TRH was also reported in patients with both chronic and stable spinal cord injury (2). TRH has therefore been receiving a great deal of attention because of its possible use as a pharmacotherapeutic agent for spinal cord injury. Although the leading model of experimental spinal cord injury is the weight-drop method designed by Allen (3), Faden et al. (4, 5) failed to find any direct action of TRH on histopathological outcome using this model; and more recently, they concluded that this was due to the relatively poor correlation between light-microscopic histological changes and motor recovery, especially with regard to pharmacological changes (6). Recently, we reported a new model of spinal cord injury in rats using a screw (7), which could be prepared more easily than the apparatus used in Allen's method. Using this model, we confirmed the dose-dependent accelerating effect of TRH on neurologic recovery when administered for 7 days starting 24 hr after injury, and demonstrated the usefulness of this model (8).
The aim of this study was to clarify the relationship between histopathological and neurologic outcome in the present model, and in addition, whether TRH exerts a direct action at the injury site, to determine the mechanism of the effect of TRH in accelerating neurologic recovery when administration is started 24 hr after injury.

MATERIALS AND METHODS

Animals

Male Wistar rats each weighing about 350 g were used. They were kept under constant environmental conditions (24 ± 1°C and a regular 12 hr light/dark cycle), given food and water ad libitum, and housed individually following spinal cord injury.

Surgical procedures and postoperative care

Spinal cord injury was produced by the new method introduced in our previous paper (7). A midline longitudinal incision was made in the skin of the back under sodium pentobarbital (30 mg/kg, i.p.) anesthesia, the fascia was cut along the midline, and the dorsal surface of the spinal column was exposed from T10 to T12 by a paramedian incision into the paraspinal muscles. The spinous process at the eleventh thoracic (T11) vertebra was dissected, and a small burr hole was made in the center of the dissected surface of the vertebra. A stainless steel screw 2 mm in diameter and 2.8 mm in length was implanted into the burr hole, just above the dura mater, and then the incision was sutured. On the following day, rats showing no neurologic dysfunction were housed individually, and any urine wetting the external skin was washed away daily with warm water and the area was dried with a paper towel.

Neurologic scoring

Neurologic scores (NS) of 0–5 based on both motor and sensory deficits were defined as follows: 0: no spontaneous movement of the hind limbs and no nociceptive response upon tail-pinching, including biting the clamps or vocalization; 1: no spontaneous movement of the hind limbs but showing a nociceptive response upon tail pinching, including biting the clamps or vocalization; 2: barely perceptible coordinated movement of the hind limbs and fore limbs; 3: well-coordinated movement of the hind limbs and fore limbs, but no weight-bearing by the four limbs; 4: ability to walk with weight-bearing on all four limbs, but with an ataxic gait; 5: normal walking. Observation of the neurologic symptoms was conducted in a blind manner at 24 hr, and at 3, 5, 7, and 14 days post-injury.

Composition of experimental groups and administration of TRH

The animals were divided into the following experimental groups: Group 1, sham-treated control; Group 2, injured but given neither saline nor TRH; Group 3, injured and saline-treated; Group 4, injured and TRH-treated. Rats with an NS of 1 at 24 hr after cord injury were started on subcutaneous injections of TRH (thyrotropin-releasing hormone tartrate monohydrate, Takeda) or saline, as described previously (8). The injections were given twice daily (9:00 AM and 3:00 PM) for 7 consecutive days; and on day 3, 5 and 7 post-injury, the first injection was given following the neurologic scoring. Saline or 22.5 mg/kg TRH dissolved in saline was injected in a volume of 1 ml/kg.

Histological and morphometric examination

After the last neurologic scoring on day 14 post-injury, all animals were sacrificed by intra-aortic perfusion fixation following admini-
istration of intraperitoneal sodium pentobarbital (50 mg/kg, i.p.). The perfusion was initiated with saline and continued until there was a clear return from the right atrium. This was followed by perfusion with neutralized and buffered 10% formalin. The spinal cord was removed and fixed in the same fixative for 7 days. Transverse slices were made 0.5 mm rostral to the center of the injury site (epicenter) and at 5 mm caudal to the epicenter. The tissue blocks were dehydrated with ethanol, cleared with xylene and embedded in "Tissue Prep" (Fisher Scientific, U.S.A). Transverse sections (4.5 μm thick) at the epicenter were obtained from several series of sections. Hematoxylin and eosin (H.E.) staining, Klüver-Barrera staining, Bodian silver staining modified by Otsuka and glial fibrillary acidic protein (GFAP) immunohistochemistry were performed on the sections for light microscopic observation and morphometric analysis. For morphometric analysis, photographs of all sections were taken using light microscopy, and then the boundaries of the white matter, gray matter and lesioned area were outlined with black ink, as shown in Fig. 1a. In the case of obscure boundaries, the dividing line was decided from the quantity of myelin, nerves and vacuoles observed in the sections. These processes were also conducted in a blind manner. Using these photographs, we measured the total cross-sectional area of the cord, as well as that of the lesion and the white and gray matter, using an image-analyzer (IBAS2000, Carl Zeiss).

**Analysis of data**

The significance of relationships between NS and the various morphometric parameters were determined by Spearman's rank correlation coefficient (r_s). The differences in NS between the saline-treated control group and the TRH-treated group were compared using the non-parametric Mann-Whitney U-test (two-tailed). Other differences between groups were compared by the parametric Student's t-test (two-tailed) following analysis of variance (ANOVA).

**RESULTS**

**Histopathological changes at the epicenter of the rat spinal cord injury model**

Eighty-two rats with spinal cord injury (45 in Group 2 and 37 in Group 3) and eight sham-treated control rats (Group 1) were used to characterize the histopathologic changes in the spinal cord injury model. Fourteen days after compression-induced spinal cord injury, the extent of the lesion was more prominent in the dorsal area than in the ventral area at the epicenter of the cord, and it covered almost the whole of the gray matter in many cases (Fig. 1a). In the lesioned area, various amounts of macrophages and fibroblasts were prominently infiltrated (Fig. 1b). The number of nerve fibers was markedly decreased in the dorsal spinal cord. The remaining white matter contained many small vacuoles and showed a decreased number of nerve fibers (Fig. 2, a and c). In these areas, a small number of nerve fibers showed axonal swelling. In the area of remaining gray matter, disappearance and degeneration of neurons and numerous GFAP-positive structures were observed (Fig. 3). As shown in Table 1, the morphometric parameters in the epicenter on day 14 post-injury were markedly changed (total cross-sectional area of the cord, -16%; area of gray matter, -95%; area of white matter, -53% as compared with the sham-treated control). When relationships between the NS and the morphometric parameters on day 14 post-injury were studied, there were significant non-parametric correlations between NS and all of the morphometric parameters, including the total cross-sectional area of the cord (r_s = 0.438, P < 0.001), area of the lesion (r_s = -0.421, P < 0.001), area of the gray matter (r_s = 0.377, P < 0.001) and area of the white matter (r_s = 0.704, P < 0.001). As shown in Fig. 4, the area of the white matter correlated especially strongly with NS.

**Effect of TRH on neurologic deficits and histopathological changes**

As shown in Fig. 5, TRH treatment starting
Fig. 1. Transverse section of a rat spinal cord, at the epicenter, 14 days after injury. a: Low magnification. The black line traced on the photograph indicates the boundary between the lesion and the white matter. The dorsal column and the gray matter are severely damaged, and many small cavities are seen in the damaged white matter. Klüver-Barrera stain. Scale bar = 250 μm. b: Higher magnification of the damaged dorsal area in Fig. 1a. Many macrophages (arrow heads) and fibroblasts (arrows) are present. Hematoxylin and eosin (H.E.) stain. Scale bar = 50 μm.

Fig. 2. Transverse sections of the spinal cord of an injured animal at the epicenter (a and c, same as Fig. 1) and at the corresponding site from a sham-treated control animal (b and d). a and b: Dorsal funiculus. Marked reduction in the number of axons is evident in the injured animal (a). c and d: Ventral funiculus. Marked vacuolar formation (arrow heads), axonal swelling (arrows) and reduction in the number of axons are evident in the injured animal (c). Bodian silver stain modified by Otsuka. Scale bar = 100 μm.
Fig. 3. The ventral gray matter at the epicenter with a mild lesion, from a spinal cord-injured animal. a: Klüver-Barrera stain. The gray matter contains many degenerated neurons and small round cells. b: Glial fibrillary acidic protein (GFAP) immunocytochemistry of the same region as that shown in Fig. 3a. Numerous GFAP-positive structures are present. Scale bar = 100 μm.
24 hr after injury accelerated the neurologic recovery, and there was a significant difference between the saline-treated control group and the TRH-treated group at each observation point from day 3 to day 14 post-injury. On the other hand, as shown in Fig. 6 and Table 2, there were no qualitative or quantitative differences in the histopathological changes at the epicenter on day 14 post-injury between the saline-treated control group and the TRH-treated group. As shown in Fig. 7, when relationships between NS and morphometric parameters on day 14 post-injury were studied, there was a significant non-parametric correlation between NS and the area of white matter in the saline-treated control group ($r_s = 0.588$, $P < 0.001$) but not in the TRH-treated group ($r_s = 0.252$, $P > 0.05$).

**DISCUSSION**

In the present study, we investigated the qualitative and quantitative histopathological changes at the epicenter 14 days after compression-induced thoracic spinal cord injury in rats. Light-microscopic examination of the epicenter showed inflammatory changes, reduction in the area of gray matter and a decrease in the number of axons. These changes resembled those described in the model pro-
duced by the weight-drop method (9–12). Morphometric examination showed a reduction in the total cross-sectional area of the cord (−16%), the area of the gray matter (−95%) and the area of the white matter (−53%), and an increase in the lesioned area. These figures indicated atrophy at the epicenter and loss of most of the gray matter and part of the white matter. In addition, correlation analysis revealed that the NS on day 14 post-injury correlated closely ($r_s = 0.704$, $P < 0.001$) with the area of the white matter at the epicenter 14 days after spinal cord injury, indicating that this parameter might represent the grade of histopathological changes in the present model. Although some investigators

![Table 2. Effect of TRH-treatment (22.5 mg/kg x 2/day, s.c.) on morphometric parameters at the epicenter in rats 14 days after spinal cord injury](image)

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>TRH</th>
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<tbody>
<tr>
<td>Total cross-sectional area</td>
<td>1.83 ± 0.05</td>
<td>1.89 ± 0.04</td>
</tr>
<tr>
<td>Area of lesion</td>
<td>1.17 ± 0.04</td>
<td>1.21 ± 0.04</td>
</tr>
<tr>
<td>Area of gray matter</td>
<td>0.02 ± 0.01</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>Area of white matter</td>
<td>0.65 ± 0.04</td>
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Data are mean areas (mm²) and ± S.E.M. from 37 saline-treated control (Group 3) and 34 TRH-treated animals (Group 4).
Fig. 6. Transverse sections of spinal cord tissue at the epicenter from a saline-treated control (a) and a TRH-treated animal (b) 14 days after spinal cord injury. Although neurologic scores in the control and TRH-treated animals were 3 and 4, respectively, there was no qualitative histopathological difference between the two. Klüver-Barrera stain. Scale bar = 250 μm.
(4, 5, 13) have reported no correlation between neurologic impairment and light-microscopic features at the epicenter produced by experimental spinal cord injury, and Blight (13) considered gross motor recovery in cats not to be a reliable index of the overall extent of axonal damage in contusive spinal cord lesions of similar intensity, other investigators (11, 12, 14–16) have reported a correlation between the above two measures of outcome. Noble and Wrathal (11, 12) demonstrated that the area of residual white matter correlated well with the neurologic score of behavioral impairment in the rat model produced by the weight-drop method. These characteristics resemble the results obtained in our rat model, despite the differences in the injury and neurologic impairment-scoring methods. This indicates that the present model could also be useful for studying spinal cord injury.

Faden et al. (6) demonstrated immunohistochemically that the quantity of serotonin fibers in the ventral gray matter in the rat lumbar cord distal to the lesioned site correlated very significantly with the severity of injury, as reflected by motor impairment. Recently, using the present model, we found that there were significant correlations between neurologic score and the levels of norepinephrine and serotonin, but not that of dopamine in the lumbar cord distal to the injury site (17). In addition, Eidelberg et al. (18) have reported that significant hindlimb motor recovery in cats after partial transection of the thoracic cord was closely linked to axonal sparing in at least one ventrolateral quadrant of the cord, suggesting that the essential elements belong to the vestibulospinal and reticulospinal systems. Moreover, Midha et al. (19) have suggested that although the number of corticospinal neurons in the rat sensorimotor cortex after spinal cord injury correlates closely with the severity of injury, neurologic recovery correlates not with the number of corticospinal neurons but rather with that of rubrospinal neurons. In the present study, we measured the area of the preserved white matter at the epicenter without any precise anatomical considerations, and demonstrated a close correlation between neurologic recovery and the area of preserved white matter. Thus, in the present model, it is conceivable that at the epicenter, the percentage of fibers passing through the tracts, which participate in neurologic recovery as described above, is decreased almost uniformly in accordance with a gross decrease in the area of the white matter.

Histopathological changes following spinal cord injury have been thought to be due not only to the initial physical insult but also to changes occurring secondarily as a result of putative pathophysiological factors (20). Faden et al. (1, 4, 5) speculated that the release of
endogenous opioids following spinal cord injury might cause a reduction of blood flow in the cord, and they reported an ameliorating effect of naloxone and TRH, both of which have anti-opioid properties, when administered within the first several hours after injury. They referred to the potential clinical usefulness of TRH not only because of its lack of anti-analgesic action but also because of its antinociceptive activity (21), although they failed to demonstrate any neural tissue-preserving effect of the above two drugs, since correlation between the neurologic and histopathological outcome was poor (6). In the present study, we re-examined the direct action of TRH on the injury site using a new spinal cord injury model which shows a close correlation between neurologic and histopathological outcome. We have already reported that once daily (5, 15, or 45 mg/kg/day, s.c.) or twice daily (2.5, 7.5, or 22.5 mg/kg X 2/day, s.c.) treatment with TRH for 7 days starting 24 hr after injury in rats with an NS of 1 dose-dependently accelerates neurologic recovery, and a twice daily dosage schedule tends to produce greater improvement in the neurologic state than a once daily schedule does in the present model of spinal cord injury (8). In the present study, although twice daily treatment with TRH (22.5 mg/kg X 2/day, s.c.) for 7 days starting 24 hr post-injury was again shown to accelerate neurologic recovery, it was found to have absolutely no effect on the qualitative or quantitative histopathological changes at the epicenter of the injury site. This suggests that the effect of TRH in accelerating neurologic recovery in rats with spinal cord injury is not due to preservation of neural tissue against secondarily occurring expansion of the damaged area at the injury site due to putative pathophysiological factors. Interestingly, significant correlations between neurologic score and morphometric parameters were no longer observed in the TRH-treated rats even though a high and significant correlation was still evident between the area of residual white matter and neurologic score in the saline-treated control rats. This indicates that neurologic recovery in TRH-treated rats correlates less with the number of axons at the injury site, and suggests that the neurologic recovery-accelerating effect of TRH is derived from action at a site other than the injured one.

Van der Berg et al. (22) have shown that ablation of spinoally projecting serotonergic pathways by intracisternal injection of 5,7-dihydroxytryptamine (5,7-DHT), a selective neurotoxin for serotonin neurons (23), in neonatal rats results in deficient recovery of plantar foot muscles which have been functionally denervated with botulinum toxin type A, suggesting the importance of serotonin neurons in functional recovery. Recently, we found that neurologic recovery after spinal cord injury was retarded by intracerebroventricular injection of 5,7-DHT, but not by p-chlorophenylalanine or reserpine (17). This led us to propose some functional significance of serotonin neurons other than serotonergic transmission in this neurologic recovery following cord injury, and the possible contribution of TRH and substance P, both of which coexist in spinoally projecting serotonin neurons (24, 25). In addition, several investigators have reported that TRH has neurotrophic activity in spinal cord tissue (26–29). Thus, it is conceivable that TRH causes an alteration in the plasticity of the neural network controlling hindlimb motor activity and accelerates functional recovery after spinal cord injury. Further study will be required to determine the precise mechanism and site of action of TRH.

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