Morphometric assessment of drug effects in experimental spinal cord injury

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The effect of large doses of methylprednisolone sodium succinate (MPSS) and two protease inhibitors, leupeptin and bestatin, on experimental acute spinal cord injury was evaluated by morphometric analysis of degenerating axons with the aid of an automated image analyzer. Spinal cord injury was produced by epidural compression with a surgical clip on the T-11 segment in rats. The extent of axonal damage was assessed in Rexed's lamina VIII in the L-6 segment by measuring the amount of silver grains, representing degenerating axons and their terminals, using the Fink-Heimer method. The severity of axonal damage was expressed as the degeneration index: that is, the amount of silver grains in experimental animals/the amount of silver grains in cord-transected animals. When examined on the 7th postoperative day, axonal degeneration in MPSS-treated rats was significantly decreased, with an average degeneration index difference of 6 (p < 0.05). Increased preservation of axons was seen in the leupeptin-treated rats sacrificed 7, 10, and 14 days after trauma. The difference in the degeneration index between the leupeptin-treated and untreated groups was 16 on Day 7 (p < 0.001), 12 on Day 10 (p < 0.001), and 13 on Day 14 (p < 0.01). Bestatin had no beneficial effect. The implications for the use of calcium-activated neutral protease inhibitors in acute spinal cord injury are discussed.

KEY WORDS • spinal cord injury • methylprednisolone • leupeptin • bestatin • axonal degeneration

With the exception of a few studies,6,9,10,22 the severity of tissue damage and the effects of therapeutic intervention in experimental spinal cord injury have generally been defined by the extent of hemorrhagic necrosis and subsequent cystic degeneration at the site of mechanical insult.6,7 Clinical grading, a method most widely used in the evaluation of spinal cord damage,7 is more likely to reflect the degree of long-tract degeneration after injury than the severity of focal tissue degeneration at the site of injury. For the assessment of drug effects on spinal cord injury, therefore, it seems more appropriate to measure the extent of axonal damage distal to the site of mechanical insult. Degeneration of axons and their terminals in the central nervous system (CNS) tissue can be selectively demonstrated by the Fink-Heimer method.6,11 The validity of the use of this technique in evaluating CNS tissue damage has recently been suggested,6,16 and, with the aid of an automated image analyzer, we have used this method to make objective measurements of the extent of spinal cord injury.14,15

In this communication, we present a morphometric assessment of the effect of a large dose of methylprednisolone sodium succinate (MPSS) on the preservation of distal axons in acute experimental spinal cord injury in rats. In addition, the effects of two protease inhibitors, leupeptin1 and bestatin,23 were also evaluated in this acute spinal cord injury model. These drugs were selected for study since, in animal experiments, the degradation of neurofilaments (principal structural components of axons) has been shown to be mediated by increased protease activity, possibly from calcium-activated proteases.5 It has also been demonstrated in in vitro experiments that neurofilaments can be degraded by calcium-activated neutral proteases derived from nervous tissues20,21 and that the degradation can be suppressed by protease inhibitors.17 Furthermore, our preliminary experiments have clearly shown the
Drug effects in experimental acute spinal cord injury

Materials and Methods

Surgical Procedures

A total of 75 adult male Wistar rats, each weighing 195 to 240 gm, were used for this study. Caution was exercised to meet the standards for animal care set by the United States Government Animal Welfare Act of 1974. Seventy rats were subjected to epidural compression injury, as described previously. In brief, under intraperitoneal sodium pentobarbital anesthesia (40 mg/kg body weight), a posterior laminectomy was performed, then epidural compression was applied for 30 seconds to the T-11 segment of the spinal cord by means of a Biemer cerebral vascular clip with a closing force of 40 gm. Under these conditions, the majority of animals will be rendered paraplegic. In five rats, the spinal cord was completely transected at the T-11 level with a surgical scalpel to provide standard samples for calculation of the degeneration index.

Drug Treatment

Methylprednisolone Sodium Succinate. Eleven rats with compression injury of the spinal cord received MPSS according to a timetable proposed by Braughler and Hall. By this protocol, a 30-mg/kg bolus of MPSS was injected into a tail vein 30 minutes and 2 1/2 hours after injury. Nine rats that received the same volume of a vehicle solution used to dissolve MPSS served as a control group. All the rats receiving MPSS or vehicle were sacrificed on the 7th postoperative day.

Leupeptin. Leupeptin was dissolved in distilled water at a concentration of 10 mg/ml and administered intraperitoneally to 15 rats at a dose of 25 mg/kg twice a day on the day of injury and on the following 2 days, starting 10 minutes before surgery. Another 15 animals with compression injury of the spinal cord received normal saline solution and served as a control group. Five rats from each group were randomly selected and sacrificed on the 7th, 10th, or 14th postoperative day.

Bestatin. Bestatin was dissolved in a normal saline solution at a concentration of 1 mg/ml and given to 10 rats at a dose of 10 mg/kg, using the same schedule as that for leupeptin. Ten rats received a normal saline solution and served as a control group. All of the bestatin-treated and control rats were sacrificed on the 7th postoperative day.

Clinical Grading

Residual hindlimb motor functions were carefully evaluated on the day of sacrifice. Although the distinction between voluntary and involuntary movements was sometimes puzzling, the motor functions of individual rats were roughly graded as follows:

- Grade 0 = no difference from normal rats
- Grade 1 = crawling with mild difficulty
- Grade 2 = some voluntary movement to support the hindquarters
- Grade 3: no voluntary movement.

Histological Study

The animals were sacrificed by transcardial perfusion with phosphate-buffered 10% formalin. For detection of degenerating axons, the spinal cord was removed with roots attached to identify the cord segments and was further fixed in formalin for at least 1 month. After completion of formalin fixation, horizontal sections 35 μ thick were made from the lumbar segments with a cryostat and then processed by the Fink-Heimer method. The remaining tissues were embedded in paraffin and stained with hematoxylin and eosin and by the Bodian and Klüver-Barrera method.

Morphometry

The L-6 vertebral segment was chosen as the site of morphometric assessment of axonal damage because of the easy identification of this segment by the presence of the nucleus of Onuf. Morphometry was carried out on Rexed's lamina VIII in the anterior horn with the aid of a Luxex 500 Tu automated image analyzer connected to a light microscope equipped with a television camera as described previously. The extent of axonal damage in each section was expressed as the percentage of the area occupied by silver grains, and the severity of cord damage in individual rats was shown by a degeneration index. The degeneration index (D.I.) was calculated as follows: D.I. = an arithmetic mean of the percent area occupied by silver grains in cord-compressed rat/the mean of the measurements of the percent area in five cord-transected rats × 100.

Results

Clinical Grading

Since the maximum development of silver grains is observed by the Fink-Heimer method on the 7th postoperative day, all the animals except some in the leupeptin experiment were sacrificed on Day 7 to establish a clinicopathological correlation. Clinical grading of individual animals is shown in Table 1. There was little individual variation in function among the control groups in each experiment. The average score for control animals on Day 7 was 2.7 for MPSS, 2.8 for leupeptin, and 2.6 for bestatin. In the MPSS and bestatin experiments, there was no obvious difference in clinical grading between the drug-treated and the con-
TABLE 1
Assessment of clinical symptoms*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day of Death</th>
<th>Clinical Grading</th>
<th>Total Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day of Death</td>
<td>Grade 1</td>
<td>Grade 2</td>
</tr>
<tr>
<td>MPSS</td>
<td>7</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>controls</td>
<td>7</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>leupeptin</td>
<td>7</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>controls</td>
<td>7</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>leupeptin</td>
<td>10</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>controls</td>
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<td>0</td>
<td>5</td>
</tr>
<tr>
<td>leupeptin</td>
<td>14</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>controls</td>
<td>14</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>bestatin</td>
<td>7</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>controls</td>
<td>7</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

* MPSS = methylprednisolone sodium succinate. For a description of the grading system see text.

trol animals. In the leupeptin experiment, in which clinical grading was done not only on Day 7 but also on Days 10 and 14, the drug-treated animals tended to show better recovery. The number of animals was, however, too small to evaluate the difference statistically.

Histological Study

All of the animals subjected to spinal cord compression had a focal but severe, partially cystic, degenerative lesion in the central portion of the cord in the lower thoracic segments (Fig. 1). The effect of drugs on the severity of local lesions, however, was not discernible by routine histology. Figure 2 shows a low-power view of a Fink-Heimer preparation of the L-6 segment of a rat subjected to cord transection at the T-11 vertebral level 7 days previously. The L-6 cord segment in rats was readily identified by the presence of the nucleus of Onuf. As shown in this figure, accumulation of silver grains, representing degenerating axons, was seen in the descending tracts in the anterolateral funiculi and in the corticospinal tracts in the posterior funiculus. In the gray matter, no apparent histopathological changes were detected by routine stains (not shown), but silver grains were accumulated in Rexed’s laminae VII and VIII, into which the terminals of the descending tracts are known to be converged (Figs. 2 and 3). 19

Figure 3 shows the Fink-Heimer preparations of Rexed’s lamina VIII of the L-6 segment of rats from the different experimental groups. The pale area in the left lower corner of these figures is a part of the nucleus of Onuf, and morphometric measurement was carried out in the area adjacent to this nucleus. The density of silver grains in the MPSS-treated rats (Fig. 3a) was slightly lower than that in the control sections (Fig. 3b); however, the difference was not impressive under a microscope. In the leupeptin experiment, the density of grains was much lower in the rats sacrificed on Day 14 than in those sacrificed on Days 7 and 10 in both the leupeptin-treated and the untreated control rats. When the leupeptin-treated rats (Fig. 3c and e) were compared with the untreated control rats (Fig. 3d and f), there was an apparent difference in the density of grains regardless of the length of survival time. Thus, it appears that the leupeptin treatment did not merely retard the development of axonal degeneration in the region distal to the site of mechanical insult but actually suppressed it. No difference was detected between the bestatin-treated and untreated rats.

Fig. 1. Longitudinal section of spinal cord at the site of epidural compression lesion in an untreated control rat on the 7th postoperative day. Arrow indicates rostral direction. The central portion of the cord is completely necrotic and the surrounding tissue is severely edematous. Kluver-Barrera method, × 25.

Fig. 2. Cross section of the L-6 cord segment of a rat subjected to cord transection 7 days previously. Descending tracts in the white matter were almost entirely replaced by silver grains. In the gray matter, silver grains were densely accumulated in Rexed’s laminae VII and VIII. Arrows indicate the nucleus of Onuf. Fink-Heimer preparation, × 27.
FIG. 3. The effects of methylprednisolone sodium succinate (MPSS) and leupeptin treatments on the density of silver grains in Rexed's lamina VIII in the L-6 cord segment of the drug-treated rats (left) and untreated control rats (right). Fink-Heimer preparations, × 150. a and b: Sections from an MPSS-treated rat and an untreated control rat at 7 days survival. c and d: Sections from a leupeptin-treated rat and an untreated control rat at 7 days survival. e and f: Sections from a leupeptin-treated rat and an untreated control rat at 14 days survival. The amount of silver grains in the MPSS-treated rat (a) was slightly lower than that in the control (b). The density of silver grains on Day 14 (e and f) was lower than that on Day 7 (c and d) in both leupeptin-treated and untreated control rats, but the amount of silver grains was apparently smaller in the leupeptin-treated rats (e and f) than in their controls (d and f) on both Days 7 and 14. Degeneration indices were 82.6 (a), 88.8 (b), 73.8 (c), 88.5 (d), 56.3 (e), and 68.8 (f).
Fig. 4. The results of the morphometric measurement of the degeneration index in individual rats treated with methylprednisolone sodium succinate (MPSS), leupeptin, and bestatin and their control groups sacrificed on the 7th postoperative day. Each point represents an arithmetic mean of the measurement of three sections made from the L-6 cord segment. Closed squares: MPSS-treated rats; open squares: controls for MPSS-treated rats; closed circles: leupeptin-treated rats; open circles: controls for leupeptin-treated rats; closed triangles: bestatin-treated rats; open triangles: controls for bestatin-treated rats. Values shown are means ± standard deviations.

Fig. 5. The results of the morphometric measurement of the rats treated with leupeptin and their controls sacrificed 7, 10, or 14 days after the spinal cord injury. Each point (closed circles: leupeptin-treated rats; open circles: control rats) represents an arithmetic mean of the measurement of three sections made from the L-6 cord segment. Values shown are means ± standard deviations.

**TABLE 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day of Death</th>
<th>No. of Rats</th>
<th>Degeneration Index†</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPSS</td>
<td>7</td>
<td>11</td>
<td>83.1 ± 4.02</td>
</tr>
<tr>
<td>controls</td>
<td>7</td>
<td>9</td>
<td>89.1 ± 6.37</td>
</tr>
<tr>
<td>leupeptin</td>
<td>7</td>
<td>5</td>
<td>71.8 ± 6.03</td>
</tr>
<tr>
<td>controls</td>
<td>7</td>
<td>5</td>
<td>88.2 ± 2.91</td>
</tr>
<tr>
<td>leupeptin</td>
<td>10</td>
<td>5</td>
<td>74.3 ± 3.20</td>
</tr>
<tr>
<td>controls</td>
<td>10</td>
<td>5</td>
<td>86.9 ± 3.89</td>
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<tr>
<td>leupeptin</td>
<td>14</td>
<td>5</td>
<td>56.5 ± 4.53</td>
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<tr>
<td>controls</td>
<td>14</td>
<td>5</td>
<td>69.5 ± 4.85</td>
</tr>
<tr>
<td>bestatin</td>
<td>7</td>
<td>10</td>
<td>89.0 ± 2.51</td>
</tr>
<tr>
<td>controls</td>
<td>7</td>
<td>10</td>
<td>89.3 ± 3.74</td>
</tr>
</tbody>
</table>

*MPSS = methylprednisolone sodium succinate. Significance (compared to the value of the control groups by Student's t-test): ² = p < 0.05; ³ = p < 0.001; ¹ = p < 0.01.
† Values represent the mean ± standard deviation.

**Morphometry**

The results of morphometric measurement of the degeneration index in individual rats treated with MPSS, leupeptin, and bestatin and their control groups sacrificed on Day 7 are plotted in Fig. 4. The average degeneration index for each of the experimental groups is summarized in Table 2. As can be seen in Fig. 4, there was a modest difference in the degeneration index between the MPSS-treated and the untreated control rats. Although there was considerable overlap, the difference between the MPSS-treated and the untreated control rats was significant (p < 0.05). In contrast, there was no overlap between the leupeptin-treated and the untreated control rats, and the difference between the groups was more marked (p < 0.001). No difference was seen between the bestatin-treated rats and the untreated control rats.

Figure 5 shows the influence of survival time on the density of silver grains in the leupeptin experiment. As noted before, the deposit of silver grains diminished as a function of time in both the leupeptin-treated and the untreated control rats. The changes in the degeneration index reflecting the reduction in the amount of silver grains demonstrable by the Fink-Heimer method were more pronounced between Days 10 and 14 than between Days 7 and 10 in both the leupeptin-treated and the untreated control rats (Table 2). Nevertheless, regardless of the length of survival time, the difference between the leupeptin-treated and the untreated control groups was highly significant. The probability of error was less than 0.001 in the rats sacrificed on Days 7 and 10 and less than 0.01 on Day 14.

**Discussion**

Corticosteroids are widely used in the treatment of acute spinal cord injury despite the lack of confirmation of their effectiveness in clinical trials. Several investigators have claimed the efficacy of a large dose of MPSS.
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in experimental acute spinal cord injury. On the other hand, a recent clinical survey on the efficacy of large and standard MPSS treatments in acute spinal cord injury disclosed no difference between two regimens in the neurological outcome of patients. In the present study, two large doses of MPSS (30 mg/kg) were administered to rats 30 minutes and 2½ hours after injury, respectively, since this regimen seemed to be effective in protecting neurofilament degradation in the injured tissue. In specimens examined 7 days after injury, significant protection of axons was seen in MPSS-treated rats (Fig. 3e and f and Table 2) even though the clinical grading of these rats was not very different from that of untreated control groups (Table 1). Since the pharmacological actions of MPSS in CNS tissue injury are known to be diverse, the mechanisms of the protection in the present study remain obscure. However, better preservation of axons on Day 7, when acute tissue destructive processes had largely subsided, may indicate the beneficial effects of MPSS on the neurological outcome of injury over the long term.

The protection of axons was more clearly seen in the rats treated with leupeptin, a specific inhibitor of calcium-activated neutral protease. In this group, there was no overlap of the degeneration index between the treated and untreated control rats at any time after injury (Fig. 4). It should also be noted that leupeptin is a potent inhibitor of thiol endopeptidases and that its pharmacological actions are not as multifactorial as those of MPSS. Thus, the protective effect of leupeptin in acute spinal cord injury is more likely due to its antiprotease activity. Complete failure of bestatin, an inhibitor of exopeptidases (particularly of aminopeptidases on the cell surface), in suppression of axonal degeneration also supports the specific requirement of neutral protease inhibitors in the treatment of acute spinal cord injury.

The limitation of the Fink-Heimer method in detecting degenerating axons in animals with longer survival periods can be seen in the experiment with leupeptin. Reduction of the degeneration index in the animals sacrificed on Day 14 is not due to the alleviation of tissue damage but rather to the changes in degenerating axons. With this method, the maximum development of silver grains is seen about 1 week after tissue damage, and the amount of silver grains rapidly diminishes afterward. In experiments comparing drug effects, therefore, the length of survival time should be the same both in experimental and control animal groups.

In the past few years, much attention has been focused on axonal damage, in particular on the degradation of neurofilaments, in the destructive process in acute spinal cord injury. In fact, the degradation of neurofilaments appears to be one of the earliest events in tissue destruction at the site of mechanical insult as witnessed by the biochemical and ultrastructural studies of Banik, et al. They suggested that the loss of neurofilament proteins occurred in accordance with granular degeneration of axons as early as 15 minutes after trauma, apparently preceding the degeneration of myelin sheaths. In in vitro experiments, the rapid degradation of neurofilaments by the proteases endogenous to the nervous tissue (possibly calcium-activated neutral proteases) has been shown by several investigators.

If one considers the fact that accumulation of calcium is known to take place quickly at the site of spinal cord trauma, it is conceivable that the calcium-activated proteases play a key role in the degradation of axons in acute spinal cord injury. Our preliminary experiment, in which leupeptin substantially reduced the extent of axonal damage in experimental acute spinal cord injury in rats, also supports this possibility.

References


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