The time course and extent of motor neuron loss following spinal cord compression in rats

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Motor deficits after spinal cord injury arise from damages to the descending spinal pathways and ventral motoneurons (VMN). In contrast to data on damages to the white matter or the descending spinal pathways, few quantitative data on damages to VMN after injury are available currently. The purpose of this study was to examine quantitatively the temporal and spatial pattern of VMN loss after spinal cord compression. Two groups of adult female Wistar rats were used in this study: rats which were subjected to spinal cord compression in short duration with an aneurysm clip (experimental group) and rats which were subjected to a sham-operation (control group). Using serial cross-sections of the spinal cord, VMN were counted up to the 7th day after surgical intervention at 0, 1, 2, and 3 mm rostral and caudal to the lesion epicenter (experimental group) or to the median of the serial sections (control group). At 15 minutes after the compression, VMN were lost only at the epicenter section and no VMN were observed there. By 8 hours, VMN loss had spread to next 1 mm rostral and caudal section to the epicenter. Virtually, no further loss was detected between 8 hours and later time points. This study showed that compression to the adult rat spinal cord in short duration led to VMN loss, which progressed acutely and expanded modestly. Our findings could be used to develop effective treatment and provide a better understanding of VMN loss after spinal cord injury.

Introduction

Spinal cord injury (SCI) leads to a loss of motor function as paraplegia or quadriplegia. This deficit is produced from damages to the descending spinal pathways and ventral motoneurons (VMN). Though there has been much quantitative data on the loss of white matter tissue or spinal axons following experimental SCI by impact or compression trauma[2, 3, 6, 14, 15], VMN loss after these traumas was not previously subjected to quantitative analysis. Recent studies demonstrated that stem cells can differentiate into VMN and these neurons can innervate muscles[7, 10]. Information on VMN loss after SCI is beneficial to develop strategies to replace lost VMN through stem cell transplantation.

Recently, some investigators have provided quantitative data on the temporal and spatial pattern of VMN loss after experimental SCI. To obtain a guideline for pharmacological protection, Grossman et al.[8, 9] gave a weight-drop impact onto the adult rat spinal cord and examined the changes of VMN number after the impact. Brown et al.[4] investigated VMN number in the young

・ラット脊髄の圧迫後における神経細胞損失の経過と範囲

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rat spinal cord after a weight-drop impact. However, to our knowledge, no quantitative data on VMN loss after compression is available currently. The purpose of the present study was to investigate quantitatively the time course and extent of VMN loss after spinal cord compression in rats.

Materials and Methods

All experimental procedures were approved by the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima University School of Medicine.

1. Operative procedures

Thirty-two female Wistar rats with an average body weight of 175 g (157-197 g) were used. The rats were anesthetized with 50 mg/kg sodium pentobarbital intraperitoneally, and a midline skin incision was made on the dorsal side. After exposing the laminae, a laminectomy was performed at the T8 as described previously[13]. For the experimental group, the spinal cords of 20 rats were extradurally compressed at T8 level for 30 seconds with an aneurysm clip with 1.2 mm width blades calibrated to deliver a closing force of 52 g (Mizuho Medical Co., Tokyo, Japan). This intensity of compression has been reported to result in moderate to severe paraplegia[17]. For the control group, the spinal cords of 12 rats were not compressed although these rats underwent the laminectomy. After these procedures, the muscles and the skin were closed in the layers. The rats were placed in a cage with wood chips and given unlimited access to food and water. They were under the circumstance of a 12 hours light/dark cycle at temperature of 23℃ and humidity of 50%. Manual bladder expression was performed twice a day in the experimental group.

2. Tissue processing

From the experimental group of 20 rats, 4 were killed at each time point (15 minutes, 8 hours, 24 hours, 3 days, and 7 days after the compression). Four rats from the control group were killed at 24 hours, 3 days, and 7 days postoperatively. All rats were anesthetized with sodium pentobarbital intraperitoneally and were perfused transcardially with 0.9% saline followed by 4% phosphate buffered paraformaldehyde. The spinal cord segment containing the compressed site or laminectomy at its center was removed and was fixed with 4% phosphate buffered paraformaldehyde overnight in 4℃. After being fixed, the spinal cord was dehydrated in a graded series of alcohol and was embedded in paraffin. Serial 10 μm cross-sections were cut from each specimen and every 20th section was mounted onto slides.

3. Counting ventral motoneurons

Klüver-Barrera's luxol fast blue staining that visualizes both myelin and neuronal cell bodies was used to count VMN. For the experimental group, after finding the section including the epicenter which was most bleeding or destroyed, 7 sections of 0, 1, 2, and 3 mm rostral and caudal to the epicenter were chosen. For the control group, 7 sections of 0, 1, 2, and 3 mm rostral and caudal to the median of the serial sections were chosen. In the present study, when we expressed a location rostral or caudal to the epicenter or the median of the serial sections, "+" or "-" were added ahead of the numerical value, respectively.

Based on a modification of the criteria reported by Grossman[9], VMN were counted in the 7 sections.

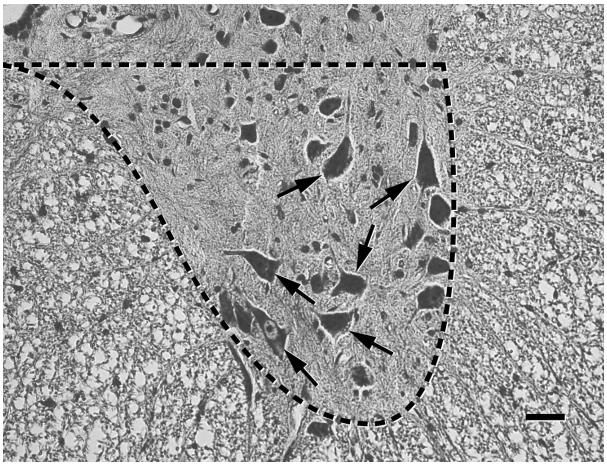

Fig. 1. Ventral motoneuron counting in the thoracic spinal cord.

Using serial cross-sections of the spinal cord stained with Klüver-Barrera's luxol fast blue staining, VMN were counted at 0, 1, 2, and 3 mm rostral and caudal to the lesion epicenter (experimental group) or to the median of the serial sections (control group). Among cell profiles in the gray matter ventral to the gray commissure (demarcated area), the profiles which had diameters larger than 20 μm were identified as VMN. Furthermore, counts required presence of a nucleolar profile. Arrows point VMN included for counting in this ventral horn. VMN number at both right and left ventral horn was summed and averaged in each section.
Scar bar = 20 μm

Firstly, cells which were located in the gray matter ventral to the gray commissure were identified. Next, among the identified cells, only cells whose diameters were larger than 20 μm measured by a scale with a light microscope were chosen. To avoid estimating VMN number excessively, counting was performed by the method of central profile counts[5]; we counted cell profiles which contained both a nuclear and a nucleolar profile as VMN. In this method, edges of cells were excluded (Fig. 1). VMN number at both right and left ventral horn was summed and averaged in each of the 7 sections.

4. Statistical analysis

All data were analyzed using the computer program SPSS 11.5J for Windows (SPSS Japan Incorporated, Tokyo). The number of VMN was compared between the groups different for the period after the operations and between the different locations in each group by two-way repeated-measures analysis of variance (ANOVA). When significant interactions were found, one-way ANOVA followed by Scheffe's post hoc test was performed to locate the level of factor(s) with significant differences in the means of the variables. Differences were considered to be statistically significant at the level of $p < 0.05$.

Results

The spinal cord from the control group was structurally intact throughout the experimental period. In the experimental group, hemorrhage was observed at and beyond the epicenter 15 minutes after the compression. At the epicenter, hemorrhage created a large hematoma, which covered the gray matter the pericentral white matter (Fig. 2A). Beyond the epicenter, hemorrhage covered predominantly the dorsal funiculi (Fig. 2B). The distribution of hemorrhage is consistent with those found by others[9, 18]. No further bleeding was seen after this time point. The spinal cord lesion

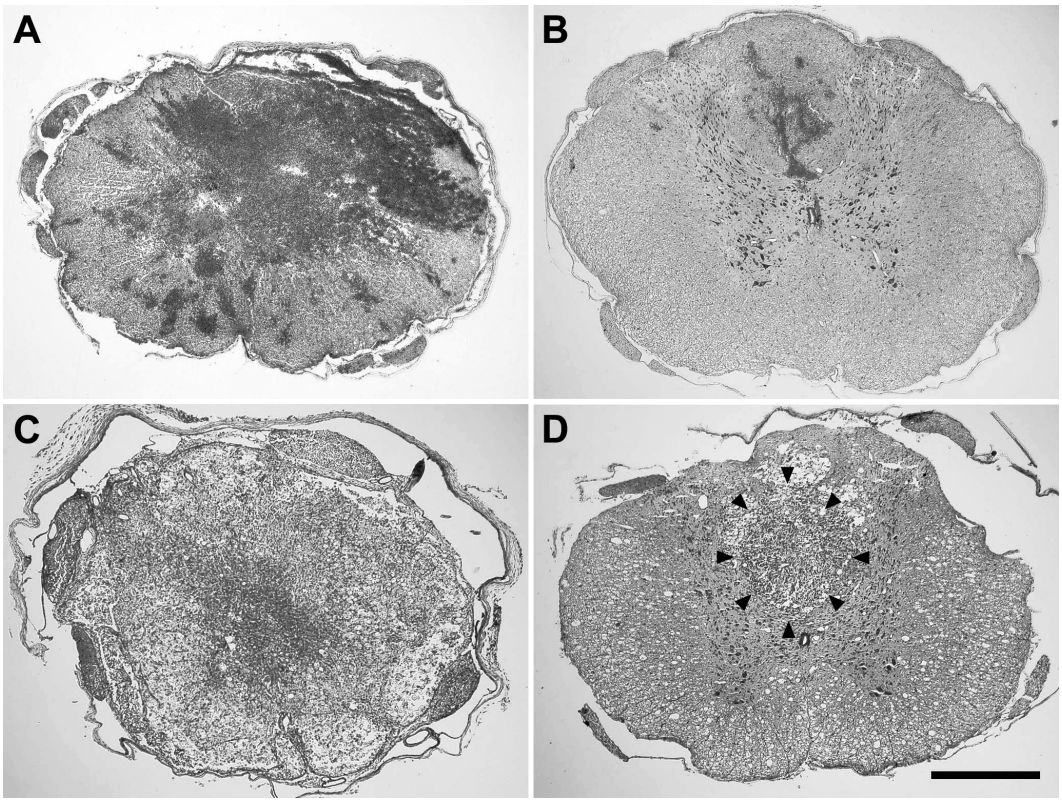

Fig. 2. Histopathology of the spinal cord after spinal cord compression.

Cross-sections of the spinal cord at 15 minutes after the compression showed a central hematoma at the epicenter (A) and hemorrhage which predominantly covered the dorsal funiculi beyond the epicenter (B). The spinal cord at 7 days after the compression indicated necrotic lesion at almost the entire cross-sectional area of the epicenter (C) and at the dorsal funiculi away from the epicenter (D; arrowheads). Klüver-Barrera's luxol fast blue staining.
Scar bar = 500 μm

developed at almost the entire cross-sectional area of the epicenter (Fig. 2C) and at the dorsal funiculi away from the epicenter over time (Fig. 2D).

Table 1 shows VMN numbers in the time points and locations examined in the control group. No significant interaction was detected between the time points and locations ($p = 0.68$). Therefore, the control rats were considered to be one group statistically, and the number of VMN in the median of the serial sections (0 mm in the control group) for their spinal cords was used as the standard.

Table 2 shows VMN numbers in the median of serial sections of all the control group and in the time points and locations examined in the experimental group. Since there was a highly significant interaction between the time points and locations ($p = 3.63 \times 10^{-60}$), the comparisons in time- and location-dependent manner were performed by one-way ANOVA followed by Scheffe's post hoc tests, respectively.

In the comparison in time-dependent manner, at 15 minutes after compression, the VMN numbers at the epicenter (0 mm section in the experimental group) was different from the standard VMN number from the control groups ($p < 0.001$). No VMN was seen at 0 mm section at this and later time points. At 8 hours and later time points, the VMN numbers at ± 1 mm sections were significantly decreased compared to those of the standard number and 15 minutes ($p < 0.05$). At 3 days, the VMN numbers at ± 1 mm sections were different from the VMN numbers at 8 hours at ± 1 mm sections ($p < 0.05$). However, except for the VMN numbers at 3 days, no further loss was detected between 8 hours and later time points. At 8 hours and later time points except 3 days, the VMN numbers at ± 1 mm sections were 21-43% of the standard number from the control groups (Table 2).

In the comparison in the location-dependent manner, the VMN number at 0 mm sections at 15 minutes was significantly different from that at other locations ($p < 0.001$). The VMN number at 0 mm and ±

Table 1. VMN number in the control group

Location / *Group*	+3 mm	+2 mm	+1 mm	0 mm	-1 mm	-2 mm	-3 mm
24 hours	7.1 ± 1.44	7.3 ± 0.96	6.6 ± 0.48	6.5 ± 1.47	6.4 ± 0.63	7.3 ± 1.76	7.0 ± 0.82
3 days	7.9 ± 1.25	7.0 ± 0.91	7.1 ± 0.25	6.6 ± 0.25	8.0 ± 1.35	7.6 ± 1.60	7.6 ± 1.25
7 days	6.3 ± 0.50	7.1 ± 1.49	6.0 ± 1.29	7.1 ± 1.31	7.6 ± 1.11	7.1 ± 1.49	7.9 ± 1.25

Comparisons between each group and between each location were performed with two-way repeaed miasures ANOVA. There are no significant difference. Values are mean ± S.D.

Table 2. VMN number after spinal cord compression

Location / *Group*	+3 mm	+2 mm	+1 mm	0 mm	-1 mm	-2 mm	-3 mm
Control				6.8 ± 1.08			
Experimental							
15 minutes	6.5 ± 1.58	7.4 ± 1.89	5.5 ± 1.00	0.0 ± 0.00[a,d]	6.3 ± 0.29	6.3 ± 1.50	7.0 ± 0.82
8 hours	6.8 ± 1.19	7.0 ± 0.58	2.9 ± 0.85[a,b,e]	0.0 ± 0.00[a,e]	2.1 ± 0.25[a,b,e]	6.9 ± 0.75	8.8 ± 1.04
24 hours	6.4 ± 0.48	6.4 ± 1.55	1.4 ± 0.48[a,b,e]	0.0 ± 0.00[a,e]	1.4 ± 0.25[a,b,e]	6.4 ± 0.48	6.9 ± 1.49
3 days	7.9 ± 0.75	5.5 ± 1.35	0.3 ± 0.50[a,b,c,e]	0.0 ± 0.00[a,e]	0.0 ± 0.00[a,b,c,e]	6.8 ± 1.19	7.4 ± 1.80
7 days	6.4 ± 0.85	7.3 ± 0.87	1.5 ± 0.41[a,b,e]	0.0 ± 0.00[a,e]	1.5 ± 1.22[a,b,e]	6.6 ± 1.11	7.0 ± 0.71

Comparisons between each group or between each location were performed with one-way ANOVA followed by Scheffe's post hoc tests, respectively.
The value of the control group is VMN number in the median of the serial sections (0 mm) of all the control groups.
Values are mean ± S.D.
a : compared to the control group.
b : compared to the same location in the 15 minutes group.
c : compared to the same location in the 8 hours group.
d : compared to other locations in the same time group.
e : compared to the ± 2 mm and ± 3 mm in the same time group.

1 mm at 8 hours and later were significantly different from those at ± 2 mm and ± 3 mm ($p < 0.001$) (Table 2).

Discussion

1. Accuracy of VMN counting

Based on the method of central profile[5], we counted cellular profiles which contained both a nuclear and a nucleolar profile as VMN. During histological sectioning, VMN might be split randomly into a part which contained a nucleus and a nucleolus and parts which did not contain them. Therefore, our results were smaller than the actual VMN number. However, since we processed all spinal cords by consistent procedures, the comparison of VMN number between the groups different from the period after the operations and between the different locations in each group is possible.

2. The time course and extent of VMN loss after spinal cord compression

Our data indicated that VMN loss developed progressively and expansively after compression to the adult rat spinal cord in short duration. At 15 minutes after the compression, while no VMN were seen at the epicenter, no changes were observed in VMN number at other locations. At 8 hours, VMN loss had spread to ± 1 mm sections and 57-69% of VMN had been lost there. We found that the VMN number at 8 hours after compression was the same as the number at 7 days.

Previous investigators have revealed the time course and extent of VMN loss after a weight-drop impact onto the rat spinal cord. Grossman et al.[8, 9] placed an impounder with a tip of 2.4 mm in diameter onto the adult rat spinal cord, dropped a 10 g weight from a height of 2.5 cm onto the impounder, and examined the changes of VMN number until 1 month after the weight-drop impact. In their study, at 15 minutes after the impact, VMN loss was the most remarkable at the epicenter (67% of VMN were lost) and tapered rostrally and caudally in a symmetrical manner, but no loss was observed at ± 4 mm. By 24 hours, complete loss of VMN was seen within ± 2 mm and 56% of VMN were lost at ± 4 mm. No difference was seen in VMN number between 24 hours and 1 month after the impact. Recently, Brown et al.[4] gave a 10 g × 2.5 or 5.0 cm weight-drop impact onto an impounder with a tip of 1.5 mm in diameter which was put on the young rat spinal cord, and investigated the changes of VMN number till 28 days. They reported that in these two intensities of impact, VMN loss was restricted within ± 2 mm at 24 hours but expanded at least to ± 4 mm at 28 days. The spinal cord of young rats elongates with growing. However, in their study, VMN were counted at 0, 1, 2, 3, and 4 mm rostral and caudal to the epicenter throughout the experimental period, such that their results include the relative gap accompanying growth. Therefore, it is not suitable to compare their results on the young rat spinal cord with our results on the adult rat spinal cord.

Comparing the results from our present study and those by Grossman et al.[8, 9], the following differences seemed to exist between them. Firstly, immediately after SCI, VMN loss at the epicenter appeared to be greater in the spinal cord which received the compression than in the spinal cord which received the weight-drop impact. While no VMN remained there in our study, one third of VMN were present in their study. Secondly, immediately after SCI, the extent of VMN loss seemed to be more restricted at the actually traumatized site in our results than in theirs. In our study, the spinal cord was compressed with an aneurysm clip with 1.2 mm width blades and VMN were lost only in the compressed site. In their studies, loss was seen at least within ± 3 mm and this extent was 2.5 times wider than the diameter of tip of impounder which gave an impact to the spinal cord. Thirdly, though progressive loss of VMN was observed in both studies, the time course of loss in our study (8 hours) was considered to be shorter than that seen in theirs (24 hours). Finally, at the time when no further VMN loss was found, the extent of loss was supposed to be narrower in our results than in theirs. In our study, VMN loss was noted within ± 1 mm and this extent was about 2 times greater than the width of the blades of aneurysm clip which compressed the spinal cord. In their study, loss had spread at least within ± 4 mm and this extent reached a 3.3-fold at least to the diameter of tip of impounder.

Immediately after SCI, our compression which caused severer VMN loss at the epicenter than the impact of Grossman et al.[8, 9] appeared to give greater force there than the latter. On the other hand, since the extent of loss in their results was more extensive than that shown in our results, the extent to which

sufficient force to lose VMN was brought seemed to be wider in their impact. Spinal cord compression can be characterized as static and continuous force to the spinal cord. In contrast, impact to the spinal cord gives it force dynamically and instantly. The differences of the mechanical kinetics in each trauma may vary the extent to which sufficient force to lose VMN is given. Unfortunately, exact mechanism can not be explained through the present study.

Mechanical force in SCI produces not only direct loss of VMN but also additional loss by inducing secondary autodestructive processes such as excitatory amino acid neurotoxisity, free radical lipid peroxydation, and apoptosis[1, 12]. Our compression brought severer force to more restricted area as compared with the impact of Grossman et al.[8, 9], such that secondary processes possibly progressed more rapidly in narrower area in the spinal cord subjected to our compression. That means that VMN loss perhaps occurred in shorter time course and in narrower extent after our compression than after their impact. We processed the spinal cord with alcohol and paraffin, so the tissue might have undergone some shrinkage by dehydration and heat. Thus, the extent of VMN loss in our study seemed to include some underestimation. However, this change is small and therefore can not deny the difference in the extent of VMN loss between our results and theirs.

3. Use of the experimental model which has VMN loss within particular area makes evaluation of therapeutic efficacy of cell transplantation easy

Our spinal cord compression in short duration resulted in VMN loss within more restricted area compared with the weight-drop impact to the spinal cord. This finding suggests that this method can bring dysfunction only to particular muscles by making loss of VMN within a selected area. Recently, a number of investigators have studied to replace lost VMN through stem cell transplantation[7, 10, 11, 16, 19]. Use of the experimental model which has dysfunction in particular muscles will make evaluation of therapeutic efficacy of cell transplantation easy.

Conclusion

Compression to the adult rat spinal cord in short duration led to progressive and expansible loss of VMN. The loss progressed until 8 hours after compression and expanded from the compressed site slightly.

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