Decreased choline acetyltransferase activity in the murine spinal cord motoneurons under chronic mechanical compression

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The tiptoe-walking Yoshimura (twy) mouse is a model of chronic spinal cord compression caused by ossification of intraspinal ligaments. Choline acetyltransferase (CAT), which is known to be a specific marker of cholinergic neurons, best reflects spinal motoneuron function. Changes in CAT immunoreactivity following chronic spinal cord compression in twy mice were investigated quantitatively in order to elucidate spinal motoneuron functional changes according to the degree and direction of compression. Thirty 24-week-old twy mice were used in this study. They were divided into three groups according to the direction of spinal cord compression (anterior, posterior, and lateral) and the CAT immunoreactivities in whole sections of their upper cervical spinal cords were investigated quantitatively using a fluorescence microphotometry system. The lateral compression group showed histological spinal motoneuron atrophy and loss on the compressed, but not the non-compressed, side. Spinal motoneuron atrophy and loss were observed when the severity of spinal canal stenosis due to the ossified lesion, expressed as the occupation rate, was 30% or more, but the spinal motoneurons appeared normal when it was below 30%. The CAT immunofluorescence intensity of the anterior horn showed a linear negative correlation with the degree of canal stenosis. When the occupation rate was below 20%, the CAT immunofluorescence intensities in the anterior horns of the compression and control groups did not differ significantly. The CAT immunofluorescence intensity of twy mice with occupation rates of 20% or more were significantly lower than that of those with occupation rates below 20%. Furthermore, the CAT immunofluorescence intensity was significantly lower on the compressed than the non-compressed side of the lateral compression group. Thus, our findings indicate that an occupation rate of about 20% may be the critical level for functional changes in the spinal motoneurons.

Keywords: cervical spinal cord; chronic compression; choline acetyltransferase; twy mouse

Introduction

Choline acetyltransferase (CAT), which is known to be a specific marker of cholinergic neurons, best reflects spinal motoneuron function. Although acetylcholine has been known as a neurotransmitter for many years, relatively little is known about the distribution or connections of spinal cholinergic neurons,¹ mainly due to technical limitations to investigating cholinergic systems at the cellular level. The recent immunohistochemical method devised for detecting CAT provides a reliable means of localizing cholinergic neurons,^{2–6} and a new fluorescence microphotometry system has been developed which is extremely useful for analyzing the distribution and amount of neurotransmitters at the cellular level.^{7–9} This system can accurately measure the fluorescence intensity of CAT immunoreactivity,

which provides a sensitive means of assessing the function of cholinergic neurons.¹⁰ It would therefore be very useful to apply these procedures to the investigation of CAT activity changes and distribution in the spinal cord of the mouse, because they should provide direct information about the gray matter. The aim of this study was to investigate CAT immunoreactivity changes following chronic spinal cord compression in mice in order to elucidate the functional changes in cholinergic neurons, especially motoneurons, according to the degree and direction of compression.

Materials and methods

Chronic spinal cord compression model (twy mouse)

The tiptoe-walking Yoshimura (twy) mouse (Central Institute of Experimental Animals, Kawasaki, Japan) is a strain characterized by hereditary abnormalities of

bone and cartilage. It exhibits contractures of the peripheral joints of its extremities and an abnormal gait described as 'walking tiptoe',¹¹ and develops chronic spinal cord compression due to ossification and/or calcification of the spinal ligaments, especially in the upper cervical region.¹²

In this study, 30 24-week-old twy mice were used as a model of chronic spinal cord compression (compression group) and 12 24-week-old Institute of Cancer Research (ICR) mice were used as controls (control group).

Immunohistochemistry

Both the twy and ICR mice were perfused intracardially with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, their cervical spines and spinal cords between C1 and C3 were removed *en bloc*, postfixed with the same solution for 48 h, decalcified with 5% ethylenediaminetetraacetate for 7 days, and then cryoprotected by incubation with 10% buffered sucrose at 4°C overnight. After quick freezing with dry ice and acetone, serial 10 µm-thick frozen sections were cut with a cryostat and stained immunohistochemically using the procedures reported previously.^{8,13} In brief, the sections were washed with PBS at 4°C, treated with anti-CAT rabbit serum, diluted 1:100 with PBS (Chemicon International, Temecula, CA, USA), for 12 h at 4°C, washed with PBS, treated with FITC-labeled anti-rabbit-IgG goat serum,^{14,15} diluted 1:20 with PBS (Tago; Biosource International, Cararillo, CA, USA), for 2 h at room temperature and the stained sections were embedded in 20% glycerin-PBS.

Fluorescence microphotometry

The CAT immunofluorescence intensity of each anterior horn was measured using a microphotometry system (Model P-101; Nikon, Tokyo),¹⁰ which enables the average fluorescence intensity of a small spinal cord region to be measured through a measuring spot. The spinal cord section was moved in the X- and Y-directions by means of a high-precision step-motor scanning stage under the objective lens of a fluorescence microscope and the fluorescence intensity of its entire surface was analyzed. The fluorescence intensity data and positions (X and Y values) were transmitted to a PC9801FA computer (NEC, Tokyo), for statistical analysis. The microphotometry system conditions were: excitation range, 420–490 nm; interference filter, 530 nm; photo-multiplier voltage, 850 V; objective lens, 20×/0.75 (magnification/aperture number) and measuring spot diameter, 40 µm. The mean fluorescence intensity of the anterior horn was determined by analyzing the data for the connected measuring points and the values presented are means ± s.e.

Histopathology

After the CAT distribution analysis had been completed, the spinal cord sections were stained with

hematoxylin-eosin and examined histologically under a light microscope. The twy mice were divided into three groups, according to the direction of spinal cord compression (anterior, posterior and lateral). If compression regions were present at more than one site, the group was assigned according to the direction of greatest compression. The degree of compression is expressed as the occupation rate ($A/B \times 100\%$; A: area of ossified ligaments, B: cross-sectional area of the spinal canal).

Statistical analysis

Statistical significance was defined as $P < 0.05$ using a non parametric test followed by the Mann-Whitney U-test.

Figure 1 (a) The lateral compression group. (Hematoxylin-eosin staining. Scale bar: 1000 µm). (b) Atrophy and loss of the anterior horn cells were observed on the compressed side, but not on the non-compressed side. (Hematoxylin-eosin staining. Scale bar: 100 µm)

Results

Histopathological findings

No histological changes were observed in the anterior horn cells of the control group. There were eight, six and 16 twy mice in the anterior, posterior and lateral compression groups respectively. In the lateral compression group, anterior horn cell atrophy and loss were observed on the compressed, but not on the non-compressed, side. There were no histological differences between the right and left sides of either the anterior or posterior compression groups (Figure 1). There were no histopathological changes in the anterior horn cells of the twy mice, with occupation rates below 20% (mild compression). Anterior horn cell loss and atrophy were observed in those with occupation rates of 30% or more (severe compression), and those with occupation rates of 20–30% (moderate compression), showed few histopathologic changes in the anterior horn cells (Figures 2, 3, 4 and Table 1).

CAT immunofluorescence intensity changes in the anterior horn

CAT-immunoreactive neurons were present in the ventrolateral anterior horns of the ICR and twy mice, and the CAT immunofluorescence intensity in

Table 1 Occupation rate and histological changes of anterior horn cells

Occupation rate	Cell atrophy	Cell loss
~ 20%	1/13	0/13
20 ~ 30%	4/13	2/13
30% ~	4/4	4/4

There were no histopathological changes in the anterior horn cells at occupation rates of less than 20%. Atrophy and loss of anterior horn cells were observed at occupation rates of 30% or more. On the other hand, at occupation rates of 20–30%, histopathological changes in the anterior horn cells were observed in four mice out of 13

Figure 2 (a) The mild compression was observed in the spinal cord. (occupation rate <20%) (Hematoxylin-eosin staining. Scale bar: 1000 μ m). (b) There were no histopathological changes of the anterior horn cells. (Hematoxylin-eosin staining. Scale bar: 100 μ m)

Figure 3 (a) The severe compression was observed in the spinal cord. (occupation rate >30%) (Hematoxylin-eosin staining. Scale bar: 1000 μ m). (b) Atrophy and loss of the anterior horn cells were observed. (Hematoxylin-eosin staining. Scale bar: 100 μ m)

the anterior horn showed a linear negative correlation with the occupation rate (Figure 5). Moreover, the CAT immunofluorescence intensity of the anterior horn in two mice with occupation rates below 20% (mild compression), did not differ significantly from that of the control group. The CAT immunofluorescence intensities of mice with occupation rates of 20% above (moderate and severe compression) were significantly lower than that of those with occupation rates below 20% (mild compression) (Figure 6). Furthermore, in the lateral compression group, the CAT immunofluorescence intensity on the compressed side was significantly lower than that on the non-compressed side (Figure 7).

Discussion

It is well known that severe chronic spinal cord compression causes histological changes, such as

Figure 5 CAT immunofluorescence intensity in the anterior horn showed a linear negative correlation with occupation rates ($r=0.839$, $P<0.0001$)

Figure 4 (a) The moderate compression was observed in the spinal cord. (occupation rate 20–30%) (Hematoxylin-eosin staining. Scale bar: 1000 μ m). (b) There was atrophy of the anterior horn cells, but the number of the anterior horn cells was kept normally. (Hematoxylin-eosin staining. Scale bar: 100 μ m)

Figure 6 (a) CAT immunofluorescence microphotometry of the anterior horn. Control group. (Scale bar: 200 μ m). (b) CAT immunofluorescence microphotometry of the anterior horn cells. Control group (Scale bar: 100 μ m)

anterior horn cell atrophy and loss.^{16,17} However, exactly what degree of chronic spinal cord compression causes functional changes in cholinergic neurons, especially spinal motoneurons, is unclear. One reason for this is that functional changes in the gray matter, especially of spinal motoneurons, could not be demonstrated, due to difficulty actually reaching the gray matter and elucidating its functions. In this study, we used a new fluorescence detection system to overcome this problem and demonstrated that the CAT immunoreactivity changes correlated with the severity of the histopathologic changes in the chronically compressed spinal cord. Another reason was the lack of availability of a satisfactory chronic spinal compression model. Although many animal models of chronic spinal cord compression have been described, most of them have proved artificial and unsatisfactory.¹ The two mouse exhibits multi-level spinal canal stenosis resulting from the natural occurrence of ossification of various spinal ligaments, including those of the upper cervical spine.^{11,12} Therefore, we chose this animal as the chronic spinal compression model for use in our study.

We found that mild chronic spinal cord compression (occupation rate <20%) caused neither histopathologic nor CAT immunoreactivity changes, whereas severe chronic spinal cord compression (occupation rate >30%) caused spinal motoneuron atrophy and/or loss and reduced CAT immunoreactivity, in the anterior horn. Some investigators showed that the transverse area was correlated with the severity of pathological changes¹⁶ and that chronic compressed spinal cord with a transverse area below 60% of normal appeared to be associated with severe and irreversible pathological changes in patients with ossification of the posterior longitudinal ligament,¹⁸ which is consistent with our findings. Furthermore, we found that moderate chronic spinal cord compression (20% < occupation rate < 30%) did not cause histologic changes, but resulted in a significant decrease in the CAT immunoreactivity in the anterior horn. In other words, moderate chronic spinal cord compression caused functional changes in the spinal motoneurons

prior to the development of histopathologic changes. These findings indicate that moderate chronic spinal cord compression (occupation rate of around 20%) may be the critical level for functional changes in spinal motoneurons.

Figure 8 (a) CAT immunofluorescence microphotometry of the anterior horn. Moderate compression group. (Scale bar: 200 μ m). (b) CAT immunofluorescence microphotometry of the anterior horn cells. Moderate compression group. (Scale bar: 100 μ m)

Figure 7 CAT immunofluorescence intensity at occupation rates of 20% and above were significantly lower than at occupation rates of less than 20%

Figure 9 In the lateral compression group, CAT immunofluorescence intensity on the compressed side was significantly lower than on the non-compressed side

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