III. 5. Biochemical, Behavioral and Immunohistochemical Study in the Brain of MPTP-treated Mouse Model


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INTRODUCTION

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is known to cause degeneration of mesencephalic dopaminergic neurons in several species including monkeys, dogs, cats and mice. The neurotoxic effects of MPTP are thought to be initiated by 1-methyl-4-phenyl-pyridinium ion (MPP⁺) which is a major metabolite formed by the monoamine oxidase (MAO) B-mediated oxidation of MPTP. MPP⁺ is taken up by high-affinity dopamine and noradrenaline uptake systems and is subsequently accumulated within mitochondria of nigrostriatal dopaminergic cells. This can lead to a number of deleterious effects on cellular function, resulting in neuronal cell death. Therefore, MPTP-treated animals are widely used as one of models for Parkinson’s disease.

Induction of parkinsonism by MPTP in mice has generated a wealth of neurochemical, pharmacological and anatomical findings. However, in order to deplete striatal dopamine in mice, large doses of MPTP and frequent injections are required. Although the magnitude of the striatal dopamine loss can be increased under some circumstances, progressive and persistent dopamine depletion over a relatively long period has yet to be demonstrated. Furthermore, little is known about the relationship between motor abnormalities and dopamine depletion in MPTP-treated mice.

In the present study, therefore, we investigated the relationship between motor deficit and dopamine depletion in mice after MPTP treatment. Furthermore, we examined immunohistochemically changes of neurons and glial cells in the striatum and substantia nigra of mice after MPTP treatment.
MATERIALS AND METHODS

Male C57BL/6 mice (Nihon SLC Co., Shizuoka, Japan), 8 weeks of age, were used in this study. All experiments were performed in accordance with Guidelines for Animal Experiments of the Tohoku University School of Medicine.

The mice were injected intraperitoneally MPTP hydrochloride (20 mg/kg in saline) four times at 2-hr intervals within a day. The mice were killed by cervical dislocation at 1, 3, 7, 14 or 21 days after MPTP treatment. The striata were rapidly dissected and were then sonicated in ice-cold 0.2 M perchloric acid containing 100 ng/ml isoproterenol as an internal standard. Dopamine, DOPAC and HVA were quantified by high-performance liquid chromatography (HPLC) with an electrochemical detector (ECD) (Eicom, Kyoto, Japan), as described previously\textsuperscript{8,9}.

To measure cataleptic symptoms such as akinesia and rigidity, bar-test catalepsy was evaluated by placing both forepaws of the mouse over a horizontal bar (diameter: 0.2 cm), elevated 15 cm from floor, as described previously\textsuperscript{10}. The time during which the animals maintained this position was recorded. To determine the degree of bradykinesia, a typical symptom of parkinsonism, pole test was performed according to the method of Ogawa et al.\textsuperscript{11} with minor modifications\textsuperscript{10,12,13}. The mouse was placed head upward on the top of a rough-surfaced pole (8 mm in diameter and 50 cm in height) which was wrapped doubly with gauze to prevent slipping; the time until it turned completely downward (Tturn) and the time until it climbed down to the floor (TLA) were examined.

For immunohistochemical study, the paraffin sections of the striatum and substantia nigra were used as described previously\textsuperscript{14,15}. For tyrosine hydroxylase (TH) and glial fibrillary acidic protein (GFAP) immunostainings, a rabbit anti-TH polyclonal antibody (Chemicon International, Inc., Temecula, CA, USA), a mouse anti-GFAP monoclonal antibody (Chemicon International, Inc., Temecula, CA, USA) and a Vectastain elite ABC kit (Vector Lab., Burlingame, USA) were used. The immunohistochemical stainings with anti-TH antibody (1:200) or anti-GFAP antibody (1:200) were performed as described previously\textsuperscript{14,15,16}.

Microglial cells were histochemically stained with alpha-D-galactosyl-specific isolectin B\textsubscript{4} conjugated with horseradish peroxidase derived from Griffonia simpliciation seeds (GSA I-B\textsubscript{4}-HRP, isolectin B\textsubscript{4}) (Sigma, St Louis, MO, USA), as described previously\textsuperscript{17}.
RESULTS

The striatal dopamine, DOPAC and HVA content showed a severe reduction from 1 day to 14 days post-treatment except for the DOPAC level 14 days post-treatment. Thereafter, the striatal dopamine level showed a significant decline (59% loss) even 21 days after MPTP treatment. A significant reduction was also found in the striatal DOPAC (48% loss) and HVA (58% loss) level 21 days after MPTP treatment (Table 1).

The measurement of motor activity had a significant prolongation of Tturn, TLA and cataleptic effect 7 days after MPTP treatment. After 14 days, a significant prolongation of TLA and cataleptic effect was still observed in these mice. However, a significant prolongation of Tturn was not evident in these mice (Table 2).

Representative photographs of TH immunostaining in the striatum and substantia nigra are shown in Fig. 1. Dopaminergic neurons with the TH antibody were easily detectable in control mice. One day after MPTP treatment, TH immunopositive fibers and cell bodies were reduced in the striatum and substantia nigra, respectively. Thereafter, the TH immunopositive fibers and cell bodies were markedly decreased in the striatum and substantia nigra up to 7 days after MPTP treatment.

Representative photographs of GFAP immunostaining in the striatum and substantia nigra are shown in Fig. 2. GFAP positive astrocytes were evident in the substantia nigra. One day after MPTP treatment, GFAP immunopositive astrocytes were increased in the striatum and substantia nigra. Thereafter, GFAP positive astrocytes were markedly increased in the striatum and substantia nigra 3 and 7 days after MPTP treatment.

Representative photographs of isolectin B4 staining in the striatum and substantia nigra are shown in Fig. 3. Isolectin B4 positive cells were evident in the striatum and substantia nigra. One day after MPTP treatment, isolectin B4 positive cells were increased in the striatum and substantia nigra. Thereafter, isolectin B4 positive cells were markedly increased in the substantia nigra 3 and 7 days after MPTP treatment.

DISCUSSION

To clarify the pathophysiological mechanisms of Parkinson’s disease, first we investigated a close correlation between neurochemical and behavioral manifestation using mice with several studies. This study is the first report using mice in several experimental studies to test the close correlation between neurochemical and behavioral manifestation.

Since the discovery that MPTP selectively destroys nigrostriatal dopaminergic neurons in humans, a wide range of animal models for parkinsonism have been extensively
studied. Mice are susceptible to MPTP neurotoxicity and the animals make excellent conventional models for Parkinson’s disease. In general, MPTP is usually administered to mice either by an acute or a subacute regimen\textsuperscript{7,18}. In the acute and subacute mice models, MPTP can produce depletions in the striatal level of dopamine and its metabolites (DOPAC and HVA) along with a reduction in the striatal synaptosomal dopamine uptake\textsuperscript{18,19}. However, when survival times in mice are extended, the neurotoxic effects of MPTP are reversible\textsuperscript{20}. Furthermore, despite evidence of dopamine reductions, animals that receive MPTP acutely or subacutely do not always exhibit motor dysfunctions or motor abnormalities\textsuperscript{4,21}. Therefore, we examined the exact neurotoxic effects of acute or consecutive treatment of MPTP in mice.

In the present study, we investigated the striatal dopamine, DOPAC and HVA levels from 1 day to 21 days after acute MPTP treatment. As shown in Table 1, the acute MPTP treatment showed a severe reduction in the striatal dopamine, DOPAC and HVA content in mice from 1 day to 14 days post-treatment. Thereafter, the striatal dopamine level showed a significant decline (59% loss) even 21 days after MPTP treatment. In our behavioral studies, the acute MPTP treatment caused severe motor deficits in mice 7 and 14 days post-treatment (Table 2). From the present findings, we suggest that the model with acute MPTP treatment can cause a severe dopamine depletion and motor deficiency in mice. Based on these present results, we speculate that the acute treatment with MPTP is a very useful model of Parkinson’s disease. Our study also suggests that continuous and excessive production of endogenous MPTP-like substrates in the brain may be one of the mechanisms in the development of Parkinson’s disease\textsuperscript{22}.

In our immunohistological study, we observed loss of TH immunoreactivity in the striatal fibers and nigral cells from 1 day after the acute MPTP treatment. Thereafter, the severe loss of TH immunoreactivity was observed in the striatum and substantia nigra up to 7 days post-treatment (Fig. 1). In contrast, 1 day after acute MPTP treatment, GFAP immunopositive astrocytes were increased in the striatum and substantia nigra. Thereafter, GFAP positive astrocytes were markedly increased in the striatum and substantia nigra 3 and 7 days after MPTP treatment (Fig. 2). These results suggest that an increase in GFAP immunostaining produced by MPTP in the striatum and substantia nigra is linked to decrements in TH immunostaining, suggesting that factors originating in the damaged dopamine neurons initiated the astrocyte reaction to MPTP. For histochemical staining of isoelectin B\textsubscript{4}, which specifically combines with terminal alpha-D-galactose residues located on the cell surface of microglia\textsuperscript{23,24}, isoelectin B\textsubscript{4} staining was weak in the striatum and
substantia nigra of control mice. One day after acute MPTP treatment, isolectin B4-positive microglia were increased markedly in the striatum and substantia nigra. Thereafter, the increase of isolectin B4-positive microglia lasted up to 7 days after MPTP treatment. It is known that activated microglia exert cytotoxic effects in the brain through two different, yet complementary processes\(^{25}\). First, activated microglia can act as phagocytes, which involve direct cell-to-cell contact. Second, they are capable of releasing a large variety of potentially neurotoxic substances\(^{25}\). Therefore, it is believed that activated microglia may sometimes be associated with beneficial effects and often they may appear to be deleterious\(^{26}\). A recent interesting study suggested that inhibition of microglial activation by minocycline can protect the nigrostriatal dopaminergic pathway against neurotoxic effects of MPTP in mice\(^{27}\). These findings seem to suggest that activated microglia plays an important role in the pathogenesis of MPTP-induced degeneration of dopaminergic neurons. In the present study, the number of activated microglia appeared in the striatum and substantia nigra earlier than that of reactive astrocytes, as shown in Fig. 3. These results were, at least in part, consistent with the previous report\(^{28}\). Based on these observations, we also suggest that microglial activation may play a key role in the MPTP neurotoxic process.

In conclusion, we have shown that the acute treatment of mice with MPTP is accompanied by sustained nigral degeneration and motor abnormalities relatively resembling Parkinson’s disease. Furthermore, our results show that continuous and excess production of endogenous MPTP-like substrates in the brain may be one of the mechanisms in the development of Parkinson’s disease. Thus our findings provide valuable information for explorations of age-related disease progression, mechanisms of neurodegeneration and neuroprotection.

REFERENCES

Table 1.  Time course effects of MPTP (20 mg/kg,i.p.) treatment four times a day at 2-hr intervals on the striatal dopamine, DOPAC and HVA levels in mice.

<table>
<thead>
<tr>
<th>Days after MPTP treatment</th>
<th>Dopamine (µg/g tissue)</th>
<th>DOPAC (µg/g tissue)</th>
<th>HVA (µg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.22±0.57</td>
<td>2.99±0.26</td>
<td>1.73±0.14</td>
</tr>
<tr>
<td>1 day</td>
<td>2.63±0.53**</td>
<td>1.21±0.25**</td>
<td>0.82±0.17**</td>
</tr>
<tr>
<td>3 days</td>
<td>3.03±0.62**</td>
<td>1.24±0.23**</td>
<td>0.56±0.06**</td>
</tr>
<tr>
<td>7 days</td>
<td>2.91±0.37**</td>
<td>1.39±0.18**</td>
<td>0.63±0.06**</td>
</tr>
<tr>
<td>14 days</td>
<td>3.46±0.32**</td>
<td>3.20±0.20</td>
<td>1.11±0.02**</td>
</tr>
<tr>
<td>21 days</td>
<td>6.58±0.51**</td>
<td>1.55±0.18**</td>
<td>0.73±0.06**</td>
</tr>
</tbody>
</table>

Values are expressed as means±S.E.M. *p<0.05, **p<0.01 compared with control (Dunnett’s multiple range test). n=5-6 mice.

Table 2.  Effects of MPTP (20 mg/kg,i.p.) treatment four times a day at 2-hr intervals on the motor activity in mice.

<table>
<thead>
<tr>
<th>Days after MPTP treatment</th>
<th>Pole test (sec)</th>
<th>Catalepsy test (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Turn</td>
<td>TLA</td>
</tr>
<tr>
<td>Control</td>
<td>1.74±0.10</td>
<td>4.02±0.29</td>
</tr>
<tr>
<td>7 days</td>
<td>2.69±0.20**</td>
<td>5.80±0.60**</td>
</tr>
<tr>
<td>Control</td>
<td>1.77±0.16</td>
<td>3.75±0.33</td>
</tr>
<tr>
<td>14 days</td>
<td>2.13±0.18</td>
<td>5.25±0.65*</td>
</tr>
</tbody>
</table>

Values are expressed as means±S.E.M. *p<0.05, **p<0.01 compared with control (Student’s t-test). n=8-10 mice.
Fig. 1. Representative microphotographs of tyrosine hydroxylase (TH) immunostaining in the striatum and substantia nigra of mice after MPTP (20 mg/kg, i.p.) treatment four times a day at 2-hr intervals within a day. (a,e): Control. (b,f): 1 day after MPTP treatment. (c,g): 3 days after MPTP treatment. (d,h): 7 days after MPTP treatment. Striatum (a-d), bar=100 mm; Substantia nigra (e-h), bar=100 µm. n=5.

Fig. 2. Representative microphotographs of glial fibrillary acidic protein (GFAP) immunostaining in the striatum and substantia nigra of mice after MPTP (20 mg/kg, i.p.) treatment four times a day at 2-hr intervals within a day. (a,e): Control. (b,f): 1 day after MPTP treatment. (c,g): 3 days after MPTP treatment. (d,h): 7 days after MPTP treatment. Striatum (a-d), bar=100 µm; Substantia nigra (e-h), bar=100 µm. n=5.
Fig. 3. Representative microphotographs of isolectin B4 immunostaining in the striatum and substantia nigra of mice after MPTP (20 mg/kg, i.p.) treatment four times a day at 2-hr intervals within a day. (a,e): Control. (b,f): 1 day after MPTP treatment. (c,g): 3 days after MPTP treatment. (d,h): 7 days after MPTP treatment. Striatum (a-d), bar=100 µm; Substantia nigra (e-h), bar=100 µm. n=5.