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# A < 10 Kilodalton Fraction of Cerebrospinal Fluid from Patients with Parkinson's Disease Decreases the Survival of Rat Mesencephalic Dopaminergic Neurons in Culture

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**Parkinson's disease (PD) is characterized by loss of dopaminergic neurons of the pars compacta in the substantia nigra. It has been suggested that environmental or intrinsic toxic factors for mesencephalic dopaminergic neurons are associated with this progressive neuronal loss. Using a primary rat mesencephalic dopaminergic culture method, we tested the impact of the cerebrospinal fluid (CSF) obtained from patients with PD on the survival of mesencephalic dopaminergic neurons. Specimens of CSF were obtained by lumbar puncture from 6 patients with PD (including 2 patients never treated with L-dopa) and control patients with other neurological diseases. On day 4 of in vitro culture of the CSF specimens, 20% of the growth medium was exchanged with the < 10 kDa fraction of CSF. The survival of dopaminergic neurons was evaluated by tyrosine hydroxylase (TH) staining on day 6 of in vitro culture. The percentage of TH-positive neurons significantly decreased in cultures treated with the < 10 kDa fraction of CSF from PD patients (3.0 ± 1.4%; mean ± SD) than in those from control subjects (6.2 ± 2.5%, $P < 0.05$), without any significant difference to the total cell number. The toxicity of CSF from PD patients not treated with L-dopa was significantly higher than that from PD patients treated with L-dopa. A dose-response curve of 1-methyl-4-phenylpyridinium ion ($MPP^+$) toxicity on this culture was tested in a range from 0.1 to 20 μM (median effective dose, 1.0 μM). The mean effect of the 20% medium replacement with the < 10 kDa fraction of CSF from PD patients was equivalent to that of 5.0 μM $MPP^+$. It is suggested that a somewhat toxic factor exists in CSF of PD patients, which is one of the causes of the development of PD.**

**Key words:** cerebrospinal fluid; mesencephalic dopaminergic neurons; microisland cultures; neurotoxicity; Parkinson's disease

Parkinson's disease (PD) is characterized by progressive degeneration of the mesencephalic dopaminergic neurons (MDNs) from the pars compacta in the substantia nigra, which causes tremors, muscle rigidity and akinesia. The cause and mechanism of the selective degeneration of MDNs in PD has not yet been fully understood. At present, the involvement of genetic factors (Smith et al., 1992; Shoffner et al., 1993), endogenous or exogenous toxic factors (Nagatsu and Yoshida, 1988; Drucker et al., 1990; Matsubara et al., 1992, 1993) and oxidative stress (Edwards, 1993) are hypothesized as variables in the development of PD. Since a neurotoxin, 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP), was found to cause

Abbreviations: 10 kDa CTL-CSF, 10 kDa fraction of CSF from control subjects; 10 kDa PD-CSF, 10 kDa fraction of CSF from PD patients; CSF, cerebrospinal fluid; CYP2D6, cytochrome p-450 deprisoquine spulteine gene; FCS, fetal calf serum; MDN, mesencephalic dopaminergic neuron; $MPP^+$, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PBS, phosphate buffered saline; PD, Parkinson's disease; TH, tyrosine hydroxylase; TIQ, 1,2,3,4-tetrahydroisoquinoline

**Table 1. Patient characteristics**

| Case No. | Age* | Sex | Duration* of illness | Yahr stage | Period* after L-dopa therapy | Other anti-Parkinson drugs |
|---|---|---|---|---|---|---|
| PD 1 | 70 | M | 6 | III | 6 (200) | Amantadine (100) |
| PD 2 | 63 | M | 2 | III | 2 (300) | Amantadine (100), pergolide [450], droxydopa (300), trihexyphenidyl (2) |
| PD 3 | 77 | F | 10 | III | 2 (400) | Amantadine (300) |
| PD 4 | 69 | F | 3 | IV | 1 (300) | Bromocriptine (5), droxydopa (300) |
| PD 5 | 66 | F | 1 | III | Free | Free |
| PD 6 | 66 | F | 3 | III | Free | Free |
| Mean ± SD | 68.2 ± 5.2 | | 4.2 | | | |

F, female; M, male; PD, Parkinson's disease.
*year(s).
( ), mg/day; [ ], μg/day.

Parkinsonism in human (Langston et al., 1983) and nonhuman primates (Davis et al., 1979; Langston et al., 1984), endogenous and exogenous toxic factors similar to MPTP have been sought in the cause of PD.

Recently, a microisland culture method for the ventral mesencephalon of 14-embryonic-day-old rats, which centers on the A8, A9 and A10 dopaminergic nuclei, has been established (Takeshima et al., 1994a, 1996). In this culture, 95% of the cells are stained positive for neuronal markers, and 20% for thyrosine hydroxylase (TH), which is a marker for dopaminergic neurons, at the early stage of culture. Less than 5% of the cells are stained positive to glial markers. This culture technique involving a high percentage of TH-positive dopaminergic neurons is suitable for the bioassay of toxic or trophic factors for dopaminergic neurons (Takeshima et al., 1994a, 1994b, 1996).

Using this technique, we verified the impact of the < and > 10 kDa fractions of CSF from patients with PD and other neurological diseases on the survival of mesencephalic dopaminergic neurons in vitro. In addition, we tested the dose-response toxicity of $MPP^+$ in a serum-priming serum-free microisland culture, and quantified the toxicity of the < 10 kDa fraction of CSF from patients with PD.

## Materials and Methods

### *CSF of PD patients and control subjects*

The CSF was collected from 6 patients with PD and from 7 patients with other neurological diseases as controls. A lumbar puncture was performed under routine clinical conditions: the initial 4 mL CSF was evaluated with the routine CSF test including cell counts and a protein and glucose assay, and the remaining 6 mL CSF was used in this study. Table 1 summarizes the backgrounds of patients with PD, and Table 2, the backgrounds of control subjects. Informed consent was fully obtained after the nature and scope of the study were explained.

**Table 2. Control characteristics**

| Case No. | Age* | Sex | Diagnosis |
|---|---|---|---|
| CTL 1 | 75 | M | Vascular dementia |
| CTL 2 | 57 | M | Abducens nerve palsy |
| CTL 3 | 78 | F | Multiple cerebral infarction |
| CTL 4 | 75 | F | Meige syndrome |
| CTL 5 | 71 | F | Alzheimer disease |
| CTL 6 | 51 | F | Motor neuron disease |
| CTL 7 | 68 | F | Abducens nerve palsy |
| Mean ± SD | 67.9 ± 10.1 | | |

CTL, control subject; F, female; M, male.
*years.

Following the collection, the CSF samples were immediatly centrifuged (1000 × *g*, 3 min) and the supernatants were frozen at –80˚C. Later, the CSF samples were thawed at room temperature and diluted twice with Dulbecco's phosphate buffered saline (PBS). The CSF samples were fractionated at 10 kDa with Centricon 10 microconcentrators (Amicon, Grace Co., Bevely, MA), then sterilized with low protein binding filters (0.22 μm) (SLGV 025 LS; Millipore, Bedford, MA) and frozen at –80˚C until tested in culture.

### *Serum-priming serum-free microisland culture of MDNs*

All procedures were carried out in accordance with the standardized methods of bioassay neurotrophic factors for dopaminergic neurons (Takeshima et al., 1996). In brief, pregnant Sprague-Dawley rats were obtained from Shimizu Jikken Doubutsu Co. (Shizuoka, Japan), and exposed to $CO_2$ on the 14th gestational day. A laparotomy was done as described previously, the uterus was removed and transferred to cold, clean PBS at pH 7.4 without $Ca^{2+}$ and $Mg^{2+}$. Subsequently, the intact brain was removed, the brain stem was isolated, and then the ventral mesencephalic region (ventral medial region; $1.0 \times 1.5 \times 0.75 \fallingdotseq 1.0$ mm$^3$) was isolated. This region centered on A8, A9 and A10 dopaminergic nuclei. Dissected tissue blocks were pooled in cold fresh medium kept at 4˚C. The serum-supplemented medium was composed of Dulbecco's modified Eagle medium/Ham's F-12 (1:1) (GIBCO, Grand Island, NY), 10% fetal calf serum (FCS; GIBCO), 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO). The tissue was triturated with a 1000 μL pipet with a blue tip, and then with a 21 G needle fitted to a 1.0 mL plastic syringe. Special care was taken to prevent the cells from touching the rubber tip of the plunger, so as not to create bubbles in the cell suspension. The dispersed cells were transfered to 1.5 mL Eppendolf tubes (1.0 mL/tube), and centrifuged at 300 × *g* for 10 min. The medium was carefully removed, and the cells were resuspended in fresh medium and counted using a hemocytometer.

Cell viability was evaluated with a 2-color fluorescence cell viability assay kit (Live/Dead Assay Kits, Molecular Probes, Inc., Eugene, OR). Cell viability just before plating was always > 90%. The cells were resuspended in a final density of $5.0 \times 10^5$/mL. A 25 μL droplet of suspension ($1.25 \times 10^4$ cells) was plated on 8-well chamber slides (Nunc, Inc, Naperville, IL). Before use, the 8-well chamber slides were coated overnight with poly-D-lysine (Sigma Chemical Co., St. Louis, MO). The droplet occupied an area up to 6.2 mm$^2$, for a final density of $2.0 \times 10^5$ cells/cm$^2$. The cultures in the covered chamber slides were incubated at 37˚C, in 5% $CO_2$ at 100% humidity, for 4 h to allow cells to attach to the coated surface. Then, 375 μL of the serum-supplement medium was added to each well. The cultures were primed with serum for 24 h, and grown in serum-free medium: Dulbecco's modified Eagle medium/Ham's F-12 (GIBCO), 1.0 mg/mL bovine albumin fraction V (Sigma), 0.1 mg/mL apo-transferrin (Sigma), 15 μg/mL insulin (Sigma), 30 nM L-thyroxine (Sigma), 20 nM progesterone (Sigma), 30 nM sodium selenite (Sigma), 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO). Every 2nd day, 50% of the medium was exchanged with the serum-free medium.

### *Treatment of test CSF*

On day 4 of in vitro culture, 20% (80 μL) of the medium was replaced with either the < or > 10 kDa fraction of the CSF from PD patients and controls, and with PBS as a vehicle. To verify the possible effect of the vehicle, the control culture without any supplementation other than the medium was also served.

### *Immunostaining of TH and evaluation of survival of MDNs in vitro*

The cultures were evaluated on day 6 of in vitro culture for the survival of MDNs using TH immunostaining as an assay. The cultures were washed with cold PBS, fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized in 1% $CH_3COOH$/95% $C_2H_5OH$ at –20˚C for 5 min, and then washed with PBS twice. Non-

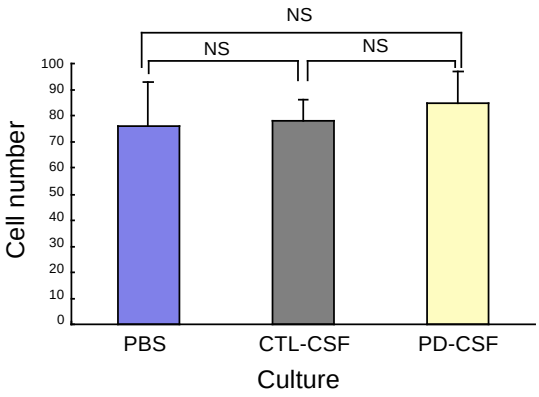


**Fig. 1.** Total number of neurons cultured in the < 10 kDa fraction of the cerebrospinal fluid (CSF) obtained from patients with Parkinson's disease (PD-CSF) and controls (CTL-CSF), and in phosphate-buffered saline (PBS). The serum-priming serum-free microisland culture of mesencephalic dopaminergic neurons obtained from 14-embryonic-day-old rats was treated with test CSF (20% of medium was replaced with the < 10 kDa fraction of CSF) on day 4 of in vitro culture, and evaluations were done on day 6 of in vitro culture. Columns and bars show mean ± SD. The data were analyzed with the analysis of variance. Differences among the 3 groups are not significant.

specific binding was blocked with 1% bovine serum albumin (Sigma) in PBS for 15 min. After aspiration of blocking buffer, 50 μL of anti-TH antibody (× 100) (Boehringer Mannheim, Mannheim, Germany) was applied, and the chamber slides were incubated for 30 min in a dark and humid chamber. After washing twice with blocking buffer, 50 μL of fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G (× 100) (Vector Laboratories, Burlingame, CA) was applied, and slides were incubated for 30 min. After washing twice with PBS, the excess fluid was aspirated, the chamber walls were taken off and 2 drops of Vectashield mounting medium (Vector) were applied, followed by a cover glass sealed with nail polish. An Olympus BX60 microscope equipped with the appropriate filters for fluorescence microscopy was used to visualize the cells. The TH-positive neurons were blindly counted in a 0.4 $mm^2$ area (2.6% of the plate area) using an eye glid. Three fields were counted, and the percentage of TH-positive neurons was calculated as (number of TH-positive neurons/number of total cells in cultures) × 100 (%). An objective of 20 magnifications was used to count cells.

### *Dose-response toxicity of $MPP^+$*

Serum-priming serum-free MDN cultures were set up as described above. On day 4 of in vitro culture, 0.5 to 20 μM $MPP^+$ was added in culture, and TH staining and counting were carried out on day 6 of in vitro culture.

### *Statistical analysis*

Statistical analyses were carried out with the one-way analysis of variance, followed by appropriate post hoc tests.

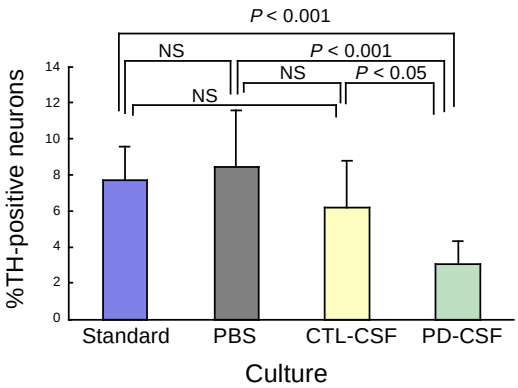


**Fig. 2.** Impact of the < 10 kDa fraction of the CSF from PD patients (< 10 kDa PD-CSF) on the survival of tyrosine hydroxylase (TH)-positive dopaminergic neurons in culture. The < 10 kDa PD-CSF decreased the survival of TH-positive dopaminergic neurons in culture. The percentage of TH-positive neurons was calculated as (number of surviving TH-positive neurons/number of total neurons) × 100 (%). Columns and bars show mean ± SD. The data were analyzed with the analysis of variance ($P < 0.05$, 0.001). NS, not significant. CTL-CSF, < 10 kDa fraction of CSF from control subjects; PBS, phosphate-buffered saline; standard, standardized control culture of serum-priming serum-free microisland mesencephalic dopaminergic neurons.

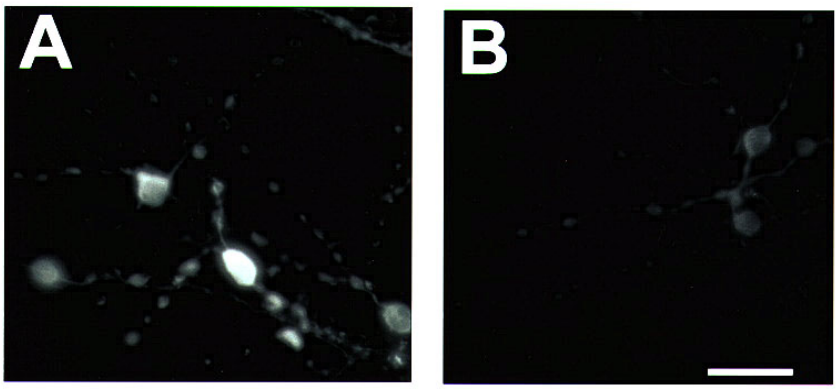


**Fig. 3.** TH immunostaining of the cultures exposed to the < 10 kDa fraction of the CSF from control subjects (< 10 kDa CTL-CSF) (**A**) and PD patients (< 10 kDa PD-CSF) (**B**), showing the growth and survival of TH-positive neurons on day 6 of in vitro culture. The number of TH-positive neurons decreases in cultures treated with < 10 kDa PD-CSF. Scale bar = 20 μm.

## Results

### *Impact of the < 10 kDa fraction of CSF from PD patients and controls and in PBS*

The total cells in culture were exposed to the < 10 kDa fraction of CSF from PD patients (< 10 kDa PD-CSF) or control subjects (< 10 kDa CTL-CSF), and to PBS (Fig. 1). There were no significant differences among the 3 test groups. Figure 2 shows the percentage of TH-positive neurons cultured in the < 10 kDa PD-CSF, < 10 kDa CTL-CSF, PBS and the standardized serum-priming serum-free medium. The ratio in the mean percentage of TH-positive neurons cultured in the < 10 kDa PD-CSF, < 10 kDa CTL-CSF and PBS to that in the standard medium, were 39.2%, 79.8% and 109.3%, respectively. The mean percentage of TH-positive neurons cultured in the < 10 kDa PD-CSF was significantly lower than that of those cultured in the < 10 kDa CTL-CSF ($P < 0.05$), PBS ($P < 0.001$) and standard medium ($P < 0.001$). Figure 3 shows the surviving TH-positive neurons cultured in the < 10 kDa PD-CSF or < 10 kDa CTL-CSF.

The mean percentage of TH-positive neurons was 3.8 ± 1.1% when cultured in the < 10 kDa PD-CSF obtained from 4 patients with PD treated with L-dopa, and 1.6 ± 0.2% when cultured in that obtained from 2 patients with PD never treated with L-dopa (de novo PD), respectively. Although the statistical comparison was not applicable because of the limitation of given CSF samples, the mean percentage of TH-positive neurons of CSF obtained from patients with de novo PD was apparently lower than that from patients with PD treated with L-dopa.

### *Dose-response toxicity of $MPP^+$*

The dose-response toxicity of $MPP^+$ in the serum-priming serum-free microisland MDN culture is shown in Fig. 4. After exposed to $MPP^+$, TH-positive neurons selectively decreased. The median effective dose of $MPP^+$ in this culture model was 1.0 μM. The mean toxicity of the 20% medium replacement with the < 10 kDa PD-CSF was determined to be equivalent to that of 5.0 μM $MPP^+$.

### *Impact of the > 10 kDa fraction of CSF from PD patients and controls*

The total cell number was 74.8 ± 21.7 cells/field (mean ± SD) when cultured in the > 10 kDa PD-CSF, and 83.9 ± 9.2 cells/field when cultured in the > 10 kDa CTL-CSF, respectively (not

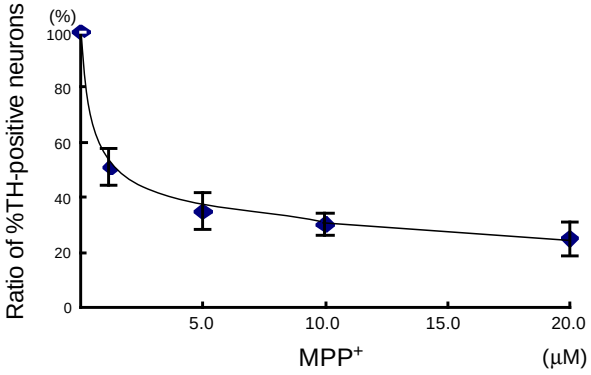


**Fig. 4.** The dose-response toxicity curve of 1-methyl-4-phenylpyridinium ion ($MPP^+$) from 0.1 to 20.0 μM on thyrosine hydroxylase (TH)-positive neurons cultured in serum-priming serum-free mesencephalic dopaminergic neurons (MDNs). The vertical axis represents the ratio to the survival of TH-positive neurons without $MPP^+$ treatment on day 6 of in vitro culture. The median effective dose of $MPP^+$ was 1.0 μM in this culture model.

significant). The percentage of TH-positive neurons was 4.9 ± 2.1% when cultured in the > 10 kDa PD-CSF, and 6.4 ± 0.6% when cultured in the > 10 kDa CTL-CSF, respectively (not significant).

## Discussion

PD is one of the most common neurodegenerative diseases, which is characterized by systematic neuronal death. The cause and mechanism of such systematic neuronal death have not been fully understood. Since MPTP was found to induce Parkinsonism (Langston et al., 1983), neurotoxins like MPTP have been suspected as the cause of PD. Rat mesencephalic cultures have been used for the bioassay of neurotoxins to dopaminergic neurons. Defazio and colleagues (1994) tested specimens of serum from PD patients using a culture of rat MDNs, and found the complementary-dependent toxicity of serum from PD to rat MDNs. Serum might be a good test material, because serum is obtained relatively easily with minimum pain to patients. However, serum contains numerous factors from many organs and may not always reflect alterations in the brain. CSF is more relevant than serum for the study of changes in the brain because the brain exists within the CSF. Although some attempts were made to test the CSF in MDN cultures, some found toxicity and others found trophic effects of CSF from PD patients with MDNs in culture (Carvey et al., 1993; Yu et al., 1994; Hao et al., 1995a, 1995b). One possibility is that the CSF from PD patients contains both trophic and toxic factors. This could be possible, because toxic factors and a damaged brain can upregulate the production of neurotrophic factors. We fractionated the CSF of 10 kDa because most trophic factors have a molecular mass higher than 10 kDa; for example, the brain-derived neurotrophic factor has a 135 kDa molecular mass (Hyman et al., 1991), and the striate-derived neurotrophic factor has a 14 kDa molecular mass (Dal Toso et al., 1988). Hao and colleagues (1995a, 1995b) reported that the < 10 kDa PD-CSF from Caucasian patients was toxic to MDNs in culture. Yu and colleagues (1994), using CSF specimens obtained at autopsy, have shown that the < 10 kDa fractions of CSF from PD patients, Alzheimer's patients and age-matched healthy controls inhibited dopaminergic neuronal growth in culture. Dying and postmortal changes in the brain might bring different results. We found that control CSF specimens did not show toxic effects on dopaminergic neuronal growth, and confirmed the toxicity of < 10 kDa PD-CSF in Japanese people. The existence of racial difference was reported in the etiology of PD, and the prevalence ratio of PD is higher in Caucasians than in Japanese (Schoenberg et al., 1985; Kusumi et al., 1996). However, we detected that the toxicity of the < 10 kDa PD-CSF in Japanese people was the same as the reported toxicity in Caucasians (Hao et al., 1995b). Increase of toxic factor(s) is considered to play an important role of development of PD in both races.

In this study, the impact of < 10 kDa PD-CSF on the survival of the total cell number in culture was not different from the impact of <

10 kDa CTL-CSF, PBS and standardized serum-priming serum-free culture. Treatment with the < 10 kDa PD-CSF more significantly decreased the number of surviving TH-positive neurons in culture than the treatment with the < 10 kDa CTL-CSF and PBS did. These results clearly mean that the toxicity of < 10 kDa PD-CSF is selective for dopaminergic neurons. Judging from the $MPP^+$ dose-response toxicity curve (Fig. 4), we determined that the 20% medium replacement with the < 10 kDa PD-CSF was equivalent in toxicity to 5.0 μM $MPP^+$ in this culture model. $MPP^+$ is produced by MPTP oxygenated with monoamine oxidase-B, whose inhibitor deprenyl improves parkinsonian symptoms in monkeys, and is regarded to delay the progression of PD (Choen et al., 1985; Youngster et al., 1989). In in vitro experiments, monoamine oxidase-B inhibitor selegiline increased the number of TH-positive neurons cultured in the < 10 kDa PD-CSF (Hao et al., 1995a). Therefore, the neurotoxin in the < 10 kDa PD-CSF may act like $MPP^+$.

$MPP^+$ or MPTP has never been found in vivo or from natural sources; however, MPTP-like neurotoxins such as 1,2,3,4-tetrahydroisoquinoline (TIQ) have been found in the human brain (Kohno et al., 1986; Niwa et al., 1989; Ohta et al., 1987). An oxidation product of TIQ by monoamine oxidase-B can damage the mitochondrial respiratory functions and cause neuronal death. Kotake and colleagues (1995) reported that the administration of 1-benzyl-TIQ, which has the same skeleton as TIQ, induced abnormal behavior in rats such as $MPP^+$ treatment, and a significantly high concentration of 1-benzyl-TIQ was found in the CSF from PD patients. The toxic factor(s) in the < 10 kDa PD-CSF might be TIQ or similar substances.

The CSF from patients with de novo PD never treated with L-dopa was more toxic to MDNs in vitro than that from patients with PD treated with L-dopa. Administration of L-dopa relieves parkinsonian symptoms in patients. Long-term or high-dose administration of L-dopa causes the down-regulation of dopamine receptors in the striatum to go down, and decreases the clinical effects of L-dopa. Steece and colleagues (1990) reported that L-dopa has a dose-dependent toxicity to MDNs in culture. Hao and colleagues (1995b) measured dopamine levels in the CSF from PD patients, and found no significant relation between L-dopa concentration in the CSF and toxicity to MDNs. Although L-dopa is toxic to MDNs in culture, the CSF from patients receiving L-dopa was less toxic to MDNs in culture. This finding is new. Based on our data, L-dopa treatment may have favorable effects not only on improving symptoms but also on preventing MDN degeneration.

Our data and earlier observations imply that somewhat neurotoxic factor(s) for MDNs exist in the CSF of PD patients. Regarding the pathogenesis of PD, such neurotoxic factors might be endogenous. Allelic associations in PD patients with cytochrome P-450 deprisoquine spulteine gene (CYP2D6) (Smith et al., 1992; Kurth et al., 1993; Sandy et al., 1996) and monoamine oxidase (Kurth et al., 1993; Shoffner et al., 1993) have been reported. CYP2D6 and monoamine oxidase are related to the degradation of MPTP and MPTP-like substances in vivo. It is possible that the abnormal metabolism of MPTP-like substances in vivo increases the concentration of neurotoxins in the brain, and that it increases the risk of PD.

The standardized microisland MDN culture technique used in this study is useful and promises further understanding of the pathogenesis of PD. The toxicity of the < 10 kDa PD-CSF, which was confirmed in this study, should be related to the development of PD. Treatment with L-dopa may protect MDNs from degeneration. Further studies are necessary to clarify the advantage of L-dopa therapy in PD progression.

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