**In Vivo Complementation of Complex I by the Yeast Ndi1 Enzyme**

**POSSIBLE APPLICATION FOR TREATMENT OF PARKINSON DISEASE**

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The mammalian proton-translocating NADH-quinone oxidoreductase (complex I)9 is located in the inner mitochondrial membranes, is composed of 46 different subunits, and bears one FMN and eight iron-sulfur clusters as cofactors (1–3). It has been known for many years that the structural and functional defects of this enzyme complex are involved in a number of human mitochondrial diseases (4–6). Therefore, it is expected that a strategy to reestablish the function of complex I would lead to the treatment of human diseases caused by the defects of this enzyme complex. The respiratory chain of certain organisms in bacteria and fungi, but not in mammals, lacks the complex I-type enzyme. Instead, the functionality of complex I, namely oxidation of NADH and reduction of quinone, is performed by structurally simpler alternatives (collectively called NDH-2). Therefore, a simple question was asked: can the NDH-2-type enzyme be implemented in mammalian mitochondria, and can it supplement malfunctioning complex I? As a therapeutic method that works irrespective of the cause of complex I deficiencies, we have proposed using the Ndi1 protein, which is one of the NDH-2-type enzymes, found in the mitochondria of *Saccharomyces cerevisiae* (7–12). The Ndi1 enzyme is composed of a single polypeptide of 53 kDa and contains noncovalently bound FAD and no iron-sulfur clusters. This enzyme is resistant to complex I inhibitors like rotenone and 1-methyl-4-phenylpyridium ion (MPP+) (9). The gene encoding the Ndi1 protein (NDI1) has been cloned and sequenced (13, 14). By using a recombinant adenovirus carrying the NDI1 gene that encodes the Ndi1 protein (rAAV-NDI1), we were able to express the Ndi1 protein in cultured mammalian cells. The expressed Ndi1 protein was correctly imported to the mitochondria of host cells with its own leading sequence (10, 12). Recently, we have shown that the expressed Ndi1 enzyme is functionally active and is able to restore NADH oxidation in the mitochondria of complex I-deficient cells (8, 11). In addition, NDI1-transduced cells can grow in the culture containing various complex I inhibitors (12).

A number of recent studies have indicated a reduced activity of complex I in Parkinson disease (PD) both in animal models and patients (15–18). In fact, it has been suggested that environmental toxins known to inhibit complex I may be the primary cause of PD. Therefore, to further test the idea of using Ndi1 as a remedy for complex I defects (19), we chose an animal model of PD to carry out *in vivo* experiments. Among the animal models available to date, mouse models involving the neurotoxin MPTP have been widely used. It is hypothesized that MPTP is oxidized to MPP+, which is then transported into dopaminergic neurons by the dopamine transporter. MPP+ is accumulated in the mitochondrial matrix and inhibits complex I. The damaged complex I appears to generate reactive oxygen species, which may induce death of neurons. Furthermore, in these models the activity of complex I is reported to be lowered (20), and therefore they are suitable as our test system. In this paper, we report that the Ndi1 protein expressed in the

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12 The abbreviations used are: complex I, the mitochondrial proton-translocating NADH-quinone oxidoreductase; Ndi1, internal rotenone-insensitive NADH-quinone oxidoreductase of *S. cerevisiae* mitochondria; PD, Parkinson disease; TH, tyrosine hydroxylase; GFAP, glial fibrillary acidic protein; SN, substantia nigra; AAV, adenovirus; rAAV, recombinant adenovirus; MPP+, 1-methyl-4-phenylpyridium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; DA, dopamine; IU, infectious unit; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; GFP, green fluorescent protein.
substantia nigra (SN) of mouse brain offered protection against the neurodegeneration caused by MPTP administration.

**EXPERIMENTAL PROCEDURES**

Recombinant AAV Vector Productions—rAAV serotype 2 (rAAV2) carrying the NDI1 gene (designated rAAV-NDI1) was prepared as described previously (10). Briefly, an rAAV proviral plasmid, pCB-NDI1, designed to express the full-length NDI1, was approved by the Institutional Animal Care and Use Committee at our in-house breeding colony) were housed three to four per cage in temperature-controlled rooms under a 12-h light/dark cycle with free access to food and water. The housing and treatment of the animals were conducted in accordance with the National Institutes of Health experimental procedures. Seven days after the MPTP administration, brain tissue was collected and subjected to immunohistochemical analysis for the assessment of neuronal degeneration.

**Histochemical Staining for NADH dehydrogenase activity** was based on the NADH-tetrazolium reductase reaction (24). Tissue sections were incubated for 10 min at room temperature and incubated with the fluorophore tyramide (1:750 dilution with amplification buffer) for 10 min at room temperature and examined using a fluorescence microscope. Ndi1 protein staining was done using the tyramide signal amplification-direct procedure following the manufacturer’s instructions (PerkinElmer Life Sciences). Briefly, the sections were incubated with the primary antibodies as described above and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000; Calbiochem) at room temperature for 2 h. The sections were then washed three times for 10 min each with PBS at room temperature and incubated with the fluorophore tyramide (1:100 dilution with amplification buffer) for 7 min at room temperature in a dark, moist chamber followed by three washes for 10 min each in PBS at room temperature and examined using a fluorescence microscope. To stain nucleic acid, the sections were treated with TO-PRO-3 iodide (1:750 dilution with amplification buffer) for 10 min at room temperature and washed three times for 5 min each in PBS.

**HPLC Analysis**—Striatal DA and its metabolite levels were determined by HPLC. The mice were sacrificed, and brains were quickly removed and placed in brain matrix (RBM 2000C; Activational Systems Inc.) on dry ice and sliced with a razor blade to 1–2-mm sections. Striatal regions from each side of the brain were isolated separately and weighed. Each sample was briefly sonicated in 4 volumes of ice-cold 0.4 m perchloric acid containing 0.15% sodium metabisulfite and 0.05% disodium EDTA and deproteinized by centrifugation at 13,000 × g for 20 min at 4 °C. Aromatic amines and their metabolites were separated using ion-paired reversed phase HPLC coupled with electrochemical detection according to Wagner et al. (25). Samples (10 μl) kept at 4 °C were injected into an HPLC system equipped with an A-314 C18 column (5 μm, 300 × 6.0 mm; YMC). The flow rate was 0.55 ml/min through a pump (LC10AD; Shimadzu) at a pressure of 100–110 kg/cm² at 30 °C. The mobile phase was composed of 75 mm sodium phosphate, 2.78 mm HCl, 3.3 mm, 300 μl of mouse brain offered protection against the neurodegeneration caused by MPTP administration.
sodium octyl sulfate, 33 mM triethylamine, and 0.1 mM EDTA with the final pH adjusted to 3.43 prior to adding 25% (v/v) methanol. The signal was detected on a graphite carbon working electrode set at +0.75 V (against the Ag/AgCl reference) (ECD-300; Eicom). The data were collected through an EPC-500 processor (Eicom), and peak areas were calculated using the PowerChrom software and quantified from a standard curve. Linearity was maintained from 10 femtol to 100 pmol for standards.

Materials—MPTP, 3,3′-diaminobenzidine tetrachloride, NADH, and Triton X-100 were purchased from Sigma; anti-TH and horseradish peroxidase were from Calbiochem; 4,6′-diamidino-2-phenylindole-containing mounting medium and ABC Elite kit were from Vector Laboratories; fluorescent microspheres were from Polysciences Inc.; tyramide signal amplification kit was from PerkinElmer Life Sciences; fluorescent microspheres were from Acros Organics (Morris Plains, NJ); Permoran Mounting Medium was from Fisher. The OCT compound was from Sakura (Torrance, CA).

RESULTS

Expression of the GFP in Mouse Brains—For a therapeutic agent to be useful, it is necessary to deliver the agent to the dopaminergic neurons in the SN. To test the efficacy of the virus in mouse brains, we used rAAV particles carrying the GFP gene (rAAV-GFP). The rAAV-GFP particles were injected unilaterally into the SN region of mice. After 6–8 weeks, a good level of GFP fluorescence was observed in the SN of the side of the brain where rAAV-GFP had been injected (Fig. 1). Furthermore, sections at the level of striatum also exhibited GFP fluorescence. The expressed GFP was observed on the left side of the brain where rAAV-GFP had been injected (Fig. 1). The opposite side gave a low level of color development that was presumably ascribed to complex I. These results strongly suggest that the Ndi1 enzyme expressed in the mouse SN is biologically active. The expression of the Ndi1 protein in mouse brain was observed for at least 7 months, which was expected because of the long term nature of AAV-based gene delivery. One potential concern may arise from the fact that Ndi1 substitution reduces the P/O ratio from 3 to 2 because the Ndi1 enzyme is not a proton pump (9). However, it should be noted that the animals that received rAAV-ND1 did not show apparent health problems.

MPTP Treatment and Immunohistochemical Assessment of Neurodegeneration—After having established experimental conditions for the Ndi1 expression in the SN, experiments were carried out that critically tested whether Ndi1 had protective effects against MPTP-induced neurodegeneration. The conditions for MPTP treatment were worked out by following the method reported in the literature (22). It turned out that four injections of MPTP at a dose of 15 mg/kg were most appropriate to achieve the desired level of degeneration in a reproducible manner keeping the survival rate high. Brain sections at the level of the SN and of the striatum were collected, and dopaminergic cells were visualized immunohistochemically using the antibody against TH. Typical results of the TH staining are presented in Fig. 3A. As expected, MPTP-treated mice showed a significant loss of the TH immunoreactivity in the SN and denervation in the striatum. The decreased level of striatal TH was retained for at least a week. Similar results have been reported by Sugama et al. (26) and Przedborski and colleagues (27, 28) by using the same MPTP injection procedure (15 mg/kg/dose and a total of four injections at 2-h intervals).

In the following experiment, 13 2-month-old mice received unilateral injections of the rAAV-ND1 particles in the left SN region. About 5–6 months after the rAAV-ND1 injection, MPTP was administered to mice as described above. Brains were collected 7 days after the MPTP injection, and the brains were processed for TH immunostaining as described above. Thionin staining of the brain sections shows a more severe denervation of the striatum (Fig. 3B) than in MPTP-treated mice (Fig. 3A).

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FIGURE 1. Delivery of the GFP gene into the SN of mouse brain. Recombinant adeno-associated virus carrying the GFP gene (rAAV-GFP, 1.0 x 10^11 IU/ml, 2 μl) was stereotaxically injected unilaterally into the left SN of the mouse brain. After 4 weeks, brain sections were examined for GFP fluorescence. The expressed GFP was observed on the left side of the SN pars compacta (SNc) but not on the opposite side (top panel). The fluorescence was also visible in the ipsilateral (left) striatum because of anterograde transport from the SN to the nerve terminals (bottom panel). The contralateral (right) striatum had no fluorescence.
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MPTP Treatment and Levels of DA and Its Derivatives in the Striatum—In the animal model employed, MPTP administration can effectively cause rapid and drastic depletion of striatal DA. To confirm the immunochemical data, we have determined the concentrations of DA and its metabolites in the striatum. The procedures for rAAV-NDI1 injections and MPTP treatment were the same as those described for the histochemistry experiments except that the striata from the two hemispheres were sampled separately. As shown in Fig. 5, the concentration of DA in the striatum of the MPTP-treated mice was <10% of that of the control mice. Similarly, both 3,4-dihydroxyphenylacetic acid and homovanillic acid significantly diminished in the MPTP-treated group. In the group of mice that had received rAAV-NDI1, there was a clear difference between the two hemispheres. All values of the ipsilateral side were 2–4 times greater than those of the contralateral side and reached ~60% of the normal level. The observed protective effect by rAAV-NDI1 is in total agreement with the immunochemical results.

DISCUSSION

Gene replacement strategies have been explored for many years as an approach to correcting mtDNA-related disorders (29, 30). The first suc-
successful work reports using a yeast system in which subunit 8 of the ATP synthase is replaced by an artificial nuclear gene designed to code for this subunit (31). More recently, allotopic expression is achieved in tissue cell lines or yeast. The present study is the first realization of such DNA-encoded subunits. These achievements were limited to tissue cultures of defects of complex I subunits are not available at the present time. Thus far employed an acute model of PD, and the next step could be to examine chronic animal models that are currently available for mouse mutants (32, 33). Our earlier experiments for restoring NADH oxidation by the ND1 gene were performed using complex I-deficient cell lines irrespective of defects of mtDNA- and nuclear DNA-encoded subunits. These achievements were limited to tissue culture cell lines or yeast. The present study is the first realization of such attempts using animals. The Nd1 enzyme expressed in dopaminergic neurons of mouse brains elicits resistance to neuronal injury caused by MPTP, clearly indicating the complementation of complex I by the Nd1 protein. Use of the Nd1 protein has advantages over the allotopic strategy. First, the Nd1 polypeptide is not as extremely hydrophobic as most of the mtDNA-encoded subunits, which may alleviate the problem of aggregation when it is expressed in the cytoplasm. Second, because Ndi1 complements functionality of complex I as a whole, issues such as proper integration of the expressed subunit and displacement of the defective subunit are not relevant. In addition, appropriate animal models of defects of complex I subunits are not available at the present time. The MPTP treatment, on the other hand, provided a good model in which to test the Nd1 strategy.

Although PD is not recognized as a mitochondrial disorder, reduced activity of complex I is no doubt involved in the early stage of the cellular events that would eventually trigger neuronal cell death. Consequences of complex I inhibition include bioenergetic disturbance such as a lowered level of cellular ATP, or more likely, an elevated production of reactive oxygen species. The principle of our strategy is to bypass complex I and to reestablish electron transfer from NADH to quinone by introducing the Nd1 enzyme. It is apparent that this approach is effective in the MPTP mouse model tested, and the findings establish a key role of complex I in the neurodegenerative process.

The protective effect reported here is obviously not optimal. First, we deliberately carried out unilateral injections of rAAV-ND1 to obtain unequivocal assessment in each individual animal. We expect improved protection with bilateral injections, which are currently being assessed. Second, it may be necessary to refine the conditions for NDI1 delivery in the SN. Factors such as infectious titer of viral particles can be adjusted for better efficacy, and/or the use of AAV of different serotype (i.e. type 5) might boost the level of transgene expression (34). Third, we have thus far employed an acute model of PD, and the next step could be to examine chronic animal models that are currently available for mouse and rat (17, 23, 35).

It would be of interest to cause the degeneration in animals first and then inject the rescue gene. This set of experiments should mimic the real situation in which the protective treatment can only be applied after the damage to the nigrostriatal system has begun. At the present time,
rodent models that resemble human PD more in terms of slow development are not available. However, in monkeys, parkinsonian features become manifest upon a chronic administration of MPTP over the period of several months and can be maintained for 2 months (36). This model may provide us with a long term, progressive state of neuronal deficiencies in which to test the potency of rAAV-NDI1.

The single subunit Ndi1 is capable of substituting for the mitochondrial complex I and thus might become a versatile tool in broader applications. The approach employed in this study may open a new avenue toward understanding disorders in which complex I is involved.

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