Functional Expression of the Internal Rotenone-Insensitive NADH-Quinone Oxidoreductase (*NDI1*) Gene of *Saccharomyces cerevisiae* in Human HeLa Cells

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ABSTRACT

Many studies propose that dysfunction of mitochondrial proton-translocating NADH-ubiquinone oxidoreductase (complex I) is associated with neurodegenerative disorders, such as Parkinson's disease and Huntington's disease. Mammalian mitochondrial proton-translocating NADH-quinone oxidoreductase (complex I) consists of at least 46 different subunits. In contrast, the *NDI1* gene of *Saccharomyces cerevisiae* is a single subunit rotenone-insensitive NADH-quinone oxidoreductase that is located on the matrix side of the inner mitochondrial membrane. With a recombinant adeno-associated virus vector carrying the *NDI1* gene (rAAV-NDI1) as the gene delivery method, we were able to attain high transduction efficiencies even in the human epithelial cervical cancer cells that are difficult to transfect by lipofection or calcium phosphate precipitation methods. Using a rAAV-NDI1, we demonstrated that the Ndi1 enzyme is successfully expressed in HeLa cells. The expressed Ndi1 enzyme was recognized to be localized in mitochondria by confocal immunofluorescence microscopic analyses and immunoblotting. Using digitonin-permeabilized cells, it was shown that the NADH oxidase activity of the NDI1-transduced HeLa cells were not affected by rotenone which is inhibitor of complex I, but was inhibited by flavone and antimycin A. The NDI1-transduced cells were able to grow in media containing rotenone. In contrast, control cells that did not receive the *NDI1* gene failed to survive. In particular, in the NDI1-transduced cells, the yeast enzyme becomes integrated into the human respiratory chain. It is concluded that the *NDI1* gene provides a potentially useful tool for gene therapy of mitochondrial diseases caused by complex I deficiency.

(Key words : complex I, Ndi1, mammalian cell, mitochondrial disease, oxidative phosphorylation, gene therapy)

INTRODUCTION

Mammalian respiratory NADH-quinone (Q) oxidoreductase (complex I) is composed of at least 46 different subunits and has the most intricate structure of the membrane-bound mitochondrial enzyme complexes (Buchanan and Walker, 1996). Of these subunits, seven are encoded by mitochondrial DNA and others are encoded by nuclear DNA (Chomyn *et al.*, 1985; Chomyn *et al.*, 1986). It has been shown in recent years that structural and functional defects of complex I are involved in numerous human mitochondrial diseases (Wallace, 1992; Shoffner and Wallace, 1994). In more current years, it became obvious that inhibitors specific to complex I [such as rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)] cause dopaminergic cell death and induce parkinsonian symptoms in animals (Betarbet *et al.*, 2000; Manning-Bog *et al.*, 2002; Sherer *et al.*, 2002). These and other findings suggest that complex I impairments may be central to the pathogenesis of dopamine neuronal demise in sporadic Parkinson's disease (Dawson and Dawson, 2003). Therefore, it is expected that a strategy to reestablish the functionality of complex I would lead to the treatment of human diseases caused by the defects of this enzyme complex. Complex I dysfunction may have genetic origins or may be triggered by environmental factors. Mutations in mitochondrial DNA as well as nuclear DNA are difficult to correct, and success has been limited (Guy *et al.*, 2002; Manfredi *et al.*, 2002).

There is another type of NADH-Q oxidoreductase that is different from complex I in that they do not contain a proton translocation site and are rotenone-insensitive. In contrast to mammalian mitochondria that are believed to contain only complex I, mitochondria of *Saccharomyces cerevisiae* lack

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complex I but instead have rotenone-insensitive NADH-Q oxidoreductases (de Vries and Grivell, 1988; Marres CAM, 1991). In *S. cerevisiae* mitochondria, at least two distinct rotenone-insensitive NADH-Q oxidoreductases are considered to be present, because in contrast to mammalian mitochondria, a malate/aspartate shuttle that allows redox equilibration of NADH between the mitochondrial matrix and the cytoplasm is absent from this organism (de Vries *et al.*, 1992). Therefore, one NADH-Q oxidoreductase faces the intermembrane space (referred to as external, rotenone-insensitive NADH-Q oxidoreductase), and the other faces the matrix (designated internal, rotenone-insensitive NADH-Q oxidoreductase) (Marres CAM, 1991). The internal, rotenone-insensitive NADH-Q oxidoreductase of *S. cerevisiae* mitochondria is a single polypeptide enzyme with noncovalently bound FAD as a cofactor and no iron-sulfur clusters (de Vries and Grivell, 1988). The enzyme is reported to be a two-electron reaction enzyme, whereas complex I is believed to be a one-electron reaction enzyme (Kitajima-Ihara and Yagi, 1998). If so, the yeast enzyme should not cause complications resulting from free radicals. The *NDI1* gene encoding the enzyme has been cloned and sequenced by de Vries *et al.* (de Vries *et al.*, 1992). The Ndi1 enzyme is believed to be attached to the inner membranes on the matrix side. It is the main entry point into the respiratory chain in this organism, just as complex I is in mammalian mitochondria (Yagi, 1993).

Recently, adeno-associated virus (AAV) expression systems have been developed for the expression of genes in nonproliferating cells (Naldini *et al.*, 1996). AAV vectors have been used to deliver a number of different genes into a variety of target tissues both *in vitro* and *in vivo*, thus demonstrating the significant potential of this virus in the treatment of human diseases (Snyder, 1999; Owen *et al.*, 2000). In contrast to the more commonly used retroviral and adenoviral vectors, AAV is a non-pathogenic human parvovirus, does not elicit antibodies against itself, and has high possibility for long-term expression of transgenes (Flotte *et al.*, 1993).

HeLa cells are a human epithelial cervical cancer and the first human cells. With a recombinant adeno-associated virus vector carrying the *NDI1* gene (rAAV-NDI1) as the gene delivery method, we were able to attain high transduction efficiencies even in the HeLa cells that are difficult to transfect by lipofection or calcium phosphate precipitation methods.

Our goal is to develop the incorporation of the yeast Ndi1 enzyme into human mitochondria as a potential remedy for complex I defects. In this paper, we demonstrate that the *S. cerevisiae NDI1* gene using rAAV was successfully expressed in HeLa cells. The NDI1-transduced cells were able to grow in media containing rotenone. In contrast, control cells that did not receive the *NDI1* gene failed to survive. Furthermore, we have shown that Ndi1 can be functionally expressed in the HeLa cells.

MATERIALS AND METHODS

1. Proviral Plasmid Construction and Packaging

An rAAV proviral plasmid, pCB-NDI1, designed to express the full-length *NDI1* gene from the cytomegalovirus/β-actin hybrid (CB) promoter, was constructed as follows. An rAAV-CB-hAAT construct was digested with *Not*I and *Eco*RI leaving the rAAV backbone and the CB promoter with *Not*I- and *Eco*RI-compatible ends. The *NDI1* gene was then released from the pPCRScript Amp SK(+) vector with *Not*I and *Eco*RI, and this fragment was ligated into the rAAV backbone to produce the pAAV-CB-NDI1 plasmid. The rAAV proviral plasmid, pAAV-CB-NDI1, was then packaged into rAAV virions by double-transfection of human embryonic kidney 293 cells and purified by iodixanol step-gradient centrifugation followed by heparin sulfate column chromatography as described previously (Zolotukhin *et al.*, 1999).

2. Cell Culture and *NDI1* Gene Infection

The HeLa cells were grown in DMEM medium (Dulbecco's modified Eagle's medium) complemented with 10% (v/v) heat inactivated fetal calf serum (FCS), 25 mm glucose, 30 units/ml of penicillin, 30 μg/ml of streptomycin, 1 mM sodium pyruvate, 1% (v/v) nonessential amino acids (100×), and 2 mM of l-glutamine. Cells were maintained at 37℃ in a 5% CO_2 atmosphere. The HeLa cells (1×10^5) in 1 ml of DMEM containing 25 mm glucose and 10% fetal bovine serum were infected with 1×10^9 infectious units of rAAV-NDI1 virions. The transduced cells were isolated by screening in the DMEM+ 10% fetal calf serum+10 nM rotenone in the presence of 5 mM galactose and were grown in the same media.

3. Growth Measurements

The HeLa cells were plated at 1×10^5 cells/ml onto 6-well plates (10^5 cells/well) in the appropriate medium (DMEM, which contains 4.5 mg of glucose/ml and 0.11 mg of pyruvate/ml, or DMEM lacking glucose and containing 0.9 mg of galactose/ml and 0.5 mg of pyruvate/ml, both supplemented with 10% dia-

lyzed FBS), and were harvested and counted on a daily base using a hemocytometer (Hayashi *et al.*, 1991). Viability was determined using trypan blue exclusion. Both floating and attached cells was counted in triplicate.

4. Digitonin-Permeabilized Cells

Digitonin, by binding to cholesterol in the eukaryotic plasma membrane, creates pores through which the soluble components of the cell can be released (Granger and Lehninger, 1982). Because the intracellular membranes have a cholesterol content substantially lower than the plasma membrane, the mitochondria and the cytoskeleton are left intact (Hofhaus *et al.*, 1996). Thus, permeabilization with digitonin allows the mitochondria ready access to the respiratory substrates and inhibitors (glutamate, succinate, etc.) to be tested. It should be noted, however, that in this assay system NADH added exogenously does not serve as a substrate for complex I because NADH provided exogenously is present on the cytoplasmic side and is unable to penetrate into the mitochondrial matrix. When malate plus glutamate are employed as respiratory substrates, the corresponding dehydrogenases generate NADH from NAD in the matrix compartment, which can then be oxidized by complex I. This assay procedure is reliable and is not disturbed by various diaphorases.

5. Isolation of Mitochondria and Mitochondrial Membrane Fraction

Mitochondria were isolated from freshly harvested cells essentially according to Trounce *et al.* (1996). The non-transduced and NDI1-transduced HeLa cells (approximately 1×10^9 cells) were washed twice with phosphate-buffered saline (PBS) and harvested by trypsinization. The pellets were suspended in 5 ml of a buffer containing 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 5 mM Hepes (pH 7.2), 0.2 mM PMSF, and 0.5% fatty acid free bovine serum albumin (isolation buffer). The cell suspensions were treated with 1~2 mg/ml of digitonin for 1 min on ice (Seo *et al.*, 1998). The digitonin-treated suspension was then diluted 10-fold with isolation buffer and centrifuged at 3,000×g for 5 min to remove excess detergent. The cell pellet was resuspended with the isolation buffer and homogenized using a tight fitting Dounce homogenizer (15~20 up/down strokes). The homogenate was centrifuged at 10,000×g for 20 min at 4℃ to separate cell membrane fragments from the remaining intact organelles. The pellet was suspended in 0.1 ml of the isolation buffer and used as the intact mitochondrial fraction. The intact mitochondria were briefly sonicated and centrifuged at 150,000×g for 30 min at 4℃. The pellets were resuspended in 0.1 ml of the isolation buffer and used as the mitochondrial membrane fraction. This fraction is designated as the crude mitochondrial fraction.

6. O_2 Consumption Measurements

The medium of the cell lines to be analyzed was replaced with fresh medium the day before the measurements. For determination of oxygen consumption, about 2×10^7 cells were resuspended in 1 ml of respiration buffer (20 mm HEPES pH 7.1, 10 mm $MgCl_2$, 250 mm sucrose), and then 100 μg of digitonin (1 μl of a 10% solution in dimethyl sulfoxide) in 1 ml of buffer were added, as previously described (Seo *et al.*, 1999). After incubation for 1 min at room temperature, the cell suspension was diluted with 9 ml of buffer. The digitonin-permeabilized cells were rapidly pelleted and resuspended in respiration buffer. Oxygen consumption was measured polarographically in 0.6 ml of a buffer containing 20 mM Hepes (pH 7.1), 250 mM sucrose, and 10 mM $MgCl_2$ by using a Clarke-type electrode in a water-jacketed chamber maintained at 37℃. The substrates (adjusted to pH 7.0 with NaOH) and inhibitors were added with Hamilton syringes. The final concentrations were as follows: malate, 5 mM; glutamate, 5 mM; succinate, 5 mM; rotenone, 5 μM; flavone, 0.5 mM; antimycin A, 5 μM.

7. Other Analytical Procedures

Protein was estimated by the bicichoninic acid (BCA) method (Pierce). SDS-polyacrylamide gel electrophoresis was carried out by the modified method of Laemmli (1970). Any variations from the procedures and other details are described in the figure legends.

8. Materials

The human HeLa 293 cells were from American Type Culture Collection; 0.4% trypan blue solution, flavone, rotenone, and antimycin A were from Sigma; anti-rabbit IgG, heavy and light chain (goat) fluorescein isothiocyanate-conjugated was from Calbiochem; fetal bovine serum and Dulbecco's modified Eagle's medium (DMEM) without glucose and sodium pyruvate were from Life Technologies, Inc.; trypsin EDTA 1× solution, 1×phosphate-buffered saline were from Irvine Scientific (Santa Ana, CA); 4',6'-diamidino-2-phenylindole-containing mounting medium was from Vector Laboratories, Inc. (Burlingame, CA). Cytochrome oxidase subunit Ⅳ antibody and Rhodamine red-x conjugated goat anti-mouse IgG (Red) were from

Molecular Probes. Acrylamide, *N,N'*-methylenebis (acrylamide), SDS, SDS- PAGE calibration marker proteins; enhanced chemiluminescence kits were from Pierce.

RESULTS

1. Expression of Human HeLa Cells with the *NDI1* Gene

We have previously reported that the yeast *NDI1* gene can be functionally overexpressed in *E. coli* membranes (Kitajima-Ihara and Yagi, 1998) and introduced the yeast Ndi1 enzyme into mitochondria of the Chinese hamster CCL16-B2 cells and HEK 293 cells (Seo *et al.*, 1998; Seo *et al.*, 1999). The expressed protein was functional and was able to restore the respiratory activities of those cells that were defective in complex I. The method of gene transfer used, however, involved use of chemicals and, thus, had some limitations. For example, the transfer efficiency was generally low. Also, by using this technique we were unable to transfect other human cells such as the human HeLa cells and human neuron cells. Therefore, establishing stable cell lines was essential for performing reliable enzymatic activity assays and also for our ultimate goal of gene therapies.

For this purpose, we used to recombinant technology and constructed a recombinant AAV proviral plasmid containing a full-length *NDI1* gene. This recombinant proviral plasmid, rAAV-NDI1, was then used to infect the human HeLa cells and the NDI1-transduced HeLa cells (rAAV-NDI1) were analyzed by confocal immunofluorescence microscopy by using antibody against the Ndi1 protein. Antibody against a cytochrome oxidase subunit was used to locate mitochondria. As shown in Fig. 1, it is clear that the Ndi1 protein was expressed and localized in mitochondria in the HeLa cells. In addition, infection efficiencies ranged from 50 to 80% in HeLa cells. For further characterization of the NDI1-transduced cells, we screened the HeLa cells after the infection in the presence of rotenone in medium containing galactose instead of glucose as the carbon source. Under these conditions, cells can survive only if the Ndi1 enzyme is being expressed at a level that is high enough to sustain oxidative phosphorylation (Seo *et al.*, 2000). The expression of the Ndi1 protein was retained after 4 months of continuous culture.

2. Immunoblotting Result of the Yeast *NDI1* in HeLa Cells

To provide additional evidence for the mitochondrial localization of the Ndi1 enzyme, we performed immunoblotting of crude mitochondrial fractions from non-transduced cells and

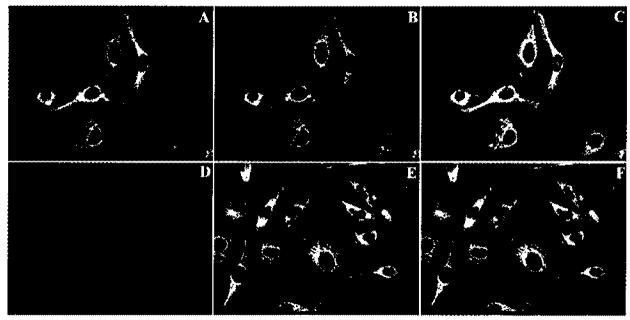

Fig. 1. Mitochondrial localization of the Ndi1 enzyme expressed in Human HeLa cells as demonstrated by confocal immunofluorescence microscopy. NDI1-transduced (A, B, and C) and non transduced (D, E, and F) HeLa cells were double-labeled with affinity-purified rabbit antibody to the Ndi1 protein and antibody to the cytochrome oxidase subunit IV. Secondary detecting reagents were fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (A and D) and rhodamine-conjugated goat anti-mouse IgG (B and E). (C and F) Overlapping images of A/B And D/E, respectively.

the NDI1-transduced HeLa cells, using antibody against the Ndi1 protein and antibody against a mitochondrial cytochrome oxidase subunit (Fig. 2). The data indicate that the antibody specific to the yeast Ndi1 enzyme reacted with mitochondria of the NDI1-transduced cells but did not react with mitochondria of non-transduced cells. The antibodies to the mitochondrial cytochrome oxidase subunit reacted with a single band of crude mitochondrial fractions of both non-transduced and NDI1-transduced HeLa cells. These results strongly suggest that the

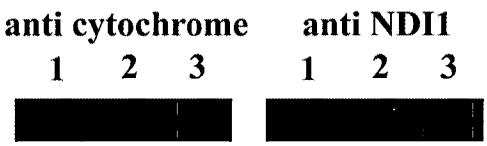

Fig. 2. Immunoblotting analyses of mitochondrial membranes from non-transduced and NDI1-transduced HeLa cells with affinity-purified rabbit antibody to the Ndi1 protein (right) and antibody to the cytochrome oxidase subunit IV (left). In both blots, lane 1 is non-transduced cells (100 mg) and lane 2 is NDI1-transduced cells (100 mg). Lane 3 (left) is bovine mitochondria (1.0 mg). Lane 3 (right) is the Ndi1 protein (20 ng) containing the leader sequence, which was expressed in and isolated from *E. coli*. The crude mitochondrial fractions were prepared as detailed under materials and methods. Immunoblotting was performed by use of the chemiluminescence kits (pierce).

leader sequence of the yeast *NDI1* gene successfully guides the product to the mitochondria in human HeLa cells.

3. Effect of the Ndi1 Expression on the Electron Transfer Activity

To investigate whether the yeast *NDI1* gene can be functionally expressed in mammalian cells, we measured respiratory chain activity using digitonin-permeabilized cells, as illustrated in Fig. 3 for the HeLa cells (Hofhaus *et al.*, 1996). Fig. 3 shows the respiratory activities of the non-transduced HeLa cells (control) and the NDI1-transduced HeLa cells. In the case of the non transduced cells, oxygen consumption in the presence of the respiratory complex I substrates malate/glutamate was inhibited by addition of rotenone, a complex I inhibitor, but enhanced by the following addition of succinate. By contrast, the respiratory activities of the NDI1-transduced cells in the presence of glutamate/malate was insensitive to rotenone but sensitive to flavone, an inhibitor of Ndi1. Furthermore, the complex Ⅲ inhibitor antimycin A inhibited this respiration completely. These results indicate that the expressed Ndi1 protein is functionally active and serve as an electron donor for the host respiratory chain. Similar results have been reported by Seo and coworkers (Chinese hamster cells) (Seo *et al.*, 1998) and Bai and coworkers (human cells) (Bai *et al.*, 2001).

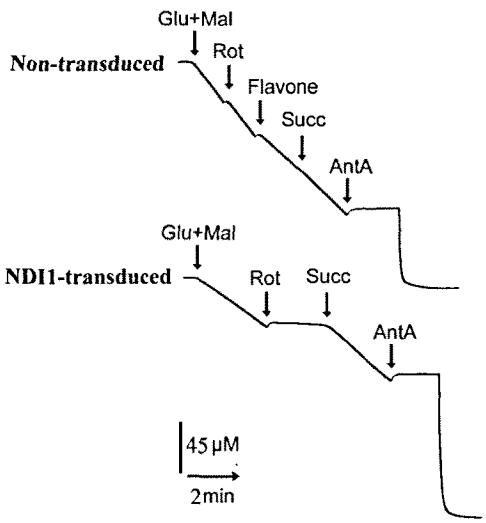

Fig. 3. Respiratory activities of non-transduced control cells and NDI1-transduced HeLa cells. The cells were harvested by trypsinization and treated with 50~150 μg of digitonin until more than 90% of cells were stained by trypan blue. Oxygen consumption was measured polarographically in 0.6 ml of a buffer containing 20 mM Hepes, pH 7.1, 250 mM sucrose, and 10 mM $MgCl_2$ by using a Clark-type electrode in a water-jacketed chamber maintained at 37℃. Upper trace, non-transduced control cells 2×10^7 cells/ml. Lower trace, NDI1-transduced cells with 2×10^7 cells/ml. Where indicated, 5 mM glutamate (Glu), 5 mM malate (Mal), 5 μM rotenone (Rot), 0.5 mM flavone, 5 mM succinate (Succ), and 5 μM antimycin (AntA) were added.

4. Effect of Rotenone on the Cell Growth of the NDI1-transduced HeLa Cells

Rotenone is known to inhibit complex I and to cause Parkinson's disease-like symptoms in rats (Singer *et al.*, 1987; Sherer *et al.*, 2003). To determine whether rotenone toxicity depended on interaction with complex I, we analyzed toxicity in the HeLa cells expressing the rotenone-insensitive single-subunit NADH dehydrogenase of *Saccharomyces cerevisiae* (*NDI1*), which acts as a "replacement" for the entire complex I in mammalian cells (Seo *et al.*, 2000; Bai *et al.*, 2001). Fig. 4 illustrates the effects of complex I inhibitors on cell growth of non-transduced and NDI1-transduced HeLa cells. In the left panel, the medium contained galactose as the carbon source with or without 10 nM rotenone as the complex I inhibitor. The cells depend on

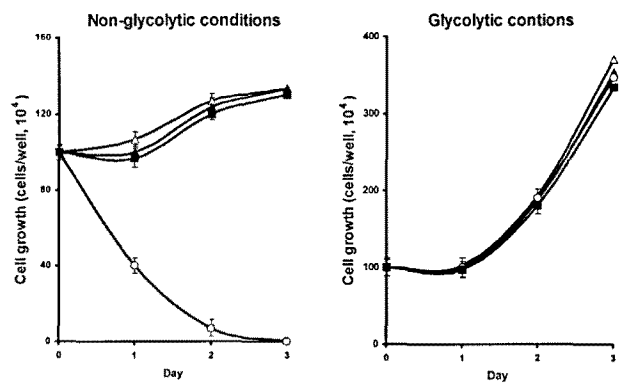

Fig. 4. Effects of rotenone on cell growth of NDI1-transduced HeLa cells under non-glycolytic and glycolytic conditions. The NDI1-transduced cells (filled symbols) and non-transduced control cells (open symbols) were cultured in the presence (■ and ○) or absence (● and △) of 10 nM rotenone. In left panels (non-glycolytic conditions) 5 mM galactose was used as the carbon source to make the cell dependent on oxidative phosphorylation for the energy source. In rigth panels (glycolytic conditions), the medium contained 25 mM glucose to allow the cell to grow under glycolysis. Cells were cultured at 37℃ in a 5% CO_2 atmosphere. Cell viability was assessed by trypan blue exclusion, and cell numbers were determined by using a hemocytometer. The experiments were carried out in triplicate.

oxidative phosphorylation for the energy source in this medium (non-glycolytic conditions), and inhibition of the electron transfer at the level of complex I leads to cell death within 2~3 days. This is clearly seen for the non-transduced control cells. On the contrary, the NDI1-transduced cells were able to grow in the presence of rotenone almost to the same extent as that of control cells cultured without rotenone. These data clearly indicate that the Ndi1 protein renders the cells resistant to complex I inhibitor by providing an alternative means to oxidize NADH. In the right panel, the same set of experiments was carried out in a medium containing high glucose to allow glycolysis. Under these conditions, all cells, regardless of the transduction, can now grow in the presence of added inhibitors. In other words, the inhibitory effects of rotenone are only observed when NADH oxidation is taking place.

DISCUSSION

It has been shown in recent years that structural and functional defects in complex I are involved in many human mitochondrial diseases (Schapira *et al.*, 1998; Robinson, 1998). In particular, mutations and deletions of both the seven mitochondrially encoded subunits and the nuclear encoded subunits have been correlated with mitochondrial diseases. Alternatively, complex I may be inhibited by chemical agents that come from the environment. Because of the diversity of the cause, repairing complex I is not easy or straightforward.

Our ultimate goal is to use the yeast *NDI1* gene to restore the NADH oxidase activity in human mitochondria with complex I deficiency. However, use of chemicals or liposomes as the gene delivery techniques, although convenient in *in vitro* experiments, may be restricted in clinical applications because of relatively low transfection efficiencies and other limitations. Viral vectors, on the other hand, have advantages in that they can be used to transduce quiescent cells such as hepatocytes, myocytes, and neurons and that the gene can be integrated into the DNA of the host cell so that it will be replicated and expressed indefinitely (Robbins and Ghivizzani, 1998; Klimatcheva, *et al.*, 1999; Wu and Ataai, 2000; Todd *et al.*, 2000). We chose recombinant AAV vector because AAV has not been implicated as the causative agent for any diseases, does not elicit antibodies against itself, and supports long term transgene expression (Muzyczka, 1994; Naldini *et al.*, 1996; Flotte *et al.*, 1993). HeLa cells are a human epithelial cervical cancer and the first human cells. With a recombinant adeno-associated virus vector carrying the *NDI1* gene (rAAV-NDI1) as the gene delivery method, we were able to attain high transduction efficiencies even in the HeLa cells that are difficult to transfect by lipofection or calcium phosphate precipitation methods.

Our earlier study showed that the *S. cerevisiae* Ndi1 enzyme can be functionally expressed and can exhibit NADH-Q oxidoreductase activity in the respiratory chains of *E. coli* membranes and complex I-deficient Chinese hamster CCL16-B2 mitochondria (Kitajima-Ihara and Yagi, 1998; Seo *et al.*, 1998). Furthermore, we have shown that the Ndi1 was functionally expressed in growth-arrested human cells (Seo *et al.*, 2000) and Ndi1 protein expression does protect SK-N-MC human neuroblastoma cells against oxidative damage caused by rotenone (Sherer *et al.*, 2003).

In this paper, we have demonstrated that the Ndi1 enzyme was successfully expressed in human HeLa cells using a rAAV-NDI1. The expressed Ndi1 enzyme was recognized to be localized to mitochondria by confocal immunofluorescence microscopic analyses and immunoblotting. The transduction efficiency is as high as 90% as judged by immunofluorescence. Using digitonin-permeabilized cells, it was also shown that the NADH oxidase activity of the NDI1-transduced HeLa cells was not affected by rotenone which is inhibitors of complex I but was inhibited by flavone and antimycin A. Despite the presence of endogenous complex I, the expressed Ndi1 accounts almost entirely for the electron transfer from NADH to UQ10 in the mitochondria of HeLa cells. This result indicated that the NDI1-transduced HeLa cells showed NADH oxidase activity which was actually higher than the non-transduced cells. Furthermore, the data showed that the NDI1-transduced cells were able to grow in media containing rotenone. In contrast, control cells that did not receive the *NDI1* gene failed to survive. In particular, in the NDI1-transduced cells, the yeast enzyme becomes integrated into the human respiratory chain. These results strongly suggest that *NDI1* gene is an excellent candidate for gene therapy of mitochondrial diseases caused by complex I deficiency. In addition, we expect that this type of strategy might be applicable for therapies of dysfunction of other enzyme complexes involved in oxidative phosphorylation.

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