Functional Expression of *Saccharomyces cerevisiae* NADH-quinone Oxidoreductase (NDI1) Gene in the AML12 Mouse Liver Hepatocytes for the Applying Embryonic Stem Cell

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ABSTRACT

Mitochondria diseases have been reported to involve structural and functional defects of complex I-V. Especially, many of these diseases are known to be related to dysfunction of mitochondrial proton-translocating NADH-ubiquinone oxidoreductase (complex I). The dysfunction of mitochondria complex I is associated with neurodegenerative disorders, such as Parkinson's disease, Huntington's disease, and Leber's hereditary optic neuropathy (LHON). Mammalian mitochondrial proton-translocating NADH-quinone oxidoreductase (complex I) is largest and consists of at least 46 different subunits. In contrast, the *NDI*1 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. The *Saccharomyces cerevisiae NDI*1 gene using a recombinant adeno-associated virus vector (rAAV-*NDI*1) was successfully expressed in AML12 mouse liver hepatocytes and the *NDI*1-transduced cells were able to grow in media containing rotenone. In contrast, control cells that did not receive the *NDI*1 gene failed to survive. 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 cells was not affected by rotenone which is inhibitor of complex I, but was inhibited by antimycin A. Furthermore, these results indicate that Ndi1 can be functionally expressed in the AML12 mouse liver hepatocytes. It is conceivable that the *NDI*1 gene is powerful tool for gene therapy of mitochondrial diseases caused by complex I deficiency. In the future, we will attempt to functionally express the *NDI*1 gene in mouse embryonic stem (mES) cell.

(Key words : *NDI*1 gene, AML12 mouse liver hepatocytes, Gene therapy, Mouse embryonic stem cell)

INTRODUCTION

The oxidative phosphorylation system in mammalian mitochondria generates ATP by means of five enzyme complexes (I through V), all localized within the inner mitochondrial membranes (Hatefi, 1985; Wallace *et al.*, 1992). Complexes I to IV constitute the respiratory chain. NADH produced from the Krebs cycle is oxidized by complex I (the mitochondrial proton-translocating NADH-quinone (Q) oxidoreductase). Alternatively succinate is oxidized by complex II (succinate-Q oxidoreductase). Complexes I and II reduce ubiquinone (UQ) to yield ubiquinol (UQH2). The electrons from UQH2 are transferred to complex III (UQH2-cytochrome *c* oxidoreductase or *bc*1 complex), which catalyzes the subsequent electron transfer to cytochrome *c* (cyt *c*), followed by transfer to complex IV (cytochrome *c* oxidase), and finally to oxygen. Coupled to electron transfer through complexes I, III and IV, protons are pumped across the inner mitochondrial membrane from the matrix to cytosol. The resulting electrochemical gradient is utilized by complex V (ATP synthase) to produce ATP from ADP and Pi in the matrix. The ATP produced in the matrix is transferred to the cytoplasmic compartment by the adenine nucleotide translocase.

Mammalian complex I is largest and composed of at least 46 different subunits and has the most intricate structure of the membrane-bound mitochondrial enzyme complexes (Buchanan and Walker, 1996). Seven subunits are encoded by mitochondrial DNA (mtDNA) and others are encoded by nuclear DNA (Chomyn *et al.*, 1985; Chomyn *et al.*, 1986).

Mitochondrial disease were first recognized in 1962 when a young woman was found to have a hypermetabolic state, structurally abnormal mitochondria, and abnormalities of oxidative phosphorylation, all signs of a rare disorder now known as Luft's disease (Luft,

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1994). Since then, mitochondria diseases have been reported to involve structural and functional defects of complex I-V (Wallace, 1993). Especially, many of these diseases are known to be related to dysfunction of complex I. These diseases include Leber's hereditary optic neuropathy (LHON), Parkinson's disease, dystonia, severe lactic acidosis, various forms of encephalomyopathies, Leigh's disease, and possibly Huntington's disease (Wallace, 1992).

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 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 complex I but instead have rotenone-insensitive NADH-Q oxidoreductases (de Vries and Grivell, 1988; Marres *et al*., 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 *et al*., 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 *NDI*1 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).

It seems likely that the *NDI*1 gene provides a potentially useful tool for treatment, in the form of gene therapy, for neurodegenerative diseases associated with complex I defects. Therefore, I began developing a gene therapy strategy involving use of the *NDI*1 gene as the rescue molecule for Parkinson's disease. To develop the therapy of mitochondria complex I defects, it is a prerequisite to functionally express the yeast *NDI*1 gene in nonproliferating cells. The liver is the major site of fatty acid metabolism and is numerous targets of gene transfer experiments. Hepatocytes are known to be nonproliferating as well as heart, neurons, hematopoietic cells. Therefore, it is important to develop the procedure to express the *NDI*1 gene in hepatocytes.

Recently, adeno-associated virus (AAV) expression systems have been developed for the expression of genes in nonproliferating cells (Naldini *et al.*, 1996). Adeno-associated virus is a non-pathogenic human parvovirus, does not elicit antibodies against it, and has high possibility for long-term expression of transgenes (Flotte *et al*., 1993).

The goal of the present study was attempted to transfer the yeast *NDI*1 gene using recombinant adeno-associated virus vector (rAAV-NDI1) to AML12 mouse liver hepatocytes that are difficult to infect in nonproliferating cells and the expressed Ndi1 protein protected cells from inhibitory effects of complex I inhibitors such as rotenone. The overall goal of this study is to seek gene therapy for mitochondrial diseases caused by complex I deficiency.

MATERIALS AND METHODS

Preparation of rAAV-NDI1

An rAAV proviral plasmid, pCB-NDI1, designed to express the full-length *NDI*1 gene from the CMV/β-actin hybrid (CB) promoter was constructed and packaged into rAAV virions as described previously by Seo *et al*. (Seo *et al*., 2000). AAV serotype 2 was used for this study.

Cell Culture and *rAAV-NDI*1 Infection

AML12 mouse liver hepatocytes were obtained from American Type Culture Collection. AML12 mouse liver hepatocytes were cultured in DMEM/Ham's F12 medium (1:1, vol/vol) supplemented with 10% heat inac-

tivated fetal bovine serum (FBS), 5 μg/ml insulin, 5 μg /ml transferrin, 5 ng/ml selenium, 40 ng/ml dexamethasone and 2.5 mM L-glutamine (Nesnow *et al*., 2011). All culture media contained antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Cells were maintained at 37℃ in humidified 5% CO_2 air atmosphere. They were routinely subcultured using trypsin-EDTA every 3~4 days. AML12 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. Cells expressing *NDI*1 were selected by culturing in DMEM-Ham's F-12 medium containing 5 mM galactose, 10% FBS and 100 nM rotenone for 1 week and and were grown in the same media.

Viability Measurements

AML12 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% FBS), and were harvested and counted on every 24 hours using a hemocytometer. Viability was determined using trypan blue exclusion. Cell number was counted in triplicate.

Immunofluorescence

The expressed Ndi1 protein was visualized by immunostaining analysis by incubating the cells with affinity-purified rabbit antibody to Ndi1 followed by secondary anti-rabbit antibody conjugated with fluorescein isothiocyanate (FITC) (Seo *et al*., 1998). Mitochondria were located using goat antibody to subunit IV of cytochrome oxidase and anti-goat antibody conjugated with rhodamine red (Molecular Probes, Eugene, OR).

Isolation of Mitochondria and Mitochondrial Membrane Fraction

Mitochondria were isolated from freshly harvested cells essentially according to Trounce *et al*. (Trounce *et al*., 1996). The non-transduced and *NDI*1-transduced AML12 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 3000×*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.

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). 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: glutamate, 5 mM; malate, 5 mM; succinate, 5 mM; rotenone, 5 μM; flavone, 0.1 mM; antimycin A, 5 μM; ascorbate, 10 mM; *N,N,N′, N′*-tetramethyl-*p*-phenylenediamine (TMPD), 10 mM ; and KCN, 1 mM.

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 (Laemmli, 1970). Any variations from the procedures and other details are described in the figure legends.

Materials

Anti-rabbit IgG, heavy and light chain (goat) fluorescein isothiocyanate-conjugated was from Calbiochem; 0.4% trypan blue solution, flavone, rotenone, antimycin A, ascorbate, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), and KCN were from Sigma; fetal bovine serum and Dulbecco's modified Eagle's medium (DMEM) without glucose and sodium pyruvate were from Life Technologies, Inc.; DMEM/Ham's F-12 medium, trypsin EDTA 1x solution, and 1x 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 Rhodam-

ine red-x conjugated goat anti-mouse IgG (Red) were from Molecular Probes. Acrylamide, *N,N'*-methylenebis (acrylamide), SDS, SDS-PAGE calibration marker proteins, and enhanced chemiluminescence kits were from Pierce.

Statistical Analysis

Statistical analysis of the data was performed using the Student's *t*-test. Results are expressed as the mean ±standard deviation (SD). Statistical significance is described in figure legend.

RESULTS

Functional Expression of the *NDI*1 Gene in AML12 Mouse Liver Hepatocytes

It is important to assess whether the expressed Ndi1 is functionally expression in AML12 mouse liver hepatocytes. Our previous papers were reported that the yeast Ndi1 enzyme was successfully introduced into mitochondria of the Chinese hamster cells and HEK 293 cells (Seo *et al*., 1998; Au *et al*., 1999; 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 infect in nonproliferating cells such as AML12 mouse liver hepatocytes. The recombinant technology and constructed a recombinant AAV proviral plasmid containing a full-length *NDI*1 gene were used for this study. This recombinant proviral plasmid, rAAV-*NDI*1, was then used to infect AML12 cells and the *NDI*1-transduced AML12 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 AML12 cells. In addition, the efficiency of transduction as observed in immunofluorescent staining ranged from 50 to 90%. For further characterization of the *NDI*1-transduced cells, we screened AML12 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.

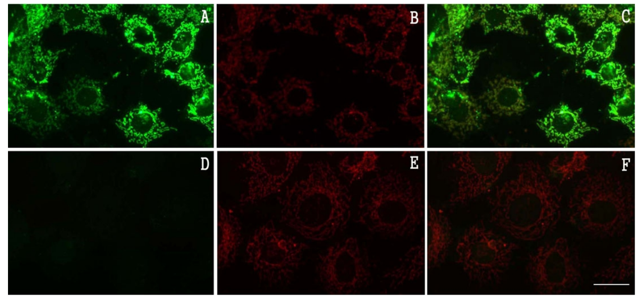

Fig. 1. Expression and mitochondrial localization of the Ndi1 protein in AML 12 infected with the rAAV-*NDI*1 particles. Cells (1×10^5) in 1 ml of growth medium were infected with 9×10^8 IU of rAAV-*NDI*1. *NDI*1-transduced (A, B, C) and non transduced (D, E, F) AML12 cells were double-labeled with affinity-purified 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, D) and rhodamine-conjugated goat anti-mouse IgG (B, E). (C, F) Overlapping images of *A*/*B* and *D*/*E*, respectively. Scale bars=2 μm.

Effect of the Ndi1 Expression on the Electron Transfer Activity

To assess the activities of the expressed Ndi1 protein, I measured respiratory chain activity using digitonin-permeabilized cells. Respiration can be measured with intact cells (Hofhaus *et al*., 1998). 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 cholesterol content substantially lower than the plasma membrane, the mitochondria, other cell organelles, and the cytoskeleton are left intact (Hofhaus *et al*., 1998). Thus, the permeabilization with digitonin allows ready access by the respiratory substrates and inhibitors (glutamate, succinate, etc.) to be tested. Fig. 2 shows the respiratory activities of the nontransduced AML 12 cells (control) and the *NDI*1-transduced AML 12 cells (rAAV-*NDI*1). In the case of the nontransduced cells, oxygen consumption in the presence of the respiratory substrates malate/glutamate was inhibited by addition of rotenone, a complex I inhibitor as described previously (De Francesco *et al*., 1976). When succinate was added, the respiration was stimulated. This stimulation was completely inhibited by antimycin A and KCN. By contrast, the respiratory activity of the NDI1-transduced cells in the presence of glutamate/malate was insensitive to rotenone but antimycin A and KCN inhibited this respiration completely. These results indicate that the expressed Ndi1 enzyme functions as an upstream member of the respiratory chain of the cell.

Western blotting Result of the Yeast NDI1 in AML12 Cells

To provide additional evidence for the mitochondrial

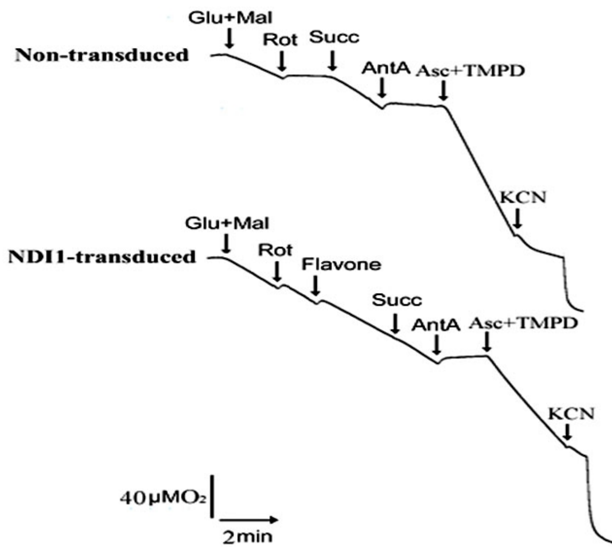

Fig. 2. Respiration measurements of non-transduced control cells and *NDI*1-transduced AML 12 cells. The cells were harvested by trypsinization and treated with 50～150 μg of digitonin until more than 90% of cells were stained by trypan blue. Oxygen consumption was measured polarographically in 0.6 ml 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, *NDI*1-transduced cells with 2×10^7 cells/ml. Where indicated, 5 mM glutamate (Glu), 5 mM malate (Mal), 5 μM rotenone (Rot), 0.1 mM flavone, 5 mM succinate (Succ), 5 μM antimycin A (AntA), 5 mM; ascorbate, 10 mM; *N,N, N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), and 1 mM KCN were added.

localization of the Ndi1 enzyme, I performed western-blotting of crude mitochondrial fractions from non-transduced AML 12 cells and the *NDI*1-transduced AML 12 cells (rAAV-*NDI*1) using antibody against the Ndi1 protein and antibody against a mitochondrial cytochrome oxidase subunit (Fig. 3). The data indicate that the antibody specific to the yeast Ndi1 enzyme reacted with mitochondria of the *NDI*1-transduced cells but did

Fig. 3. Western blot analysis of mitochondrial membranes from non-transduced and *NDI*1-transduced AML12 mouse hepatocytes 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 and lane 2 is *NDI*1-transduced cells. Lane 3 (Left) is bovine mitochondria. Lane 3 (Right) is the Ndi1 protein 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" and the products were analyzed by polyacrylamide gel electrophoresis.

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 *NDI*1-transduced AML 12 cells. These results strongly suggest that the leader sequence of the yeast *NDI*1 gene successfully guides the product to the mitochondria in the AML12 mouse liver hepatocytes.

Effects of Complex I Inhibitors on Cell Growth

To determine whether rotenone toxicity depended on interaction with complex I, I analyzed toxicity in AML-12 cells expressing the rotenone-insensitive single-subunit NADH dehydrogenase of *Saccharomyces cerevisiae* (*NDI*1). Fig. 4 shows the effects of rotenone which is known to inhibit mitochondria complex I and to cause Parkinson's disease-like symptoms in rats (Singer *et al*., 1987; Sherer *et al*., 2003) on cell growth of non-transduced and *NDI*1-transduced AML12 cells. The medium contained 5 mM galactose and 0.6 mM glucose as the carbon source with or without 100 nM rotenone as the complex I inhibitor. The cells depend on oxidative phosphorylation for the energy source in this medium (non-glycolytic conditions), and inhibition of the electron tr-

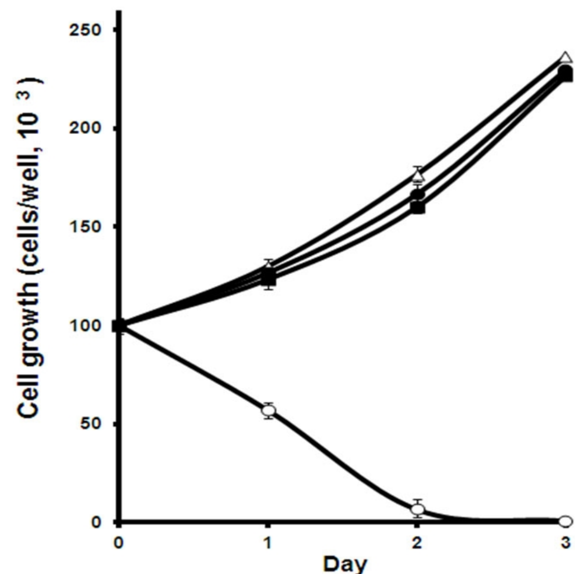

Fig. 4. Effects of rotenone on cell growth of *NDI*1-transduced AML12 mouse hepatocytes cells under non-glycolytic conditions. The *NDI*1-transduced cells (filled symbols) and non-transduced control cells (open symbols) were cultured in the presence (■ and ○) or absence (● and △) of 10 nM rotenone. 5 mM galactose was used as the carbon source to make the cell dependent on oxidative phosphorylation for the energy source. 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.

ansfer at the level of complex I leads to cell death within 2∼3 days. This is clearly shown for the non-transduced control cells. On the contrary, the *NDI*1-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 results clearly indicate that the Ndi1 protein cause to be the cells resistant to complex I inhibitor by providing an alternative means to oxidize NADH.

The same set of experiments was tried in a medium containing high glucose (25 mM) to allow glycolysis (data not shown). Under these conditions, all cells, regardless of the transduction, can now grow in the presence of added rotenone. Therefore, the inhibitory effect of rotenone is only observed when NADH oxidation is taking place.

DISCUSSION

We have previously shown 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 reported that the Ndi1 was functionally expressed in growth-arrested human cells and Ndi1 protein expression does protect SK-N-MC human neuroblastoma cells (Seo *et al*., 2000) against oxidative damage caused by rotenone (Sherer *et al*., 2003).

Viral vectors have advantages in that they can be used to transduce quiescent cells such as neurons, hepatocytes, and myocytes 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). The use of recombinant-associated adeno virus (rAAV) has been widely explored as a gene therapy tool for the past 20 years (Burger *et al*., 2005). Recombinant AAV vector was chosen 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). With a recombinant adeno-associated virus vector carrying the *NDI*1 gene (rAAV-*NDI*1) as the gene delivery method, it was able to attain high transduction efficiencies even in the AML12 mouse liver hepatocytes that are difficult to transfect by lipofection or calcium phosphate precipitation methods.

One concern might be that the Ndi1 protein is foreign to humans, possibly leading to host immune response. In a previous paper (Mathieu *et al*., 2011), we have reported that Ndi1 expression did not trigger any inflammatory or immune response in rats. These results push forward the Ndi1-based molecular therapy and also expand the possibility of using foreign proteins that are directed to subcellular organelle such as mitochondria.

In this paper we demonstrated that the gene using a recombinant adeno-associated virus vector (rAAV-*NDI*1) was successfully expressed in AML12 mouse liver hepatocytes. The expressed Ndi1 enzyme was recognized to be localized in mitochondria by confocal immunofluorescence microscopic analyses and the transduction efficiency is as high as 90% as judged by confocal immunofluorescence. The *NDI*1-transduced cells were able to grow in media containing rotenone. In contrast, control cells that did not receive the gene failed to survive. Using digitonin-permeabilized cells, it was shown that the NADH oxidase activity of the NDI1-transduced cells was not affected by rotenone which is inhibitor of complex I, but was inhibited by antimycin A. Furthermore, these results indicate that Ndi1 can be functionally expressed in the AML12 mouse liver hepatocytes.

Our ultimate goal is to use the yeast *NDI*1 gene as a gene therapy for the treatment of PD and the possibility for use in other mitochondrial complex I deficient diseases. The data presented here clearly suggest that Ndi1 can be functionally expressed in the AML12 mouse liver hepatocytes. It is believable that the *NDI*1 gene is powerful tool for gene therapy of mitochondrial diseases caused by complex I deficiency and of the treatment of Parkinson's disease. In the future, we will attempt to functionally express the *NDI*1 gene in mouse embryonic stem (mES) cell.

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