CREATION OF CYBRIDS FOR INVESTIGATING MITOCHONDRIAL ELECTRON TRANSPORT CHAIN FUNCTIONS IN PARKINSON’S DISEASE

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Abstract: Existence of mitochondrial dysfunction is reported in the blood and brain of parkinsonian patients. Cybrids are cytoplasmic hybrids created by the fusion of anucleate cells with rho0 (ρ0) cell line that has been deprived of its mitochondrial DNA. Present study describes production of normal and parkinsonian cybrids and differences observed in the mitochondrial dysfunction in relation to the expression of certain nuclear encoded mitochondrial subunits and the mitochondrial electron transport chain complex activities. Platelets from patients and age- and gender-matched controls were used to create cybrids by fusion of these platelets with the ρ0 cells. Parkinson’s disease (PD) cybrids showed significant decline in activities of mitochondrial complex I and IV as analyzed spectrophotometrically and increased expression of one of the subunits of complex I, NDUFA2 as detected by densitometric analysis following western blot. These cybrids exhibiting the disease mitochondrial gene and functional expression is a reliable cellular model of PD, and is an invaluable tool for mitochondrial research in this disease, and has been achieved for the first time in this part of the world. It is expected that the cybrids created in the laboratory would enable elucidation of mitochondrial machinery underlying PD pathology.

Key words: Cybrids, Electron transport chain, Mitochondrial genes, Complex-I subunits

INTRODUCTION

Mitochondria, referred to as the ‘powerhouses of cell’, are the most sensitive organelles vulnerable to cellular aging, excitotoxicity and oxidative stress. Mitochondrial defect in Parkinson’s disease (PD) was first identified in 1989 in substantia nigra (SN) region of the brain [1]. A decreased expression of some subunits of mitochondrial complex I in the striata of PD patients was observed by Mizuno et al. [2] and Parker et al. [3]. Our group recently reported complex I functional deficits in the platelets of PD patients [4]. Several groups have confirmed the findings of regional specific complex I activity decline in PD brains [5-8]. Defects in complex I activity have also been reported from skeletal muscle [9-13], platelets [4,14], lymphoblasts [15] and fibroblasts [16].

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replication in comparison to nuclear DNA replication. Our study is aimed to create and characterize a series of control and PD cybrids from Indian population and to further investigate the mitochondrial functions in these newly created cell lines.

MATERIALS AND METHODS

Cell lines and media: The human neuroblastoma cell line, SH-SY5Y was obtained from American Type Culture Collection, USA. Control and PD human cybrids created were grown in Dulbecco’s modified Eagle’s medium (GIBCO, Invitrogen Corporation, CA, USA) supplemented with 10% characterized fetal bovine serum (Hyclone, UT, USA), 50 Units/ml penicillin, 50 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 0.10 mg/ml pyruvate and 0.05 mg/ml uridine. The cells were maintained at 37 °C, in a humidified 5% CO₂ atmosphere (Series II water-jacketed carbon dioxide incubator, Thermo-Forma, OH, USA) and were routinely passaged by trypsinization under aseptic conditions in a Class II A2 biological safety cabinet (Thermo-Forma, OH, USA).

Creation of cybrids: Cybrid cell lines were created by fusing platelets from individuals suffering from PD or disease-free control subjects, with ρ₀ cells created from SH-SY5Y neuroblastoma cells following long-term exposure to ethidium bromide (EtBr, Sigma, MO, USA) to selectively deplete mitochondrial DNA (mtDNA) (Fig. 1). The resulting transformed cybrid cells were selected from untransformed cells by pyruvate/uridine withdrawal from the medium for two months [18]. The ρ₀ cells put through platelet-free mock fusions died in the selection media while the fused cybrids (both control and PD, Fig. 2) survived and were further cultured and maintained for the isolation of their mitochondria and for the detailed study of the electron transport chain (ETC) activity. We have created cybrids from more than a dozen control and PD subjects, and the present study involved four control and four PD cybrids.

Validation of ρ₀ and cybrid creation: by long-template PCR: The primer sets (Sigma-Genosys, Bangalore, India) for PCR were designed as per Trimmer et al. [19], so that the internal primers amplified a 5.8 kb mtDNA-specific sequence. Since the amplicons of these primer sets were large, a long-template PCR method was used. The ethidium bromide-treated cells were harvested by trypsinization and washed in phosphate buffered saline. Whole genomic DNA was prepared from the cells by phenol-chloroform extraction followed by ethanol precipitation [20]. DNA content was estimated at 260 nm spectrophotometrically (Cintra 10e spectrophotometer, GBC Cintra, Melbourne, Australia). The PCR mix was set up with 1.5 mM deoxyribonucleotide triphosphate mix, 100 ng each of the forward and reverse primers per reaction, template DNA (12.5 ng of SH-SY5Y and cybrid DNA or 250 ng of ρ₀ DNA per reaction), assay buffer 20B and 3 U XT-20 polymerase from the XT-20 system (Bangalore Genei, Bangalore, India) and water to make up to 25 µl volume and overlaid with mineral oil. For each template being analyzed, there was an internal and an external primer reaction. The PCR was programmed for 30 cycles in a Px2 thermocycler (Thermo-Forma, OH, USA) to include a start step at 92 °C for 1 min 30 s, followed by ten cycles of denaturation at 92 °C for 30 s, annealing at 62 °C for 30 s and extension at 68 °C for 5 min 15 s. For the last 20 cycles, the extension time was incremented by 10 s for each cycle. The PCR was stopped by a 10 min final extension step at 70 °C, followed by a hold step at 4 °C. The PCR products were analyzed along with a DNA molecular weight marker in a 0.6% agarose gel containing ethidium bromide (Fig. 3). The amplicons were visualized by UV trans-illumination using the ChemiDoc XRS gel documentation system (BioRad, CA, USA) and the gel image was photographed.

Isolation of mitochondria for its complex I and IV activities: The cells were harvested by trypsinization, washed in homogenization buffer (mannitol 210 mM, sucrose 70 mM, HEPES 5 mM, EGTA 1 mM, pH 7.4) and resuspended in the same buffer. The cells were homogenized by 60-90 strokes in an all-glass homogenizer, and cell disruption was confirmed under the microscope. The homogenate was centrifuged at 900 x g for 10 min at 4 °C to obtain the P₁ pellet. The supernatant from the first step was subjected to centrifugation at 5000 x g for 10 min at 4 °C to get the P₂ pellet. The supernatant was again centrifuged at 15,000 x g for 10 min at 4 °C to give the P₃ pellet. The pellets were resuspended in the homogenization buffer and used for the assays of mitochondrial ETC activity.
Mitochondrial ETC activity: Analysis of NADH dehydrogenase or complex I activity was performed on mitochondrial fractions of cybrid cells (control and PD) with respect to that in SH-SY5Y and ρ0 cells by a modified procedure [21,22]. Complex IV activity was measured in cybrid cells using spectrophotometric assay, a modification of published methods [22-24]. The mean activities were compared using Student’s t test.

SDS-PAGE and western blot: The mitochondria isolated (as previously described) from SH-SY5Y, ρ0, and control and PD cybrids were separated by SDS-PAGE and immunoblotted for NDUFS3, NDUFS7, NDUFS5 and NDUFA2, some of the nuclear encoded mitochondrial complex I subunits, and visualized using chemiluminescence.

RESULTS

Cybrid creation: After the fusion of platelets with the ρ0 cells the transformed cell lines were separated from the untransformed by the selection procedure depriving the medium off pyruvate/uridine for two months. A mock containing only the ρ0 cells was studied in parallel to the fused group as a negative control. After two months of treatment the mock group did not contain any healthy or surviving cells, while the transformed control and PD cybrids survived and multiplied even in pyruvate−/uridine− condition (Fig. 2).

Long-template PCR: Long template PCR was carried out to determine and confirm the formation of cybrids. The ρ0 cells which lack the mtDNA did not show any band in the presence of an external primer which indicates that the cell does not contain any mtDNA. While the cybrids show the band after amplification with the external primer which validates the success of cybrid creation (Fig. 3).

Complex I & IV activities: Among the three fractions studied, as expected the P2 fraction exhibited the maximum activity of both the enzymes studied (Fig. 4). For both complex I and IV, the activities of the ρ0 were greatly reduced as compared to those of the SH-SY5Y. The activity levels were restored in the cybrids in all the fractions. However, in PD cybrids, complex I activity in the P1 fraction (Fig. 4B) and complex IV activity in the P1 and P2 fractions (Fig. 4D,E) did not recover to the level of SH-SY5Y. The complex I activity of PD cybrids was significantly lower than that of the control group in the P2 fraction (Fig. 4B). The P1 and P2 fractions of PD cybrids showed reduced activity of complex IV in comparison to the control cybrids (Fig. 4D,E).

Expression of mitochondrial complex I subunit: The expression of the mitochondrial complex I subunits NDUFS3, NDUFS7, NDUFS5 and NDUFA3 in SH-SY5Y, ρ0 and the cybrids were analyzed by western blot (Fig. 5A). The level of NDUFS3 was variable, that of NDUFS7 was reduced in the ρ0 cells as compared to SH-SY5Y and the other subunits were not detected in ρ0. The NDUFS7 levels were restored in the cybrids. Cybrids showed decreased NDUFS3 level in controls and PD, lower NDUFS7 and NDUFS5 subunit levels in controls and less NDUFA2 in the PD cybrids. A significant increase in the expression of the NDUFA2 subunit was observed in the PD cybrids when compared to the control cybrids (Fig. 5B).

DISCUSSION

The major finding of our study is the characterization of cybrids in comparison with their host (ρ0) and with SH-SY5Y from which the host has been created. These cybrids express the mitochondrial genes from patients with PD or the age- and sex-matched control subjects, and would provide information on the cause(s) of mitochondrial dysfunction, and would be the foundation of PD pathology. We created the cybrid model (Fig. 2) and characterized the cell lines to counter check the transfer of mtDNA in the host ρ0 cells by long template PCR method (Fig. 3).

PD cybrid cell lines are currently the only human based models available to study PD pathology. Not only the cybrids recreate mitochondrial dysfunctions as seen in PD, but also the key neuropathological indications of the disease, such as cytoplasmic inclusions Lewy bodies and neurites are also reproduced in these cells [25]. Schapira et al. [1] first reported the mitochondrial defect in the SN of postmortem brain samples of PD patients. In Indian population the NADH dehydrogenase activity in the platelets of PD patients was shown to be lower than that in healthy age- and gender-matched controls [4]. In our study we found a significant decrease in complex I activity in PD cybrids too (Fig. 4). There was decrease in expression of three of the four nuclear encoded complex I subunits in the ρ0 cells which have defective mitochondria (Fig. 5). Similar
Fig. 1: Schematic representation of creation of control and PD cybrids by fusing ρ0 cells with control or PD platelets, respectively.

Fig. 2: Selection procedure for cybrids: (A) Mock (B) Control cybrid and (C) PD cybrid. After the withdrawal of sodium pyruvate and uridine from the growth medium for two months, only the transformed or the fused cells (B) and (C) are able to survive. The mock i.e., only ρ0 cells, are unable to survive in the selection medium as shown in (A).

Fig. 3: Long template PCR for the validation of ρ0 cells and cybrids: The amplicons from the long-template PCR amplification of the DNA from SH-SY5Y, ρ0 and cybrid cells were run on a 0.6% agarose gel and stained with EtBr. The absence of mtDNA in the ρ0 cells was confirmed by the absence of any band in the external primer reaction. The internal primer reaction gave a band in all the groups. The mtDNA band was present in the cybrids (Cy1-3).

results have been reported in MRC5 ρ0 cells where the expression of mitochondrial and nuclear COX-VIc subunits of cytochrome c oxidase were found to decline, while COX-IV and COX-Va were still detectable [26]. The changes in expression of the nuclear encoded subunits of complex I in the cybrids may be a response to the changed mtDNA content and expression (Fig. 5). Mitochondrion to nucleus signaling has been implicated in the changes in stability and expression of chromosomal DNA [27], and similar activities may regulate the altered expression of nuclear encoded mitochondrial proteins in cybrids. Thus the cybrid model provides a strong and worthy stage for the study of defective mtDNA or the proteins encoded in the human samples by providing additional insights into pathogenesis, and hence can serve as a reliable PD therapeutics development platform. Cybrids could therefore be used for screening novel antiparkinsonian molecules prior to any animal experimentation, and our laboratory has standardized procedures for differentiation of the cybrids into neurons (unpublished...
observations), and testing known and new putative drugs for PD.

REFERENCES


Fig. 4: Mitochondrial complex I and IV activities: Mitochondria were isolated from SH-SY5Y, ρ0, control and PD cybrids by differential centrifugation and the pellets from the three centrifugation steps were analyzed separately for the activities by spectrophotometric assay. Specific activity of complex I in (A) P1, (B) P2 and (C) P3 as nmol NADH oxidized/min/mg protein. Specific activity of complex IV in (D) P1, (E) P2 and (F) P3 was expressed as nmol cytochrome c oxidized/min/mg protein. *p < 0.05 as compared to SH-SY5Y, @p ≤ 0.05 as compared to ρ0 and #p < 0.05 as compared to control cybrid.