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Respiratory Chain Complex Disorganization Impairs Mitochondrial and Cellular Integrity

Phenotypic Variation in Cytochrome c Oxidase Deficiency

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The relationships between the molecular abnormalities in mitochondrial respiratory chain complexes and their negative contributions to mitochondrial and cellular functions have been proved to be essential for better understandings in mitochondrial medicine. Herein, we established the method to identify disease phenotypic differences among patients with muscle histopathological cytochrome *c* oxidase (COX) deficiency, as one of the representative clinical features in mitochondrial diseases, by using patients' myoblasts that are derived from biopsied skeletal muscle tissues. We identified two obviously different severities in molecular diagnostic criteria of COX deficiency among patients: structurally stable, but functionally mild/moderate defect and severe functional defect with the disrupted COX holoenzyme structure. COX holoenzyme disorganization actually triggered several mitochondrial dysfunctions, including the decreased ATP level, the increased oxidative stress level, and the damaged membrane potential level, all of which lead to the deteriorated cellular growth, the accelerated cellular senescence, and the induced apoptotic cell death. Our cell-based *in vitro* diagnostic approaches would be widely applicable to understanding patient-specific pathomechanism in various types of mitochondrial diseases, including other respiratory chain complex deficiencies and other mitochondrial metabolic enzyme deficiencies. *(Am J Pathol 2016, ■: 1–12; http://dx.doi.org/10.1016/j.ajpath.2016.09.003)*

Cytochrome *c* oxidase (COX) is a terminal protein in the mitochondrial electron transport system with oxidative phosphorylation and comprises its 13 structural subunits. The three largest, most hydrophobic catalytic core subunits are encoded in mitochondrial DNA (mtDNA), and the others are encoded in nuclear DNA (nDNA). In addition, COX also requires several nDNA-encoded assembly factors for its holoenzyme organization and maintenance. COX deficiency is widely recognized as one of the representative clinical phenotypes in mitochondrial diseases and presents muscle histopathological diversity among patients (focally, diffusely, or completely deficient). Although disease-causative mutations in nDNA-encoded assembly factors are mostly inherited as autosomal recessive,[1] only a few detrimental mutations in nDNA-encoded COX structural subunits have been reported.[2,3] Other genetic defects in mtDNA-encoded COX structural subunits[4–10] or in several mitochondrial tRNA genes are also responsible for COX deficiency; moreover, infantile reversible COX deficiency (alias reversible infantile respiratory chain deficiency), which is caused by homoplasmic m.14674T>C or T>G mutations in *MT-TE* gene, has recently been identified as a new disease subtype with rare, distinct disease outcome.[11,12] To date, the relationships between pathogenic mutations in COX-associating components and the aberrant COX

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holoenzyme organization become evident at a molecular level. However, there still remains no reasonable explanation how such gene-specific defects actually affect widespread mitochondrial and cellular functions, resulting in the variation and the severity of disease phenotypes at tissue and organ levels.

To overcome this problem, the use of cells derived from the affected tissues and organs is advantageous, because such cells faithfully recapitulate cell type–specific pathophysiology in a patient-specific manner. Herein, we established the method to identify disease phenotypic differences in patients exhibiting mitochondrial diseases by using a comprehensive functional analysis at mitochondrion and cell levels. We demonstrated that severely disrupted COX holoenzyme integrity (its function and structure) actually triggered several mitochondrial dysfunctions, including the decreased ATP level, the increased oxidative stress level, and the damaged membrane potential level, followed by the injured cellular homeostasis like the deteriorated cellular growth, the accelerated cellular senescence, and the induced apoptotic cell death. Therefore, COX holoenzyme disorganization determines the variation and the severity in clinical phenotypes of patients exhibiting mitochondrial diseases with muscle histopathological COX deficiency, and our proposed molecular diagnostic criteria may also be suggestive for effectively exploring disease-causative genetic defects, which are responsible for patient-specific pathology.

Materials and Methods

Patients

This study was approved by our institutional review board and was stringently conducted in accordance with the ethical principles of the Declaration of Helsinki. Patient skeletal muscle biopsy was performed for diagnostic purposes only after we received written informed consent. Note that 10 control subjects were also used in this study.

mtDNA Mutation Analysis

A long PCR-based whole mtDNA sequence in each patient was performed to eliminate any adverse results associating with pseudosequences in nDNA, as described elsewhere[13] with modifications: Extracted DNA from cultured patients' myoblasts (100 ng) was amplified by mtDNA-specific long-range PCR and the following mtDNA-specific nested PCR using a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). The amplified mtDNA fragments were sequenced using DNA analyzer (ABI PRISM 3130xl; Applied Biosystems). The obtained mtDNA sequence data in each patient were compared with the WEB databases of Human Mitochondrial Genome Database (MITOMAP) and Human Mitochondrial Genome Polymorphism (mtSNP)[14] to find any genetic variants.

RT-PCR

One-step RT-PCR was performed with the PrimeScript II High Fidelity RT-PCR kit (TaKaRa Bio), according to the manufacturer's instructions. Extracted total RNA from cultured patients' myoblasts (100 ng) was applied for RT-PCR using a thermal cycler (GeneAmp PCR system 9700; Applied Biosystems). Amplified PCR products were electrophoresed, stained with ethidium bromide, and detected using UV transilluminator (GelDoc-It Imaging System; UVP).

Primers used were as follows: *MT-CO1*, 5′-TTAGCTGACTCGCCACACTCC-3′ (forward) and 5′-AGTCAGGCCACCTACGGTGA-3′ (reverse); *MT-CO2*, 5′-CTCATGAGCTGTCCCCACATTAG-3′ (forward) and 5′-TTGACCGTAGTATACCCCCGG-3′ (reverse); *COX4*, 5′-CGGCAGAATGTTGGCTACCA-3′ (forward) and 5′-AGCGAAAAGTCTTCGCTCTTCAC-3′ (reverse); *COX5B*, 5′-TGGCATCTGGAGGTGGTGTT-3′ (forward) and 5′-TGCCTGAAGCTCCCTTTGG-3′ (reverse); and *GAPDH*, 5′-CAATGACCCCTTCATTGACCTC-3′ (forward) and 5′-CTCGCTCCTGGAAGATGGTG-3′ (reverse).

Cell Culture

Small portions of biopsied skeletal muscle tissues from the patients' biceps brachii were minced with surgical scissors and forceps, enzymatically digested with collagenase-trypsin solution [400 μg/mL collagenase (Wako), 5× trypsin-EDTA (Gibco)] at 37°C for 1 hour, and centrifuged at 1500 rpm for 5 minutes to collect myoblasts. Cells were resuspended and seeded onto tissue culture dishes and were maintained at 37°C under humidified atmosphere of 5% CO_2. Myoblast culture medium used was as follows: Dulbecco's modified Eagle's medium with F12 nutrient mixture (Gibco) supplemented with 20% fetal bovine serum (Gibco), 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco). During primary culture, 0.5 μg/mL MC210 (DS Pharm) as a mycoplasmacidal reagent and 2.5 μg/mL fungizone (Gibco) as a fungicidal reagent were also added into myoblast culture medium.

For the cellular proliferation experiment, patients' myoblasts (100 cells/mm^2) were seeded onto 96-well culture plates and were maintained at 37°C under humidified atmosphere of 5% CO_2. After 3 days in culture, cells were treated with bromodeoxyuridine chemiluminescence-based cell proliferation enzyme-linked immunosorbent assay kit (Roche), according to the manufacturer's instructions, and cellular proliferation potential was measured on chemiluminescent multiwell plate reader (Centro LB 960; Berthold Technologies).

For cellular growth experiment, patients' myoblasts (50 cells/mm^2) were seeded onto 6-well culture plates and were maintained at 37°C under humidified atmosphere of 5% CO_2. Cells were observed under phase contrast microscope (IX71 System; Olympus) at predetermined time

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