Mitochondrial DNA Mutations and Disturbances of Energy Metabolism in Myocardium

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Since mitochondria occupy a pivotal position in energy metabolism, mitochondrial dysfunction is directly linked with disturbances in cellular function. Mitochondria possess their own DNA, which codes 13 subunits of the mitochondrial energy transducing system; the other subunits are coded by nuclear DNA. Recent advances in gene technology, especially the polymerase chain reaction (PCR), permit us to analyze mitochondrial DNA mutations in a small quantity of tissue. We devised rapid and accurate methods to detect mitochondrial DNA mutations, i.e., the primer shift PCR method and the PCR-Southern method. We also developed a method to determine DNA sequences directly without cloning. Using these methods, we revealed that multiple mitochondrial DNA mutations exist in the myocardium of patients with cardiomyopathy. One mutation was based on the following directly repeated sequence: 5'-CATCAAACCG-3'. This sequence exists in both the ATPase6 gene and the D-loop region, and pseudo-recombination occurs at that directly repeated sequence resulting in a 7.4 kbp deletion. Accordingly, some subunits of the mitochondrial energy transducing system can not be biosynthesized by these deleted mitochondrial DNA, and energy transduction is substantially depleted. Even without reduction of blood supply, mitochondrial DNA mutations can induce a chronic ischemia-like state in the myocardium, which might be a factor in the genesis of cardiomyopathy.

MITOCHONDRIA are involved in the Krebs cycle and the β-oxidation pathway for fatty acids. These catabolic sequences essentially remove hydrogen from metabolic fuels and transfer it via coenzymic carriers to the mitochondrial respiratory chain. The chain then transfers these reducing equivalents eventually to react with molecular oxygen with the production of H₂O. This latter process is associated with the inner mitochondrial membrane, and transduces redox energy into the driving force for ATP synthesis, which is itself carried out by an enzymic apparatus also associated with that membrane. ATP is the direct driving force for virtually all energy-demanding processes of living cells, including muscle contraction. Accordingly, deterioration of the mitochondrial electron transport chain leads directly to cellular dysfunction. In cardiac muscle, this might mean heart failure.

The mitochondrial electron transport chain is primarily composed of 4 complexes (Complex I—IV). These complexes together with complex V (ATPase) are all embedded in the mitochondrial inner membrane and are responsible for the overall process of oxidative phosphorylation, i.e., ATP production, as shown in Fig. 1. Each complex consists of various numbers of subunits. Some

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subunits are synthesized in the mitochondrion according to information encoded within mitochondrial DNA (mtDNA), and others are specified by nuclear DNA, and synthesized in the cytoplasmic compartment. The mutation rate of mtDNA is many times higher than that of nuclear DNA! and mtDNA deletions induce severe damage in ATP production.

In the present paper, we present evidence that might categorize cardiomyopathy as a mtDNA disease at least in some cases.

MATERIALS AND METHODS

Patients: Cardiac tissue specimens were obtained from 3 patients (49 years old, female, 47 years old, male, and 53 years old, male) with hypertrophic or dilated cardiomyopathy of unknown etiology, and from a person who died in an accident as a normal control (41 years old, female).

Preparation of DNA: The autopsied heart muscles (5 mg) were homogenized using a Physocotron Handy Micro Homogenizer (Niti-on Tokyo) for 30 sec, and were digested in 1 ml of 10 mM Tris-HCl, 0.1M EDTA(pH 7.4) containing 0.1 mg/ml proteinase K and 0.5% sodium dodecyl sulfate (SDS). DNA was extracted twice with equal volumes of phenol/chloroform/isoamyl alcohol (25:25:1), and once with chloroform/isoamyl alcohol (25:1). DNA was precipitated with a one-fiftieth volume of 5M NaCl and two volumes of ethanol at −80°C for 2 h, and rinsed with 70% ethanol. The precipitated DNA was recovered in 30 μl of 10 mM Tris-HCl, 0.1 mM EDTA (pH 8.0).

Southern blot analysis: Southern blot analysis was performed as follows. DNA (100 ng) was digested with 12 units of PvuII and PstI obtained from Toyobo, Osaka, Japan, and separated electrophoretically on 0.6% agarose gels. Size standards employed were lambda phage DNA digested with Hind III and phage X174 DNA digested with Hae III from Nippon Gene, Toyama, Japan. DNA in the gels was denatured and transferred onto Hybond-N+ membranes from Amersham, UK. Hybridization using the PCR-amplified mtDNA fragments as the probes was carried out with the Enhanced Chemiluminescence Gene Detection System (ECL kit) from Amersham, UK.

Polymerase chain reaktion (PCR) amplification: Primers for PCR were synthesized using a Shimazu model NS-1 DNA synthesiz-
Fig. 2. The schema and results of the primer shift PCR method of three patients with cardiomyopathy.

PCR amplification was carried out using primers L731–H60 (lane A), primers L853–H60 (lane B), and primers L853–H38 (lane C). Sizes of amplified fragments are indicated in kbp. The shift in the sizes of the amplified fragments parallels the shift in the position of the primers from L853 to L731 (1.2 kbp), and from H60 to H38 (0.2 kbp), respectively.

PCR amplification was carried out on 1 µl of the DNA solution (ca. 10 ng of total DNA) in a final volume of 100 µl which included 200 µM of each dNTP, 2.5 units of Taq DNA polymerase (AmpliTaq, Cetus) and PCR buffer (50 mM Tris-HCl, pH 8.4, containing 50 mM KCl, 1.5 mM MgCl₂, and 0.01% gelatin) with 1 µM each of primers. The reactions were carried out for a total of 35 cycles, with the use of a Perkin-Elmer/Cetus Thermal Cycler. The cycle times were as follows: denaturation 15 sec at 94 °C; annealing, 15 sec at 45 °C; and primer extension, 80 sec at 72 °C. Amplified fragments were separated by electrophoresis on 1% agarose gels and were detected fluorographically after staining with ethidium bromide.

**Primer shift PCR method:** In PCR, misannealing of primers sometimes results in amplification of abnormal fragments. In order to ascertain that an amplified fragment is not due to misannealing of primers to an unexpected position of mtDNA, we identified the deletion of mtDNA in the patients by the primer shift PCR method. The schematically presented principle of the method and the results are illustrated in Fig. 2. In principle, PCR amplifications of the same mtDNA with different primer pairs should produce different sizes of mtDNA fragments corresponding to the distance between the primers, if there is no misannealing of the

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primer(s). In our survey, amplified fragments from mtDNA of three patients with cardiomyopathy were detected using a primer pair, L731 and H60, L853 and H60 and L853 and H38 as shown in lanes A, B, and C, respectively.

**PCR Southern method**: PCR-Southern analysis is another method which can be used to confirm the existence of mtDNA deletions. The principle is presented schematically in Fig. 3. First, three kinds of mtDNA probes are prepared by PCR. A pair of probes, A and C, are located at either end of a suspected deletion. Probe B is located in the middle of the deletion. In a Southern blot analysis probes A and C will hybridize to abnormal fragments, but probe B will not.

In the case of mtDNA of patients with cardiomyopathy, a PCR amplified-fragment from the normal mtDNA using a primer pair of L853 and H884 was used as the probe A (of 330 bp), that using L1167 and H1189 as the probe B (of 240 bp) and that using L1641 and H12 as the probe C (of 299 bp), as shown in Fig. 3. These three probe were tested with the PCR fragments using a primer pair, L853 and H38, 8.4 kbp long.

**Asymmetric PCR amplification**: PCR reamplification was carried out on 2.5 μl of the primary PCR product in a final volume of 100 μl which included the reagents described above with 0.01 μM of one primer and 1 μM of another primer, essentially according to the method of Gyllensten and Erlich. The combination of primers used for patients is shown in Table I. PCR was performed for a total of 35 cycles as above. The PCR product containing single-stranded DNA was precipitated with 0.6 volumes of

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**Fig. 3**: The schema and result of the PCR-Southern method of three patients with cardiomyopathy.

Three different fragments are amplified by PCR from the normal mtDNA and used as the probes: probe A (spanning positions 8,531–8,860), amplified with primers L853 and H884; probe B (spanning positions 11,671–11,910), amplified with primers L1167 and H1189; and probe C (spanning positions 16,411–140), amplified with primers L1641 and H12. Probe A and C hybridize to small fragments, but probe B does not hybridize to those fragments.
TABLE I COMBINATIONS OF PRIMERS USED FOR ASYMMETRIC AMPLIFICATION OF MITOCHONDRIAL DNA AND FOR SEQUENCING OF THE TEMPLATES

<table>
<thead>
<tr>
<th>Primers for amplification</th>
<th>Primer for sequencing</th>
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</thead>
<tbody>
<tr>
<td>L853 (0.01)+H38 (1)</td>
<td>L853</td>
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Figures in parentheses are concentration of primers in μM used for asymmetric amplification.

Fig.4. Mitochondrial DNA fragments amplified by PCR from specimens obtained from patients with cardiomyopathy.

In mtDNA fragments obtained from patients 1, 2, and 3 (lanes 1, 2, and 3), multiple fragments shorter than an 8.4 kbp fragment were detected, whereas only the 8.4 kbp fragment exists in the control specimen (lane C).

![DNA Fragments Image]

Fig.5. Direct sequencing of the deleted mitochondrial DNA from heart muscle.

Shown is a portion of an autoradiograph of sequencing gel of the amplified DNAs from the deleted mtDNA. mtDNA fragment with 7,436 bp deletion was sequenced and the direct repeat was identified as 5'-CATCAACAACCG-3', which was located in both the ATPase6 gene and the D-loop region.

The pellet was rinsed with 0.5 ml of 70% ethanol, dried in a vacuum for 15 min, and dissolved in 10 μl of distilled water. The removal of dNTP and primers by this PEG precipitation step is essential for reduction of the background in the subsequent sequencing.

DNA sequencing: DNA was sequenced by Sanger's dideoxynucleotide chain ter-

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mination method using the incorporation of α-[32P]dCTP as the radiolabeling extension method. Sequenase reactions were performed using a kit supplied by United States Biochemicals. For the labeling reaction, the amplified single-stranded DNA-enriched template (7.5 µl, 0.5–1.0 pmol) was mixed with 1 µl of 10 µM sequencing primer (Table I), and the primer-template mixture was heated to 100°C for 10 min and immediately placed on ice. Reactions were initiated by adding 8.8 µl of this mixture to 5.2 µl of dideoxy G, A, T, and C reaction mixtures composed of reagents provided in the “Sequencing” kit as follows: 2 µl of five-times diluted labeling mix, 0.25 µl of Sequenase (3.25 units), 1.75 µl of “dilution buffer”, 2.2 µl of “5×Sequence buffer”, and 1 µl of 0.1 M DTT. The mixture was incubated at 37°C for 2 min. The product (3.5 µl) was transferred to four tubes containing 2.5 µl of one of four “termination mixes” and incubated at 37°C for 2 min. After addition of 4 µl of “stop solution”, the mixture was heated to 100°C for 3 min, and 3 µl was loaded onto a 6% polyacrylamide/7M urea sequencing gel.

RESULTS

Figure 4 shows the PCR amplification of mtDNAs from the heart muscle using primers L853 and H38. Multiple abnormal fragments, which were derived from a deleted mtDNA, were detected in patients with cardiomyopathy, and a 1.0 kbp fragment was commonly observed. As shown in Fig. 2, coincident with the distance 1.4 kbp between L731–H60 and L853–H38, a major fragment of 1.0 kbp in lane C is shifted to 2.4 kbp. By using the other primer pair, L853–H60, 0.2 kbp longer than the pair used in lane C, 1.0 kbp fragment is shifted to 1.2 kbp as shown in lane B. That is primer shift PCR method clearly indicates that the PCR amplified abnormal fragments are not the products of misannealing of PCR primer(s) to mtDNA, but are fragments derived from real deletions of mtDNA. As shown in Fig. 3, both probes A and C hybridized to several deleted mtDNA fragments beside the normal 8.4 kbp fragment. Probe B hybridized only to the 8.4 kbp fragment. These results demonstrate that probe B region is really deleted in these mtDNAs in patients with cardiomyopathy. Therefore, the 1.0 kbp fragment was revealed not to be an artifact, and we amplified this mtDNA fragment with PCR and sequenced directly. The direct repeats of this fragment are presented in Fig. 5. The crossover sequence was demonstrated to be a 12 bp directly repeated sequence of 5′-CATCAACACCG-3′, which was located on the boundaries of the deletion between the ATPase6 gene and the D-loop region. The deletion spanned 7, 436 bp.

DISCUSSION

MtDNA comprises about 0.1–0.2% of the total DNA. The human mitochondrial genome is a closed circular DNA strand of 16,569 base pairs and contains protein-coding genes specifying hydrophobic subunits of the mitochondrial electron transport chain: seven subunits of complex I, the apocytochrome b of complex III, and three subunits of complex IV. Two subunits of complex V are also encoded in mtDNA. Together with other protein subunits coded by nuclear DNA and synthesized in and imported from the extramitochondrial cytoplasm, these mitochondrial translation products are assembled into functional enzyme complexes of the respiratory chain. The rest of the mitochondrial genome contains genetic information essential for the assembly of the mitochondrial protein-synthesizing machinery required for the expression of the protein-coding genes; genes specifying two mitochondrial rRNAs and 22 organelle specific tRNAs.

The rate of mutation of mtDNA in humans is expected to be very high, which may be due to the fact that mtDNA has neither histons nor a repair system, and mtDNA is directly susceptible to attack by oxygen radicals leaked from the mitochondrial electron transport chain. Another reason for the mutational susceptibility of mtDNA might arise from its highly economical packaging of information. MtDNA is “small, beautiful and essential”; the expression of the whole mitochondrial genome is needed for maintenance of the mitochondrial energy transducing system, whereas only about 7% of the nuclear genome is ever expressed at any particular differentiate stage. Thus, whilst it is
probable that a mutational event in the nuclear DNA will affect a non-expressed region of the genome, any mutation in the mtDNA will involve a functionally important part of the genome.

MitDNA is inherited maternally and deletion is also inherited maternally? Furthermore, non-inherited (i.e. acquired) deletion of mtDNA might also occur. Hence, both familial and sporadic cardiomyopathy associated with mtDNA mutations might occur. We have therefore attempted to elucidate whether or not mtDNA mutations exist in patients with cardiomyopathy. By PCR amplification of mtDNA, multiple abnormal bands were detected in patients with cardiomyopathy. It was noted that one mtDNA with a 7.4 kbp deletion exists commonly among these specimens. This mutation arises because the directly repeated 5'-CATCAACAACCG-3' sequence is found in both the ATPase6 gene and the D-loop region, and pseudo-recombination between the directly repeated sequences in these two regions results in a 7.4 kbp deletion. As a result of the deletion, the mitochondrial energy transducing system cannot be biosynthesized by this mtDNA. As a result, cellular energy metabolism is substantially depleted, and a chronic ischemia-like state is induced in the myocardium. This might be an important contributor to idiopathic cardiomyopathy.

To mitigate the inhibition of energy metabolism, we proposed redox therapy. Mitochondria produce ATP using energy transduced from exogenic redox reactions. Bypassing blockage in the mitochondrial energy producing system by using substances which have redox potentials permitting interaction with relevant complexes in the mitochondrial energy producing system might mitigate disturbances of mitochondrial energy production caused by mtDNA mutations. Although the rate of electron flow which can be induced by presently available redox therapy is much lower than that of the normal activity of the mitochondrial electron transport chain, the beneficial effects of redox therapy were confirmed. To improve the efficacy of this kind of redox therapy, the development of substances which have even more suitable redox properties is expected.

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