Oligomycin Induces a Decrease in the Cellular Content of a Pathogenic Mutation in the Human Mitochondrial ATPase 6 Gene*

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A T → G mutation at position 8993 in human mitochondrial DNA is associated with the syndrome neuropathy, ataxia, and retinitis pigmentosa and with a maternally inherited form of Leigh’s syndrome. The mutation substitutes an arginine for a leucine at amino acid position 156 in ATPase 6, a component of the F0 portion of the mitochondrial ATP synthase complex. Fibroblasts harboring high levels of the T8993G mutation have decreased ATP synthase activity, but do not display any growth defect under standard culture conditions. Combining the notions that cells with respiratory chain defects grow poorly in medium containing galactose as the major carbon source, and that resistance to oligomycin, a mitochondrial inhibitor, is associated with mutations in the ATPase 6 gene in the same transmembrane domain where the T8993G amino acid substitution is located, we created selective culture conditions using galactose and oligomycin that elicited a pathological phenotype in T8993G cells and that allowed for the rapid selection of wild-type over T8993G mutant cells. We then generated cytoplasmic hybrid clones containing heteroplasmatic levels of the T8993G mutation, and showed that selection in galactose-oligomycin elicited a significant increase in the fraction of wild-type molecules (from 16 to 28%) in these cells.

A T → G transversion at human mtDNA1 position 8993 (1) is associated with a multisystemic syndrome characterized clinically by neuropathy, ataxia, and retinitis pigmentosa (NARP) (2), and with a maternally inherited form of Leigh’s syndrome (MILS) (3, 4), a fatal encephalopathy of infancy. The mutation results in the conversion of leucine to arginine at amino acid 156 in ATPase 6, one of the two mtDNA-encoded subunits of the F0 portion of the mitochondrial ATPase complex (complex V). It has been reported that very high levels of mutant mtDNA are required for the clinical phenotype to be expressed (3), suggesting that this mutation is “recessive” in nature.

Cultured cells harboring high levels (>95%) of the T8993G mutation have decreased ATP synthase activity (5, 6), implying that a defect in oxidative ATP production is the most likely cause of both disorders. Nevertheless, cultured fibroblasts from NARP and MILS patients do not display any growth abnormalities under standard culture conditions, despite often being homoplasmic for the mutation (i.e. containing 100% mutant mtDNA). Nevertheless, we hypothesized that we could identify culture conditions that would not only allow for selection against mutant cells, but would also affect the intracellular proportion of mutant mtDNAs.

We took advantage of two factors that eventually allowed us to develop such a system. First, fibroblasts from patients with diverse mitochondrial disorders grow poorly when glucose is replaced by galactose as the sole carbon source (7). Second, resistance to the antibiotic oligomycin, an inhibitor of mitochondrial function, is associated with at least two mutations in the yeast ATPase 6 gene (8) and with at least one mutation in the hamster ATPase 6 gene (9); interestingly, not only are all three mutations located within a single transmembrane domain of the polypeptide, but the T8993G (L156R) mutation is located within this domain as well (Fig. 1).

We show here that, when grown in galactose-containing medium, cells harboring the T8993G mutation are more susceptible to oligomycin than are normal cells. We also show that galactose-oligomycin medium can be used as a tool to cause shifts in heteroplasmy among cells.

MATERIALS AND METHODS

Cells—Human fibroblasts were cultivated from a skin biopsy from a patient with MILS syndrome who harbored the T8993G mutation (patient 1 in Ref. 6) and from a healthy age-matched control (6). Transmitochondrial hybrids (cybrids) were generated by fusion of platelets containing 20% T8993G mutant mtDNA (obtained from the asymptomatic grandmother of a patient with MILS) with human osteosarcoma 143B cells lacking mtDNA (ρ0 cells) as described by King and Attardi (10). Briefly, platelets were isolated from plasma by centrifugation at 2,000 × g for 10 min; the platelet pellet was washed three times in DMEM; 1 × 106 ρ0 cells were added; cells and platelets were pelleted together, and all traces of medium were removed; 0.5 ml of a 50% solution of polyethylene glycol in DMEM was added for 40 s; and the cells were diluted in 40 ml of DMEM supplemented with 10% fetal bovine serum, 50 μg/ml uridine, and 100 μg/ml bromodeoxyuridine. After 24 h the cells were grown in uridine-lacking medium, to select those that had been repopulated with platelet mtDNA. Approximately 100 clones survived in selective medium. The colonies were trypsinized in a cloning ring when they reached approximately 1 mm in diameter, and expanded in a 100-mm plate. DNA was extracted from each clone, and the mtDNA was analyzed as described below.

Cell Culture—Fibroblasts were grown either in DMEM supple-
membrane domain is highlighted; the oligomycin resistance (in *B. romyces cerevisiae*, Ref. 9) and the human T8993G mutations are indicated. *B*, the mutations are also shown on a Kytle-Doolittle hydrophobicity plot.

*Oligomycin Selection and mtDNA Heteroplasmy*  

Nucleotide 3' of the T8993G mutation was cleaved by *Hpa*II, which cleaves mutant mtDNA into three fragments (301, 130, and 92 bp) but wild-type mtDNA into only two fragments (393 and 130 bp). The digestion products were electrophoresed through a 10% non-denaturing polyacrylamide gel and quantitated as described above.

**Single Cell PCR**—Cells were diluted serially in DMEM to an average 1 cell/μl. 1-μl aliquots of the cell suspension were transferred to 0.5-ml Eppendorf tubes. The cells were subjected to alkaline lysis as described by Sciacco et al. (12) and then to PCR amplification. The PCR reaction was carried on as described above for cybrid cells, except that the number of cycles was increased to 40. RFLP analysis and quantitation of the mutant molecules was as above. The data were analyzed using Student’s paired *t* test.

**Biochemical Assays**—ATP synthesis in fibroblasts was assayed as reported (6) on isolated mitochondria prepared as described by Millis and Pious (13). ATP synthesis in cybrid cell lines was measured in living cells after digitonin permeabilization (14), using the luciferin-luciferase system to report ATP content. In brief, 1 × 10⁶ cells were suspended in 150 mM KCl, 25 mM Tris-HCl, pH 7.4, 10 mM KH₂PO₄, 2 mM EDTA, 5 mM MgCl₂, and 0.1% (w/v) bovine serum albumin. The cells were first incubated with 25 μg/ml digitonin (14) at room temperature for 15 min, and then incubated in a final concentration of 7 μM luciferin, 7 mg/ml luciferase, 40 mM ADP, 100 mM succinate, and placed in a recording luminometer (MGM Instruments). Mitochondrial-specific ATP synthesis was measured as the difference between the light produced in the absence and the presence of atractyloside.

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**RESULTS**

**T8993G Mutation**—The patient’s fibroblasts were homoplasmic for the mtDNA T8993G mutation (i.e. 100% mutant), whereas control fibroblasts contained only wild-type mtDNA.

The three cybrid lines used in the oligomycin selection experiments contained 100% wild-type mtDNA (clone JCP213), 100% mutant mtDNA (clone JCP261), and 84 ± 4% mutant mtDNA (clone JCP239).

**Growth Characteristics**—The analysis of the growth rates revealed essentially no difference between patient and control fibroblasts when glucose medium was used (not shown), and there was only a modest reduction in the growth rate of the patient’s fibroblasts when they were grown in galactose medium (Fig. 2A). However, we observed a dramatic decrease in the growth rate of the patient’s fibroblasts, as compared with control, when the cells were grown in medium containing galactose plus oligomycin present at concentrations of 0.05 and 0.1 ng/ml (Fig. 2, B and C); both cell lines died in galactose medium containing 0.2 ng/ml oligomycin (Fig. 2D). Importantly, oligomycin concentrations of 0.05 and 0.1 ng/ml in medium containing glucose did not affect the growth rate of the cell lines (not shown) and, in this medium, an oligomycin concentration of at least 1 ng/ml was necessary to kill both cell lines (not shown). These results are similar to those reported previously on hamster cells (15).

Cybrid cell lines JCP213 (0% mutant), JCP239 (84% mutant), and JCP261 (100% mutant) were grown in galactose medium containing oligomycin for 5 days, followed by recovery for periods of up to 5 days in glucose medium. All cell lines showed a clear decrease in growth rate during the selection period. In 0.1 ng/ml oligomycin, cybrid JCP213 (100% wild-type mtDNA) divided at a rate slower than in glucose medium for the first two days, and cell division stopped entirely thereafter. In the same medium, cybrids JCP239 (84% mutant) and JCP261 (100% mutant) stopped dividing immediately (Fig. 3A). The number of cells attached to the plate plus those floating in the medium (i.e. dead cells) were approximately equal to the number of cells originally plated (not shown), indicating that little or no cell division had occurred during the selection period. Whereas the wild-type JCP213 cells resumed growing in...
log phase immediately after shifting to glucose medium, cybrid JCP239 took about 3–4 days to resume a normal rate of division, and cybrid JCP261 did not recover at all (Fig. 3A).

Respiratory Chain Activities—The activities of succinate cytochrome c reductase, NADH cytochrome c reductase, NADH dehydrogenase, and cytochrome c oxidase have been described previously (6) and were normal in the patient’s fibroblasts as compared with controls. ATP synthesis activity was markedly decreased in the patient’s fibroblasts as compared with control fibroblasts with all substrates tested, but oligomycin-sensitive ATP hydrolysis was only mildly decreased, as reported by Vazquez-Memije et al. (6).

ATP synthesis in cybrid clones JCP261 (100% mutant) and JCP239 (84% mutant), as measured by our luciferin/luciferase assay on four replicate samples from each line, was reduced to 20 ± 3% (S.E.) and 24 ± 3%, respectively, of that in clone JCP213 (100% wild-type) (Fig. 3B). After selection in galactose-oligomycin, residual ATP synthesis in clone JCP239 (now 72% mutant; see below), was increased from 24 ± 3% to 36 ± 1%. This increase in ATP synthesis was statistically significant (p < 0.025).

Intercellular Selection Against Mutant mtDNAs—Patient and control fibroblasts were mixed in different proportions, cultured in galactose medium plus oligomycin (0, 0.05, or 0.1 ng/ml) for 6 days, and subsequently allowed to recover for 6 days in nonselective glucose medium. PCR/RFLP analysis with AvaI showed that in the presence of oligomycin, a rapid intercellular selection in favor of fibroblasts containing wild-type mtDNA occurred (Fig. 4). With 0.05 ng/ml oligomycin, the overall percentage of mutant mtDNA measured in a mixture of 80% mutant cells and 20% wild-type cells decreased from 80 to 20% at the end of the 6-day recovery period; when starting from an initial mixture of 95% mutant and 5% wild-type cells, the proportion decreased from 95 to 63%. In the presence of 0.1 ng/ml oligomycin, the overall percentage of mutant mtDNA measured in a mixture of 80% mutant cells and 20% wild-type cells decreased from 80 to 20% at the end of the 6-day recovery period; when starting from an initial mixture of 95% mutant and 5% wild-type cells, the proportion decreased from 95 to 63%. In the presence of 0.1 ng/ml oligomycin and the same initial mixtures of mutant and wild-type cells, the percentages of mutant mtDNAs decreased from 80 to 10% and from 95 to 55%, respectively.

Intracellular Selection Against Mutant mtDNAs—We also asked if growth in galactose-oligomycin medium of cellular clones derived from single cybrid cells harboring a heteroplasmic population of T8993G mutated mtDNAs could affect the proportion of mutated mtDNAs over time. Cybrid cell lines
JCP213, JCP239, and JCP261 were grown in galactose medium containing 0.1 ng/ml oligomycin (Fig. 3A). As expected, no change in the proportion of mutant mtDNA was observed in the homoplasmic clones JCP261 (100% mutant) and JCP213 (100% wild type) in either concentration of oligomycin, nor did growth of JCP239 (84% mutant) in medium containing glucose and oligomycin (at 0.1 and 0.2 ng/ml) have any effect on the heteroplasmy (data not shown).

However, after 5 days of selection in galactose-oligomycin medium plus one day of recovery in glucose medium, we observed a significant reduction in the content of mutant mtDNA in clone JCP239 (Fig. 3A), from 84% to approximately 72%. As shown in Fig. 5, PCR/RFLP analysis with HpaII (the PCR fragment contains a naturally occurring HpaII site at nt-9293, which was used as an internal control for completeness of digestion) showed that the heteroplasmy was reduced from the original value of 84% (lane 2) to 74% after 5 days of selection in 0.1 ng/ml oligomycin (lane 3). The proportion of mutant mtDNA remained stable at 72 ± 1% in the 5 days of recovery (lanes 4–7). This result was reproduced in three independent experiments. Importantly, no further change in mutant mtDNA content was observed up to three months after the selection.

Significantly, the substitution of oligomycin with rotenone, an inhibitor of respiratory chain complex I, at 0.6 nM (a concentration that kills 143B parental osteosarcoma cells in galactose medium in 7–8 days,3 inhibited the growth rates of all three cell lines in an identical manner but did not change the proportion of mutant mtDNA in any of them after 6 days of selection followed by up to 10 days of recovery in glucose medium.

Cells from clone JCP239, which after selection in galactose-oligomycin medium contained 72% mutant mtDNA, were again grown in the selective medium for 5 days followed by 5 further days of recovery. This time the growth rate during selection and following recovery was identical to that of wild-type cells (not shown), and there was no change in the content of mutant mtDNA (i.e. it was still 72%).

**Analysis of Heteroplasmy in Single Cells**—To better understand the intracellular distribution of the mtDNAs in a heteroplasmic cell line, we performed single cell PCR/RFLP on cybrid JCP239 cells before and after selection. Before selection, the amount of mutant mtDNA was the same in each cell line (Fig. 4A, lanes P1 and P2). After selection, the proportion of mutant mtDNA was reduced to 74% in the JCP239 clone (Fig. 4B, lane 2), while the control remained unchanged (Fig. 4B, lane 3).

**DISCUSSION**

The lack of an observable phenotype in cultured cells harboring the T8993G mtDNA mutation makes it impossible to select for “less affected” cells (i.e. those containing a lower amount of mutant mtDNA), despite the fact that cells harboring this mutation are deficient in oxidative phosphorylation (5, 6, 16). We reasoned that a mitochondrial inhibitor such as oligomycin might be such a selective agent, because of its known interaction with the ATPase 6 subunit of complex V (8, 9, 15). Oligomycin belongs to a family of antibiotics produced by Streptomyces diastatochromogenes and is toxic to both eucaryotic and procaryotic organisms. It has been suggested that high concentrations of oligomycin cause apoptotic death of mammalian cultured cells (17). It is noteworthy that amino acid substitutions conferring oligomycin resistance in yeast (8), Chinese hamster ovary cells (9), and NARP/MLHS (17), are all localized in the fourth transmembrane domain of ATPase 6 (Fig. 1), suggesting that this portion of the polypeptide is most directly involved in the interaction with the antibiotic.

It is not clear why the mutant cells are selectively vulnerable to galactose/oligomycin treatment. According to secondary structure predictions (18), the T8993G mutation causes a structural change in the fourth transmembrane α-helix domain,
growth, a mixed population of mutant and wild-type fibroblasts shifted rapidly toward a predominantly wild-type genotype. However, in this case the competition was between two populations of cells, one homoplasmic wild-type and the other homoplasmic mutant. Thus, the selection was at the intercellular level.

A more interesting question, of greater relevance to the clinical situation, is whether selection can also occur at the intracellular level. To study the effect of the galactose-oligomycin selection on a heteroplasmic population of cells (i.e. containing both mutant and wild-type genomes within the same cell, and potentially within the same organelle) and which more closely resembles the situation in patient tissues, we generated clones of cybrids with different proportions of the T8993G mutation. The effect on cell growth of galactose/oligomycin medium on the cybrids was comparable with that observed with fibroblasts (compare Fig. 2C with Fig. 3, the days 0–5 portion).

Upon removal of the selection, the recovery of the homoplasmic wild-type and the heteroplasmic cells was much faster than that of the homoplasmic mutant cells (Fig. 3, day 5–8). Strikingly, we observed a reproducible decrease in the proportion of mutant mtDNA in the heteroplasmic cells after selection (and which was stable over a period of several months even in the absence of a selective pressure), and a concomitant increase in the rate of ATP synthesis. Importantly, the substitution of oligomycin (a complex V inhibitor) with rotenone (a complex I inhibitor) did not cause a shift in heteroplasm, suggesting that the effect of oligomycin is specific for the mutation (i.e. affecting its own target respiratory chain complex), rather than being a selection at the level of the entire respiratory chain.

We measured the fraction of mutant mtDNA in single cells before and after selection. The amount of mutant mtDNA in the population of cells followed a normal distribution, both before selection (average content of 84% mutant) and after selection (average content of 71% mutant). This difference was statistically highly significant (p < 0.001) and was in agreement with the values obtained from analysis of the mass culture. Because the heteroplasmic cybrid cells did not appear to undergo any significant degree of cell division during the 5-day selection period, these results imply that, besides some degree of intercellular selection, an intracellular selection between mtDNAs had also occurred. We note that the percentage of heteroplasmy in two of the 29 cells analyzed after selection was well below the average value (Fig. 6). A likely explanation for this finding is that during the recovery phase, when the cells were dividing rapidly, there was mitotic segregation of mtDNAs such that a few cells segregated stochastically to heteroplasmies much lower (and sometimes higher) than the average.

The mechanism by which intracellular selection occurs is poorly understood. Perhaps the organelles containing a lower proportion of mutant molecules divide more rapidly or replicate their genomes more efficiently than do those containing virtually homoplasmic mutant mtDNA, resulting in an overall reduction of the mutation load in the cell. A second possibility is that selection at the level of the organelle may be enhanced when energy deprivation promotes the formation and subsequent elimination of organelar elements containing a disproportionately high level of mutated mtDNAs.

The hypothesis that the observed shift in heteroplasmy was intracellular is also consistent with the findings of Tønsgard et al. (20), who showed that resistance to rutamycin, another ATPase inhibitor, could be selected within heteroplasmic cybrids far more rapidly than could be accounted for merely by cellular growth rate or by organellar segregation alone. It is also consistent with the results of King and Attardi (21), who were able to select for chloramphenicol resistance soon after
the introduction of only a few chloramphenicol-resistant mitochondria into chloramphenicol-sensitive cells.

Spelbrink and colleagues (22) were also able to induce an intracellular selection in favor of wild-type mitochondrial genomes in lymphoblast cell lines containing a heteroplasmic mtDNA deletion, by treating them for a prolonged period of time with doxycycline, a mitochondrial protein synthesis inhibitor. However, there are several differences between our results and Spelbrink and colleagues (22). First, the mtDNA abnormality was a large scale deletion in their cells and a point mutation in ours, which have vastly different effects on respiratory chain function (23). Second, the heteroplasmic shift in their system took months, not days, to occur. Finally, the heteroplasmic shift of the deleted molecules was inherently unstable and reverted to increased levels of deletion after removal of the selection, as opposed to our observations with a point mutation, in which the shift remained stable. We do not know the reason for these differences.

The fact that a treatment with lower levels of oligomycin did not cause a shift in the percentage of mutant mtDNA and that the cells which had been selected behaved like the homoplasmic wild-type ones when grown in galactose-oligomycin for a second time, suggest that at least in our tissue culture system, 72% mutation and 38% residual ATP synthesis are approximately the functional threshold values. We note that heteroplasmic values below 70% are generally associated with asymptomatic members of T8993G pedigrees (2, 3, 24–26).

In conclusion, the combination of galactose and oligomycin creates selective culture conditions that elicit an abnormal growth phenotype in cells containing the T8993G mutation. These conditions can also induce a shift in heteroplasm in cells, presumably via interorganellar selection. This finding could form the basis for pharmacological approaches for the therapy of mitochondrial disorders that target directly the underlying genetic defect.

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