

Defects in maintenance of mitochondrial DNA are associated with intramitochondrial nucleotide imbalances

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Defects in mtDNA maintenance range from fatal multisystem childhood diseases, such as Alpers syndrome, to milder diseases in adults, including mtDNA depletion syndromes (MDS) and familial progressive external ophthalmoplegia (AdPEO). Most are associated with defects in genes involved in mitochondrial deoxy-nucleotide metabolism or utilization, such as mutations in thymidine kinase 2 (TK2) as well as the mtDNA replicative helicase, Twinkle and gamma polymerase (POLG). We have developed an *in vitro* system to measure incorporation of radiolabelled dNTPs into mitochondria of saponin permeabilized cells. We used this to compare the rates of mtDNA synthesis in cells from 12 patients with diseases of mtDNA maintenance. We observed reduced incorporation of exogenous α ³²P-dTTP in fibroblasts from a patient with Alpers syndrome associated with the A467T substitution in POLG, a patient with dGK mutations, and a patient with mtDNA depletion of unknown origin compared to controls. However, incorporation of α ³²P-dTTP relative to either cell doubling time or α ³²P-dCTP incorporation was increased in patients with thymidine kinase deficiency or PEO as the result of TWINKLE mutations compared with controls. The specific activity of newly synthesized mtDNA depends on the size of the endogenous pool diluting the exogenous labelled nucleotide. Our result is consistent with a deficiency in the intramitochondrial pool of dTTP relative to dCTP in cells from patients with TK2 deficiency and TWINKLE mutations. Such DNA precursor asymmetry could cause pausing of the replication complex and hence exacerbate the propensity for age-related mtDNA mutations. Because deviations from the normal concentrations of dNTPs are known to be mutagenic, we suggest that intramitochondrial nucleotide imbalance could underlie the multiple mtDNA mutations observed in these patients.

INTRODUCTION

Eukaryotic mitochondria maintain a 16 569 bp circular multi-copy genome present between 1000 and 100 000 copies per nucleated cell. The replication and maintenance of these genomes are distinct from that of the nucleus and continues

even in postmitotic cells, a state termed 'relaxed replication' (1,2). Despite a degree of replicative autonomy, mtDNA is entirely dependent on enzymes encoded by the nuclear genome for its repair and replication. Recent years have shown that defects of mtDNA maintenance are an important cause of neurological disease, including autosomal dominant

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or recessive progressive external ophthalmoplegia (PEO, affecting mainly the extra-ocular muscles) and mtDNA depletion syndromes (MDS, affecting primarily the muscle, liver and brain). These disorders are characterized by multiple large-scale deletions or point mutations of mtDNA, and/or a general tissue-specific reduction of mtDNA copy number. The best characterized disorder is associated with late onset mtDNA depletion: recessive mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) (3) associated with mutations in the gene for thymidine phosphorylase (TP). Mutations in three different genes underlying PEO-disease with multiple mtDNA deletions have been identified in recent years: mitochondrial DNA polymerase (POLG) (4); the heart/muscle specific mitochondrial adenine nucleotide translocator (ANT1) (5) and the mitochondrial replicative DNA helicase, Twinkle (6). Furthermore, mutations in proteins directly or indirectly affecting the mitochondrial nucleoside pools have also been found underlying MDS: thymidine kinase 2 (TK2) (7), deoxyguanosine kinase (dGK) (7) and ADP-Forming Succinyl-CoA Synthase (8). Alterations in the intramitochondrial nucleotide pool could affect the accuracy or rate of mtDNA synthesis (9). Hence, almost all of the genes implicated in defects of mtDNA maintenance appear to be involved directly or indirectly in the mtDNA replication or repair.

It is hard to investigate of the mechanism of autosomal mtDNA disorders, because the diseases affect postmitotic cells, and mtDNA rearrangements have not been found in cultured cells. Direct measurement of intramitochondrial dNTP levels is technically challenging, and cannot readily be used as a screening test in disorders where they may be deranged (10–12).

We have developed a radiolabelling technique that reflects intramitochondrial dNTP levels by assessing rates of mtDNA synthesis in permeabilized cells (13), hereafter called permeabilized cell labelling (PCL). Studying mtDNA synthesis in living cells has the advantage that the conditions resemble the cellular milieu. For instance, the rates of physiological processes involving nuclear replication and transcription are minimally affected (14,15). The level of newly synthesized mtDNA relative to steady-state mtDNA can be used to compare rates of synthesis in different conditions. Here, we show that patients with defects in TK, TP and Twinkle, but not POLG, show abnormally increased incorporation of exogenous dTTP using this technique. This suggests that the intramitochondrial dTTP pool is decreased in these disorders and that mitochondrial nucleotide balance may be central to the disease mechanism.

RESULTS

We investigated mtDNA synthesis in cell lines derived from 12 patients with defects in mtDNA maintenance, in one of whom there was a substantially reduced cytochrome oxidase activity (16). In three further cell lines, there were cells with low or absent mtDNA content judged by Picogreen fluorescence microscopy (Supplementary Material, Figure S2) and/or real-time PCR (Table 1). In order to compare nucleotide utilization in the mitochondria of cell lines derived from

the patients and controls, we labelled the cells with either α - ^{32}P -dTTP or α - ^{32}P -dCTP in the presence of aphidicolin to block nuclear DNA replication (Fig. 1A). Because of the need for multiple (usually triplicate) loadings at several time points for each cell line on the same gel, in each experiment comparisons were made between a small number of cell lines. In each experiment, the signal due to incorporation of radioisotope (or PCL for permeabilized cell labelling) was compared to the signal when probed with full-length mtDNA (CSH for conventional Southern hybridization), because this reflects the rate of mtDNA synthesis. Differences between patients and controls reflect both the rate of mtDNA synthesis and the size of the pool of unlabelled intramitochondrial nucleotides (Fig. 1B). All patient samples (Table 1) were compared with the same control line (control1) that was used as a reference for the other experiments.

mtDNA synthesis is reduced in fibroblast lines from MDS patients, especially in lines that have reduced mtDNA copy number

Incorporation of α - ^{32}P -dTTP into the mtDNA of two cell lines from MDS patients with POLG mutations was slow. Figure 2 shows that newly synthesized mtDNA was barely detectable in MDS-POLGP1, confirming previous investigation of MDS-POLGP1 using BrdU (16). The probed gel in Figure 2 shows a lower signal for mtDNA compared to control, despite comparable signals on the ethidium stained gel (not shown) because mtDNA copy number in this cell line was 22% (± 2) of that of the value in the control line. For this cell line, the signal on the PCL gels was at the limit of detection, so it was not possible to quantitate the ratio of PCL to CSB signal accurately. In MDS-POLGP2, mtDNA copy number was within the normal range, and the ratio of the signals on the PCL and CSB gels (I/S) was lower than that for two control lines (22 or 64%, uncorrected for cell doubling time, see Supplementary Material, Experiment S1). Hence, the incorporation was slower in lines MDS-POLGP1 and MDS-POLGP2 compared with controls, and this reduction was more than expected for the mtDNA copy number in MDS-POLGP2.

Reduced rate of mtDNA synthesis in MDS patient lines is not due to permeabilization of mitochondria

As this result might reflect intramitochondrial nucleotide availability, we sought to determine whether the experimental procedure had affected the permeability of the inner mitochondrial membrane (IMM). Previous studies have shown that the mitochondrial nucleotide pool is distinct from the cytoplasmic pool because of the low permeability of the IMM (17). While the deoxynucleotide carrier (DNC) in the IMM transfers dNDPs between the two pools (18), recent studies suggest that other routes may be more critical to intramitochondrial nucleotide supply (19).

Because saponin selectively attacks cholesterol rich membranes, it should permeabilize only the plasma membrane, leaving the cholesterol poor mitochondrial membranes intact. To estimate the intactness of the IMM, we utilized a citrate synthase activity assay. Citrate synthase is an enzyme of the

Table 1. Molecular characterization of patients in this study

Patient cell line	Patient molecular characteristics Gene and nucleotide or amino acid change(s)	Experimental data		Reference or source	Phenotype	dTTP incorporation relative to			
		MtDNA content by QPCR ^a	PicoGreen staining			Cell doubling	dCTP	dGTP	Experiment ^b
MDS-POLGP1 (Fig. 2)	POLG T914P and R1096C	22–55%	Mosaic depletion	(16)	Alpers	–			
MDS-POLGP2 (Fig. 2)	POLG E873TAG (stop) and A467T	Not depleted	Mosaic depletion	(50–52)	Alpers	–			1
MDS-TK2.1 (Fig. 2)	TK2 K171del/R152G	Not depleted	Not depleted	(47)	Myopathy		+++		2
MDS-TK2.2 (Fig. 4)	TK2 R161K and T77M	Not depleted	Not depleted		Myopathy			+	
MDS-dGK1 (Fig. 4)	dGK GAdel563–564 Transvn at -8 intIV	34%	Mosaic depletion		Hepatocerebral degeneration			–	
MDS-dGK2 (Fig. 4)	dGK GAdel563–564 Transvn at -8 intIV	64%	Mosaic depletion		Hepatocerebral degeneration			±	
MNGIE1	Thymidine phosphorylase A1453G and T2306C	Not depleted	Not depleted	Patient 1 (53)	MNGIE	+	+		3,5
MNGIE2	Thymidine phosphorylase A3371C and G3868C	Not depleted	Not depleted	Patient 2 (53)	MNGIE	±	±		
PEO-TW1	TWINKLE T1100C	Not depleted	Not depleted	Patient 4 (6)	CPEO	+	+	±	4,6
PEO-TW2	TWINKLE Dup1053_1092	Not depleted	Not depleted	Patient 12 (6)	CPEO	+	+	±	
PEO-POLGP1	POLG G2869T	Not depleted	Not depleted	(54)	CPEO		±		
PEO4	Unknown	Not depleted	Not depleted		CPEO	–			
Control1	Wild-type	Not depleted	Not depleted	Skin	NA				1 to 7
Control2	Wild-type	Not depleted	Not depleted	Skin	NA				7
Control3	Wild-type	Not depleted	Not depleted	Muscle	NA				1
Control4	Wild-type	Not depleted	Not depleted	Skin	NA				

^aNormal 68–193%.^bExperiment number in supplementary information.

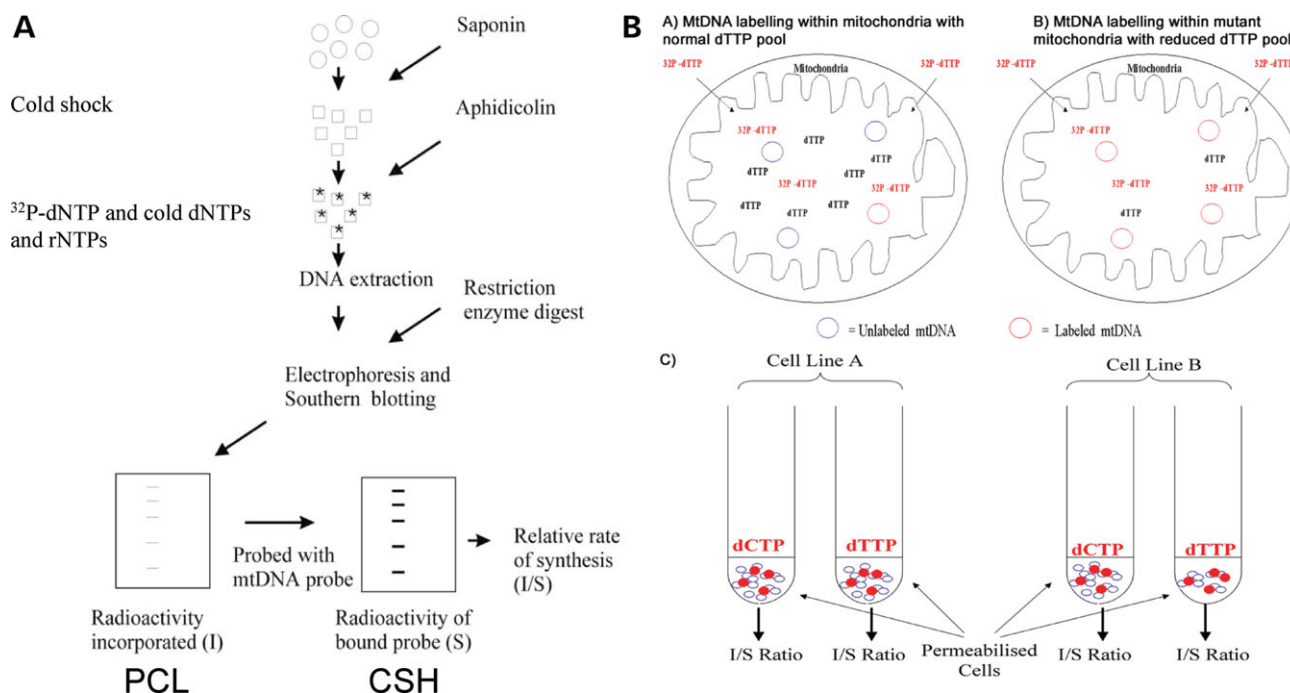


Figure 1. (A) Cartoon illustrating the permeabilized cell labelling assay. The radioactive signal that is incorporated into mtDNA (I) on the Southern blot (the PCL signal) is compared with the signal (S) on the same filter probed using conventional Southern hybridization (CSH). The ratio I/S enables comparison of the rate of mtDNA labelling between samples on the same gel. (B) Cartoon illustrating indirect analysis of intra-mitochondrial dNTPs using permeabilized cell labelling. Within the intact mitochondria, the radiolabel competes with endogenous unlabelled dTTP to become incorporated into mtDNA (a). In cells with a reduced intramitochondrial dTTP pool, the labelled dTTP comprises a higher proportion of the intramitochondrial pool (b) than the control (a). For dual labelling, permeabilized cells from a patient with intramitochondrial dTTP deficiency were split into two tubes and incubated with either radiolabelled dTTP or dCTP, and the labelling of mtDNA compared with a control (c). The proportion of newly synthesized mtDNA that is labelled with α - ^{32}P -dTTP is greater than the control in the cell line where the intramitochondrial dTTP pool is small. Comparison can be made with α - ^{32}P -dCTP incorporation or cell doubling time.

inner mitochondrial matrix and so an intact IMM will hinder entry of the reaction reagent to the site of citrate synthase activity. By comparing the reaction in the presence and absence of 0.2% triton X (which achieves full mitochondrial permeabilization), we were able to assess the intactness of the IMM (20). Using this approach, the citrate synthase activity in the PB buffer suspending either unpermeabilized or saponin permeabilized cells was undetectable, despite the fact we used a saponin concentration that was more than three times higher than that required to achieve full cellular permeabilization (as judged by trypan blue dye exclusion). However, addition of 0.02% triton X or lauryl maltoside increased the rate of reaction more than 20-fold, indicating permeabilization of the IMM (see Supplementary Material, Figure S1).

We also sought to visually assess the intactness of mitochondria using Calcein-AM loading. Once esterified, calcein is trapped within membranous compartments including mitochondria, only released by permeabilization of the IMM (21,22). Addition of calcein-AM to fibroblasts results in rapid formation of a diffuse green fluorescence distributed across the whole cell (Fig. 3A). However, after saponin permeabilization, the diffuse green calcein signal is lost and only semi-elongated and punctate structures remain (Fig. 3B). Co-staining with the mitochondrion specific probe, Mitotracker Red, showed that these saponin resistant structures were intact mitochondria (Fig. 3C and D). The mitochondria continued to retain significant amounts of calcein even after incubation for 180 min

in PB buffer at 37°C (Fig. 3E). Addition of 0.05% triton X or lauryl maltoside leads to a large reduction in the mitochondrial calcein signal (Fig. 3F).

Collectively, the lack of citrate synthase activity in the supernatant of permeabilized cells and their retention of calcein indicate that the IMM of mitochondria in saponin-permeabilized cells remains intact. Such cells are thus capable of maintaining their intramitochondrial nucleotide pools.

The reduced incorporation of α - ^{32}P -dTTP into mtDNA in MDS-POLGP2 is not explained by prolonged cell doubling time

Because mitochondrial DNA copy number remains relatively stable with sequential passage in fibroblasts (unpublished observations), the rate of mtDNA synthesis should depend on cell doubling time. In order to determine whether differences between the PCL:CSB signal ratios for different cell lines is explained by differences in cell proliferation, we compared MDS-POLGP2 with two control cell lines whose doubling times differed markedly. The doubling time of control 2 was twice that of MDS-POLGP2, and the doubling time of control 1 less than half. The rate of α - ^{32}P -dTTP incorporation of MDS-POLGP2 was 36 and 28% of controls 1 and 3, respectively, when corrected for cell doubling ($P = 0.01$ and 0.04 , respectively, Fig. 2). Controls were not significantly different. A subsequent experiment using controls 1 and 2,

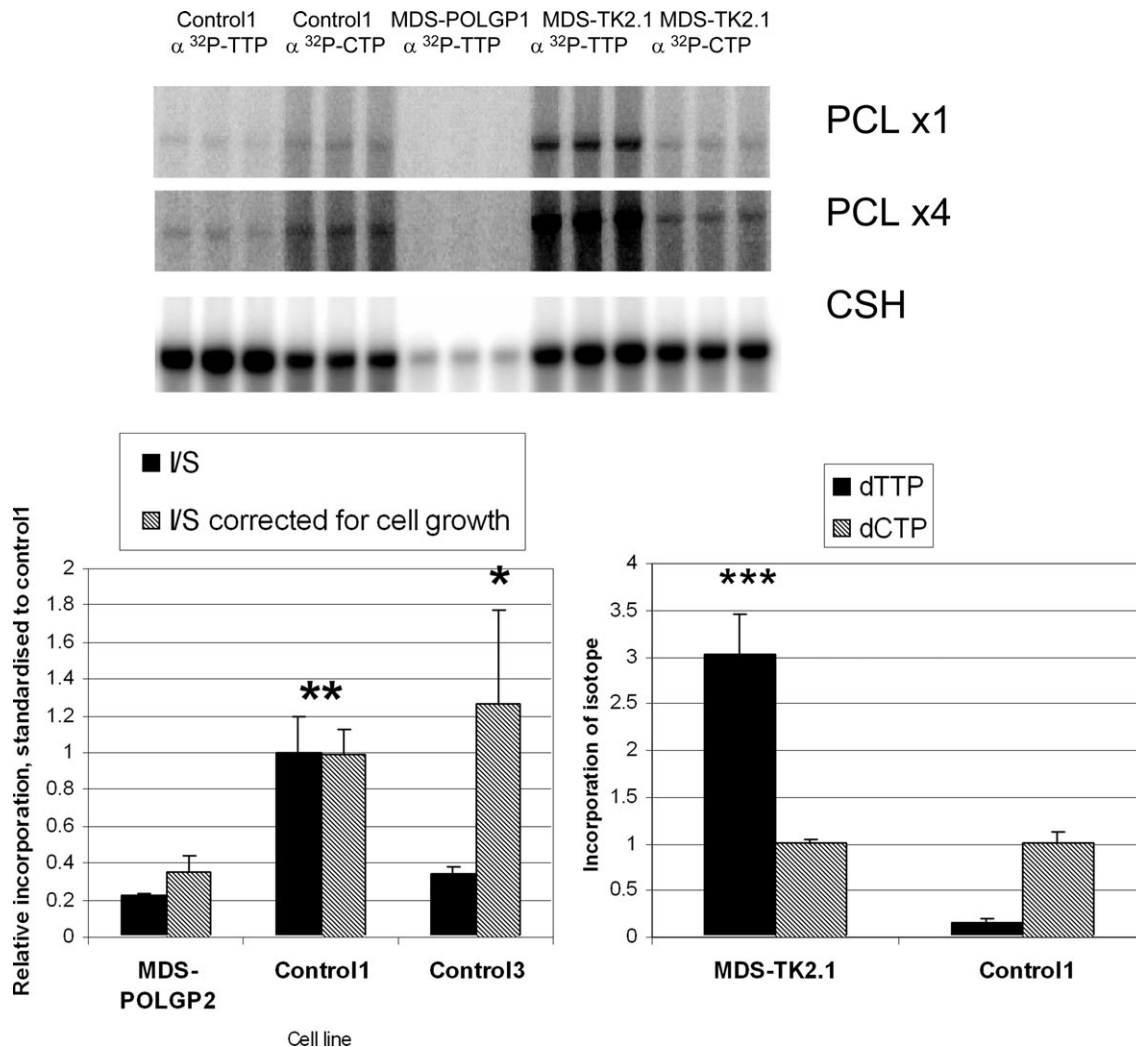


Figure 2. Permeabilized cells from MDS patients incorporate less label than controls. Top panel: Cells were labelled with either α ^{32}P -dTTP or α ^{32}P -dCTP. Comparable quantities of labelled DNA were then digested with PvuII and Southern blotted (two settings of the phosphorimager gain shown, differing 4-fold, PCLx1 and PCLx4). The signal from MDS-POLGP1, labelled with α ^{32}P -dTTP, was much less than control 1. The same filter was then hybridized with a total mtDNA probe (CSH) showing that there is mtDNA depletion in MDS-POLGP1, but the reduction is qualitatively less than in the PCL. This was consistent with the progressive depletion of mtDNA from this line. Bottom left panel: A similar experiment was carried out with MDS-POLG2 and two controls. The ratio of the newly synthesized to steady state mtDNA (I/S, that is PCL signal, I, divided by CSH signal, S) was higher in the controls than MDS-POLG2. When corrected for cell doubling time, the MDS incorporation was significantly less than either control (*denotes $P < 0.05$, **denotes $P < 0.01$). Bottom right panel: Quantitation of α ^{32}P -dTTP relative to α ^{32}P -dCTP incorporation in the TK2.1 mutant cell line shows a clear increased incorporation of labelled dTTP in MDS-TK2 patient compared with control. This indicates intramitochondrial dTTP deficiency. ***denotes $P < 0.001$. The incorporation of α ^{32}P -dTTP has been standardized relative to α ^{32}P -dCTP.

whose doubling times differed by a factor of 2.0 (± 0.4), showed that the corrected ratio (2.5-fold (± 0.2)) was not significantly different. Incorporation differed by a factor of 1.67 in a comparison of early and late passage fibroblasts from the same individual where the doubling time differed by 1.64.

Measurement of α ^{32}P -dTTP relative to α ^{32}P -dCTP incorporation in a TK2 mutant cell line

We were interested in clarifying whether alterations in nucleotide incorporation were specific to a single nucleotide, and therefore we compared α ^{32}P -dTTP with α ^{32}P -dCTP incorporation into mtDNA. We therefore labelled permeabilized MDS-TK2.1 and control cells with α ^{32}P -dTTP in parallel

with α ^{32}P -dCTP. Incorporation of exogenous α ^{32}P -dTTP relative to α ^{32}P -dCTP was increased 11–18-fold in the MDS-TK2.1 cells compared with the control (Fig. 2, $P < 0.001$), suggesting that our method reflects intramitochondrial dTTP availability. In addition, this avoids the need to correct for cell doubling time and to determine whether the alterations are specific for α ^{32}P -dTTP.

Measurement of α ^{32}P -dTTP relative to α ^{32}P -dGTP incorporation in cell lines with mutations in dGK or TK2

To confirm that the increased rate of α ^{32}P -dTTP incorporation reflected a deficiency of intramitochondrial dTTP in TK2 deficiency, we labelled cells from patient MDS-TK2.2 with

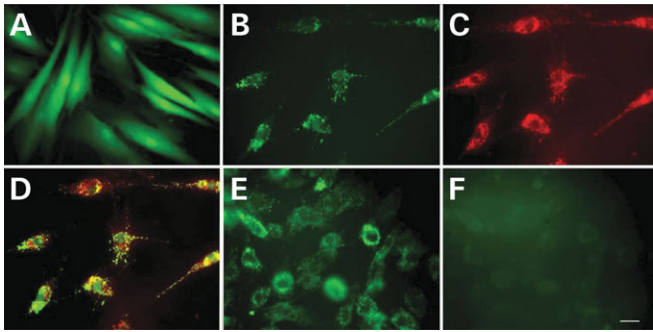


Figure 3. Visual demonstration of mitochondrial intactness using calcein-AM fluorescence. Intact fibroblasts after incubation with calcein-AM form fluorescent calcein which is retained within the cells, which fluoresce green (A). After cells are permeabilized with cold saponin, most of the calcein fluorescence is lost revealing elongated and punctate structures which retain calcein (B). The same cells were co-stained with the mitochondrial probe mitotracker red to visualize mitochondria (C). Co-localization of the calcein (green) and mitotracker (red) showed a high degree of overlap (yellow) confirming these saponin resistant structures are intact mitochondria (D). The mitochondria retained the calcein after 180 min in PB at 37°C, showing they remained intact (E). However, this fluorescence was rapidly lost after addition of 0.5% triton X (F). Bar 40 μ m.

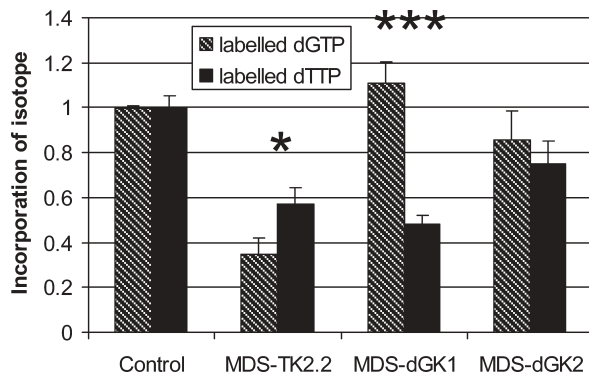


Figure 4. Measurement of α 32 P-dTTP relative to α 32 P-dGTP incorporation in the MDS-dGK1 and 2 and MDS-TK2.2 mutant cell lines. Significant differences in dGTP and dTTP incorporation in MDS-TK2.2 and MDS-dGK1 lines * denotes $P = 0.05$ and *** $P = 0.001$ respectively, standardized to the incorporation of control. The trend towards slower incorporation in patient lines than the control was consistent with their growth rates.

α 32 P-dTTP in parallel with α 32 P-dGTP (Fig. 4). α 32 P-dTTP incorporation was increased 1.7-fold compared with control ($P = 0.05$), confirming a deficiency of dTTP relative to dGTP. In the same experiment, we labelled cells from two patients with mutations in dGK. In both cases, incorporation of α 32 P-dGTP was greater than α 32 P-dTTP by 2.3- and 1.2-fold ($P = 0.001$ and NS, respectively, Fig. 4).

Measurement of α 32 P-dTTP incorporation in patients with TWINKLE and TP mutations relative to cell doubling time and to α 32 P-dCTP incorporation

Previous investigators have suggested that point mutations may arise from pausing of the replication complex in both TWINKLE and TP mutants, and it was therefore appropriate

Table 2. α 32 P-dTTP incorporation in patients with defects in mitochondrial biogenesis compared with control (corrected for cell doubling time)

	dTTP incorporation relative to cell doubling as a ratio of the control	<i>P</i> -value
MNGIE1	5.1	0.012
PEO-TW1	2.8	0.028
PEO-TW2* experiment 1	2.4	0.029
PEO-TW2* experiment 2	2.3	0.03
PEO-TW2 experiment 3	2.0	0.014

Incorporation of α 32 P-dTTP relative to cell doubling time is significantly increased in cell lines from two Twinkle and a MNGIE patient relative to controls (see Supplementary Material, Experiments 3 and 4). PEO-TW2 indicates the primary fibroblasts, PEO-TW2* were fibroblasts from the same patient but transformed with E6E7 retrovirus (compared to E6E7 retrovirus transformed control).

to investigate incorporation of exogenous α 32 P-dTTP into the mtDNA in these lines. Incorporation of exogenous α 32 P-dTTP relative to doubling time was consistently greater in lines PEO-TW1 and 2 than in controls. Experiments where this difference was statistically significant are listed in Table 2 ($P = 0.012$ to 0.03 Student's *t*-test to compare the ratio of the gradients of regression lines for the time course of α 32 P-dTTP incorporation and log cell count). While the reverse would be predicted in cells in which the intramitochondrial dTTP pool was abnormally large, the trend was towards similarly increased dTTP incorporation in cells from both MNGIE patients. This result reached statistical significance on one occasion.

The current analysis measures the radionucleotide signal in bands of a Southern blot, and hence largely in full-length mtDNA. We reasoned that the rapid rate of α 32 P-dTTP incorporation might be due either to a high failure rate in mtDNA replication, hence rapid turnover of stalled intermediates or to a decreased intra-mitochondrial dTTP pool. We investigated the possibility that there might be large differences in fragmented molecules arising from replication intermediates in two ways. Firstly, degradation of stalled replication intermediates should increase the intensity of the background smear in each lane of the PCL gel. There was no evidence of any clear difference either by eye or by quantitation with a phosphorimager. Secondly, incorporation of other α 32 P-dNTPs should be similarly increased relative to doubling time if stalling of replication intermediates is increased.

Incorporation of exogenous α 32 P-dTTP relative to α 32 P-dCTP was increased in TWINKLE mutants compared with controls. Differences were smaller when α 32 P-dTTP was compared with α 32 P-dGTP incorporation, not reaching statistical significance (not shown). There was no significant difference between controls (with ratios ranging from 0.66 to 1.0, $n = 3$). While an excess of intramitochondrial dTTP should result in reduced α 32 P-dTTP incorporation relative to α 32 P-dCTP, this was not evident in cell lines from patients with MNGIE. Indeed, the trend was the reverse (Tables 2 and 3). The ratio of α 32 P-dTTP to α 32 P-dCTP incorporation in PEO-POLGP1 fibroblasts, derived from a patient with the A957S mutation of POLG, was similar to that of controls (Table 3).

Table 3. The incorporation of α 32 P-dTTP was compared with α 32 P-dCTP in patients relative to controls

	dTTP compared with dCTP incorporation relative to control	P-value
MNGIE1 experiment 1	2	0.034
MNGIE1 experiment 2	2.5	0.065
MNGIE1 experiment 3	1.4	0.114
PEO-TW1 experiment 1	1.91	0.024
PEO-TW1 experiment 2	1.67	0.01
PEO-POLGP1	1.17	0.40

Incorporation of α 32 P-dTTP relative to α 32 P-dCTP is significantly increased in cell lines from a MNGIE patient and a TWINKLE patient relative to controls (see Supplementary Material, Experiments 5 and 6).

Effect of excess unlabelled dCTP and dTTP and other variables on measurement of α 32 P-dTTP and α 32 P-dCTP incorporation

As is routine in assays of this sort (L. Kaguni, personal communication), we used a much lower molar concentration of radionucleotide than of cold nucleotides which were present in excess (for example 12.5 μ M of α 32 P-dTTP and 250 μ M of unlabelled dTTP, the K_m of wild type POLG polymerase activity being 5.7 ± 1.5 for dTTP (21)). Because of concern that the level of cold nucleotide might be limiting, the rate of α 32 P-dCTP incorporation was compared when the concentration was increased from the normal 250 to 375 μ M or reduced to 125 μ M. For both patients and controls, this only had a small effect but 250 μ M was found to be optimum (not shown).

The effect of plating density was also compared on the rate of mtDNA synthesis. There was no difference in rate of α 32 P-dTTP incorporation, the cells that had been over-confluent for 2 days being identical to the control. This suggests that any reduction in the rate of mtDNA synthesis caused by contact inhibition by confluent cells rapidly disappears after they are trypsinized.

DISCUSSION

Abnormal intramitochondrial dNTP pools are likely to be critically important to defects of mtDNA maintenance, because such alterations are known to be mutagenic in both human and yeast cells (23). We have developed an established method for investigating mtDNA synthesis in cell lines (13) to demonstrate intramitochondrial nucleotide imbalance in patients with defects in mtDNA maintenance. Because previous investigators have shown that there is a clear, mitochondrial dTTP deficiency in patients with mutations in TK2, we used cells from two such patients to validate our method. We showed that incorporation of α 32 P-dTTP, relative to α 32 P-dCTP or α 32 P-dGTP incorporation, is greater in TK2 mutant than in control cells. The specific activity of newly synthesized mtDNA depends on the size of the endogenous dNTP pool diluting the exogenous labelled nucleotide (Fig. 1B). Our result is thus consistent with intramitochondrial dTTP deficiency relative to dCTP levels in TK2 mutant cells.

This is presumably accompanied a deficiency of its precursors dTDP and dTMP. Similarly, intramitochondrial dGTP deficiency may be a feature of dGK mutant cell lines.

Cells from two patients with mutations in POLG did not have increased incorporation of α 32 P-dTTP. Firstly, the rate of α 32 P-dTTP incorporation relative to cell doubling was slowed in MDS-POLGP2 from a patient with mono-allelic expression of the A467T substitution in POLG. Biochemical expression studies show that the A467T mutant has only ~4% of wild-type polymerase activity (24). Secondly, there were no differences in α 32 P-dTTP incorporation relative to α 32 P-dCTP incorporation in cell line PEO-POLGP1 with the common POLG A957S mutation. This mutation reduces the binding affinity for all four nucleoside triphosphates (25). Further, the reduced α 32 P-dTTP incorporation relative to cell doubling time that we demonstrated in patient PEO1 suggests that he may have a POLG mutation that we have not yet characterized.

Cell lines with TWINKLE and TP mutations both had moderately but consistently increased α 32 P-dTTP incorporation into mtDNA relative to α 32 P-dCTP incorporation and to cell doubling, compared with controls. This observation suggests that intramitochondrial dTTP concentrations may be limiting in these cells.

Cell lines PEO-TW1 and TW2 were both derived from patients with AdPEO (26), associated with mutations in the linker region of the TWINKLE gene (6). TWINKLE is a ~72 kDa mitochondrial protein that is homologous to the phage T7 primase-helicase. Korhonen *et al.* (27) have purified and characterized the properties of human TWINKLE, and found that it has DNA helicase activity with 5' to 3' directionality. The helicase activity has an absolute requirement for hydrolysis of a nucleoside 5'-triphosphate and any of UTP, GTP and ATP can be utilized (26). Our method could be used to determine whether the apparent deficiency of dTTP relative to dCTP that we documented is due to an imbalance that is specific to pyrimidines. The vectorial movement of the T7 phage primase helicase T7 gp4 on DNA is coupled to the hydrolysis of 2'-deoxythymidine triphosphate (dTTP). This helicase, which has a close homology to TWINKLE, has been termed a molecular motor (28). Pathogenic TWINKLE mutations, such as those identified in our patients, are generally located in linker region (6). While the mechanism for the mutations in our patients is not yet clear, both are located in the linker region close to the region corresponding to the A257T mutation of T7 gp4 that exhibits enhanced dNTPase activity, most marked with dTTP, in the absence of substrate DNA (29). It is conceivable that nucleotidase activity is increased in our patients, causing a selective loss of dTTP and subsequent intramitochondrial nucleotide deficiency.

Similarly, we found a moderate but consistent increase in α 32 P-dTTP incorporation into mtDNA relative to α 32 P-dCTP and to cell doubling in the MNGIE1 and 2 cells compared with the control. TP mutations have generally been found in patients with mitochondrial neuropathy gastrointestinal encephalopathy (MNGIE), a subgroup associated with tissue specific mtDNA depletion and multiple deletions. In these patients, the low TP activity leads to a several-fold elevation of both thymidine (3) and deoxyuridine in blood. Fibroblasts derived from MNGIE patients have been shown to excrete

excess thymidine into culture medium, while normal controls actively metabolize the thymidine present (30). Previous workers have shown that excess thymidine is able to generate mtDNA mutations in cultured cells (31) (11) (32) and that cellular dTTP pools are increased in such cells. They have suggested that mtDNA mutations are generated in MNGIE as a result of an excess of dTTP in the mitochondria (30) but have not yet demonstrated this directly. The nucleotide imbalance in MNGIE is complex, with pleiotropic effects (30). For instance, the substantial increase in deoxyuridine derivatives is a potential problem for mtDNA synthesis (33,34). If either dUMP or dUDP level increase, they may potentially compete with thymidine derivatives for their intramitochondrial kinases. Furthermore, thymidine excess is known to reduce the activity of TK by negative cooperativity (35).

Our studies have not demonstrated the expected excess of intramitochondrial dTTP. Indeed, they suggest that there is an intramitochondrial dTTP deficiency in MNGIE cells. Thymidine is rapidly replaced after extensive dialysis of MNGIE patients, suggesting that the renal tubules are actively compensating by reabsorbing thymidine (30). While it is clear that reduction of intramitochondrial dTTP is able to inhibit mtDNA synthesis, the evidence that increased dTTP could do the same is based on exposure of normal cells to excess thymidine, [held to be a good cellular model of MNGIE (31,32)]. These studies provided evidence of abnormal dCTP pools (11) as well as an increase in dTTP pools (31). Studying intramitochondrial dNTP levels in cells from patients with MNGIE directly, to our knowledge has not been reported, and is technically difficult, as crudely purified preparations of mitochondria are likely to be substantially contaminated with nuclear or cytoplasmic deoxynucleotides, including dTTP. The small mitochondrial pools would be certainly prone to such contamination, particularly if extramitochondrial nucleotides were elevated. Furthermore, analyses of the mtDNA point mutations that are increased in MNGIE patients demonstrate that TA to CG mutations are common (12). Such a mutation pattern would be consistent with a deficiency in intramitochondrial dTTP, as would the renal conservation of thymidine observed in MNGIE patients (30).

The PCL assay allowed us to investigate mtDNA replication in saponin permeabilized cultured fibroblasts *in situ*, using an exogenous radionucleotide that enters the mitochondria and specifically labels mtDNA. This approach overcomes measurement problems by using intact mitochondria in the near physiological state of a permeabilized cell. Our observations agree with previous studies in that saponin or digitonin permeabilization of plasma membranes have a minimal impact on cellular function and do not appear to impair respiratory chain activity (14) or mitochondrial transcription (15) and we have now demonstrated that the IMM remains relatively intact (Fig. 3). Thus, we infer that our method preserves the intramitochondrial nucleotide pool which is retained by the intact IMM. Because exogenous radionucleotide has access to the cytoplasm and then enters the mitochondrial pools, we infer that the label probably enters the mitochondria via carriers such as the DNC transporter and is then incorporated into replicating mtDNA.

Labelling of cell lines from patients with MDS demonstrated that α 32 P-dTTP incorporation was clearly reduced

compared to controls, consistent with previous investigators (10) and with progressive loss of mtDNA on fibroblast culture (36,37). In order to correct for differences in the quantity of DNA loaded in each lane of the Southern blot, we compared the ratio of labelled to steady state (probed) mtDNA in the same band (13). This has the advantage that it corrects for variation in factors such as efficiency of restriction enzyme digestion, provided that there is no major difference between newly synthesized and older mtDNA molecules. Hence, it enables comparison of the rate of radionucleotide incorporation in different cell lines. It also enables us to take variation in mtDNA copy number into account. Using this approach, we found that incorporation of exogenous α 32 P-dTTP into mtDNA was reduced in fibroblasts from two patients with MDS, consistent with frank depletion of mtDNA compared to controls (Fig. 2).

In non-senescent control fibroblasts, mitochondrial DNA copy number remains relatively constant, even though cell doubling slows with successive passage (not shown). Hence, mtDNA synthesis rate depends on cell doubling time. To correct for this, we used two approaches. As an alternative to correcting for cell doubling time (Table 1), we compared incorporation of α 32 P-dTTP into mtDNA with α 32 P-dCTP incorporation (Table 2). Using both approaches has the additional advantage that it should be possible to detect major alterations in mtDNA turnover. However, the results from both approaches were consistent, suggesting that any increase in turnover in our patient-derived cell lines is relatively small. Comparison of the ratio of α 32 P-dTTP to α 32 P-dCTP is similar in control lines, but suggests preferential incorporation of exogenous dTTP relative to dCTP in a thymidine kinase mutant cell line. This suggests that endogenous intramitochondrial dTTP is depleted, compared to dCTP in MDS-TK2 cells, because the exogenous radionucleotide would compete more effectively with a smaller endogenous intramitochondrial nucleotide pool, and its incorporation into mtDNA would be reduced. Our finding of an 11-18-fold increase in incorporation of exogenous α 32 P-dTTP into mtDNA relative to α 32 P-dCTP in the MDS-TK2.1 mutant cells compared with the control (Fig. 2) thus suggests that there is an intramitochondrial dTTP deficiency in the mutant. This is consistent with the expected intramitochondrial dTTP deficiency that has been demonstrated by other workers (10), who have measured deoxythymidine triphosphate (dTTP) content directly in quiescent fibroblasts from patients with TK2 deficiency (10,38). It is also consistent with the increased α 32 P-dTTP relative to α 32 P-dGTP incorporation that we demonstrated in the MDS-TK2.2 cells, though it is not clear why this was less marked. Low intramitochondrial dTTP levels relative to dCTP and dGTP potentially limit the rate and accuracy of mtDNA synthesis.

Hence our data suggest that intramitochondrial nucleotide imbalance, particularly limiting levels of dTTP, may play a critical role in defects of mtDNA maintenance. Three lines of evidence suggest that nucleotide imbalance might be an important factor in this group of patients. Firstly, the majority of genes so far implicated in disorders of mtDNA maintenance appear to be involved, directly or indirectly in the regulation of mitochondrial nucleotide metabolism, including TK2 (7), dGK (7), TP (3) and ADP-forming Succinyl-CoA Synthase

(8). One clear exception is mutant POLG in patients with CPEO and Alpers (4,25), consistent with no evidence of nucleotide imbalance in cell lines MDS-POLGP2 and PEO-POLGP1. Another exception may be mutant MPV17 in patients with MDS (39), which is implicated in regulation of antioxidant levels (40).

Secondly, the error rate of wild type POLG is increased by cellular nucleotide imbalances (41,42), and consequent misincorporation is believed to induce stalling and slippage of DNA polymerases (43). Replication stalling has been inferred from analysis of mutations in patients with adPEO due to mutations in either POLG or TWINKLE (44). Dissociation of the polymerase may follow stalling or failure of the exonucleotide activity to excise a misincorporated base, and consequential strand separation of the terminal portion of the newly synthesized strand (25) could underlie the propensity for mtDNA deletions (43). Hence while POLG dysfunction may explain the increased point mutation and multiple deletions seen in patients with POLG mutations, we suggest that nucleotide imbalance in the form of dTTP deficiency may contribute to POLG errors in patients with mutations in TP or TWINKLE, as presumably occurs in TK2 deficiency.

Thirdly, several lines of evidence suggest that mitochondria possess their own separate nucleotide pools, that are replenished both from cytoplasmic *de novo* synthesis and from nucleotide salvage (45), and are probably insufficient to support even one round of mtDNA replication (45). Hence mtDNA synthesis relies on dNTP import from the cytoplasm (45), where levels increase massively during S phase. Intramitochondrial dTTP levels may be critically important, because they are consistently lower than the other three dNTPs, by as much as 100-fold lower than dGTP levels in some studies (46).

In conclusion, we have identified consistent and reproducible alterations in the incorporation of α ³²P-labelled dNTPs into mtDNA in cells from patients with defects in mtDNA maintenance compared to normal controls. These included cells harbouring mutations in TK2, dGK and TWINKLE genes. We suggest that nucleotide imbalance, particularly intramitochondrial dTTP deficiency, is likely to contribute to impaired mtDNA maintenance in the mitochondria of these patients.

MATERIALS AND METHODS

Patients

We compared fibroblast lines from 12 patients with defects in mitochondrial mtDNA maintenance with 4 controls (summarized in Table 1). This included six patients with MDS of whom two (MDS-POLGP1 and P2) had mutations in, and one a documented enzyme deficiency in POLG (MDS-POLGP2), another two in TK2 (MDS-TK2.1 and MDS-TK2.2) (47) and two in dGK (MDS-dGK1 and 2). The latter were unusual in manifesting mtDNA depletion without the need for serum starvation (36), perhaps because differences in their underlying mutations (Table 1). Two cell lines were investigated from patients with MNGIE and TP deficiency (MNGIE1 and 2), four patients with PEO and multiple mtDNA deletions of whom two had TWINKLE mutations (6) (PEO-TW1 and 2) and one patient with a POLG defect (PEO-POLGP1). In one

patient (PEO1) the phenotype was typical of PEO with multiple mtDNA deletions respectively, but sequencing of candidate genes have not yet revealed a causative mutation. In addition, we have previously demonstrated both mtDNA depletion (16) and delayed recovery from ddC treatment in cell line from MDS-POLGP1 (48). For one patient two cell lines were available, one primary fibroblasts (PEO-TW2) and one transformed with E6E7 retrovirus (PEO-TW2* and its control). The remaining 15 cell lines were primary fibroblast lines. The degree of mtDNA depletion was quantitated in live cells by labelling with the fluorescent stain PicoGreen (16) (supplementary information Experiment 9 Supplementary Material, Fig. S2).

Methods

Cells were grown in Dulbecco's Modified Eagle's Medium (Sigma), with 4.5 g/l glucose supplemented with 10% Fetal Bovine Serum, 110 mM pyruvate, 2 mM glutamate, and 100 Units/ml Penicillin/streptomycin and 50 μ M uridine. All cells were grown at log phase in culture flasks (Falcon) fitted with filter caps at 37°C in a humidified 5% CO₂ incubator. Cells were routinely passaged at 90% confluence by trypsinisation and resuspended in fresh medium. Cells were routinely assayed for mycoplasma infection using the Hoescht staining method described in the Sigma mycoplasma test kit.

Primary human fibroblast cells were grown to 90% confluence before harvesting and resuspension in DMEM. An aliquot was taken for cell counting using a haemocytometer. Cells were allowed to recover for 1 hour before centrifugation followed by gentle resuspension in 10–20 ml ice cold 'physiological buffer' (PB) consisting of 1 mM DTT, 1 mM ATP, 130 mM KCl, 10 mM Na₂HPO₄, 1 mM MgCl₂, buffered to pH 7.4 with KH₂HPO₄. The scheme for labelling mtDNA is shown in Figure 1. Cellular permeabilization was accomplished by addition of between 30–120 μ g saponin/ml to the PB buffer before the cells were resuspended. The amount of saponin used was determined by the minimal amount required to achieve >95% cell permeabilization, as judged by trypan blue dye uptake. Cells were incubated on ice for 6 minutes and then centrifuged at low speed. The cell pellet was washed twice with ice cold PBS (phosphate buffered saline) to remove residual saponin before re-suspension into pre-warmed reaction mixture(s). Triton staining indicates that the cells remain permeabilized for the duration of the experiments.

Reaction mixtures consisted of physiological buffer containing a final concentration of 250 μ M of each dNTP, omitting the deoxynucleotide corresponding to the labelled precursor which is present at 12.5 μ M, and 100 μ M of each rNTP (A,G,U,C), and 5 μ g/ml of aphidicolin. The re-suspended cells were then incubated at 37°C for 5 minutes to recover before addition of a specified volume of radio labelled dNTP with gentle mixing. The cells were then incubated for a further 3 h at 37°C with gentle agitation, during which aliquots of radiolabelled cells were removed at specified time points. Cells were resuspended in a large (20 ml) volume of the PB (ice cold) and pelleted at 1000 rpm. The supernatant containing un-incorporated nucleotides was removed and the cell pellet subjected to total DNA

extraction either by proteinase K/SDS digestion and isopropanol precipitation or by using Qiagen DNAmP columns, as per manufacturers protocol. The isolated DNA was quantitated fluorometrically by Hoescht 33 241 staining and then restriction digested by PvuII. The DNA was then precipitated by ethanol/NaOAc washed several times with 70% ethanol and resuspended in TE plus gel loading buffer. The DNA was then subjected to electrophoresis through a 0.6–0.8% agarose gel at 60 V overnight, and the DNA visualized by ethidium bromide staining. DNA was transferred to Hybond N+ nylon membranes by neutral Southern blotting protocols in 10XSSC. After transfer was complete, the membranes were baked dry to fix the DNA and the radiolabelled bands visualized by exposure to phosphor image screens. The total amount of mtDNA (or 'steady state' S) on the membrane was then quantified by hybridization with a random primed total mtDNA probe labelled with α 32 P-dCTP. The probe was created with an Amersham Megaprime kit utilizing a 16 500 bp mtDNA template generated by long-range PCR (49). Unincorporated radionucleotide was removed by centrifugation through a Sigma-spin post-reaction cleanup column an excess of probe was added to ensure that the probed signal would be proportional to the quantity of mtDNA on the membrane. Membrane hybridization was performed overnight in a rotating oven at 65°C and unhybridized probe washed off as per manufacturers protocols. The probed membranes were then re-exposed to phosphor image screens and the resulting membrane images were analysed and quantitated with ImageQuant software. For each DNA band, the total signal representing the amount of incorporated radiolabel (I) was quantitated and compared to its corresponding mtDNA probed signal to generate a ratio, I/S. The I/S ratio provides a simple comparison of the ratio of labelled (i.e. newly synthesized mtDNA) to total mtDNA (steady-state mtDNA amount) in each sample.

The amount of radionucleotide incorporation into mtDNA (I) can be directly compared to the total amount of mtDNA (S) to provide a ratio (I/S). The I/S ratio can then be used to compare labelling of mtDNA in different cell cultures and under different reaction conditions. Linear regression of the I/S ratio against time was used to calculate the rate of increase of ratio of radionucleotide incorporation relative to loading and its standard error. Linear regression of logarithmic growth curves was similarly used to calculate cell doubling time and its standard error. The ratio for patient cell lines relative to control was compared with the ratio of their cell doubling times, and this ratio of ratios was tested for statistical significance using a *t*-test.

Real-time QPCR

Single colour molecular beacon PCR reactions were used to quantify mtDNA levels relative to nuclear DNA, primer and primer sequences as described previously (16), each probe being labelled with FAM at the 5' end and TAMRA at the 3' end. Real-time PCR amplifications were performed with triplicate 25 μ l reactions, containing 1X TaqMan Universal PCR Master Mix (Applied Biosystems), 250 nM each of either mitochondrial or nuclear primers, 100 nM of the corresponding nuclear or mitochondrial probe and 8 ng of target DNA.

Amplification reactions were performed in an GeneAmp 5700 sequence detection system (Applied Biosystems).

Citrate synthase activity assay

Intactness of the IMM after saponin treatment was estimated as previously described (20) with minor adaptations, by measuring citrate synthase activity within the inner mitochondrial matrix in the presence and absence of detergent. Cells were cultured and harvested, rinsed and re-suspended in ice cold PB as described above. The cell suspension was then divided equally into two fresh tubes and kept on ice. Saponin was added to one of the tubes, and mixed gently to permeabilize the cells for 6 min. The cells were then rinsed and resuspended in fresh ice cold PB. A 20 μ l aliquot of each cell suspension was then used for the citrate synthase assay. The cells were resuspended into a volume of 950 μ l in reaction buffer in a 1 ml polystyrene cuvette. The reaction buffer consisted of PB containing 0.3 mM acetyl-CoA, and 0.1 mM 5'5'-dithiobis (2-nitrobenzoic acid) (DTNB). The reaction was measured at 24°C by measuring the change in adsorption at 412 nm after the addition of 0.5 mM Oxalacetate, using a Cecil Spectrophotometer. Triton X was added to a concentration of 0.02% to fully permeabilize the mitochondria. The linear rate of reaction (Δ A412/min) in the absence of oxalacetate was deducted from the linear reaction rate (Δ A412/min) to calculate the net citrate synthase activity both in the presence and absence of detergent.

Mitotracker red CMTXros/Clacein-AM loading to determine mitochondrial inner membrane intactness and morphology

The method is adapted from that of Petronilli *et al.* (19), the molecular weight of Calcein being close to that of dNTPs. Cells were incubated with 2 μ M Calcein-AM (Sigma) and/or 100 nM Mitotracker red, diluted directly into the culture medium and the cells incubated under standard condition for 20 min. The cells were then rinsed 2X in PBS placed on ice and permeabilized with PB containing 500 μ g/ml saponin for 6 min. The saponin was then aspirated and the cells rinsed 2X in PB before addition of fresh PB, and the cells visualized directly using a Leica epifluorescent microscope maintained at 37°C. Cells were visualized at various time-points, up to 3 h after incubation.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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